Aggregated Low-Density Lipoprotein Induces LRPI
Stabilization Through E3 Ubiquitin Ligase CHFR
Downregulation in Human Vascular Smooth Muscle Cells

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Objective—Low density lipoprotein retention and aggregation low-density lipoprotein (agLDL) in the arterial intima are key processes in atherosclerosis. AgLDL is taken up through low-density lipoprotein receptor-related protein 1 (LRP1) by human vascular smooth muscle cells (VSMC). AgLDL increases LRPI expression, at least in part, by downregulation of sterol regulatory element-binding proteins. It is unknown whether agLDL has some effect on the ubiquitin-proteasome system, and therefore on the LRPI receptor turnover. The objective of this study was to analyze the effect of agLDL on the degradation of LRPI by the ubiquitin-proteasome system in human VSMC.

Methods and Results—Human VSMC were isolated from the media of human coronary arteries. Ubiquitylated LRPI protein levels were significantly reduced in human VSMC exposed to agLDL (100 μg/mL) for 20 hours (agLDL: 3.70±0.44 a.u. versus control: 9.68±0.55 a.u.). Studies performed with cycloheximide showed that agLDL prolongs the LRPI protein half life. Pulse-chase analysis showed that LRPI turnover rate is reduced in agLDL-exposed VSMC. Two-dimensional electrophoresis shows an alteration in the proteomic profile of a RING type E3 ubiquitin ligase, CHFR. Real-time PCR and Western blot analysis showed that agLDL (100 μg/mL) decreased the transcriptional and protein expression of CHFR. CHFR silencing increased VSMC, but not macrophage, LRPI expression. However, CHFR silencing did not exert any effect on the classical low-density lipoprotein receptor protein levels. Furthermore, immunoprecipitation experiments demonstrated that the physical interaction between CHFR and LRPI decreased in the presence of agLDL.

Conclusion—Our results demonstrate that agLDL prolongs the half life of LRPI by preventing the receptor ubiquitylation, at least in part, through CHFR targeting. This mechanism seems to be specific for LRPI and VSMC. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: aggregated LDL ▪ CHFR ▪ LRPI ▪ ubiquitin-proteosomal system ▪ VSMC

Lipoprotein retention and aggregation favored by electrostatic interactions with extracellular matrix proteoglycans in the human arterial intima is the main process responsible for lipoprotein deposition during atherosclerosis.1,2 Cholesterol accumulates extracellularly and intracellularly in both macrophages and vascular smooth muscle cells (VSMC) of the arterial intima. 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 VSMCs.3,4 Contrary to native LDL (nLDL), agLDL was shown to be a strong inducer of intracellular cholesteryl ester (CE) accumulation.5,7 These findings are related to differences in the internalization mechanisms; whereas nLDL is taken up by the classical LDL receptor (LDLR), which is downregulated by intracellular cholesterol, agLDL is taken up through LDL receptor-related protein 1 (LRP1).5,7 Uptake of agLDL through LRPI allows high-intracellular CE accumulation not only because of its high capacity to bind and internalize agLDL, but also because LRPI is transcriptionally upregulated by intracellular cholesterol. Our group reported that LRPI is upregulated at transcriptional level by hypercholesterolemia through sterol regulatory element-binding proteins (SREBP) downregulation in human VSMC,5–10 and by hypoxia through hypoxia-inducible factor 1α upregulation in human VSMC11 and cardiomyocytes.12,13 Other groups have reported that insulin promotes the presence of LRPI in the plasma membrane without influencing mRNA expression levels.14,15 LRPI cellular turnover is regulated by the ubiquitin-proteasome system,16 an important mechanism for targeting membrane proteins to destruction, and therefore key in the modulation of protein levels.17,18 Ubiquitylation of proteins...
is an essential step in this process and involves a multiple enzyme cascade composed of ubiquitin-activating (E1), conjugating (E2), and ligating (E3) enzymes.\textsuperscript{17,18} E3 ubiquitin ligases determine the substrate specificity and are classified into 2 groups depending on the presence of either a HETC or a RING domain.\textsuperscript{18} Once a protein is ubiquitylated, it is targeted for degradation by the 26S proteasome complex.\textsuperscript{17,18}

It has been previously reported that agLDL modulates an ubiquitin-conjugated enzyme in human macrophages.\textsuperscript{19} The aim of this study was to analyze the effect of agLDL on LRP1 degradation by the ubiquitin-proteasome system in human VSMC. We report that one of the proteins differentially expressed in agLDL-exposed VSMC is CHFR. CHFR (checkpoint protein with FHA and RING finger domains) is a RING type E3 ubiquitin ligase, first described as a mitotic checkpoint that delays cell-cycle progression to metaphase in response to mitotic stress.\textsuperscript{20} Our results show that agLDL stabilizes LRP1 protein expression through CHFR downregulation.

Materials and Methods

VSMC Isolation and Culture

Primary cultures of human VSMCs were obtained from the media layer of macroscopically healthy coronary artery segments collected from patients undergoing cardiac transplantation at Hospital de la Santa Creu i Sant Pau, Barcelona. VSMC were isolated by a modification of the explant technique, as previously described.\textsuperscript{21} The explants were incubated at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}. After 1 week, the cells have started to migrate from the explants and have proliferated, covering the floor of the culture well. The medium was exchanged every 3 days after the onset of cell outgrowth. A significant outgrowth was reached after 10 days. Tissue fragments were collected with forceps and placed in a new dish with fresh medium. The cells that remain in the dish were cultured until confluency. For cell characterization, cells were seeded in coverslips and grown to confluence. Cells used in the present experiments were between the fourth and sixth passage. VSMC at these passages appeared as a relatively homogeneous cell population, showing a hill-and-valley pattern at confluence. Western blot analysis for specific differentiation markers revealed a clear positive band for α-actin (45 kDa) and calponin (33 kDa). Cell monolayers were grown in medium M199 (GIBCO) supplemented with 20% fetal bovine serum and 2% human serum, 2 mM L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin. The study was approved by the institutional ethics committee at Hospital de la Santa Creu i Sant Pau and conducted in accordance with the Declaration of Helsinki.

Small Interfering RNA Gene Silencing in Human VSMC

To inhibit LRP1 expression in human VSMC, cells were transiently transfected with annealed small interfering RNA (siRNA) (siRNA-LRP1) synthesized by Ambion, according to our previously published LRP1 target sequences.\textsuperscript{22} siRNA-LRP1 was a 15-mer (5’-CGCCGGGCTGACAGAT-3’) oligonucleotide complementary to nucleotides 466 to 481 of LRP mRNA. A siRNA-random was used as a negative control (Ambion AM 4636) in cellular transfections. Fasta analysis (in the Genetic Computer Group package) indicated that these sequences would not hybridize to other receptor sequences (including LDL receptors) in the GenBank database. CHFR-specific siRNA (siRNA-CHFR) was acquired from Applied Biosystems (siRNA ID s31393). The VSMC were transfected with siRNA by the nucleofection technique using the Human AoSMC Nucleofector Kit from Lonza (VPC 1001), according to the manufacturer’s instructions. The final siRNA transfection concentration for siRNA-LRP1, as for siRNA-CHFR, was 0.9 μmol/L. After 48 hours of transfection, cells were exposed to agLDL (100 μg/mL) for the tested time points and harvested by scraping in TriPure Isolation Reagent (Roche Molecular Diagnostics) for PCR and Western blot analysis.

Isolation and Differentiation of Human Monocyte-Derived Macrophages

Human monocyte-derived macrophages isolation, culture, and siRNA gene silencing was performed, as explained in the online-only Data Supplemental methods.

LDL Isolation, Modification, and Characterization

Human LDL (d\textsubscript{1.019–1.063} g/mL) was obtained from pooled sera of normocholesterolemic volunteers by sequential ultracentrifugation. LDLs were dialyzed against 3 doses of 200 vol of 150 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.4, overnight, and once against 150 mM NaCl. LDL protein concentration was determined by the bicinchoninic acid method, and cholesterol concentration was determined by a commercial kit (Boehringer). The average total cholesterol content of human LDL was 4.2 mg/mL. LDLs used in the experiments were <48 hours old. The purity of LDLs was assessed by agarose gel electrophoresis (Paragon System, Beckmann). TBARS were measured as an indirect evaluation of lipid peroxidation and were <1.2 mmol malondialdehyde per milligram of LDL protein. AgLDL was prepared by vortexing LDL in PBS at room temperature. The formation of LDL aggregates by vortexing was monitored by measuring the turbidity (absorbance at 680 nm), as previously described.\textsuperscript{4} The percentage of LDL in aggregated form was calculated by measuring the fraction of protein recovered in the pellet obtained after centrifugation at 10.000g for 10 minutes.\textsuperscript{3,12} The different fractions were analyzed by agarose electrophoresis (Paragon system, Beckmann). No significant alterations of TBARS levels against nLDL were detected after LDL aggregation.

Protein Extraction and Proteomic Analysis

Protein Extracts

Samples were homogenized in Tris-base buffer (40 mM/L Tris-base), incubated for 15 minutes at room temperature, and centrifuged at 16000g for 20 minutes. Protein pellets were further extracted with a urea/chaps buffer (7 mol/L urea, 2 mol/L thiourea, 4% chaps, and 40 mM/L Tris-base) for 15 minutes at room temperature, as described above. Protein concentration in the extracts was measured with a 2D-Quant Kit (GE-HealthCare).

Two-Dimensional Gel Electrophoresis

For analytical and preparative gels, respectively, a protein load of 120 and 300 μg protein of the urea/chaps soluble extracts was applied to 17-cm dry strips (pH 3–10 linear range, Bio-Rad). Gels were developed by fluorescent staining (Flamingo, BioRad).\textsuperscript{21} For each independent experiment, 2-dimensional gel electrophoresis for protein extracts from the control, nLDL, and agLDL groups were processed in parallel to guarantee a maximum of comparability. Each 2-dimensional gel electrophoresis run was at least repeated twice. Analysis for differences in protein patterns was performed with the PD-Quest software (Bio-Rad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared with the volume of all spots in the gel, after background extraction and normalization between gels.

Mass Spectrometry Analysis

Proteins were identified after in-gel tryptic digestion and extraction of peptides from the gels pieces, as described,\textsuperscript{23} by matrix-assisted laser desorption/ionization time-of-flight using an AutoFlex III Smartbeam MALDI-ToF/ToF (Bruker Daltonics). Samples were applied to Prespotted AnchorChip plates (Bruker Daltonics) surrounding the calibrants provided on the plates. Spectra were acquired with
flexControl on reflector mode (mass range 850–4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77 kV; ion source 1 voltage: 19 kV; ion source 2: 16.5 kV; detection gain 2.37x), with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (Version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3 and applying statistical calibration. For identification, peaks between 850 and 1000 were not considered, as in general only matrix peaks are visible on this mass range. After processing, spectra were sent to the interface BioTools (Bruker Daltonics, version 3.2) and, with no further modifications, Mascot search on Swiss-Prot 57.15 database was done (Taxonomy: Homo Sapiens, Mass Tolerance 50–100, up to 2 miss cleavage, Global Modification: Carbamidomethyl [C], Variable Modification: Oxidation [M]). Identification was accepted with a score higher than 56.

Real-Time PCR

Total RNA was isolated by TriPure isolation Reagent (Roche), according to the manufacturer’s instructions. Gene expression of LRP1 and CHFR mRNA was assessed by real-time PCR using the assays on demand Hs00233899_m1 and Hs00943495_m1 (Applied Biosystems), respectively. Human GAPDH (4326317E) (Applied Biosystems) was used as endogenous control. Taqman real-time PCR was performed with 1 μL of RT products (1 μg total RNA) in 10 μL of TaqMan PCR Master Mix (PE Biosystem), with the primers at 300 nmol/L and the probe at 200 nmol/L. PCR was performed at 95°C for 10 minutes (for AmpliTaq Gold activation), and then run for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The threshold cycle (Ct) values were determined and normalized to endogenous control.

Western Blot Analysis

Total protein was isolated by TriPure isolation Reagent (Roche), according to the manufacturer’s instructions. Proteins were analyzed by Western blot, as previously described.4,15 Blots were incubated with monoclonal antibodies against LRP1 (Epitomics, 2703-1, dilution 1:7000) or CHFR (Cell Signaling, 4297, dilution 1:1000). LDLR was analyzed using anti-LDLR (Epitomics, 1956-1, dilution 1:7000) and anti-ubiquitin (Calbiochem, 662099, dilution 1:5000) antibodies.

Measurement of LRP1 Stability

After Exposure to AgLDL

To measure protein stability, we used cycloheximide, a translation inhibitor in eukaryotic cells. Human VSMC were preexposed to agLDL (100 μg/mL) for 18 hours before addition of cycloheximide (Sigma, 100 μmol/L). Cells were harvested at various time points after cycloheximide treatment (6, 12, 24, and 32 hours) and collected in lysis buffer, and protein processed for Western blot analysis. LRP1 protein stability was assessed as the proportion of the initial protein remaining after cycloheximide treatment.

Pulse-Chase and Immunoprecipitation of LRP1

Human VSMC were incubated with DMEM (Gibco) without methionine and cysteine for 2 hours, and then pulsed with 250 μCi of L-[35S]-methionine (PerkinElmer Life Sciences) for 30 minutes at 37°C. After 2 hours of chase in complete medium containing 10-fold increased concentration of methionine, cells were washed with methionine-free medium and harvested at the indicated times in lysis buffer. The [35S]-labeled LRP1 was immunoprecipitated, as previously described, and separated by electrophoresis on SDS-polyacrylamide gels. Gels were fixed with 25% methanol and 7.5% acetic acid, dried, and bands visualized by autoradiography.

Statistical Analysis

Results were expressed as mean±SEM, and the number of experiments is shown in every case. Statistical differences between control and treated groups were analyzed by the non-parametric Mann-Whitney test for paired data. A probability value of ≤0.05 was considered significant.

Results

AgLDL Decrease LRP1 Ubiquitinylation in Coronary Human VSMC

On the basis that LRP1 is degraded by the proteasomal system,16 we investigated whether agLDL could modulate LRP1 ubiquitinylation. As shown in Figure 1A, ubiquitinylated LRP1 protein levels were strongly reduced in VSMC exposed to agLDL for 20 hours (agLDL 50 μg/mL: 6.87±0.32 a.u. and agLDL 100 μg/mL: 3.70±0.44 a.u. versus control: 9.6±0.55 a.u.). However, ubiquitinylated LRP1 protein levels were not significantly altered in VSMC exposed to nLDL for 20 hours (agLDL 50 μg/mL- and 100 μg/mL: 20 hours). AgLDL did not show any significant effect on LRP1 ubiquitinylation at 4 or 8 hours of agLDL exposure. As expected, exposure of human VSMC to MG132, a proteasomal inhibitor, also strongly reduced ubiquitinylated LRP1 protein levels in human coronary VSMC. No differences were observed in the low-ubiquitinylated LRP1 protein levels, when mock (-) beads were used (data not shown).

As shown in Figure 1B, agLDL (100 μg/mL) and MG132 (10 μmol/L)-exposed VSMC showed higher LRP1 protein levels in membrane (agLDL: 3.98±0.75 a.u., MG132: 6.29±1.02 a.u. versus control: 2.33±0.32 a.u.) and cytoplasm (agLDL: 4.24±0.26 a.u., MG132: 7.91±0.54 a.u. versus control: 2.26±0.33 a.u.). Confocal microscopy images (Figure 1C) showed areas of colocalization (in yellow) of LRP1 (in green) and ubiquitin (in red) in control and nLDL-exposed, but not in agLDL-exposed, VSMC.
AgLDL Increases LRP1 Stability and Decreases LRP1 Turnover Rate in Coronary Human VSMC

To analyze the effect of agLDL on protein stability, human VSMC were unexposed (control) or preexposed to nLDL or agLDL followed by addition of cycloheximide to block translation. In agreement with previous results, LRP1 protein levels remained constant during 12 hours of cycloheximide treatment, and decayed to 30±4.4% and to 36±3.2% in control and VSMC exposed to nLDL for 32 hours. In contrast, LRP1 protein expression slightly decayed to 80±1.8% in agLDL-exposed VSMC (Figure 2A). These findings demonstrate that agLDL increases the half life of LRP1 protein in human coronary VSMC.

AgLDL Increase Turnover Rate in Coronary Human VSMC

To further analyze the effect of agLDL on LRP1 protein stability, we analyzed LRP1 turnover by pulse-chase experiments. VSMC were metabolically labeled for 2 hours with [35S]-labeled methionine, followed by a chase period in the absence or presence of nLDL and agLDL (100 μg/mL). Cell lysates were then immunoprecipitated with anti-LRP1 antibodies, and the amount of radiolabeled-LRP1 estimated by autoradiography. As shown in Figure 2B, LRP1 protein levels declined more in control and nLDL than in agLDL-exposed VSMC at 16- and 32-hour chase.

AgLDL Induce Modifications in the Ubiquitin-Proteasome System of Human Coronary VSMC

Differential proteomic analysis was used to identify changes in the protein signature of agLDL-treated VSMCs. A significant change induced by agLDL in the ubiquitin-proteasome system appears to be in the proteomic profile of a RING type E3 ubiquitin ligase, CHFR protein (red circle; Figure 3, B versus A). This alteration in CHFR protein by agLDL was not observed in LRP1-deficient VSMC (Figure 3, D versus C). Surprisingly, the spot identified as CHFR had an observed molecular weight of 28.5 kDa instead of the expected 73 kDa. The difference in molecular weights, a result of degradation or cleavage, suggested that the observed CHFR spot was not the intact and active
form of the protein, but that the protein was significantly changed.

Real-time PCR results demonstrated that agLDL (100 μg/mL) significantly reduced CHFR mRNA at 8 and 20 hours of exposure in human VSMC (Figure IA in the online-only Data Supplement), but not in human monocyte-derived macrophages (Figure IB in the online-only Data Supplement). The nLDL did not exert any significant effect on CHFR mRNA expression either in VSMC or macrophages. As shown in Figure 4A, Western blot analysis demonstrated that agLDL, but not nLDL, decreased CHFR protein expression to 33% after 20 hours of exposure. As expected,8–10 LRP1 protein expression was upregulated by approximately 2.5-fold in human VSMC exposed to agLDL (100 μg/mL, 20 hours). AgLDL did not exert any significant effect on VSMC-CHFR or LRP1 protein levels at 4 or 8 hours of exposure.

LRP1 Is Required for the Effect of AgLDL on CHFR Protein Expression

Previous studies from our group have consistently demonstrated that LRP1 mediates agLDL binding and internalization in human VSMC.6,7 To evidence the role of LRP1 on the CHFR supression by agLDL, we analyzed the effects of nLDL and agLDL on CHFR protein expression in siRNA-LRP1-treated VSMC. As shown in Figure 4B, silencing LRP1 mRNA showed a strong efficacy to inhibit LRP1 protein expression in control, nLDL-, or agLDL-exposed VSMC (Figure 4B, left). In siRNA-LRP1-treated VSMC, agLDL was unable to reduce CHFR protein expression (Figure 4B, right).

AgLDL Stabilizes LRP1 by Decreasing CHFR Binding to LRP1 β-Chain in Human Coronary VSMC

To know whether CHFR physically interacts with LRP1, we performed immunoprecipitation experiments with anti-LRP1 β-chain antibodies. LRP1 immunoprecipitates from control, nLDL (100 μg/mL), agLDL (100 μg/mL), and MG132 (10 µmol/L)-exposed VSMC were analyzed by Western blot and immunoblotted with anti-CHFR antibodies. As shown in Figure 5, agLDL and MG132 strongly reduced the amount...
of CHFR bound to LRPI cytoplasmic chain. In addition, to confirm the presence of CHFR peptides in the LRPI immunoprecipitate, bands that were positive for CHFR from control VSMC were digested and subjected to mass spectrometry analysis. Figure II in the online-only Data Supplement shows the spectrum of a band identified as CHFR, the peaks corresponding to CHFR peptides, and their position in the protein sequence. These results evidence that CHFR is present in the LRPI immunoprecipitate, and that therefore CHFR interacts with LRPI cytoplasmic chain.

To further explore the role of CHFR on the regulation of LRPI protein levels, we analyzed the effect of siRNA-CHFR on LRPI protein levels in human VSMC. Western blot showed that CHFR protein levels were reduced to 56% by agLDL, to 39% by siRNA-CHFR treatment, and to 27% by both treatments (Figure 6A and 6B). As previously reported by our group, LRPI protein expression was upregulated by agLDL.8–10 Interestingly, LRPI protein expression was also upregulated by siRNA-CHFR (1.90-fold), and synergically by both agLDL and siRNA-CHFR treatments (by 2.26-fold; Figure 6A and 6D). As expected, agLDL strongly reduced LDLR protein expression levels. However, siRNA-CHFR did not exert any significant effect on LDLR protein expression (Figure 6A and 6D), suggesting a specific effect of CHFR on LRPI protein levels. The lack of effect of siRNA-CHFR on macrophage LRPI protein levels (Figure III in the online-only Data Supplement) suggests that CHFR specifically modulates LRPI protein levels in human VSMC.

Discussion

The main findings of the present study are that (1) agLDL decreases CHFR protein levels, (2) CHFR modulates LRPI ubiquitinylation, and (3) agLDL stabilizes LRPI protein by decreasing CHFR binding to LRPI. This mechanism (summarized in Figure 6E) seems to work in human VSMC, but not in human macrophages.
LRP1 ubiquitinylation in different cell types. LRP1 ligands may control LRP1 degradation by regulating ubiquitin-proteasomal degradation, and on LRP1 stability. Prompted us to analyze the effect of agLDL on LRP1 in HepG2 cells and J774 macrophages. These results suggested that agLDL, by reducing CHFR levels, decreases the ubiquitinylation of LRP1-β-chain and induces the stabilization of LRP1 protein levels in human coronary VSMC (summarized in Figure 6E). Melman et al already demonstrated that CHFR silencing specifically increases LRP1 protein expression in human VSMC. Taken together, these results demonstrate that CHFR silencing in macrophages. The specific contribution of VSMC-LRP1 expression to atherogenesis has been studied in a mice model lacking smooth muscle LRP1. Loss of vascular LRP1 has been shown to result in greater VSMC proliferation, deficient contractile protein expression, impairment of vascular contractility, and promotion of denudation-induced neointimal hyperplasia. Deletion of macrophage LRP1 also increased atherogenesis in fat-fed LDLR-deficient mice, revealing a role for LRP1 in monocyte recruitment, regulation of inflammatory responses, and MMP activity. These effects can be, at least in part, attributed to the pivotal role of LRP1 in signal transduction. In summary, results from the present study show that agLDL, one of the main LDL modifications in the arterial intima, prolongs the half life of LRP1 by preventing LRP1 ubiquitinylation through CHFR targeting.
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Disclosures

None.

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Material and Methods

VSMC isolation and culture

Primary cultures of human VSMCs were obtained from the media layer of macroscopically healthy coronary artery segments collected from patients undergoing cardiac transplantation at Hospital de la Santa Creu i Sant Pau, Barcelona. VSMC were isolated by a modification of the explant technique as previously described. The explants were incubated at 37°C in a humidified atmosphere of 5% CO₂. After 1 week, the cells have started to migrate from the explants and have proliferated covering the floor of the culture well. The medium was exchanged every 3 days after the onset of cell outgrowth. A significant outgrowth was reached after 10 days. Tissue fragments were collected with forceps and placed in a new dish with fresh medium. The cells that remain in the dish were cultured until confluence. For cell characterization, cells were seeded in coverslips and grown to confluence. Cells used in the present experiments were between the fourth and sixth passage. VSMC at these passages appeared as a relatively homogeneous cell population, showing a hill-and-valley pattern at confluence. Western blot analysis for specific differentiation markers revealed a clear positive band for α-actin (45 kDa) and calponin (33 kDa). Cell monolayers were grown in medium M199 (GIBCO) supplemented with 20% fetal bovine serum (FBS) and 2% human serum, 2 mmol/L L-glutamine, 100 U/mL penicillin G, and 100 µg/mL streptomycin. The study was approved by the institutional ethics committee at Hospital of Santa Creu i Sant Pau and conducted in accordance with the Declaration of Helsinki.

Small-interfering RNA (siRNA) gene silencing in human VSMC

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**Isolation and differentiation of human monocyte-derived macrophages (HMDM)**

Human monocyte-derived macrophages (HMDM) were isolated by standard protocols from buffy coats (35–40 mL) of healthy donors. The study was approved by the Reviewer Institutional Committee on Human Research of the Hospital of Santa Creu i Sant Pau that conforms to the Declaration of Helsinki. Cells were applied on 15 mL of Ficoll–Hypaque and centrifuged at 400\times g, 40 min, 22°C with no brake. Mononuclear cells were obtained from the central white band of the gradient, exhaustively washed in PBS, and resuspended in RPMI medium supplemented with 10% human AB serum, 1% P/S, 1% Hepes. Cells were allowed to differentiate into macrophages by exposure to 10% human AB serum for 7 days changing medium every other day. HMDM were arrested, incubated in absence or presence of native (nLDL) or agLDL (100 µg/mL) for the indicated times, washed and collected to analyze CHFR mRNA expression.
Small-Interfering RNA (siRNA) gene silencing of CHFR in human macrophages

In brief, HMDM were transfected with siRNA-random (as a control) and siRNA-CHFR (siRNA ID s31393) (200 nmol/L) using Hiperfect (Qiagen) in serum-free DMEM medium (1% glutamine) according to the manufacturer’s instructions. This medium with siRNA-CHFR was maintained for 18 hours and it was then replaced by a new medium containing native LDL (nLDL) and aggregated LDL (agLDL) (100 μg/mL). After 18h, cells were exhaustively washed and harvested to test LRP1 and CHFR protein expression. The cells did not take up trypan blue and their morphology was not altered by the procedure.

LDL isolation, modification and characterization

Human LDL (d_{1.019-d_{1.063}} g/mL) was obtained from pooled sera of normocholesterolemic volunteers by sequential ultracentrifugation. LDLs were dialyzed against 3 dosages of 200 vol of 150 mmol/L NaCl, 1 mmol/L EDTA, and 20 mmol/L Tris-HCl, pH 7.4, overnight and once against 150 mmol/L NaCl. LDL protein concentration was determined by the bicinchoninic acid (BCA) method, and cholesterol concentration was determined by a commercial kit (Boehringer). The average total cholesterol content of human LDL was ≈2 mg/mg LDL protein. LDLs used in the experiments were <48 hours old. The purity of LDLs was assessed by agarose gel electrophoresis (Paragon System, Beckmann). TBARS were measured as an indirect evaluation of lipid peroxidation and were <1.2 mmol malonaldehyde per milligram of LDL protein. AgLDL was prepared by vortexing LDL in PBS at room temperature. The formation of LDL aggregates by vortexing was monitored by measuring the turbidimetry (absorbance at 680 nm) as previously described. The percentage of LDL in aggregated form was calculated by measuring the fraction of protein recovered in the pellet obtained after centrifugation at 10,000g for 10 minutes. The different fractions were analyzed by agarose electrophoresis (Paragon system, Beckmann). No significant alterations of TBARS levels against nLDL were detected after LDL aggregation.
Protein extraction and proteomic analysis

Protein extracts

Samples were homogenized in Tris-base buffer (40 mmol/L Tris-base), incubated for 15 min at room temperature and centrifuged at 16000xg for 20 min. Protein pellets were further extracted with a urea/chaps buffer (7 mol/L urea; 2 mol/L thiourea; 4% CHAPS; and 40 mmol/L Tris-base) for 15 min at room temperature, as described above. Protein concentration in the extracts was measured with 2D-Quant Kit (GE-HealthCare).

Two-dimensional gel electrophoresis

For analytical and preparative gels, respectively, a protein load of 120 and 300 µg protein of the urea/chaps soluble extracts was applied to 17 cm dry strips (pH 3–10 linear range, Bio-Rad). Gels were developed by fluorescent staining (Flamingo, BioRad). For each independent experiment, two-dimensional gel electrophoresis (2-DE) for protein extracts from the control, native LDL (nLDL) and agLDL groups were processed in parallel to guarantee a maximum of comparability. Each 2-DE run was at least repeated twice. Analysis for differences in protein patterns was performed with the PD-Quest software (Bio-Rad), using a single master that included all gels of each independent experiment. Each spot was assigned a relative value that corresponded to the single spot volume compared with the volume of all spots in the gel, following background extraction and normalization between gels.

Mass spectrometry analysis

Proteins were identified after in-gel tryptic digestion and extraction of peptides from the gels pieces, as described, by matrix – assisted laser desorption/ionization time-of-flight (MALDI-TOF) using an AutoFlex III Smartbeam MALDI-ToF/ToF (Bruker Daltonics). Samples were applied to
Prespotted AnchorChip plates (Bruker Daltonics) surrounding the calibrants provided on the plates. Spectra were acquired with flexControl on reflector mode, (mass range 850 - 4000 m/z, reflector 1: 21.06 kV; reflector 2: 9.77kV; ion source 1 voltage: 19 kV; ion source 2: 16.5kV; detection gain 2.37x) with an average of 3500 added shots at a frequency of 200 Hz. Each sample was processed with flexAnalysis (Version 3.0, Bruker Daltonics) considering a signal-to-noise ratio over 3 and applying statistical calibration. For identification, peaks between 850 to 1000 were not considered as in general only matrix peaks are visible on this mass range. After processing, spectra were sent to the interface BioTools (Bruker Daltonics, version 3.2) and, with no further modifications, MASCOT search on Swiss-Prot 57.15 database was done (Taxonomy: Homo Sapiens, Mass Tolerance 50 to 100, up to 2 miss cleavage, Global Modification: Carbamidomethyl (C), Variable Modification: Oxidation (M)). Identification was accepted with a score higher than 56.

**Real time PCR**

Total RNA was isolated by TriPure™ isolation Reagent (Roche) according to the manufacturer’s instructions. Gene expression of LRP1 and CHFR mRNA was assessed by real time PCR using the assays on demand Hs00233899_m1 and Hs00943495_m1 (Applied Biosystems) respectively. Human GAPDH (4326317E) (Applied Biosystems) was used as endogenous control. Taqman real-time PCR was performed with 1 µL/well of RT products (1 µg total RNA) in 10 µL of TaqMan PCR Master Mix (PE Biosystem) with the primers at 300 nmol/L and the probe at 200 nmol/L. PCR was performed at 95°C for 10 minutes (for AmpliTaq Gold activation) and then run for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The threshold cycle (Ct) values were determined and normalized to endogenous control.

**Western blot analysis**
Total protein was isolated by TriPure™ isolation Reagent (Roche) according to the manufacturer’s instructions. Proteins were analyzed by Western blot as previously described. Blots were incubated with monoclonal antibodies against LRP1 (Epitomics, 2703-1, dilution 1:7000) or CHFR (Cell Signaling, 4297, dilution 1:1000). Low density lipoprotein receptor (LDLR) was analysed using anti-LDLR (Epitomics, 1956-1, dilution 1:500). Equal protein loading for the different samples was ensured by Ponceau staining of the blots and by performing in parallel β-tubulin (Abcam ab6046, dilution 1:500) blot. In some experiments, cell monolayers were washed with PBS and lysed with Subcellular Proteome Extraction kit (Calbiochem) to obtain membrane and cytoplasmic protein. The protein fractions were cleaned using clean-up kit (Amersham) according to manufacturer's instructions. Proteins were analyzed by Western blot as previously described.

**Immunoprecipitation of total LRP1 protein**

50 µL of Dynabeads (Novex, Life technologies) were incubated with anti-LRP1 antibodies (Epitomics, 2703-1, dilution 1:50) for 10 minutes at RT in an orbital shaker. Cellular protein extracts were incubated with the Dynabeads-LRP1 antibody complex for 30 minutes at RT in an orbital shaker. The Dyanbeads- Antibody-Antigen (LRP1 protein) complex was then washing four times until the elution of target protein. Samples were separated by SDS-PAGE and transferred blots were incubated with Ab against LRP1 (Epitomics, 2703-1, dilution 1:7000). In parallel, immunoprecipitated samples were run in a polyacrilamide gel, stained with Coomasie, the obtained bands were cut from the gel and subjected to trypsin digestion and mass spectrometry analysis.

**Immunoprecipitation of ubiquitinylated LRP1 protein**
Polyubiquitinylated proteins were immunoprecipitated by using the Ubiquitinylated Protein Enrichment Kit (Calbiochem 662200). In brief, one aliquot of protein extracts from VSMC unexposed (control) or exposed to nLDL or agLDL (50 and 100 µg/mL) for 4, 8 and 20 hours were applied to polyubiquitinylated-affinity beads and other aliquot to control beads. After immunoprecipitation, samples were examined by Western blot using anti-LRP1 (Epitomics, 2703-1, dilution 1:7000) and anti-Ubiquitin (Calbiochem, 662099, dilution 1:5000) antibodies.

**Measurement of LRP1 protein stability after exposure to agLDL**

To measure protein stability we used cycloheximide, a translation inhibitor in eukaryotic cells. Human VSMC were pre-exposed to agLDL (100 µg/mL) for 18 hours before addition of cycloheximide (Sigma, 100 µmol/L). Cells were harvested at various time points after cycloheximide treatment (6, 12, 24 and 32 hours) and collected in lysis buffer and protein processed for western blot analysis. LRP1 protein stability was assessed as the proportion of the initial protein remaining after cycloheximide treatment.

**Pulse-chase and immunoprecipitation of LRP1**

Human VSMC were incubated with DMEM (Gibco) without methionine and cysteine for 2 hours and then pulsed with 250 µCi of L-[³⁵S]-methionine (PerkinElmer Life Sciences) for 30 min at 37°C. After 2 h of chase in complete medium containing 10-fold increased concentration of methionine, cells were washed with methionine-free medium and harvested at the indicated times in lysis buffer. The [³⁵S]-labeled LRP1 was immunoprecipitated as previously described and separated by electrophoresis on SDS-polyacrylamide gels. Gels were fixed with 25% methanol and 7.5% acetate, dried and bands visualized by autoradiography.

**Statistical analysis**
Results were expressed as mean±SEM and the number of experiments is shown in every case. Statistical differences between control and treated groups were analyzed by the nonparametric Mann-Whitney test for paired data. A probability value of 0.05 or less was considered significant.

References


Online Figure I. Effect of nLDL and agLDL on CHFR mRNA expression in human VSMC and HMDM. Quiescent human VSMC (A) or HMDM (B) were exposed to nLDL or agLDL (100 µg/mL) for increasing times (4, 8 and 20 hours) and harvested to test CHFR mRNA expression. Cells not exposed to LDL were also collected at each time point (controls). B) Bar graphs show real-time PCR quantification of CHFR mRNA expression. Data were processed with a specially designed software program based on Ct values of each sample and normalized to gapdh in VSMCs and to 18s rRNA in HMDM. Results are expressed as mean±SEM of two experiments performed in triplicate. *P<0.05 vs control cells.
Online Figure II

(A) Spectrum of trypsin in-gel digestion of CHFR positive band from LRP1 immunoprecipitate. Peaks corresponding to the m/z of CHFR peptides present in the samples are indicated (*).

(B) List of observed, expected and calculated m/z of the peptides and their suggested sequence.

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</table>

(C) Sequence of CHFR (CHFR_HUMAN (Q96EP1) - Uniprot/SwissProt). The sequences of the observed peptides are shown in red.

Online Figure II. A) Spectrum of trypsin in-gel digestion of CHFR positive band from LRP1 immunoprecipitate. Peaks corresponding to the m/z of CHFR peptides present in the samples are indicated (*). B) List of observed, expected and calculated m/z of the peptides and their suggested sequence. C) Sequence of CHFR (CHFR_HUMAN (Q96EP1) - Uniprot/SwissProt). The sequences of the observed peptides are shown in red.
Online Figure III. Small anti-CHFR-interfering RNA (siRNA-CHFR) did not exert any significant effect on LRP1 protein expression in HMDM. HMDM were treated with siRNA-random or siRNA-CHFR and then exposed to agLDL (100 µg/mL) as detailed in Methods. Representative Western blot shows CHFR, LRP1 and β-tubulin protein bands.