Aggregated Low-Density Lipoprotein Induces LRP1 Stabilization Through E3 Ubiquitin Ligase CHFR Downregulation in Human Vascular Smooth Muscle Cells

Roi Cal, Maisa García-Arguinzonis, Elena Revuelta-López, José Castellano, Teresa Padró, Lina Badimon, Vicenta Llorente-Cortés

Objective—Low density lipoprotein retention and aggregation in the arterial intima are key processes in atherogenesis. Aggregated LDL (agLDL) is taken up through low-density lipoprotein receptor-related protein 1 (LRP1) by human vascular smooth muscle cells (VSMC). AgLDL increases LRP1 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 LRP1 receptor turnover. The objective of this study was to analyze the effect of agLDL on the degradation of LRP1 by the ubiquitin-proteasome system in human VSMC.

Methods and Results—Human VSMC were isolated from the media of human coronary arteries. Ubiquitinylated LRP1 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 LRP1 protein half life. Pulse-chase analysis showed that LRP1 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, LRP1 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 LRP1 decreased in the presence of agLDL.

Conclusion—Our results demonstrate that agLDL prolongs the half life of LRP1 by preventing the receptor ubiquitylation, at least in part, through CHFR targeting. This mechanism seems to be specific for LRP1 and VSMC. (Arterioscler Thromb Vasc Biol. 2013;33:369-377.)

Key Words: aggregated LDL ■ CHFR ■ LRP1 ■ 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 diverse 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 LRP1 allows high-intracellular CE accumulation not only because of its high capacity to bind and internalize agLDL, but also because LRP1 is transcriptionally upregulated by intracellular cholesterol. Our group reported that LRP1 is upregulated at transcriptional level by hypercholesterolemia through sterol regulatory element-binding proteins (SREBP) downregulation in human VSMC,5–7 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 LRP1 in the plasma membrane without influencing mRNA expression levels.14,15 LRP1 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

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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.

\textbf{Materials and Methods}

\textbf{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{4–8} The explants of the excised coronary arteries 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 surface 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 \(\alpha\)-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 mmol/L L-glutamine, 100 U/mL penicillin G, and 100 \(\mu\)g/mL streptomycin. The study was approved by the institutional ethics committee at Hospital de Santa Creu i Sant Pau and conducted in accordance with the Declaration of Helsinki.

\textbf{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{15} siRNA-LRP1 was a 15-mer (5’-CGCGGGGTCGACAGAT-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 \(\mu\)mol/L. After 48 hours of transfection, cells were exposed to agLDL (100 \(\mu\)g/mL) for the tested time points and harvested by scraping in TriPure Isolation Reagent (Roche Molecular Diagnostics) for PCR and Western blot analysis.

\textbf{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.

\textbf{LDL Isolation, Modification, and Characterization}

Human LDL (\(d_{1.019–1.063} \leq 0.8 \text{mg/mL})\) was obtained from pooled sera of normocholesterolemic volunteers by sequential ultracentrifugation. LDLS were dialyzed against 3 dosages of 200 \(\mu\)l 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 method, and cholesterol concentration was determined by a commercial kit (Boehringer). The average total cholesterol content of human LDL was \(\approx 2 \text{mg/mL}\) LDL protein. LDLS used in the experiments were <48 hours old. The purity of LDLS was assessed by agarose gel electrophoresis (Paragon System, Beckmann). Thiobarbituric acid reactive substances 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 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{17} Different fractions were analyzed by agarose electrophoresis (Paragon system, Beckmann). No significant alterations of thiobarbituric acid reactive substances levels against nLDL were detected after LDL aggregation.

\textbf{Protein Extraction and Proteomic Analysis}

\textbf{Protein Extracts}

Samples were homogenized in Tris-base buffer (40 mmol/L Tris-base), incubated for 15 minutes at room temperature, and centrifuged at 16 000g 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 mmol/L Tris-base) for 15 minutes at room temperature, as described above. Protein concentration in the extracts was measured with 2D-Quant Kit (GE-HealthCare).

\textbf{Two-Dimensional Gel Electrophoresis}

For analytical and preparative gels, respectively, a protein load of 120 and 300 \(\mu\)g 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.

\textbf{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: 9.77kV; 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 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 TRIzol 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 reverse transcription 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 activa-
tion), 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 TRIzol isolation Reagent (Roche),
according to the manufacturer’s instructions. Proteins were ana-
lyzed by Western blot, as previously described.8–10 Blots were incu-
bated with monoclonal antibodies against LRP1 (Epitomics, 2703-1,
dilution 1:7000) or CHFR (Cell Signaling, 4297, dilution 1:1000).
LRP1 was analyzed using anti-β-Actin (Santa Cruz Biotechnology,
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
fractions. 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 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 Dulbecco’s modified eagle medium
(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 immuno-
precipitated, as previously described, and separated by electrophore-
sis 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 experi-
ments 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 was con-
sidered 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 modu-
late 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: 9.68±0.55 a.u.). However, ubiquitinylated
LRP1 protein levels were not significantly altered in VSMC
exposed to nLDL (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 stronger 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 con-
trol: 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 LRPI Stability and Decreases LRPI 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, LRPI 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, LRPI protein expression slightly decayed to 80±1.8% in agLDL-exposed VSMC (Figure 2A). These findings demonstrate that agLDL increases the half life of LRPI protein in human coronary VSMC.

To further know the effect of agLDL on LRPI protein stability, we analyzed LRPI 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-LRPI antibodies, and the amount of radiolabeled-LRPI estimated by autoradiography. As shown in Figure 2B, LRPI 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 Stabilizes LRP1 by Decreasing CHFR Binding to LRP1 β-Chain

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

Figure 2. Aggregation low-density lipoprotein (agLDL) increases LDL receptor-related protein 1 (LRP1) protein stability and decreases LRP1 protein turnover. A, AgLDL increases LRP1 protein stability. Vascular smooth muscle cells (VSMC) were unexposed (control) or preexposed to native LDL (nLDL) or agLDL (100 μg/mL, 24 hours), followed by incubation with cycloheximide (100 μmol/L) for the indicated times. Unchanged levels of β-tubulin are shown as loading control. Line graphs show the percentage of the value before the addition of cycloheximide. Results are expressed as mean±SEM of 2 independent experiments performed in triplicate. *P<0.05 vs control cells. B, AgLDL decreases LRP1 protein turnover. Human VSMC were pulse-labeled with 250 μCi of [35S]methionine for 2 hours in the absence of LDL and chased for the indicated times in absence or presence of nLDL or agLDL (100 μg/mL). Cells were then lysated with lysis buffer and immunoprecipitated with an anti-LRP1 antibody. The immunoprecipitates were run in sodium dodecyl sulfate polyacrylamide gel electrophoresis and detected by autoradiography.

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 LRP1 cytoplasmic chain. In addition, to confirm the presence of CHFR peptides in the LRP1 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 LRP1 immunoprecipitate, and that therefore CHFR interacts with LRP1 cytoplasmic chain.

To further explore the role of CHFR on the regulation of LRP1 protein levels, we analyzed the effect of siRNA-CHFR on LRP1 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, LRP1 protein expression was upregulated by siRNA-CHFR (1.90-fold), and synergically by both agLDL and siRNA-CHFR treatments (by 2.26-fold; Figure 6A and 6C). 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 LRP1 protein levels. The lack of effect of siRNA-CHFR on macrophage LRP1 protein levels (Figure III in the online-only Data Supplement) suggests that CHFR specifically modulates LRP1 protein levels in human VSMC.

Discussion

The main findings of the present study are that (1) agLDL decreases CHFR protein levels, (2) CHFR modulates LRP1 ubiquitinylation, and (3) agLDL stabilizes LRP1 protein by decreasing CHFR binding to LRP1. This mechanism (summarized in Figure 6E) seems to work in human VSMC, but not in human macrophages.
We found that LRPI has extremely long half life (approximately 24 hours) in human VSMC compared with other cell types, such as HepG2 (3.5 hours) or U87 (higher than 8 hours). Our results demonstrate that the half life of VSMC-LRPI is even larger in the presence of agLDL. Moreover, pulse-chase experiments showed that agLDL reduces LRPI protein turnover in human VSMC. These results evidence that agLDL slows down LRPI degradation and turnover.

By means of 2-dimensional gel electrophoresis analysis, we could identify CHFR as the main protein of the ubiquitin-proteasome system altered by exposure of human coronary VSMC to agLDL. The spot identified as CHFR was upregulated in agLDL-treated cells. However, the molecular weight was considerably smaller than expected, suggesting that the identified spot was not the intact protein form, and, in consequence, the total expression and the activity of CHFR could be impaired. Real-time PCR and Western blot analysis showed that agLDL decreases both CHFR mRNA and protein levels in control, but not in LRPI-deficient VSMC. Immunoprecipitation assays combined with mass spectrometry and Western blot analysis showed that CHFR binds LRPI β-chain and that LRPI-CHFR binding is significantly lower in agLDL-exposed VSMC. Moreover, CHFR silencing specifically increases LRPI protein expression in human VSMC. Taken together, these results demonstrate that agLDL, by reducing CHFR levels, decreases the ubiquitinylation of LRPI β-chain and induces the stabilization of LRPI protein levels in human coronary VSMC (summarized in Figure 6E). Melman et al already demonstrated that a short region (residues 60–78) of the cytoplasmic chain is required for LRPI proteosomal degradation, although these authors could not demonstrate the direct ubiquitinylation of the cytoplasmic tail. The inhibitory effect of agLDL on CHFR expression may have consequences for several cellular processes, as CHFR modulates the activity of certain transcription factors such as HLFT26 or NF-Kα. In the present study, we showed that the effect of CHFR is selective for LRPI, as the protein levels of other receptors from the same family such as LDLR were not influenced by CHFR silencing. The effect of CHFR on LRPI is also cell type-specific, as LRPI protein levels were not affected by CHFR silencing in macrophages.

The specific contribution of VSMC-LRPI expression to atherogenesis has been studied in a mice model lacking smooth muscle LRPI. Loss of vascular LRPI 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 LRPI also increased atherogenesis in fat-fed LDLR-deficient mice, revealing a role for LRPI in monocyte recruitment, regulation of inflammatory responses, and matrix metalloproteinases activity. These effects can be, at least in part, attributed to the pivotal role of LRPI 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 LRPI by preventing LRPI 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.\(^1\)\(^-\)\(^4\) The explants were incubated at 37°C in a humidified atmosphere of 5% CO\(_2\). 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 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

To inhibit LRP1 expression in human VSMC, cells were transiently transfected with annealed siRNA (siRNA-LRP1) synthesized by Ambion according to our previously published
LRP1 target sequences. siRNA-LRP1 was a 15-mer (5'-CGCGCGGTCAGCAT-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 (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×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.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.2-4 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-[\textsuperscript{35}S]-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 [\textsuperscript{35}S]-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 18srRNA 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. **C)** Sequence of CHFR (CHFR_HUMAN (Q96EP1) - Uniprot/SwissProt). The sequences of the observed peptides are shown in red.

### Table B

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<th>Mr (calc)</th>
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### Table C

1. HREDPEKQFS PDQPNQGSL DLLGASEEGPH VLLREBERTI G3RRCIMLSF
2. FGSLVQDSG CRYYTVSDE GQYALEPTSF GIVYHRLNV HKRTCLPQDG
3. 151 SVIILYRHG EESDVAVLY ESLEQRENT QSGREANDN VPLLQVFTSG
4. AGAEGQGDRV VPDSSFQEATQV CFSAQFTSTS TSDLPTPTSA SADSTPEAFSR
5. 301 RSQSCSOSR SG13PRHSVF SVADSEVSY TSALPFRTA SSSILEPQQQ
6. GLREKPDQGQH QSOGQLESGQ QHCLQGSRQH AQTVQMOVHA AQKPGGDQK
7. 301 LTICQICQDL LHCVSDQCQC HSICTACGYS NHGERSLCT FGCPVHCRIC
8. 351 NRSILNLTVAYLIQHFDK RSDDAQGMD RKHRTPQUML QOPVRPHFSD
9. KESQSHNEE QGQGQGQGQ QGQGQGQGQ QGQGQGQGQ QGQGQGQGQ QGQGQGQGQ QGQGQGQGQ
10. 451 RG9PGQAAQG PSTVSTGQ VQQTVIPQHQ QMALCTQCT PKRFAQRE
11. 501 GQDVADQGQ AHQGQGQGQ QGQGQGQGQ QGQGQGQGQ QGQGQGQGQ QGQGQGQGQ
12. QSSQSDQGQ QGQGQGQGQ QGQGQGQGQ QGQGQGQGQ QGQGQGQGQ QGQGQGQGQ
13. 601 QYCOIXSFR ACTTYQRYQI PASAEIPVAVT SPACQGSRBH QDIQVHADIA
14. 651 KHNHTCQDQ RPAV
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.