Adipocyte differentiation-related protein is induced by LRP1-mediated aggregated LDL internalization in human vascular smooth muscle cells and macrophages

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Abstract **Aggregated LDL (agLDL) is internalized by LDL receptor-related protein (LRP1) in vascular smooth muscle cells (VSMCs) and human monocyte-derived macrophages (HMDMs). AgLDL is, therefore, a potent inducer of massive intracellular cholesteryl ester accumulation in lipid droplets. The adipocyte differentiation-related protein (ADRP) has been found on the surface of lipid droplets. The objectives of this work were to analyze whether agLDL uptake modulates ADRP expression levels and whether the effect of agLDL internalization on ADRP expression depends on LRP1 in human VSMCs and HMDMs. AgLDL strongly upregulates ADRP mRNA (real-time PCR) and protein expression (Western blot) in human VSMCs (mRNA: by 3.06-fold; protein: 8.58-fold) and HMDMs (mRNA: by 3.5-fold; protein: by 3.71-fold). Treatment of VSMCs and HMDMs with small anti-LRP1-interfering RNA (siRNA-LRP1) leads to specific inhibition of LRP1 expression. siRNA-LRP1 treatment significantly reduced agLDL-induced ADRP overexpression in HMDMs (by 69%) and in VSMCs (by 53%). Immunohystochemical studies evidence a colocolocalization between ADRP/macrophages and ADRP/VSMCs in advanced lipid-enriched atherosclerotic plaques. These results demonstrate that agLDL-LRP1 engagement induces ADRP overexpression in both HMDMs and human VSMCs and that ADRP is highly expressed in advanced lipid-enriched human atherosclerotic plaques. Therefore, LRP1-mediated agLDL uptake might play a pivotal role in vascular foam cell formation.**—Llorente-Cortés, V., T. Royo, O. Juan-Babot, and L. Badimon. **Adipocyte differentiation-related protein is induced by LRP1-mediated aggregated LDL internalization in human vascular smooth muscle cells and macrophages.** ***J. Lipid Res.*** **2007.** 48: **2133–2140.**

Supplementary key words adipocyte differentiation-related protein • aggregated LDL • LDL receptor-related protein • adipophilin • human vascular smooth muscle cells • macrophages

One of the main events in the atherogenic process is the accumulation of lipids, mainly cholesteryl esters (CEs) from LDL, in the subendothelial space of the vascular wall (1, 2). In the extracellular intima, extracellular matrix proteoglycans favor LDL retention and aggregation (3, 4). LDL aggregates have been isolated from the arterial intima (5). Unlike native LDL (nLDL), aggregated LDL (agLDL) is a potent inducer of massive intracellular CE accumulation in both macrophages (6–8) and vascular smooth muscle cells (VSMCs) (9–13). Whereas unmodified LDL is taken up by the endocytic LDL receptor (LDLR), which is downregulated by intracellular cholesterol, agLDL is taken up through mechanisms not downregulated by cholesterol. In human VSMCs, we described for the first time that LDL receptor-related protein (LRP1) is the receptor that mediates agLDL-CE-selective uptake (10–13). Some authors have suggested a phagocytic process for agLDL internalization in macrophages (6), whereas others have described a new process named patocytosis (7, 8). We have recently demonstrated that LRP1 also mediates agLDL uptake by macrophages (14), in agreement with previous results showing that LRP1 participates in the uptake of matrix-retained LDL and of LDL degraded by sphyngomyelinase, mainly agLDL (15, 16). LRP1-mediated agLDL uptake can be considered a high-capacity mechanism that allows the uptake of large amounts of ligand, because LRP1, unlike LDLR, has multiple binding sites (17, 18) and it is not downregulated by intracellular cholesterol (19, 20). We have previously demonstrated that large lipid vacuoles filled with CE derived from LRP1-mediated agLDL-selective uptake colocalize with adipose differentiation-related protein (ADRP) in human VSMCs (13). ADRP is a 50 kDa protein encoded by a gene initially isolated from differentiated adipocytes that shares

Abbreviations: ADRP, adipocyte differentiation-related protein; agLDL, aggregated LDL; CE, cholesteryl ester; HMDMs, human monocyte-derived macrophages; LDLR, LDL receptor; LRP1, LDL receptor-related protein; nLDL, native LDL; siRNA-LRP1, small anti-LRP1-interfering RNA; VSMCs, vascular smooth muscle cells.

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sequence similarity with perilipin (21). ADRP expression increases in the early stage of adipose differentiation (22). ADRP selectively increases the uptake of long-chain fatty acids (23), regulates fatty acid mobilization (24), and may contribute to the transfer of lipid between cells (25). ADRP is considered a specific marker of lipid droplet formation, because it is localized on the surface monolayer of the lipid droplet (26) and actively stimulates lipid accumulation and lipid droplet formation (27). ADRP probably plays a role in atherosclerosis, because it has been found to be associated with macrophage-derived foam cells in atherosclerotic lesions (28). However, it is unknown whether agLDL, one of the most important modifications of LDL in the arterial intima, has the ability to modulate ADRP expression in smooth muscle cells and macrophages, the two key cellular sources of foam cells in the vascular wall.The objectives of this study were to analyze whether agLDL uptake might modulate ADRP mRNA and protein expression in both macrophages and human VSMCs and whether agLDL effects on ADRP depend on LRP1. Our results demonstrate that: *1*) there is a colocalization of ADRP with both VSMCs and macrophages in advanced lipid-enriched human atherosclerotic plaque; *2*) agLDL induces ADRP mRNA and protein overexpression in both macrophages and VSMCs; and *3*) agLDL-induced ADRP overexpression depends on LRP1 expression in VSMCs and macrophages. Taken together, these results demonstrate that LRP1-mediated agLDL uptake induces ADRP overexpression in both macrophages and VSMCs. Because ADRP is highly expressed by both macrophages and VSMCs in advanced lipid-enriched atherosclerotic plaques, LRP1-mediated agLDL uptake probably plays a pivotal role in vascular foam cell formation associated with atherosclerotic lesion progression.

MATERIALS AND METHODS

LDL isolation and modification

Human LDLs ($d_{1.019}$–$d_{1.063}$ g/ml) were obtained from pooled sera of normocholesterolemic volunteers, isolated by sequential ultracentrifugation, and dialyzed. LDL protein concentration was determined by the bicinchoninic acid method, and cholesterol concentration was determined using a commercial kit (Boehringer). The purity of LDLs was assessed by agarose gel electrophoresis. The model system of agLDL was generated by vortexing LDL (1 mg/ml) for 4 min at room temperature at maximal speed. AgLDL was then centrifuged at 10,000 *g* for 10 min, and the precipitable fraction composed of 100% agLDL was added to cell cultures (10–12). LDL preparations were <48 h old, nonoxidized (less than 1.2 mmol malonaldehyde/mg protein LDL), and without detectable levels of endotoxin.

Lipid extraction and determination of free and esterified cholesterol

After LDL incubation, cells were exhaustively washed (twice with PBS, twice with PBS/1% BSA, and twice with PBS/1%BSA/heparin 100 U/ml) before harvesting into 1 ml of 0.15 mol/l NaOH. The intracellular lipid content was analyzed by lipid extraction followed by TLC performed as previously described (10–12). The spots corresponding to free cholesterol and CEs were quantified by densitometry against the standard curve of cholesterol and cholesterol palmitate, respectively, with the use of a computing densitometer (Molecular Dynamics).

VSMC culture

Primary cultures of human VSMCs were obtained from nonatherosclerotic areas of human coronaries from explanted hearts at transplant operations at the Hospital de la Santa Creu i Sant Pau, as previously described (10–12). The study was approved by the Reviewer Institutional Committee on Human Research at the Hospital of Santa Creu i Sant Pau that conforms to the Declaration of Helsinki. To analyze the effect of LDL on adipophilin and LRP1 expression, quiescent VSMCs were preincubated in the absence or presence of nLDL or agLDL (100 μg/ml) for increasing time periods.

Isolation and differentiation of human monocyte-derived macrophages

Human monocyte-derived macrophages (HMDMs) were obtained by standard protocols from buffy coats (35–40 ml) from healthy donors. Cells were applied on 15 ml of Ficoll-Hypaque and centrifuged at 400 *g* for 40 min at 22°C, with no brake. Mononuclear cells were obtained from the central white band of the gradient, exhaustively washed in Dulbecco's phosphate buffer saline, and resuspended in RPMI medium (Gibco) supplemented with 20% human serum AB (Immunogenetics). Cells were allowed to differentiate into macrophages by the addition of complete medium with 20% human serum AB and macrophage colony-stimulating factor (M-CSF) (R and D Systems) at 100 ng/ml for 3 days. HMDMs were incubated with nLDL or agLDL (100 μg/ml) and *N*-acetyl-leu-leu-norleucinal (25 μmol/l) for 24 h. HMDMs were then exhaustively washed and collected for either mRNA/protein or lipid extraction.

Treatment of VSMCs and HMDMs with small anti-LRP1-interfering RNA

To inhibit LRP1 expression in VSMCs and HMDMs [small anti-LRP1-interfering RNA (siRNA-LRP1)-VSMCs or siRNA-LRP1-HMDMs], human cells were transiently transfected with annealed siRNA. LRP1-specific siRNA was synthesized by Ambion according to our previously published LRP1 target sequences (10–12). Sense and random oligodeoxynucleotides did not exert any effect on LRP1 expression (10). Fasta analysis (Genetic Computer Group Package) indicated that these sequences would not hybridize to other receptor sequences (including LDLR) in the GenBank database. In agreement, siRNA-LRP1 treatment did not alter LDLR expression (13) or nLDL uptake by vascular cells (10). In brief, VSMCs and HMDMs were transfected with siRNA-LRP1 (50 nM) using siPORT NeoFx in serum-free DMEM medium (1% glutamine) according to the kit instructions (Silencer™ siRNA Transfection Kit; Ambion no. 4511). This medium with siRNA-LRP1 was maintained for 48 h, and it was then replaced by a new medium containing LDL (100 μg/ml). After 18 h, cells were exhaustively washed and harvested to test LRP1 and ADRP expression. Extra wells were used in order to test the specificity of siRNA-LRP1 treatment by analyzing LRP1, LDLR, CD36 and ABCA1 mRNA expression by real-time PCR. The cells did not take up Trypan Blue, and their morphology was not altered by the procedure.

Real-time PCR

Total RNA and protein were isolated by Tripure™ isolation Reagent (Roche Molecular Biochemicals) according to the ma-

nufacturer's instructions. LRP1, LDLR, and adipophilin levels were analyzed by real-time PCR. TaqMan fluorescent real-time PCR primers and probes (6'FAM-MGB) for LRP1 were designed by use of Primer Express software from PE biosystems and were as follows: LRP1 forward: 5′-gagctgaaccacgcctttg-3′; LRP1 reverse: ggtagacactgccactccgatac-3′; LRP1 probe: 5′-ttgccatggtgacacag-3′; LDLR forward: 5′-tgacaatgtctcaccaagctctg-3′; LDLR reverse: 5′-ctcacgctactgggcttcttct-3′; LDLR probe: 5′-ctgccagcaacgtcg-3′. Assays on demand (Applied Biosystems) were used for ADRP (Hs 00357608 m1), CD36 (Hs 00169627 m1) and ABCA1 (Hs 01059118 m1). Human *gapdh* (4326317E) was used as endogenous control for human VSMCs and 18srRNA (4319413E) for HMDMs. Taqman real-time PCR was performed as previously described (19, 20, 29).

Western blot analysis

Proteins were analyzed by Western blot analysis as previously described (19, 29). Blots were incubated with monoclonal antibodies against human LRP1 (β-chain; Research Diagnostics; clone 8B8 RDI 61067) and human ADRP (Research Diagnostics RDI-PROGP40). Equal loading of protein in each lane was verified by staining filters with Pounceau and also by incubating blots of human VSMCs with monoclonal antibodies against human α-actin (MAB 1682; Chemicon International) and blots of HMDMs with monoclonal antibodies against β-actin (clone AC-15; Sigma). Western blot bands were quantified with a Chemidoc (BioRad) using the Quantity One 1-D Analysis Software. Results are expressed as arbitrary units that refer to units of intensity × millimeters.

Immunohistochemistry

Human coronary arteries were obtained from explanted hearts immediately after surgical excision. All procedures were approved by the Institutional Review and Ethics Committee. Paraffin-embedded specimens were cut into 5-μm-thick serial sections, placed on poly-L-lysine-coated slides, deparaffinized, and stained with Masson's trichrome or processed for immunohistochemistry. Lesions were characterized in Masson's trichrome-stained sections according to American Heart Association criteria (30).

In brief, consecutive sections were deparaffinized, after target retrieval, and the sections were hydrated and blocked. We applied the indirect double-immunofluorescence method. The primary antibodies were as follows: monoclonal mouse anti-human smooth muscle actin (α-SMC, clone 1A4; Dako); monoclonal mouse anti-human macrophage, CD68 (CD68, EBM11; Dako); and guinea pig polyclonal to ADRP (Research Diagnostics RDI-PROGP40), for 2 h (dilution 1:100) at room tem-

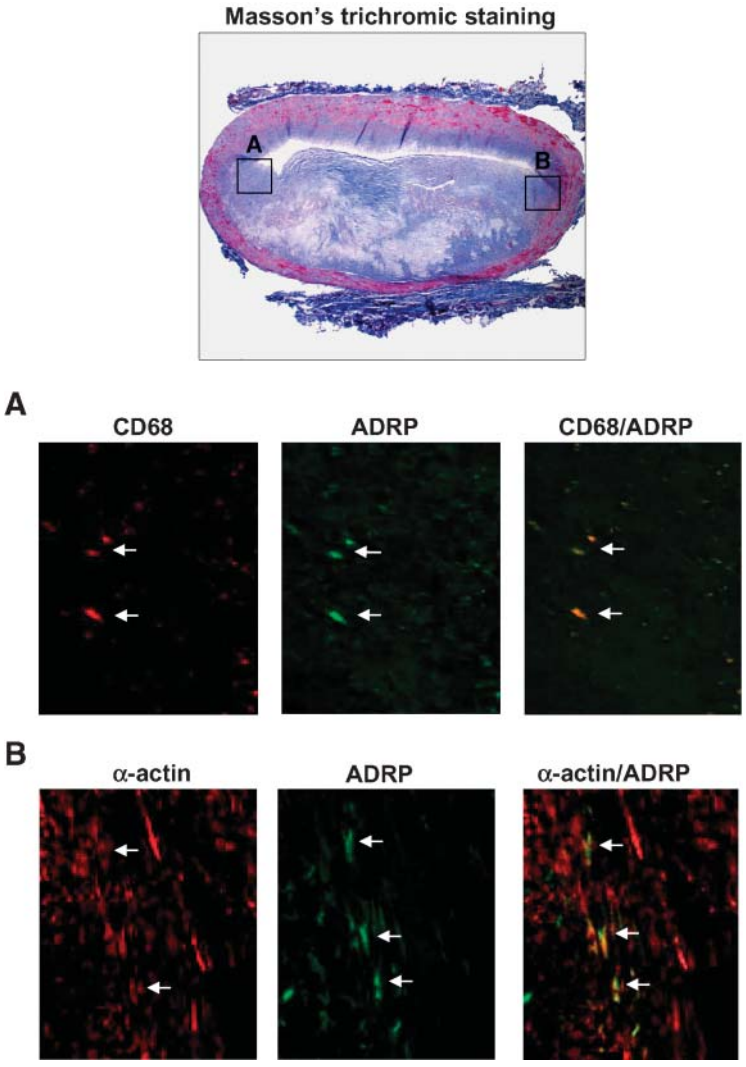

Fig. 1. Analysis of the colocalization between adipocyte differentiation-related protein (ADRP) and CD68 or α-actin-positive cells in the vascular wall. Colocalization of ADRP (in green) and CD68 (marker of macrophages) (in red) (A) and colocalization of ADRP (in green) and α-actin (in red) (B) in type V atherosclerotic lesion (Masson's trichromic staining). Bar: 100 μm. Arrows indicate a high degree of colocalization betwen ADRP and CD68 or α-actin-positive cells.

TABLE 1. Intracellular CE accumulation, LRP1, LDLR, CD36, and ABCA1 mRNA levels in VSMCs and HMDMs

	24 h			48 h		
	Control	nLDL	agLDL	Control	nLDL	agLDL
HMDM						
CE accumulation	Undetectable	10.25 ± 0.54^{a}	$77.09 \pm 1.2^{a,b}$	Undetectable	11.54 ± 1.98^{a}	$100.4 \pm 2.3^{a,b}$
LRP1 mRNA	1 ± 0	1.11 ± 0	1.04 ± 0.045	1 ± 0	1.0 ± 0.13	1.02 ± 0.10
LDLR mRNA	1 ± 0	0.12 ± 0.01^{a}	$0.042 \pm 0.07^{a,b}$	1 ± 0	0.068 ± 0.03^{a}	$0.050 \pm 0.009^{a,b}$
CD36 mRNA	1 ± 0	1.06 ± 0.06	1.16 ± 0.1	1 ± 0	0.89 ± 0.1	0.98 ± 0.04
ABCA1 mRNA	1 ± 0	0.98 ± 0.15	$1.34 \pm 0.10^{a,b}$	1 ± 0	1.29 ± 0.17	$1.67 \pm 0.04^{a,b}$
VSMC						
CE accumulation	Undetectable	25.42 ± 2.24^{a}	$75 \pm 3^{a,b}$	Undetectable	26.35 ± 1.20^{a}	$95 \pm 2.5^{a,b}$
LRP1 mRNA	1 ± 0	1.50 ± 0.35^{a}	$2.20 \pm 0.30^{a,b}$	1 ± 0	1.69 ± 0.31^{a}	$2.70 \pm 0.25^{a,b}$
LDLR mRNA	1 ± 0	0.15 ± 0.02^{a}	0.16 ± 0.05^{a}	1 ± 0	0.16 ± 0.04^{a}	0.25 ± 0.02^{a}
CD36 mRNA	1 ± 0	0.92 ± 0.03	0.89 ± 0.018	1 ± 0	0.87 ± 0.03	0.97 ± 0.02
ABCA1 mRNA	1 ± 0	0.97 ± 0.07	$1.38 \pm 0.18^{a,b}$	1 ± 0	1.11 ± 0.08	$1.40 \pm 0.11^{a,b}$

CE, cholesteryl ester; HMDMs, human monocyte-derived macrophages; LDLR, LDL receptor; LRP1, LDL receptor-related protein; nLDL, native LDL; VSMCs, vascular smooth muscle cells. HMDMs and VSMCs were incubated in the absence or presence of nLDL or agLDL (100 μg/ml) for 24 h or 48 h. At the end of each period, CE accumulation was analysed by TLC after lipid extraction, and LRP1 and LDLR mRNA expression levels were analysed by real-time PCR, as described in Materials and Methods. CE accumulation results were expressed as microgram of CE per milligram of protein. LRP1, LDLR, CD36, and ABCA1 mRNA levels were expressed as arbitrary units normalized to the housekeeping gene *gapdh* in human VSMCs or to the housekeeping gene 18srRNA in HMDMs. Results are shown as mean ± SEM of three experiments performed in duplicate.

[a] $P < 0.05$ versus control cells.

[b] $P < 0.05$ versus nLDL-exposed cells.

perature. After several washes, the sections were incubated with anti-mouse IgG TRITC conjugate (T1659; Sigma) and goat anti-guinea pig IgG FITC (106-095-006; Jackson ImmunoResearch) for 1 h (dilution 1:50). The sections were washed in PBS 100 mM, pH 7.4, mounted in Glicergel® mounting medium (DAKO A/S), and analyzed using an Eclipse 80i Nikon microscope. The images were captured by the Visilog 5.4 Noesis(c) program. Controls were run with each set of specimens.

Data analysis

Data were expressed as mean ± SEM. A statview (Abacus Concepts) statistical package for the Macintosh computer system was used for all analyses. Multiple groups were compared by ANOVA or Wilcoxon test as needed. Statistical significance was considered when $P < 0.05$.

RESULTS

ADRP is highly expressed by macrophages and smooth muscle cells in lipid-enriched human advanced atherosclerotic lesions

Figure 1 shows a representative type V lesion classified according to the American Heart Association. The immunostaining with CD68 (marker of macrophages) and ADRP showed a high degree of colocalization between ADRP (in green) and CD68 (in red) (Fig. 1A). Figure 1B showed a high degree of colocalization between ADRP (in green) and α-actin (marker of VSMCs) (in red). Thus, ADRP colocalizes with both macrophages and VSMCs in advanced lipid-enriched atherosclerotic plaques (n = 3).

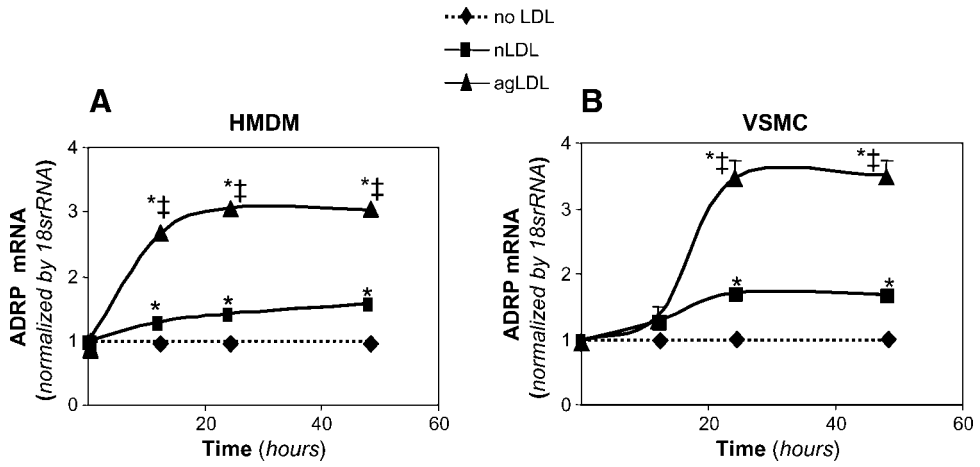

Fig. 2. Time course response of ADRP mRNA expression in native LDL (nLDL) and aggregated LDL (agLDL). Human monocyte-derived macrophages (HMDMs) (A) or vascular smooth muscle cells (VSMCs) (B) were incubated in the absence (romboid), or presence of nLDL (squares) or agLDL (triangles) (100 μg/ml) for increasing times. Graphs show real-time PCR quantification of ADRP mRNA. Data were processed with a specially designed software program based on Ct values of each sample and normalized to *18srRNA* mRNA in HMDMs or to *gapdh* in VSMCs (n = 3). *$P < 0.05$ versus control cells; ‡$P < 0.05$ versus nLDL-exposed cells.

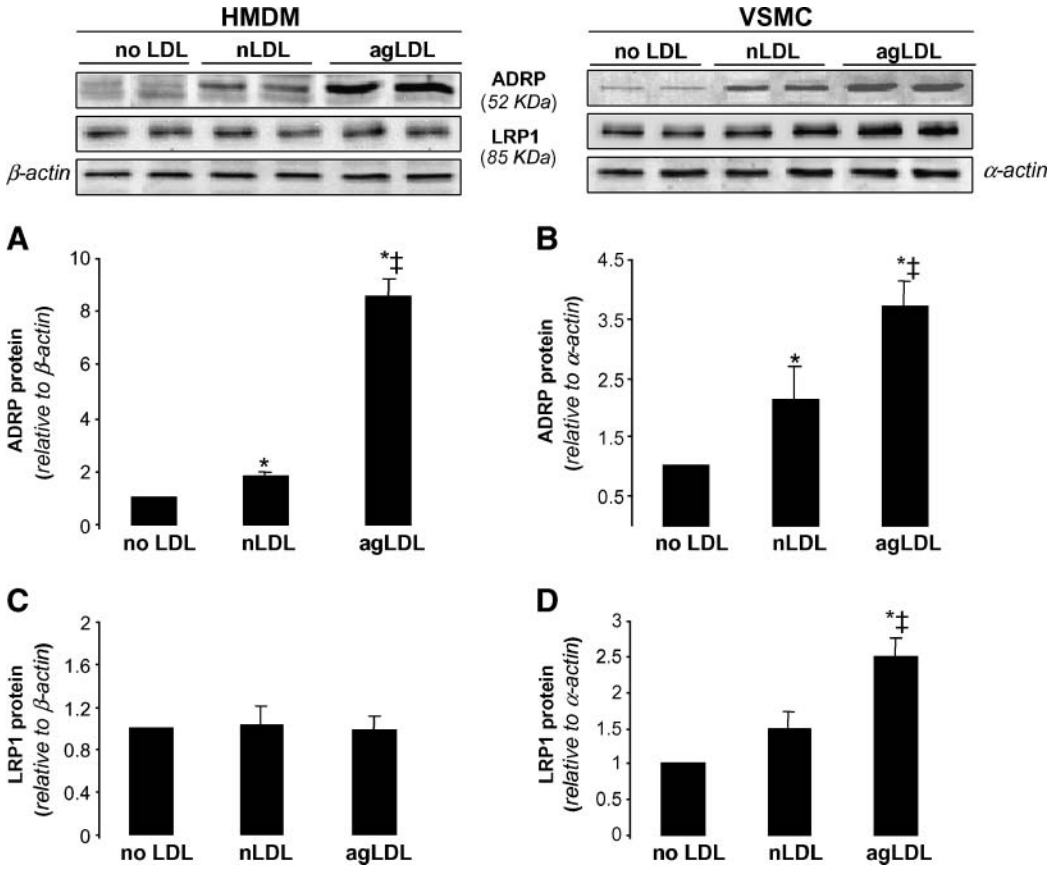

Fig. 3. Effect of nLDL and agLDL on ADRP and LDL receptor-related protein (LRP1) expression. Representative Western blot shows ADRP, LRP1 (β-chain), and actin protein levels in HMDMs or VSMCs incubated with nLDL or agLDL (100 μg/ml, 24 h). Bar graphs show the quantification of ADRP bands in HMDMs (A) and VSMCs (B), and the quantification of LRP1 bands in HMDMs (C) and VSMCs (D) incubated in the absence or presence of nLDL or agLDL. Results are expressed as a percentage of control cells (incubated in the absence of LDL) and are shown as mean ± SEM (n = 3). *$P < 0.05$ versus control cells; ‡$P < 0.05$ versus nLDL-exposed cells.

Effect of nLDL and agLDL on CE accumulation, LRP1, LDLR, CD36, and ABCA1 expression in human HMDMs and VSMCs

By TLC, we analyzed the effect of nLDL and agLDL on intracellular CE accumulation in both HMDMs and VSMCs. Strong differences in CE levels induced by nLDL or agLDL internalization were observed in both VSMCs and HMDMs (**Table 1**). While agLDL (100 μg/ml; 24 h) strongly induced intracellular CE accumulation from undetectable levels up to 77.09 ± 1.2 μg/mg protein in HMDMs and up to 75 ± 3 μg/mg protein in VSMCs, nLDL induced a significant but slight increase up to 10.25 ± 0.54 μg/mg protein in HMDMs and 25.42 ± 2.24 μg/mg protein in VSMCs during the same LDL exposure time.

By real-time PCR, we analyzed LRP1 and LDLR, CD36, and ABCA1 mRNA expression levels in HMDMs and VSMCs (Table 1). LDLR mRNA expression was strongly downregulated by both nLDL and agLDL in both HMDMs and VSMCs. In contrast, LRP1 mRNA was not regulated by LDL in HMDMs and it was significantly upregulated by nLDL and agLDL in VSMCs (nLDL: 1.69 ± 0.31-fold vs. agLDL: 2.70 ± 0.25-fold at 48 h), as previously described (18, 19, 28). The scavenger receptor CD36 was not significantly modified by nLDL or agLDL in HMDMs or VSMCs. Interestingly, ABCA1 was slightly but significantly upregulated by agLDL in both HMDMs (by 1.34 ± 0.10-fold at 24 h, by 1.67 ± 0.04-fold at 48 h) and VSMCs (by 1.38 ± 0.18-fold at 24 h, by 1.40 ± 0.11-fold at 48 h).

Effect of agLDL on ADRP mRNA and protein expression in HMDMs and VSMCs

AgLDL shows a high capacity to induce ADRP mRNA overexpression in HMDMs (2.67-fold at 6 h; 3.06-fold at

TABLE 2. Effect of siRNA-LRP1 treatment on LRP1, LDLR, CD36, and ABCA1 mRNA levels in HMDMs and VSMCs

	HMDM		VSMC	
	siRNA-LRP1$^-$	siRNA-LRP1$^+$	siRNA-LRP1$^-$	siRNA-LRP1$^+$
LRP1 mRNA	4.75 ± 0.25	1.22 ± 0.17[a]	3.21 ± 0.15	0.86 ± 0.05[a]
LDLR mRNA	1.60 ± 0.10	1.67 ± 0.2	0.60 ± 0.02	0.64 ± 0.04
CD36 mRNA	2.24 ± 0.13	2.0 ± 0.14	1.17 ± 0.04	0.96 ± 0.09
ABCA1 mRNA	1.11 ± 0.05	0.83 ± 0.02	0.83 ± 0.14	0.81 ± 0.17

SiRNA-LRP1, small anti-LRP1-interfering RNA. HMDMs and VSMCs were treated with siRNA-LRP1 as discussed in Materials and Methods. LRP1, LDLR, CD36, and ABCA1 mRNA expression levels were analysed by real-time PCR and expressed as arbitrary units normalized to the housekeeping gene 18srRNA in HMDMs and to the housekeeping gene *gapdh* in human VSMCs. Results are shown as mean ± SEM of four experiments performed in duplicate.

[a] $P < 0.05$ versus siRNA-LRP1-untreated cells.

12 h) (**Fig. 2A**). In human VSMCs, agLDL also induced ADRP mRNA expression but at later exposure times than in HMDMs (1.36-fold at 12 h, by 3.5-fold at 24 h) (Fig. 2B). ADRP upregulation by agLDL in HMDMs and VSMCs was maintained along the time. ADRP was slightly upregulated by nLDL in both HMDMs (by 1.6-fold at 24 h) (Fig. 2A) and human VSMCs (by 1.68-fold at 24 h) (Fig. 2B).

ADRP protein expression was strongly upregulated by agLDL (100 μg/ml; 24 h) in both HMDMs (8.58-fold) (**Fig. 3A**) and VSMCs (3.71-fold) (Fig. 3B). In contrast, nLDL only slightly induced ADRP protein expression in these cells (HMDMs: 1.84-fold; VSMCs: 2.11-fold). In agreement with our previous results obtained in human VSMCs (18, 28) and with the ability of nLDL and agLDL to induce LRP1 mRNA expression (Table 1), agLDL strongly induced LRP1 protein expression by 2.50-fold (Fig. 3D), concomitantly with the induction of ADRP. In contrast, the induction of ADRP in HMDMs was not related to a concomitant induction of LRP1 protein expression (Fig. 3C).

AgLDL-induced ADRP overexpression depends on LRP1 expression in both HMDMs and VSMCs

To evidence the role of LRP1 in the induction of ADRP by agLDL, we analyzed the effects of agLDL in siRNA-LRP1-treated HMDMs and siRNA-LRP1-treated VSMCs. As shown in **Table 2**, siRNA-LRP1 treatment reduced LRP1 mRNA expression by 74% in HMDMs and by 73% in VSMCs. However, LDLR, CD36, and ABCA1 mRNA expression levels were not significantly altered by siRNA-LRP1 treatment. Moreover, siRNA-LRP1 significantly decreased LRP1 protein expression in control HMDMs, in nLDL-exposed HMDMs, and in agLDL-exposed HMDMs by more than 85% (**Fig. 4A**). In human VSMCs, in agreement with our previous results (18, 28), siRNA-LRP1 treatment decreased LRP1 protein expression in control VSMCs

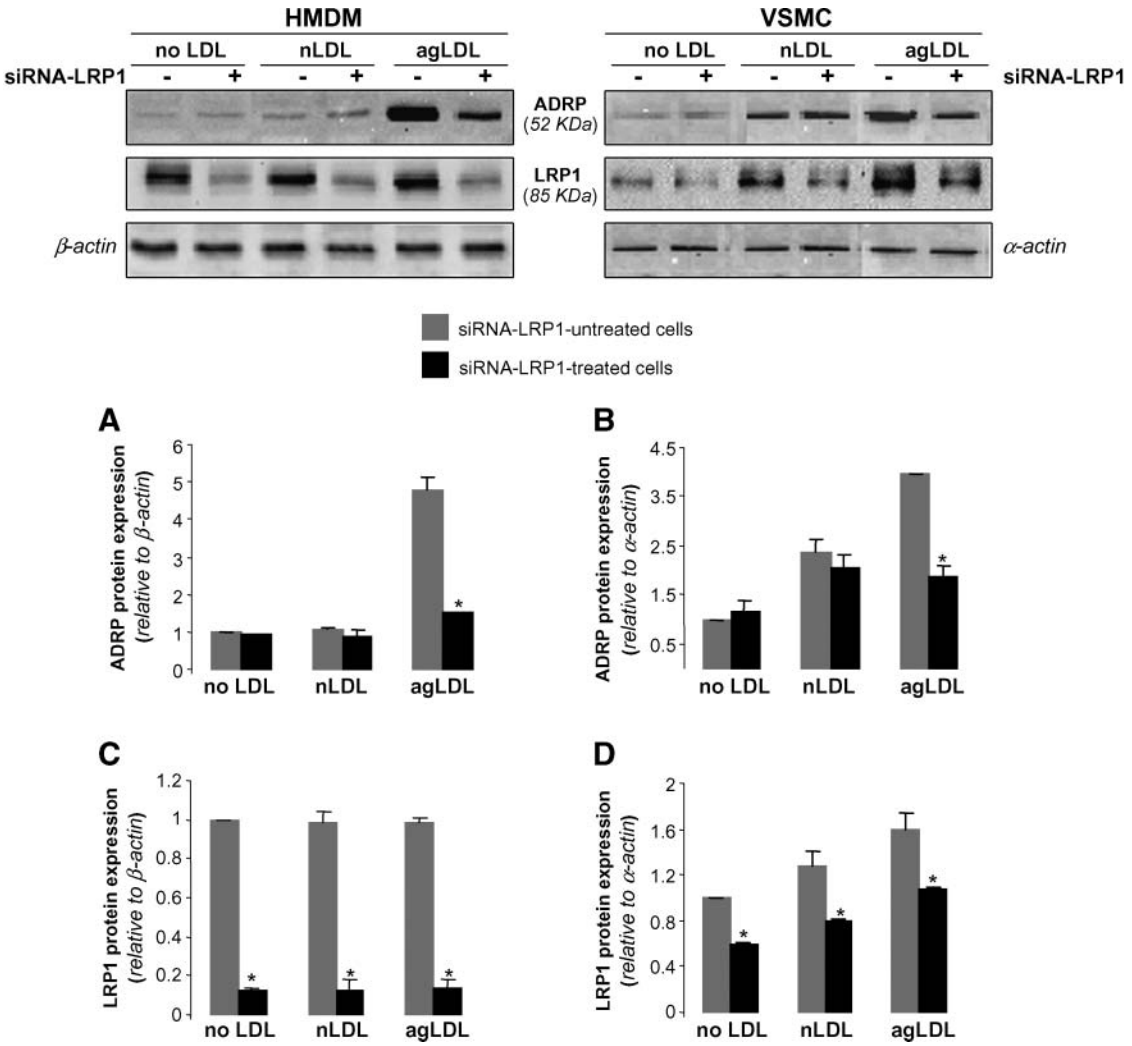

Fig. 4. Small anti-LRP1-interfering RNA (siRNA-LRP1) treatment prevents agLDL-induced ADRP overexpression in HMDMs and VSMCs. HMDMs (A) and VSMCs (B) were treated with siRNA-LRP1 as detailed in Materials and Methods. VSMCs were then harvested for measurement of LRP1, ADRP, and actin protein expression levels. Representative Western blot shows LRP1, ADRP, and actin protein bands in untreated (black bars) or siRNA-LRP1-treated cells (gray bars). Bar graphs show the quantification of ADRP bands in HMDMs (A) or VSMCs (B), and the quantification of LRP1 bands in HMDMs (C) and VSMCs (D). Results are shown as mean ± SEM (n = 3). *$P < 0.05$ versus siRNA-LRP1-untreated cells.

by 40%, in nLDL-exposed VSMCs by 38%, and in agLDL-exposed VSMCs by 33% (Fig. 4B).

siRNA-LRP1 treatment did not show any significant effect on nLDL-induced ADRP protein expression in either HMDMs or VSMCs. However, siRNA-LRP1 treatment significantly reduced agLDL-induced ADRP overexpression by 69% in HMDMs (Fig. 4C) and by 53% in human VSMCs (Fig. 4D).

DISCUSSION

LDL aggregation is one of the most important LDL modifications in the vascular wall, because most LDL has been found to be associated with extracellular matrix proteoglycans in the arterial intima (2–5). AgLDL is able to induce massive intracellular CE accumulation in two crucial cell types involved in atherosclerosis, human VSMCs and macrophages (6–16), and LRP1 is involved in the uptake of agLDL in both VSMCs (11–13) and HMDMs (14–16). In accord with the pivotal role of LRP1 in agLDL binding and internalization (11–16), siRNA-LRP1 treatment, which specifically inhibits LRP1 expression, almost completely prevented agLDL-induced ADRP overexpression in HMDMs and VSMCs. siRNA-LRP1 treatment did not show any significant effect on nLDL-induced ADRP expression, in agreement with the lack of effect of LRP1 inhibition of nLDL uptake previously published by our group (10). The expression of other genes related to foam cell formation, such as CD36 and ABCA1, were not affected by siRNA-LRP1 treatment in HMDMs or VSMCs. These results demonstrate that LRP1 is crucial for agLDL-induced ADRP overexpression in vascular cells. In fact, in HMDMs, where LRP1 basal expression levels are higher than in human VSMCs, the upregulatory effect of agLDL on ADRP expression requires less time. The higher LRP1 basal expression levels in macrophages are due, at least in part, to induction of LRP1 expression during monocyte-to-macrophage differentiation (31) and to the fact that M-CSF, used in our experimental setting to differentiate monocytes from macrophages, also upregulates LRP1 expression (32).

In HMDMs and, interestingly, also in human VSMCs, ADRP overexpression induced by agLDL was much higher than that induced by unmodified LDL, suggesting an important relationship between ADRP overexpression and intracellular CE accumulation induction by agLDL uptake in both vascular cell types. The high ability of agLDL to induce ADRP overexpression in macrophages is shared by other modified lipoproteins, such as oxidized LDL (28) or enzymatically modified LDL (E-LDL) (33), which have been described to strongly induce CE accumulation in macrophages. Therefore, oxidized LDL, E-LDL, and agLDL, which are taken by unrelated internalization mechanisms, exert a common upregulatory effect on ADRP expression. ADRP overexpression in macrophages thus seems to be more related to the high capacity of modified lipoproteins to carry CE into the cells than to the specific mechanism involved in modified LDL uptake. In contrast to the high capacity of differently modified LDL to induce intracellular cholesterol accumulation in macrophages, LDL aggregation is the only modification of LDL able to induce CE accumulation in VSMCs (9). AgLDL uptake by human VSMCs is thus the main mechanism for VSMC foam cell formation. AgLDL-induced ADRP overexpression in human VSMCs took place concomitantly with an increase in LRP1 expression (19, 29). However, agLDL did not significantly alter the expression of CD36 scavenger receptor in either HMDMs or VSMCs. In fact, CD36 seems to be positively regulated by oxidized LDL through protein kinase-specific signal pathways (34). LRP1 and ADRP induction by agLDL may lead, therefore, to a progressive intracellular CE accumulation in VSMCs. AgLDL also significantly induced the expression of ABCA1, a protein that mediates the generation of HDL, in HMDMs and VSMCs. Our results are in agreement with the positive regulation of ABCA1 gene transcription by cellular cholesterol (35). ABCA1 upregulation by cholesterol has been described to be mediated through SREBP-2 downregulation (36). We have previously described that agLDL reduced the active form of SREBP-2 in both HMDMs (14) and VSMCs (19); therefore, agLDL might upregulate ABCA1 through SREBP downregulation.

Although an abundance of ADRP mRNA has been demonstrated in macrophage-rich areas of atherosclerotic lesions (28, 37), little is known about the possible role of ADRP overexpression in VSMC foam cell formation. Our immunohistochemical studies demonstrate a significant colocalization between ADRP and macrophages but also between ADRP and VSMCs in lipid-enriched advanced human atherosclerotic plaques.

In summary, agLDL induces ADRP mRNA and protein overexpression, and this effect depends on LRP1 in VSMCs and macrophages. Because ADRP is highly expressed by both macrophages and VSMCs in advanced lipid-enriched atherosclerotic plaques, LRP1-mediated agLDL uptake probably plays a pivotal role in vascular foam cell formation associated with atherosclerotic lesion progression.

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