Low-Density Lipoprotein Upregulates Low-Density Lipoprotein Receptor–Related Protein Expression in Vascular Smooth Muscle Cells

Possible Involvement of Sterol Regulatory Element Binding Protein-2–Dependent Mechanism

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Background—Low-density lipoprotein (LDL) receptor–related protein (LRP) is highly expressed in vascular smooth muscle cells (VSMCs) of both normal and atherosclerotic lesions. However, little is known about LRP regulation in the vascular wall.

Methods and Results—We analyzed the regulation of LRP expression in vitro in human VSMCs cultured with native LDL (nLDL) or aggregated LDL (agLDL) by semiquantitative reverse transcriptase–polymerase chain reaction, real-time polymerase chain reaction, and Western blot and in vivo during diet-induced hypercholesterolemia by in situ hybridization. LRP expression in human VSMCs is increased by nLDL and agLDL in a time- and dose-dependent manner. Maximal induction of LRP mRNA expression was observed after 24 hours of exposure to LDL. However, agLDL induced higher LRP mRNA expression (3.0-fold) than nLDL (1.76-fold). LRP mRNA upregulation was associated with an increase on LRP protein expression with the greatest induction by agLDL. VSMC-LRP upregulation induced by nLDL or agLDL was reduced by an inhibitor of sterol regulatory element binding protein (SREBP) catabolism (N-acetyl-leucyl-leucyl-norleucinal). In situ hybridization analysis indicates that there is a higher VSMC-LRP expression in hypercholesterolemic than in normocholesterolemic pig aortas.

Conclusions—These results indicate that LRP expression in VSMCs is upregulated by intravascular and systemic LDL. (Circulation. 2002;106:3104-3110.)

Key Words: arteriosclerosis ■ lipoproteins ■ receptors ■ hypercholesterolemia ■ vasculature
an SRE in the promoter, an intriguing SRE-1 site has been found in the unusually long 5'-untranslated region of LRP. 26

The aim of this work was to analyze the effect of agLDL on LRP expression in vitro in cultured human VSMCs and in vivo in a hypercholesterolemic porcine model. Here, we show that agLDL upregulates LRP expression in a dose- and time-dependent manner in human VSMCs. In agreement, LRP expression was significantly enhanced in the vascular wall of hypercholesterolemic animals.

**Methods**

**VSMC Culture**

Primary cultures of human VSMCs were obtained from nonatherosclerotic areas of macroscopically healthy ascending aortas of explanted hearts removed from nonischemic cardiomyopathy patients transplanted at the Hospital de la Santa Creu i Sant Pau with the permission of the ethics committee of the Hospital. Histological analysis (Masson Trichromatic stain) of these arteries did not show atherosclerotic changes, although proatherosclerotic molecular abnormalities in these human arteries cannot be excluded. VSMCs were obtained by a modification of the explant technique as we described previously. 13,14 VSMCs were identified morphologically using light microscopy and by their growth behavior and were characterized by immunological staining of cytoskeleton proteins. Primary and subcultured VSMCs with a low in vitro age had a spindle-shaped appearance. Confluent VSMC cultures showed the characteristic "hill-and-valley" growth pattern. Mouse monoclonal antibodies specific for human α-SM actin (clone 1A4), human von Willebrand factor (clone F8/86), and human fibroblast surface protein (clone 1B10) were used. VSMCs were used between passages 2 and 4. Cells showed positive immunostaining with anti-SMC α-actin. No staining was observed with antibodies against a human fibroblast cell-surface antigen or von Willebrand factor. Cells were arrested 48 hours in M199 supplemented with 0.2% FCS and incubated with different concentrations of nLDL and agLDL for the indicated times (0 to 48 hours). In some experiments, cells were pretreated for 24 hours with N-acetyl-leu-leu-norleucinal (ALLN) (25 μmol/L).

**Animals**

Female pigs (body weight at initiation, 32±4 kg) were divided into two groups: normocholesterolemic animals (n=6), which were fed a normal chow diet, and hypercholesterolemic animals (n=10), which were fed a cholesterol-rich diet (2% cholesterol, 1% cholic acid, 20% beef tallow) for 100 days. 27 After 100 days, the animals were killed with a thiopental overdose. Plasma cholesterol levels and hemato logical parameters were measured at baseline and at death. Because the porcine model of atherosclerosis initially develops lesions in the abdominal aorta, rings from this vessel were collected and fixed in 4% paraformaldehyde in PBS 0.1 mol/L (pH 7.4), cryoprotected in 30% saccharose in PBS 0.1 mol/L (pH 7.4), embedded in OCT, and frozen on dry ice. All procedures were in accordance with institutional guidelines and followed the American Physiological Society guidelines for animal research.

Plasma total cholesterol was determined with an automatic analyzer (Kodack Ektachem DT System). Plasma LDL-cholesterol was analyzed using the validated methods of the Lipid Research Clinic Program 28 and quantified spectrophotometrically (Kontron Instruments).

**LDL Preparation and Determination of the Free Cholesterol and Cholesteryl Esters Content**

Human LDLs (d 1.006–d 1.063 mol/L) were obtained and modified as previously described. 23,24 LDL preparations were <48 hours old, nonoxidized (<1.2 mmol malondialdehyde/mg protein LDL), and without detectable levels of endotoxin (Limulus Amebocyte Lysate test, Bio Whittaker).

Arrested cells were incubated with nLDL or agLDL (100 μg/mL) for 6, 12, 24, and 48 hours. In some experiments, cells were pretreated for 24 hours with ALLN (25 μmol/L) before adding LDL (50, 100 μg/mL). Cells were then exhaustively washed and harvested into 1 mL of 0.10 N NaOH. Lipid extraction and TLC were performed as previously described. 25,26

**Semiquantitative and Real-Time PCR**

Arrested cells were incubated with nLDL or agLDL for 6, 12, 24, and 48 hours. RNA and protein were isolated by using the Tripure isolation Reagent (Roche Molecular Biochemicals) according to the manufacturer. LRP and LDL receptor mRNA levels were analyzed by semiquantitative RT-PCR as previously described. 24 The specific oligonucleotides for SREBP were SREBP-1 forward primer: 5'-agtgtggctgctgccgcgtc-3'; SREBP-1 reverse primer: 5'tgtgacctgctgcagctcgc-3'; SREBP-2 forward primer: 5'-tgtgagctcgctgccagcgc-3'; and SREBP-2 reverse primer: 5'-tgtgagctcgctgccagcgc-3'. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize results. Amplification was carried out by 20 (GAPDH), 20 (LRP), 25 (LDL receptor), 20 (SREBP-1), and 22 (SREBP-2) cycles. PCR products (10 μL) were resolved by electrophoresis in 1.5% to 2% agarose gels and transferred onto nylon membranes by the standard capillary technique. Blots were UV cross-linked. Detection of digoxigenin (DIG)-labeled nucleic acids was performed with an anti-DIG antibody linked to alkaline phosphatase, and CSPD (Disodium 3-(4-methoxyspiro(1.2-dioxetane-3,2'-15'-chloro) tricyclo(3.3.1.17)-decane)-4-yl) phenylphosphate (Roche Molecular Biochemicals) was used as substrate.

TagMan fluorescent Real-Time PCR primers and probes (6′FAM-MGB) for LRP and LDL receptor were designed by use of Primer Express software from PE biosystems and were as follows: LRP forward: 5'-gagctgaacacgctgcgctg-3'; LRP reverse: 5'-tgccagctgtgcaacgc-3'; LDL receptor forward: 5'-tgaactctctacaacgtctc-3'; LDL receptor reverse: 5'-tcgacatctgccttcctc-3'; and LDL receptor probe: 5'-tcgacatctgccttcctc-3'. Human gapdh (4326317E) was used as endogenous control. The specificity and the optimal primer and probe concentrations were tested. Taqman real-time PCR was performed with 2-μL/well of RT products (1 μtg total RNA) in 25 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 on the ABI PRISM 7000 Detection System. The threshold cycle (Ct) values were determined and normalized to the housekeeping gene gapdh. Because the targets have similar amplification efficiency as endogenous controls, we have used the comparative Ct method (delta delta Ct) to perform relative quantification of LRP and LDL receptor.

**Western Blot Analysis**

SDS/PAGE was run as previously described. 14 Blots were incubated with monoclonal antibodies against human LRP (β-chain; Research Diagnostics, clone 8B8 RDI 61067, dilution 1:40). To test equal protein loading for the different samples, blots were also incubated with monoclonal antibodies against human α-actin (Chemicon International, MAB 1682, dilution 1:10000).

**LRP In Situ Hybridization**

Abdominal aorta sections (n=3) from the same segment of normocholesterolemic and hypercholesterolemic animals were analyzed. Conventional histology (Masson Trichrome) was performed to identify the lesion type.

For riboprobe synthesis, human LRP cDNA corresponding to nucleotides 2221 to 2764 (544 mer) was cloned in plasmid vectors using standard techniques. Antisense and sense riboprobe were synthesized using T3 and T7 RNA polymerase (Promega), respectively, and DIG-labeled nucleotides (Roche Molecular Biochemi-
In situ hybridization studies were performed on a set of 20-μm-thin sections. Sections were fixed before being permeabilized by proteinase K. Proteinase K was then deactivated and the sections were treated with TEA, postfixed again, and blocked with 2 mg/mL glycine in PBS. After wash with SSC/H2O, sections were prehybridized with a solution of 50% formamide, SSC/H2O, 50 g/mL heparin, 5% dextran sulfate, 0.01% SDS, Denhardt’s solution/H2O, 100 g/mL polyAmRNA, 25 μg/mL tRNA, 25 μg/mL salmon sperm DNA. Sections were then incubated with the probe at 1 g/mL in the prehybridization solution overnight at 53°C. Digoxigenin labeled nucleotides were detected by an alkaline phosphatase conjugated anti-digoxigenin antibody provided by the Dig-labeled immunodetection kit, using NBT/BCIP as substrate (Vector Laboratory). Results were evaluated with an Olympus Vanox fluorescence microscope. Images were digitalized using a Sony 3CCD camera. Controls were performed by the incubation of the section with the sense riboprobe and with the empty-plasmid riboprobes.

**Data Analysis**

Data were expressed as mean±SEM. A statview (Abacus Concepts) statistical package for the Macintosh computer system was used for all analysis. Multiple groups were compared by nonparametric tests, Wilcoxon, or Mann-Whitney U, as needed. Statistical significance was considered when P<0.05.

**Results**

**nLDL and agLDL Downregulate LDL Receptor and Upregulate LRP mRNA Expression in a Time- and Dose-Dependent Manner**

Differences in cholesteryl ester (CE) levels between VSMCs incubated with nLDL or agLDL were highly significant (P<0.05) (Figure 1). VSMC-CE accumulation derived from agLDL increased from undetectable levels to 103±5.2 μg/mg protein at 48 hours. On the contrary, VSMC-CE accumulation from nLDL was slightly increased (from undetectable levels to 32.2±3.16 μg/mg protein at 48 hours), reaching a plateau after 12 hours of incubation. Free cholesterol content was not changed by either agLDL or nLDL.

LRP was slightly upregulated by nLDL and strongly by agLDL in a time-dependent (Figure 2A) and dose-dependent (Figure 2C) manner. AgLDL was able to upregulate LRP expression at the lowest concentration tested (50 μg/mL). Maximal LRP induction by both nLDL and agLDL (100 μg/mL) was observed after 24 hours; however, agLDL induced a much higher LRP expression than nLDL (agLDL, 3±0.12 versus nLDL, 1.76±0.32; P<0.05). In absence of LDL (control), LRP expression remained unaltered along the tested times. Both nLDL and agLDL downregulated LDL receptor mRNA expression in a time-dependent (Figure 2B) and
dose-dependent (Figure 2C) manner. The lowest concentration tested of both nLDL and agLDL (50 μg/mL) completely reduced LDL receptor expression. The time-course assays performed with the highest LDL concentration (100 μg/mL) showed a complete LDL receptor downregulation after 6 hours with nLDL and after 12 hours with agLDL. A slight reversion of the LDL downregulation was observed after 24 and 48 hours of LDL incubation. In absence of LDL (control), LDL receptor expression remained unaltered along the tested times.

Western blot analysis showed that both nLDL and agLDL (100 μg/mL, 24 hours) were able to induce LRP protein synthesis. However, agLDL induced higher LRP protein expression than nLDL (agLDL: 8.3±1.2 versus nLDL: 3.3±0.5; P<0.05) (Figure 3).

nLDL and agLDL Downregulated SREBP-2 mRNA Levels in a Time- and Dose-Dependent Manner

We evaluated SREBPs mRNA expression levels in VSMCs exposed to increasing concentrations of nLDL and agLDL, and although SREBP-1 remained unaltered, SREBP-2 levels were downregulated in a dose-dependent (Figure 4A) and time-dependent (Figure 4B) manner. The lowest concentration tested of both nLDL and agLDL (50 μg/mL) decreased SREBP-2 mRNA expression by ~50%, and a complete SREBP-2 downregulation was observed at 100 μg/mL. The time-course assays performed with the highest LDL concentration (100 μg/mL) showed that, as observed with the LDL receptor mRNA expression, there was a delay on the SREBP-2 downregulation induced by agLDL (12 hours) compared with that induced by nLDL (6 hours). In addition, similarly to the LDL receptor expression, a slight reversion of the SREBP-2 downregulation was observed after 24 and 48 hours of incubation with nLDL or agLDL.

ALLN Prevents the LDL Receptor Downregulation and the LRP Upregulation Caused by nLDL and agLDL

To analyze the possible involvement of SREBPs in LDL-mediated upregulation of LRP expression, we analyzed the effect of ALLN (25 μmol/L), an inhibitor of SREBP catabolism, on LRP mRNA levels. ALLN blocked LRP upregulation induced by LDL not only at mRNA (Figure 5B) but also at the protein level (Figure 5D). As expected, ALLN almost completely prevented the downregulation on LDL receptor mRNA expression induced by nLDL and agLDL (100 μg/mL, 24 hours) (Figure 5C). ALLN also decreased the CE accumulation derived from agLDL by 36±4% at 50 μg/mL agLDL and by 46±6.8% at 100 μg/mL agLDL (Figure 5E), indicating that LRP function is affected by ALLN. Taken together, these results suggest the involvement of SREBP-2 on the LRP upregulation.
caused by nLDL and agLDL. ALLN did not show any effect on SREBP-2 mRNA expression in control or LDL-incubated VSMCs (Figure 5A).

**LRP mRNA Levels Were Higher in VSMCs of Hypercholesterolemic Pigs**

To determine whether hypercholesterolemia influences LRP expression in VSMCs in vivo, we performed in situ hybridization analysis. As shown in the Table, animals fed the hypercholesterolemic diet showed higher cholesterol plasma levels. Masson Trichromic staining shows very incipient lesions in hypercholesterolemic pigs (Figure 6B). In situ hybridization analysis revealed that there is an increase in LRP expression (arrows) in the intima-media layer (just below developing plaques) of hypercholesterolemic (Figure 6D) compared with normocholesterolemic pigs (Figure 6C). Controls with sense riboprobes were negative for normocholesterolemic (Figure 6E) and hypercholesterolemic (Figure 6F) pigs.

**Plasma Lipid Profile in Normolipemic and Hyperlipemic Pigs**

<table>
<thead>
<tr>
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<th>Normolipemic</th>
<th>Hyperlipemic</th>
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<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>65.1±16</td>
<td>463±202*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>34.0±10.9</td>
<td>333±120*</td>
</tr>
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Results are mean±SD. *P<0.01 vs normolipemic animals.

**Discussion**

In human VSMCs, LRP is the lipoprotein receptor that internalizes agLDL obtained by vortexing or incubation with versican. Because vortexing generates agLDL similar to that isolated from the arterial wall and versican is one of the main proteoglycans interacting with the LDL in the arterial intima, the LRP-mediated agLDL uptake could have a crucial role in VSMC-lipid deposition in atherosclerotic plaques.

In this work, we demonstrate that agLDL strongly upregulates LRP at transcriptional level. The increase on mRNA LRP transcription leads to a large increase in LRP protein expression. In these cells, both nLDL and agLDL completely downregulate LDL receptor expression. Consequently, by inducing LRP expression in human VSMC, agLDL could lead to a progressive intracellular accumulation of cholesterol in these cells. The equal capacity of nLDL and agLDL to downregulate LDL receptor expression can be explained by their identical capacity to downregulate SREBP-2 mRNA levels. LDL receptor downregulation is prevented by ALLN, an inhibitor of SREBP-2 catabolism, confirming the expected response of cells to cholesterol loading. Interestingly, nLDL and agLDL were unable to upregulate LRP expression in ALLN-treated VSMCs. Furthermore, LRP upregulation, like LDL receptor downregulation, seems to be dependent on SREBP-2 downregulation. However, other factors besides SREBP-2 must be involved on LRP upregulation, because...
agLDL has much more capacity than nLDL to increase LRP expression levels, whereas both lipoproteins have equal capacity to completely downregulate SREBP-2 levels. These results suggest that the large amount of cholesterol internalized from agLDL into cells could modulate other transcription factors likely involved in the LRP upregulation. Although the LRP gene does not have SRE-1 sequences in its promoter, an SRE-1 site in the unusually long 5' untranslated region has been described. Other genes such as microsomal triglyceride transfer protein or 7α-hydroxylase have been described to be upregulated through SREBPs downregulation. Our results obtained in human VSMCs are in agreement with those obtained in macrophages, because LRP upregulation has been observed in cells incubated with cholesterol and 25-hydroxycholesterol. In addition, the LRP upregulation observed in vitro has been corroborated in vivo; in situ hybridization analysis revealed that LRP expression is upregulated in the vessel wall of hypercholesterolemic animals. LRP upregulation in hypercholesterolemic aortas is concomitant with the SREBP-2 downregulation previously described by our group. Our results in vivo are in agreement with those obtained in blood mononuclear cells, in which dietary cholesterol has been shown to increase LRP mRNA levels, and in aortas of Watanabe rabbits, although the main cellular component at both early and late stages of atherosclerosis in this animal model are infiltrated macrophages.

To our knowledge, this is the first demonstration that exposure to high LDL concentration and cellular accumulation of CE increases LRP expression in VSMCs. Our results suggest that hypercholesterolemia might increase the capacity of VSMCs to take up LDL from the intima by regulating cellular LRP levels. In addition, LRP upregulation may influence other pathways involved in atherothrombosis, because LRP mediates the degradation of molecular complexes involved in thrombogenesis and fibrinolysis.

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