Low Density Lipoproteins Downregulate Lysyl Oxidase in Vascular Endothelial Cells and the Arterial Wall

Cristina Rodríguez, Berta Raposo, José Martínez-González, Laura Casaní, Lina Badimon

Objective—Hypercholesterolemia induces endothelial dysfunction, a hallmark of the atherosclerotic process, modulating the expression of key genes in vascular endothelial cells.

Methods and Results—By differential display analysis, we have studied the effect of high concentrations of native low density lipoprotein (LDL) on endothelial gene expression. mRNA levels of lysyl oxidase (LOX), an enzyme involved in collagen and elastin cross-linking, were downregulated by LDL treatment in endothelial cells in a dose- and time-dependent manner (80% of inhibition by 180 mg/dL LDL for 24 hours). This reduction of LOX expression was associated with a decrease in LOX activity (40% and 54% of inhibition after 24 and 48 hours of LDL treatment, respectively). LOX mRNA half-life was not modified by LDL, but transcriptional inhibition blocked the effect of LDL. Inhibition of LOX activity by either LDL or β-aminopropionitrile, an inhibitor of LOX, increased endothelial permeability (192±0.19- and 3.37±0.74-fold, respectively). Interestingly, a reduction in LOX expression (3.5-fold) was observed in vivo in the vascular wall of hypercholesterolemic pigs.

Conclusions—These findings suggest that LDL downregulation of LOX could contribute to the endothelial dysfunction caused by hypercholesterolemia, thus contributing to atherosclerotic plaque formation. (Arterioscler Thromb Vasc Biol. 2002;22:1409-1414.)

Key Words: endothelial cells ■ low density lipoproteins ■ lysyl oxidase ■ vascular permeability ■ hypercholesterolemia

Vascular endothelium acts as a selective barrier, controlling the exchange of macromolecules between blood and the underlying tissues and regulating vascular tone and the thrombogenic/fibrinolytic balance. Endothelial dysfunction associated with hypercholesterolemia is one of the earlier events in the atherosclerotic process. The alteration of endothelial gene expression by high concentrations of LDL leads to a decrease in NO bioavailability1-3 and to an increase in leukocyte recruitment by an induction of adhesion molecule expression.4

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It has been reported that atherogenic concentrations of LDL alter the composition and permeability of the endothelial barrier by inducing changes in the basement membrane.5 Thus, the alteration in endothelial extracellular matrix (ECM) could play a key role in the endothelial dysfunction associated with hypercholesterolemia. One of the key enzymes involved in ECM maturation is lysyl oxidase (LOX). This enzyme, a copper-containing semicarbazide-sensitive amine oxidase,6 initiates the covalent cross-linking of collagen and elastin that is essential in maintaining ECM structure. LOX oxidatively deaminates peptidyl lysine residues of collagen and elastin, leading to the synthesis of peptidyl aldehydes, which condense spontaneously to form the mature and insoluble ECM.7 LOX has also been associated with tumor suppression8,9 and chemotaxis,10 and in the last few years, different LOX isoforms, probably with different substrate specificity and function, have been identified.11-15 Alterations in normal LOX expression have been linked to human diseases. Reduced LOX expression has been described in type IX Ehlers-Danlos syndrome and Menkes’ disease,16 whereas fibrotic diseases, such as hepatic, lung, or kidney fibrosis, have been associated with increases in LOX.17 The role of this enzyme in atherosclerosis remains to be elucidated because it has been suggested that increases18 and decreases19-21 in LOX activity could be associated with this pathology.

The aim of the present study was to analyze the mechanisms involved in the initiation of atherosclerosis, targeting the endothelial functions regulated by LDL. We have identified LOX as a gene differentially regulated by LDL in endothelial cells. LOX expression and activity are downregulated by LDL in a time- and concentration-dependent manner. Moreover, LOX inhibition was associated with an increase in endothelial permeability in vitro. Finally, in pigs fed an atherogenic diet, a significant downregulation of vascular LOX expression was observed. Considering the multiple cell functions in which LOX could be involved and its potential...
role in the atherogenic process, the effect of downregulation exerted by LDL could be crucial in endothelial dysfunction.

Methods

Cell Culture
Porcine aortic endothelial cells (PAECs) were obtained from adult normolipemic animals as described.22 Cells (10^3 cells per well in a 6-well plate) were grown in medium 199 ( Gibco), supplemented with 10% FCS ( Biological Industries), antibiotics (0.1 mg/mL streptomycin and 100μg/mL penicillin G), and 2 mmol/L l-glutamine. Forty-eight hours after the seeding, the cells were placed in serum-deprived (2% FCS) medium for 24 hours. Then, LDLs (180 mg/dL) were added for an additional 24 hours of incubation. Human umbilical vein endothelial cells were obtained by collagenase digestion and cultured as described.23 No cytotoxicity, analyzed by the trypan blue exclusion test and the XTT-based assay for cell viability (Roche), was observed after LDL (180 mg/dL for 48 hours) and β-aminopropionitrile (BAPN, 100 μmol/L for 16 hours) incubation.

In Vivo Animal Model
Female pigs (Landrace/Large white [Piensos Victoria SA, Barcelona, Spain], mean body weight at initiation 32 ± 2 kg) were randomized into 2 groups: normolipemic animals (n = 6) that were fed a normal chow and hyperlipemic animals (n = 10) that were fed a cholesterol-rich diet (2% cholester, 1% cholic acid, and 20% beef tallow) for 100 days.23,24 At the end of the dietary period, the animals were euthanized with a thiopental overdose. Plasma lipoproteins (HDL cholesterol, LDL cholesterol, and VLDL cholesterol) were fractionated by sequential ultracentrifugation (density 1.019 to 1.063 g/mL).

LDL Isolation
Porcine or human LDLs were obtained from fresh nonfrozen plasma by sequential ultracentrifugation (density 1.019 to 1.063 g/mL). Plasma total cholesterol was determined with an automatic analyzer (Kodak Ektachem DT System). Plasma lipoproteins (HDL cholesterol, LDL cholesterol, and VLDL cholesterol) were fractionated by agarose gel electrophoresis (Paragon System, North Reading, Mass). LDLs was assessed by agarose gel electrophoresis (Paragon System, North Reading, Mass). LDL used in the experiments were isolated by sequential ultracentrifugation (density 1.019 to 1.063 g/mL).

Plasma Biochemistry
Plasma total cholesterol was determined with an automatic analyzer (Kodak Ektachem DT System). Plasma lipoproteins (HDL cholesterol, LDL cholesterol, and VLDL cholesterol) were fractionated by agarose gel electrophoresis (Paragon System, North Reading, Mass). LDLs was assessed by agarose gel electrophoresis (Paragon System, North Reading, Mass). LDL used in the experiments were isolated by sequential ultracentrifugation (density 1.019 to 1.063 g/mL).

mRNA-DD Analysis
Cells grown in a 6-well plate, as indicated above, were incubated with LDL (180 mg/dL for 24 hours). Total RNA was isolated by using a QuickPrep total RNA kit (Pharmacia) or Ultraspec (Biotec) according to the manufacturer’s instructions. mRNA–differential display (DD) analysis was performed with a Delta RNA Fingerprinting kit (Clontech) by using 1 μg of total RNA as described previously.22 Reproducible DNAs from 3 independent experiments upregulated or downregulated by LDL were cloned and sequenced. Comparison of DNA homology with databases (GenBank) was performed by the use of BLAST, a National Center for Biotechnology Information server program.

Northern Blot Analysis
Total RNA was obtained as described above. RNA samples were fractionated in 1.2% agarose–2% formaldehyde gels. RNA was transferred by capillarity to Nytran Supercharge (Schleicher & Schuell) membranes and UV–cross-linked. Filters were hybridized by using the reamplified product from the DD–polymerase chain reaction (PCR) assay labeled with [α –32P]dATP as the LOX probe. A 28S ribosomal cDNA was used to normalize the results, as described previously.3 Filters were exposed to Agfa Curix RP2 X-ray films and quantified by densitometric analysis (Molecular Dynamics).

RT-PCR
Reverse transcription (RT)-PCR analysis was performed as described previously.22 The specific oligonucleotides selected were as follows: porcine LOX upper primer, 5′-gta aat age tgc tt ggt-3′; human LOX upper primer, 5′-cag gta aga ttc act gct-3′; porcine LOX lower primer, 5′-tct gta gat gcc agt ctc-3′. Amplification was carried out by 20 cycles of 94°C for 1 minute, 57°C for 1 minute, and 72°C for 30 seconds, followed by a final extension of 72°C for 7 minutes. Levels of GAPDH were used to normalize the results.3,22

LOX Activity
LOX activity was measured by a high-sensitivity fluorescence assay as previously described.22 Briefly, PAECs were plated (500 000 cells per dish) on 100-mm-diameter tissue culture dishes. Thirty-six hours after plating, cells were serum-depleted and fed with phenol red–free medium 199 for 18 hours. Cells were then reincubated with fresh medium containing 180 mg/dL LDL every 24 hours. LOX activity was measured in cell culture medium. The medium (200 μL) was incubated in the presence and absence of 500 μmol/L BAPN at 37°C for 30 minutes with 1 μmol/L horseradish peroxidase, 10 μmol/L Amplex red (Molecular Probes), and 10 mmol/L 1,5-diaminopentane in 1.2 mol/L urea and 0.05 mol/L sodium borate (pH 8.2). The reaction was stopped on ice, and differences in fluorescence intensity (563-nm excitation wavelength and 587-nm emission wavelength) between samples with and without BAPN were determined. Results were normalized by cell protein content.

Transendothelial Exchange
Endothelial permeability was determined by the exchange of FITC-dextran (M, 40 000, Sigma Chemical Co) through the endothelial monolayer as described previously.22 In brief, PAECs (10^4) were seeded on Transwell–collagen porous membranes (3-μm pore size and 0.33-cm² area, Costar) and cultured for 2 weeks to obtain a highly confluent culture. After 36 hours of incubation with LDL (140 mg/dL) or 16 hours with BAPN (100 μmol/L), FITC-dextran (10 μmol/L) was added to the upper compartment. After 6 hours, samples (50 μL) from the lower compartment were taken, and dextran transfer was determined in a fluorimeter (495-nm excitation wavelength and 530-nm emission wavelength).

Statistical Analysis
Data are expressed as mean±SD. Means were compared by ANOVA. For the in vivo study, statistical differences between groups were analyzed by the Mann–Whitney U test. Differences were considered significant at P<0.05.

Results

DD Analysis
The effect of atherogenic concentrations of LDL (180 mg/dL for 24 hours) on PAEC expression patterns was assessed by DD-PCR analysis using cDNAs from independent experiments performed with PAECs and LDLs from 3 different animals. A DD-PCR assay performed with primers T8 and P8 showed a downregulated band in a reproducible manner (Figure 1A). This cDNA was cloned, sequenced, and compared with the GenBank database by the National Center for Biotechnology Information server program BLAST. This cDNA shared high homology (90%) with the human LOX.
gene (exon 7) between positions 1649 and 1975 (GenBank accession No. L16895). To confirm the downregulation produced by LDL treatment, new experiments were performed in which PAECs were incubated with LDL (180 mg/dL for 24 hours, n=5), and Northern blot assays were carried out with the LOX-cloned product used as a probe. As shown in Figure 1B, LDL significantly downregulated LOX mRNA levels (P<0.001). Similar results were obtained by RT-PCR assays with specific oligonucleotides (data not shown).

**Time- and Dose-Dependent Effect of LDL on LOX Expression in Endothelial Cells**

Although DD experiments were performed in serum-deprived cells (2% FCS), the downregulation of LOX by atherogenic levels of LDL was also observed in the presence of 10% FCS, conditions that did not affect LOX basal expression (please see online Figure I, available at http://www.ahajournals.org). Thus, subsequent experiments were performed in serum-deprived cells. LOX mRNA levels decreased in a dose-dependent manner. As shown in Figure 2, LOX expression was significantly reduced by low LDL concentrations (74% inhibition by 50 mg/dL). To determine the time course of LOX mRNA expression, total RNA was isolated at different times from PAECs incubated with LDL (180 mg/dL). The LOX mRNA level, analyzed by RT-PCR, decreased after 12 hours of incubation with LDL, and expression levels remained low after 24 hours. Similar LDL-mediated downregulation effects on LOX expression were observed in human umbilical vein endothelial cells (please see online Figure II, available at http://www.ahajournals.org).

**Effect of LDL on LOX Activity**

LOX activity was determined in PAECs incubated with LDL (180 mg/dL). LDL significantly decreased LOX activity in the cell culture medium (40% and 54% of inhibition after 24 and 48 hours, respectively; Figure 3A).

**Effect of LDL on Endothelial Barrier Function**

Modulation of transendothelial exchange by LDL and BAPN, an inhibitor of LOX activity, was analyzed. As shown in Figure 3B, LDL and BAPN caused a significant increase in FITC-dextran transfer compared with control cultures (~2- and 3-fold, respectively), an effect that became apparent 6 hours after the addition of FITC-dextran. These results suggest that LOX inhibition disturbs endothelial barrier integrity.

**Effect of DRB and Cycloheximide**

To characterize the mechanisms involved in the downregulation of LOX expression by LDL, we analyzed the effect of 5,6-dichlorobenzimidazole (DRB), a transcriptional inhibitor, and cycloheximide, a protein synthesis inhibitor. PAECs were incubated with or without LDL for 24 hours, and then transcription was inhibited by DRB (50 μmol/L, time 0). LOX mRNA levels were analyzed by Northern analysis at different times (0, 6, 9, 12, and 24 hours). LDL-treated cells showed a half-life (~24 hours) similar to that of control cells; thus, the effect of LDL could not be attributable to a decrease in LOX mRNA stability (data not shown). DRB (Figure 4A) and cycloheximide (Figure 4B) blocked the effect of LDL on the downregulation of LOX expression.

![Figure 1](http://www.ahajournals.org/)

**Figure 1.** LOX expression is downregulated by atherogenic LDL concentrations in PAECs. A, PAECs were incubated with LDL (180 mg/dL) for 24 hours, and mRNA-DD analysis was performed as described in Methods. Only reproducible differentially displayed bands from 3 independent experiments performed with PAECs from different animals were considered for subsequent analysis. The arrow shows a band downregulated by LDL. This cDNA was cloned and sequenced, showing identity with the human LOX gene (exon 7). B, Northern blot is shown from PAECs incubated with LDL (180 mg/dL, 24 hours) with the use of LOX cDNA obtained in the mRNA-DD assay as a probe. Blots were quantified, normalized by 28S ribosomal RNA levels, and expressed as mean±SD (n=5). CT indicates control. *P<0.001.

![Figure 2](http://www.ahajournals.org/)

**Figure 2.** Dose-dependent effect of LDL on LOX mRNA levels. PAECs were treated with increasing LDL concentrations for 24 hours. Representative autoradiographs from 2 different assays performed in duplicate are shown. Blots were quantified, normalized by GAPDH mRNA levels, and expressed as percentage of control.
LOX mRNA levels, suggesting that transcriptional and post-transcriptional mechanisms are involved in the LDL-mediated effect.

**In Vivo Effects of Hypercholesterolemia on LOX Expression in the Vessel Wall**

LOX expression was analyzed by RT-PCR in porcine abdominal aortas from normolipemic and hypercholesterolemic pigs. Animals fed the hypercholesterolemic diet, compared with animals fed the normolipemic diet, showed higher plasma LDL cholesterol levels (333 ± 1100 versus 34 ± 10.9 mg/dL, respectively; P < 0.01). Vascular LOX mRNA levels were significantly reduced (∼3-fold) by the hypercholesterolemic diet (P < 0.01, Figure 5).

**Discussion**

We have identified LOX by DD-PCR analysis as a gene downregulated by LDL in a dose- and time-dependent manner in porcine and human endothelial cells. This enzyme catalyzes a key step in the cross-linking of collagen and elastin, leading to maturation of the ECM. Although LOX regulation has been extensively studied in fibroblast and smooth muscle cells, little is known about its role in endothelial cells.28 The observed decrease in PAEC LOX expression and activity by LDL led us to hypothesize that this effect could be associated with alterations in endothelial ECM processing and vascular permeability in hypercholesterolemia-mediated endothelial dysfunction.

We have analyzed the mechanisms involved in LOX regulation by LDL. Altogether, the experiments performed in the presence of cycloheximide and DRB suggest the involvement of a transcriptional mechanism, mainly because a decrease in LOX mRNA stability was not observed after LDL incubation. The long LOX mRNA half-life observed in our

![Figure 3](image1.png)

**Figure 3.** Effect of LDL on LOX activity and transendothelial exchange. A. LOX activity was measured in culture media from PAECs after 24 and 48 hours of incubation with LDL (180 mg/dL). Results were normalized by cell protein content and are expressed as the mean ± SEM of 3 assays performed in triplicate. A.U. indicates arbitrary units; open bars, control; and solid bars, LDL treatment. *P < 0.02 and **P < 0.01. B. PAECs were incubated with LDL (140 mg/dL for 36 hours) or BAPN (100 μmol/L for 16 hours), and transendothelial exchange of FITC-dextran was evaluated after 6 hours. Results are mean ± SEM of 3 assays performed in duplicate. *P < 0.001 and **P < 0.03.

![Figure 4](image2.png)

**Figure 4.** Mechanisms involved in downregulation of LOX expression by LDL. A, PAECs were incubated with LDL (180 mg/dL) alone or in combination with 50 μmol/L DRB for 12 hours. B, PAECs were incubated with LDL (180 mg/dL) alone or in combination with cycloheximide (CHX, 2 μg/mL) for 12 hours. LOX mRNA levels were determined by RT-PCR. Results were normalized by GAPDH mRNA levels. Representative RT-PCR assays (n=2) are shown.

![Figure 5](image3.png)

**Figure 5.** Effect of hypercholesterolemia on vascular LOX mRNA levels. Densitometric analyses of LOX mRNA levels in abdominal aorta samples from normolipemic pigs (NORMO) and hyperlipemic pigs (HYPER) are shown. Results were normalized by GAPDH mRNA levels. A representative RT-PCR is shown.
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culture conditions (2% FCS) is in agreement with that reported in other cell types, such as smooth muscle cells maintained in low serum culture or quiescent IMR90 fibroblasts. However, in PAECs, serum deprivation did not alter LOX endothelial expression, and the reported superinduction of LOX mRNA by cycloheximide was not observed. Thus, differential regulation of LOX expression could be observed depending on the cell type. In this sense, it has been speculated that cell type–specific mechanisms could be involved in LOX regulation, probably mediated by different promoter elements. Supporting the data, we observed similar LOX downregulation by LDL in vascular cells from human and porcine origin. In addition, we observed that LOX downregulation by LDL is accompanied by a reduction in LOX activity. This inhibition was produced in the absence of any effect on bone morphogenetic protein (bMP) 1 gene expression (authors’ unpublished data, 2002), the C-proteinase that processes pro-LOX to its active form with the highest LOX activity. This inhibition was produced in the absence of any effect on bone morphogenetic protein 1 gene expression (authors’ unpublished data, 2002), the C-proteinase that processes pro-LOX to its active form with the highest activity.

The increased endothelial permeability induced by BAPN, a specific inhibitor of LOX activity, and LDL suggests a role of LOX in the alteration of endothelial barrier function caused by LDL. Alterations in endothelial ECM composition by atherogenic concentrations of LDL have been reported and have been associated with an increase in endothelial permeability. However, there are some conflicting reports in the literature. Mildly oxidized LDL but not native LDL, at low concentrations and short incubation times (2 minutes), have been reported to increase endothelial permeability. Other authors testing nonatherosclerotic LDL concentrations concluded that LDL did not alter endothelial permeability.

In the present study, we report that LOX downregulation is associated with an increased permeability. Moreover, not only does the inhibition of LOX maintain collagen in a soluble form, but this soluble form is also more susceptible to metalloproteinase degradation. On the other hand, because LOX activates the transcription of collagen III, LOX downregulation by LDL could trigger an additional decrease in some ECM components. Thus, LOX by different mechanisms could modulate ECM integrity and could play a key role in the modulation of endothelial function. Interestingly, in vivo, in a model of early atherosclerosis, vascular LOX mRNA levels were downregulated by the hypercholesterolemic diet. No changes or even increases in vascular LOX activity have been previously reported in other animal models; however, differences in LOX regulation between species and even differences in diet composition and types of lesions in these models could explain these discrepancies. Indeed, we have chosen the porcine animal model for the present study because it develops lesions resembling those in humans. After 100 days on a cholesterol-rich diet, pigs develop lesions that can be classified from arterial intimal thickening to type I and II according to the American Heart Association classification.

Although the LDL-mediated LOX downregulation mechanism is largely unknown, preliminary data suggest that sterol regulatory element–binding proteins are not involved because no sterol regulatory element has been identified in the LOX promoter and because ALLN, an inhibitor of sterol regulatory element–binding protein catabolism, did not alter the LDL effect. On the other hand, LOX downregulation does not seem to be mediated by NO because endothelial NO synthase inhibition by Nω-nitro-L-arginine methyl ester did not affect LOX expression (authors’ unpublished data, 2002).

In addition to hypercholesterolemia, other atherosclerotic risk factors, such as hyperhomocysteinemia, could be associated with decreases in LOX activity, inasmuch as homocysteine thiolactone and other analogues are irreversible inhibitors of LOX. Thus, LOX downregulation could be involved in the pathogenesis of atherosclerosis. In the present study, we have observed that hypercholesterolemia downregulates vascular LOX expression, an effect that could be related to an increase in endothelial permeability in the early steps of the atherosclerotic process. Further experiments focusing on the regulation of LOX activity in the course of LDL-induced vascular changes are necessary to clarify the role of LOX in this process.

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References


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Figure I. *Effect of culture conditions on LOX downregulation by LDL.* LOX mRNA levels from PAEC incubated in the presence (+) or in the absence (-) of LDL (180 mg/dL, 24 h) in different culture conditions (10% or 2% FCS). Representative autoradiographies from 2 different assays performed in duplicate are shown.

Figure II. *Time-dependent effect of LDL on LOX mRNA levels in vascular endothelial cells.* PAECs or HUVECs were incubated with LDL from pig or human origin respectively (180 mg/dL), during the times indicated. LOX mRNA levels were determined by RT-PCR. Representative autoradiographies from 2 different assays performed in duplicate are shown. Blots were quantified, normalized by GAPDH mRNA levels and expressed as percent of controls. Black diamonds and white squares correspond to PAECs and HUVECs respectively.