Electronegative LDL From Normolipemic Subjects Induces IL-8 and Monocyte Chemotactic Protein Secretion by Human Endothelial Cells

Conxita De Castellarnau, José Luis Sánchez-Quesada, Sonia Benítez, Roser Rosa, Luis Caveda, Luis Vila, Jordi Ordóñez-Llanos

Abstract—The presence in plasma of an electronegative LDL subfraction [LDL(−)] cytotoxic for endothelial cells (ECs) has been reported. We studied the effect of LDL(−) on the release by ECs of molecules implicated in leukocyte recruitment [interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1)] and in the plasminogen activator inhibitor-1 (PAI-1). LDL(−), isolated by anion-exchange chromatography, differed from nonelectronegative LDL [LDL(+)1] in its higher triglyceride, nonesterified fatty acid, apoprotein E and apoprotein C-III, and sialic acid contents. No evidence of extensive oxidation was found in LDL(−); its antioxidant and thiobarbituric acid–reactive substances contents were similar to those of LDL(+)1. However, conjugated dienes were increased in LDL(−), which suggests that mild oxidation might affect these particles. LDL(−) increased, in a concentration-dependent manner, the release of IL-8 and MCP-1 by ECs and was a stronger inducer of both chemokines than oxidized LDL (oxLDL) or LDL(+)1. PAI-1 release increased slightly in ECs incubated with both LDL(−) and oxLDL but not with LDL(+)1. However, no cytotoxic effects of LDL(−) were observed on ECs. Actinomycin D inhibited the release of IL-8 and MCP-1 induced by LDL(−) and oxLDL by up to 80%, indicating that their production is mediated by protein synthesis. Incubation of ECs with N-acetyl cysteine inhibited production of IL-8 and MCP-1 induced by LDL(−) and oxLDL by >50%. The free radical scavenger butylated hydroxytoluene slightly inhibited the effect of oxLDL but did not modify the effect of LDL(−). An antagonist (BN-50730) of the platelet-activating factor receptor inhibited production of both chemokines by LDL(−) and oxLDL in a concentration-dependent manner. Our results indicate that LDL(−) shows proinflammatory activity on ECs and may contribute to early atherosclerotic events. (Arterioscler Thromb Vasc Biol. 2000;20:2281-2287.)

Key Words: LDL ■ electronegative LDL ■ interleukin-8 ■ monocyte chemotactic protein ■ endothelial cells

Evidence from both in vitro and in vivo studies suggests that oxidative modification of LDL plays a key role in the development of atherosclerosis and the onset of coronary artery disease (CAD).1 In vivo LDL oxidation has been demonstrated by the presence of oxidized LDL (oxLDL) and oxidized lipids in atherosclerotic lesions,2 circulating autoantibodies to modified forms of LDL3,4 and higher plasma levels of oxLDL and malondialdehyde-modified LDL in patients with CAD than in healthy subjects.4 The extent to which LDL is oxidized has a fundamental influence on its physical and biological properties. Extensively oxidized LDL contains abundant lipid peroxidation products and displays atherogenic properties, such as its uptake by scavenger receptors, cytotoxicity, vasoconstriction, and chemotaxis for circulating leukocytes.5,15 In contrast, when LDL is mildly oxidized, ie, minimally modified LDL (MM-LDL), it contains fewer lipid peroxidation products and is recognized by the native LDL receptor; however, it also stimulates monocyte chemotaxis and adherence to and transmigration through endothelial cells (ECs).5–8 Chemotaxis of leukocytes is known to play a key role in the initiation and development of the atheromatous lesion.6 The monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) are the major chemoattractant cytokines for monocytes and T lymphocytes6–10 and for neutrophils,6,10,11 respectively. Both chemokines are released by ECs, smooth muscle cells, and macrophages in response to modified LDLs7–11 and proinflammatory cytokines.12 The activation of ECs by native and modified LDLs also modulates, among other factors involved in inflammation and thrombosis,13 the production of plasminogen activator inhibitor-1 (PAI-1).14

Despite the pivotal role of oxLDL in the development of atherosclerosis, the existence in blood of significant amounts of modified forms of LDL with atherogenic properties is a matter of controversy. In the past few years, a minor electronegative subfraction of LDL has been isolated from human plasma by anion-exchange chromatography.15 This subfraction is found in a concentration great enough to play a significant role in the pathogenesis of atherosclerosis. It has been suggested that electronegative LDL [LDL(−)] was the

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2281
result of peroxidative processes, because these particles had a higher content of lipoperoxides and cholesterol oxides and a lower content of a-tocopherol than native LDL. However, the oxidative nature of LDL(−) has been questioned by other authors, because they found no lipid oxidation markers.

Enhanced contents in triglyceride, sialic acid, and apoproteins (apo) E and apoC-III have been reported to be the main determinants for the negative charge of LDL(−). Controversy also exists regarding its interaction with LDL receptors, with findings indicating lower, similar, or higher affinity to the LDL receptor. Nevertheless, all authors agree that these particles are cytotoxic on ECs, suggesting that LDL(−) could affect EC function and be atherogenic. A relevant role in atherogenesis for this fraction is supported by the observation that the relative proportion of LDL(−) is elevated in subjects at high risk of atherosclerosis, such as familial hypercholesterolemia and type 1 diabetic patients. In addition, a positive association between serum cholesterol levels and LDL(−) has been reported. The role of LDL(−) in endothelial function remains unknown. Given the importance of elevated MCP-1 and IL-8 levels in the recruitment, extravasation, and migration of leukocytes toward the inflammatory loci and the role of PAI-1 as a local regulator of fibrinolysis, the present study examined the effect of LDL(−) isolated from normolipemic subjects in the release of these molecules from cultured human vascular ECs. In addition, the implication of oxidative processes was also studied by the use of the free radical scavenger butylated hydroxytoluene (BHT), the platelet activating factor (PAF) antagonist BN-50730, and the glutathione donor N-acetyl cysteine (NAC).

Methods

Isolation of LDL

Plasma samples from healthy normolipemic donors (age 22 to 47 years, 14 male, 19 female, all nonsmokers) were obtained in EDTA-containing Vacutainer tubes and stored at -80°C until analysis. Aliquots of plasma were pooled, and LDL (1.020 < d < 1.050 g/mL) was isolated by sequential ultracentrifugation and filtered through 0.45-μm filters. This range of densities was chosen to avoid coisolation of LDL with lipoprotein (a) (Lp(a)). Ultrafiltration was performed at 4°C with 1 mmol/L EDTA. During LDL manipulation, general precautions to avoid lipopolysaccharide (LPS) contamination were taken, and regular controls against buffer A, its content on apoB was quantified, and 20 to 30 mg (10 mmol/L Tris-HCl, 1 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4) was added to PBS-dialyzed LDL with lipoprotein (a) [Lp(a)]. LDL was dialyzed against buffer A by gel filtration chromatography (FPLC) system (Amersham Pharmacia). The counterion was introduced by washing of the column with 300 mL of elution buffer B (10 mmol/L Tris-HCl, 1 mol/L NaCl, 1 mmol/L EDTA, pH 7.4) and then equilibration with 300 mL of buffer A.

Isolation of LDL(−) by Anion-Exchange Chromatography

LDL(−) was isolated by anion exchange chromatography as described, with modifications. A preparative Q-Sepharose High Performance 35/100-mm column was adapted to a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia). The counterion was introduced by washing of the column with 300 mL of elution buffer B (10 mmol/L Tris-HCl, 1 mol/L NaCl, 1 mmol/L EDTA, pH 7.4) and then equilibration with 300 mL of buffer A.

Endothelial Cell Culture and Incubation With LDL Subfractions

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described. Briefly, cells were grown in medium 199 containing 20% bovine serum, 2 mmol/L L-glutamine, 20 mmol/L HEPES, antibiotics (Biological Industries), 30 mg/L endothelial cell growth supplement (ECGS), and 100 mg/L heparin (Sigma). Confluent first- and second-passage cells grown in 12-well culture plates (except when indicated) were used. Twenty-four hours before the experiment, growth medium was removed from the wells and replaced by heparin- and ECGS-free medium 199 with 4% inactivated human serum (maintenance medium). Cells were then incubated at 37°C for 24 hours with or without the addition of LDL(+), LDL(−), or oxLDL at different concentrations (0, 17.5, 35, 70, 140, and 210 mg apoB/L). Tumor necrosis factor-α (TNF-α) at 20 μg/L was used as a positive control for chemokines and PAI-1 induction.

Figure 1. Representative chromatogram of LDL(+) and LDL(−) isolation. Procedure was as described in Methods. LDL(+) subfraction eluted at 0.22 mol/L of NaCl, and LDL(−) subfraction eluted at 0.5 mol/L NaCl. LDL(−) was 8.6% (measured by peak area), 9.0% (measured as cholesterol), or 8.9% (measured as apoB) of total LDL. Inset, Electrophoretic mobility in agarose gels of 1, LDL(+) 2, LDL(−); and 3, oxLDL.
The time-dependent effect of LDLs on IL-8, MCP-1, and PAI-1 production was analyzed in HUVECs incubated with 140 mg apoB/L for 2, 4, 6, 24, and 48 hours. Before cell incubation, LDLs were dialyzed against PBS containing 1 mmol/L CaCl₂ and 0.5 mmol/L MgCl₂, pH 7.4 (PBS+). Then, apoB was measured, filtered through 0.22-μm filters, and diluted at the indicated final concentration with PBS+ and maintenance medium. Control experiments in the presence of polymyxin B (50 mg/L) in the medium were performed to exclude the possibility that trace amounts of LPS present in LDL samples were responsible for the IL-8, MCP-1, and PAI-1 release. To determine whether free radicals or oxidized phospholipids were involved in IL-8 and MCP-1 production, NAC, BHT, and the PAF antagonist BN-5073035 (kindly donated by Dr Braquet, Institut Henri Beaufour Research Laboratories, Paris, France) were used. HUVECs were preincubated for 2 hours with 10 mmol/L NAC or for 15 minutes with 1, 2.5, 5, and 10 μmol/L of BN-50730 before addition of LDL (140 mg apoB/L) and for 24 hours of incubation. BHT was preincubated for 2 hours at 40°C until analysis. Experiments in the presence of polymyxin B (50 mg/L) in the medium were performed to exclude the possibility that trace amounts of LPS present in LDL samples were responsible for the IL-8, MCP-1, and PAI-1 release.

The cytotoxicity of LDLs was examined by incubating cells in 96- or 210 mg apoB/L for 2, 4, 6, 24, and 48 hours. Before cell incubation, LDLs were preincubated for 2 hours at 40°C and 24 hours of incubation. The time-dependent effect of LDLs on IL-8, MCP-1, and PAI-1 was measured.37 Briefly, propidium iodide (Molecular Probe) fluorescence scanner (530 nm excitation and 645 nm emission) and results were expressed as ng/10⁵ cells, ng/10⁵ cells, and μg/10⁵ cells, respectively.

Density of LDL

At the end of incubation, supernatants from each well were collected, centrifuged to eliminate debris, and frozen at −80°C. Before cell incubation, LDLs were added (final concentration 4 mg/L) to the media of confluent HUVECs in 24-well plates treated with LDLs 1 hour before the end of incubation. Fluorescence was quantified with a Cytofluor 2350 (Millipore) fluorescence scanner (530 nm excitation and 645 nm emission), and results were expressed as percentage of living cells.38

**Composition, Antioxidants, TBARS, and Conjugated Diene Contents of LDL(+) and LDL(−) Subfractions**

<table>
<thead>
<tr>
<th></th>
<th>LDL(+)</th>
<th>LDL(−)</th>
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<tbody>
<tr>
<td>Total cholesterol, %</td>
<td>43.8±0.3</td>
<td>43.8±0.3</td>
</tr>
<tr>
<td>Esterified cholesterol, %</td>
<td>31.1±0.6</td>
<td>31.0±2.2</td>
</tr>
<tr>
<td>Free cholesterol, %</td>
<td>12.5±1.7</td>
<td>12.8±1.5</td>
</tr>
<tr>
<td>Triglycerides, %</td>
<td>5.9±1.1</td>
<td>7.3±1.2*</td>
</tr>
<tr>
<td>Phospholipids, %</td>
<td>27.2±2.4</td>
<td>26.7±2.6</td>
</tr>
<tr>
<td>ApoB, %</td>
<td>27.3±1.3</td>
<td>25.8±1.7</td>
</tr>
<tr>
<td>NEFAs, mol/mol apoB</td>
<td>3.1±4.2</td>
<td>12.9±10.5*</td>
</tr>
<tr>
<td>ApoE, mol/mol apoB</td>
<td>0.013±0.010</td>
<td>0.147±0.160*</td>
</tr>
<tr>
<td>Lipoprotein(a), mol/mol apoB</td>
<td>0.034±0.040</td>
<td>0.160±0.120*</td>
</tr>
<tr>
<td>ApoB-III, mol/mol apoB</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ApoC-II, mol/mol apoB</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Sialic acid, mol/mol apoB</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>α-Tocopherol, mol/mol apoB</td>
<td>17.0±6.4</td>
<td>22.1±6.4*</td>
</tr>
<tr>
<td>α-Carotene, mol/mol apoB</td>
<td>8.2±1.2</td>
<td>8.5±1.9</td>
</tr>
<tr>
<td>β-Carotene, mol/mol apoB</td>
<td>0.04±0.04</td>
<td>0.04±0.03</td>
</tr>
<tr>
<td>Lycopene, mol/mol apoB</td>
<td>0.14±0.04</td>
<td>0.12±0.03</td>
</tr>
<tr>
<td>TBARS, μmol/g apoB</td>
<td>0.22±0.15</td>
<td>0.35±0.18</td>
</tr>
<tr>
<td>Conjugated dienes, μmol/g apoB</td>
<td>117±38</td>
<td>144±56*</td>
</tr>
</tbody>
</table>

ND indicates not detected. Results are the mean±SD of 14 independent experiments. *P<0.05.

LDL Composition

Differences in composition between LDL(+) and LDL(−) are shown in the Table. Concerning lipids, LDL(−) was significantly enriched in triglycerides compared with LDL(+). NEFAs, apoproteins, and sialic acid content also differed significantly between LDL(+) and LDL(−). NEFAs associated with LDL were increased 4-fold in LDL(−) compared with LDL(+). ApoA-I and apoC-II were not detected either in LDL(−) or in LDL(+); nevertheless, apoE and apoC-III were 11- and 5-fold higher in LDL(−) than in LDL(+), respectively. Sialic acid content was increased 30% in LDL(−).

Antioxidant content (α-tocopherol and carotenoids) was similar in both LDL fractions (Table). TBARS were slightly increased in LDL(−), although significant differences were not obtained with respect to LDL(+). Conjugated diene content was significantly higher in LDL(−) than in LDL(+). These data suggest the presence of a low amount of oxidized lipids in LDL(−), but they indicate that this LDL subfraction was not extensively oxidized, because higher contents of TBARS (13.8±5.7 μmol malondialdehyde/g protein) and conjugated dienes (429±379 μmol/g protein) were found in oxLDL than in either LDL subfraction (P<0.05), and no detectable amounts of α-tocopherol or carotenoids were observed.

**Electrophoretic Studies**

LDL(−) had slightly higher relative motility than LDL(+) (Rf 1.1±0.1, P<0.05, n=14) but lower than that of oxLDL
ApoB integrity was demonstrated in both LDL(-) and LDL(+), but not in oxLDL.

**Effect of LDL(-) on IL-8, MCP-1, and PAI-1 Release**

Treatment of HUVECs with oxLDL and LDL(-), but not with LDL(+), resulted in a significantly increased release of IL-8 into the culture supernatants compared with media from untreated HUVECs after 24 hours of treatment (Figure 2A). This incubation time was chosen after preliminary time-course experiments in which the chemokine release was detectable after 4 hours of incubation, increased up to 6 to 8 hours, and remained at a high level for 24 hours (data not shown). MCP-1 and PAI-1 were also measured at 24 hours of incubation. The LDL(-)-induced release of IL-8 was concentration-dependent. Differences versus untreated or LDL(+)-treated cells reached significance at 35 to 210 mg apoB/L for LDL(-) and oxLDL (Figure 3) and >70% when induced by TNF-α (data not shown). In contrast, preincubation of LDLs with BHT had a slight effect on IL-8 and MCP-1 release (Figure 3), which was statistically significant only when induced by oxLDL. No inhibition of IL-8 and MCP-1 induction was observed when treated with BHT (free radical scavenger), NAC (free radical scavenger and glutathione precursor), and BN-50730 (PAF antagonist) were assessed to determine whether free radicals or PAF-like oxidized phospholipids were involved in chemokine release induced by LDL(-).

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**Inhibition of the Effect of LDL(-) on IL-8 and MCP-1 Release**

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BHT was simultaneously mixed with HUVECs and LDL (data not shown).

Pretreatment of cells with increasing amounts of BN-50730 before LDL addition inhibited, in a concentration-dependent manner, the production of chemokines in LDL(−) and oxLDL-treated cells (Figure 3). Approximately 50% of inhibition was obtained at 10 μmol/L.

Actinomycin D treatment inhibited LDL(−) and oxLDL-induced IL-8 (60% to 80% inhibition) and MCP-1 (70% to 80% inhibition) generation from HUVECs to levels close to those of LDL(+).

**Cytotoxic Effects of oxLDL, LDL(−), and LDL(+)**

No toxic effects were found with the LDL(+) and LDL(−) subfractions at a protein concentration of up to 210 mg/L after 24 and 48 hours of incubation with ECs (please see www... Figure III). In contrast, some morphological changes, such as EC gaps and cell detachment, occurred after 24 hours of incubation with oxLDL at 210 mg/L, and significant cytotoxicity was found after 48 hours of incubation. Finally, exposure of HUVECs to 7β-hydroxycholesterol resulted in concentration- and time-dependent damage, as observed at 50 μmol/L with the cytotoxicity tests. At higher concentrations (100 μmol/L), this oxysterol produced cell detachment and was highly toxic (results not shown).

**Discussion**

In the present study, we report that LDL(−), a plasma modified subfraction of LDL, causes IL-8 and MCP-1 release in HUVECs. Induction of chemotactic activity in ECs has been demonstrated by in vitro–modified LDL, such as MM-LDL7–9 and oxLDL,10,11 but not by native LDL.8,31 However, to the best of our knowledge, this is the first report of a proinflammatory action of a minimally modified LDL present in the circulation.

It has been suggested that LDL(−) could be the in vivo counterpart of in vitro–obtained MM-LDL, because variable amounts of lipoperoxides have been reported in LDL(−).16–19

The present study shows that major compositional differences between LDL(−) and LDL(+) consist of the higher triglyceride, sialic acid, apo E, and apoC-III content. These data concur with those reported when similar FPLC procedures were used to isolate LDL(−),21–23 In addition, we observed a relative enrichment of the NEFA content in LDL(−) compared with LDL(+) (data not shown). Whether or not these components, which are responsible for the electronegative charge,39–41 could mediate the proinflammatory effect of LDL(−) remains unknown. Our data indicate that LDL(−) was not extensively oxidized because its antioxidant and TBARS contents were normal, apoB was not degraded, and electrophoretic motility in agarose was only slightly increased compared with LDL(+) (data not shown). Only increased conjugated dienes indirectly suggest that LDL(−) might present low amounts of oxidized lipids.

OxLDL, but not native LDL, has been reported to increase IL-8 production in the EA.hy 926 endothelial cell line41 and in monocytes.6,10 Mildly oxidized LDLs stimulate monocyte chemotaxis, adherence to and transmigration through the endothelium, and expression of growth factors and cytokines.5–9 The effect of native LDL and oxLDL observed in the present study concur with these observations. However, the fact that LDL(−) is more active and proinflammatory to human ECs than oxLDL is in agreement with the greater biological activity of MM-LDL compared with oxLDL5,8,13 and with the proinflammatory effect described for all plasma apoB-containing lipoproteins after minimal modifications.42

The use of oxidation inhibitors supports the role of oxidized lipids for the enhanced IL-8 and MCP-1 production induced by LDL(−). NAC, which acts as a glutathione precursor and a free radical scavenger,33,44 was the strongest inhibitor of IL-8 and MCP-1 synthesis induced by both LDL(−) and oxLDL in HUVECs. This observation suggests that oxidative stress participates in this response, as has been described for IL-8 synthesis induced by oxLDL in the EA.hy 926 EC line.11 Thus, the response of endothelium to proinflammatory stimuli may be strongly regulated by the redox status of the cell.11,43 In contrast, the lipophilic antioxidant BHT, which neutralizes the lipoperoxide-derived free radicals contained in oxLDL, partially inhibited MCP-1 and IL-8 production induced by oxLDL but showed no effect on LDL(−)-stimulated cells. This finding suggests that chemokines could be released through different mechanisms by oxLDL and LDL(−). Conversely, the observation that stimulation of both IL-8 and MCP-1 by LDL(−) was partially inhibited by a PAF-receptor antagonist suggests that LDL(−) activity could result in part from the presence of PAF or PAF-like molecules able to activate the PAF receptor.5,42,45

PAI-1 release was slightly induced by LDL(−) and oxLDL, but no differences were found between these particles and LDL(+) (data not shown), which showed a slight concentration-dependent increase; these results concur with previous data with native and modified LDLs.14 The findings that LDL(−) strongly induced IL-8 and MCP-1 synthesis by ECs and that only a small effect was observed with PAI-1 could be explained by a possible effect of LDL(−) on the nuclear transcription factor-κB (NF-κB). In fact, IL-8 and MCP-1, but not PAI-1, genes contain NF-κB binding sites in their promoters.5,46,47 NAC prevents the synthesis of cytokines induced by TNF-α in HUVECs through the inhibition of NF-κB.43 This observation is in accordance with our finding that NAC inhibits the release of IL-8 and MCP-1 induced by LDL(−), oxLDL, and also TNF-α (data not shown).

In contrast, it has been reported that NAC has no effect on TNF-α-induced PAI-1 synthesis, which is induced in an NF-κB–independent manner.47 These results suggest that intracellular free radicals could be involved in the effects of LDL(−) on chemokine release by ECs. Another possibility is that NEFAs, which are increased in LDL(−), could mediate the proinflammatory response, because it has been reported that NEFAs induce NF-κB activation.46,48 It is tempting to speculate, however, that the effects of LDL(−) on IL-8 and MCP-1 could be indirectly mediated through endogenous production of first-wave cytokines, such as TNF-α or IL-1.10,16 Further experiments are necessary to characterize the mediators and/or the signal transduction pathways involved in LDL(−) action.

Our study showed LDL(−) proinflammatory action to be specific and not due to LPS contamination, because (1) neither maintenance media nor LDL(+) derived from the same plasma source as LDL(−) increased chemokine production; (2) LPS inhibitor polymyxin B did not prevent LDL(−)- and oxLDL-induced chemokine release; and (3) undetectable levels of LPS were found in LDL preparations.
In contrast to studies reporting that the LDL(−) subfraction is cytotoxic in HUVECs21 and in rabbit aortic ECs,17 we found no toxic effect in LDL(−)-treated HUVECs. This result indicates that small amounts of circulating LDL(−) could locally initiate the synthesis of inflammatory chemokines, a process more evident in some conditions, such as diabetes mellitus or hypercholesterolemia, in which the relative proportion of LDL(−) is strongly increased.24,25 However, because the composition of LDL(−) in these patients may differ from that of healthy subjects, investigations into the effect of LDL(−) from these patients are required. Furthermore, it should be stated that the in vitro response of HUVECs to LDL(−) may not represent the in vivo situation in human vessels. Thus, further studies evaluating in parallel the proportion of LDL(−) and endothelial dysfunction are necessary to confirm the proatherogenic role of this electronegative LDL subfraction in vivo. In conclusion, results obtained in the present study indicate that LDL(−)-mediated release of chemokines by activated endothelium occurs without apparent toxic effects and may participate in the adherence of leukocytes to ECs, an early event in atherogenesis and inflammation.

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References


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Figure I. Concentration-dependent effect of LDL(-), LDL(+) and oxLDL on PAI-1 secretion from HUVEC. Cells were treated with different concentrations of LDL(-), LDL(+) or oxLDL for 24 hours. Levels of PAI-1 antigen in the media were determined by ELISA. Each point represents the mean ± SD of 6 independent experiments.

* P < 0.05 vs untreated HUVEC

*C de Castellarnau et al; please see www...Figure I*
**Figure II.** Cytotoxic effect of LDLs measured by propidium iodide staining and MTT test in HUVEC. Confluent cells were incubated 24 and 48 hours in maintenance medium (M199 with 4% v/v human serum) with or without 210 mg apoB/L of LDL(-), LDL(+), and oxLDL or 50 µmol/L of 7β-hydroxycholesterol. Propidium nuclei staining (upper panel) was expressed as percentage of the staining increase obtained after treatment with 0.2 % Triton X-100. The MTT assay (lower panel) was expressed as the percentage of the absorbance at 490 nm for cells incubated without LDLs. Results are mean ± SD of 4 separate experiments in triplicate.

* P < 0.05 vs control

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**Figure II A**

- **Legend:**
  - LDL (+)
  - LDL (-)
  - ox -LDL
  - 7β-hydroxycholesterol

**Figure II B**

- **Legend:**
  - MTT test (% of control)

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C de Castellarnau el al; please see www...... Figure II