Oxidized Low Density Lipoprotein Stimulates Prostacyclin Production by Adult Human Vascular Endothelial Cells

J. Eric Triau, Simin Nibkin Meydani, and Ernst J. Schaefer

Interactions between vascular endothelium and low density lipoprotein (LDL) have been implicated in the development of atherosclerosis. The effect of normal and oxidized LDL (Ox-LDL) on prostaglandin release by cultured adult human saphenous vein endothelial cells was investigated. Ox-LDL induced a rapid release of prostacyclin (PGI₂) to levels which were several-fold higher than those observed with control LDL. PGI₂ release was concentration-dependent and was biphasic, with a first peak occurring within 30 minutes (followed by a decrease), and a second peak occurring after several hours of incubation. PGI₂ production was inhibited by lipoprotein-depleted serum and by indomethacin, an antagonist of cyclooxygenase activity. These cells produced mainly PGF₁α, with some PGE₂ and PGI₂ when stimulated by the ionophore A23187 at confluency. However, among these prostanoids, mainly PGI₂ was produced in response to Ox-LDL. The data indicate that Ox-LDL induces the production of PGI₂ by human vascular endothelial cells. Since Ox-LDL is cytotoxic, this phenomenon may be a manifestation of an early response to injury.

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Tissue Culture

Human saphenous vein endothelial cell (HSVEC) cultures were prepared by collagenase digestion of unused saphenous vein sections obtained during coronary artery bypass surgery. The use of normally discarded human tissues was approved by the Human Investigation Review Committee of the New England Medical Center, Boston, MA. The cells were grown on human fibronectin-coated dishes (1.5 μg/cm²) in Morgan’s M-199 medium containing 25 mM HEPES, and supplemented with 5% fetal bovine serum (FBS), 50 μg/ml bovine endothelial cell growth factor, 100 μg/ml porcine heparin, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37°C in a humid atmosphere containing 5% CO₂. All experiments with HSVEC were performed with cultures at passages 2 to 4, 3 to 4 weeks after isolation. Under these culture conditions, monolayers have several characteristics of vascular endothelium, such as typical morphology, expression of von Willebrand factor mRNA and protein, as well as production of glycoconjugates specific for the binding of Ulex europaeus I lectin. Human monocytes were isolated by Ficoll hypaque centrifugation followed by adherence to plastic Petri dishes covered with FBS. Monocytes obtained were more than 95% pure as indicated by esterase staining.

Preparation of Lipoprotein-depleted Serum and Low Density Lipoprotein

Lipoprotein-depleted serum (LPDS) was prepared by ultracentrifugation of FBS at a hydrated density of 1.25 g/ml; cholesterol and triacylglycerol were undetectable in this fraction, as measured by enzymatic assays previously described. Following phlebotomy of healthy human donors into 0.2% ethylenediamine tetraacetate (EDTA)
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measuring radioactivity in the medium after treatment of the cells with 2% Nonidet P-40.

Quantitation of Lipid Oxidation

Oxidation of LDL lipids was estimated by assaying for thiobarbituric acid reactive substances (TBARS) by using a modification of the flurometric method of Yagi. Briefly, 50 µg of LDL protein (0.5 mg/ml) was precipitated with 2 ml of 20% trichloroacetic acid containing 0.1% BHT; 1 ml of a freshly dissolved solution (0.67%) of thiobarbituric acid was added and the mixture was boiled for 45 minutes, cooled with running water, and cleared by centrifugation. Fluorometric measurements were made at 553 nm with 515 nm excitation. The amount of malondialdehyde (MDA) equivalent was determined against a standard curve obtained using freshly diluted 1,1,3,3-tetramethoxy propane. The degree of LDL oxidation was expressed in nanomoles of MDA equivalent per milligram of LDL protein. Under these conditions, the TBARS values for control LDL preparations were typically 0 to 0.2 nmol/mg, while the values for Ox-LDL preparations were 3 to 18 nmol/mg, depending on the length of the fourth dialysis. Highly oxidized LDL (TBARS above 18 nmol/mg) was degraded, as evidenced by a trail of lipid staining (fat red 7B) after agarose electrophoresis. In our experiments, the maximum level of oxidation in Ox-LDL used had a TBARS value of 16 nmol/mg.

Incubation of Low Density Lipoprotein with Cells

To prevent oxidation of control LDL, incubation media were combined with concentrated DMEM just before incubation. Such preparations were found to have a level of gram-negative bacterial endotoxin below 20 pg/ml with the chromatographic elution system used to separate mouse amebocyte lysate (MA Bioproducts, Walkersville, MD). Concentrations of nonlipoprotein species (including BHT, EDTA, and Cu²⁺) were identical in both control LDL and Ox-LDL preparations.

HSVEC were grown on 2 cm² wells until confluency (defined as the absence of open space on the monolayer), were washed twice in HBSS, and were incubated once in 300 µl of medium. Before experimental incubation, each well was examined by inverted phase contrast microscopy, and any discontinuity in the monolayer resulted in the elimination of the well from the experiment. Human monocytes (3 x 10⁶) were incubated in 0.32 cm² wells containing 250 µl of LDL preparation. In each experiment, control LDL and Ox-LDL isolated from the same lipoprotein donor were used on the cells obtained from another single donor.

Measurements of Prostaglandin Production

Radioimmunoassay

Prostaglandin production was measured by radioimmunoassay (RIA) of 6-keto-PGF₁α (a stable PGI₂ metabolite) or PGE₂ in the medium. After each incubation, 200 µl of medium collected from HSVEC monolayers or from the centrifuged monocyte suspensions were frozen at -80°C until analysis. Rabbit antiserum specific for 6-keto-PGF₁α or PGE₂ was pre-precipitated overnight at 4°C with sheep anti-IgG in the presence of nonimmune rabbit serum and PBS-EDTA. Serial dilutions of unlabeled prostaglandin standard were then incubated overnight at 4°C with tritiated and the antibodies. After decantation, the radioactivity was measured by liquid scintillation spectrometry, and quenching was monitored using an external source of ¹³⁷Cs. The sensitivity of the assay was 20 pg and a 50% displacement was obtained at about 300 pg. The specificities of our antisera have been previously reported; however, Ox-LDL did react with the antisera (see Results) and therefore, in all experiments, background values (obtained from RIA on parallel incubations of media in the absence of cells) were subtracted from total values.

High Performance Liquid Chromatographic Analysis

Further characterization of PGI₂ production was made by reversed-phase high performance liquid chromatography (RP-HPLC) analysis of the medium after [³H]-ara-
chidonic acid metabolic labeling. Tritiated arachidonic acid (240 Ci/mmol, New England Nuclear, Boston, MA) was adsorbed to polypropylene under a stream of purified N₂ and was transferred to FBS by a 45-minute incubation at 37°C. After washes with HBSS, subconfluent monolayers were incubated with 10 µCi/ml of ³H-arachidonic acid in fully supplemented M-199 medium. After 48 hours, approximately 80% of the radioactivity was associated with the cells; the monolayers were washed in HBSS-albumin (4%) and then HBSS and were incubated with the lipoprotein or the ionophore A23187. After incubation, the entire medium was collected and acidified. The prostanoids were adsorbed onto an octadeccysilysilica (C18) cartridge (Sep-pak, Millipore Corporation), were washed with a mixture of ethanol and water (15:85) and then with ether and were eluted with methyl formate, as described by Powell. Extraction efficiencies were greater than 95% in all cases. The solvent was evaporated under N₂ and the residue was redissolved in ethanol. The prostanoids were fractionated by RP-HPLC in a 4.6 mmx15 cm C18 column (3 µm, Adsorbosphere, Alltech Association, Incorporated, Deerfield, MA) guarded with a C18 precolumn (Brownlee Labs Incorporated, Santa Clara, MA). The mobile phase was Terragno’s biphasic solvent (17.3 mM H₂PO₄/C₆H₂CN, 7:3) used at a flow rate of 1.7 ml/min.²² Aliquots (1 ml) were collected and radioactivity was measured. Identification of the elution peaks was based on retention volume of authentic tritiated standards (6-keto-PGF₁α, PGF₂α, and PGE₂) and on relative retention volume of the authentic standard 6,15-diketo-PGF₁α.

Results

**Incubation Conditions**

Adult human venous endothelial cells require a complex culture medium for growth. However, confluent monolayers of these cells can be maintained for at least 24 hours in serum-free media such as DMEM or Eagle’s minimum essential medium (MEM). When LDL was incubated in either media without cells, no significant oxidation of LDL was observed (Table 1). However, in the presence of cells, the use of MEM induced several-fold more LDL oxidation than the use of DMEM over a 24-hour period. An increase of BHT concentration from 1.7 µM (average concentration after medium reconstitution) to 20 µM in DMEM did not significantly protect LDL against oxidation.

Since EDTA (0.01 to 0.05 mM) was present in the LDL preparations used for tissue culture, the extent of EDTA toxicity was assessed by specific 111-indium release. When HSVEC were incubated in DMEM containing 1 mM EDTA, a significant specific release of ¹¹¹In was observed, indicating cytotoxicity (Table 2). When the cells were incubated with 0.1 mM EDTA, a concentration at least twice the concentration used during LDL incubation experiments, little toxicity was seen.

Confluent HSVEC can be maintained with control LDL in serum-free medium for at least 24 hours (Figure 1); under those conditions the monolayers displayed a cobblestone phenotype characteristic of quiescent endothelial cells. In contrast, when the monolayers were incubated with Ox-LDL, the cells displayed an “activated” phenotype; the presence of debris in the medium was an indication of cell injury.

**Prostacyclin Production**

Ox-LDL, compared to control LDL, caused a marked increase in 6-keto-PGF₁α release from HSVEC. The increase in PGF₁α, measured by RIA of the medium, was concentration-dependent (Figure 2). Ox-LDL induced a rapid rise in 6-keto-PGF₁α concentrations in the media of HSVEC, followed by a decrease, and a subsequent increase after several hours of incubations (Figure 3). The degree of LDL oxidation clearly affected this response, and the addition of LPDS decreased this response (Figure 3). Indomethacin inhibited the production of 6-keto-PGF₁α by HSVEC (Figure 4). Ox-LDL (500 µg/ml, TIBARS 2.9 nmol/mg) induced a response similar to the response induced by the calcium ionophore A23187 (Figure 4).

The antisera for 6-keto-PGF₁α cross-reacted with LDL and especially with Ox-LDL at a given degree of oxidation. This cross-reactivity was linearly related to the amount of

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**Table 1. Oxidation of Low Density Lipoprotein in Culture Media Alone and in Presence of Cells**

<table>
<thead>
<tr>
<th>Concentration of BHT in medium (µM)</th>
<th>Concentration of LDL in medium (mg/ml)</th>
<th>Oxidation in MEM (TBARS in nmol/mg)</th>
<th>Oxidation in DMEM (TBARS in nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without cells</td>
<td>With cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µM</td>
<td>20 µM</td>
<td>0.5±0.09</td>
<td>3.2±0.18</td>
</tr>
<tr>
<td>1.7 µM</td>
<td>1.7 µM</td>
<td>0.4±0.18</td>
<td>0.3±0.14</td>
</tr>
</tbody>
</table>

Values are the means±SD of triplicate determinations on duplicate incubations.

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**Table 2. Cytotoxicity of EDTA on Human Saphenous Vein Endothelial Cells**

<table>
<thead>
<tr>
<th>Concentration of EDTA in medium (mM)</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>111-indium specific release</td>
<td>0.1</td>
<td>2.3</td>
<td>18.1</td>
</tr>
</tbody>
</table>

Values are the means of triplicate incubations.

Confluent human saphenous vein endothelial cells were metabolically labeled with ¹¹¹In-oxine and were incubated with Dulbecco's modified Eagle’s medium containing various concentrations of EDTA. After 24 hours, EDTA cytotoxicity was assessed by measuring the specific release of ¹¹¹In in the medium, as described in the Methods section.
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Figure 1. Inverted phase-contrast microscopic pictures of confluent human saphenous vein endothelial cells incubated for 24 hours with control (A) or oxidized low density lipoprotein (Ox-LDL) (B). Incubation media were prepared with serum-free Dulbecco’s modified Eagle’s medium as described in the Methods section. The level of LDL in A and B was 200 μg/ml; the degree of LDL oxidation in B was 12.6 nmol of malondialdehyde equivalent per milligram of LDL protein. ×98

Figure 2. The effect of various levels of control and oxidized low density lipoprotein (Ox-LDL) incubated with human saphenous vein endothelial cells on prostacyclin production. Various protein concentrations of LDL and Ox-LDL (TBARS 5.4 nmol/mg) were incubated with confluent monolayers for 24 hours. Prostacyclin production was assessed by radioimmunoassay of 6-keto-PGF₁α in the medium as described in the Methods section. Values are the means±SD of duplicate incubations with control LDL (△) or Ox-LDL (○).

Ox-LDL within a range of 3.5 to 105 μg of LDL protein (Table 3). Control or Ox-LDL, however, did not quench RIA detection of 6-keto-PGF₁α (data not shown). The data indicate that parallel incubations of LDL without cells must be performed to correct for cross-reactivity.

In similar experiments with human monocytes, cross-reactivity of Ox-LDL (100 μg/ml, TBARS 10.3 nmol/mg) with PGE₂ antiserum was observed (data not shown). In this system, the same kind of prostanoid response was noted, with Ox-LDL inducing a sixfold higher level of PGE₂ in the media than did control LDL over a 20-hour incubation (579±29 pg/ml vs. 99±14 pg/ml).

Because of the cross-reactivity with the antisera, and our interest in examining the entire spectrum of prostanoids produced by HSVEC in response to LDL, we also used RP-HPLC methodology for assessing the presence of these constituents in the media after metabolic labeling. PGI₂ is not produced uniformly by cells isolated from various vascular beds. To our knowledge, there is no available data on the HPLC profile of the prostanoids produced by adult human saphenous vein endothelial cells. Thus, we stimulated HSVEC with the ionophore A23187 to compare the prostanoid response to that induced by Ox-LDL. Confluent monolayers of HSVEC produced PGE₂ with some PGE₂ and little 6-keto-PGF₁α (Figure 5). This prostanoid profile was identical to the profile reported by others in porcine aortic endothelial cells stimulated with A23187 after preincubation with ¹⁴C-arachidonate-labeled LDL. In contrast, when HSVEC were incubated with Ox-LDL for 14 hours, the monolayers produced mainly PGI₂ metabolites as shown by the presence of 6-keto-PGF₁α and another PGI₂ degradation product, which has a relative retention volume similar to the relative retention volume of 6,15-diketo-PGF₁α (Figure 6). Using metabolic labeling and HPLC analysis, no PGI₂ metabolites were detected until after several hours of incubation. These data indicate that the initial release of this constituent detected by RIA was derived from a pool of unlabeled arachidonic acid, either intracellular or extra-
Figure 3. Time dependence of prostacyclin (PGI2) production by human saphenous vein endothelial cells during incubation with control or oxidized low density lipoprotein (Ox-LDL) (A), and Ox-LDL or Ox-LDL with lipoprotein-depleted serum (LPDS) (B). In A, levels of LDL were 500 μg/ml (TBARS for Ox-LDL: 2.9 nmol/mg). In B, levels of Ox-LDL were 200 μg/ml (TBARS 6.8 nmol/mg), and 5% LPDS (1.25 mg protein/ml) was used. PGI2 production was assessed by radioimmunoassay of 6-keto-PGF1α in the medium, as described in the Methods section. The values are the means±SD of duplicate incubations with control LDL (A), Ox-LDL (•), Ox-LDL and LPDS (○).

Discussion

In characterized adult HSVEC, concentration and time-dependence experiments demonstrated that Ox-LDL versus control LDL increased PGI2 synthesis (Figures 2 and 3). This observation was confirmed by using a cyclooxygenase antagonist, which inhibited production of PGI2 (Figure 4). Further demonstration of this effect was provided by HPLC analysis after metabolic labeling, which showed that PGI2 was the major prostanoid synthesized in response to Ox-LDL, despite the ability of these cells to also produce PGF2α and PGE2 when exposed to the ionophore A23187 (Figures 5 and 6). Although it was not possible to observe early cytopathological effects (within minutes) when the monolayers were incubated with Ox-LDL, cell morphology was drastically altered after several hours of incubation with Ox-LDL (Figure 1). These observations indicate a link between Ox-LDL cytotoxicity and late PGI2 release, and suggest that early PGI2 release may also be a manifestation of Ox-LDL-mediated cytotoxicity.

A number of investigators have examined the effects of LDL on PGI2 production by vascular tissue.36-39 Beitz and Forster36 have reported that LDL decreases PGI2 production in microsomal fractions of pig aorta. Szczeklik and Gryglewski37-38 have noted that Ox-LDL decreases PGI2 production in rat aorta slices as compared to control LDL. In contrast to these data, Spector et al.39 have reported...
that LDL stimulates PGI₂ production by bovine aortic and human umbilical vein endothelial cells. In these latter studies, oxidation of LDL was not assessed. Our own experiments are not directly comparable with studies carried out in pig and rat aorta, since the preparations studied contained a variety of different cell types.36,37,38 However, in viable and well-characterized tissue cultures of endothelial cells, our results are consistent with those of Spector et al. We have extended these studies by examining the effects of Ox-LDL and control LDL.

Precise control of the experimental conditions under which incubations of LDL with endothelial cells are performed is critical. Endothelial cells can modify LDL in a manner similar to copper-induced oxidation.7 Certain media favor cell-induced LDL oxidation. Endothelial cells incubated in Ham's F10 medium (which contains 10 nM Cu²⁺) induce rapid LDL oxidation, which can be prevented by BHT.7 BHT has similar antioxidant properties in DMEM. In our cell preparation and in the presence of BHT, more LDL oxidation was seen with MEM than with DMEM (Table 1). Such a difference may be due to the higher levels of cysteine (a primary antioxidant) and tryptophan (an oxygen radical quencher) in DMEM than in MEM (2- and 1.6-fold, respectively). The presence of EDTA in the medium has also been reported to inhibit LDL oxidation by endothelial cells, probably because of its high affinity for Fe²⁺, Cu²⁺, and Zn²⁺.7 However, too much EDTA will cause the chelator to complex significantly with Ca²⁺ and Mg²⁺ (which have a lower affinity constant than transition metals).7 Using specific release of 111-indium as an index of cytolsis, we have shown that up to 100 µM of EDTA can be tolerated by confluent HSVEC in DMEM over a 24-hour period (Table 2). In our experiments, the level of EDTA was always 100-fold higher than the level of Cu²⁺, but it never exceeded 100 µM. Such concentrations of EDTA (0.01 to 0.05 mM) with BHT in the media are not toxic but still prevent oxidation of LDL by endothelial cells. HEPES was excluded from the final serum-free media since this buffer has been reported to stimulate endothelial cells to produce reactive oxygen metabolites.40 The use of chloramphenicol during all steps of LDL purification protects against contamination with gram-negative bacterial endotoxins, which are known to induce PGI₂ production in endothelial cells.41 During LDL preparation, NaN₃ was not used because this common bactericidal agent has been reported to promote LDL oxidation and subsequent apo B-100 degradation.42

Ox-LDL can seriously affect radioimmunoassay of prostaglandins (Table 3). There are several possible explanations for this effect. One possibility is that Ox-LDL cross-reacts with the ligand-binding region of the pre-precipitated IgG and thus decreases the extent of competition between radiolabeled and unlabeled prostaglandins. A net decrease of recovered radioactivity would result in an increase of the calculated amount of prostaglandin. Another possibility is that Ox-LDL binds prostaglandins; this phenomenon is, however, unlikely since Ox-LDL does not quench RIA detection of 6-keto-PGF₁α (data not shown). To our knowledge, such cross-reactivity, which can be considerable at elevated levels of highly oxidized LDL, has not been previously reported. Thus, particular care needs to be...
taken when analyzing prostaglandins by RIA in media containing significant amounts of Ox-LDL.

The rapid initial increase of 6-keto-PGF$_{1a}$ in the medium followed by a longer period of disappearance (Figure 3) suggests that the epitope becomes shielded from the antisem. A priori, such a phenomenon could be due to binding of 6-keto-PGF$_{1a}$ to the cells or to the culture dishes. Also, the prostanoi could bind to a cellular factor released in the medium. Since we did not find any evidence of 6-keto-PGF$_{1a}$ binding to the cells or to the culture dishes (data not shown), it is possible that a binding factor is released from the cells and sequesters 6-keto-PGF$_{1a}$ from the antisem. Binding of PGI$_2$ to albumin and a Cohn fraction VI glycoprotein has been reported. Such findings raise the possibility that an endothelium-derived factor binds early metabolites of PGI$_2$, perhaps to allow specific targeting to tissues equipped for further catabolism of these compounds. It is also possible that metabolizing enzymes are actively released and induce a modification of the epitope recognized by our antisem. A third possibility is the leaking of metabolizing enzymes; this could occur at late stages of Ox-LDL cytotoxicity as indicated by the presence, after 14 hours of incubation, of 6,15-diketo-PGF$_{1a}$, a product of the cytoplasmic enzyme 15-hydroxy-prostaglandin dehydrogenase found in bovine arteries and veins. Another explanation is that, as Ox-LDL is metabolized by the cells and disappears from the medium, the correction used for the RIA would then no longer be applicable and thus would reduce artefactually the final value of 6-keto-PGF$_{1a}$. Such a phenomenon is unlikely, because we estimate that less than 1% of Ox-LDL (100 ug/ml) is metabolized by HSVEC over 24 hours (calculations based on reported data about MDA- and acetylated-LDL catabolism by endothelial cells). The mechanisms by which Ox-LDL stimulates PGI$_2$ production in HSVEC are not clear. Endothelial cells express the LDL receptor and the scavenger receptor, which recognizes a more negatively charged apo B. Ox-LDL is recognized exclusively by the scavenger receptor; such a phenomenon could be due to the intimal space being much more susceptible to oxidation because of the lack of serum antioxidants and the ability of endothelial cells to oxidize LDL. High plasma levels of LDL, presumably resulting in a larger LDL infiltration into the intima, would exacerbate this effect. The intimal presence of Ox-LDL is suggested by several lines of evidence. LDL isolated from human aorta is more electronegative than plasma LDL, a feature of oxidized LDL. Moreover, LDL isolated from human atherosclerotic lesions is degraded and induces cholesterol esterification in macrophages; both features are shared with Ox-LDL. Therefore, once Ox-LDL is present in the intima, it may trap macrophages, thereby inducing foam cell formation and thus initiating and amplifying the injury events of atherosclerosis. Remarkably, PGI$_2$ can antagonize these events by inhibiting leukocyte adhesion and cholesterol accumulation in aortic smooth muscle cells. These observations, interpreted with the injury hypothesis for the pathogenesis of atherosclerosis, suggest that oxidized LDL-induced production of PGI$_2$ by endothelial cells could be an in vivo mechanism of defense against the injury inflicted by modified lipoprotein.

Acknowledgments

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