Chronic Exposure of Cultured Bovine Endothelial Cells to Oxidized LDL Abolishes Prostacyclin Release

Eric Thorin, Carlene A. Hamilton, Marek H. Dominiczak, John L. Reid

Abstract We investigated the effect of chronic exposure (3 days) with low-density lipoprotein (LDL) and oxidized (Ox)-LDL on the unstimulated and stimulated formation of prostacyclin (6-keto-prostaglandin [PGF]_1) and total inositol phosphates (IPs) by cultured bovine aortic endothelial cells. Neither basal nor bradykinin-stimulated (1 to 10 nmol/L) formation of 6-keto-PGF_\text{1a} was affected by LDL, except at the highest concentration of bradykinin tested (100 nmol/L). In the presence of the antioxidants N-acetyl-L-cysteine (NAC, 10 \text{ \mu mol/L}) or vitamin E (100 \text{ \mu mol/L}), basal and bradykinin-stimulated formation of 6-keto-PGF_\text{1a} was potentiated by 20 \text{ \mu g protein/mL} of LDL. Ox-LDL decreased unstimulated formation of the eicosanoid from 3.1±0.2 pg/\text{\mu g protein} in control cells to 1.6±0.1 and 0.5±0.1 pg/\text{\mu g protein} after 3-day incubation with 5 and 20 \text{ \mu g protein/mL} of Ox-LDL, respectively (P<.05). As in the basal state, Ox-LDL decreased bradykinin-induced 6-keto-PGF_\text{1a} formation. NAC or vitamin E did not influence Ox-LDL-induced endothelial cell changes in eicosanoid production. IPs formation by endothelial cells increased to a similar extent in the presence of 20 \text{ \mu g protein/mL} of either LDL or Ox-LDL. However, no change was apparent in the bradykinin (10 \text{ \mu mol/L})-induced increase in total IPs formation after incubation with the lipoproteins. The data indicate that chronic exposure to Ox-LDL abolishes the production of prostacyclin by cultured endothelial cells. The oxidatively modified lipoprotein seems to more specifically affect the prostacyclin pathway. This phenomenon could be involved in the progression of the fatty streak to a more advanced lesion. (Arterioscler Thromb. 1994;14:453-459.)

Key Words • cultured endothelial cells • oxidized LDL • prostacyclin • antioxidants

For many years, it was accepted that injury to the endothelium triggered the atherosclerotic lesion, but studies in a number of laboratories show that fatty-streak lesions can and do develop under an intact endothelial layer. Circulating monocytes penetrate between endothelial cells, enter the intima, and there become loaded with lipoprotein-derived lipids. The low-density lipoproteins (LDLs) have to undergo oxidative modification (Ox-LDL) to be taken up by macrophages. One may postulate that LDL uptake serves a protective function, preventing endothelial damage that otherwise might cause thrombosis even sooner. However, Ox-LDL can be highly cytotoxic. Antioxidant therapy in an animal model of hypercholesterolemia prevents the formation of fatty streaks, demonstrating that the oxidative modification of LDL gives it an atherogenic role. Once the macrophages in the artery are heavily loaded with lipids, they may possibly become nonfunctional or die, releasing their contents. Ox-LDL, through its cytotoxicity, may then cause injury to the endothelial cells overlying the fatty streak. What is the role of chronic exposure to Ox-LDL? Could Ox-LDL have a role in the progression of the fatty-streak lesion to a more complicated lesion? One attractive hypothesis is that Ox-LDL could first interfere with the cellular protective mechanism and thereby accelerate the progression of the atherosclerotic lesion by increasing monocyte adhesion and cellular proliferation.

Prostacyclin, an arachidonic acid (AA)-derived product, is a potent cytoprotective agent, inhibiting cell proliferation, platelet adhesion and aggregation, and monocyte adhesion. Its release from cultured endothelial cells, which seems to be dependent on formation of inositol phosphates (IPs), can be stimulated by low concentrations of hydrogen peroxide or short-term exposure to Ox-LDL. Potentially, even the cell's own oxidative metabolism could be a stimulus. However, the production of prostacyclin is decreased in atherosclerotic tissues and smooth muscle cells from atheromatous lesions in culture. The observation that high concentrations of hydrogen peroxide can alter the production of prostacyclin by endothelial cells suggests that chronic exposure with pro-oxidative reactive species like Ox-LDL could alter the endothelial AA pathway and thereby, the endothelial protective function.

In this study, we used bovine aortic endothelial cells in culture to investigate the effect of chronic exposure to LDL and Ox-LDL on the unstimulated and stimulated release of prostacyclin. The results obtained demonstrate that, at a very low concentration, Ox-LDL inhibits eicosanoid release without affecting the formation of IPs. Part of these results have been presented to the British Pharmacological Society.

Methods

Endothelial Cell Culture

Bovine aortas were obtained from a local abattoir. After removal from the animal (within 30 minutes of death), the
Measurement of Phosphoinositide Hydrolysis

Measurement of Prostacyclin

Measurement of Phosphoinositide Hydrolysis

Preparation of LDL and Oxidation

Quantification of Lipid Oxidation

Cytotoxicity Studies
Protocols

The bovine aortic endothelial cells were incubated for 3 days without or with LDL or Ox-LDL at a concentration of 5 or 20 μg protein/mL. In the control group of cells, PBS was added to the culture medium. The antioxidants N-acetyl-L-cysteine (NAC, 10 μmol/L final concentration, Sigma) or vitamin E (100 μmol/L final concentration, Sigma) were added to the cells for the 3 days of incubation with PBS, LDL, or Ox-LDL.

Statistical Analysis

Results are expressed as the mean±SEM, and comparisons were made by one-way ANOVA, followed by a Student’s t test analysis. In the case of nonparametric distribution of the data, results are expressed as median and range, and the comparisons between groups were done by the Kruskal-Wallis test. Differences among means or medians were considered significant at P<.05.

Results

Cytotoxicity Assays

The growth rate of endothelial cells was not affected by Ox-LDL at a concentration of 20 μg protein/mL. After a 5-day incubation period, cells reached confluence in both Ox-LDL–treated cells and PBS-treated control cells. The number of cells was not significantly affected by Ox-LDL (2.7±0.1×10⁶ cells/mL) compared with control (3.2±0.4×10⁶ cells/mL). Further incubation of the cells in the presence of M199 (which does not contain growth factors) had no influence on the number of cells in either Ox-LDL–treated cells (2.8±0.3×10⁶ cells/mL) or PBS-treated cells (2.8±0.3×10⁶ cells/mL).

The vital dye exclusion remained stable for endothelial cells incubated with PBS or 20 μg LDL or Ox-LDL protein/mL (n=12 per group). At confluence, 94.6±1.8%, 96.7±0.8%, and 94.8±0.7% of cells were viable after 3 days of incubation in the presence of PBS, LDL, or Ox-LDL, respectively.

Total Protein Content

Three days of incubation of endothelial cells with 5 μg protein/mL LDL (n=24) had no effect on total protein content (59±3 μg/well) compared with control (58±2 μg/well) but increased slightly in the presence of 5 μg protein/mL Ox-LDL (70±2 μg/well, P<.05 versus control and LDL groups).

In seven independent experiments, 3 days of incubation of endothelial cells with 20 μg protein/mL LDL did not affect the protein content of the wells compared with control but was significantly decreased, by 45±7%, by 20 μg protein/mL Ox-LDL compared with control. Protein contents per well (in micrograms) were, in the control group, (1) 103±3 (n=20), (2) 101±3 (n=18), (3) 104±2 (n=16), (4) 108±2 (n=18), (5) 105±2 (n=16), (6) 107±2 (n=18), and (7) 103±2 (n=16); in the LDL groups, (1) 116±4 (n=20), (2) 115±4 (n=18), (3) 118±4 (n=16), (4) 120±4 (n=18), (5) 121±4 (n=16), (6) 122±4 (n=18), and (7) 118±4 (n=16); and in the Ox-LDL groups, (1) 123±5 (n=20), (2) 124±5 (n=18), (3) 126±5 (n=16), (4) 128±5 (n=18), (5) 129±5 (n=16), (6) 131±5 (n=18), and (7) 127±5 (n=16). In the presence of 20 μg/mL LDL protein, the protein contents were 37±1, 33±1, and 41±1 μg/well, respectively (P<.05 compared with the results obtained in the absence of vitamin E).

Oxidation of LDL

The TBARS values for native LDL preparations were below the sensitivity of the assay, whereas the mean value for Ox-LDL preparations was 9.6±0.3 nmol/mg. The incubation medium being changed every day, the TBARS value of the medium containing PBS, LDL, or Ox-LDL was measured after 18 hours of incubation with or without endothelial cells (n=7 independent experiments per group). The medium by itself, with or without 20 μg LDL protein/mL, had no TBARS value.

After an 18-hour incubation period without cells, 19±4% of LDL was spontaneously oxidized. This value was not affected by NAC (20±5%).

In the presence of cells, 66±5% of LDL was oxidized. When NAC (10 μmol/L) was added to the medium, however, only 36±2% of LDL was oxidatively modified (P<.05). The cell-dependent oxidative process represents 46±2% of LDL oxidation (total minus spontaneous oxidation), an effect that was reduced by 63% by the antioxidant to represent only 16±3% of LDL being oxidatively modified by the endothelial cells (P<.05).

Unstimulated Release of Prostacyclin

Basal formation of 6-keto-PGF₁α was not affected by 3 days of incubation of the endothelial cells with 5 or 20 μg/mL LDL protein (Tables 1 and 2). Oxidized LDL decreased the basal formation of 6-keto-PGF₁α by >50% and 80% at 5 and 20 μg/mL, respectively (Tables 1 and 2). NAC had no effect on the unstimulated formation of prostacyclin in cells incubated with 5 μg/mL LDL protein, but both NAC and vitamin E increased the basal formation of 6-keto-PGF₁α in the presence of 20 μg/mL LDL protein (Tables 1 and 2).

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**Table 1. Effect of Chronic Exposure of Endothelial Cells With Two Concentrations of LDL and Ox-LDL on Unstimulated Formation of 6-Ketoprostaglandin F₁α**

<table>
<thead>
<tr>
<th>N-Acetyl-L-Cysteine, 10 μmol/L</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS control</td>
<td>3.1±0.2</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>LDL 5 μg/mL</td>
<td>3.4±0.8</td>
<td>3.6±0.4</td>
</tr>
<tr>
<td>LDL 20 μg/mL</td>
<td>3.3±0.5</td>
<td>4.7±0.1*</td>
</tr>
<tr>
<td>Ox-LDL 5 μg/mL</td>
<td>1.6±0.1*</td>
<td>1.6±0.3*</td>
</tr>
<tr>
<td>Ox-LDL 20 μg/mL</td>
<td>0.5±0.1*</td>
<td>0.5±0.1*</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; Ox, oxidized; PBS, phosphate-buffered saline. Results are mean±SEM of 10 to 12 independent experiments. *P<.05 statistically different from results in the absence of N-acetyl-L-cysteine (one-way ANOVA).
Both antioxidants had no influence in Ox-LDL groups (Tables 1 and 2).

A short incubation period of 1 hour with either LDL or Ox-LDL (20 μg protein/mL) did not affect the basal formation of 6-keto-PGF₁₀ (4.2±0.2 and 3.8±0.3 pg/μg protein) compared with PBS-treated cells (4.1±0.5 pg/μg protein).

### Stimulated Release of Prostacyclin

Bradykinin dose-dependently stimulated the release of prostacyclin by endothelial cells. Added to the culture medium, the antioxidant NAC (10 μmol/L) decreased the stimulated formation of 6-keto-PGF₁₀ at the highest concentration of bradykinin tested (100 nmol/L) from 43.9±3.1 to 30.6±2.4 pg/μg protein (P<.05), whereas vitamin E (100 μmol/L) had no significant effect on bradykinin-induced prostacyclin release (Table 2).

LDL at a concentration of 5 μg/mL, in either the absence or presence of NAC (10 μmol/L), had no influence on bradykinin-induced prostacyclin release (Figure, A and B). However, incubation of the cells with 20 μg/mL LDL decreased the bradykinin-stimulated formation of 6-keto-PGF₁₀ at the highest concentration used (100 nmol/L) from 43.9±3.1 to 29.9±2.3 pg/μg protein (P<.05) compared with PBS-treated cells (4.1±0.5 pg/μg protein).

### Phosphoinositide Hydrolysis

IP₃ may be involved in prostacyclin release. We investigated the effect of the higher concentration of lipoproteins on total IP₃ formation. The technique used being less sensitive than the radioimmunoassay of 6-keto-PGF₁₀, a bradykinin concentration of 100 nmol/L to 10 μmol/L was used to reach a maximum stimulation of IP₃ formation.

Basal formation of total [3H]IP₃ was increased by 3 days of incubation with 20 μg protein/mL LDL and Ox-LDL by 59% and 76%, respectively (Table 4). When stimulated with bradykinin, the resulting total IP₃ formation remained increased for the lowest concentration of agonist (100 nmol/L) but not significantly altered by LDL or Ox-LDL for the highest (10 μmol/L) concentration of bradykinin tested compared with control (Table 4).

In another series of experiments (n=8 per group), the effect of NAC (10 μmol/L) on basal IP₃ production was studied. As reported above, native LDL and Ox-LDL stimulated the basal formation of IP₃ (7.4±0.2% and 10.8±1.4% of total PI, respectively, P<.05) compared with control (6.1±0.3% of total PI). Basal IP₃ production was not significantly affected by NAC in control cells (7.1±0.5% of total PI). Treatment with the antioxidant also had no effect in LDL-treated cells (8.4±0.5% of total PI), but it was no longer significantly different from the NAC-treated control group (P=.101). Finally, basal IP₃ formation remained significantly increased in Ox-LDL-treated cells when incubated with NAC (10.5±1.2% of total PI, P<.05 compared with NAC-treated control cells).

### Discussion

This study suggests that Ox-LDL can inhibit prostacyclin release from cultured endothelial cells. The atherogenicity of Ox-LDL is now accepted as being one of the key events in the formation of the fatty streak. Before its uptake by the macrophages, LDL undergoes an oxidative modification, partly catalyzed by endothelial cells, leading to an increase in its uptake and degradation rates. This endothelium-dependent ox-
Oxidation of LDL has been demonstrated in vitro in cultured endothelial cells. Therefore, experiments with LDL were carried out in the absence or presence of an antioxidant. NAC (10 μmol/L) was chosen because we have shown it to inhibit LDL oxidation in plasma ex vivo. Some experiments were carried out in the presence of vitamin E, an endogenous antioxidant.

LDL had no effect on basal prostacyclin production when alone but increased production in the presence of antioxidant. Bradykinin-stimulated production was also modified by NAC and vitamin E in the presence of LDL. Bradykinin-induced 6-keto-PGF₁α formation was slightly decreased by LDL, whereas in the presence of antioxidants, the stimulated production of prostacyclin rose. These effects of the antioxidants in the presence of the higher concentration of LDL tested (20 μg/mL) suggest that an endothelium-dependent oxidative mechanism does occur in our experimental system. This hypothesis is confirmed by the increase in TBARS value in the medium of LDL-treated cells, an effect partially reversed by NAC, as previously described by others.

In contrast to bradykinin-stimulated 6-keto-PGF₁α formation, the facilitatory effect of LDL on ATP-induced 6-keto-PGF₁α formation occurred in the absence of NAC. A recent study shows that, in contrast to thrombin (another agonist inducing prostacyclin release), ATP-induced 6-keto-PGF₁α formation can be abolished by activation of protein kinase C. Different regulatory mechanisms of the prostacyclin synthetic pathway could account for the difference of effect of LDL observed during different receptor-dependent prostacyclin release mechanisms.

Table 3. Effect of Chronic Exposure of Endothelial Cells With LDL or Ox-LDL (20 μg/mL) on ATP- and Arachidonic Acid-Induced 6-Ketoprostaglandin F₁α Formation (pg/μg protein)

<table>
<thead>
<tr>
<th></th>
<th>ATP, 1 mmol/L</th>
<th>AA, 0.01 μmol/L</th>
<th>AA, 1 μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.9±2.7</td>
<td>12.3±4.8</td>
<td>42.4±4.8</td>
</tr>
<tr>
<td>LDL</td>
<td>39.0±2.6*</td>
<td>28.1±3.2*</td>
<td>67.1±7.2*</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>0.4±0.1*</td>
<td>8.0±1.6</td>
<td>9.5±1.1*</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; Ox, oxidized; and AA, arachidonic acid. Results are mean±SEM of six independent experiments.

*P<.05 statistically different from control phosphate-buffered saline group (one-way ANOVA).
concentration antagonized it. Finally, it has been shown that lipoperoxides resulting from lipid accumulation in atherosclerosis destroyed the prostacyclin synthase by oxidative deactivation. Taken together, all these elements suggest that Ox-LDL itself could trigger the decrease in prostacyclin synthase observed in atherosclerotic lesions.

As well as inhibiting basal formation of prostacyclin, Ox-LDL chronically incubated with endothelial cells almost abolished the receptor-dependent stimulated release of prostacyclin, suggesting that the alteration is not specific for one class of receptor. Moreover, AA-induced 6-keto-PGF\(_1\alpha\) formation did not appear to be dose-dependently related. Those two latter observations, in conjunction with the absence of modification of phospholipase C maximum activity, suggest that the Ox-LDL-induced decrease in prostacyclin release may be related to a decrease in enzyme activities and/or quantities within the cells rather than a receptor-dependent alteration.

High concentrations of oxidized lipoprotein can be cytotoxic. The higher concentration of Ox-LDL used in this study (20 \(\mu g/mL\)) decreased the total amount of protein. This effect, however, cannot explain the decrease in prostacyclin release observed at the lower concentration of Ox-LDL or at the higher concentration in the presence of vitamin E, since this phenomenon still occurred despite a significant increase or a normalization in the protein content per well, respectively. The stimulated phospholipase C activity, moreover, was not altered after 3 days of incubation with 20 \(\mu g/mL\) Ox-LDL, nor was cell number or cell viability. At the concentration used, Ox-LDL appears not to be cytotoxic.

In conclusion, the data presented here indicate that endothelial cells chronically exposed to Ox-LDL have a severely impaired capacity to release prostacyclin. Such interference with AA utilization in aortic endothelial cells may be involved in the progression of the fatty streak to a more advanced atherosclerotic lesion.

Acknowledgment
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