Oxidized LDL Enhances Lipopolysaccharide-Induced Tissue Factor Expression in Human Adherent Monocytes

Korbinian Brand, Carole L. Banka, Nigel Mackman, Robert A. Terkeltaub, Sao-Tah Fan, Linda K. Curtiss

Abstract Oxidized low-density lipoprotein (oxLDL) has been characterized as an atherogenic molecule responsible for the induction of a variety of gene products. One such gene, tissue factor (TF), the cellular initiator of the coagulation cascade, is not expressed in normal vascular tissue but is expressed by monocytes and foam cells in atherosclerotic lesions. Therefore, we examined the effect of oxLDL on TF expression in cultured human adherent monocytes. Endotoxin-free oxLDL alone did not induce TF expression in adherent monocytes. However, oxLDL significantly enhanced TF expression induced by the inflammatory mediator, bacterial lipopolysaccharide (LPS), in a time- and dose-dependent manner. In contrast, oxLDL did not alter LPS-mediated production of interleukin-8 and actually inhibited LPS-induced secretion of tumor necrosis factor-α, suggesting that some aspects of the signaling pathways for TF induction differ from those of other LPS-responsive monocyte/macrophage gene products. Thus, this study documents specific modulation of the expression of LPS-inducible genes in mononuclear cells by oxLDL. Factors that enhance TF expression in monocyte/macrophage cells present in atheroma may contribute to the severity of thrombotic episodes and complications observed in atherosclerosis. (Arterioscler Thromb. 1994;14:790-797.)

Key Words • oxidized LDL • lipopolysaccharide • tissue factor • atherosclerosis • interleukin-8 • tumor necrosis factor-α

Oxidized low-density lipoprotein (oxLDL) plays an important role in the development of atheroma. OxLDL has been detected in atherosclerotic lesions, and human and rabbit sera contain antibodies against oxLDL. Treatment of rabbits with potent antioxidants such as probucol, which may act by inhibiting LDL oxidation, substantially reduces the development of atherosclerotic lesions. The three major cell types of the arterial wall, endothelial cells, macrophages, and smooth muscle cells, oxidize native LDL in vitro. During oxidative modification LDL acquires biological properties, not present in the native molecule, that promote atherogenesis. For example, oxLDL is a chemotractant for monocytes, increases monocyte adhesion to the endothelium, enhances macrophage lipid accumulation, and induces production of macrophage and other colony-stimulating factors. In contrast, oxLDL inhibits the synthesis of platelet-derived growth factor and bacterial lipopolysaccharide (LPS)- or maleylated albumin-induced mRNA expression of interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α.

The transmembrane glycoprotein tissue factor (TF) serves as a receptor and essential cofactor for plasma coagulation factors VII/VIIa. The TF-VIIa complex activates coagulation factors IX and X by limited proteolysis, which ultimately leads to thrombin formation and fibrin deposition. Normally, TF is not expressed within the vasculature. However, TF expression can be induced in monocytes and endothelial cells by a variety of agonists, including LPS and cytokines. More recently, acetylated LDL, malondialdehyde-modified LDL, and oxLDL have been reported to induce TF activity in monocytes/macrophages and endothelial cells. Pathological activation of the coagulation cascade by aberrant expression of TF on the surface of monocytes or endothelial cells is implicated in the pathogenesis of septic shock and various thromboembolic disorders. In atherosclerotic plaques, TF antigen has been localized to monocyte-derived foam cells and vascular smooth muscle cells, suggesting a role for this receptor in atherogenesis.

In the present study, human plasma-derived adherent monocytes were chosen as a model to elucidate the possible physiological effect of oxLDL on TF expression. During the course of these studies, we observed significant levels of endotoxin contamination in preparations of oxLDL that induced TF expression in monocytes. Results of subsequent experiments with essentially "endotoxin-free" lipoproteins indicated that oxLDL alone had no significant effect on TF activity in adherent monocytes but significantly enhanced the inductive effect of low or intermediate doses of LPS in a specific and dose-dependent manner.

Materials

Butylated hydroxytoluene (BHT), 2-thiobarbituric acid, E-Toxa-Clean, tetramethoxypropane, and cupric acetate were purchased from Sigma Chemical Co. Low-endotoxin, high-

Received October 25, 1993; revision accepted February 4, 1994.

From the Department of Immunology, The Scripps Research Institute, Department of Immunology, IMM-17, 10666 N Torrey Pines Rd, La Jolla, CA 92037.

Reprint requests to Linda K. Curtiss, PhD, The Scripps Research Institute, Department of Immunology, IME-17, 10666 N Torrey Pines Rd, La Jolla, CA 92037.
were cultured at a density of 1×10^6 cells in 1 mL per well in Ham’s F-10 medium by exposure to 10 μmol/L, cupric acetate at room temperature. Oxidation was terminated by addition of BHT to a final concentration of 20 μmol/L. Because dialysis had no significant effect on the biological activity of oxLDL (see “Results”) and increased the risk of endotoxin contamination, oxLDL preparations were not dialyzed. Precautions taken to prevent endotoxin contamination during lipoprotein isolation and oxidation included the use of pyrogen-free sterile water for all reagents. All glassware was washed with 1% E-Toxase-Clean in sterile water, rinsed with sterile water, and baked at 185°C for 5 hours. Endotoxin contamination of reagents and lipoproteins was monitored with the chromogenic LAL assay using E. coli 0111:B4 endotoxin supplied with the kit for the standard curve. Method Lipoprotein Isolation and Oxidation LDL (d=1.019 to 1.063 g/mL) was isolated from normal human plasma by sequential ultracentrifugation as described and stored in DMEM/0.3 mmol/L EDTA for no longer than 4 weeks. LDL was oxidized at a concentration of 200 μg/mL in Ham’s F-10 medium by exposure to 10 μmol/L, cupric acetate at room temperature. Oxidation was terminated by addition of BHT to a final concentration of 20 μmol/L. Because dialysis had no significant effect on the biological activity of oxLDL (see “Results”) and increased the risk of endotoxin contamination, oxLDL preparations were not dialyzed. Precautions taken to prevent endotoxin contamination during lipoprotein isolation and oxidation included the use of pyrogen-free sterile water for all reagents. All glassware was washed with 1% E-Toxase-Clean in sterile water, rinsed with sterile water, and baked at 185°C for 5 hours. Endotoxin contamination of reagents and lipoproteins was monitored with the chromogenic LAL assay using E. coli 0111:B4 endotoxin supplied with the kit for the standard curve.

LPS Association With Lipoprotein Biosynthetically tritiated Re595-LPS was kindly provided by Drs Peter S. Tobias and John C. Mathison. [3H]Re595-LPS (2.8 μg/mL) and LDL or oxLDL (80 μg/mL) were coinoculated in 66% lipoprotein-depleted human plasma for 12 hours at 37°C. The lipoproteins were floated to the top of a KBr gradient by a single ultracentrifugation step (d>1.21 g/mL). Gradient fractions were analyzed for radioactivity. Overall recovery of [3H]Re595-LPS from the gradients ranged from 73.5% to 90.5%.

Thiobarbituric Acid–Reactive Substance (TBARS) Assay TBARS content of the lipoproteins was used as a measure of lipid peroxidation and was assayed by a modification of the method of Steinbrecher et al. Briefly, 0.2 mL of sample containing 40 μg LDL protein and BHT (20 μmol/L) was assayed by the addition of 1.0 mL 20% trichloroacetic acid followed by 1.0 mL 2-thiobarbituric acid (0.67% in 0.05N NaOH). Samples were heated for 30 minutes at 90°C, cooled, and centrifuged, and the absorbance at 532 nm was compared with that of tetramethoxypropane standards.

Electrophoresis OxLDL was examined for apoprotein fragmentation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with use of the Phast System (Pharmacia LKB Biotechnology). LDL and oxLDL (0.4 μg protein per lane) were separated on 4% to 15% polyacrylamide gradient gels containing 5% endotoxin-free fetal calf serum, 20 mmol/L glucose, Dulbecco’s modified Eagle medium (DMEM) was prepared in the media kitchen at The Scripps Research Institute. The L. amebocyte lyase (LAL) assay kit for endotoxin and Ham’s F-10 medium were purchased from Whittaker MA Bioproducts. LPS (Escherichia coli 0111:B4) was obtained from Calbiochem.

Results

Endotoxin Contamination in Lipoprotein Preparations When adherent monocytes were incubated for 12 hours with increasing concentrations of our initial preparations of oxLDL (the Table, Preparation No. 1), we observed a dose-dependent induction of TF activity that exhibited a maximal induction of 87-fold at 80 μg/mL (Fig 1). Studies in our laboratory indicate that TF expression is induced in human monocytes exposed to LPS concentrations as low as 10 to 100 pg/mL, although higher concentrations (100 ng/mL) are required to induce maximal TF expression (Steinmann et al, unpublished observations). Therefore, it was possible that contaminating endotoxin present in the oxLDL was partly responsible for the observed induction of TF activity. Lipoprotein preparation No. 1 (the Table) contained such significant levels of endotoxin contami-
Characterization of Lipoprotein Preparations

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Lipoprotein</th>
<th>Endotoxin Contamination, pg/µg Protein</th>
<th>TF Activity, Fold Induction*</th>
<th>TBARS, nmol/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LDL</td>
<td>1.07</td>
<td>1.1</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>oxLDL</td>
<td>15.2</td>
<td>87.1</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>LDL</td>
<td>0.028</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>oxLDL</td>
<td>0.048</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>LDL</td>
<td>0.064</td>
<td>1.1</td>
<td>3.0</td>
</tr>
<tr>
<td>6</td>
<td>oxLDL</td>
<td>0.045</td>
<td>1.3</td>
<td>18.8</td>
</tr>
<tr>
<td>7</td>
<td>LDL</td>
<td>0.705</td>
<td>None</td>
<td>5.9</td>
</tr>
<tr>
<td>8</td>
<td>oxLDL</td>
<td>0.781</td>
<td>None</td>
<td>20.8</td>
</tr>
<tr>
<td>9</td>
<td>LDL</td>
<td>0.625</td>
<td>1.1</td>
<td>2.9</td>
</tr>
<tr>
<td>10</td>
<td>oxLDL</td>
<td>0.346</td>
<td>1.2</td>
<td>12.3</td>
</tr>
<tr>
<td>11</td>
<td>LDL</td>
<td>0.391</td>
<td>None</td>
<td>7.2</td>
</tr>
<tr>
<td>12</td>
<td>oxLDL</td>
<td>0.257</td>
<td>None</td>
<td>37.9</td>
</tr>
<tr>
<td>13</td>
<td>LDL</td>
<td>0.245</td>
<td>1.1</td>
<td>2.6</td>
</tr>
<tr>
<td>14</td>
<td>oxLDL</td>
<td>0.223</td>
<td>1.2</td>
<td>45.6</td>
</tr>
</tbody>
</table>

TF indicates tissue factor; TBARS, thiobarbituric acid-reactive substance; LDL, low-density lipoprotein; oxLDL, oxidized LDL; and NA, not assayed.

*TF activity was measured after 12 hours in response to 80 µg/mL oxLDL.

nation that maximal doses of lipoprotein produced an endotoxin concentration of 85 pg/mL and 1.22 ng/mL for LDL and oxLDL, respectively. Nevertheless, this contaminating endotoxin alone did not appear to account for the observed 87-fold induction of TF activity, because exogenously added LPS (10 ng/mL) led to only a 21-fold induction (Fig 2). One possible explanation was that endotoxin and oxLDL acted in concert to induce TF activity in monocytes. Therefore, we made great efforts to prepare LDL and oxLDL with virtually no endotoxin contamination (<1 pg/µg lipoprotein, the Table). No induction of TF activity was observed in adherent monocytes incubated for 12 hours with 80 µg/mL of endotoxin-free oxLDL or native LDL (Fig 1). These data indicated that oxLDL, in the absence of significant levels of contaminating endotoxin, did not induce TF activity in adherent monocytes.

OxLDL Enhances LPS-Induced TF Activity

To verify that addition of oxLDL could enhance the induction of TF activity in monocytes exposed to LPS, we incubated monocytes for 12 hours with increasing...
concentrations of LPS (0.01 to 10 ng/mL) in the absence or presence of 80 μg/mL endotoxin-free LDL or oxLDL (Fig 2). The concentrations of LPS in these experiments were calculated to be similar to those of contaminating endotoxin observed in lipoprotein preparation No. 1 (the Table). Unstimulated monocytes expressed very low levels of TF activity. As expected, LPS alone induced TF activity in a dose-dependent manner, with a minimal effective concentration of 1 ng/mL. However, induction of TF activity in cells incubated with both LPS (1 to 10 ng/mL) and oxLDL (80 μg/mL) was significantly higher than that observed with LPS alone. Ox-LDL caused a 3.5±1.0-fold (n=3) enhancement of the effect of 10 ng/mL LPS. In contrast, the presence of native LDL (80 μg/mL) derived from the identical lipoprotein preparation had no effect on induction of TF activity by exogenously added LPS (Fig 2). After dialysis against Ham's F-10 medium, oxLDL lost 65% of its TBARS but effected a 2.8-fold enhancement of TF activity induced by 10 ng/mL LPS (data not shown). Cupric acetate alone had no effect on LPS induction of TF activity (data not shown).

Because LPS is known to associate with lipoproteins,23 enhancement of LPS-induced TF activity by oxLDL might be explained by a preferential association of LPS with oxLDL. Therefore, we coincubated [3H]Re595-LPS (2.8 μg/mL) with LDL and oxLDL (80 μg/mL) under conditions similar to those in our culture system (12 hours, 37°C, in the presence of lipoprotein-depleted plasma). LDL and oxLDL were reisolated by ultracentrifugal flotation in KBr at 1.21 g/mL. The lipoproteins were recovered in the top 10% of the KBr gradient. Furthermore, the majority of the radioactivity associated with the [3H]Re595-LPS was recovered in this lipoprotein fraction (62.0±2.8% and 56.0±1.4% for LDL and oxLDL, respectively).

Time Course of TF Induction

The kinetics of TF induction in monocytes by 10 ng/mL LPS alone and in the presence of oxLDL or native LDL (80 μg/mL) was examined. Adherent LPS-stimulated monocytes exhibited a 15-fold increase in functional TF activity, which was maximal at 12 hours (Fig 3). This LPS induction of TF was enhanced 3.4-fold by oxLDL, with a similar maximal effect at 12 hours (Fig 3). TF activity in cells coincubated with LPS and oxLDL decreased at 18 hours but remained elevated in comparison with the TF activity observed in cells treated with LPS alone. In contrast, native LDL (80 μg/mL) had no effect on the LPS-stimulated increase in TF activity. Monocytes incubated in medium alone exhibited no increase in TF expression. When monocytes were exposed to oxLDL (80 μg/mL) for 12 hours before the 12-hour incubation with LPS (10 ng/mL), TF activity was again enhanced 2.6-fold (data not shown).

Dose Response of TF Induction by OxLDL in the Presence of LPS

We examined the dose response of oxLDL for enhancement of LPS-induced TF activity. As shown in Fig 4, monocytes were incubated for 12 hours with 10 ng/mL LPS and increasing concentrations of oxLDL or native LDL (10 to 80 μg/mL). LPS-induced TF activity was significantly enhanced in a dose-dependent manner by oxLDL, with a maximal effect at the highest dose
LPS-induced TF activity (Fig 5). Oxidation of the lipoproteins was characterized by SDS-PAGE and the TBARS assay that revealed a time-dependent loss or fragmentation of apolipoprotein (apo) B-100, as evidenced by Coomassie Blue R staining (Fig 5, upper panel), and a time-dependent increase in TBARS, a measure of lipid peroxidation (Fig 5, center panel). In addition, conjugated-diene generation in oxLDL (increases of 0.137, 0.375, and 0.595 absorbance units at 1, 2, and 4 hours, respectively, as monitored by UV absorbance at 234 nm) indicated a faster rate of LDL modification than was evidenced by apo B fragmentation or TBARS formation. LPS (10 ng/mL) alone induced TF activity 15-fold, which was not changed by the presence of sham-oxidized LDL (control LDL containing copper and BHT; "0 h" oxidation in Fig 5, lower panel). In contrast, LDL oxidized for as little as 1 hour enhanced LPS-induced TF activity 3.1-fold, and maximal enhancement was observed for LDL samples oxidized for 9 hours (4.8-fold). Oxidation for 24 or 48 hours did not further increase the effect of oxLDL on LPS-induced TF activity, although it did increase apo B-100 protein fragmentation and TBARS content of the lipoprotein. It is interesting that a 1-hour exposure to Cu²⁺ was sufficient to initiate significant oxidation as measured by the TF response, suggesting that induction of TF expression, apo B fragmentation, and conjugated-diene formation are more sensitive measures of oxLDL activity than the TBARS assay. It is important to note that the degree of oxidation measured by SDS-PAGE, TBARS, and conjugated-diene formation represented LDL modifications that existed before it was added to the adherent monocyte cultures. Further cell-mediated modification of LDL may have occurred during the 12-hour culture period despite the presence of serum and BHT in the cultures. This experiment also served as a control for endotoxin contamination, because all samples were derived from a single LDL sample and yet, after oxidation, the samples had different abilities to enhance LPS-induced TF activity.

Effect of OxLDL on LPS-Induced Production of TNF-α and IL-8

To document the specificity of the enhancing effect of oxLDL on TF induction by LPS, we examined the effect of this modified lipoprotein on monocyte expression of two other LPS-inducible pro-inflammatory cytokines, TNF and IL-8. TNF has been implicated as playing a role in atherosclerotic plaque development. Furthermore, oxLDL inhibits LPS-induced expression of TNF mRNA in murine macrophages. In a separate study, we have documented induction of IL-8 in human monocytes and monocytic THP-1 cells by endotoxin-free oxLDL. IL-8 has the potential to affect atherogenesis by virtue of its ability to chemoattract T lymphocytes and to induce angiogenesis. Both cytokines were measured in the 12-hour supernatants obtained from the LPS dose-response experiment shown in Fig 2. In agreement with the previous report, oxLDL dramatically inhibited LPS-induced production of TNF (Fig 6). In contrast, oxLDL did not modulate LPS-stimulated IL-8 production, although in agreement with our previous data, oxLDL alone induced IL-8 expression (Fig 7).

Discussion

It has been suggested that oxLDL modulates functions of monocytic cells within atherosclerotic lesions. This study demonstrated that in addition to reported direct effects, oxLDL also modulated LPS-induced gene expression in human adherent monocytes. Whereas oxLDL alone, which was free of significant detectable levels of LPS contamination, did not induce TF activity, this oxLDL consistently enhanced LPS-induced TF activity in human adherent monocytes in a time-and
OxLDL Enhances LPS-Induced TF Expression

It emphasizes the importance of accurate and sensitive methods for the expression of LPS-sensitive genes in monocytic cells. This is the first report to our knowledge of an enhancement of LPS-inducible gene expression by oxLDL.

Three lines of evidence support our conclusion that enhancement of LPS-induced TF activity by oxLDL is not due to additional contaminating LPS but rather to direct or indirect cooperation between oxLDL and LPS. First, the levels of contaminating LPS in the oxLDL preparations were in all cases below those required to increase TF activity in monocytes under our culture conditions. Second, the endotoxin levels in control LDL were comparable with those in oxLDL, but control lipoproteins had no enhancing effect on LPS-induced TF expression. Third, in studies designed to determine the effect of the duration of oxidation of LDL, all samples were removed from a single oxidation mixture (and therefore had identical LPS levels), yet induction of TF activity was dependent on the time of oxidation.

Modulation of the effects of LPS by oxLDL may result from a direct extracellular interaction between LPS and oxLDL. However, we were unable to document a preferential association between [3H]Re595-LPS and oxLDL. It is equally possible that cooperative interactions between oxLDL and LPS occur intracellularly. Both oxidative stress and LPS activate the NF-κB/Rel family of transcription factors. These proteins play an essential role in the transcriptional regulation of several LPS-inducible genes expressed in monocytic cells. Recently we have demonstrated that a κB-like DNA element in the human TF promoter mediates LPS induction of TF expression in THP-1 monocytic cells. Furthermore, minimally modified LDL injected into atherosclerosis-susceptible mice increases liver expression of a set of inflammatory gene products involved in atherosclerosis that may be regulated by NF-κB. OxLDL may exert oxidative stress on the cells and thereby contribute to intracellular signals that increase the magnitude of activation of some or all of the NF-κB/Rel family of transcription factors. Further studies are necessary to characterize the cellular, biochemical, and molecular mechanisms responsible for oxLDL amplification of the TF response to LPS.

No direct effect of oxLDL on TF activity was observed in adherent monocytes when LPS contamination of our lipoprotein preparations was reduced to <0.8 pg/μg lipoprotein. Our results differ from an earlier report that demonstrated induction of TF expression in monocytes/macrophages by chemically modified LDL at doses of 25 to 750 μg/mL. In addition, our preliminary studies with the human monocytic cell line THP-1 indicated that oxLDL directly induces TF mRNA and TF activity. Minimally modified LDL and oxLDL also directly induce TF expression in endothelial cells. These discrepancies may be due to differences in the differentiation state of the monocytic cells, differences in TF regulation in monocytes versus endothelial cells, or the type and degree of modification of LDL. However, the rigorous precautions we found necessary to reduce endotoxin contamination of lipoproteins during isolation and particularly during oxidation suggest that endotoxin contamination may account for some of the conflicting results. Thus, our study should serve as a caveat for all experiments designed to examine the effect of lipophilic molecules, such as lipoproteins, on the expression of LPS-sensitive genes in monocytic cells. It emphasizes the importance of accurate and sensitive methods for the expression of LPS-sensitive genes in monocytic cells.
screening for endotoxin at all stages of lipoprotein isolation and modification.

In summary, our study demonstrated that oxLDL significantly enhanced LPS-induced TF activity in adherent monocytes. LPS is responsible for many of the cellular responses to Gram-negative bacterial infections, including induction of TF. Therefore, these studies indicate that an in vitro relationship exists between LPS and oxLDL and may parallel a pathogenic relationship in vivo. Monocytes are exposed to endotoxins in plasma and to oxLDL after recruitment into the artery wall. In addition, endotoxins may leak through the defective endothelial barrier in atherosclerotic lesions. Finally, endotoxins may be carried within the LDL or oxLDL particle, so cells would be exposed to the two pathogenic factors simultaneously. Thus, monocytes exposed to endotoxins and oxLDL in the intimal lesion may express increased levels of TF that can contribute to a hypercoagulable state and to thrombotic events associated with atherosclerosis.

Acknowledgments
This work was supported by National Institutes of Health grants HL-35297 (to Dr. Curtis), HL-45872 (to Dr. Mackman), and DK-36702 (to Dr. Terkeltaub); the Veterans Administration Research Service (to Dr. Terkeltaub); a fellowship from the Deutsche Forschungsgemeinschaft (to Dr. Brand); and a fellowship from the American Association of University Women (to Dr. Banka). This is manuscript 7972-IMM from The Scripps Research Institute. We acknowledge David J. Bonnet, Marsha Hansen, Denise Santoro, and Joell Solan for technical assistance; Audrey Black for graphics; andAnna Meyers for preparing the manuscript.

References


Oxidized LDL enhances lipopolysaccharide-induced tissue factor expression in human adherent monocytes.

K Brand, C L Banka, N Mackman, R A Terkeltaub, S T Fan and L K Curtiss

Arterioscler Thromb Vasc Biol. 1994;14:790-797
doi: 10.1161/01.ATV.14.5.790

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/14/5/790