Oxidized Low-Density Lipoprotein Downregulates Endothelial Basic Fibroblast Growth Factor Through a Pertussis Toxin–Sensitive G-Protein Pathway
Mediator Role of Platelet-Activating Factor–Like Phospholipids

Po-Yuan Chang, MD, PhD; Sherry Luo, BS; Tao Jiang, PhD; Yuan-Teh Lee, MD, PhD; Shao-Chun Lu, PhD; Philip D. Henry, MD; Chu-Huang Chen, MD, PhD

Background—Oxidized LDL (oxLDL) inhibits angiogenesis in part by downregulating endothelial basic fibroblast growth factor (bFGF). To determine the mechanism of the downregulation, we investigated the signal transduction pathway involving potential phospholipid mediators.

Methods and Results—Cultured bovine aortic endothelial cells were incubated with PBS (lipoprotein-free control), LDL, or copper oxLDL under serum-free conditions. At 24 hours, oxLDL (50 μg/mL) decreased bFGF mRNA (Northern blot), bFGF protein (Western blot and ELISA), and concomitant DNA synthesis, all by 40% to 50% compared with PBS. LDL had no effect. Pretreating the cells with 100 ng/mL pertussis toxin (PTX) for 18 hours before oxLDL exposure almost completely blocked the inhibitory effects of oxLDL. In contrast, inhibiting other major cellular signal transduction pathways with PD-98059 (mitogen-activated protein kinase inhibitor), HA-1004 (inhibitor of cGMP- and cAMP-dependent protein kinase), or Ro-31-8220 (protein kinase C inhibitor) or chelating intracellular Ca²⁺ with BAPTA-AM failed to attenuate any of the oxLDL effects assayed. Addition to the cultures of WEB 2086, a specific antagonist of the PTX-sensitive G protein–coupled platelet-activating factor (PAF) receptor, blocked the action of oxLDL. Whereas PAF dispersed in the culture medium failed to produce oxLDL-like effects, degradation of PAF and PAF-like phospholipids accumulated in oxLDL with a recombinant human PAF acetylhydrolase eliminated the inhibitory effects of oxLDL on bFGF expression and DNA synthesis.

Conclusions—OxLDL suppresses endothelial bFGF expression and DNA synthesis through a PTX-sensitive heterotrimeric G-protein pathway involving mediator phospholipids similar, but not identical, to PAF. (Circulation. 2001;104: 588-593.)

Key Words: phospholipids ■ lipoproteins ■ growth substances ■ endothelium ■ genes

The cell growth and survival factor basic fibroblast growth factor (bFGF) directly or, by inducing other genes, indirectly regulates a wide range of functions in vascular endothelial cells (ECs).¹–³ Massive apoptosis and greatly impaired angiogenic responses occur in ECs deprived of bFGF.⁴–⁶ In arterial explants, EC proliferation and growth of capillary-like microtubules are inhibited by oxidized LDL (oxLDL)–induced bFGF downregulation.⁶⁷ Delineation of the signal transduction pathway mediating the downregulation may reveal clues to the nature of the components in oxLDL responsible for the inhibition. Characterization of the mediator components in copper oxLDL may suggest a method useful for the identification of similar mediators in the oxLDL-like lipoproteins present in the plasma of patients with hypercholesterolemia.

Signal transduction pathways described to date in oxLDL effects include involvement of G protein–coupled receptors, mitogen-activated protein kinase (MAPK), MAPK kinase (MEK), cGMP- and cAMP-dependent protein kinase (PK), and PKC.⁸–¹² Some oxLDL activities have been associated with excessive release of Ca²⁺ in the cytoplasm.¹³ The lack of uniformity in the signaling pattern suggests that different LDL components modified during oxidation may participate in mediating cellular effects. Our data suggest that oxLDL-induced bFGF downregulation is sensitive to pertussis toxin (PTX). One of the PTX-sensitive G protein–coupled mem-

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brane receptors implicated in the mediation of oxLDL signals is platelet-activating factor (PAF) receptor (PAFR). 14–17 Some oxLDL effects have been attributed to mediator phospholipids accumulated in the modified lipoproteins. 14–18–20 The phospholipids may share structural similarities with PAF, because they exhibit sensitivity to PAFR antagonists. 14–17 Here, we addressed the question of whether oxLDL down-regulates endothelial bFGF via a signal pathway that involves PAFR.

Methods

Cells and LDL Preparations

Primary cultures of bovine aortic ECs (BAECs) were derived and purified as described. 7 Cells at 8 to 12 passages, maintained in DMEM supplemented with 10% FBS and antibiotics (streptomycin 100 μg/mL, penicillin 100 IU/mL, amphotericin B 0.25 μg/mL), were used. LDL particles (d=1.019 to 1.063 g/mL) from pooled human plasma anticoagulated with EDTA (0.5 mg/mL) were isolated by sequential ultracentrifugation. 18 For oxLDL preparation, LDL was exposed at 37°C for 24 hours to 5 μmol/L CuSO4 in PBS. After the incubation was terminated by EDTA (0.5 mg/mL), the preparation was dialyzed and preserved in nitrogen-filled tubes. 9 Precautions previously described were taken to prevent endotoxin contamination. 6 Protein in LDL preparations was estimated by the Lowry method, and thiorbituric acid-reactive substances (TBARS) contained in LDL preparations were assayed as a measure of oxidative lipid modification. 18 At 24 hours, the TBARS values in LDL were 18 to 22 μg/mg protein with and without CuSO4 exposure, respectively.

Protocol

Cell cultures grown to subconfluence were washed 3 times with serum-free medium and maintained under serum-free conditions for 6 hours before treatment with various signal transduction inhibitors, PAF receptor blockers, or PBS, according to protocols determined by preliminary experiments. After another 18 hours, cells were incubated with PBS (lipoprotein-free control), native LDL (50 μg/mL), or oxLDL (50 μg/mL) for 24 hours.

Inhibitors of Signal Transduction Pathways

To determine the involvement of major signal transduction pathways, cells were treated with PTX (a G protein inhibitor), PD-98059 (an MEK/MAPK inhibitor), Ro-31-8220 (a PKC inhibitor), and BAPTA-AM (an intracellular Ca2+ chelator) before exposure to oxLDL. All agents were purchased from Calbiochem. Protocols for individual agents were determined on the basis of the maximal doses individual agents were determined on the basis of the maximal doses

PAFR Antagonists, PAF-16, and PAF-Acetylhydrolase

To determine whether the signal was transmitted through PAFR, the competitive PAFR antagonist WEB 2086 (10 μmol/L; gift from Boehringer Ingelheim) was added to the medium and allowed to equilibrate for 1 hour before addition of oxLDL. To test whether PAF was equivalent to oxLDL in inhibiting bFGF expression and DNA synthesis, some cultures were incubated with 0.1 to 1 μmol/L PAF-16 (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine; Calbiochem), a synthetic, water-soluble PAF, instead of oxLDL. To degrade the sn2 acetyl group of phospholipids accumulated in oxLDL, some oxLDL preparations were preincubated with 50 to 200 μg/mL of a recombinant PAF-acetylhydrolase (rPAF-AH; gift from ICOS, Bothell, Wash) for 1 hour at 37°C.

Northern Blot Analysis

After isolation from cultured cells, 50 μg total RNA was subjected to electrophoresis in each lane of 1% agarose/2.2 mol/L formaldehyde gel and transferred to Nytran membranes (Schleicher & Schuell) as described. 7 The bFGF cDNA insert was excised from plasmid constructs with EcoRI and gel-purified (Geneclean Kit, Bio 101, Inc) for use as templates. After labeling with [α-32P]dCTP to a high specific activity (~10 6 cpm/μg) by a random-priming method, probes were hybridized to the blots (10 4 cpm/mL) overnight at 42°C in a solution containing 40% formamide, 5% SSC, 5% Denhardt’s solution, 0.5% SDS, 250 μg/mL salmon sperm DNA, and 10% dextran sulfate. The blots were washed under high stringency at 65°C in 0.2× SSC plus 0.1% SDS. For autoradiographic detection, films were exposed at ~70°C for 3 days with 2 intensifying screens. A 1.2-kb Paf1 fragment of human GAPDH cDNA, cloned according to a described method, 13 was 32P-labeled by random priming and used as an internal control. A PhosphorImager was used to determine bFGF expression.

Western Blot Analysis

Protein concentrations were determined according to the method of Bradford (BioRad) in cells lysed in a pH 7.5 buffer containing 20 mmol/L Tris-HCl, 0.5% SDS (wt:vol), 10% glycerol, and 50 mmol/L dithiothreitol. Protein samples (180 μg) were resolved by electrophoresis on denaturing 15% SDS–polyacrylamide gels for 2 hours at 100 V with a Mighty Small Gel System (Hoefer). After electrophoresis, the samples were transferred to a polyvinylidene difluoride membrane in a buffer containing 25 mmol/L Tris base, 192 mmol/L glycine, and 20% methanol, pH 8.3. Nonspecific binding sites were blocked with nonfat skim milk, and the membranes were incubated with a monoclonal antibody against bovine bFGF (Upstate Biotechnology). Antigen-antibody complexes were visualized with a horseradish peroxidase chemiluminescent system (ECL kit, Pharmacia). PhosphorImager quantification was used to determine bFGF expression.

ELISA and DNA Synthesis

For ELISA and DNA synthesis, 100×10 4 cells were inoculated in each well of 12-well Corning cell culture plates. Intracellular bFGF concentrations in cell lysates prepared with Nonidet P-40 (Sigma) were measured by ELISA with a Quantikine kit (R&D Systems) and estimated spectrophotometrically at 450 nm, as described. 7 To evaluate treatment effects on DNA, DNA synthesis was assayed by addition of 3 μCi/mL [3 H]thymidine (Moravek Biomedicals) to the medium during the final 4 hours of incubation. Incubation was terminated by decanting the medium and fixing the cells with 1 mL of 10% (wt:vol) cold trichloroacetic acid for 15 minutes at 4°C. [3 H]Thymidine incorporated in extracted DNA was assayed by scintillation spectrometry. 6,7

Statistical Analysis

The significance of differences between the means of treatment and control (PBS) groups was assessed by a 2-sided Student’s t test with Bonferroni correction. Values of P<0.05 were considered significant. Results are expressed as mean±SEM values. A GB-STAT program (Dynamic Microsystems) was used.

Results

Signal Transduction of oxLDL-Induced bFGF Downregulation

At 24 hours, oxLDL (50 μg/mL) decreased bFGF mRNA by 40% to 50% compared with PBS, whereas LDL had no effect (Figure 1), findings similar to those reported previously. 7 The inhibitory effect was almost completely blocked in cells incubated with the G-protein inhibitor PTX (100 ng/mL) for 18 hours before oxLDL exposure. In contrast, pretreating the cells with the MEK/MAPK inhibitor PD-98059 (20 μmol/L)
for 18 hours or the PKC inhibitor Ro-31-8220 (3.5 μmol/L) for 1 hour failed to exhibit any inhibitory effects (Figure 1). At the Western blot–assayed protein level as well, the inhibitory effect of oxLDL (40% to 50% reduction) was sensitive to PTX but not to PD-98059 or Ro-31-8220 (Figure 2). Blocking the cGMP- and cAMP-dependent PK with HA-1004 (10 μmol/L) for 18 hours or chelating the intracellular Ca2+ with BAPTA-AM (16 μmol/L) for 1 hour failed to attenuate the reduction caused by oxLDL. In parallel, the effect of oxLDL in reducing intracellular bFGF concentration measured by ELISA was preventable only by PTX pretreatment, among the treatments tested (Figure 3). The concomitant 50% reductions in DNA synthesis (n=3; P<0.05) at 24 hours were also sensitive to PTX but not other signal inhibitors or the Ca2+ chelator (data not shown).

**Mediator Role of PAF-Like Phospholipids**

Compared with PBS, WEB 2086 (10 μmol/L) pretreatment for 1 hour prevented the reduction in bFGF mRNA in cells incubated with oxLDL (Figure 4). In addition, WEB 2086 pretreatment greatly attenuated oxLDL-induced reductions in intracellular bFGF concentrations and DNA synthesis (Table). PAF-16 (1 μg/mL) did not induce significant reductions in intracellular bFGF concentrations or DNA synthesis (Table). Cells incubated with rPAF-AH alone (200 μg/mL) exhibited no differences in bFGF concentrations or DNA synthesis compared with PBS at 24 hours. The inhibitory effects of oxLDL on bFGF expression and DNA synthesis, however, were abolished by pretreatment with rPAF-AH (Figure 4 and Table). Sham pretreatment of oxLDL with PBS did not alter its effects on bFGF expression or DNA synthesis (data not shown).

**Discussion**

Induced angiogenesis has the potential to become an important therapy for ischemia from atherosclerotic vascular disease. Key to its pursuit is understanding factors that may render the ECs unresponsive to angiogenic stimuli. Copper oxLDL inhibits EC proliferation and angiogenesis in vitro,6,7 and modified LDL occurring in arterial walls and circulating in plasma may have similar effects.22–24 The angiostatic effects of oxLDL are attributable to suppressed expression of bFGF, a growth factor involved in all phases of angiogenesis: EC proliferation, EC migration, and vascular differentiation.25 Here, we demonstrated that oxLDL downregulates endothelial bFGF through a PTX-sensitive heterotrimeric G-protein pathway. The mediators—culprit components accumulated in oxLDL—are characterized by their sensitivity to rPAF-AH, an enzyme that degrades phospholipids at the glycerol sn2 position.14–16 Their actions are attenuated by WEB 2086,17 a selective antagonist of PAFR, a heptahelical G protein–coupled receptor.26

**Figure 1.** Effects of signal transduction inhibitors on oxLDL-induced reduction in bFGF mRNA assessed by Northern blot analysis and densitometry in cultured BAECs. Cells were incubated with 50 μg/mL oxLDL for 24 hours alone or with preceding exposure to MEK/MAPK inhibitor (PD-98059), PKC inhibitor (Ro-31-8220), or G-protein inhibitor (PTX) according to protocols described in text. PBS and LDL (50 μg/mL) were used as lipoprotein-free and oxidation-free controls. Data are representative of 3 separate experiments with similar results, normalized to GAPDH standards.

**Figure 2.** Effects of signal transduction inhibitors on oxLDL-induced reduction in bFGF protein assessed by Western blot analysis in cultured BAECs. Cells were incubated with 50 μg/mL oxLDL for 24 hours alone or with preceding exposure to MEK/MAPK inhibitor (PD-98059), PKC inhibitor (Ro-31-8220), PK inhibitor (HA-1004), Ca2+ chelator (BAPTA-AM), or G-protein inhibitor (PTX) according to protocols described in text. PBS was used as a lipoprotein-free control. Data are representative of 3 separate experiments with similar results.

**Figure 3.** Effects of signal transduction inhibitors on oxLDL-induced reduction in intracellular bFGF protein concentrations assessed by ELISA in cultured BAECs. Cells were incubated with 50 μg/mL oxLDL for 24 hours alone or with preceding exposure to MEK/MAPK inhibitor (PD-98059), inhibitor of cGMP- and cAMP-dependent PK (HA-1004), PKC inhibitor (Ro-31-8220), Ca2+ chelator (BAPTA-AM), or G-protein inhibitor (PTX) according to protocols described in text. PBS and LDL (50 μg/mL) were used as lipoprotein-free and oxidation-free controls. Values are mean±SEM (n=3). Each well contained 100×104 cells at inoculation. *P<0.05 vs PBS.
Angiogenesis involves multiple growth factors/cytokines. Like bFGF, vascular endothelial growth factor (VEGF) improves collateral-dependent tissue perfusion in hypercholesterolemic rabbits. Previously, we showed that oxLDL downregulates endothelial bFGF without affecting the FGF receptor FGFR-1; the associated reductions in DNA synthesis and capillary-like microtubule growth in arterial explants are reversed by exogenous bFGF, but not by VEGF or transforming growth factor-β. Because concentrations of circulating material reactive to anti-oxLDL monoclonal antibody are found to be highly increased in subjects with coronary events, bFGF downregulation by oxLDL may be an important mechanism in impaired angiogenic responses in atherosclerosis.

Signal transduction pathways in oxLDL-induced growth factor modulation have not been demonstrated. In this study, the inhibitory effect of oxLDL on endothelial bFGF expression at both mRNA and protein levels was nearly completely abolished by PTX, suggesting a role of G, and probable involvement of a PTX-sensitive G protein–coupled receptor. He signal pathway for this particular action of oxLDL did not involve the activation of MEK/MAPK, cGMP- and cAMP-dependent PK, or PKC: their respective inhibitors—PD98059, HA-1004, and Ro-31-8220—failed to alter the effect of oxLDL. In addition, the inhibition was largely independent of intracellular Ca²⁺ release, because chelating the cytosolic Ca²⁺ with BAPTA-AM failed to stop the action of oxLDL.

Our results complicate understanding of how oxLDL exerts various bioactivities through different signaling pathways. In contrast to our finding in bFGF modulation, it was recently reported that oxLDL upregulates endothelial monocyte chemoattractant protein 1 via a lectin-like receptor pathway that involves activation of MAPK but not PTX-sensitive G proteins. Yet in canine vascular smooth muscle cells, mitogenic effects of oxLDL are mediated through a PTX-sensitive G protein–coupled receptor mechanism that involves activation of the Ras/Raf/MEK/MAPK pathway.

### Effects of LDL Preparations, PAF-16, rPAF-AH, OxLDL, and WEB 2086+OxLDL on Intracellular bFGF Protein Levels and DNA Synthesis at 24 Hours

<table>
<thead>
<tr>
<th>Assessment</th>
<th>PBS (Control)</th>
<th>LDL 100 µg/mL</th>
<th>OxLDL 50 µg/mL</th>
<th>PAF-16 1 µg/mL</th>
<th>rPAF-AH 200 µg/mL</th>
<th>OxLDL+ rPAF-AH 50 µg/mL+ 200 µg/mL</th>
<th>WEB + OxLDL 10 µmol/L+ 50 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular bFGF level, pg/mg protein×10⁻⁶/dish</td>
<td>300±26</td>
<td>304±24</td>
<td>205±44*</td>
<td>272±50</td>
<td>326±42</td>
<td>284±45</td>
<td>278±53</td>
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<tr>
<td>[³H]-Thymidine incorporation, cpm×10⁻⁶/dish (DNA)</td>
<td>90±10</td>
<td>88±12</td>
<td>58±11*</td>
<td>74±12</td>
<td>100±12</td>
<td>82±16</td>
<td>80±15</td>
</tr>
</tbody>
</table>

OxLDL + rPAF-AH indicates that oxLDL was pretreated with rPAF-AH at 37°C for 1 hour before being used in cell culture. WEB + OxLDL indicates that cells were treated with WEB 2086 1 hour before the addition of oxLDL. Values are mean±SEM; n=3 in all treatments; 100×10⁻⁶ cells/well at inoculation.

*P<0.05 vs PBS.
derivatives of oxidized PAPC, including POVPC, GPGC, and an m/z 828.6 (M+H+) molecule. Some of the lipid components, including lysophosphatidylcholine, PAF, and those derived from MM-LDL, have been shown to exert their effects by means of the PAFR, which is known to be coupled with PTX-sensitive G proteins. The nearly complete suppression of oxLDL-induced bFGF downregulation in the presence of the PAFR-specific antagonist WEB 2086 substantiates the importance of the PAFR pathway. WEB 2086 and WEB 2170, both PAFR antagonists of pyrrolothiazole-derivative origin, have been shown to attenuate various oxLDL or MM-LDL bioactivities, such as monocyte-EC adhesion and smooth muscle cell proliferation. The oxLDL effects may also be blocked by structurally different PAFR antagonists, such as L659,989, a naturally occurring compound.

Whereas the oxLDL effects were prevented by WEB 2086, PAF itself failed to elicit oxLDL-like effects. Although PAF-16, a potent, water-soluble PAF analogue, was administered within the dose range (0.1 to 1 μg/mL) capable of inducing maximal gallbladder muscle contraction, it failed to exhibit oxLDL-like effects in suppressing bFGF expression and DNA synthesis. The seemingly paradoxical results raise 2 possibilities: that WEB 2086 antagonizes oxLDL by a mechanism other than binding to PAFR or that PAFR acts as a receptor for phospholipids other than PAF. Although the issue warrants further investigation, complete inactivation of oxLDL by rPAF-AH suggests a mediator role of PAF-like phospholipids other than PAF. Although the issue warrants further investigation, complete inactivation of oxLDL by rPAF-AH suggests a mediator role of PAF-like phospholipids. PAF-AH is an enzyme that degrades PAF and PAF-like phospholipids at the glycerol sn2 position; a loss of PAF-AH activity in LDL during oxidative modification of such phospholipids may be important in generating lipoproteins with oxLDL-like properties. Protective effects of HDL against atherosclerosis may be mediated by the PAF-AH activity of HDL acting on phospholipids accumulated in oxidatively modified LDL. Depleting apolipoprotein B-containing lipoproteins (LDL, IDL, VLDL) of their intrinsic PAF-AH activity increases their potency in stimulating monocyte chemotaxis and adhesion.

Thus, many oxLDL bioactivities appear to be mediated by a common pathway that involves PAFR. The oxLDL pathway mediating bFGF downregulation exhibits a striking similarity to that mediating phospholipase D activity stimulation, which also goes through the PTX-sensitive G protein–coupled PAFR without involving PKC. Accordingly, we propose a possible signal transduction pathway (Figure 5) that mediates the inhibitory effect of oxLDL on endothelial bFGF expression. The schematic pathway includes the finding that oxLDL reduces intracellular bFGF concentration in part by destabilizing mRNA posttranscriptionally. The cytosolic messengers and the mechanisms of transcription factor–bFGF promoter complex modulation are yet to be identified or delineated. Because other mediators remain to be identified, other possible pathways cannot be excluded.

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