Oxidized Low-Density Lipoproteins Inhibit Endothelial Cell Proliferation by Suppressing Basic Fibroblast Growth Factor Expression

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Background—Hyperlipidemia inhibits proliferation of endothelial cells (ECs) in culture and angiogenesis in vivo and in arterial explants. Elucidation of the mechanisms may suggest novel therapies against atherosclerosis.

Methods and Results—Basic fibroblast growth factor (bFGF) expression and mitogenic effects were assessed in bovine aortic ECs incubated with oxidized LDL (ox-LDL). Compared with native LDL and lipoprotein-free controls, ox-LDL reduced bFGF mRNA levels in a time- and concentration-dependent manner, 100 μg/mL producing a maximum reduction of 40% to 50% within 24 to 48 hours. There were commensurate reductions in intracellular and extracellular bFGF concentrations, DNA and total RNA syntheses, and cell replication. FGF receptor 1 and β-actin mRNA levels were unchanged. Ox-LDL accelerated bFGF mRNA degradation in actinomycin D–treated cells. However, inhibition of bFGF expression by ox-LDL was attenuated by cyclohexamide, indicating a requirement for continuous new protein synthesis for posttranscriptional destabilization. Reduced syntheses of DNA and total RNA were completely restored by bFGF but not by vascular endothelial growth factor. Inhibition of total RNA synthesis achieved by exposing cells to a bFGF-neutralizing antibody was similar in magnitude to that induced by ox-LDL.

Conclusions—Cytotoxic effects of ox-LDL on ECs are attributable in part to suppression of bFGF expression. **(*Circulation*. 2000;101:171-177.)**

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

Hyperlipidemia impairs function of both large-vessel and microvascular endothelium. We have shown that compensatory macrovascular and microvascular growth is impaired in rabbits with diet-induced hypercholesterolemia.[1] Impaired capillary-like microtube growth in arterial explants exposed to hypercholesterolemia in vivo or to oxidized LDL (ox-LDL) in vitro is associated with reduced basic fibroblast growth factor (bFGF) concentrations in the culture medium. The impairment can be partially reversed by exogenous bFGF.[2,3] Here, we determined whether impairment of proliferative and angiogenic responses induced by ox-LDL is due to suppressed endothelial expression of bFGF or of its receptor, FGFR-1. The effects of bFGF on DNA and total RNA syntheses in the presence of ox-LDL were compared with those of vascular endothelial growth factor (VEGF), an endothelial cell (EC)–specific mitogen and potent angiogenic factor.[4,5]

Methods

Cells

Primary cultures of bovine aortic ECs (BAECs) were derived by a previously reported procedure.[2] Cell purity was assessed by uptake of acetylated LDL labeled with 1,1′-dioctadecyl-1,3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Biomedical Technologies), immunocytochemical staining for von Willebrand factor–related antigen with FITC-labeled monoclonal antibody (Incstar), and negative staining for α-actin with HHF35 antibody (Enzo).[2,6] More than 98% of the cells exhibited responses typical of cells of endothelial origin. 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.

Preparation of Native LDL and Ox-LDL

Native LDLs (d=1.019 to 1.063 g/mL) from pooled human plasma anticoagulated with EDTA 0.5 mg/mL were isolated by sequential ultracentrifugation.[7] Ox-LDLs were prepared by exposing native LDL for 24 hours at 37°C to 5 μmol/L $CuSO_4$ in PBS.[8] Mildly oxidized LDLs were prepared by exposing native LDLs to $CuSO_4$ for

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4 hours. Oxidation was terminated by EDTA 0.5 mg/mL, and LDL preparations were dialyzed against PBS. Precautions were taken to prevent endotoxin contamination during lipoprotein isolation and oxidation, including monitoring by the limulus amoebocyte lysate assay (Associates of Cape Cod, Inc).[3] The endotoxin concentration of the ox-LDL preparations was <0.1 EU $\cdot$ mL^{-1} $\cdot$ mg protein^{-1}. Protein in LDL preparations was estimated by the Lowry method. Thiobarbituric acid–reactive substances contained in LDL preparations were assayed as a measure of oxidative lipid modification.[7] Ox-LDL contained thiobarbituric acid–reactive substance concentrations of 18 to 22 nmol/mg LDL protein; mildly oxidized LDL contained 6 to 8 nmol/mg LDL protein.

Protocol

Cell cultures grown to subconfluence were washed 3 times with serum-free medium and maintained under serum-free conditions for another 24 hours before experiments. To demonstrate a concentration-dependent effect of ox-LDL on bFGF and on FGFR-1 expression, cells were incubated with 25 to 200 μg/mL ox-LDL for 24 hours. In other experiments, the effect of mildly oxidized LDL was compared with that of ox-LDL. To determine reaction time course, cells were treated with 100 μg/mL ox-LDL for 6, 12, 24, and 48 hours. To ascertain whether the effects of ox-LDL on DNA and RNA syntheses could be reversed by recombinant human bFGF or VEGF-165 (R&D Systems), cells were treated with each in the presence or absence of ox-LDL. In some experiments, bFGF-neutralizing antibody (R&D Systems) was added.

For the study of DNA and RNA syntheses, cell growth, and intracellular bFGF protein, 100×10^4 cells were seeded in each well of 12-well Corning cell culture plates.

Reverse Transcriptase–Polymerase Chain Reaction

To determine bFGF mRNA levels, total RNA was extracted from control or ox-LDL–treated cells.[9] One microgram of the extracted total RNA was reverse transcribed in 10 μL reaction mixture containing 2.5 U Moloney murine leukemia virus reverse transcriptase (RT) for 45 minutes at 42°C. A fraction of the synthesized cDNA was subjected to polymerase chain reaction (PCR) amplification (GeneAmp, Perkin-Elmer Cetus Co). The bFGF primers were 5′-GGAGTGTGTGCTAACCGTTACCTGGCTATG-3′ (upstream) and 5′-TCAGCTCTTAGCAGACATTGGAAGAAAAAG-3′ (downstream). The FGFR-1 primers were 5′-AAGGACAAACCC-AACCGTGTGACC-3′ (upstream) and 5′-CCCAAAGTCTGCTA-TCTTCATCAC-3′ (downstream).[10] The cDNA mixture supplemented with the primers, *Taq* DNA polymerase (AmpliTaq), PCR buffer, dNTP, and $MgCl_2$ was then incubated in a PTC-100 programmable thermal cycler (step-cycle file for 30 cycles at 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1 minute, followed by a time delay file at 72°C for 10 minutes). As an internal control, levels of β-actin mRNA were estimated under the same cycling conditions as used in bFGF amplification with the following primers: 5′-AACCGCGAGAAGATGACCCAGATCATGTTT-3′ (upstream) and 5′-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3′ (downstream).[11] The PCR profile was optimized in preliminary experiments to ascertain linearity of amplification for bFGF, FGFR-1, and β-actin genes. A fraction of each PCR product (10 μL) was electrophoresed in 2% agarose gel, and DNA bands stained with ethidium bromide were visualized by ultraviolet transillumination.

RNase Protection Assay

RNase protection assays were performed with a Guardian kit (Ambion)[12] with the bFGF RNA probe prepared by in vitro transcription from a bFGF cDNA insert previously cloned in the TA vector (Invitrogen). An RT-PCR product from BAECs using bFGF primers as above was inserted into the TA cloning vector and completely sequenced. This plasmid was linearized with *Eco*RV, and the antisense RNA was synthesized with SP6 RNA polymerase and [α-^{32}P]uridine triphosphate (DuPont NEN), followed by purification through a G-50 column (Boehringer). Labeled probe (150 000 cpm) was then mixed with 20 μg total RNA extracted from cells subjected to selected treatments and incubated at 42°C overnight. After hybridization, the mixture was treated with ribonuclease to degrade single-strand, unhybridized probes. Samples of the mixture were separated by electrophoresis in urea polyacrylamide gel and exposed to x-ray films at −70°C with an intensifying screen. Relative mRNA levels were quantified by densitometry with an Ultro Scan densitometer (LKB Produkter).

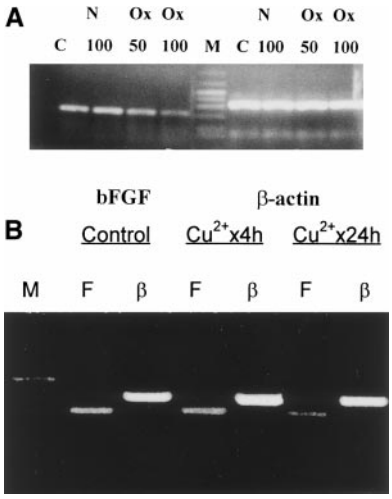

Figure 1. Effects of LDL preparations on bFGF and β-actin expression evaluated by RT-PCR. A, Relative bFGF mRNA levels were expressed in BAECs incubated for 24 hours with PBS (C, control), 100 μg/mL native LDL (N), or 50 or 100 μg/mL ox-LDL (Ox). M denotes marker. B, Cells were incubated for 24 hours with PBS (control), 100 μg/mL mildly oxidized LDL (Cu^{2+} for 4 hours), and 100 μg/mL ox-LDL (Cu^{2+} for 24 hours). F and β denote bFGF and β-actin, respectively.

Northern Blot Analysis

Total RNA was isolated from cultured cells with RNAzol (Biotecx Laboratories, Inc). Twenty micrograms of total RNA was subjected to electrophoresis in each lane of 0.7% agarose/2.2 mol/L formaldehyde gel, then transferred to Nytran membranes (Schleicher & Schuell).[13] The bFGF cDNA insert was excised from plasmid constructs with *Eco*RI and gel-purified (Geneclean Kit-Bio 101, Inc) to be used as probes. After labeling with [α-^{32}P]dCTP to a high specific activity ($\approx10^9$ cpm/μg) by a random priming method, probes were hybridized to the blots (10^6 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 then washed under high stringency at 65°C in 0.2×SSC plus 0.1% SDS. Films were exposed at −70°C for 3 days with 2 intensifying screens for autoradiography. β-Actin was used in each experiment as internal control (not shown).

Actinomycin D and Cycloheximide

To inhibit RNA transcription, cells were treated with 50 ng/mL actinomycin D, which decreased [^{3}H]uridine uptake by $>95\%$. To

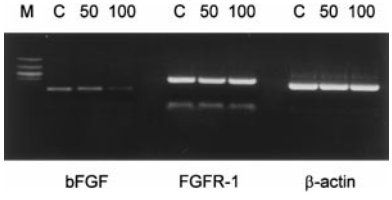

Figure 2. Effects of ox-LDL on bFGF, FGFR-1, and β-actin expression evaluated by RT-PCR. BAECs were incubated for 24 hours with PBS (C, control) or 50 or 100 μg/mL ox-LDL. M denotes marker.

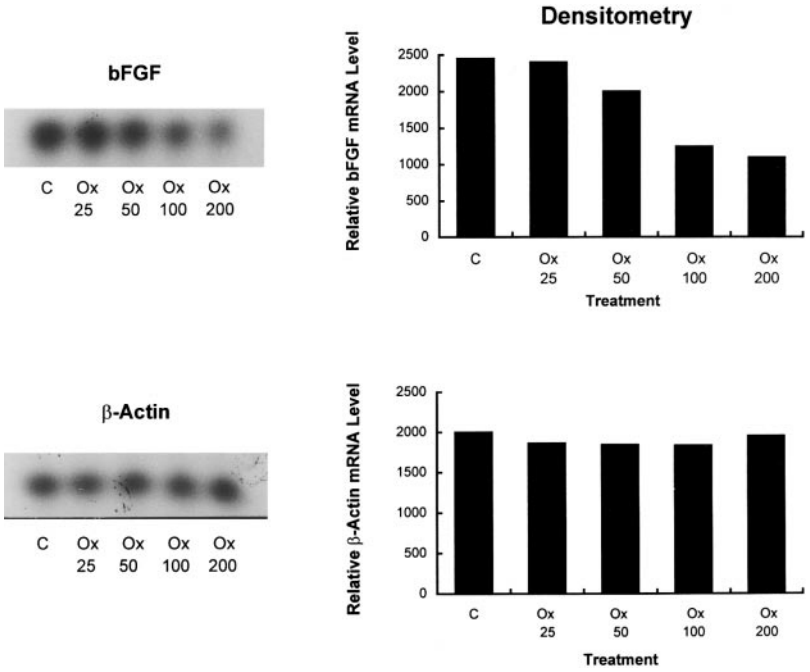

Figure 3. Effects of increasing concentrations of ox-LDL on bFGF and β-actin mRNA levels assessed by RNase protection assay and densitometry in BAECs incubated for 24 hours with 25, 50, 100, or 200 μg/mL ox-LDL, relative to PBS control (C).

determine whether ox-LDL accelerated posttranscriptional degradation of bFGF mRNA, ox-LDL was included in the medium of actinomycin D–treated cells.[14] To determine whether bFGF mRNA degradation required new protein synthesis, cyclohexamide 1.5 μg/mL was used.[14]

Enzyme-Linked Immunosorbent Assay

Extracellular bFGF concentrations in culture medium collected during the serum-free period were measured by ELISA with a Quantikine Kit (R&D Systems). Medium samples and bFGF standards were incubated at room temperature for 2 hours in wells of the microtiter plate coated with a murine bFGF monoclonal antibody. After a washing, the cells were incubated for 2 hours with a rabbit polyclonal antibody against bFGF conjugated to horseradish peroxidase. The bFGF concentration in each well was estimated spectrophotometrically at 450 nm by use of standard curves.[3] The bFGF concentrations in cell lysates prepared with Nonidet P-40 (Sigma) were assayed similarly.

DNA and Total RNA Syntheses

To evaluate treatment effects on DNA synthesis, 3 μCi/mL [^{3}H]thymidine (Moravek Biomedicals) was included in 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.[2,3] To evaluate total RNA synthesis, 5 μCi/mL [^{3}H]uridine was included during the final 2 hours of incubation. Cells were fixed with trichloroacetic acid, and RNA was extracted for detection of incorporated [^{3}H]uridine.[15]

Statistical Analysis

The significance of the differences between group means was assessed by a 2-sided Student's *t* test for single comparisons and Bonferroni's test for multiple comparisons. Probability values <0.05 were considered significant. Results are expressed as mean±SD. A GB-STAT program (Dynamic Microsystems, Inc) was used.

Results

Effects of Ox-LDL on bFGF and on FGFR-1 Expression

Incubation of the cells with 50 and 100 μg/mL ox-LDL suppressed, in a concentration-dependent manner, the expression of bFGF mRNA as assessed by RT-PCR but had no effect on "housekeeping" β-actin expression (Figure 1A). Native LDL and mildly oxidized LDL had no effect on these expressions (Figure 1A and 1B). Expression of FGFR-1 was not inhibited by ox-LDL at concentrations up to 100 μg/mL (Figure 2). At ox-LDL concentrations from 25 to 200 μg/mL, RNase protection assays demonstrated a concentration-dependent decrease in bFGF mRNA (Figure 3). The reductions were clearly demonstrable at 50 μg/mL and were ≈50% at 100 μg/mL ox-LDL, a concentration that did not increase

Effects of LDL Preparations on DNA and Total RNA Syntheses and on Cell Proliferation at 24 Hours

Assessment	PBS (Control)	LDL 100 μg/mL	Ox-LDL 25 μg/mL	Ox-LDL 50 μg/mL	Ox-LDL 100 μg/mL	Ox-LDL 200 μg/mL
[^{3}H]thymidine incorporation (DNA), cpm×10^{-4}/well	86±8	84±9	75±10	65±10*	60±15*	29±15†
[^{3}H]uridine incorporation (RNA), cpm×10^{-4}/well	53±3	52±4	48±5	46±3*	42±2*	25±10†
Cell count, cells×10^{-4}/well	210±13	202±11	194±14	186±16	152±14*	102±22†
Dead cells, %/well	14±10	13±12	15±10	17±12	17±13	56±13†

n=6 in all treatments; 100×10^4 cells/well at inoculation.
*$P<0.05$, †$P<0.01$ vs PBS.

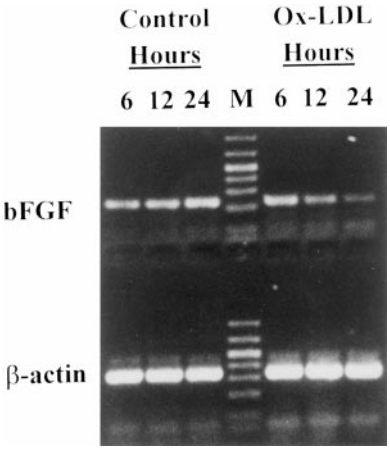

Figure 4. Time-course effects of ox-LDL on bFGF and β-actin expression evaluated by RT-PCR. BAECs were incubated with PBS (control) or 100 μg/mL ox-LDL for 6, 12, or 24 hours.

the percentage of dead cells (Table). At higher concentrations, up to 200 μg/mL, there was an increase in cell death but no further reduction in bFGF mRNA (Figure 3).

In time-course experiments performed with an ox-LDL concentration of 100 μg/mL, reduction of bFGF mRNA became apparent at 12 hours and reached the maximum of 40% to 50% at 24 to 48 hours (Figures 4, 5, and 6).

Posttranscriptional Destabilization

Actinomycin D 50 ng/mL decreased [^{3}H]uridine uptake by >95% (from 33.5±2.5 to 1.0±0.5 cpm, n=6, $P<0.001$). In the presence of actinomycin D, bFGF mRNA decreased by 60% and cell viability was detectably impaired after 24 hours (Figure 6). Combined treatment with actinomycin D and ox-LDL accelerated bFGF mRNA degradation; message reductions reached 50% by 12 hours (n=4). Reductions with combined treatment exceeded those seen with ox-LDL or actinomycin D alone and resulted in barely visible messages at 24 hours. In the presence of cyclohexamide (n=4), the inhibitory effect of ox-LDL was appreciably attenuated. Only minimal reduction in bFGF expression was demonstrable at 24 hours (Figure 6). In preliminary experiments, cyclohexamide alone did not affect DNA synthesis, bFGF protein, or bFGF mRNA levels (data not shown). These results indicate that ox-LDL induced posttranscriptional destabilization of bFGF mRNA that required sustained synthesis of new protein(s).

Intracellular and Extracellular bFGF Concentrations

Ox-LDL treatment produced concentration-dependent decreases in intracellular bFGF peptide detectable by ELISA. In cells exposed to 0, 25, 50, or 100 μg/mL ox-LDL for 24 hours, the intracellular bFGF concentration (n=4) averaged 316±24, 177±60, 153±53, and 115±32 pg/mg protein, respectively (Figure 7). These peptide reductions paralleled those of bFGF mRNA, although bFGF mRNA reductions at the lowest ox-LDL concentration were not detectable. The average extracellular bFGF concentration was 7.6±0.8 pg/mL (n=4) for PBS controls. After 24-hour incubation with 50 μg/mL ox-LDL, assayable bFGF was reduced to 5.2±0.4 pg/mL ($P<0.05$); with 100 μg/mL ox-LDL, bFGF was not detectable (<5 pg/mL). At an ox-LDL concentration of 200 μg/mL, most cells ruptured, and the bFGF concentration in the medium surged to 32±14 pg/mL ($P<0.01$).

Effects of Ox-LDL on DNA and Total RNA Syntheses and Cell Proliferation

Ox-LDL decreased DNA and total RNA syntheses in a concentration-dependent manner (Table). At 50 μg/mL, synthesis reductions were moderate but statistically significant. At 100 and 200 μg/mL, synthesis decreases were accompanied by reductions in total cell count. At 100 μg/mL, few trypan blue–positive cells were detectable, but at 200 μg/mL, many cells exhibited disrupted membranes and >50% showed positive staining.

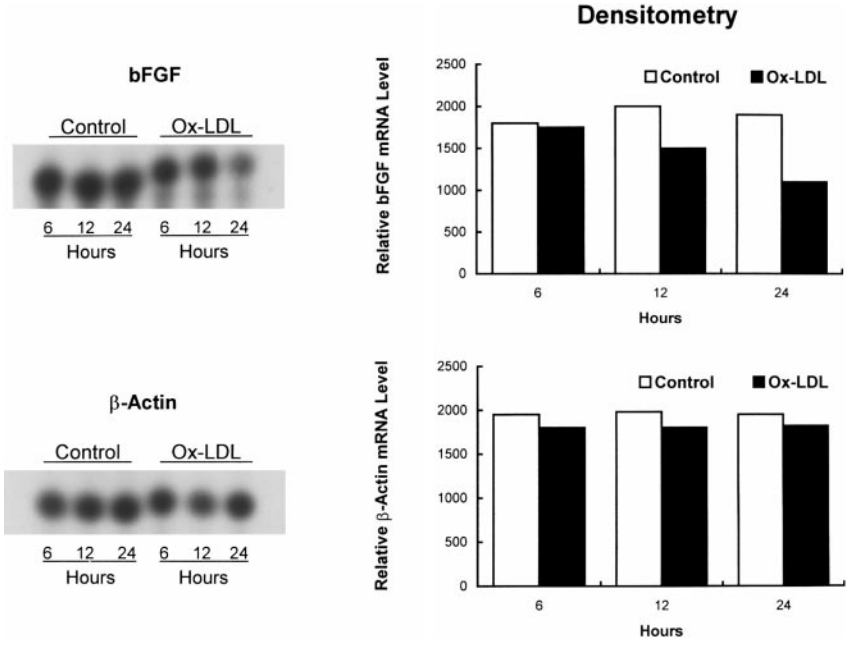

Figure 5. Time-course effects of ox-LDL on bFGF and β-actin mRNA levels assessed by RNase protection assay and densitometry in BAECs incubated with 100 μg/mL ox-LDL for 6, 12, or 24 hours relative to PBS control (C).

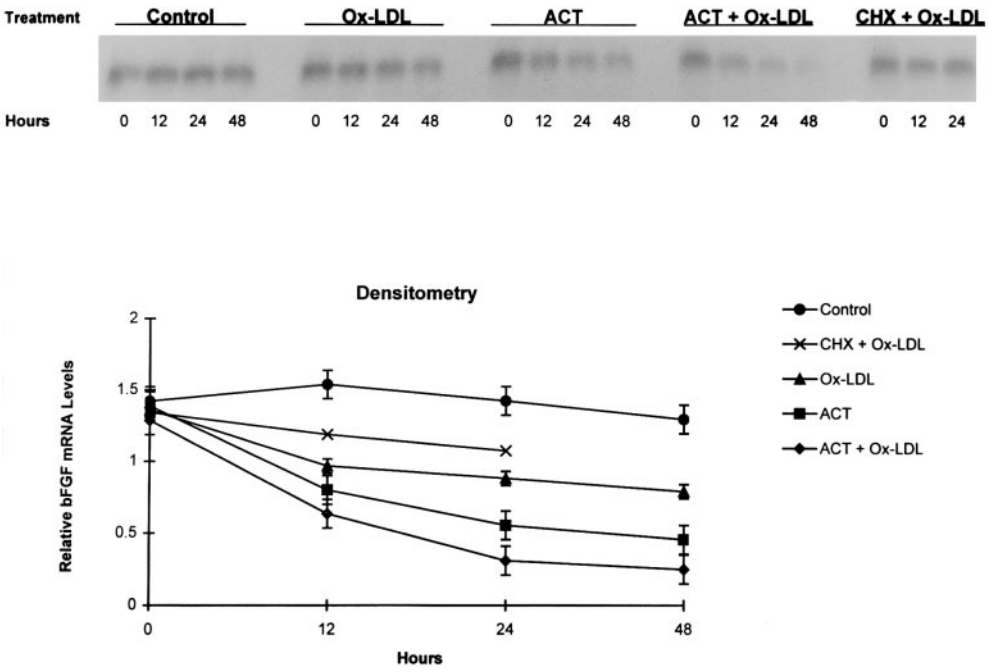

Figure 6. Effects of actinomycin D (ACT, 50 ng/mL, 0 through 48 hours) and cyclohexamide (CHX, 1.5 μg/mL, 0 through 24 hours) in presence or absence of 100 μg/mL ox-LDL on bFGF expression, compared with PBS (control), assessed by Northern blot analysis and densitometry.

bFGF and VEGF Efficacies in Counteracting Ox-LDL Inhibition of DNA and Total RNA Syntheses

Applied alone, bFGF 10 ng/mL increased DNA synthesis 2-fold, and VEGF-165 50 ng/mL evoked an increase of lesser magnitude (n=6; Figure 8). VEGF-165 and VEGF-121 had similar effects; maximal stimulation was at 50 ng/mL (data not shown). DNA synthesis was increased to a similar extent by bFGF whether ox-LDLs were present or absent, which suggests that bFGF prevented inhibition by ox-LDL 100 μg/mL. In contrast, VEGF-stimulated DNA synthesis was sensitive to ox-LDL inhibition.

bFGF increased total RNA synthesis to the same extent in the presence or absence of ox-LDL (n=6; Figure 9). VEGF-165 applied alone failed to increase total RNA synthesis or to counteract its inhibition by ox-LDL. bFGF-neutralizing antibody 10 μg/mL produced a maximal decrease in total RNA synthesis, very close to that obtained with 100 μg/mL ox-LDL.

Discussion

We demonstrated that ox-LDLs reduce steady-state bFGF mRNA levels in cultured vascular ECs. The time- and concentration-dependent suppression of bFGF expression was indicated by decreases in both intracellular and extracellular bFGF concentrations. Expression of 2 important reference genes, FGFR-1 and β-actin, was preserved, consistent with selective bFGF gene inhibition. This would explain why exogenous bFGF was capable of completely restoring DNA and total RNA syntheses, even in the presence of ox-LDL. The restorative effects of bFGF were not matched by those of VEGF, an EC-specific mitogen.[4,5] These results support findings that hyperlipidemic impairment of angiogenesis is associated with reduced availability of bFGF and can be corrected by exogenous bFGF.[2,3]

The progressive reduction in bFGF expression reflected, in part, accelerated posttranscriptional mRNA degradation. Ox-LDL shortened the bFGF half-life from 24 to ≈12 hours after transcription was inhibited by actinomycin D. Sensitive to

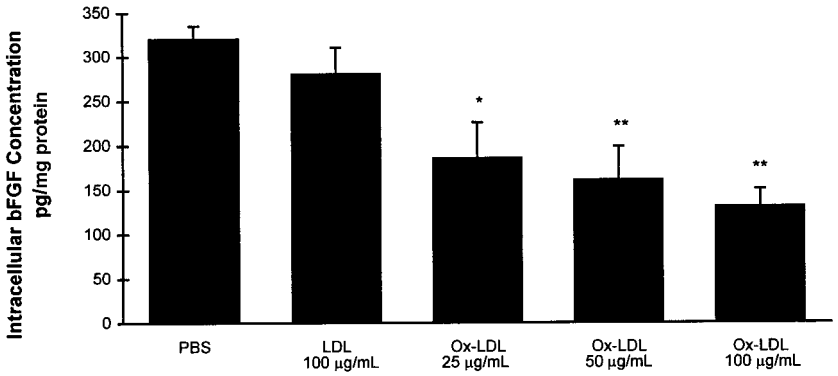

Figure 7. Effects of LDL preparations on intracellular bFGF concentration (24-hour incubations). *$P<0.05$, **$P<0.01$ vs PBS control.

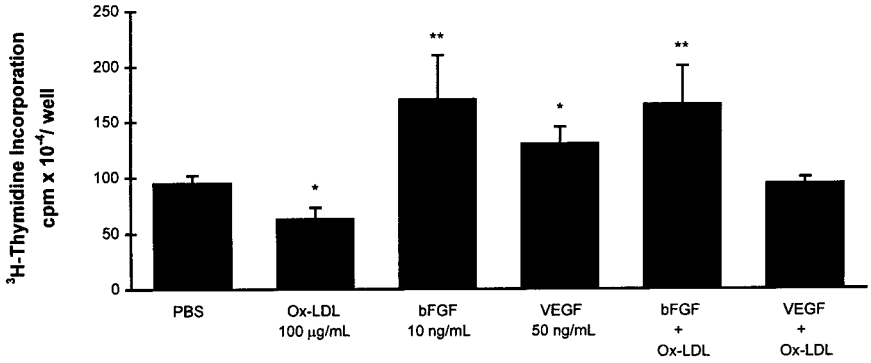

Figure 8. Effects of ox-LDL, bFGF, and VEGF-165, alone or in combination (24-hour incubations). *$P<0.05$, **$P<0.01$ vs PBS control.

cyclohexamide, enhanced degradation of bFGF mRNA depended on newly synthesized protein(s). The effects resemble endothelial nitric oxide synthase expression by ox-LDL, attributed to combined posttranscriptional mRNA degradation and early transcriptional inhibition.[14]

Maximal inhibition of bFGF expression required 24 to 48 hours of incubation with ox-LDL, but significant inhibition was evident by 12 hours. Similar early and progressive reductions induced by ox-LDL were reported for the nitric oxide synthase gene.[14] bFGF message level reductions correlated with reductions in immunoassayable bFGF concentration in both cytoplasm and culture medium. This may have functional implications, because bFGF exerts its effects through both intracellular and extracellular mechanisms. Ox-LDL decreased DNA synthesis, and to a lesser extent total RNA synthesis, in a concentration-dependent fashion. With concentrations up to 100 μg/mL, only reductions in total cell number were noted, but with higher concentrations, increases in dead cells were evident. Also, concentration-dependent effects of ox-LDL on assayable extracellular bFGF were observed only up to 100 μg/mL. At 200 μg/mL, extracellular bFGF surged, reflecting release of peptide from irreversibly damaged cells.

In many systems, ox-LDLs exert biphasic effects: low concentrations or brief incubations are stimulatory, and high concentrations or prolonged incubations are inhibitory. Complex, concentration-dependent effects of ox-LDL may reflect actions of distinct stimulatory or inhibitory mediators such as platelet-activating factor (PAF)[16,17] or oxysterols.[18] However, single mediators, such as lysophosphatidylcholines, may by themselves exert biphasic effects.[19] The present experiments further illustrate the complex actions of ox-LDL. As noted, ox-LDL suppressed bFGF expression without apparent effect on FGFR-1 and β-actin, although suppressant effects of ox-LDL have been reported for other genes such as nitric oxide synthase[14] and thrombomodulin.[20] Yet ox-LDLs tend to activate vasoconstrictor, proinflammatory, and procoagulatory genes.[21–24] Suppressant effects on DNA and total RNA syntheses as observed here may reflect inflammatory responses leading to apoptosis.[25]

In contrast to bFGF, VEGF failed to reverse the inhibitory effects of ox-LDL on DNA and total RNA syntheses. In the absence of ox-LDL, high-dose VEGF increased DNA but not RNA synthesis. VEGF has been shown to increase DNA synthesis in bovine ECs[26] and to improve collateral development indices in the ischemic limbs of Watanabe heritable hyperlipidemic rabbits.[27] The role of VEGF in RNA synthesis, however, has not been investigated. In contrast, bFGF acts as an autocrine stimulator and can stabilize RNA in the presence of actinomycin D. Also, it increases total cellular RNA synthesis in neuropeptide-producing 44-2C cells treated with αA, an inhibitor of RNA polymerase II.[15] Although [^{3}H]uridine uptake has limitations as an index, its increase or decrease yields useful qualitative information on RNA synthesis.

Although bFGF has been used to induce angiogenesis,[28,29] its role in regulating angiogenesis remains incompletely defined. Other factors, including VEGF and its receptors, angiopoietin and the Tie2 receptor, transforming growth factor-α, and platelet-derived growth factor B, have also been recognized as important.[30,31] The present findings and those of our previous reports with the explant model do not provide mechanistic information. The data clearly indicate, however, that vascular ECs of various species (human, rabbit, bovine) are unable to replicate or form microvessels when the cells are deprived of bFGF.[2,3] We and others have emphasized as a potential mechanism the importance of phospholipid mediators contained in

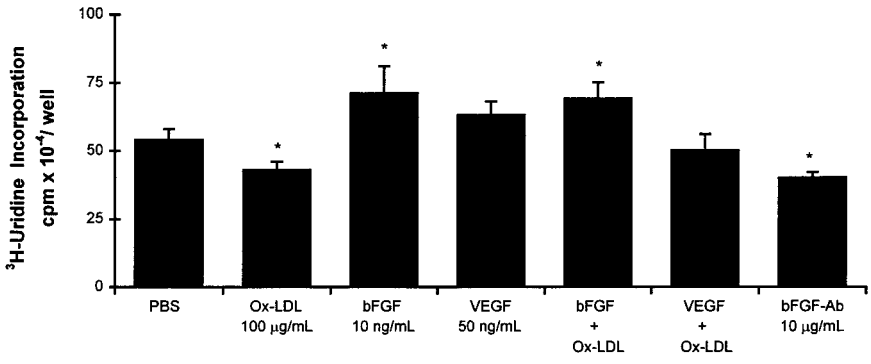

Figure 9. Effects of ox-LDL, bFGF, and VEGF-165, alone or in combination, and of bFGF-neutralizing antibody (bFGF-Ab) on total RNA synthesis (24-hour incubations). *$P<0.05$ vs PBS control.

modified LDL.[8,16,17,23,32–34] There is evidence that these mediators, including PAF and lysophosphatidylcholines and their phospholipid analogues, act by means of PAF receptors.[35] Recently, ox-LDLs have been shown to downregulate PAF receptor expression, consistent with the action of ox-LDL phospholipids through the PAF receptor pathway. It is clear, however, that other lipids in ox-LDLs, in particular oxysterols,[18] are likely to play roles in mediating its effects.

Acknowledgments

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