Oxidized LDL Suppresses NF-κB and Overcomes Protection from Apoptosis in Activated Endothelial Cells

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Abstract. Atherosclerosis is a chronic inflammatory disease associated with enhanced apoptotic cell death in vascular cells, partly induced by oxidized low-density lipoprotein (OxLDL). However, proinflammatory stimuli such as lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α) activate endothelial cells (EC) and inhibit apoptosis through induction of nuclear factor κB (NF-κB)-dependent genes. This study therefore investigated whether OxLDL or its component, lysophosphatidylcholine (LPC), interacts with the effect of LPS or TNF-α on cell survival. Human EC were incubated with LPS, TNF-α, OxLDL, or LPC alone or in combinations. OxLDL (100 to 200 μg/ml) and LPC (100 to 300 μM) induced apoptosis dose-dependently. LPS and TNF-α had no effect on cell survival in the presence or absence of OxLDL or LPC. LPS and TNF-α both induced the antiapoptotic gene A20, whereas OxLDL and LPC suppressed its induction. Expression of A20 is regulated by NF-κB. OxLDL and LPC dose-dependently suppressed NF-κB activity. For functional analysis, bovine EC were transfected with A20 encoding expression constructs in sense and antisense orientation. Bovine EC that overexpressed A20 were protected against OxLDL-induced apoptosis, whereas expression of antisense A20 rendered cells more sensitive to OxLDL. These results suggest that OxLDL not only induces cell death, as has been shown before, but also compromises antiapoptotic protection of activated EC. OxLDL sensitizes EC to apoptotic triggers by interfering with the induction of A20 during the inflammatory response seen in atherosclerotic lesions. This inhibition is based on repression of NF-κB activation. The effect may be caused by the OxLDL component LPC.

Having a high level of blood lipids is a known risk factor for atherosclerosis that leads to the formation of atherosclerotic plaques and inflammatory reactions. For example, formation of fatty streaks in C57BL/6 mice caused by a high-cholesterol diet is accompanied by the expression of several inflammation and oxidative stress responsive genes, including the activation of nuclear factor κB (NF-κB) (1). Some of these in vivo effects were also observed in vitro after endothelial cells (EC) were activated with lipoproteins. Human EC (HUVEC) respond to modified LDL by upregulating the inflammatory markers MCP-1, VCAM-1, and ICAM-1 (2–5).

Upon proinflammatory activation, EC become protected against cell death by the induction of antiapoptotic genes, including the zinc-finger protein A20 (6,7). However, apoptotic bodies can be found in atherosclerotic plaques (8–10). Therefore, we were interested how the protection against apoptosis that is usually conferred by proinflammatory agonists is compromised in atherosclerosis. In vitro experiments point to a role for atherogenic lipoproteins in the induction of apoptosis, e.g., stimulation of EC with oxidized low-density lipoprotein (OxLDL) results in enhanced apoptosis (11–14).

There are conflicting results as to the role of NF-κB in the stimulation of EC by OxLDL. Takahara et al. (15) and Rajavashisth et al. (16) found an enhanced activity of NF-κB, whereas Ares et al. (17) reported suppression of NF-κB activity upon treatment with OxLDL. Experiments with lysophosphatidylcholine (LPC), which is enriched in OxLDL, point to a biphasic dose effect that might explain the opposing results (18). Because NF-κB is essential for the inhibition of tumor necrosis factor (TNF-α)-induced apoptosis (19–21), its up- or downregulation by OxLDL might affect cell survival. Inhibition of apoptosis through upregulation of NF-κB might in part be mediated by A20 because its expression can be activated by NF-κB (22–24).

In this study, we investigated the reactions of cultured HUVEC toward different proinflammatory stimuli. We compared stimulation with OxLDL or LPC with stimulation with LPS or TNF-α and analyzed cell viability, expression of A20, and DNA binding of NF-κB. The functional role of A20 in OxLDL-induced apoptosis was studied by overexpression of exogenous and inhibition of endogenous gene product in EC.

Materials and Methods

Isolation of LDL

Human LDL was isolated as described recently (25). Pooled plasma from healthy donors was centrifuged at a density of 1.065 g/ml at 242,000 × g for 8 h followed by density gradient centrifugation at
242,000 \times g for 3 h. Isolated LDL was dialyzed three times against 300 vol of 150 mM NaCl and 1 mM ethylenediaminetetraacetic acid (EDTA; pH 7.4) and subsequently analyzed for purity by 0.6% agarose gel electrophoresis as well as 4% sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Protein content of lipoproteins was measured using a commercially available kit (Sigma protein kit, Deisenhofen, Germany), which is based on a modification of the Lowry method (26). Lipoprotein concentrations are given as micrograms of protein per milliliter of solution.

**Oxidation of LDL**

LDL was oxidized as described recently (25). Briefly, antioxidant-free LDL (300 μg protein/ml) was incubated with CuSO₄ (5 μM) in phosphate-buffered saline for 30 h at 23°C. The degree of oxidation was quantified by two different methods: (i) the increase in relative mobility on agarose gel, indicating an enhanced negative charge of oxidized lipoprotein, and (ii) the formation of thiobarbituric acid-reactive substances (27). Homogeneity of lipoproteins was tested by agarose gel electrophoresis (REP-HDL-plus cholesterol electrophoresis, Helena Diagnostika, Hartheim, Germany). The relative mobility of OxLDL on agarose gel electrophoresis as an index for lipoprotein oxidation was 1.7 to 2.1 compared with native LDL. Levels of thiobarbituric acid-reactive substances, determined in samples that contained 300 μg of lipoprotein/ml, were 0.2 ± 0.01 μM in native LDL and 3.9 ± 0.7 μM in OxLDL. Lipoproteins were prepared freshly every 2 wk. During this period, the apolipoprotein B did not degrade.

**Cell Culture**

HUVEC were purchased from Clonetics (Walkersville, MD) and cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml, Sigma), bovine brain extract (12 μg/ml), gentamycin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% fetal calf serum (FCS; Life Technologies, Karlsruhe, Germany) until the fifth passage. Cells (approximately 10⁶) were incubated with native or OxLDL, LPS, or TNF-α and taken for detection of apoptosis (after 18 h of incubation) or RNA analysis (after 4 h of incubation).

Bovine EC (BPEC) were isolated from fresh bovine pulmonary arteries by collagenase digestion (2 min, 0.1% collagenase) and subse-
quent scraping. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for up to the fifth passage. For apoptosis assays, cells were incubated with OxLDL for 12 h.

**Reverse Transcription-PCR**

RNA was prepared according to Chomczynski and Sacchi (28). For reverse transcription-PCR (RT-PCR), 1 μg of total RNA was reverse-transcribed in 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM each dATP, dCTP, dGTP, and dTTP, 5 μM specific reverse primer, and 100 U mouse moloney leukemia virus RT (Promega, Madison, WI). The RT product was used as template in a PCR reaction. With an annealing temperature of 60°C, 30 cycles of PCR were performed in 50 mM Tris/HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 2 mM dithiothreitol, 0.1% TritonX-100, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 1 μM specific primers, and 0.75 32 P dATP (ICN, Eschwege, Germany). Reactions were incubated for 30 min at room temperature and analyzed through electrophoresis through a 6% nondenaturating polyacrylamide gel. To determine NF-κB–specific signals, in some experiments, unlabeled probe or unrelated oligonucleotide sequences were added before the addition of labeled DNA for competition analysis. Further specificity controls have been published elsewhere (31,32).

**Transient Transfection**

BPEC in the fourth to fifth passages were transfected using FuGENE 6 (Boehringer Mannheim) following the manufacturer’s protocol. Briefly, cells were seeded at 10⁵/cm² 24 h before transfection.

**Detection of Apoptosis and Necrosis**

Apopotosis was detected by staining with Annexin-V-FLUOS (Boehringer Mannheim, Mannheim, Germany). Cells (1 × 10⁶) were washed with phosphate-buffered saline, removed from the culture dish with Trypsin/EDTA, centrifuged at 200 × g, and resuspended in 150 μl of 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 5 mM CaCl₂ containing 1 μg/ml propidium iodide and 20 μl/ml Annexin-V-FLUOS. After a 15-min incubation period at room temperature, 500 μl of cell culture medium were added and cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ). Propidium iodide–stained cells were considered necrotic. Cells that were Annexin-V-FLUOS positive and propidium iodide negative were considered apoptotic.

**Preparation of Nuclear Extracts**

Cells were stimulated with the indicated agents for 2 h before nuclear proteins were extracted as described elsewhere (29,30). Briefly, the cells were lysed by nonionic detergents that preserved the nuclear membranes. Nuclei were collected by centrifugation and sonicated for 10 s on ice. All buffers contained protease inhibitors (Complete, Boehringer Mannheim). Protein concentrations of nuclear extracts were determined using the BioRad protein assay with bovine serum albumin as the reference standard.

**Electrophoretic Mobility Shift Assay**

A double-stranded probe containing the second NF-κB binding site in the IkB-α promoter 5‘-AATTCGGCTTGGAAATTCCCCGAG CG-3’ was end-labeled with [α-32P]dATP (ICN, Eschwege, Germany), with the use of the Klenow fragment of DNA polymerase I. Binding reactions of 25 μl total volume contained 10 μg of nuclear protein, 10⁵ cpm of oligonucleotide, 3 μg of poly(dIdC) in 20 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM EDTA, 1 mM β-mercapto-
ethyl, and 5% glycerol. Reactions were incubated for 30 min at room temperature and analyzed through electrophoresis through a 6% nondenaturating polyacrylamide gel. To determine NF-κB–specific signals, in some experiments, unlabeled probe or unrelated oligonucleotide sequences were added before the addition of labeled DNA for competition analysis. Further specificity controls have been published elsewhere (31,32).
Western Blot

Proteins were extracted from sedimented cells with the use of 40 μM Tris-HCl (pH 8), 0.276 M NaCl, 20% glycerol, 2% tergitol NP-40, 4 mM EDTA (pH 8), 20 mM NaF, 3% aprotinin, 2 mM Na$_2$VO$_4$, 0.1 g/L phenylmethylsulfonyl fluoride, and 20 mg/L leupeptin. One hundred μg of protein from each sample were fractionated on a sodium dodecyl sulfate–10% polyacrylamide gel. Proteins were transferred onto polyvinyliden difluoride membranes (NOVEX, San Diego, CA). After blocking with 5% nonfat milk, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.1% Tween 20 at room temperature, the membranes were exposed to a 1:1000 dilution of rabbit anti-A20 antiserum (kindly provided by Dr. Yong Li, University of Michigan Medical School, Ann Arbor, MI) for 1 h at room temperature followed by exposure to a 1:3000 dilution of swine anti-rabbit IgG (DAKO Diagnostics, Hamburg, Germany) for 1 h at room temperature. The proteins were visualized using the ECL Western blotting protocol (Amersham, Freiburg, Germany).

Statistical Analyses

Data are presented as means ± SEM of n experiments. Dose-effect curves in the plots in Figure 1 were analyzed using one-way ANOVA for repeated measurements. The statistical difference in Figure 5 was determined using Student-Newman-Keuls Test and Dunn’s Test (Sigma Stat Software Program, Jandel Scientific, Erkrath, Germany). Differences were considered significant at an error probability of $P < 0.05$.

Results

OxLDL and LPC Induce Apoptosis in EC

To investigate the effects of different inflammatory stimuli on endothelial cell survival, we incubated HUVEC with native or oxidized LDL and LPS (Figure 1A) or with LPC and LPS (Figure 1B) for 18 h and tested for apoptosis. OxLDL and LPC both induced apoptosis dose-dependently between 100 and 250 μg/ml or 50 μM to 300 μM, respectively. Concentrations of up to 300 μg/ml native LDL or 10 μg/ml LPS had no apparent effect on cell survival. Preincubation with LPS for 1 h before adding OxLDL did not suppress the induction of apoptosis either (data not shown). For illustration of the effect of 200 μM LPC and 250 μg/ml OxLDL, representative histograms are shown in Figure 1C.

OxLDL Suppresses the Expression of A20

TNF-α and LPS enhance the expression of A20 in EC and protect against apoptosis. To test whether OxLDL or LPC...
inhibited A20 expression and thereby overcame protection, RT-PCR was performed with RNA obtained from HUVEC that had been stimulated for 4 h with proinflammatory stimuli together with increasing doses of OxLDL (Figure 2A) or LPC (Figure 2B).

Constitutive expression of A20 was below detectable levels. Stimulation of HUVEC with TNF-α or LPS induced expression of A20 that was suppressed by high doses of OxLDL or LPC (250 μg/ml or 200 μM, respectively). Actin as control for equal amounts of mRNA in the reactions was consistently positive.

**OxLDL and LPC Suppress Binding Activity of NF-κB**

The expression of A20 is known to depend at least in part on the activation of the transcription factor NF-κB. Therefore, binding of NF-κB proteins to a known NF-κB binding site was investigated by electrophoretic mobility shift assay. In comparison to nonstimulated cells, in nuclear extracts derived from cells that were treated for 2 h with either LPS (Figure 3A) or TNF-α (Figure 3B), NF-κB binding was markedly induced. The signal could be blunted by adding an excessive amount of non–radioactive-specific oligonucleotide but not by adding nonspecific oligonucleotide (Figure 3A, spec. and nonspec. competition lanes, respectively). Both OxLDL and LPC suppressed NF-κB binding dose-dependently.

**Figure 2.** Suppression of A20 gene expression by OxLDL. Reverse transcription-PCR (RT-PCR) of HUVEC (A) co-stimulated with 10 μg/ml LPS and increasing concentrations of OxLDL and (B) co-stimulated with 10 nM tumor necrosis factor (TNF-α) and increasing concentrations of LPC. RT-PCR was performed with specific primers for A20 and actin. Representative experiment of three independent cell preparations.

**Figure 3.** Inhibition of nuclear factor κB (NF-κB) activation in HUVEC by OxLDL and LPC. HUVEC were co-stimulated with 10 μg/ml LPS and increasing concentrations of OxLDL (A) and 10 nM TNF-α and increasing concentrations of LPC (B). Binding of NF-κB to DNA was analyzed by electrophoretic mobility shift assay. Representative experiments of two independent cell preparations.
Because bovine cells were used for transfection experiments, they were also subjected to electrophoretic mobility shift assay analysis for NF-κB binding after treatment with OxLDL (Figure 4). In BPEC, OxLDL suppressed NF-κB binding dose-dependently.

Cellular location and DNA binding of NF-κB is regulated by its inhibitor IκB-a. IκB-a protein abundance was analyzed by Western blot (data not shown). Upon stimulation with TNF-α protein, concentrations of IκB-a in HUVEC were reduced to 20% of the levels in unstimulated cells, indicating its release from NF-κB and subsequent degradation. In contrast, treatment of HUVEC with OxLDL increased the amount of IκB-a protein dose-dependently; 300 μg/ml OxLDL restored 40% of the IκB-a control level.

**A20 Suppresses OxLDL-Induced Apoptosis**

To investigate whether the observed suppression of A20 gene expression correlated with the increased sensitivity toward OxLDL-induced apoptosis, we modulated A20 gene expression in BPEC by transient transfection experiments using expression vectors encoding A20 cDNA either in sense or antisense orientation. Transfected BPEC were incubated with OxLDL, and cell viability was determined subsequently (Figure 5A). In mock-transfected BPEC, incubation with OxLDL resulted in 15% of apoptotic cells at 100 μg/ml and in 23% at 200 μg/ml of OxLDL, respectively. At a concentration of 250 μg/ml OxLDL, there was no further increase in apoptosis but a slight enhancement of necrosis. In cells that were transfected with an A20 expression vector, both apoptosis and necrosis were reduced to background level. Transfection with A20 antisense had the opposite effect: 100 μg/ml OxLDL caused an apoptosis rate of 25%. Concentrations of 200 μg/ml OxLDL or higher resulted in a sharp increase in necrosis. Figure 5B shows a Western blot analysis of A20 expression in antisense- and sense-transfected BPEC. Sense-transfected cells contain two-fold more A20 protein than antisense-transfected cells.

**Discussion**

Induction of apoptosis and protection from apoptosis use different gene products. In the case of TNF-α (recently reviewed by Rath and Aggarwal (34)), signal transduction through Fas-associated death domain and caspase 8 leads to cell death. This pathway is counteracted by signal transduction through tumor necrosis factor receptor associated factor, NF-κB–inducing kinase, and NF-κB, leading to protection. If both pathways are activated, the fate of the cell depends on the TNF-α concentration: high concentrations of TNF-α will lead to cell death despite NF-κB activation. For OxLDL, the signal transduction is still obscure. However, several groups have demonstrated that OxLDL can induce apoptosis (11). The goal of this study is not to explain the mechanism by which OxLDL induces cell death. The results presented here show how OxLDL contributes to the loss of protection of activated EC.

EC, activated by the proinflammatory stimuli LPS or TNF-α, are protected against apoptosis. This protection is conferred in part through expression of the antiapoptotic gene A20 (6,7). We could confirm these findings in our experiments.
using HUVEC. Upon incubation with TNF-α or LPS, the expression of A20 was enhanced, whereas apoptosis was not induced by those proinflammatory stimuli. In contrast, incubation of HUVEC in the presence of OxLDL induced apoptosis, as we and others reported previously (11–14). Antiapoptotic protection provided by stimulation with LPS or TNF-α (data not shown) did not prevent apoptosis caused by OxLDL. OxLDL-induced apoptosis was not even slightly diminished by co-stimulation or prestimulation with A20 inducing agents.

To address the question of how protection from apoptosis is compromised by OxLDL, we analyzed the expression of A20 by RT-PCR. Although RT-PCR allows only semiquantitative estimation of mRNA abundance, it is useful in detecting gross differences in gene expression.

Quiescent EC express very low constitutive levels of A20. When challenged with LPS or TNF-α, EC responded with induced expression of A20 and survived. Co-stimulation with OxLDL suppressed expression of A20, and the cells died. However, higher concentrations of OxLDL were needed for complete suppression of A20 than for induction of apoptosis, suggesting that suppression of A20 is not the mechanism to induce apoptosis but sensitizes cells to proapoptotic triggers.

The role of NF-κB in OxLDL stimulation of EC has been unclear. Takahara et al. (15) and Rajavashisth et al. (16) found an enhanced binding of NF-κB upon treatment with modified LDL. In contrast, OxLDL decreased binding activity of NF-κB in our experiments, which is in agreement with the results of Ares et al. (17). The groups of Takahara and Rajavashisth worked with mildly oxidized preparations of LDL, either directly isolated from blood of diabetic patients or minimally modified in vitro. Ares et al., conversely, used OxLDL that had been treated with copper for up to 16 h, similar to the treatment that we used. In their hands, the degree of NF-κB inhibition directly correlated with the degree of oxidation. In BPEC, higher concentrations of OxLDL were needed for inhibition of NF-κB binding than in HUVEC, suggesting that loss of NF-κB protection may not be a major pathway of OxLDL signaling in bovine cells.

Phosphatidylycholine is the major phospholipid in LDL. Approximately 50% of the phosphatidylycholine can be converted into LPC during oxidation (35). In earlier work, we could show an increase of LPC in our OxLDL preparations as compared with native LDL (36). Similar effects on induction of ICAM-1 and VCAM-1 expression have been observed upon treatment of EC with LPC or modified LDL (37,38). In EC, NF-κB binding shows a biphasic response after treatment with LPC (18). Low doses of LPC increase NF-κB activity, whereas higher doses decrease NF-κB activity. Given that the LPC content of the LDL preparation increases with the degree of oxidation, both the stimulatory and the inhibitory effects of modified LDL could be accounted for by the action of LPC. We therefore questioned whether LPC could substitute for OxLDL in our experiments. Indeed, LPC dose-dependently induced apoptosis. LPC blunted the A20 signal in RT-PCR, and LPC reduced the binding activity of NF-κB.

To functionally test the role of A20 in OxLDL-induced apoptosis, we used A20 sense and antisense constructs in a transient transfection system. As the transfection efficiencies with HUVEC were low, we used BPEC that resulted in transfection efficiencies of up to 20% (data not shown), enough to see effects without enrichment of transfected cells. Overexpression of A20 protected EC from apoptosis as other groups have shown in tumor cells (7,39). Inhibition of endogenous A20 via expression of A20 antisense RNA sensitized EC for OxLDL, showing that A20 confers some protection even to quiescent EC. A20 antisense-transfected cells show an increased rate of apoptosis at 100 and 150 μg/ml compared with control cells. In these samples, the necrosis rate is low and unaffected by OxLDL. At higher OxLDL doses, there is no difference in the number of apoptotic cells between A20 antisense-transfected and control cells, but there is a difference in the total number of dead cells. In A20 antisense-transfected cells, the rate of necrosis increases at the highest doses of OxLDL. We speculate that this might be secondary necrosis of cells having died of apoptosis. The conclusion we can draw with certainty is that the sensitivity to OxLDL is enhanced and the viability is diminished after transfection with A20 antisense.

OxLDL compromises protection from apoptosis conferred to EC during inflammatory activation. Our mechanistic explanation for this is based on two findings: (1) the activation of NF-κB and A20 gene expression, both essential for antiapoptotic protection of EC, are reduced in response to OxLDL, and (2) the ability of OxLDL to induce apoptosis is inversely correlated to the amount of A20 protein in the cell. We therefore propose that OxLDL in atherosclerotic lesions not only induces apoptosis but also counteracts proinflammatory stimulation of antiapoptotic gene expression. Because, experimentally, LPC can replace OxLDL in triggering apoptosis and reducing NF-κB binding activity, it might be the OxLDL component that is responsible for apoptosis induction and the loss of protection.

In conclusion, the data presented in this study show how OxLDL contributes to the loss of protection of activated EC from apoptosis in EC. Because OxLDL accumulates not only in the vascular wall but also in glomerulosclerotic lesions (40), regions that share similarities with atherosclerotic lesions, it is tempting to speculate that OxLDL may induce similar effects on cell death in the setting of chronic glomerular diseases. Apoptotic loss of renal tissue may be associated with the progressive deterioration of renal function that occurs in patients with chronic renal diseases. However, verification of this assumption will require future studies.

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