JCI The Journal of Clinical Investigation

Oxidized low density lipoprotein inhibits lipopolysaccharide-induced binding of nuclear factor-kappaB to DNA and the subsequent expression of tumor necrosis factor-alpha and interleukin-1beta in macrophages.

B G Ohlsson, … , G Bondjers, O Wiklund

J Clin Invest. 1996;98(1):78-89. https://doi.org/10.1172/JCI118780.

Research Article

A large body of evidence suggests that oxidized LDL (oxLDL) has a role in atherogenesis. One effect is the impact on macrophage function. We have studied the effects of oxLDL and oxysterols on the binding of the transcription factors nuclear factor (NF)-kappaB and AP-1 to DNA. These transcription factors are involved in the regulation of several genes and expressed during activation of macrophages, for example by endotoxin (LPS). OxLDL did not induce binding of NF-kappaB. However, the LPS-induced response to NF-kappaB was substantially reduced after preincubation with oxLDL. Medium and highly oxidized LDL also decreased the constitutive DNA-binding of AP-1. Similar effects on AP-1-binding were seen with the oxysterols, 7beta-hydroxycholesterol, 24- hydroxy-, 25-hydroxy-, and 27-hydroxy-cholesterol. Our data therefore suggest an effect of oxLDL on the DNA-binding of AP-1, which might be mediated by the oxysterol content of oxLDL. A decreased LPS-induced TNF-alpha and IL-1beta mRNA and protein expression were found in macrophages incubated with oxLDL before LPS-exposure. These observations suggest that macrophages that internalize extensively oxidized LDL are suppressed in their response to inflammatory stimulation.

Oxidized Low Density Lipoprotein Inhibits Lipopolysaccharide-induced Binding of Nuclear Factor–κB to DNA and the Subsequent Expression of Tumor Necrosis Factor–α and Interleukin-1β in Macrophages

Bertil G. Ohlsson, Mikael C.O. Englund, Anne-Li K. Karlsson, Ellen Knutsen, Cecilia Erixon, Henriette Skribeck, Yani Liu, Göran Bondjers, and Olov Wiklund

The Wallenberg Laboratory for Cardiovascular Research, Department of Medicine I, Sahlgren's Hospital, University of Göteborg, S-413 45 Göteborg, Sweden

Abstract

A large body of evidence suggests that oxidized LDL (oxLDL) has a role in atherogenesis. One effect is the impact on macrophage function. We have studied the effects of oxLDL and oxysterols on the binding of the transcription factors nuclear factor (NF)-κB and AP-1 to DNA. These transcription factors are involved in the regulation of several genes and expressed during activation of macrophages, for example by endotoxin (LPS). OxLDL did not induce binding of NF-κB. However, the LPS-induced response to NF-κB was substantially reduced after preincubation with oxLDL. Medium and highly oxidized LDL also decreased the constitutive DNA-binding of AP-1. Similar effects on AP-1–binding were seen with the oxysterols, 7β-hydroxycholesterol, 24-hydroxy-, 25-hydroxy-, and 27-hydroxy-cholesterol. Our data therefore suggest an effect of oxLDL on the DNA-binding of AP-1, which might be mediated by the oxysterol content of oxLDL. A decreased LPS-induced TNF-α and IL-1β mRNA and protein expression were found in macrophages incubated with oxLDL before LPS-exposure. These observations suggest that macrophages that internalize extensively oxidized LDL are suppressed in their response to inflammatory stimulation. (*J. Clin. Invest.* 1996. 98:78–89.)

Key words: atherosclerosis • modified low density lipoproteins • oxysterols • transcription factors • gene expression

Introduction

Much attention has been given to the role of oxidized LDL (oxLDL)[1] and oxidized lipids in atherosclerosis. Oxidized lipids have been shown in atherosclerotic lesions, and immunohistochemistry suggests that oxLDL is deposited in the lesions. How oxidized lipid products and oxLDL affect the initiation and progression of atherosclerotic lesion still is largely unknown. OxLDL has been shown to have a pleiotropic effect on cellular functions. In a series of studies, the biological effects of minimally modified LDL (MM-LDL) have been studied. These studies show that MM-LDL induces IL-1β in monocytes. In endothelial cells MM-LDL may induce the expression of colony stimulating factor, monocyte chemoattractant protein 1, and tissue factor (1-4). In endothelial cells it has also been shown that MM-LDL induces the transcription factor nuclear factor (NF)-κB to bind to its binding site on DNA (5).

More extensively oxidized LDL seems to have different effects than MM-LDL on cellular functions. It stimulates the production of monocyte chemotactic factor in endothelial cells and potentiates monocyte-endothelial cell adhesion (6). Other suggested effects are proliferation of smooth muscle cells, differentiation of macrophages, induction of endothelial cell expression of granulocyte and macrophage colony-stimlulating factors, and recruitment and retention of macrophages during atherogenesis (3, 7). At higher concentration oxLDL is cytotoxic (6, 8–10).

In macrophages oxLDL inhibits the lipopolysaccharide-induced *IL-1β* and *TNF-α* gene expression (11,12). The mechanism for this inhibition is unknown. The regulation of the TNF-α gene is regulated by the transcription factor NF-κB, which binds to regulatory elements in the promotor of the *TNF-α* gene (13). These regulatory elements are conserved in mouse, rabbit, and human and might therefore play an important role in the regulation of the *TNF-α* gene expression in several species (14). Lipopolysaccharide (LPS) induces binding of NF-κB to DNA and expression of the *TNF-α* gene is therefore used to induce an inflammatory response in macrophages (13, 15). The *IL-1β* promotor has one binding site for NF-κB, but also two AP-1–binding sites and one serum responsive element (SRE) (16).

In this report we have studied the effects of oxLDL on the LPS-induced DNA-binding of the transcription factors NF-κB, AP-1 and the transcription factor complex of serum responsive factor (SRF), and serum responsive factor accessory protein-1a (SAP-1) to DNA. We have also studied the LPS-induced mRNA and protein expression of the *TNF-α* and *IL-1β* genes in macrophages exposed to oxLDL. NF-κB is located in the cytosol in a complex with its inhibitor I-κB (17, 18). This complex dissociates when I-κB is phosphorylated by extracellular signals and NF-κB is then translocated into the nucleus, where it binds to DNA (19–22). We have also studied the DNA-binding of the transcription factor AP-1, since it regulates transcription of the *IL-1β* gene and several other genes that are also induced by LPS (23). AP-1 appears both as a heterodimer

Address correspondence to Bertil Ohlsson, The Wallenberg Laboratory for Cardiovascular Research, Sahlgren's Hospital, University of Göteborg, S-413 45 Göteborg, Sweden. Phone: 46-31-602 933; FAX: 46-31-823 762.

Received for publication 20 March 1995 and accepted in revised form 10 April 1996.

1. *Abbreviations used in this paper:* EMSA, electrophoretic mobility shift assay; MM-LDL, minimally modified LDL; NE, nuclear protein extracts; oxLDL, oxidized LDL; SAP-1, serum responsive factor accessory protein-1a; SRE, serum responsive element; SRF, serum responsiveness factor.

J. Clin. Invest.

0021-9738/96/07/78/12 $2.00
Volume 98, Number 1, July 1996, 78–89

of the transcription factors c-jun and c-fos and a homodimer of c-jun via hydrophobic interactions (23). The binding of AP-1 to DNA as well as the transcriptional activity of the AP-1 complex are regulated both by phosphorylation and dephosphorylation (23, 24). The *IL-1β* promotor contains both two AP-1–binding sites and one serum responsive element (16). The *c-fos* promotor contains an AP-1–binding site located adjacent to SRE (25). The transcription factors SRF and SAP-1 bind to SRE and are both necessary for a proper induction of the transcription of the *c-fos* gene (26, 27). The SRF + SAP-1 complex becomes transcriptionally active after SAP-1 has been phosphorylated (28).

An oligonucleotide, from the *c-fos* promotor that contains the AP-1–binding site adjacent to SRE was used for electrophoretical mobility shift assay (EMSA) to investigate the binding of both AP-1 and SRF + SAP-1 to DNA (25). The transcription factor c-fos is a protein component in the AP-1 complex and is therefore able to bind to the *c-fos* promotor. The SRF + SAP-1 complex upregulates the transcription of the *c-fos* gene (26–28). A decreased binding of the SRF + SAP-1 complex to the *c-fos* promotor, would probably lead to a decreased transcription of the *c-fos* gene, followed by a subsequent decrease of the DNA-binding of AP-1 to the *c-fos* promotor. The effect of a change in binding of NF-κB to DNA, caused by oxLDL, was studied on the TNF-α and IL-1β mRNA expression. In addition, the TNF-α and IL-1β protein synthesis were also monitored.

Oxidized lipids, among these oxysterols, are formed during oxidation of LDL (29). Oxysterols are biologically active substances, which can alter vascular permeability (30, 31), inhibit the LPL mRNA expression in human foam cells (32) and the LDL receptor function in human fibroblasts (33). Furthermore, oxysterols have been identified in tissue from atherosclerotic human aortas (34–39). We have therefore studied the effects of oxysterols on the binding of NF-κB and AP-1 to DNA to identify components in oxLDL that could mediate changes in the binding of transcription factors to DNA. Here we report that oxidized LDL decreased the LPS-induced DNA-binding of NF-κB in human macrophages. This decreased binding of NF-κB was followed by a low TNF-α and IL-1β mRNA and protein expression. In addition, we found that oxysterols decreased the binding of AP-1 to DNA.

Methods

Macrophage culture condition. Human monocyte-derived macrophages were isolated by Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) in a discontinous gradient from buffy coats, which were obtained from the Blood Bank, Sahlgren's Hospital (Göteborg, Sweden) (40). The mononuclear cell preparations were washed five times with PBS, pH 7.2, without calcium and magnesium, but containing 10 mM EDTA. The cell preparation was performed at room temperature. Mononuclear cells were resuspended and seeded at a density of 10^7 cells per each 100-mm plastic dish in a serum-free medium (Macrophage-SFM; GIBCO BRL, Grand Island, NY), supplemented with penicillin 100 U/ml and streptomycin 100 μg/ml. Monocytes were allowed to adhere for 1 h. Nonadhered cells were eliminated with three washes with PBS. Adherent monocytes were cultured in Macrophage-SFM medium with antibiotics and supplemented with human granulocyte macrophage colony stimulating factor (GM-CSF), 70 U/ml (R&D Systems Europe Ltd., Abingdon, UK). The medium was discarded after 3 d and the cells were washed once with PBS. The macrophages were then allowed to grow for another 3 d in the same medium, but without GM-CSF.

Preparation of human LDL. Human LDL (*d* 1.019–1.063 g/ml) was prepared by sequential ultracentrifugation (4°C) of plasma from healthy, fasted male volunteers, in the presence of EDTA (0.2% wt/vol) (41). The LDL preparations were filtered through 0.22-μm filters, stored at 4°C, and used within 1 wk of preparation.

Oxidation of low density lipoprotein. After extensive dialysis against PBS for 24 h, oxidation of LDL was performed by incubating 0.1 mg LDL protein/ml in PBS containing 5 μM $CuSO_4$ for 2.5, 4, and 13 h at 37°C, as described by Steinbrecher et al. (42). The oxidation was stopped by adding butylated hydroxytoluene(2,6-di-*t*-butyl-*p*-creso-1) (Sigma Chemical Co., St. Louis, MO) to a final concentration of 0.1 mM. Oxidized LDL was separated from $CuSO_4$ and equilibrated into the cell culture medium over a PD-10 column (Pharmacia Fine Chemicals). The protein content of LDL was determined according to Bradford (43). TBAR levels after oxidation typically were 17, 38, and 55 nmol MDA/mg protein after 2.5, 4, and 13 h oxidation, respectively. In agreement with these results an increasing shift in electrophoretical mobility was seen.

The different degrees of oxidation were also tested by analyzing the uptake of oxLDL into macrophages in culture. These experiments were performed as described by Goldstein and Brown (44) using human monocyte-derived macrophages under the conditions described in the present study. Iodinated (^{125}I; Amersham International, Amersham, UK) oxLDL was added to the cell culture medium at a protein concentration of 50 μg/ml, sp act 50–100 cpm/ng. After 4 h incubation the degradation of LDL was estimated from the chloroform-extracted, acid soluble radioiodine in the medium (45). Cellular uptake was determined as cell-associated radioactivity. In all experiments parallel wells without cells were incubated as controls for nonspecific adsorption or degradation. These experiments were performed in quadruplicates and repeated four times. The results obtained showed that the macrophage uptake of low oxLDL, oxidized for 2.5 h, was on an average 25.3 ng ^{125}I-LDL/mg cell protein, while the uptake of medium oxLDL, oxidized for 4 h, was on an average 51.3 ng ^{125}I-LDL/mg cell protein. When highly oxidized LDL was used, oxidized for 13 h, an uptake of an average of 120 ng ^{125}I- LDL/mg cell protein was observed. The results showed a significant difference with respect to macrophage uptake between low, medium, and high oxLDL. The same figures for degradation were 11.8, 21.4, and 34.0 ng ^{125}I-LDL/mg cell protein, for oxLDL oxidized 2.5, 4, and 13 h respectively.

In conclusion, the degree of oxidation was studied with three different methods. Increased electrophoretical mobility was observed after LDL was submitted to oxidation. Also, the TBAR levels increased after oxidation. Finally, an increased macrophage uptake and

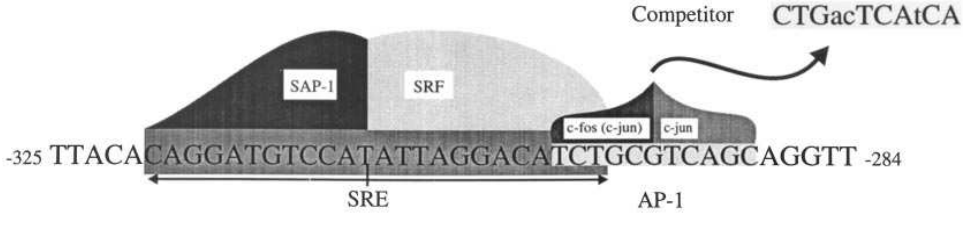

Figure 1. Binding of the transcription factors SRF + SAP-1 and AP-1 to the promotor sequence, −325 to −284, of the human *c-fos* gene. The sequence homology of the *c-fos* promotor to the oligonucleotide competitor with the consensus sequence to the AP-1–binding site is indicated by capital letters.

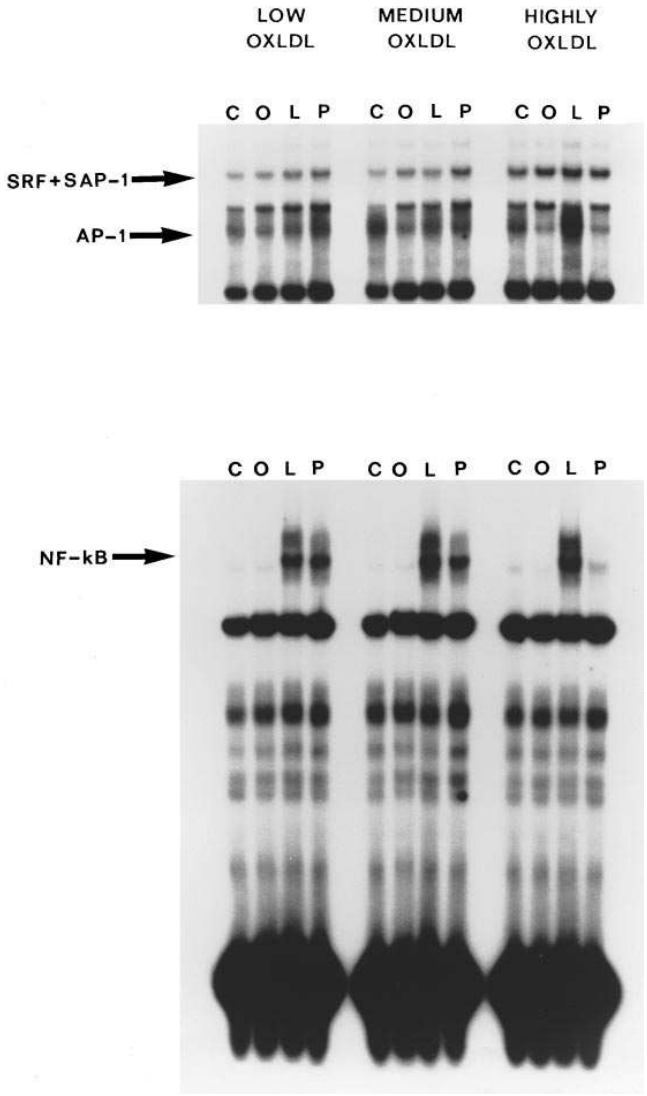

Figure 2. Nuclear protein extracts (NE) from human monocyte-derived macrophages, analyzed for binding to α^{32}P-labeled DNA oligonucleotides by electrophoretic mobility shift assay. An oligonucleotide from the human *c-fos* promotor −325 to −284 containing the SRE and AP-1–binding site was used to identify binding of SRF + SAP-1 and AP-1. Binding of NF-κB was analyzed by an oligonucleotide from the human *TNF-α* promotor, −646 to −613. An equal protein amount of NE was added to each binding reaction. NE from untreated macrophages (*C*), macrophages treated with oxLDL, 50 μg/ml, for 24 h (*O*), treated with LPS, 1 μg/ml, for 1 h (*L*), and macrophages treated with oxLDL, 50 μg/ml for 24 h before exposure of LPS, 1 μg/ml, for 1 h (*P*). The identified transcription factors SRF + SAP-1, AP-1, and NF-κB are indicated. Unbound oligonucleotide is at the bottom of the lanes.

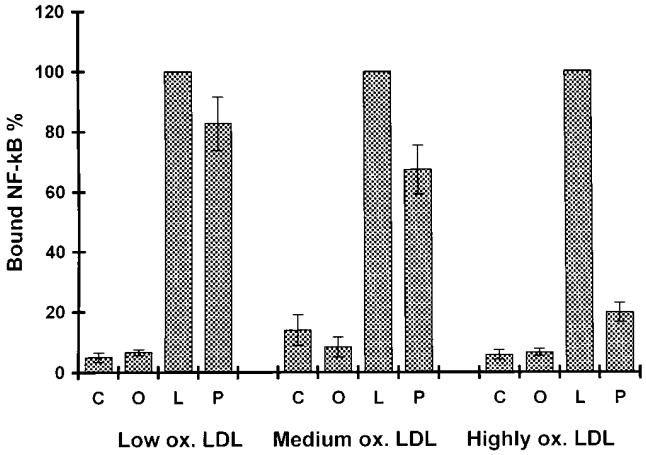

Figure 3. Effects of oxLDL on the LPS-induced binding of NF-κB. NE from human macrophages were analyzed with electrophoretic mobility shift assay. Binding of NF-κB was analyzed by an oligonucleotide from the human TNF-α promotor −646 to −613. An equal protein amount of nuclear extract (NE) was added to each binding reaction. NE from untreated macrophages (*C*), macrophages treated with oxLDL, 50 μg/ml, for 24 h (*O*), treated with LPS, 1 μg/ml, for 1 h (*L*), and macrophages treated with oxLDL, 50 μg/ml for 24 h before exposure of LPS, 1 μg/ml, for 1 h (*P*). The results obtained after laser densitometry scans of the shifted bands corresponding to NF-κB are shown. LPS-induced DNA-binding of NF-κB was set to 100%. The results are represented as mean values from five experiments and SEM is indicated.

degradation of oxidized LDL was found. Using the Cell proliferation kit II (XTT; Boehringer Mannheim, Mannheim, Germany) no differences in cell viability were found between untreated macrophages and cells treated with the different oxLDL preparations.

The presence of endotoxins in the lipoprotein preparations were analyzed by the Coatest endotoxin kit (Chromognix, Mölndal, Sweden) according to the manufacturer's instructions. Only preparations of oxLDL, which had < 8 pg LPS/ml were used for the experiments.

Experimental procedure. 6 d after plating of the monocytes, from one blood donor, the medium was replaced with the same medium, but now supplemented with either oxLDL, 50 μg protein per ml, or oxysterols, 5 μg/ml. 7-ketocholesterol, 7β-hydroxycholesterol, 25-hydroxycholesterol purchased from Sigma Chemical Co., and 24-hydrocholesterol and 27-hydroxycholesterol kindly provided by Dr. U. Diczfalusy, Huddinge University Hospital (Huddinge, Sweden) were used. The oxysterols dissolved in ethanol were added to the medium in the final concentration of 1 μl per ml medium. To control cells only the ethanol vehicle was added. This medium was replaced after 24 h incubation, by a medium with or without LPS, 1 μg/ml (Sigma Chemical Co.). 1 h after incubation with LPS, the preparations of nuclear protein extracts were started.

Preparation of nuclear protein extract. The macrophages were washed three times with cold PBS and collected by scraping the dish with a cell lifter, in 2 ml hypotonic buffer, pH 7.9, containing 10 mM Hepes, pH 7.9, 0.1 mM EDTA, 10 mM KCl, powdered nonfat milk 5 mg/ml, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT (Boehringer Mannheim Biochemical), pepstatin 50 μg/ml, leupeptin 50 μg/ml, chymostatin 50 μg/ml, antipain 50 μg/ml, benzamidine 78 μg/ml, and 0.5 mM PMSF (Sigma Chemical Co.). The macrophages were homogenized by 8 strokes with a B pestle in a Dounce homogenizer. The nuclei were collected by centrifugation at 30,000 *g* at 4°C for 10 min. The pellet containing the nuclei was resuspended in 250 μl eluting buffer; 20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and supplemented with the proteinase inhibitors in the same concentrations as in the hypotonic buffer. After 45 min extraction at 4°C, nuclei were sedimented at 30,000 g at 4°C for 1 h, and the nuclear proteins were collected and stored in aliquotes at −70°C. Protein concentrations of the nuclear proteins obtained from the macrophages were measured according to Bradford (43).

Recombinant human c-jun that forms homodimers of the AP-1 complex was purchased from Promega Corp., Madison, WI.

Constructions of oligonucleotides. Oligonucleotides were synthesized on an oligonucleotide synthesizer (Applied Biosystems Inc., Foster City, CA). Single-stranded oligonucleotides were, after purification,

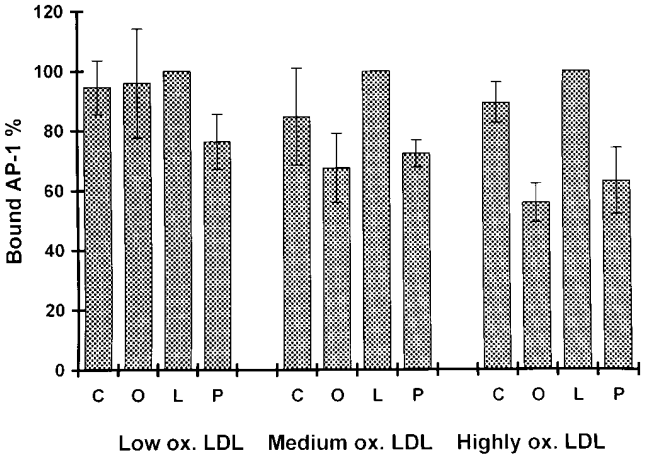

Figure 4. Effects of oxLDL on the constitutive binding of AP-1. NE from human macrophages were analyzed with electrophoretical mobility shift assay. DNA-binding of AP-1 was analyzed by an oligonucleotide from the human *c-fos* promotor −325 to −284. An equal protein amount of nuclear extract (NE) was added to each binding reaction. NE from untreated macrophages (*C*), macrophages treated with oxLDL, 50 μg/ml, for 24 h (*O*), treated with LPS, 1 μg/ml, for 1 h (*L*), and macrophages treated with oxLDL, 50 μg/ml for 24 h before exposure of LPS, 1 μg/ml, for 1 h (*P*). The results obtained after laser densitometry scans of the shifted bands corresponding to AP-1 are shown. LPS-induced DNA-binding of AP-1 was set to 100%. The results are represented as mean values from five experiments and SEM is indicated.

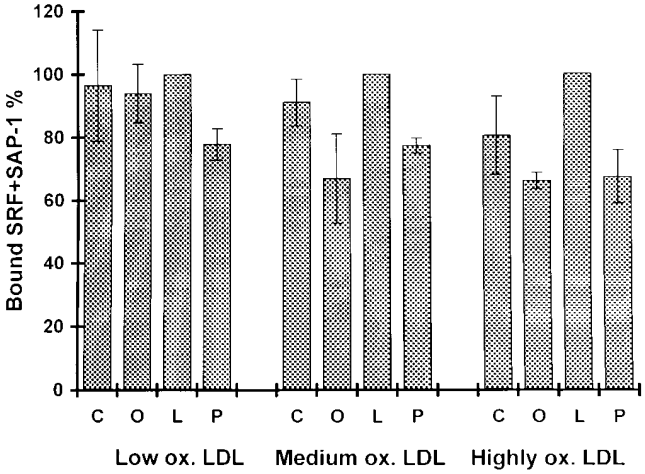

Figure 5. Effects of oxLDL on the constitutive binding of SRF + SAP-1. NE from human macrophages were analyzed with electrophoretical mobility shift assay. DNA-binding of the SRF + SAP-1 complex was analyzed by an oligonucleotide from the human *c-fos* promotor −325 to −284. An equal protein amount of NE was added to each binding reaction. NE from untreated macrophages (*C*), macrophages treated with oxLDL, 50 μg/ml, for 24 h (*O*), treated with LPS, 1 μg/ml, for 1 h (*L*), and macrophages treated with oxLDL, 50 μg/ml for 24 h before exposure of LPS, 1 μg/ml, for 1 h (*P*). The results obtained after laser densitometry scans of the shifted bands corresponding to SRF + SAP-1 complex are shown. LPS-induced DNA-binding of the SRF + SAP-1 complex was set to 100%. The results are represented as mean values from five experiments, and SEM is indicated.

annealed overnight. The annealed product was separated by a polyacrylamide gel electrophoresis and electroeluted from the gel at 50 V overnight. A double-stranded oligonucleotide identical to the sequence from −646 to −613 in the human *TNF-α* promotor, containing a κB-site, was used to identify binding of NF-κB (14). As a specific competitor to this κB-site a double-stranded oligonucleotide, containing a κB-site from −223 to −190 in the same *TNF-α* promotor was used (14).

The *c-fos* gene promotor sequence from -325 to -284, illustrated in Fig. 1, containing the serum responsive element (underlined) and the AP-1 binding site, indicated by bold letters, (TTACACAGGATG-TCCATATTAGGACATC**TGCGTCAGCAG**GTT) was constructed.

A double-stranded oligonucleotide containing the consensus sequence of the AP-1 motif, with the sequence TTCCGGCTGACT-

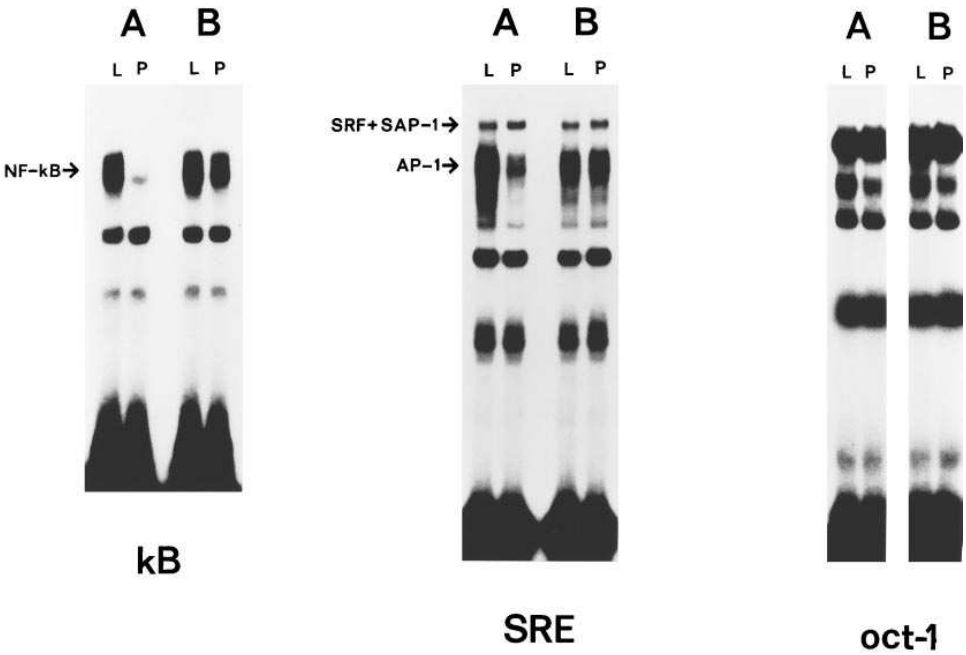

Figure 6. The reversible effect of oxLDL on the DNA-binding of NF-κB and AP-1. NE from human macrophages were analyzed with electrophoretic mobility shift assay. Binding to an α^{32}P-labeled oligonucleotide from the *c-fos* promotor containing a SRE and an AP-1–binding site and the NF-κB–binding site from the *TNF-α* promotor and a consensus sequence of the oct-1–binding motif were investigated. An equal protein amount of NE was added to each binding reaction. *A* indicates NE from macrophages treated with LPS, 1 μg/ml, for 1 h (*L*) and NE from cells incubated with highly oxidized LDL, 50 μg/ml, for 24 h before LPS exposure, 1 μg/ml, for 1 h (*P*). *B* indicates macrophages that have been incubated for 3 d; macrophages treated with LPS, 1 μg/ml, for 1 h (*L*); macrophages incubated with the same highly oxidized LDL, 50 μg/ml, for 24 h as under *A*, followed by an incubation without oxLDL for another 2 d before exposure of LPS, 1 μg/ml, for 1 h (*P*). The identified transcription factors NF-κB, SRF + SAP-1 and AP-1 are indicated. Unbound oligonucleotide is at the bottom of the lanes.

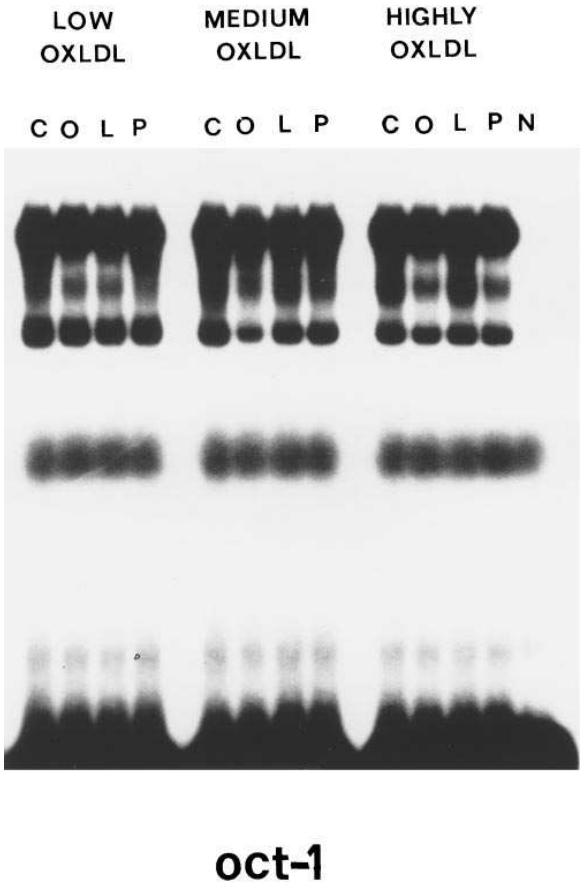

Figure 7. Effect of oxLDL and LPS on the DNA-binding of Oct-1. NE from human monocyte-derived macrophages treated with oxLDL were analyzed for protein binding to the octamer consensus sequence by electrophoretic mobility shift assay. An equal protein amount of NE was added to each binding reaction. NE from untreated macrophages (*C*), macrophages treated with oxLDL, 50 μg/ml, for 24 h (*O*), treated with LPS, 1 μg/ml, for 1 h (*L*), and macrophages treated with oxLDL, 50 μg/ml for 24 h before exposure of LPS, 1 μg/ml, for 1 h (*P*); only oligonucleotide (*N*). Unbound oligonucleotide is at the bottom.

CAAGCG, was used to identify specific binding of AP-1 (Fig. 1) (46, 47). The binding motif is underlined. A double-stranded oligonucleotide, TGTCGAATGCAAATCACTAGAA, containing the consensus sequence of the octamer binding motif (underlined) was used to identify specific binding of the transcription factor oct-1. This transcription factor regulates transcription of a number of so-called housekeeping genes (48).

Electrophoretic mobility shift assay. For gel mobility shift assays, double-stranded oligonucleotides radiolabeled with α^{32}P-labeled deoxynucleotide, using Klenow polymerase (Boehringer Mannheim Biochemical), were used. The protein-DNA binding reaction was carried out in a volume of 20 μl containing 20 mM Tris-HCl, pH 7.9, 75 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 5% glycerol and 5 μg of poly(dI-dC)poly(dI-dC) (Pharmacia Biotech). Approximately 80,000 Cerenkov cpm, corresponding to 2 ng of the probe, was added to each reaction, which proceeded at room temperature for 20 min after the same amount of protein extracts had been added to every reaction. In some experiments, rabbit polyclonal IgG towards the p65 subunit of NF-κB and towards SAP-1 and c-fos (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the binding reactions. Competitions were performed by inclusion molar excess of unlabeled double-stranded oligonucleotides.

The reaction mixtures were then electrophoresed through a native 5% polyacrylamide gel containing 0.25 × TBE (2.2 mM Tris borate, 2.2 mM boric acid, 0.5 mM EDTA) and 3% glycerol to separate DNA-protein complexes from unbound DNA-probe. Gels were vacuum dried and exposed to a photographic film at −80°C. The retarded bands, obtained after EMSA, corresponding to NF-κB, AP-1 and SRF + SAP-1 were quantitated by scanning the autoradiographs in an LKB Ultrascan laser densitometer. To compare these quantitative data between experiments from different donors, the binding obtained for the transcription factors NF-κB, AP-1, and SRF + SAP-1 to the DNA-oligonucleotides after macrophages were treated with LPS was set to 100% for each experiment.

Preparation and analysis of RNA. Total cellular RNA was isolated from differentiated macrophages by guanidinium-hydrochloride/isothiocyanate extraction and CsCl gradient ultracentrifugation (49). A quantitative RT-PCR method described by Wang et al. (50) and modified by Mattsson et al. (51) was used to quantitate mRNA for TNF-α and IL-1β. The reverse transcription reaction was performed with the Gene Amp RNA PCR kit (Perkin Elmer, Foster City, CA) using 1–100 ng of total RNA isolated from macrophages and 10^3–10^5 molecules of pAW 109 cRNA per reaction and incubated for 10 min at room temperature followed by 15 min at 42°C, 5 min at 99°C, and cooled at 5°C for 5 min. The oligonucleotide primer pairs for TNF-α and IL-1β mRNA suggested by Wang (50) were used. The amplification profile for TNF-α cDNA involved heating to 95°C for 2 min, denaturation at 95°C for 1 min, and annealing-extension at 64°C for 31 cycles in Biometra Tri-Thermoblock (Biometra, Göttingen, Germany). The amplification profile for IL-1β cDNA was performed under the same conditions as TNF-α, except that the annealing-extension reaction was performed at 65°C for 32 cycles. The PCR products were separated on 4% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME) for 2–5 h. The gel was stained with ethidium bromide (Merck, Darmstadt, Germany), and appropriate bands were excised, and radioactivity was determined by liquid scintillation counting. In the exponential phase of the amplification the amount of target mRNA was quantitated against the internal standard curve. The LPS-induced TNF-α and IL-1β mRNA expression was set to 100%.

Expression of TNF-α and IL-1β in macrophages. Synthesis of TNF-α and IL-1β was evaluated with an immunoassay using ELISA kits from Immunotech, Marseille, France. Expression of TNF-α was followed in the medium, while expression of IL-1β was followed in the cytosol. 3 h of LPS-exposure to the macrophages was allowed before monitoring the expression of TNF-α and IL-1β. The samples were assayed in duplicates. The LPS-induced TNF-α and IL-1β protein expression was set to 100%.

Cell viability. Macrophage growth in culture was determined using the cell proliferation kit II (XTT; Boehringer Mannheim) according to the manufacturer's instructions.

Results

OxLDL inhibits LPS-induced binding of NF-κB and the constitutive binding of AP-1 and SRF + SAP-1 to DNA. LDL from two donors was pooled and oxidized for 2.5, 4, and 13 h. These three variants of oxidized LDL were incubated with macrophages from three donors. This experiment was repeated five times to study the variations in the experimental results in relation to the variability in LDL with macrophages obtained from different donors.

Proteins in nuclear extracts from human macrophages treated for 24 h with oxLDL, 50 μg/ml, followed by LPS, 1 μg/ml, for 1 h were analyzed by EMSA for binding to an α^{32}P-labeled oligonucleotide from the *c-fos* promotor containing both the serum responsive element and the AP-1–binding site and an α^{32}P-labeled oligonucleotide containing a NF-κB–bind-

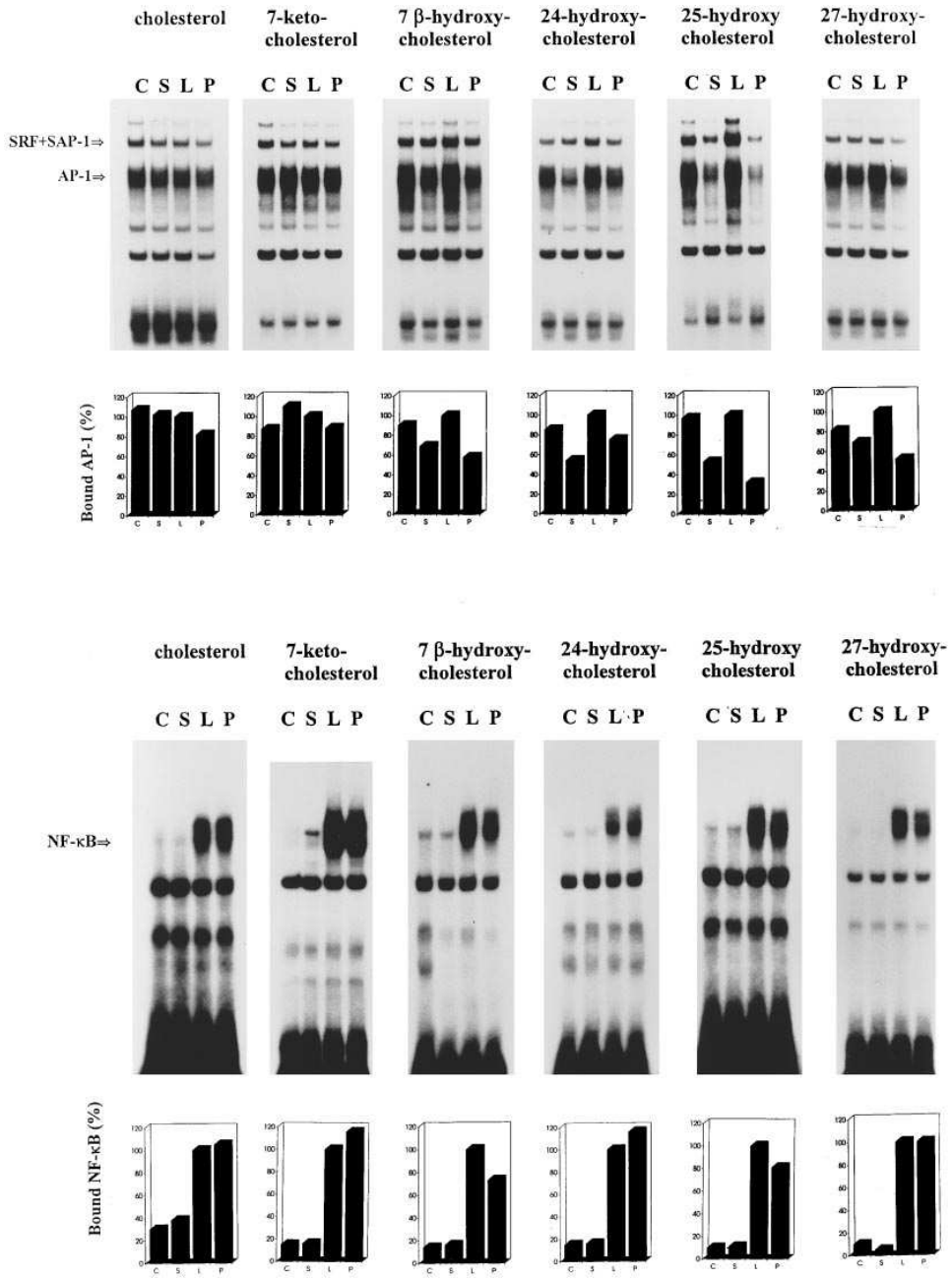

Figure 8. Effects of oxysterols on the DNA-binding of AP-1, SRF + SAP-1 and NF-κB. NE from human monocyte-derived macrophages analyzed by electrophoretic mobility shift assay for binding to the same α^{32}P-labeled oligonucleotides as in Fig. 2. An equal protein amount of NE was added to each binding reaction. NE from untreated macrophages (*C*) and macrophages treated with oxysterols, 5 μg/ml, for 24 h (*S*) and with LPS, 1 μg/ml, for 1 h (*L*), and macrophages treated with oxysterols for 24 h before exposure of LPS, 1 μg/ml for 1 h (*P*). The individual oxysterols used are indicated on top. The identified transcription factors SRF + SAP-1, AP-1, and NF-κB are indicated.

ing motif from the *TNF-α* promotor. The DNA-binding of the transcription factors NF-κB, AP-1, and SRF + SAP-1 found after EMSA, from macrophages exposed to LPS only was set to 100% (see Methods).

A typical effect of oxLDL on the LPS-induced binding of NF-κB, AP-1, and SRF + SAP-1 after EMSA is shown in Fig. 2. The results obtained after laser densitometry scanning of the autoradiographs from EMSA from five experiments with monocyte-derived macrophages and LDL from several donors are summarized in Figs. 3, 4, and 5.

The results in Figs. 2 and 3 show that almost no binding to the κB site was found in nuclear protein extracts from untreated macrophages, since NF-κB is a LPS-inducible transcription factor. Similar results was found with nuclear protein extract from macrophages incubated with oxLDL, demonstrating that oxLDL did not in itself induce binding of NF-κB to the *TNF-α* promotor (Figs. 2 and 3.). Treatment of macrophages with LPS, 1 μg/ml, for 1 h induced binding of NF-κB to the *TNF-α* promotor, which was set to 100% (Fig. 3). The response of NF-κB to LPS was reduced to 67%, when macrophages were pre-treated with medium oxidized LDL for 24 h (Fig. 3). Highly oxidized LDL even further reduced the LPS-induced binding of NF-κB to the *TNF-α* promotor to 20% (Fig. 3). However, low oxLDL, in contrast to highly oxLDL, had only a small impact on the inhibition of the LPS-induced response on NF-κB (Fig. 3). Apparently, the inhibiting effect of oxLDL on the LPS-induced binding of NF-κB to the *TNF-α* promotor was related to the degree of the oxidation of LDL.

AP-1 was, in contrast to NF-κB, constitutively bound to the oligonucleotide used for EMSA (Figs. 2 and 4). Medium and

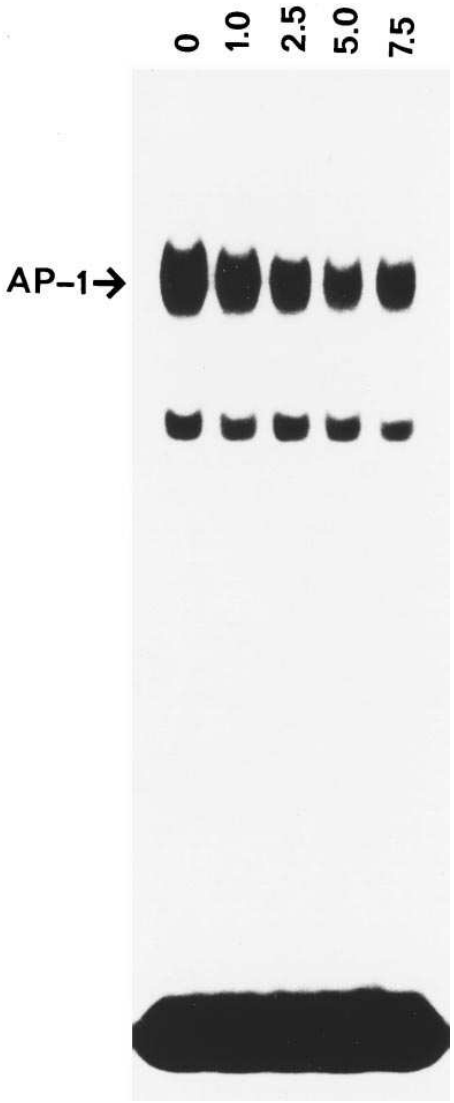

Figure 9. Concentration-dependent effect of 7β-hydroxycholesterol on the DNA-binding of AP-1. NE from human monocyte-derived macrophages incubated with increasing concentration of 7β-hydroxycholesterol for 24 h. The nuclear protein extracts were analyzed by electrophoretic mobility shift assay for binding to the consensus sequence of the AP-1 motif. An equal protein amount of NE was added to each binding reaction. The concentrations of 7β-hydroxycholesterol used, in μg/ml, are indicated on top. The identified transcription factor AP-1 is indicated. Unbound oligonucleotide is at the bottom.

highly oxidized LDL, decreased the constitutive binding of AP-1 to the *c-fos* promotor to 68 and 56% respectively (Fig. 4), while no effect was found with LPS.

The SRF + SAP-1 complex, like AP-1, was found to be constitutively bound to SRE (Fig. 5). The binding of SRF + SAP-1 to SRE after LPS exposure was set to 100%. Medium and highly oxidized LDL decreased the constitutively bound SRF + SAP-1 complex to 78 and 66%, respectively (Fig. 5). This pattern was similar to that obtained with AP-1.

To study whether the inhibiting effects on the binding of NF-κB and AP-1 to DNA were reversible, the following experiment was performed: Macrophages were exposed to highly oxidized LDL for 24 h under the same conditions as previously described. Only 9% of the LPS-induced DNA-binding of NF-κB was found in macrophages incubated with highly oxLDL (Fig. 6), which is in agreement with our other previous results shown in Fig. 2. For the AP-1–binding only 36% of the LPS-induced binding was found. After 24 h treatment with highly oxLDL, the medium was replaced with the same medium for another 2 d but without oxidized LDL. When the macrophages after these 2 d were exposed to LPS, 1 μg/ml, a 70% response of the NF-κB–binding to DNA was found, in comparison to cells treated only with LPS. No difference was found for the AP-1–binding, in comparison to macrophages exposed to LPS only, since 104% binding was found in macrophages that previously had been exposed to highly oxLDL. The binding to the octamer motif did not change during these experiments (Fig. 6).

These results taken together demonstrate that the inhibiting effects on the binding of AP-1 and NF-κB to DNA in macrophages treated with highly oxLDL before LPS-exposure were reversible and probably due to specific effects of oxidized LDL on these transcription factors, since no differences in binding to the octamer motif on DNA were found (Figs. 6 and 7).

Effects of oxysterols on the binding of AP-1 and NF-κB to DNA. We examined a number of oxysterols, indicated in Fig. 8, to investigate whether they could mediate the effects found with oxidized LDL on the binding of AP-1, SRF + SAP-1, and NF-κB to DNA. Macrophages were incubated with increasing concentrations of 7β-hydroxycholesterol to investigate at which level the maximal inhibition of the constitutive binding of AP-1 to DNA was found. The results illustrated in Fig. 9 show a dose-dependent effect of 7β-hydroxycholesterol on the binding of AP-1 to DNA up to 5.0 μg/ml. The concentration of the oxysterols used was therefore chosen to 5.0 μg/ml. The macrophages were incubated with oxysterols, 5 μg/ml, for 24 h, before LPS-exposure for 1 h. Macrophages were incubated with cholesterol, 5 μg/ml, as control to the oxysterols.

The results obtained after EMSA showed that no binding of NF-κB to DNA was found in the cells treated with cholesterol and oxysterols (Fig. 8). LPS induced binding of NF-κB to DNA in untreated macrophages as well as in cells pretreated with oxysterols. These results demonstrate that oxysterols in oxLDL are not able to inhibit the LPS-induced binding of NF-κB, found in human macrophages incubated with highly oxidized LDL. Similar results were observed in two separate experiments.

A decreased DNA-binding of AP-1 to ∼ 50–70% was found in macrophages incubated with 7β-hydroxycholesterol, 24-hydroxycholesterol, 27-hydroxycholesterol, and 25-hydroxycholesterol, while no effect was found with LPS. These results indicate an inhibiting effect on the constitutive DNA-binding of AP-1 by oxysterols. Similar results were observed in two separate experiments.

Identification of the transcription factors NF-κB, AP-1, and SRF + SAP-1. A specific antibody to the p65 subunit of NF-κB was used to identify the electrophoretical band corresponding to NF-κB after EMSA with the oligonucleotide from the *TNF-α* promotor containing a NF-κB–binding site. Only the electrophoretic band induced after LPS was supershifted with the specific antibody against NF-κB (results not shown).

To further demonstrate that the retarded band, obtained after EMSA, was NF-κB, a competition experiment was performed. A specific competitor including a κB motif from the human *TNF-α* promotor from −223 to −190 was added in molar excess. A nonspecific competitor was also added, which did

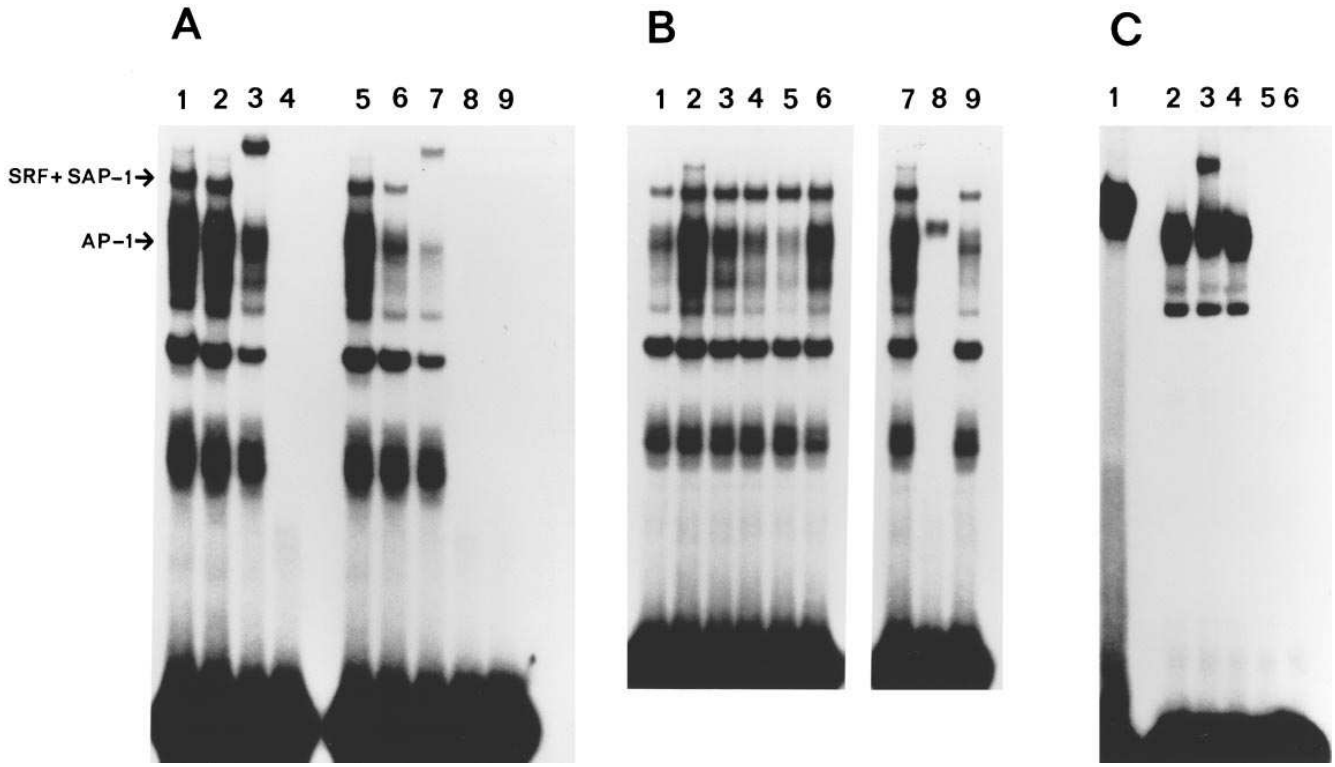

Figure 10. Identification of the transcription factors SRF + SAP-1 and AP-1. NE from human macrophages were analyzed for binding to the α^{32}P-labeled oligonucleotide from the *c-fos* promotor containing the SRE and AP-1–binding site. (*A*) NE from untreated macrophages lane *1* and macrophages treated with low oxLDL (lane *2*). Specific antibodies against SAP-1 added to the same NE as in lane *2* (lane *3*). Specific antibodies against SAP-1 added but without NE (lane *4*). NE from untreated macrophages (lane *5*) and macrophages treated with highly oxLDL (lane *6*). Specific antibodies against SAP-1 added to the same NE as in lane *6* (lane *7*). Specific antibodies against SAP-1 added, but without NE (lane *8*). Only DNA probe added (lane *9*). (*B*) Binding of AP-1 was competed with an oligonucleotide, containing the AP-1–binding consensus sequence (Fig. 1). NE from macrophages treated with highly oxLDL (lane *1*) and low oxLDL (lane *2*). Molar excess of the unlabeled specific competitor was added to the binding reaction with NE from macrophages treated with low oxLDL. 200 molar excess (lane *3*), 400 molar excess (lane *4*) and 800 molar excess (lane *5*). An unlabeled nonspecific competitor, containing the binding site for NF-κB added in 400 molar excess (lane *6*). NE from macrophages treated with low oxLDL (lane *7*). Binding of recombinant c-jun (lane *8*). NE from macrophages treated with highly oxLDL (lane *9*). (*C*) Binding to the oligonucleotide containing the consensus sequence for AP-1 (Fig. 1), using recombinant c-jun (lane *1*); NE from macrophages treated with low oxidized LDL (lane *2*); NE from macrophages treated with low oxidized LDL and a specific antibody against c-fos (lane *3*); NE from macrophages treated with low oxidized LDL and a specific antibody against p65 in NF-κB (lane *4*); only antibody against c-fos (lane *5*); only antibody against p65 in NF-κB (lane *6*).

not have any binding sites for NF-κB, and therefore did not compete with the specific oligonucleotide. The specific oligonucleotide containing the κB motif competed with the band earlier identified as NF-κB (results not shown). Thus the competition experiment confirmed the results obtained with the specific antibody. The κB motif in the oligonucleotide used as a competitor had an apparently lower affinity for NF-κB than the κB motif in the labeled oligonucleotide, since 200 molar excess of competitor was needed to abolish binding.

The transcription factor SRF is known to bind to SRE either by itself or in a complex with SAP-1 (25–28). SAP-1 is, in contrast to SRF, not able to bind to SRE by itself (27). Therefore, SRF is a prerequisite for binding of SAP-1 to DNA. To identify the SRF + SAP-1 complex bound to SRE, a specific antibody against SAP-1 was added to the protein–DNA-binding reaction. A supershifted band was found after EMSA with the antibody against SAP-1, demonstrating the SRF + SAP-1 complex (Fig. 10).

To identify the AP-1–binding site flanking the serum responsive element on the *c-fos* promotor, shown in Fig. 1, a competition experiment was performed, using the consensus sequence of the AP-1–binding site (46) (Fig. 1). When molar excess of the oligonucleotide containing the AP-1–binding consensus sequence was added to the binding reaction, a specific protein–DNA-binding complex was competed with the *c-fos* promotor (Fig. 10 *B*). 400 molar excess of the NF-κB–binding sequence did not affect any of the protein-DNA complex on the oligonucleotide from the *c-fos* promotor, demonstrating that the competetion with the AP-1–binding site was specific (Fig. 10 *B*). To further verify the electrophoretical localization of the AP-1-DNA complexes, recombinant c-jun, forming a homodimer of the AP-1 complex, was allowed to bind to the oligonucleotide from the *c-fos* promotor. The c-jun homodimer form of the AP-1 complex was shown to have similar electrophoretical mobility as the protein-DNA complex, which was competed with the oligonucleotide containing the consensus sequence for AP-1 (Fig. 10 *B*) (46).

The oligonucleotide containing the consensus sequence of the AP-1–binding site was used to study the binding of AP-1 from macrophage nuclear protein extracts in detail. With this

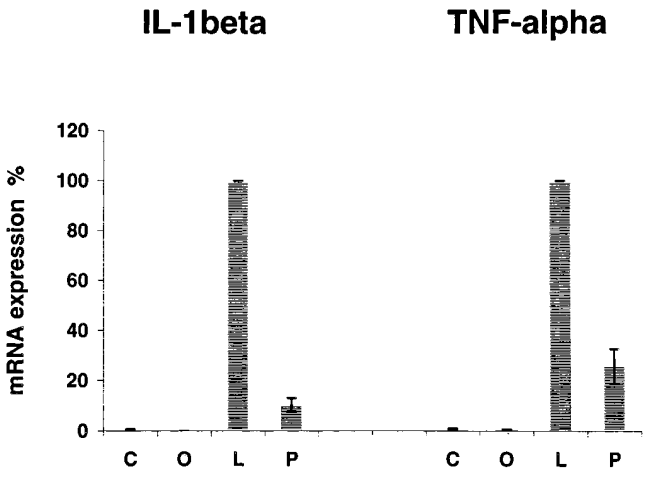

Figure 11. Effect of highly oxidized LDL on the LPS-induced IL-1β and TNF-α mRNA expression in human macrophages. Total cellular RNA was extracted for quantitative RT-PCR analyses of IL-1β and TNF-α mRNA as described under Methods. Expression in untreated (*C*) macrophages; in macrophages treated with oxLDL, 50 μg/ml for 24 h (*O*); in macrophages exposed to LPS, 1 μg/ml, for 3 h (*L*) (set to 100%) and mRNA expression in macrophages treated with oxLDL, 50 μg/ml for 24 h before exposure of LPS, 1 μg/ml, for 3 h (*P*). The results are represented as mean values from three experiments, and SEM is indicated.

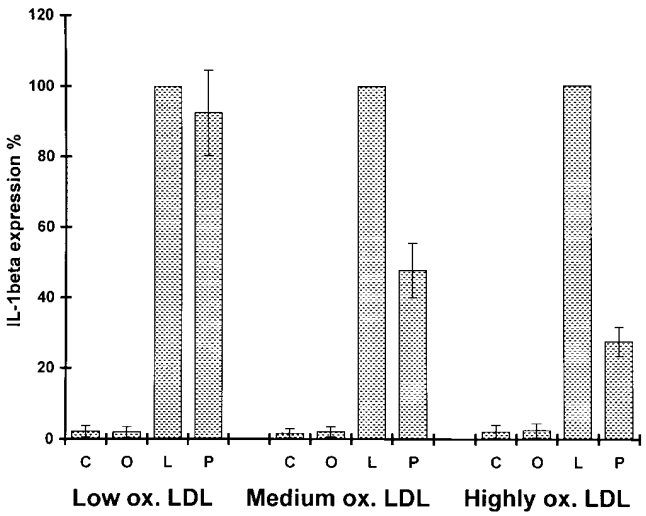

Figure 13. Effect of oxidized LDL on the LPS-induced IL-1β protein expression. The intracellular IL-1β protein expression, measured by ELISA, in untreated macrophages (*C*); in macrophages treated with oxLDL, 50 μg/ml for 24 h (*O*); in macrophages exposed to LPS, 1 μg/ml, for 3 h (*L*) (set to 100%) and the IL-1β protein expression in macrophages treated with oxLDL, 50 μg/ml, for 24 h before exposure of LPS, 1 μg/ml, for 3 h (*P*). The results are represented as mean values from three experiments, and SEM is indicated.

oligonucleotide only one retarded band was found with the nuclear protein extract from macrophages treated with low oxidized LDL (Fig. 10 *C*). This electrophoretic band had a similar mobility as the band obtained, when recombinant c-jun formed a homodimer of AP-1 and was bound to the same AP-1 oligonucleotide (Fig. 10 *C*). A specific antibody against c-fos in the AP-1 complex was also used to identify the electrophoretical, shifted band obtained with the nuclear protein extract. A supershifted band was observed when nuclear protein extract from macrophages treated with low oxidized LDL was analyzed with this antibody (Fig. 10 *C*). In conclusion, these results demonstrate that AP-1 in macrophage nuclear protein extract was bound to the AP-1 probe.

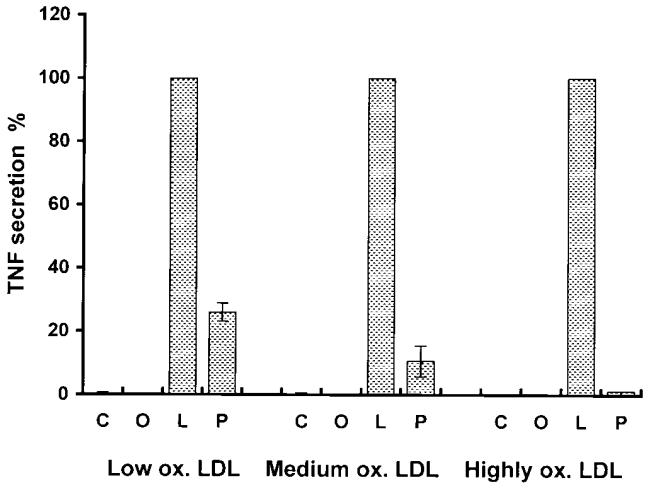

Figure 12. Effect of oxidized LDL on the LPS-induced TNF-α protein secretion. The TNF-α protein secretion was measured in cell culture medium, by ELISA, from untreated macrophages (*C*); macrophages treated with oxLDL, 50 μg/ml for 24 h (*O*); macrophages exposed to LPS, 1 μg/ml, for 3 h (*L*) (set to 100%) and TNF-α secretion from macrophages after treatment with oxLDL 50 μg/ml for 24 h before exposure of LPS, 1 μg/ml, for 3 h (*P*). The results are represented as mean values from three experiments, and SEM is indicated.

Highly oxidized LDL decreased the LPS-induced TNF-α and IL-1β mRNA expression. LPS-induced gene expression of TNF-α and IL-1β is mediated by DNA-binding of NF-κB to the *TNF-α* and *IL-1β* promotors (13). Total RNA from macrophages was isolated to investigate the effect of oxLDL on the LPS-induced TNF-α and IL-β mRNA expression. Results from three experiments are summarized in Fig. 11. These results showed that LPS-induced IL-1β and TNF-α mRNA expression was reduced to 10 and 26%, respectively, in macrophages incubated with highly oxLDL before LPS-exposure. Highly oxidized LDL in itself did not induce mRNA expression of TNF-α and IL-1β. These results are in agreement with the DNA-binding of NF-κB and AP-1 in nuclear protein extracts from macrophages incubated with highly oxLDL before LPS-exposure.

Oxidized LDL inhibits LPS-induced TNF-α and IL-1β protein synthesis. The TNF-α and IL-1β protein synthesis were followed to further study the consequences of the decreased LPS-induced binding of NF-κB and AP-1 to the *TNF-α* and *IL-1β* promotors. The results obtained for the TNF-α protein expression from three experiments, illustrated in Fig. 12, demonstrate that low oxLDL decreased the LPS-induced synthesis of TNF-α to 26%, while medium and highly oxidized LDL decreased the LPS-induced synthesis to 11 and 1%, respectively. The results for the LPS-induced IL-1β protein synthesis are shown in Fig. 13. Low oxLDL decreased the LPS-induced expression of IL-1β to 93%, while medium oxLDL decreased this expression to 48% and highly oxLDL decreased

the LPS-induced IL-1β expression to 28%. No TNF-α and IL-1β protein expression were found in untreated macrophages or in macrophages incubated with oxLDL. These results are therefore in line with those results obtained for the TNF-α and IL-1β mRNA expression.

Discussion

In this study we have demonstrated that the LPS-induced binding of NF-κB to the *TNF-α* promotor in human monocyte-derived macrophages exposed to oxidized LDL decreases. We have also shown that oxLDL, in itself, does not affect the DNA-binding of NF-κB. The effect of the decreased LPS-induced DNA-binding of NF-κB was also investigated at the level of mRNA and protein expression of TNF-α and IL-1β. After LPS-exposure both TNF-α and IL-1β mRNA and protein expression were found. However, no TNF-α or IL-1β expression were found in untreated macrophages or in macrophages incubated with oxLDL. These observations are in agreement with previous results, which also include other genes (11, 12, 52). When, after exposure to oxLDL, the LPS-induced binding of NF-κB to the *TNF-α* promotor dropped to 20% the corresponding mRNA expression was reduced to 26%. These results demonstrate a correlation between the binding of NF-κB to its binding sites on the human *TNF-α* promotor and the subsequent TNF-α mRNA expression. Also the LPS-induced IL-β mRNA expression was reduced after exposure of the cells to highly oxidized LDL. In oxLDL-treated cells the LPS-induced mRNA for IL-1β, after 3 h, was only 10% of the level in cells exposed to only LPS. The LPS-induced IL-1β mRNA expression is known to have its peak after 2–4 h (53). When macrophages were treated with oxLDL before LPS-exposure, a decrease in TNF-α and IL-1β protein expression was also observed. The simultaneous decrease of the binding of NF-κB to DNA, the mRNA, and protein expression indicate that oxLDL inhibits the transcriptional activity of the *TNF-α* and *IL-1β* genes. It has been shown by others (52) that the LPS-induced TNF-α mRNA expression is due to the transcriptional activity of the *TNF-α* gene, while no transcriptional activity was found in control cells or cells exposed to oxLDL (52). Although a decreased transcriptional activity probably explains the decreased response to LPS after exposure to oxLDL, we cannot exclude the possibility that oxLDL may also affect the translation and the posttranslational regulation of the TNF-α and IL-1β expression. Little is known concerning the stability of TNF-α mRNA in primary macrophage cultures and possible variation in mRNA stability in parallel with changes in translation efficiency (54).

In this study we have also shown that highly oxLDL inhibited the constitutive binding of AP-1 and SRF + SAP-1 to their binding sites to 56 and 66%, respectively. The low LPS-induced IL-1β mRNA expression in these cells could therefore be due to the decreased binding of these two transcription factor complexes, since the IL-1β promotor contains two AP-1–binding sites and one serum responsive element (16).

The drop in the SRF + SAP-1–binding to SRE was followed by a similar decrease in the AP-1–binding to the *c-fos* promotor in macrophages incubated with oxLDL. The c-fos protein is a constituent of the AP-1 complex, as shown with the specific antibody against c-fos in Fig. 10 *C*. These results might therefore indicate that oxLDL inhibits the *c-fos* gene expression, by decreasing the constitutive binding of the SRF + SAP-1 complex to SRE on the *c-fos* promotor, since the binding of this complex to SRE is necessary for transcription of the *c-fos* gene (26–28). In addition, both c-fos, c-jun and SRF + SAP-1 need to be phosphorylated to induce transcription (23, 24, 26).

A common factor for the described effects of oxLDL on NF-κB, AP-1, and SRF + SAP-1 is that these transcription factors are dependent on phosphorylation for their activation (17–19, 23, 24, 28). In contrast, the transcription factor Oct-1, which is not induced by phosporylation, was unaffected by oxLDL (Figs. 6 and 7)(48).

Which factor in oxLDL is responsible for the observed effects on macrophages is unknown. Lipid uptake into macrophages via scavenger receptors can also be generated by AcLDL (55). AcLDL, however, has no effect on the LPS-induced secretion of IL-1β (11). Among the constituents of oxLDL, lipid oxidation products have been suggested to have important biological effects (37–39). One group of such products are the oxysterols, generated by oxidation of cholesterol (56, 57). Oxysterols are biologically active molecules which, for example, inhibit the LDL receptor function in human fibroblasts (33), reduce the LPL mRNA expression in human macrophage cells (51), and decrease the synthesis of PGI_2 in endothelial cells (58). In addition, oxysterols have also been found to be cytotoxic to endothelial cells and smooth muscle cells in vitro (59–62). Furthermore, oxysterols have been identified in tissue from atherosclerotic human aortas (34–39). To investigate if the observed effects of oxLDL were mediated through oxysterols we investigated a series of different oxysterols and their effect on the transcription factors NF-κB and AP-1. In this study we found a decreased constitutive DNA-binding of AP-1 to $\sim$ 50–70% in macrophages incubated with 7β-hydroxy, 24-hydroxy, 25-hydroxy, and 27-hydroxy-cholesterol, while almost no effects of the oxysterols were found on the LPS-induced DNA-binding of NF-κB. Apparently, constituents other than the oxysterols studied are responsible for the inhibition of LPS-induced activation of NF-κB. Several such components have been suggested, such as various aldehydes (63), lysophosphatidylcholine (64, 65), or other degradation products of phospholipids (6). As shown by others, the components in oxLDL that conduct the inhibiting effect of the LPS-induced expression of IL-1β in macrophages are bound to the LDL particle (11, 52).

In the present study we have used human monocytes allowed to differentiate in a serum-free medium in the presence of GM-CSF. This system was found to give very reproducible results, even with cells from different donors. The variability involved in using different batches of human serum was hereby avoided, and in using a serum-free medium the risk of contamination with endotoxin could be minimized.

Similar results to those obtained by us have been found in studies on macrophages incubated with glucocorticoids (66, 67). Glucocorticoids were found to reduce LPS-induced binding of NF-κB as well as the constitutive binding of AP-1 to DNA, while binding of the transcription factor Oct-1 to DNA was unaffected. These authors found that dexamethasone induced the expression of IκB in the cytoplasm (66, 67). When macrophages were exposed to LPS, the NF-κB-IκB complex dissociated after IκB was phosphorylated, but NF-κB was then bound to another unphosphorylated IκB. The excess of IκB in comparison to NF-κB inhibited the translocation of NF-κB from the cytoplasm to the nucleus. In our experiment only 20% of the LPS-activated NF-κB was found in the nuclear

protein extract from macrophages exposed to highly oxidized LDL. Our results might suggest that oxLDL may inhibit the translocation of NF-κB from the cytoplasm to the nucleus and the subsequently transcriptional expression of the *TNF-α* gene.

The most extensive studies have been performed regarding the effects of MM-LDL on the induced inflammatory responses in endothelial cells as well as on the induced monocyte and endothelial interactions (5, 6). These studies suggest that MM-LDL activates the endothelial cells in several respects and that MM-LDL actually induces a binding of NF-κB to DNA (5). In human vascular smooth muscle cells MM-oxLDL had no effect on the DNA-binding activity of NF-κB, while highly oxLDL inhibited a LPS-induced DNA-binding of NF-κB (68). In contrast to our results both MM-oxLDL and highly oxLDL induced DNA-binding of AP-1 (68). MM-oxLDL and more extensively oxidized LDL have different constituents (29, 64). The biological effects of MM-oxLDL and oxLDL are, therefore, probably very different. In addition, various cell types in culture apparently react differently on MM-oxLDL and on highly oxLDL.

In the atherosclerotic lesion, LDL is supposed to be bound to the extracellular matrix and then modified by oxidation (45, 69). Oxidized LDL is then taken up by macrophages leading to an accumulation within the foam cells of cholesterol as well as lipid oxidation products, such as oxysterol (32). The results from this study might indicate that oxidized LDL could inhibit or minimize a normal inflammatory response. It has been speculated that the suppression of cytokine expression in foam cells may be an important contributing factor of the pathophysiologic process in the formation of fatty streaks before they convert to mature atheromas (52). The capacity of macrophages in lesions to be activated might also be crucial for the stability of the atherosclerotic plaque as well as for the thrombosis formation on the plaque surface. To further elucidate macrophage function in atherosclerosis, therefore, is essential for the understanding of plaque formation as well as for the development of complicated lesions with plaque rupture and thrombosis formation.

Acknowledgments

Oxysterols were kindly provided by Dr. Ulf Diczfalusy at Huddinge University Hospital. The authors thank Aira Lidell and Ulla-Britt Rignell for technical assistance.

This work was supported by grants from the Swedish Medical Research Council (project 4531) and the Swedish Heart-Lung Foundation.

References

1. Berliner, J.A., M. Territo, M. Navab, A. Andalibi, F. Parhami, F. Liao, J. Kim, S. Estworthy, A.J. Lusis, and A.M. Fogelman. 1992. Minimally modified lipoproteins in diabetes. *Diabetes.* 41(Suppl. 2):74–75.

2. Navab, M., S.S. Imes, S.Y. Hama, G.P. Hough, L.A. Ross, R.W. Bork, A.J. Valente, J.A. Berliner, D.C. Drinkwater, H. Laks, and A.M. Fogelman. 1991. Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein. *J. Clin. Invest.* 88:2039–2046.

3. Rajavashisth, T.B., A. Andalibi, M.C. Territo, J.A. Berliner, M. Navab, A.M. Fogelman, and A.J. Lusis. 1990. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature (Lond.).* 344:254–257.

4. Fei, H., J.A. Berliner, F. Parhami, and T.A. Drake. 1993. Regulation of endothelial cell tissue factor expression by minimally oxidized LDL and lipopolysaccharide. *Arterioscler. Thromb.* 13:1711–1717.

5. Parhami, F., T.Z. Fang, A.M. Fogelman, A. Andalibi, M.C. Territo, and J.A. Berliner. 1993. Minimally modified low density lipoprotein–induced inflammatory responses in endothelial cells are mediated by cyclic adenosine monophosphate. *J. Clin. Invest.* 92:471–478.

6. Berliner, J.A., M.C. Territo, A. Sevanian, S. Ramin, S.A. Kim, B. Bamshad, M. Esterson, and A.M. Fogelman. 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* 85: 1260–1266.

7. Quinn, M.T., S. Parthasarathy, G.L. Fong, and D. Steinberg. 1987. Oxidatively modified low density lipoprotein: a potential role in recruitment and retention of mononcyte/macrophages during atherogenesis. *Proc. Natl. Acad. Sci. USA.* 84:2995–2998.

8. Hessler, J.R., D.W. Morel, L.J. Lewis, and G.M. Chisholm. 1983. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Arteriosclerosis.* 3:215–222.

9. Hughes, H., B. Mathews, M.L. Lenz, and J.R. Guyton. 1994. Cytotoxicity of oxidized LDL to porcine aortic smooth muscle cells is associated with the oxysterols 7-ketocholesterol and 7-hydroxycholesterol. *Arterioscler. Thromb.* 14: 1177–1185.

10. Henriksen, T., S.A. Evensen, and B. Carlander. 1979. Injury to human endothelial cells induced by low density lipoproteins. *Scand. J. Clin. Lab. Invest.* 39:361–368.

11. Fong, L.G., T.A.T. Fong, and A.D. Cooper. 1991. Inhibition of lipopolysaccharide-induced interleukin-1β mRNA expression in mouse macrophages by oxidized low density lipoprotein. *J. Lipid. Res.* 32:1899–1909.

12. Hamilton, T.A., M.A. Gouping, and G.M. Chisholm. 1990. Oxidized low density lipoprotein suppresses the expression of tumor necrosis factor-α mRNA in stimulated murine peritoneal macrophages. *J. Immunol.* 144:2343–2350.

13. Collart, M.A., P. Baeuerle, and P. Vassalli. 1990. Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four κB-like motifs and of constitutive and inducible forms of NF-κB. *Mol. Cell. Biol.* 10: 1498–1506.

14. Shakov, A.N., M.A. Collart, P. Vassalli, S.A. Nedospasov, and C.V. Jongeneel. 1990. κB-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor α gene in primary macrophages. *J. Exp. Med.* 171:35–47.

15. Sen, R., and D. Baltimore. 1986 Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslation mechanism. *Cell.* 47:921–928.

16. Zhang, Y., and W.N. Rom. 1993. Regulation of interleukin-1β (IL-1β) gene by myobacterial components and lipopolysaccharide is mediated by two nuclear factor-IL-6 motifs. *Mol. Cell. Biol.* 13:3831–3837.

17. Baeuerle, P.A., and D. Baltimore. 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. *Cell.* 53:211–217.

18. Baeuerle, P.A., and D. Baltimore. 1988. IκB: a specific inhibitor of the NF-κB transcription factor. *Science (Wash. DC).* 242:540–546.

19. Shirakawa, F., and S.B. Mizel. 1989. In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Mol. Cell. Biol.* 9:2424–2430.

20. Ghosh, S., and D. Baltimore. 1990. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. *Nature (Lond.).* 344:678–682.

21. Kerr, L.D., J. Inoue, N. Davis, E. Link, P.A. Baeuerle, H.B. Bose Jr., and I.M. Verma. 1991. The rel-associated pp40 protein prevents DNA binding of rel and NF-κB: relationship with IκBβ and regulation by phosphorylation. *Genes & Dev.* 5:1464–1476.

22. Liou, H-C., and D. Baltimore. 1993. Regulation of the NF-κB/rel transcription factor and IκB inhibitor system. *Curr. Opin. Cell Biol.* 5:477–487.

23. Angel, P., and M. Karin. 1991. The role of jun, fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta.* 1072: 129–157.

24. Karin, M. 1994. Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. *Curr. Opin. Cell. Biol.* 6: 415–424.

25. Shaw, P.E., S. Frasch, and A. Nordheim. 1989. Repression of *c-fos*.transcription is mediated through $p67^{SRF}$ bound to the SRE. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2567–2574.

26. Norman, C., M. Runswick, R. Pollock, and R. Treisman. 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the *c-fos* serum responsive element. *Cell.* 55:989–1003.

27. Dalton, S., and R. Treisman. 1992. Characterization of SAP-1, a protein recruited by serum response factor to the *c-fos* serum response element. *Cell.* 68:597–612.

28. Marais, R., J. Wynne, and R. Treisman. 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell.* 73:381–393.

29. Zhang H., H.J.K. Basra, and U.P. Steinbrecher 1990. Effects of oxidatively modified LDL on cholesterol esterfication in cultured macrophages. *J. Lipid Res.* 31:1361–1369.

30. Holmes, R.P., and N.L. Yoss. 1984. 25-hydroxycholesterols increase the permeability of liposomes to Ca^{2+} and other actions. *Biochim. Biophys. Acta.*

770:15–21.

31. Theunissen, J.J.H., R.L. Jackson, H.J.M. Kempen, and R.A. Demel. 1986. Membrane properties of oxysterols, interfacial orientation, influence on membrane permeability and redistribution between membranes. *Biochim. Biophys. Acta.* 860:66–74.

32. Mattsson Hultén, L., H. Lindmark, U. Diczfalusy, I. Björkhem, M. Ottosson, Y. Liu, G. Bondjers, and O. Wiklund. 1996. Oxysterols present in atherosclerotic tissue decrease the expression of lipoprotein lipase messenger RNA in human monocyte-derived macrophages. *J. Clin. Invest.* 97:461–468.

33. Brown, M.S., and J.L. Goldstein. 1975. Cholesterol ester formation in cultured human fibroblasts: stimulation by oxygenated sterols. *J. Biol Chem.* 250:4025–4027.

34. Brooks, C.J.W., G. Steel, J.D. Gilbert, and W.A. Harland. 1971. Characterization of a new group of polar sterol from human atherosclerotic plaques. *Atherosclerosis.* 13:223–237.

35. Harland, W.A., J.D. Gilbert, G. Steel, and C.J.W. Brooks. 1971. The occurence of a new group of polar sterol esters in various stages of human atherosclerosis. *Atherosclerosis.* 13:239–246.

36. Smith, L.L., and J.E. van Lier. 1970. Sterol metabolism IX. 26-hydroxycholesterol levels in the human aorta. *Atherosclerosis.* 12:1–14.

37. Jacobson, M.S., P.P. Nair, S.M. Naseem, T.D. Smith, and F.P. Heald. 1981. Oxygenated cholesterols: new risk factors for atherosclerosis? *Pediatr. Res.* 15:535.

38. Chisholm, G.M., G. Ma, K.C. Irwin, L.L. Martin, K.M. Gunderson, L.F. Linberg, D.W. Morel, and P.E. Dicorleto. 1994. 7-βhydroperoxycholest-5-en-3β-ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidized human low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* 91: 11452–11456.

39. Carpenter, K.L.H., S.E. Taylor, C. van der Veen, B.K. Williamsson, J.B. Ballantine, and M.J. Mitchinson. 1995. Lipids and oxidized lipids in human atherosclerotic lesions at different stages of development. *Biochim. Biophys Acta.* 1256:141–150.

40. Böyum, A. 1976. Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* 5(Suppl. 5):9–15.

41. Olofsson, S.-O., K. Boström, U. Svanberg, and G. Bondjers. 1980. Isolation and partial characterization of polypeptide belonging to apolipoprotein B from low density lipoproteins in human plasma. *Biochemistry.* 19:1059–1064.

42. Steinbrecher, U.P., S. Parthasarathy, D.S. Leake, J.L. Witzum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* 81:3883–3887.

43. Bradford, M.M. 1976. A rapid and a sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

44. Goldstein, J.L., and M.S. Brown. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts: comparison of cells from a normal and from patient with homozygous familial hypercholesterolemia. *J.Biol.Chem.* 249:5153–5162.

45. Hurt-Camejo, E., G. Camejo, B. Rosengren, F. Lopez, C. Ahlström, G. Fager, and G. Bondjers. 1992. Effect of arterial proteoglycans and glycosaminoglycans on low density lipoprotein oxidation and its uptake by human macrophages and arterial smooth muscle cells. *Arterioscler. Thromb.* 12:569–583.

46. Meyer, M., R. Schreck, and P.A. Baeuerle. 1993. H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 12: 2005–2015.

47. Angel, P., M. Imagawa, R. Chiu, B. Stein, R.J. Imbra, H.J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell.* 49:729–739.

48. Cockerill, P.N., and S.P. Klinken. 1990. Octamer-binding proteins in diverse hemopoietic cells. *Mol. Cell. Biol.* 10:1293–1296.

49. Chirgwin, J.M., A.M. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5298.

50. Wang, A.M., M.V. Doyle, and D.F. Mark. 1989. Quantification of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA.* 86:9717–9721.

51. Mattsson, L., H. Johansson, M. Ottosson, G. Bondjers, and O. Wiklund. 1993. Expression of lipoprotein lipase mRNA and secretion in macrophages isolated from human atherosclerotic aorta. *J. Clin. Invest.* 92:1759–1765.

52. Hamilton, T.A., J.A. Major, and G.M. Chisholm. 1995. The effects of oxidized low density lipoproteins on inducible mouse macrophage gene expression are gene and stimulus dependent. *J. Clin. Invest.* 95:2020–2027.

53. Newton, R.C. 1990. The production of human interleukin-1beta by blood monocytes. *In* Cytokines and Lipocortins in Inflammation and Differentiation. M. Melli and L. Parente, editors. Wiley-Liss, Inc., New York. 349:217–228.

54. Beutler, B. 1990. Regulation of cachectin biosynthesis occurs at multiple levels. *In* Cytokines and Lipocortins in Inflammation and Differentiation. M. Melli and L. Parente. Editors. Wiley-Liss, Inc., New York. 349:229–240.

55. Brown, M.S., and J.L. Goldstein. 1983. Lipoprotein metabolism in macrophage: implications for the cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* 52:223–261.

56. Bhadra, S., M.A.Q. Arshad, Z. Rymaszewski, E. Norman, R. Wherley, and M.T.R. Subbiah. 1991. Oxidation of cholesterol moiety of low density lipoprotein in the presence of human endothelial cells or Cu2+ ions: identification of major products and their effects. *Biochem. Biophys. Res. Commun.* 176:431–440.

57. Jialal, I., D.A. Freeman, and S.M. Grundy. 1991. Varying susceptibility of different low density lipoproteins to oxidative modification. *Arterioscler. & Thromb.* 11:482–488.

58. Hu, B., S. Peng, D. Brubaker, and R.J. Morin. 1990. Influence of cholesterol oxides and antioxidants on prostacyclin production by cultured endothelial cells. *FASEB J.* 4:3734.

59. Pettersen, K.S., K.M. Boberg, A. Stabursvik, and H. Prydz. 1991. Toxicity of oxygenated cholesterol derivatives toward cultured human umbilical vein endothelial cells. *Arteroscler. & Thromb.* 11:423–428.

60. Sevanian, A., J. Berliner, and H. Peterson. 1991. Uptake, metabolism and cytotoxicity of isomeric cholesterol-5, 6-epoxides in rabbit aortic endothelial cells. *J. Lipid Res.* 32:147–155.

61. Zhou, Q., T.L. Smith, and F.A. Kummerow. 1993. Cytotoxicity of oxysterols on cultured smooth muscle cells from human umbilical arteries. *Proc. Soc. Exp. Biol. Med.* 202:75–80.

62. Clare, K., S.J. Hardwick, K.L.H. Carpenter, N. Weeratunge, and M.J. Mitchison. 1995. Toxicity of oxysterols to human monocyte-macrophages. *Atheriosclerosis.* 118:67–75.

63. Thomas, C.E., R.L. Jackson, D.F. Ohlweiler, and G. Ku. 1994. Multiple lipid oxidation products in low density lipoproteins induce interleukin-1beta release from human blood mononuclear cells. *J. Lipid. Res.* 35:417–427.

64. Steinberger, D., S. Parthasarathy, T.E. Carew, J.C. Khoo, and J.L. Witzum. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* 320:915–924.

65. Sugiyama, S., K. Kugiyama, M. Ohgushi, K. Fujimoto, and H. Yasue. 1994. Lysophosphatidylcholine in oxidized low-density lipoprotein increases endothelial susceptibility to polymorphonuclear leucocyte-induced endothelial dysfunction in porcine coronary arteries. Role of protein kinase C. *Circ. Res.* 74:565–575.

66. Scheinman, R.I., P.C. Cogswell, A.K. Lofquist, and A.S. Baldwin. 1995. Role of transcriptional activation of IκBα in mediation in immunosuppression by glucocorticoids. *Science (Wash. DC).* 270:283–286.

67. Auphan, N., J.A. DiDonato, C. Rosette, A. Helmberg, and M. Karin. 1995. Immunosuppression by glucocorticoids: Inhibition of NF-κB activity through induction of IκB synthesis. *Science (Wash. DC).* 270:286–290.

68. Ares, M.P.S., B. Kallin, P. Eriksson, and J. Nilsson. 1995. Oxidized LDL induces transcription factor acivator protein-1 but inhibits activation of nuclear factor-κB in human vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* 15:1584–1590.

69. Williams, K.J., and I. Tabas. 1995. The response-to-retention hypothesis of early atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* 15:551–561.