Role of endocytosis in the transactivation of nuclear factor-κB by oxidized low-density lipoprotein

Chang-Yeop HAN, Soo-Young PARK and Youngmi Kim PAK[1]

Division of Metabolic Disease Research, Department of Biomedical Sciences, National Institute of Health, Korea, Eunpyung-Ku, Nokbun-Dong, #5, Seoul 122-701, Korea

Oxidized low-density lipoprotein (oxLDL) has been shown to modulate transactivation by the peroxisome proliferator-activated receptor (PPAR)-γ and by nuclear factor-κB (NF-κB). In the present study, the oxLDL signalling pathways involved in NF-κB transactivation were investigated by utilizing a reporter construct driven by three upstream NF-κB binding sites, and various pharmacological inhibitors. OxLDL and its constituent lysophophatidylcholine (lysoPC) induced a rapid and transient increase in intracellular calcium and stimulated NF-κB transactivation in resting RAW264.7 macrophage cells in an oxidation-dependent manner. NF-κB activation by oxLDL or lysoPC was inhibited by inhibitors of protein kinase C or by a chelator of intracellular calcium. Tyrosine kinase or phosphatidylinositol 3-kinase inhibitors did not block NF-κB transactivation. Furthermore, oxLDL-induced NF-κB activity was abolished by PPAR-γ ligands. When the endocytosis of oxLDL was blocked by cytochalasin B, NF-κB transactivation by oxLDL was synergistically increased, while PPAR transactivation was blocked. These results suggest that oxLDL activates NF-κB in resting macrophages via protein kinase C- and/or calcium-dependent pathways, and that this does not involve the endocytic processing of oxLDL. The endocytosis-dependent activation of PPAR-γ by oxLDL may function as a route of inactivation of the oxLDL-induced NF-κB signal.

Key words: calcium, endocytosis, PKC, PPAR-γ, signal transduction.

INTRODUCTION

The formation of an atherosclerotic lesion is a complex process involving intracellular lipid accumulation and various signal-transduction pathways in vascular cells, such as endothelial cells, smooth muscle cells and monocytes/macrophages. Each vascular cell is capable of oxidatively modifying low-density lipoprotein (LDL) to generate oxidized LDL (oxLDL) [1], which is taken up by macrophages through scavenger receptors (SRs) [2]. This oxidative modification results in a number of important biological activities of LDL, including the induction of a number of genes encoding cytokines or growth factors [3,4]. Among growth factors and cytokines that are secreted from vascular cells, a regulatory network may exist so that a lesion results from their collective action [5].

Unlike the LDL receptor, the expression of which is finely regulated by the intracellular cholesterol level, the expression of the SR is stimulated by internalized oxLDL [6]. Recently it was reported that ligands for peroxisome proliferator-activated receptor-γ (PPAR-γ) and 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HODE) were generated from oxLDL through SR-mediated endocytosis followed by intracellular processing [7]. The endocytosis- and ligand-dependent activation of PPAR-γ by oxLDL was responsible for regulating the expression of various macrophage genes, including that encoding the SR.

Several reports indicate that oxLDL activates signalling pathway(s) upon ligation to the SR. Nuclear factor-κB (NF-κB) has been suggested to be one of the possible mediators involved in oxLDL-induced signalling. Supporting evidence includes the finding of an activated p65 component of NF-κB in an atherosclerotic lesion [8], and the observation of a retarded band on a NF-κB gel-shift assay [9,10]. In contrast, Ohlsson et al. [11] demonstrated that oxLDL did not induce the binding of NF-κB, whereas it suppressed the lipopolysaccharide (LPS)-induced response to NF-κB. Many other investigators have also shown that oxLDL blocks LPS-induced gene expression [12,13].

In the present study, RAW264.7 macrophages were stably transfected with a luciferase construct containing three upstream NF-κB binding sites of E-selectin, in order to quantify the effects of oxLDL on NF-κB activation. This assay system allows us to investigate the regulatory connection between NF-κB and other possible signalling mediators [14], such as intracellular calcium [9] and protein kinase C (PKC) [15,16], in resting macrophages. Using pharmacological inhibitors we show that oxLDL activates NF-κB via PKC- and/or calcium-dependent pathways. This process does not require the endocytic processing of oxLDL, unlike the oxLDL-induced PPAR-γ pathway. Furthermore, a specific ligand for PPAR-γ suppresses oxLDL-induced NF-κB transcriptional activity. Thus we demonstrate for the first time the regulation of NF-κB by oxLDL in resting macrophages.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, fetal bovine serum and interferon-γ were purchased from Gibco BRL (Grand Island, NY, U.S.A.), and paraformaldehyde was from Electron Microscopy Science (Fort Washington, PA, U.S.A.). 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), wortmannin, BAPTA/AM [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester] and bisindolylmaleimide I (GF109203X) were purchased from CalBiochem (San Diego, CA, U.S.A.), and anti-(NF-κB p65) polyclonal antibody was from Santa Cruz Biotechnology (Santa

Abbreviations used: BAPTA/AM, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acetoxymethyl ester; $[Ca^{2+}]_i$, cytosolic free calcium concentration; DiI, 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine percholate; fura 2/AM, fura 2 acetoxymethyl ester; 13-HODE, 13-hydroxyoctadecadienoic acid; I-κB, inhibitor of NF-κB; LDL, low-density lipoprotein; lysoPC, lysophosphatidylcholine; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; oxLDL, oxidized LDL; $oxLDL_{24h}$, LDL oxidized for 24 h; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-responsive element; SR, scavenger receptor.

[1] To whom correspondence should be addressed (e-mail ymkimpak@nih.go.kr).

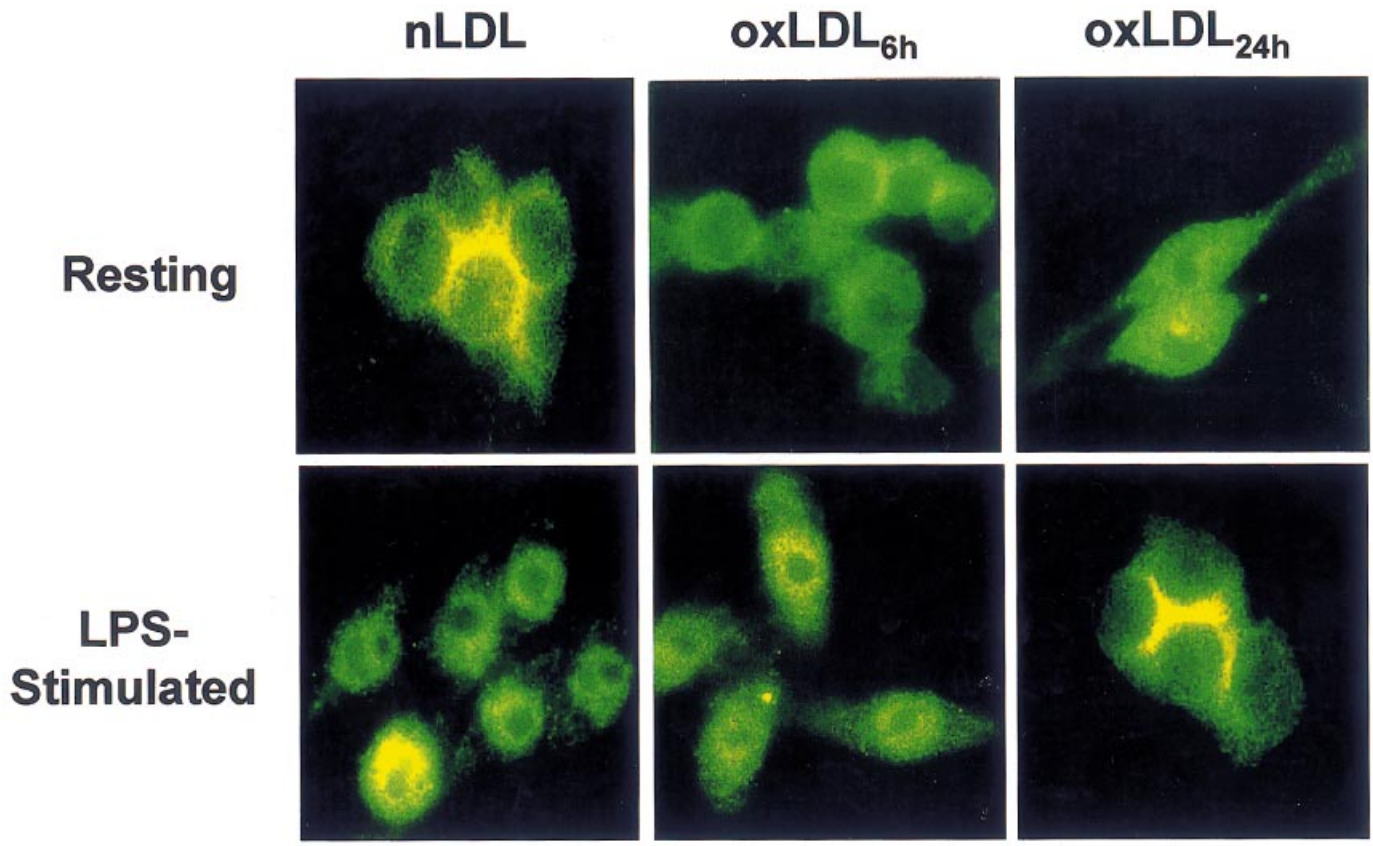

Figure 1 Effects of oxLDL on the localization of NF-κB

Resting (upper panels) or LPS-stimulated (lower panels) RAW264.7 cells were incubated for 4 h with 100 μg/ml native LDL (nLDL) or with oxLDL which had been oxidized with $CuSO_4$ for 6 h or 24 h ($oxLDL_{6h}$ and $oxLDL_{24h}$ respectively). The treated cells were fixed, stained with anti-(NF-κB p65) antibody and photographed using a fluorescence microscope, as described in the Materials and methods section. In resting cells, oxLDL changed the cell morphology from round to spindle-shaped and localized part of NF-κB into the nuclei in an oxidation-dependent manner. In contrast, oxLDL reversed the cell morphology from spindle-shaped to round in LPS-stimulated cells and inhibited NF-κB nuclear localization. The bright green fluorescence indicates the location of the NF-κB p65 subunit. Original magnification ×1000.

Cruz, CA, U.S.A.). Fura 2/AM (fura 2 acetoxymethyl ester) and SYTOX Green were purchased from Molecular Probes (Eugene, OR, U.S.A.). LPS, copper(II) sulphate, palmitoyl lysophosphatidylcholine (lysoPC; $C_{16:0}$), 7-oxocholesterol (7-ketocholesterol), cytochalasin B, staurosporine, genistein, DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine percholate) and all other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). 13-HODE was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.), and troglitazone was generously donated by Dr S. S. Yim (Natural Product Science Research Center, Seoul National University, Korea).

Preparation and oxidation of LDL

Human LDL (1.019–1.063 g/ml) was isolated from the plasma of healthy volunteers by sequential ultracentrifugation [17]. After ultracentrifugation, LDL was dialysed extensively at 4 °C against PBS containing 0.5 mM EDTA. Concentrations of LDL are expressed as LDL protein, measured by the modified Lowry method using the DC protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). LDL (1 mg/ml) was oxidized by 5 μM copper sulphate in PBS (pH 7.4) at 37 °C for the indicated time period [18] (note that $oxLDL_{24h}$ represents LDL oxidized for 24 h). Oxidation was terminated by adding EDTA (pH 8.5) and butylated hydroxytoluene at final concentrations of 0.5 mM and 50 μM respectively. OxLDL preparations were dialysed extensively at 4 °C against PBS containing 0.5 mM EDTA, and were tested for endotoxin level using an endotoxin assay kit (Sigma). The degree of LDL oxidation was routinely determined by Paragon Lipo® agarose-gel electrophoresis and Oil Red O staining (Beckman).

Labelling of oxLDL with DiI

OxLDLs were labelled with the fluorescent lipophilic dye DiI as described in [19]. Briefly, 2 ml of human lipoprotein-deficient serum (20 mg/ml) was added to oxLDL (1 mg/ml). The solution was gently shaken, and 50 μl of DiI in DMSO (3 mg/ml) was added, followed by incubation for 8–16 h in the dark at 37 °C. The oxLDLs were re-isolated from the incubation mixture by ultracentrifugation (120000 ***g*** for 18 h at 4 °C) at a KBr density of 1.063 g/ml. After centrifugation, DiI-labelled oxLDL was dialysed extensively against PBS containing 0.5 mM EDTA at 4 °C.

Cell culture and stable transfection

The mouse macrophage cell line RAW264.7 (A.T.C.C. TIB-71) was cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C/5% CO_2. RAW264.7 cells were transfected

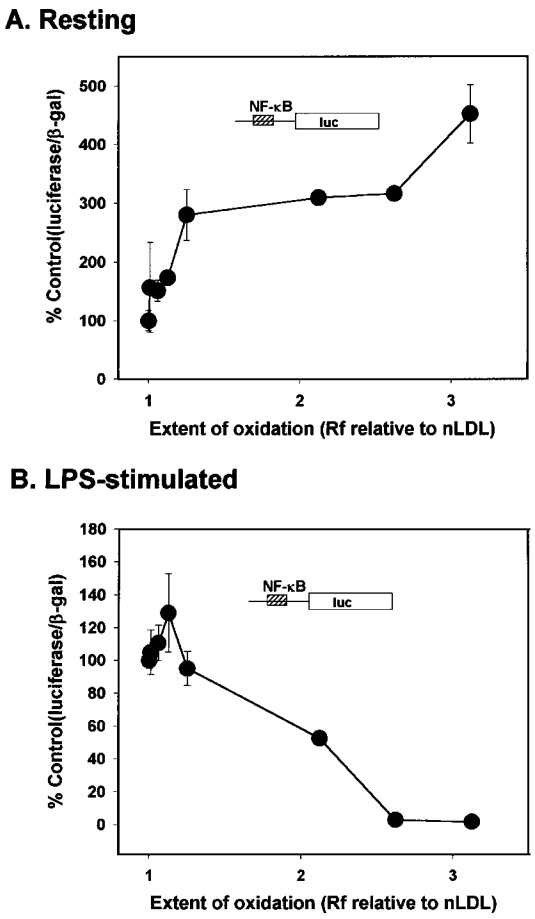

Figure 2 OxLDL activates NF-κB in resting macrophages in an oxidation-dependent manner

RAW264.7 cells were transfected with pNF-κB-luc and pHbNeo-β-gal, and the neomycin-resistant cells were selected using G418 (400 μg/ml) as described in the Materials and methods section. The pools of resistant cells were either (**A**) stimulated for 4 h with oxLDL (100 μg/ml) that had been oxidized for different time periods, or (**B**) co-treated for 4 h with LPS (100 ng/ml), interferon-γ (100 units/ml) and oxLDL (100 μg/ml). The cells were harvested and assayed for luciferase activity. The extent of oxidation is expressed as an R_F value relative to native LDL (nLDL), determined using Lipo® gels. Values are means ± S.D. of three independent experiments performed in triplicate.

with pNF-κB-luc, a luciferase reporter construct with an E-selectin promoter (−730 to +52) containing three upstream NF-κB binding elements [20], and pHbNeo-β-gal [21] by calcium phosphate co-precipitation. The neomycin-resistant cells were selected using G418 (400 μg/ml). The resistant cells were pooled and used in the assay of NF-κB-mediated transcriptional activation. For assay of PPRE (PPAR-responsive element)-mediated transactivation, RAW264.7 cells were transiently transfected with pPPRE-luc, a PPAR-responsive reporter containing three copies of the acyl-CoA oxidase PPRE linked to the thymidine kinase promoter, a human PPAR expression vector (generously provided by Dr B. H. Jhun, Pusan National University, Korea) and pcDNA3.1/lacZ (Invitrogen)

Luciferase assay

The transfected cells were stimulated with oxLDL or other reagent(s) in serum-free medium for 4 h and harvested. The cell extracts were assayed for luciferase activity using the luciferase assay kit (Promega) and a luminometer (Berthold, Badwildbad, Germany). The measured luciferase activity was normalized with regard to β-galactosidase activity [21] or protein concentration.

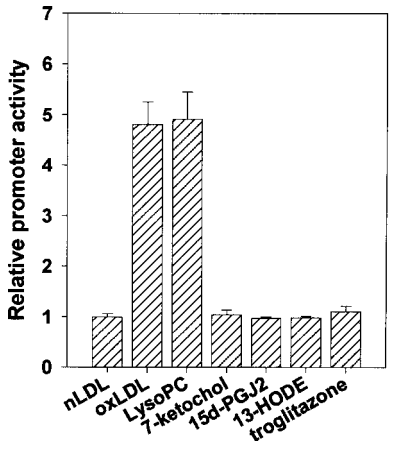

Figure 3 LysoPC simulates the effects of oxLDL in resting cells

pNF-κB-luc-transfected RAW264.7 cells were incubated for 4 h with native LDL (nLDL; 100 μg/ml), $oxLDL_{24h}$ (100 μg/ml), lysoPC (10 μM), 7-oxocholesterol (7-ketochol; 25 μM), 15d-PGJ_2 (1 μM), 13-HODE (20 μg/ml) or troglitazone (15 μM) in the resting state. Then cells were harvested and luciferase activity was measured. Relative promoter activity represents the luciferase activity relative to that in the nLDL-treated resting cells. Values are means ± S.D. of three independent experiments performed in triplicate.

Measurement of cytosolic free calcium concentration ($[Ca^{2+}]_i$)

The fluorescent calcium indicator fura 2 was used to monitor changes in $[Ca^{2+}]_i$ in RAW264.7 cells [9]. The cells were washed three times with Krebs/Ringer Hepes solution (128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM Na_2HPO_4, 10 mM glucose and 20 mM Hepes, pH 7.4), followed by exposure to 5 μM fura 2/AM in Krebs/Ringer Hepes solution for 20 min at 25 °C. After three washes with Krebs/Ringer Hepes solution, fluorescence signals in the cells were monitored at 37 °C at excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm using the MERLIN calcium-imaging system (EG&G Wallac, Cambridge, U.K.). Calibration of the fura 2 signal in terms of $[Ca^{2+}]_i$ was performed at the end of the experiments using 5 μM ionomycin.

In situ immunohistochemical staining

RAW264.7 cells grown on coverslips were incubated for 4 h with oxLDL (100 μg/ml) in the absence or presence of LPS (100 ng/ml) with interferon-γ (100 units/ml), fixed with 4% paraformaldehyde for 5 min, and permeabilized for 15 min with 0.4% Triton X-100. The specimens were blocked with 3% (w/v) BSA in PBS for 1 h and incubated with rabbit polyclonal antibody against the p65 subunit of NF-κB (Santa Cruz Biotechnology) in 1% BSA at 4 °C overnight. Then the specimens were washed with PBS and incubated with biotinylated goat anti-rabbit IgG (Jackson Immunoresearch Laboratory, West Grove, PA, U.S.A.) for 1 h. After washing twice with PBS, the specimens were incubated with FITC-conjugated streptavidin (Jackson Immunoresearch Laboratory) for 1 h and then washed twice with PBS. Coverslips with the stained cells were mounted in 80% glycerol in PBS and photographed using a fluorescent microscope (Carl Zeiss).

Localization of DiI-labelled oxLDL

Cells grown on coverslips were incubated with DiI-labelled oxLDL (100 μg/ml) for 4 h in the presence or the absence of 5 μM cytochalasin B. The nuclei were stained with 50 nM SYTOX Green nucleic acid stain in DMSO. To confirm the localization of DiI-labelled oxLDL on the cell surface, DiI-labelled oxLDL was stripped from the cell surface by incubating the cells for 1 h at 4 °C with proteinase K (500 μg/ml in PBS). The localization of oxLDL and nuclei were visualized by fluorescent microscopy, as described above.

Statistical analysis

Data are expressed as mean $\pm$ S.D., and statistical differences ($P < 0.05$) between mean values were determined by Student's t test. Experiments were performed in triplicate on three separate occasions.

RESULTS

Stimulation of NF-κB nuclear localization by oxLDL

When macrophages were treated with oxLDL for 4 h in the resting state, most NF-κB was found in the nuclei, as demonstrated by immunohistochemical staining, and the amount of NF-κB in the nuclei appeared to be proportional to the degree of LDL oxidation (Figure 1, upper panels). It should be noted that the shape of the macrophages changed from round to spindle-shaped on oxLDL treatment, implying that oxLDL activated them. Since oxLDL had been reported to suppress LPS-induced cytokine production and gene expression via NF-κB inactivation [9,11], oxLDL was also added to LPS-stimulated cells. The lower panels of Figure 1 show that oxLDL indeed inhibited LPS-induced NF-κB nuclear localization and macrophage activation in an oxidation-dependent manner, as had been reported previously by others.

OxLDL increases NF-κB-mediated transcriptional activity in resting macrophages

In order to quantify NF-κB-mediated transcriptional activity, RAW264.7 cells were stably transfected with pNF-κB-luc [20] and pools of G418-resistant cells were obtained. This reporter construct contains three NF-κB-binding sites, and oxLDL was able to induce this promoter-mediated transcriptional activity. The NF-κB activity increased proportionally as a function of the degree of LDL oxidation at a concentration of 100 μg/ml, while it did not change at 200 μg/ml (Figure 2A). High concentrations of oxLDL induced cell death of macrophages after prolonged incubation [22], and LPS-induced NF-κB transactivation was suppressed by oxLDL (100 μg/ml) in an oxidation-dependent manner (Figure 2B), as reported previously.

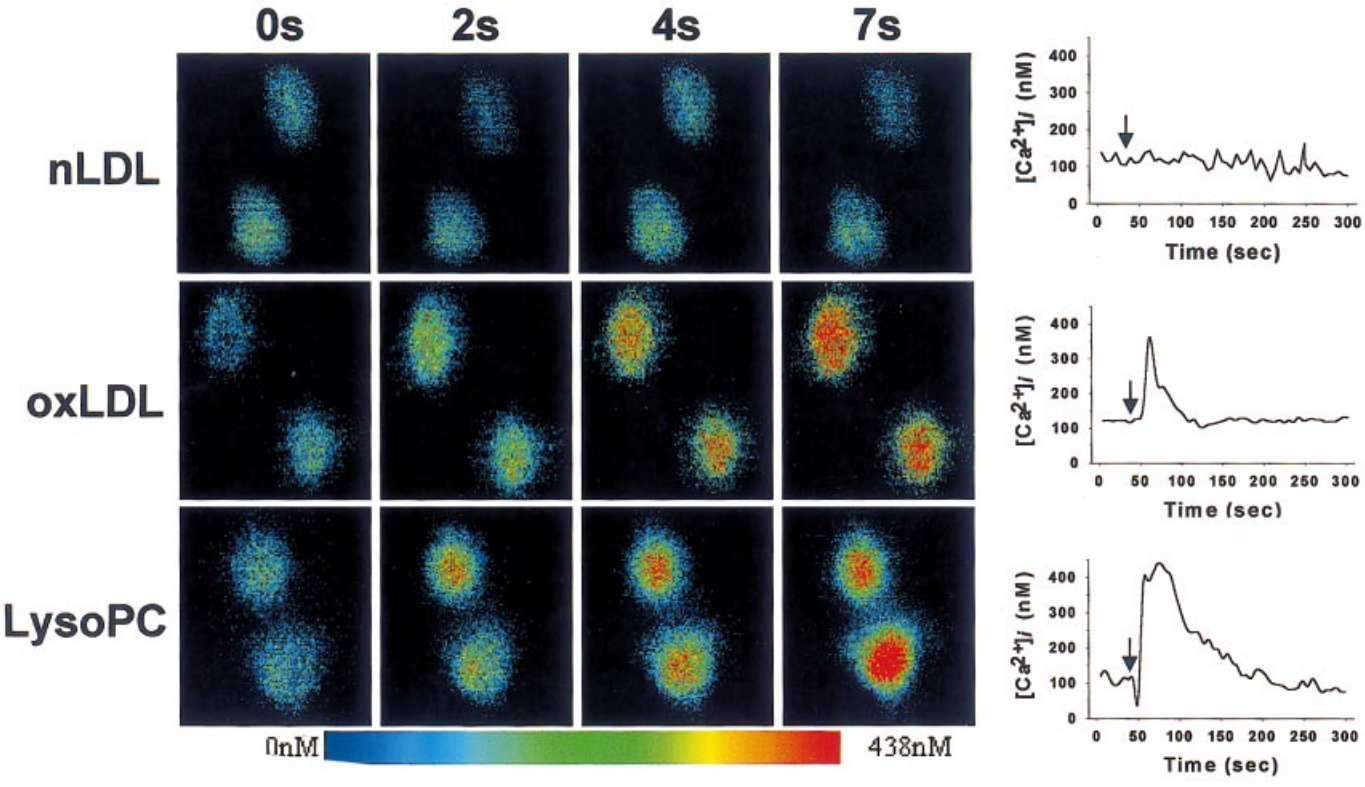

Figure 4 Rapid and transient increase in $[Ca^{2+}]_i$ induced by oxLDL

Fura 2/AM-loaded RAW264.7 cells were exposed to native LDL (nLDL; 100 μg/ml), $oxLDL_{24h}$ (100 μg/ml) or lysoPC (10 μM). Fluorescence images were obtained at alternating excitation wavelengths of 340 and 380 nm. The images with a ratio of 340/380 nm were recorded at intervals of 50 ms, and are shown at 0 s, 2 s, 4 s and 7 s after addition of the samples. The blue to red colour band shows the scale of calcium concentration from 0 to 438 nM. Calibrated calcium concentrations are plotted against time on the right side of the figure. Arrows indicate the time of oxLDL addition to the cells.

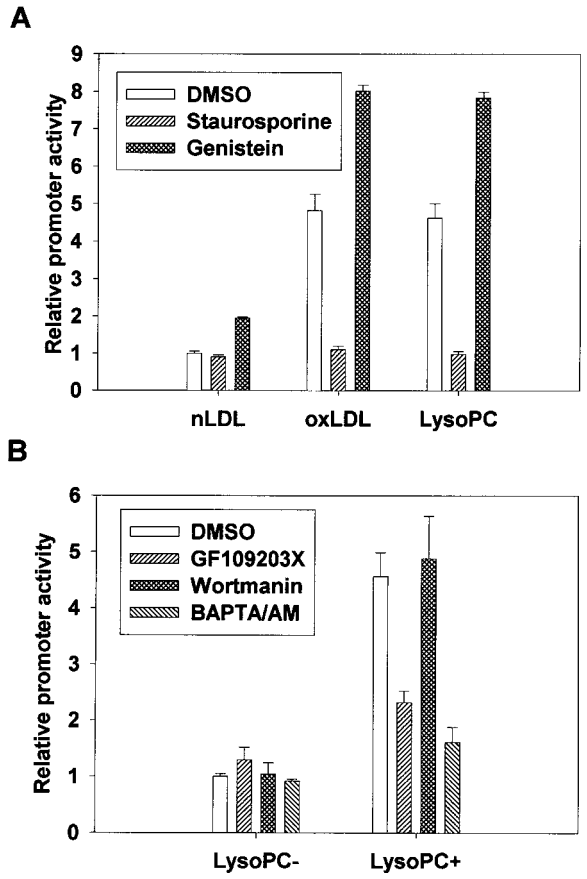

Figure 5 OxLDL activates NF-κB in resting cells through PKC- and calcium-mediated pathways

(**A**) pNF-κB-luc-transfected RAW264.7 cells were incubated for 4 h with native (nLDL; 100 μg/ml), $oxLDL_{24h}$ (100 μg/ml) or lysoPC (10 μM) in the absence or the presence of kinase inhibitors (100 nM staurosporine, a PKC inhibitor; 25 μM genistein, a tyrosine kinase inhibitor) in DMSO. (**B**) pNF-κB-luc-transfected RAW264.7 cells were co-incubated for 4 h with lysoPC and/or signalling inhibitors [GF109203X (5 μM), a PKC inhibitor; wortmannin (100 nM), a phosphatidylinositol 3-kinase inhibitor; BAPTA/AM (10 μM), a intracellular calcium chelator] in DMSO. The cells were harvested and the relative promoter activity was measured. Values are means ± S.D. of three independent experiments performed in triplicate.

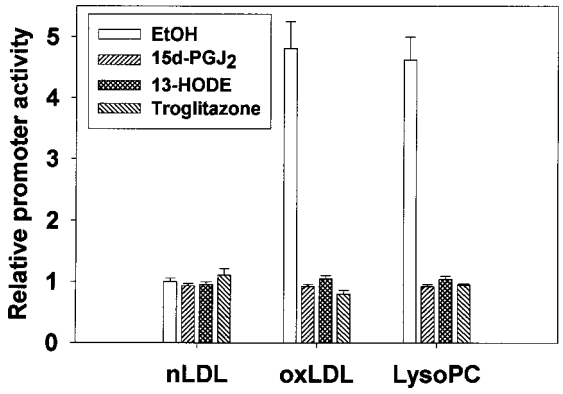

Figure 6 Suppression of oxLDL activation of NF-κB by 15d-PGJ_2

pNF-κB-luc-transfected RAW264.7 cells were incubated for 4 h with native LDL (nLDL; 100 μg/ml), $oxLDL_{24h}$ (100 μg/ml) or lysoPC (10 μM) in the absence or the presence of PPAR-γ ligands [13-HODE (20 μg/ml), 15d-PGJ_2 (1 μM) or troglitazone (15 μM)] in ethanol (EtOH). The cells were harvested and the relative promoter activity was measured. Values are means ± S.D. of three independent experiments performed in triplicate.

Free lysoPC mimics oxLDL-induced NF-κB activation

To identify which component of oxLDL is responsible for the activation of NF-κB, we tested lysoPC, 7-oxocholesterol and the PPAR-γ ligands 13-HODE, 15d-PGJ_2 and troglitazone. In resting macrophages, lysoPC (10 μM) activated NF-κB to a similar extent as oxLDL (100 μg/ml), whereas none of 7-oxocholesterol (25 μM), 13-HODE (20 μg/ml), 15d-PGJ_2 (1 μM) or troglitazone (15 μM) did so, suggesting that only lysoPC is responsible for the NF-κB activation induced by oxLDL (Figure 3).

Induction of a rapid and transient increase in $[Ca^{2+}]_i$ by oxLDL

To elucidate whether oxLDL-induced NF-κB activation is mediated by calcium, changes in $[Ca^{2+}]_i$ following oxLDL treatment were monitored by a calcium-imaging system connected to a digital fluorescent microscope. Addition of $oxLDL_{24h}$ (100 μg/ml) to cells resulted in a very rapid (in 7 s) increase (up to 400 nM) in $[Ca^{2+}]_i$, followed by a return to the baseline level with 5 min (Figure 4). LysoPC (10 μM) also initiated an instantaneous rise in $[Ca^{2+}]_i$, while native LDL had no effect.

OxLDL-induced NF-κB activation is PKC- and calcium-dependent

To activate NF-κB upon stimulation, inhibitor-κB (I-κB) protein needs to be phosphorylated and degraded [23]. Since many different kinases have been reported to be involved in I-κB phosphorylation and NF-κB activation [24], several kinase inhibitors were added with either oxLDL or lysoPC in order to identify which kinase plays a role in the oxLDL system. As shown in Figure 5, staurosporine and GF109203X, inhibitors of PKC, blocked both oxLDL- and lysoPC-induced NF-κB activation completely. BAPTA/AM, a specific intracellular calcium chelator, also abolished lysoPC-induced NF-κB activity (Figure 5B). These results suggest that activation of PKC and an increase in $[Ca^{2+}]_i$ should precede NF-κB activation by oxLDL. In contrast, tyrosine kinase and phosphatidylinositol 3-kinase do not appear to be involved in this process, since genistein and wortmanin did not inhibit lysoPC-induced or oxLDL-induced NF-κB transcriptional activity (Figure 5).

Activation of PPAR-γ antagonizes oxLDL-induced NF-κB activation

The oxidized linoleic acid compounds (9-HODE and 13-HODE) of oxLDL have been reported to activate PPAR-γ to increase macrophage gene transcription [7]. Separately, it was reported that 15d-PGJ_2, a PPAR-γ ligand, antagonized the transcriptional activity of STAT1 (signal transduction and activator of transcription 1), AP1 (activator protein 1) and NF-κB [25]. To study whether oxLDL-induced NF-κB activity is regulated by the PPAR-γ pathway, 13-HODE (20 μg/ml), troglitazone (15 μM) or 15d-PGJ_2 (1 μM) was co-added with oxLDL or lysoPC. Figure 6 shows that all three PPAR-γ ligands completely blocked the oxLDL- or lysoPC-mediated activation of NF-κB, indicating

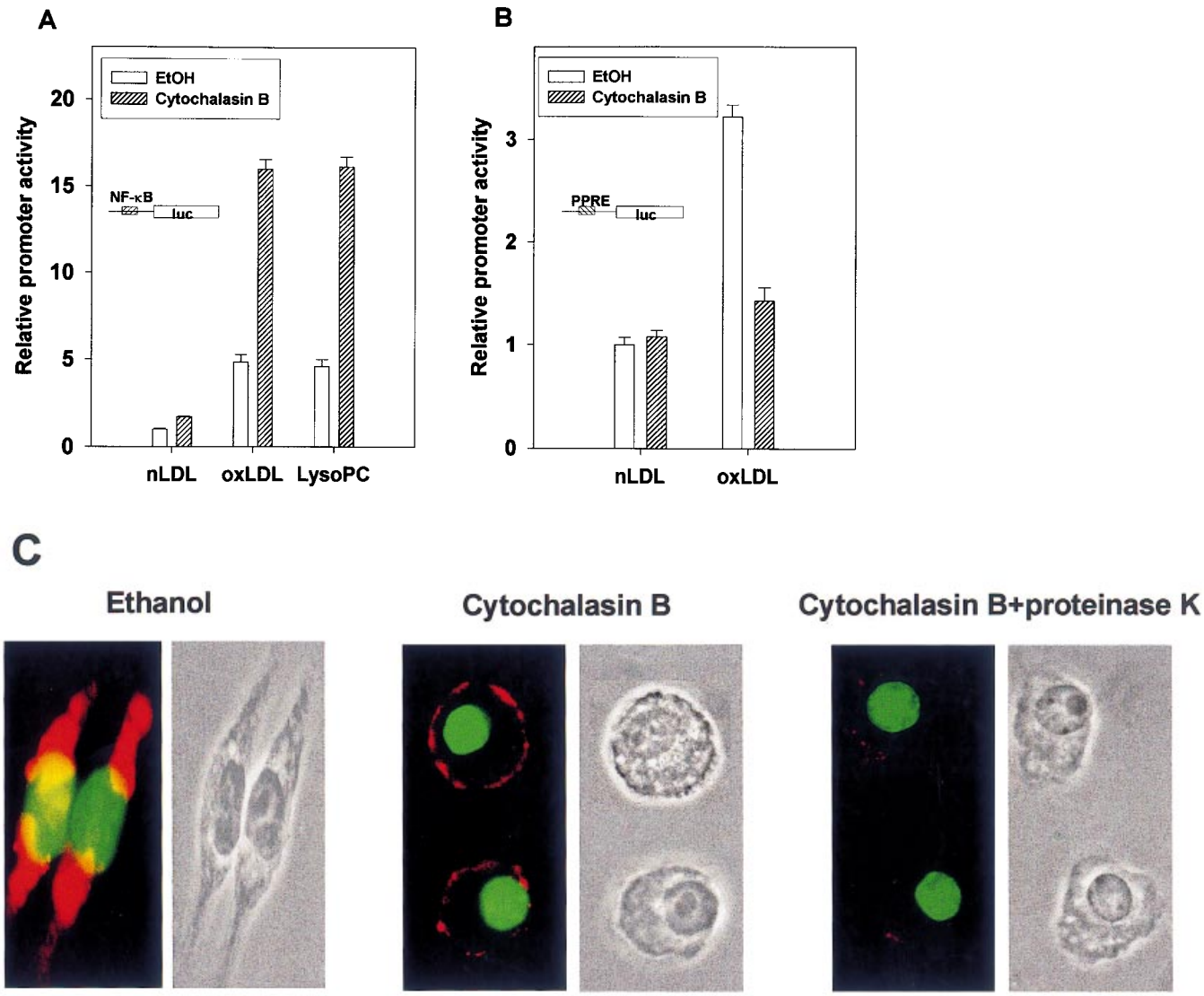

Figure 7 Endocytosis-independent activation of NF-κB by oxLDL

Resting pNF-κB-luc-transfected (**A**) or pPPRE-luc-transfected (**B**) RAW264.7 cells were incubated for 4 h and 8 h respectively with native LDL (nLDL; 100 μg/ml), $oxLDL_{24h}$ (100 μg/ml) or lysoPC (10 μM) in the absence or presence of cytochalasin B (5 μM) in ethanol (EtOH). The cells were harvested and the relative promoter activity was measured. Values are means ± S.D. of three independent experiments performed in triplicate. (**C**) To confirm that cytochalasin B blocked the uptake of oxLDL, cells were incubated with DiI-labelled oxLDL (100 μg/ml) for 4 h in the presence or the absence of cytochalasin B (5 μM). DiI-labelled oxLDL on the cell surface was stripped by proteinase K treatment. The localization of the DiI-labelled oxLDL was visualized by fluorescence microscopy (red), and nuclei stained with SYTOX (green) were overlaid. A phase micrograph of the same cells is shown with the fluorescent picture. Original magnification × 1000.

that the activation of PPAR-γ antagonizes NF-κB activation by oxLDL.

Endocytosis of oxLDL is not necessary for oxLDL-mediated NF-κB transactivation

The SR-mediated cellular endocytosis and lysosomal processing of oxLDL are required to activate PPAR-γ [7]. To test whether NF-κB activation by oxLDL in resting cells also requires SR-mediated endocytosis, 5 μM cytochalasin B, an endocytosis inhibitor [26], was incubated with cells for 1 h prior to oxLDL or lysoPC addition. Although cytochalasin B treatment resulted in morphological changes of the cells, oxLDL still activated NF-κB, regardless of the treatment (Figure 7A). Interestingly, the treatment synergistically increased the NF-κB-mediated transcriptional activity induced by oxLDL or lysoPC, implying that endocytosis might antagonize NF-κB transactivation. We checked the PPAR-γ ligand activity of our oxLDL preparation using pPPRE-luc reporter. The exposure of oxLDL for 8 h to cells transiently transfected with pPPRE-luc stimulated luciferase activity, which was blocked by cytochalasin B (Figure 7B). This result confirmed not only the presence of a PPAR-γ ligand in oxLDL, but also the blockage of endocytosis by cytochalasin B.

To rule out the possibility that oxLDL penetrated into the cells in the presence of cytochalasin B, oxLDL was labelled with DiI and visualized for the localization of oxLDL before and after treatment. As shown in Figure 7(C), DiI-labelled oxLDL was found on the surface of the cells in the presence of cytochalasin B, whereas it was observed in the cytosol in its absence. Treatment with proteinase K digested the DiI-labelled oxLDL on the cell

surface (Figure 7C). These results demonstrate that the signalling pathway for oxLDL-mediated NF-κB modulation does not require oxLDL uptake via endocytosis. The endocytosis-dependent signalling pathway may function as a route of inactivation of the oxLDL-induced NF-κB signal.

DISCUSSION

The transcription factor NF-κB, which forms a dimeric complex, has been reported to be implicated in atherogenesis, a chronic inflammatory process. NF-κB is located in the cytosol as an inactive complex bound to I-κB. Once I-κB is phosphorylated and dissociates from NF-κB upon the presence of the activation signal, NF-κB is translocated into the nucleus, where it binds to DNA to activate the expression of genes involved in inflammation and proliferation. OxLDL, one of the important risk factors for atherosclerosis, has been reported to modulate NF-κB activity in opposite directions, depending on whether or not the cells are activated. That is, oxLDL activated the DNA-binding activity of NF-κB in resting cells [10], while it suppressed NF-κB activity in LPS-stimulated cells [8,9,11]. Despite the fact that NF-κB is a common regulator in both situations, oxLDL seems to transduce the signals towards NF-κB through different pathways, depending on the state of the cells. The present study also demonstrated the opposite regulation of NF-κB by oxLDL (Figure 1), in good agreement with previous reports [10,12,13,27–29], although the exact pathways are not understood. We characterized the signal-transduction pathway involved in the activation of NF-κB by oxLDL in resting macrophages, and focused especially on the relationship with other signalling molecules.

The oxidation of LDL is a complex process, involving both the protein and the lipid moieties of the particle [2]. Each of the lipid components can undergo oxidation, including sterols, phospholipids, cholesterol esters and triacylglycerols. It is possible that one or more of the oxidized lipid components of oxLDL may activate NF-κB in resting macrophages. We categorized the lipid components of oxLDL into three groups: lysoPC, oxidized cholesterols and oxidized fatty acids. Representative components belonging to these groups, i.e. palmitoyl lysoPC [30,31], 7-oxocholesterol [32] and HODEs [7], were tested to determine the one responsible for the activation of NF-κB by oxLDL. Since HODE has been identified as an endogenous PPAR-γ ligand [7], the PPAR-γ ligands troglitazone and 15d-PGJ_2 were studied in addition to 13-HODE. Clearly, only lysoPC (10 μM) activated NF-κB to the same extent as 100 μg/ml $oxLDL_{24h}$ (Figure 3). This activation was dependent on the extent of oxidation of oxLDL (Figure 2) at 100 μg/ml, but oxLDL did not alter NF-κB activity at 200 μg/ml. This is in good agreement with the work of Sugiyama et al. [30] showing that a low concentration of lysoPC increased NF-κB activity, but that a high concentration did not. Although it is difficult to estimate how much lysoPC is present in oxLDL, the concentration of lysoPC may increase as LDL oxidation progresses. Therefore the activation of NF-κB by oxLDL may result from the lysoPC content in oxLDL.

LysoPC has been shown to induce the expression of cell-adhesion molecules and growth factors [33], and to be internalized through SRs to induce macrophage proliferation [34]. Because NF-κB is usually involved in proliferation, it was hypothesized that lysoPC may be internalized to activate NF-κB and consequently increase the number of macrophages. However, NF-κB activation by lysoPC or oxLDL in resting macrophages appears to be independent of endocytosis, since oxLDL still activated NF-κB-mediated transactivation in the presence of the endocytosis blocker cytochalasin B (Figure 7). Cytochalasin B treatment stimulated NF-κB transactivation synergistically with oxLDL or lysoPC co-treatment. This implies that the endocytic processing of oxLDL may function as an inactivation route for the oxLDL-induced NF-κB signal. It is noteworthy that PPAR-γ activation by oxLDL is endocytosis-dependent, and that the ligand-dependent activation of PPAR-γ antagonizes oxLDL-induced NF-κB activation. Therefore we suggest that SR ligation

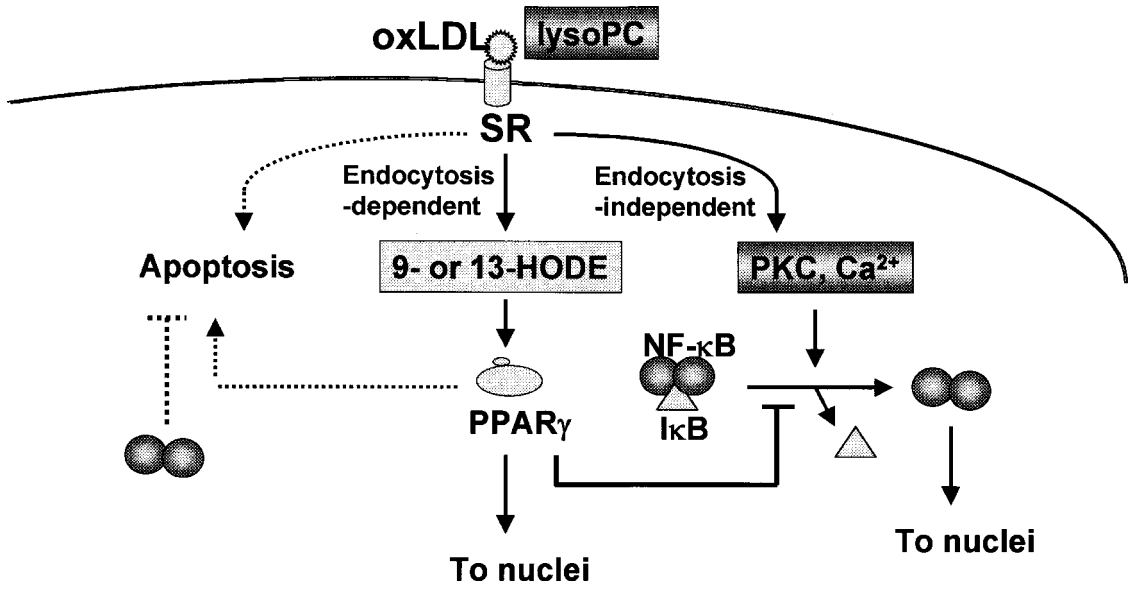

Figure 8 OxLDL-mediated signalling pathways in resting macrophages

Broken lines represent possible, but not proven, links between signalling molecules. Arrows and blunt lines represent the signals for activation and inhibition respectively.

of lysoPC activates NF-κB through surface-receptor-mediated signalling without endocytosis, while HODEs generated by endocytosis of oxLDL turn off the NF-κB signalling. Since lysoPC is a pure compound, this explanation is not applied to lysoPC. Therefore it is also possible that lysosomal processing degrades or modifies lysoPC so that it does not function inside cells. This will be addressed in future studies.

OxLDL has been reported to induce a rapid and transient increase in $[Ca^{2+}]_i$ and the hydrolysis of PtdIns(4,5)P_2, which were inhibited by pertussis toxin [9]. Our present study shows that NF-κB transactivation induced by oxLDL is calcium- and PKC-dependent. The question of which pathway is used by a variety of stimuli to activate NF-κB via the phosphorylation of I-κB has been the subject of much research [23]. With regard to oxLDL signalling, the most interesting pathways are PKC and calcium/calcineurin, since they stimulate NF-κB activation synergistically in T cells [23]. Thus I-κB kinase may be under the control of the PKC and calcium/calcineurin pathways. Further studies will be needed to examine whether both the PKC and calcium/calcineurin pathways are involved in the control of I-κB kinase in oxLDL-activated signalling.

OxLDL was reported to induce SR expression through CD36 [6], and tyrosine kinases were associated with CD36 activation in platelets [35]. In our study with macrophages, however, tyrosine kinases did not appear to be involved in NF-κB transactivation, suggesting that SR gene expression might not be mediated by NF-κB. For the NF-κB activity assay we utilized the luciferase reporter construct bearing the E-selectin promoter [20], which contains three upstream NF-κB binding sites; thus the expression of E-selectin should be induced by oxLDL or lysoPC through NF-κB activation. It should also be noted that the regulatory pathway of NF-κB activation observed in the present study might be limited to the case of E-selectin or to genes bearing multi-NF-κB binding sites in their promoter.

All currently known oxLDL-induced signalling pathways in resting macrophages are summarized in Figure 8. Studies of the oxLDL-mediated signalling pathway in resting macrophages may contribute to our understanding of atherogenesis and provide possible therapeutic interventions.

This study was supported by grants #HMP-97-M-2-0026 of Good Health 21 Program, Ministry of Health and Welfare, and #98-4-8 of National Institute of Health, Korea.

REFERENCES

1 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med. **320**, 915–924

2 Steinberg, D. (1997) Low density lipoprotein oxidation and its pathobiological significance. J. Biol. Chem. **272**, 20963–20966

3 Quinn, M. T., Parthasarathy, S. and Steinberg, D. (1985) Endothelial cell-derived chemotactic activity for mouse peritoneal macrophages and the effects of modified forms of low density lipoprotein. Proc. Natl. Acad. Sci. U.S.A. **82**, 5949–5953

4 Hamilton, T. A., Major, J. A. and Chisolm, G. M. (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

5 Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature (London) **362**, 801–809

6 Han, J., Hajjar, D. P., Febbraio, M. and Nicholson, A. C. (1997) Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. J. Biol. Chem. **272**, 21654–21659

7 Nagy, L., Tontonoz, P., Alvarez, J. G., Chen, H. and Evans, R. M. (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell **93**, 229–240

8 Brand, K., Page, S., Rogler, G., Bartsch, A., Brandl, R., Knuechel, R., Page, M., Kaltschmidt, C., Baeuerle, P. A. and Neumeier, D. (1996) Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion. J. Clin. Invest. **97**, 1715–1722

9 Schackelford, R. E., Misra, U. K., Florine-Casteel, K., Thai, S. F., Pizzo, S. V. and Adams, D. O. (1995) Oxidized low density lipoprotein suppresses activation of NF kappa B in macrophages via a pertussis toxin-sensitive signaling mechanism. J. Biol. Chem. **270**, 3475–3478

10 Draczynska-Lusiak, B., Chen, Y. M. and Sun, A. Y. (1998) Oxidized lipoproteins activate NF-kappaB binding activity and apoptosis in PC12 cells. Neuroreport **9**, 527–532

11 Ohlsson, B. G., Englund, M. C., Karlsson, A. L., Knutsen, E., Erixon, C., Skribeck, H., Liu, Y., Bondjers, G. and Wiklund, O. (1996) 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. J. Clin. Invest. **98**, 78–89

12 Fong, L. G., Fong, T. A. and Cooper, A. D. (1991) Inhibition of lipopolysaccharide-induced interleukin-1 beta mRNA expression in mouse macrophages by oxidized low density lipoprotein. J. Lipid Res. **32**, 1899–1910

13 Hamilton, T. A., Ma, G. P. and Chisolm, G. M. (1990) Oxidized low density lipoprotein suppresses the expression of tumor necrosis factor-alpha mRNA in stimulated murine peritoneal macrophages. J. Immunol. **144**, 2343–2350

14 Auge, N., Andrieu, N., Negre-Salvayre, A., Thiers, J. C., Levade, T. and Salvayre, R. (1996) The sphingomyelin-ceramide signaling pathway is involved in oxidized low density lipoprotein-induced cell proliferation. J. Biol. Chem. **271**, 19251–19255

15 Claus, R., Fyrnys, B., Deigner, H. P. and Wolf, G. (1996) Oxidized low-density lipoprotein stimulates protein kinase C (PKC) and induces expression of PKC-isotypes via prostaglandin-H-synthase in P388D1 macrophage-like cells. Biochemistry **35**, 4911–4922

16 Matsumura, T., Sakai, M., Kobori, S., Biwa, T., Takemura, T., Matsuda, H., Hakamata, H., Horiuchi, S. and Shichiri, M. (1997) Two intracellular signaling pathways for activation of protein kinase C are involved in oxidized low-density lipoprotein-induced macrophage growth. Arterioscler. Thromb. Vasc. Biol. **17**, 3013–3020

17 Fong, L. G., Albert, T. S. and Hom, S. E. (1994) Inhibition of the macrophage-induced oxidation of low density lipoprotein by interferon-gamma. J. Lipid Res. **35**, 893–904

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

19 Stephan, Z. F. and Yurachek, E. C. (1993) Rapid fluorometric assay of LDL receptor activity by Dil-labeled LDL. J. Lipid Res. **34**, 325–330

20 Pak, Y. K., Kanuck, M. P., Berrios, D., Briggs, M. R., Cooper, A. D. and Ellsworth, J. L. (1996) Activation of LDL receptor gene expression in HepG2 cells by hepatocyte growth factor. J. Lipid Res. **37**, 985–998

21 Schindler, U. and Baichwal, V. R. (1994) Three NF-kappa B binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. Mol. Cell. Biol. **14**, 5820–5831

22 Baeuerle, P. A. and Baltimore, D. (1996) NF-kappa B: ten years after. Cell **87**, 13–20

23 Thurberg, B. L. and Collins, T. (1998) The nuclear factor-kappa B/inhibitor of kappa B autoregulatory system and atherosclerosis. Curr. Opin. Lipidol. **9**, 387–396

24 Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J. and Glass, C. K. (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature (London) **391**, 79–82

25 Nishikawa, K., Arai, H. and Inoue, K. (1990) Scavenger receptor-mediated uptake and metabolism of lipid vesicles containing acidic phospholipids by mouse peritoneal macrophages. J. Biol. Chem. **265**, 5226–5231

26 Lipton, B. A., Parthasarathy, S., Ord, V. A., Clinton, S. K., Libby, P. and Rosenfeld, M. E. (1995) Components of the protein fraction of oxidized low density lipoprotein stimulate interleukin-1 alpha production by rabbit arterial macrophage-derived foam cells. J. Lipid Res. **36**, 2232–2242

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

28 Cominacini, L., Garbin, U., Pasini, A. F., Davoli, A., Campagnola, M., Pastorino, A. M., Gaviraghi, G. and Lo, C. V. (1998) Oxidized low-density lipoprotein increases the production of intracellular reactive oxygen species in endothelial cells: inhibitory effect of lacidipine. J. Hypertens. **16**, 1913–1919

29 Sugiyama, S., Kugiyama, K., Ogata, N., Doi, H., Ota, Y., Ohgushi, M., Matsumura, T., Oka, H. and Yasue, H. (1998) Biphasic regulation of transcription factor nuclear factor-kappaB activity in human endothelial cells by lysophosphatidylcholine through protein kinase C-mediated pathway. Arterioscler. Thromb. Vasc. Biol. **18**, 568–576

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

31 Lizard, G., Monier, S., Cordelet, C., Gesquiere, L., Deckert, V., Gueldry, S., Lagrost, L. and Gambert, P. (1999) Characterization and comparison of the mode of cell death, apoptosis versus necrosis, induced by 7beta-hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. Arterioscler. Thromb. Vasc. Biol. **19**, 1190–1200

32 Cominacini, L., Garbin, U., Fratta, P. A., Paulon, T., Davoli, A., Campagnola, M., Marchi, E., Pastorino, A. M., Gaviraghi, G. and Lo, C. V. (1997) Lacidipine inhibits the activation of the transcription factor NF-kappaB and the expression of adhesion molecules induced by pro-oxidant signals on endothelial cells. J. Hypertens. **15**, 1633–1640

33 Sakai, M., Matsumura, T., Biwa, T., Hakamata, H., Yi, D., Shichiri, M. and Horiuchi, S. (1997) Role of the macrophage scavenger receptor for internalization of lysophosphatidylcholine in oxidized low density lipoprotein-induced macrophage growth. Ann. N.Y. Acad. Sci. **811**, 378–384

34 Liu, Z. G., Hsu, H., Goeddel, D. V. and Karin, M. (1996) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell **87**, 565–576

35 Huang, M. M., Bolen, J. B., Barnwell, J. W., Shattil, S. J. and Brugge, J. S. (1991) Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn, and Yes protein-tyrosine kinases in human platelets. Proc. Natl. Acad. Sci. U.S.A. **88**, 7844–7848

Received 17 February 2000/7 June 2000; accepted 5 July 2000