

7 β -Hydroxycholesterol and 25-hydroxycholesterol-induced interleukin-8 secretion involves a calcium-dependent activation of c-fos via the ERK1/2 signaling pathway in THP-1 cells

Oxysterols-induced IL-8 secretion is calcium-dependent

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Abstract Oxysterols found in oxidized low-density lipoproteins are probably involved in the appearance of atheroma; some are cytotoxic and some able to induce cytokine secretion. An oxysterol-induced interleukin-8 (IL-8) secretion in human monocytes/

macrophages has been previously noticed, but the mechanisms remained unclear. In this paper, we investigated the signaling pathways leading to the induction of IL-8 secretion in monocytic THP-1 cells treated with 7 β -hydroxycholesterol, a cytotoxic oxysterol, or with 25-hydroxycholesterol, an oxysterol non-cytotoxic toward this cell line. The oxysterol-induced IL-8 secretion appears to be a calcium-dependent phenomenon as shown by the use of calcium channel blockers, which strongly decreased IL-8 secretion and IL-8 messenger RNA (mRNA) levels. Fluo-3 staining used in flow cytometry and video microscopy revealed an oxysterol-induced Ca²⁺ influx, varying according to the oxysterol studied, leading to the activation of the MEK/ERK1/2 pathway as demonstrated by Western blot analysis. ERK activation led to an increase of c-fos mRNA and/or an activation of c-fos. Luciferase reporter gene assay using constructs of the human IL-8 gene promoter and Transam assay revealed the involvement of the AP-1 transcription factor in oxysterol-dependent IL-8 secretion. These results demonstrate that oxysterol-induced IL-8 secretion is a calcium-dependent phenomenon involving the MEK/ERK1/2 pathway leading to the activation of IL-8 gene via AP-1 (c-fos).

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Abbreviations

AP-1	activator protein-1
ERK1/2	extracellular response kinase 1/2
IL-8	interleukin-8
JNK	c-jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MEK	mitogen extracellular kinase
NFκB	nuclear factor kappa B
PP2B	protein phosphatase 2B

Introduction

Oxidized low-density lipoproteins play an important role in the development of atherosclerosis. They contain various components as lipid hydroperoxides, aldehydes, and cholesterol oxidation products, oxysterols. Among these products, oxysterols are suspected to be involved in the physiopathology of atheroma, as well as in the initiation of the progression of lesions (Ross 1993). They are known to be cytotoxic against the different cell populations found in atherosclerotic plaques (Morel et al. 1983; Colles et al. 1996; Christ et al. 1993; Lizard et al. 1997). They also induce the secretion of interleukin-8 (IL-8), a cytokine belonging to the C–X–C chemokine family, produced by macrophages, isolated from human atherosclerotic plaques, and probably involved in the physiopathology of atherosclerosis (Liu et al. 1997). Indeed, IL-8 appears to be chemotactic and mitogenic for T lymphocytes and smooth-muscle cells (Larsen et al. 1989) and to induce endothelial cell proliferation (Koch et al. 1992) and angiogenesis (Simonini et al. 2000). Cytotoxicity and IL-8 secretion are different according to the cell type and the oxysterol species. In this way, 7β-hydroxycholesterol induces apoptosis in numerous cell types (Yin et al. 2000; Ares et al. 1997; Monier et al. 2003), whereas 7α-hydroxycholesterol appears devoid of cytotoxicity. The involvement of oxysterols in IL-8 synthesis by human monocytes/macrophages has been previously investigated (Liu et al. 1997; Rydberg et al. 2003; Lemaire–Ewing et al. 2005). Among oxysterols, 7β-hydroxycholesterol and 25-hydroxycholesterol appear to be the most potent inducers of IL-8 secretion, but some other oxysterols

are also able to trigger significant secretion of IL-8 (Liu et al. 1997).

However, little is known on the mechanisms underlying the oxysterol-induced secretion of IL-8. In this work, we demonstrate that IL-8 synthesis is related to a Ca²⁺ influx leading to activation of c-fos via the MEK/ERK1/2 pathway. Involvement of calcium as vector of the biological effects of oxysterol has been previously described. Recently, we demonstrated that 7-ketocholesterol-induced apoptosis was also a calcium-dependent phenomenon. Incorporation of 7-ketocholesterol into lipid raft domains of the plasma membrane induces translocation of Trpc1, a component of the store-operated Ca²⁺ entry channel from non-raft structures to lipid raft domain, leading to the opening of this channel. Consequent to the rise of cytoplasmic calcium, calcineurin (PP2B) activation induces the dephosphorylation of the proapoptotic protein BAD and consequently triggers apoptosis (Berthier et al. 2004). Thus, it appears that the main biological effects of oxysterols, although different and varying relative to the type of oxysterol and cell species, could be related to disorders in calcium homeostasis after oxysterol incorporation in plasma membrane. In this work, we investigated on IL-8 secretion induced by two oxysterols, 7β-hydroxycholesterol and 25-hydroxycholesterol. The possible relationships between oxysterol-induced cytotoxicity, apoptosis, and IL-8 secretion are discussed.

Materials and methods

Reagents and antibodies

The THP-1 cell line was purchased from the American Tissue and Culture Collection (Manassas, VA, USA). 25-Hydroxycholesterol, verapamil, nifedipine, fluo-3/AM, propidium iodide, pluronic F-127, probenecid, pepstatin A, aprotinin, trypsin inhibitor, leupeptin, phenylmethylsulfonylfluoride (PMSF), Ige-Pal, dimethylsulfoxide, NaF, β-glycerophosphate, ionomycin, and Triton X100 were purchased from Sigma (Sigma-Aldrich, L'Isles d'Abeau-Chesnes, France). 7β-Hydroxycholesterol was purchased from Steraloids Inc. (Newport, RI, USA). The anti-ERK1/2, anti-ERK1/2 phospho thr202/tyr204, and U0126 were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

THP-1 cells were grown in suspension in RPMI 1640 with glutamax-I and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin; Gibco, Eragny, France), supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco). The THP-1 cells were incubated at 37°C under a 5% CO₂/95% air atmosphere.

Cell treatment

For all experiments, 7β-hydroxycholesterol, and 25-hydroxycholesterol stock solutions were prepared at a concentration of 800 µg/ml as previously described (Lemaire-Ewing et al. 2005). Sterols were added to the culture medium for a final concentration of 20 µg/ml (50 µM). This oxysterol concentration was in the range of levels measured in human plasma after a meal rich in fat (Emanuel et al. 1991). Controls were performed with the same content of solvent (ethanol) as in the oxysterol-containing experiment. Nifedipine and verapamil, two L-type calcium channel inhibitors were added to the medium at a final concentration of 50 and 100 µmol/l, respectively. The MEK inhibitor U0126 was used at a final concentration of 10 µmol/l. In all experiments, verapamil, nifedipine and U0126 were introduced in the culture 1 h before oxysterols. Calcium-free experiments were performed with a calcium-free medium and with fetal calf serum that has been dialyzed against an iso-osmotic phosphate buffer salt.

Flow cytometric measurement of cytosolic calcium with the Fluo-3 dye

THP-1 cells were washed with PBS (pH=7.4) and then incubated with Fluo-3/AM (6 µmol/l; λEx_{max}=506 nm; λEm_{max}=526 nm) for 30 min at 37°C in Hank's balanced salt buffer (pH=7.2) with pluronic F-127. After loading, cells were suspended in 4-2-hydroxyethyl-1-piperazineethanesulfonic acid buffer (pH=7.4) supplemented with probenecid (5 mmol/l) to prevent leakage of the dye (Merritt et al. 1990). Fluorescence was measured by flow cytometry with a Galaxy flow cytometer (Partec) using a 524/44 nm band pass filter. For each sample, events were acquired for 60 s, and the data were analyzed with Flomax software (Partec).

Detection of calcium oscillations with the Fluo-3 dye

Fluo-3 fluorescence was visualized using the Cell Observer Station (Zeiss). Briefly, this station consists of an Axiovert 200M inverted fluorescence microscope equipped with an incubator where temperature and CO₂ were controlled and a cooled charged coupled device camera controlled with the AxioVision software (Zeiss). Labeled THP-1 cells were put in the incubator maintained at 37°C with 5% CO₂. 25-Hydroxycholesterol was added, and images acquisition was started. The resulting video is composed of one image every 500 ms over 30 min. The graph of fluo-3 intensity versus time was determined using Axio Vision Software by measuring the gray level of some cells for each image of the video and reported in Excel. Visilog 6.3 software was used to construct a video file showing the calcium waves.

IL-8 assay

To determine the secretion level of IL-8, THP-1 cells were treated for 24 h with oxysterols at the concentration of 20 µg/ml (50 µM). In some experiment, cells were pretreated for 1 h with 100 µM of verapamil, 50 µM of nifedipine, or 10 µM of U0126 before the addition of oxysterols. After incubation, the samples were centrifuged at 2,000 rpm for 5 min, and the supernatants were collected. All samples were assayed for levels of IL-8 by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's procedure (working protocol for IL-8 module set, Bender Medsystems™, Vienna, Austria).

Determination of cell permeability with propidium iodide and characterization of nuclear morphology by staining with Hoechst 33342

Cell permeability after staining with the phenanthrene dye propidium iodide, which only enters into dead cells, and the nuclear morphology of cells after staining with Hoechst 33342 (cells with condensed and/or fragmented nuclei are considered as apoptotic cells) were determined as previously described (Monier et al. 2003).

RNA extraction, and semi-quantitative reverse transcriptase and real-time polymerase chain reaction

THP-1 cells were incubated with oxysterols at 20 µg/ml (50 µM) for 1, 3, or 6 h. In some experiments, cells were

pretreated for 1 h with 100 μ M of verapamil, 50 μ M of nifedipine, or 10 μ M of U0126 before the addition of oxysterols. Total RNA was isolated using Qiagen RNeasy mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations.

Briefly, 2 μ g of denatured total RNA were reverse-transcribed using the Omniscript reverse transcriptase kit (Qiagen). One microliter of complementary DNA (cDNA) was amplified by polymerase chain reaction (PCR) using Hotstar Taq polymerase (Qiagen) using the following primers: for IL-8, 5'-ACAGCAGAGCACACAAGCTT-3' (forward) and 5'-CTGGCAACCCTACAACAGAC-3' (reverse), and for c-fos, 5'-GGATAGCCTCTCTTACTACCAC-3' (forward) and 5'-TCCTGTCATGGTCTTCACAACG-3' (reverse). β -actin gene amplification was used as an internal standard with the following primers: 5'-TGCTATCCAGGCTGTGCTAT-3' (forward) and 5'-GATGGAGTTGAAGGTAGTTT-3' (reverse). The amplified DNA products were loaded onto a 0.8% of agarose gel in 1 \times of Tris-acetate-ethylenediaminetetraacetic acid buffer for electrophoresis. Gels were stained with ethidium bromide and digitalized with a Biocom analyzing system.

Real-time PCR total RNA (100 to 300 ng) was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase, random primers and RNaseOUT inhibitor (Fischer Bioblock) in a final reaction volume of 20 μ l according to the manufacturer's instructions. SYBR[®] Green real-time PCR using the LightCycler 2.0 detection system (Roche Diagnostics, Meylan, France) was used to quantify messenger RNA (mRNA) expression levels. The various reverse primers (Fischer Bioblock) were selected according to intron spanning to prevent amplification of genomic DNA. Each reaction was run in triplicates and contained 1.5 μ l of cDNA template along with 50 ng/ μ l bovine serum albumin and 100 nM primers in a final reaction volume of 15 μ l. Cycling parameters were 50°C for 2 min, 95°C for 2 min to activate DNA polymerase, then 40 cycles of 94°C for 5 s, 55°C for 10 s, and 72°C for 10 s. Melting curve analysis was performed immediately after amplification at a linear temperature transition rate of 0.1°C/s from 55°C to 95°C with continuous fluorescence acquisition. Relative mRNA levels were calculated using the $\Delta\Delta$ Ct method. Values were normalized to cyclophilin A levels. The primers for cyclophilin A are 5'-GCAAGTCCATCTATGGGGA

GA-3' (forward) and 5'-TTGTCCACAGTCAGCAATGGT-3' (reverse).

Luciferase reporter gene assay

IL-8 promoter fragments of different sizes were amplified from human genomic DNA with appropriate sets of primers and cloned into XhoI/SacI sites of the promoterless luciferase reporter vector pGL3-Basic (Promega, San Luis Obispo, CA, USA). All constructs were verified by sequencing. For reporter assay, THP-1 cells (10⁶ cells per well in six-well plates) were transiently transfected using the Amaxa nucleofection technology (Amaxa, Koeln, Germany) with 0.5 μ g of the different pIL-8-LUC constructs. The transfection efficiency was normalized by assaying for β -galactosidase activity from a constitutively expressed LacZ plasmid that was co-transfected with the reporter construct. Twenty-four hours after transfections, cells were treated or not with 20 μ g/ml of 7 β -hydroxycholesterol or 25-hydroxycholesterol for a period of 12 h. Then, the cells were harvested, and the luciferase and β -galactosidase activities were measured. The luciferase reporter assay system and the β -galactosidase enzyme assay from Promega were used following the users manual. Luciferase activity was measured with a luminometer lumat LB9507 (EG&G Berthold).

AP-1 activation analysis

To detect and quantify AP-1 activation in THP-1 cells, we used ELISA-based Trans-AM transcription factor kits (Active Motif, Carlsbad, USA). The active form of c-fos in whole-cell extracts can be detected using an antibody specific for epitope that is accessible only when the nuclear factor is activated and bound to its target DNA. Preparation of cell extract was done according to the manufacturer's instructions.

Western blotting

Cells were resuspended in radio-immuno precipitation assay lysis buffer [0.1% sodium dodecyl sulfate (SDS), 1% IgePal, 0.5% Na-desoxycholate, 50 mmol/l Tris-HCl at pH=8.0, 150 mmol/l NaCl] containing a mixture of protease and phosphatase inhibitors (0.1 mmol/l PMSF, 2.5 μ g/l aprotinin,

10 µg/l pepstatin A, 2.5 µg/l trypsin inhibitor, 2.5 µg/l leupeptin, 0.1 mmol/l Na-orthovanadate, 40 mmol/l β-glycerophosphate, 100 mmol/l NaF). After 30 min of incubation at 4°C in the lysis buffer, the cell debris were eliminated by centrifugation for 20 min at 10,000×g, and the supernatant was collected. The protein concentrations were measured by using bicinchoninic acid reagent (Pierce, Rockford, IL, USA) according to the method of Smith (Smith et al. 1985). Seventy micrograms of protein was incubated in loading buffer (125 mmol/l Tris-HCl, pH=6.8, 10% β-mercaptoethanol, 4.6% SDS, 20% glycerol, and 0.003% bromophenol blue), boiled for 3 min, separated by SDS-polyacrylamide gel electrophoresis, and electroblotted onto a polyvinylidene difluoride membrane (BioRad, Ivry sur Seine, France). After blocking nonspecific binding sites for 2 h at room temperature in Triton X-100 in phosphate-buffered saline (TPBS; PBS, 0.1% Tween-20), the membranes were incubated overnight at 4°C with the primary antibody diluted in TPBS. After three 10-min washes with TPBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:2,500 for 1 h at room temperature and washed three times in TPBS for 10 min. Autoradiography of the immunoblots was performed using an enhanced chemiluminescence detection kit (Amersham, Les Ulis, France). Each experiment was repeated three times.

Statistical analyses

Statistical analyses were performed with Statview software (Cary, NC, USA) using a one-way analysis of variance (ANOVA) followed by a Dunnett *t* test.

Results

Oxysterol-induced IL-8 secretion is a calcium-dependent phenomenon

Previous work (Liu et al. 1997) demonstrated an oxysterol-induced IL-8 secretion in human monocytes and THP-1 cells. In THP-1 cells, this secretion varies according to the oxysterol used, and 7β-hydroxycholesterol appears to be the most potent inducer of IL-8 secretion with 1,038 pg/ml±282 pg/ml versus 311 pg/ml±112 pg/ml for 25-hydroxycholesterol,

whereas cholesterol appears unable to induce such a secretion (Fig. 1a). As we previously described that treatment of THP-1 cells with another oxysterol, 7-ketocholesterol, induces increase of cytoplasmic calcium concentration by opening of calcium channels (Berthier et al. 2004), we then investigated the possible involvement of calcium in oxysterol-induced IL-8 secretion. The use of Ca²⁺ channel blockers, nifedipine and verapamil, induced a dramatic decrease of oxysterol-induced IL-8 secretion (Fig. 1a); nifedipine decreasing by 90% the secretion for the two oxysterols used (7β-hydroxycholesterol and 25-hydroxycholesterol), whereas verapamil induces a decrease of 66% of the secretion in 25-hydroxycholesterol-treated cells and 77% in 7β-hydroxycholesterol-treated cells. Finally, treatment of THP-1 cells by several concentrations of ionomycin (which induces an intracellular calcium elevation) clearly shows a dose dependence of IL-8 secretion, confirming the calcium dependence of the cytokine secretion (Fig. 1b). Moreover experiments performed with calcium-free medium are also in agreement of the calcium dependence of the phenomenon; the persistence of an IL-8 secretion in 25-hydroxycholesterol-treated cells, where slight calcium oscillations are sufficient to induce IL-8 secretion (see below), could be explained by the impossibility to eliminate completely calcium of fetal calf serum used in culture cell medium. Thus, oxysterol-induced IL-8 secretion appears mainly a calcium-dependent phenomenon. These results are corroborated by reverse transcriptase PCR (RT-PCR) studies showing increased amount of IL-8 mRNA after oxysterol treatments (Fig. 1c).

Analysis of intracellular contents of Ca²⁺ using the dye fluo-3 only demonstrated a significant increase of intracellular cytosolic-free calcium in 7β-hydroxycholesterol-treated cells, whereas 25-hydroxycholesterol exhibited non-significant variations (Fig. 2a). However, at the cellular level, oscillations of the calcium were detected using video microscopy. Figure 2b shows intracellular oscillations of calcium content induced by 25-hydroxycholesterol in THP-1 cells in agreement with the calcium dependence of the 25-hydroxycholesterol-induced IL-8 secretion demonstrated above. These oscillations are only detectable during the first 90 s; they are not detected later and do not exist in 7β-hydroxycholesterol-treated cells. In experiments performed with nifedipine and verapamil or without extracellular calcium, neither

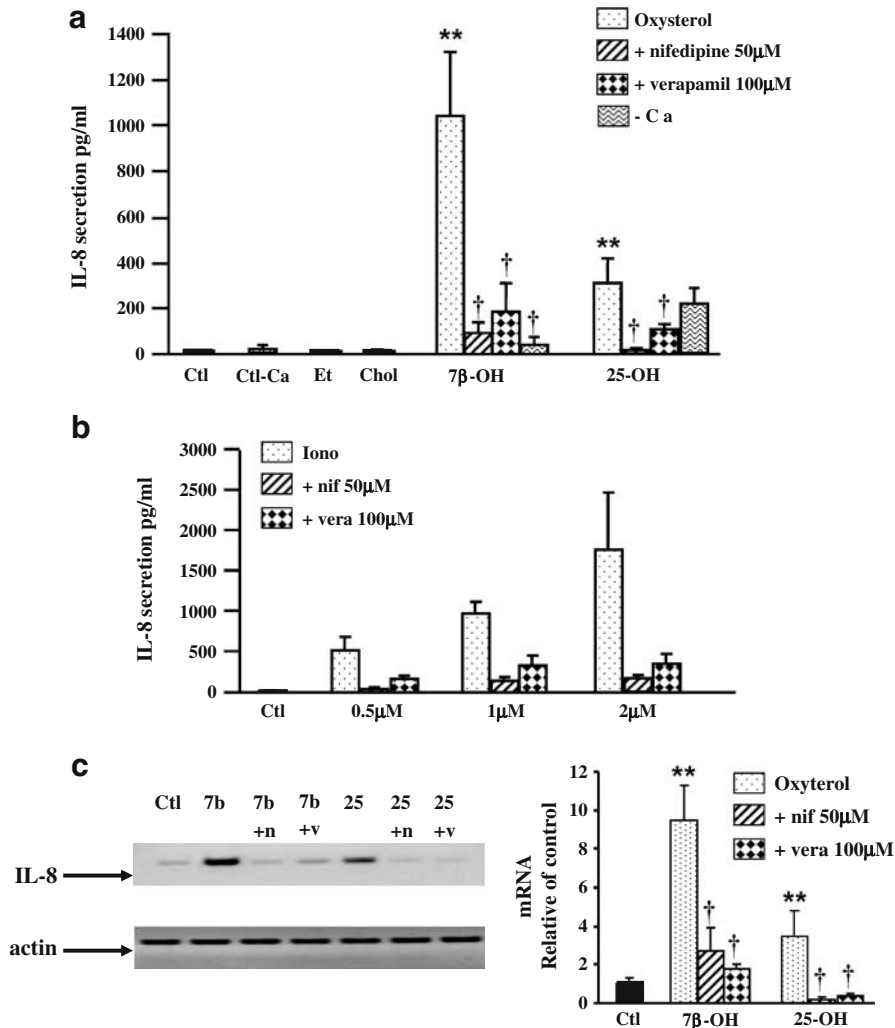


Fig. 1 a 7 β -Hydroxycholesterol and 25-hydroxycholesterol induce an increase of IL-8 secretion and mRNA level inhibited by L-type calcium channel blockers. THP-1 cells were incubated in the presence of 7 β -hydroxycholesterol (20 μ g/l, 7 β -OH) or 25-hydroxycholesterol (20 μ g/ml, 25-OH), with or without nifedipine (50 μ mol/l) or verapamil (100 μ mol/l), or in the absence of oxysterol (Ctl), with ethanol (Et) or with cholesterol (20 μ g/l, Chol), **b** with ionomycin (0.5, 1, and 2 μ mol/l, Iono) in the presence or not of nifedipine and verapamil. Cells were cultivated for 24 h in RPMI or in a calcium-free medium (-Ca), and aliquots of the culture medium

were assayed for IL-8 by ELISA. Data are the mean values \pm SD ($n=5$). **c** Expression of IL-8 and actin mRNA was determined by RT-PCR after 6 h of treatment. Data are representative of three independent experiments. Graph on **c** represents the results of real-time PCR. Real-time PCR was performed as control of semi-quantitative RT-PCR, and IL-8 mRNA expression was normalized to cyclophilin mRNA expression in the same sample. Relative stimulation represents comparison to untreated control. † $p<0.001$ vs. the oxysterol-treated group, ** $p<0.001$ vs. the control group

oscillations nor increase of cytosolic-free calcium were detected (Fig. 2c).

Oxysterol-induced IL-8 secretion needs calcium activation of the MEK ERK1/2 pathway

As calcium signals could be involved in MAPK activation and because we have previously described

a calcium activation of ERK1/2 (Berthier et al. 2005), the phosphorylation of ERK1/2 and its relationship with IL-8 secretion were investigated. Figure 3a shows that oxysterol treatments induced phosphorylation of ERK1/2 and that the kinetics of ERK1/2 activation varied according to oxysterol tested. ERK1/2 activation of 7 β -hydroxycholesterol-treated cells peaked at 6 h and rapidly decreased, whereas 25-

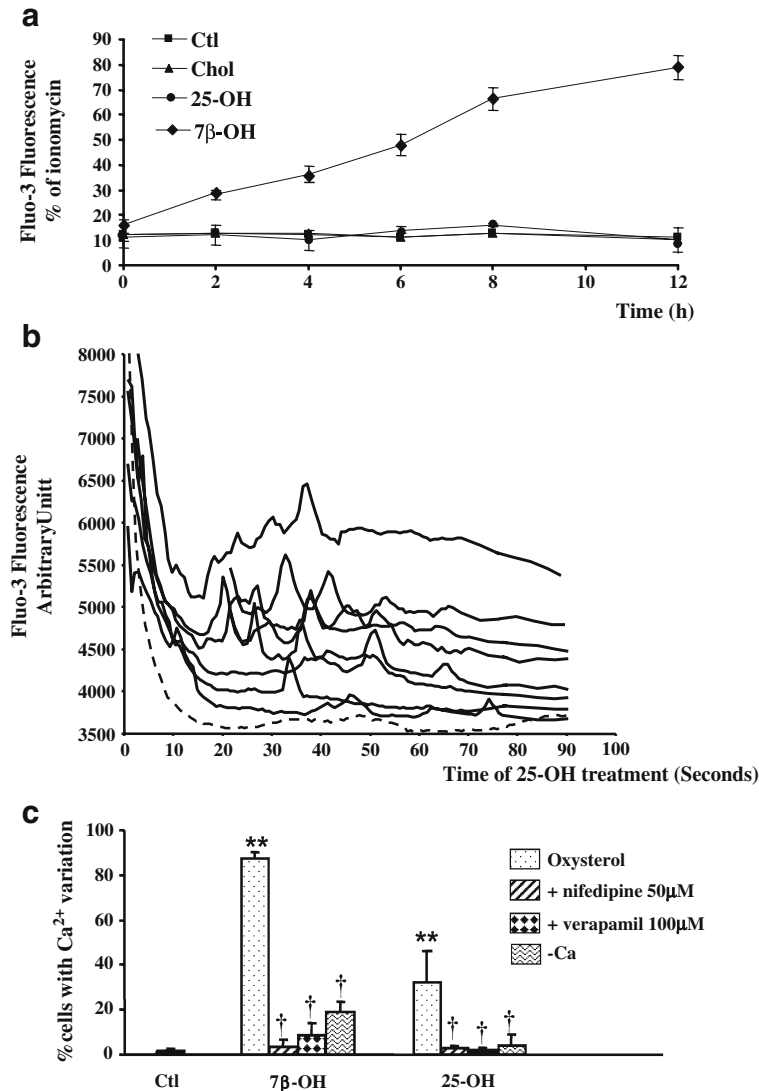


Fig. 2 a 7β-Hydroxycholesterol induces an increase of intracellular Ca²⁺. THP-1 cells were incubated in the presence of 7β-hydroxycholesterol (20 μg/ml, 7β-OH), 25-hydroxycholesterol (20 μg/ml, 25-OH), and cholesterol (20 μg/ml, Chol) or in the absence of oxysterol (Ctl). After different incubation times, cells were loaded with fluo-3/AM, and the dye fluorescence was measured by flow cytometry. Each fluorescent data point is normalized to the maximal fluo-3 fluorescence induced by cell treated with ionomycin (2 μmol/l). Data are the mean values ± SD (n=5). **b** 25-Hydroxycholesterol induces Ca²⁺ oscillations. THP-1 cells were loaded with fluo-3/AM, and 25-hydroxycholesterol (20 μg/ml, 25-OH) was added, and the fluo-3 fluorescence was visualized using the Cell Observer Station. The resulting video is composed of one image every 500 ms over 30 min. To realize the

graph of fluo-3 intensity versus time, for each image of the video, gray level of some cells was determined and reported (each line represents one cell oscillations); the dotted line represents a cell treated with the vehicle only. **c** 25-Hydroxycholesterol-induced Ca²⁺ oscillations and 7β-hydroxycholesterol-induced calcium increase are inhibited by nifedipine and verapamil. THP-1 cells were treated or not with nifedipine (50 μM) or verapamil (100 μM) for 30 min and were loaded with fluo-3/AM. 25-Hydroxycholesterol (20 μg/ml, 25-OH) or 7β-hydroxycholesterol (20 μg/ml, 7β-OH) were added, and the fluo-3 fluorescence was visualized using the Cell Observer Station. The resulting graph shows the percentage of cells presenting Ca²⁺ variation. Medium without calcium (-Ca) was used as control. †p<0.001 vs. oxysterol-treated group, **p<0.001 vs. the control group

hydroxycholesterol-treated cells exhibited a more sustained ERK1/2 activation. Interestingly, calcium is also involved in the 7 β -hydroxycholesterol-induced cytotoxicity. Indeed treatment of THP-1 cells with 7 β -hydroxycholesterol induced cell death as assayed

by PI staining viability test, and this death was mainly apoptotic as assessed by measurement of Hoechst 33342 staining; this cytotoxicity becomes detectable after 18 h of treatment (Fig. 3b). This cytotoxicity appeared clearly calcium-dependent be-

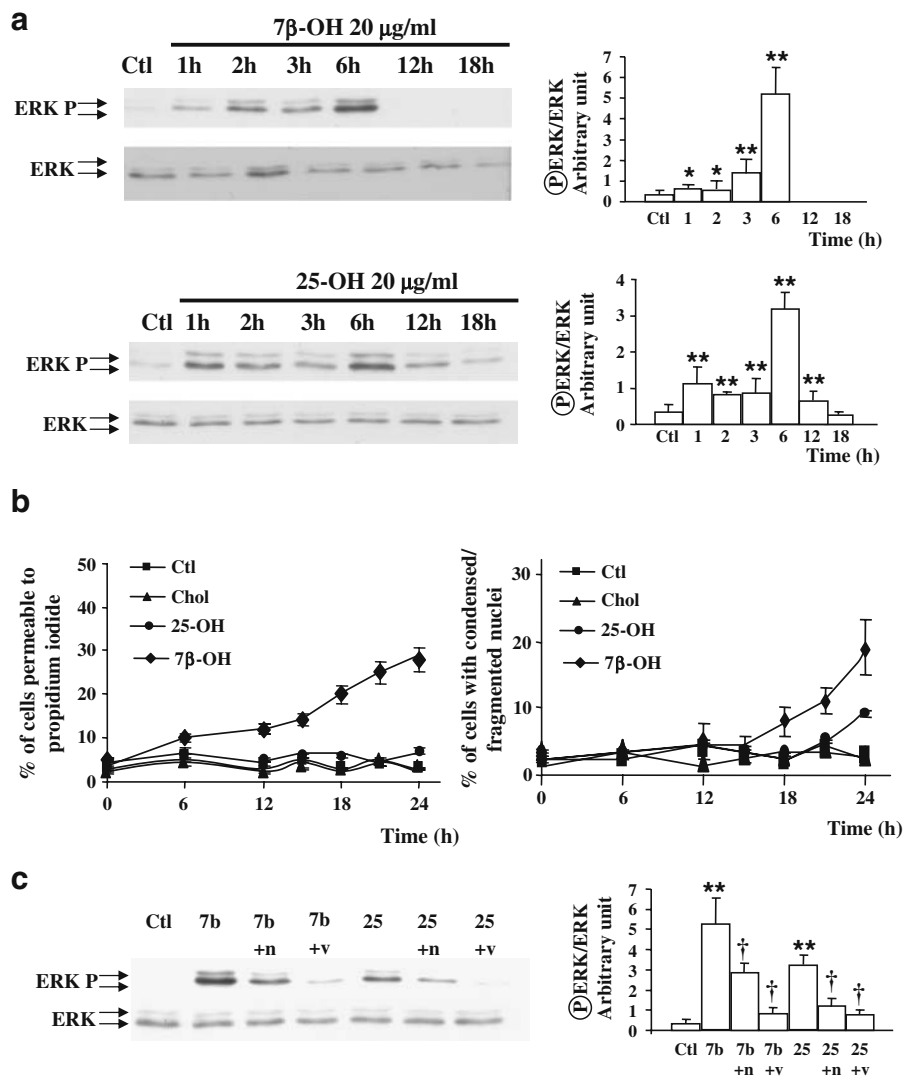


Fig. 3 **a** 7 β -Hydroxycholesterol and 25-hydroxycholesterol induce ERK activation. THP-1 cells were either untreated (*Ctl*) or treated with 7 β -hydroxycholesterol (20 μ g/ml, 7 β -OH) and 25-hydroxycholesterol (20 μ g/ml, 25-OH) for various incubation times. Cell extracts were collected, subjected to SDS/polyacrylamide gel electrophoresis (PAGE) and immunoblotted with ERK1/2 and phospho-ERK1/2 antibodies. Data are representative of three independent experiments. **b** Effect of 7 β -hydroxycholesterol and 25-hydroxycholesterol on cellular permeability to propidium iodide and morphological nuclear changes in THP-1 cells. THP-1 cells were cultured at different time points in the absence (*Ctl*) or presence of 7 β -hydroxycholesterol (7 β -OH), 25-hydroxycholesterol (25-OH), or cholesterol (*Chol*) at 20 μ g/ml.

Cellular permeability was evaluated by the percentage of propidium iodide-positive cells, and morphological nuclear changes were defined by the percentage of the cells with condensed and/or fragmented nuclei (apoptotic cells). **c** Oxysterol-induced ERK activation is inhibited by L-type calcium channel blockers. THP-1 cells were either untreated (*Ctl*) or incubated with nifedipine ($n=50$ μ mol/l) or verapamil ($v=100$ μ mol/l) for an hour before the addition of oxysterols for an additional 6 h. Cell extracts were collected, subjected to SDS/PAGE, and immunoblotted with ERK1/2 and phospho-ERK1/2. Data are representative of three independent experiments. * $p < 0.05$ and ** $p < 0.001$ vs. the control group, † $p < 0.001$ vs. the oxysterol-treated group

cause treatment with nifedipine or verapamil induced significant decrease of the 7β -hydroxycholesterol-induced cytotoxicity (data not shown). ERK1/2 phosphorylation is inhibited by co-treatment with nifedipine or verapamil and then appeared to be calcium-dependent (Fig. 3c).

Moreover, inhibition with U0126 of MEK, the kinase that phosphorylates ERK1/2, induced an inhibition of IL-8 secretion (from $1,038 \pm 282$ pg/ml to 330 ± 139 pg/ml for 7β -hydroxycholesterol and from 311 ± 112 to 37 ± 22 pg/ml for 25-hydroxycholesterol) and mRNA (Fig. 4a,b), suggesting the involvement of a

MEK-dependent pathway, in oxysterol-induced IL-8 secretion. A control blot (Fig. 4c) confirmed the inhibition of ERK phosphorylation by U0126.

ERK1/2 activation leads to increase of c-fos mRNA

It has been previously described that the AP-1 transcription factor, composed either as a homodimer of c-jun or as heterodimer of c-jun and c-fos, is implicated in IL-8 gene transcription (Angel and Karin 1991). Several members of the family of MAPK are implicated in the regulation of AP-1. C-

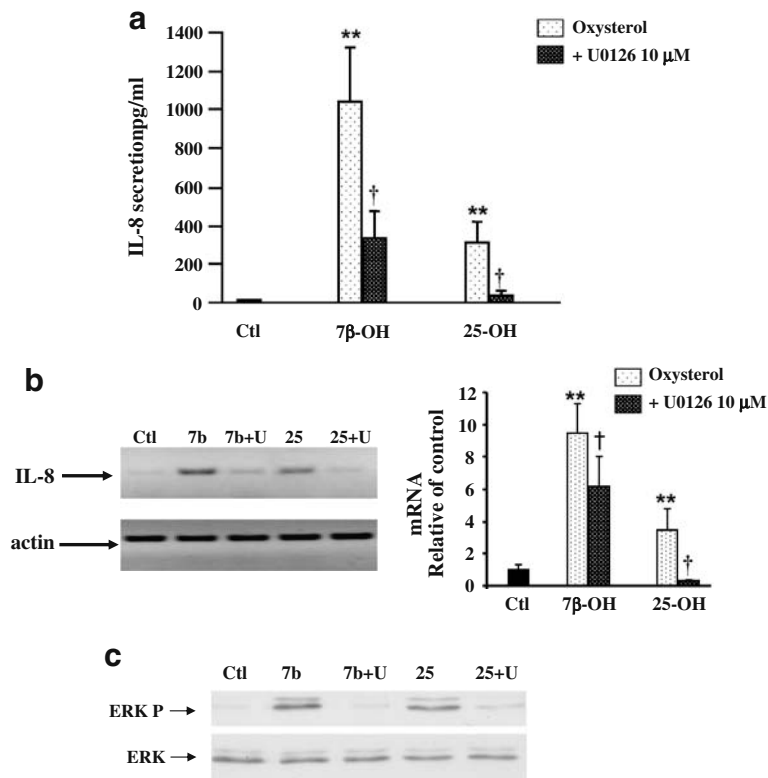


Fig. 4 7β -Hydroxycholesterol- and 25-hydroxycholesterol-induced increase of IL-8 secretion and mRNA level is inhibited by the MEK blocker U0126. **a** THP-1 cells were incubated or not with U0126 (10 μ mol/l) for an hour before the addition of 7β -hydroxycholesterol (20 μ g/ml, 7 β -OH) or 25-hydroxycholesterol (20 μ g/ml, 25-OH), or in the absence of oxysterol (Ctl). Cells were cultivated for an additional 24 h, and aliquots of the culture medium were assayed for IL-8 by ELISA. Data are the mean values \pm SD ($n=5$). $\dagger p < 0.001$ vs. the oxysterol-treated group and $**p < 0.001$ vs. the control group. **b** Expression of IL-8 and actin mRNA was determined by RT-PCR after 6 h of treatment with the oxysterols, with or without pretreatment of the cells for an hour with U0126 (U, 10 μ mol/l). Graph represents the results

of real-time PCR performed as control experiment of semi-quantitative RT-PCR. Real-time PCR was performed, and IL-8 mRNA expression was normalized to cyclophilin mRNA expression in the same sample. Relative stimulation represents comparison to untreated control. $\dagger p < 0.001$ vs. the oxysterol-treated group, $**p < 0.001$ vs. the control group, and $p < 0.05$ vs. the oxysterol-treated group. Data are confirming inhibition representative of three independent experiments. **c** Control blot by of ERK phosphorylation U0126. THP-1 cells were either untreated (Ctl) or incubated with U0126 (U, 10 μ mol/l) for an hour before the addition of oxysterols for an additional 6 h. Cell extracts were collected, subjected to SDS/PAGE, and immunoblotted with ERK1/2 and phospho-ERK1/2

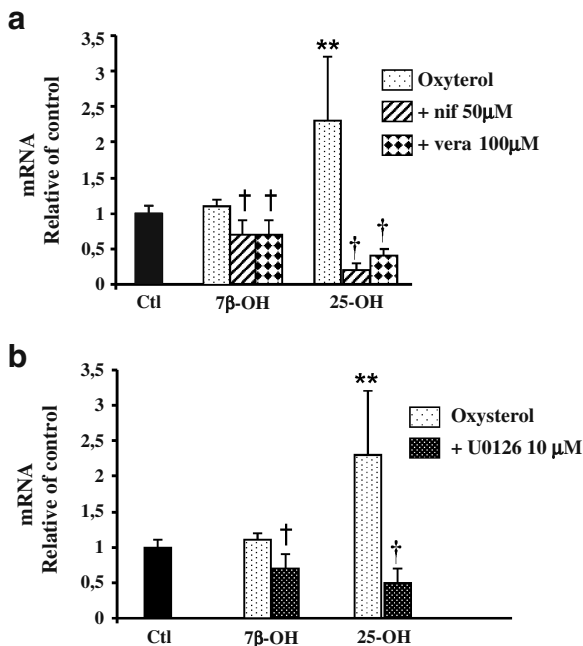


Fig. 5 25-Hydroxycholesterol induces an increase of c-fos mRNA level inhibited by L-type calcium channel blockers and the MEK blocker. THP-1 cells were incubated in the presence of 7 β -hydroxycholesterol (20 μ g/ml, 7 β) or 25-hydroxycholesterol (20 μ g/ml, 25), **a** with or without pretreatment for an hour with nifedipine (*n*, 50 μ mol/l) or verapamil (*v*, 100 μ mol/l), or **b** U0126 (*U*, 10 μ mol/l), or in the absence of oxysterol (*control*). Graphs on **a** and **b** represent the results of real-time PCR. Real-time PCR was performed, and c-fos mRNA expression was normalized to cyclophilin mRNA expression in the same sample. Relative stimulation represents comparison to the untreated control. Data are representative of three independent experiments. † p <0.001 vs. the oxysterol-treated group, ** p <0.001 vs. the control group, and ‡ p <0.05 vs. the oxysterol-treated group

Jun N-terminal kinase (JNK), ERK, and p38 kinases are involved in the regulation of c-jun (Marinissen et al. 1999) and c-fos expression (Karin 1995). RT-PCR performed on treated and non-treated cells showed an oxysterol-dependent increase of c-fos mRNA levels in 25-hydroxycholesterol-treated cells; this increase was calcium-dependent and MEK to ERK1/2-dependent, as demonstrated by the lack of increase of c-fos mRNA in nifedipine, verapamil, or U0126-treated cells (Fig. 5a,b). No important quantitative changes were detected in 7 β -hydroxycholesterol-treated cells suggesting, as c-fos and c-jun proteins activity could be regulated by phosphorylation (Hill and Treisman 1995; Monje et al. 2003), the involvement of c-fos phosphorylation by ERK1/2. However, the lack of change in phosphorylation of the P38 MAP kinase or

JNK kinase, which has been detected using Western blotting in THP-1 cells, are not in agreement with the involvement of c-jun (data not shown).

AP-1 transcription factor is involved in oxysterol-induced IL-8 secretion

To ascertain the involvement of AP-1 in IL-8 gene transcription, THP-1 cells were transiently transfected with different constructs containing a truncated part of the IL-8 promoter. Only constructs containing an AP-1 site exhibited luciferase activity showing the involvement of AP-1 in oxysterol-induced IL-8 secretion; NF κ B did not appear implicated in oxysterol-induced IL-8 secretion. To ascertain the non-involvement of NF κ B in oxysterol-induced IL-8 secretion, experiment with construct containing mutated NF κ B NF κ B site were performed, and they showed no decrease in luciferase activity (Fig. 6a). To confirm AP-1 activation and to ascertain the calcium-dependence and the MAPK pathway of the oxysterol-induced IL-8 secretion, activation of c-fos has been investigated in presence and in absence of verapamil, nifedipine, and U0126 with the Trans AM method (Fig. 6b). The low level of AP1 activation in the presence of U0126, nifedipine, or verapamil confirms the MEK and calcium dependence of oxysterol-induced IL-8 secretion. The almost complete inhibition of AP1 in response to 25-hydroxycholesterol by U0126, whereas c-fos expression exhibited only a 40% decrease (Fig. 5b), could be explained by the ability of ERK to control c-fos activity by two mechanisms: one regulating c-fos expression and another by a direct activation of c-fos by phosphorylation (Monje et al. 2003).

Discussion

Some studies had previously described the ability of oxysterols to induce IL-8 secretion in monocytes/macrophages (Liu et al. 1997; Rydberg et al. 2003). However, very little was known about the signaling pathways leading to IL-8 secretion. In this paper, we describe the calcium dependence of oxysterol-induced IL-8 secretion. IL-8 secretion appeared to be related to an increase of intracellular calcium in 7 β -hydroxycholesterol-treated cells as demonstrated by the use of calcium channel inhibitors, nifedipine and verapamil. However, in 25-hydroxycholesterol-treated cells, the involvement

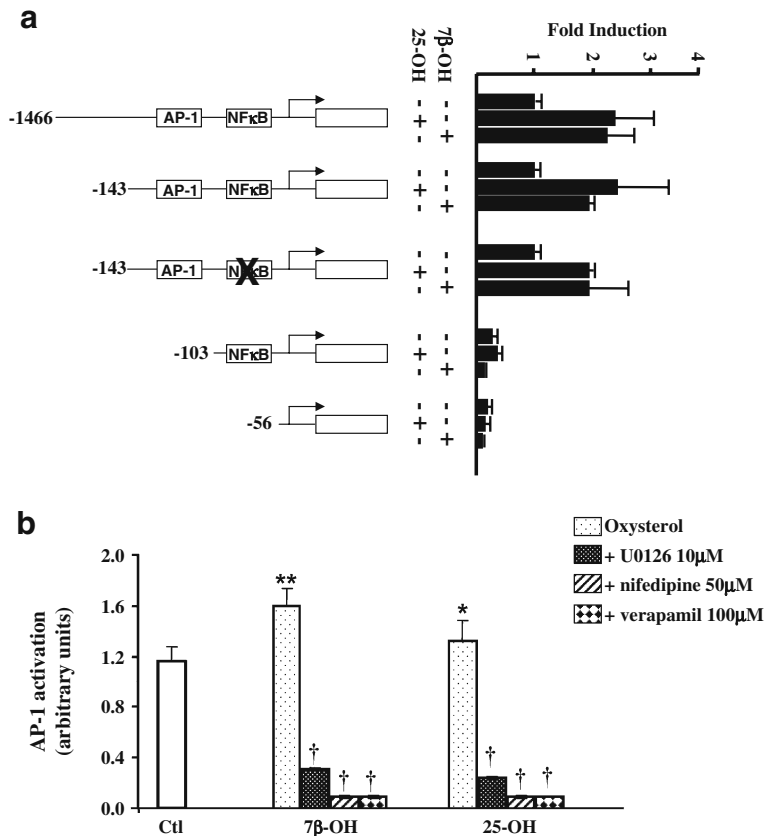


Fig. 6 Oxysterols increase IL-8 gene transcription involving AP-1. **a** THP-1 cells were transfected with the indicated IL-8 promoter constructs (0.5 μg) and treated with 7β-hydroxycholesterol (7β-OH, 20 μg/ml) or 25-hydroxycholesterol (25-OH, 20 μg/ml) for 24 h. Data are the mean values ± SD (n=5). The number designating each IL-8 promoter fragment is the number of pb upstream the initiation of transcription site. **b** L-type calcium channel blockers and the MEK blocker inhibit AP-1 activation via

c-fos induced by oxysterols. AP-1 activity was assayed in untreated and 7β-hydroxycholesterol- or 25-hydroxycholesterol-treated (7β-OH, 25-OH, 20 μg/ml) THP-1 cells in the presence or absence of verapamil (100 μmol/l), nifedipine (50 μmol/l), or U0126 (10 μmol/l), using an ELISA-based TransAM kit. Data are the mean values ± SD (n=3). *p<0.05 and **p<0.001 vs. the control group, †p<0.001 vs. the oxysterol-treated group

of calcium appeared different, although no persistent increase in cytosolic Ca²⁺ could be demonstrated. The observed Ca²⁺ oscillations could induce similar effects as the more persistent Ca²⁺ elevation, with the possible exception of apoptosis; such calcium oscillations have been previously described by Ares et al. (1997) in human aortic smooth-muscle cells.

Concerning the oxysterols studied here, change in calcium homeostasis could lead to the activation of the calcium-dependent kinase PYK2 and, consequently, the MAPK ERK1/2 (Berthier et al. 2005; Dikic et al. 1996). Interestingly, kinetics of ERK1/2 activation varied according to the oxysterol studied. In 7β-hydroxycholesterol-treated cells, the lengths of activation were considerably shorter than in 25-hydrox-

cholesterol-treated cells, which exhibited a more sustained activation of ERK1/2. This difference could be compared with the cytotoxic properties of 7β-hydroxycholesterol, which triggers apoptosis (Miguet-Alfonsi et al. 2002). We have previously described a transient activation of ERK1/2 (weaker than with 7β-hydroxycholesterol) with another proapoptotic oxysterol, the 7-ketocholesterol. In 7-ketocholesterol-treated cells, the oxysterol-induced increase of cytosolic Ca²⁺ triggered activation of the proapoptotic proteins Bim (Berthier et al. 2005) and Bad (Berthier et al. 2004) and, consequently, cell death by apoptosis. The engagement of the apoptotic process might inactivate ERK1/2, probably via activation of phosphatases. We then hypothesized that, in 25-hydroxycholesterol-treated cells,

the weak change in calcium homeostasis was unable to trigger apoptosis but sufficient to activate ERK1/2, whereas 7 β -hydroxycholesterol treatment of THP-1 cells induced both activation of a calcium-dependent apoptotic pathway, as previously described (Berthier et al. 2004; Wang et al. 1999), and of a calcium-dependent, ERK1/2-dependent cytokine secretion pathway described in this paper.

Here, we showed the involvement of activator protein-1 (AP-1) in oxysterol-induced IL-8 secretion for both the 7 β -hydroxycholesterol and the 25-hydroxycholesterol. This involvement of AP-1 has been previously reported in the control of the IL-8 promoter (Murakami et al. 2003; Rydberg et al. 2003; Kikuchi et al. 2002; Okamoto et al. 1994). The transcription factor AP-1 is formed either as a homodimer of c-jun or as a heterodimer of c-fos and c-jun. In our study, contrary to previous published findings (Rydberg et al. 2003), we did not find any change in JNK phosphorylation in agreement with the lack of involvement of the c-jun activation pathway in 25-hydroxycholesterol-induced IL-8 secretion. Moreover, their results, excluding the involvement of c-fos in 25-hydroxycholesterol-induced IL-8 secretion, were only assessed by a lack of supershift with a c-fos antibody. Interestingly, the transcriptional factor NF- κ B, which was often described to be involved in IL-8 secretion (Zhu et al. 2003; Sharma et al. 1998; Kim et al. 2005), did not appear to be involved in oxysterol-induced IL-8 secretion in THP-1 cells as demonstrated by the luciferase reporter gene assay. However, IL-8 production by a MAPK-dependent, NF- κ B-independent pathway has been previously described in epithelial cells (Akhtar et al. 2003).

In this study, we described for the first time the signaling pathway connecting oxysterol-induced changes in calcium homeostasis with IL-8 secretion, an activation of the MEK/ERK1/2 pathway leading to the activation of the AP-1 transcription via increase of c-fos mRNA and/or activation of c-fos. Hence, oxysterols and particularly 7 β -hydroxycholesterol are able to trigger two biological events involved in the development of atheromatous lesions, apoptosis and cytokine secretion, via an increase of cytosolic calcium. As we have previously described for 7-ketocholesterol, this could be due to incorporation of the oxysterol into lipid raft domain at the plasma membrane followed by the redistribution of calcium channel to rafts (Berthier et al. 2004).

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