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Lycopene prevention of oxysterol-induced proinflammatory cytokine cascade in human macrophages: inhibition of NF-κB nuclear binding and increase in PPARγ expression

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Abstract

It is now well accepted that oxysterols play important roles in the formation of atherosclerotic plaque, involving cytotoxic, pro-oxidant and proinflammatory processes. It has been recently suggested that tomato lycopene may act as a preventive agent in atherosclerosis, although the exact mechanism of such a protection is not clarified. The main aim of this study was to investigate whether lycopene is able to counteract oxysterol-induced proinflammatory cytokines cascade in human macrophages, limiting the formation of atherosclerotic plaque. Therefore, THP-1 macrophages were exposed to two different oxysterols, such as 7-keto-cholesterol (4-16 μM) and 25-hydroxycholesterol (2-4 μM), alone and in combination with lycopene (0.5–2 μM). Both oxysterols enhanced pro-inflammatory cytokine [interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor α) secretion and mRNA levels in a dose-dependent manner, although at different extent. These effects were associated with an increased reactive oxygen species (ROS) production through an enhanced expression of NAD(P)H oxidase. Moreover, a net increment of phosphorylation of extracellular regulated kinase 1/2, p-38 and Jun N-terminal kinase and of nuclear factor kB (NF-κB) nuclear binding was observed. Lycopene prevented oxysterol-induced increase in pro-inflammatory cytokine secretion and expression. Such an effect was accompanied by an inhibition of oxysterol-induced ROS production, mitogen-activated protein kinase phosphorylation and NF-κB activation. The inhibition of oxysterol-induced cytokine stimulation was also mimicked by the specific NF-κB inhibitor pyrrolidine dithiocarbamate. Moreover, the carotenoid increased peroxisome proliferator-activated receptor γ levels in THP-1 macrophages. Taken all together, these data bring new information on the anti-atherogenic properties of lycopene, and on its mechanisms of action in atherosclerosis prevention.

Keywords: Lycopene; Pro-inflammatory cytokine cascade; Oxysterols; ROS; NF-κB; PPARγ

1. Introduction

Oxysterols constitute an important family of molecules resulting from the auto-oxidation of cholesterol in air, enzymatic transformation of cholesterol in various cell species [1] or cholesterol peroxidation of low-density lipoproteins (LDL) [2]. It is known that oxysterols accumulate in the subendothelial level of the arterial wall during the atheromatous process [3]. It is now accepted that oxysterols play important roles in atherosclerosis [4]. Indeed, some of these compounds were found to be abnormally elevated in the plasma and atherosclerotic plaques of hypercholesterolemic patients [5], and increased plasma levels of oxysterols were recently associated with an increased risk of atherosclerosis in humans [6]. Moreover, the ability of some oxysterols to trigger cytotoxic, pro-oxidative and/or pro-inflammatory reactions, which are major events involved in vascular dysfunction and atherogenesis [7], has also been frequently reported. Thus, enhanced production of superoxide anions (O_2^-) and ROS [8] were observed in the presence of oxysterols [9–11]. Moreover, oxysterols, directly or through a ROS-mediated mechanism involving nuclear factor kB (NF-κB) activation, have been also reported to enhance pro-inflammatory cytokines secretion and expression at both transcriptional and post-transcriptional levels, although at different extent. In particular, 7-ketocholesterol (7-KC)

Abbreviations: 7-CHO, 7-ketocholesterol; 25OHC, 25hydroxycholesterol; ERK, extracellular regulated kinase; JNK, Jun N-terminal kinase; IL-1β, interleukin1β; IL-6, interleukin 6; IL-8, interlerleukins-8 liver X receptor (LXR); MAPK, mitogen-activated proteinkinase; NF-κB, nuclear factor-kappa B; PPAR, peroxisome proliferator-activated receptor; PDTC, pyrrolidine dithiocarbamate; PMA, phorbol 12-myristate 13-acetate; ROS, reactiveoxygen species; RSz, thiyl radical sulphonyl; RSO2z, reactive oxygen sulphynates; THF, tetrahydrofuran; TNFα, tumor necrosis factor α.

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was described mainly to enhance interleukin (IL)-1β secretion in vascular endothelial cells [12], while 25-hydroxycholesterol (25-OHC) was reported to modulate the secretion of IL-8 (a pro-atherogenic cytokine involved in firm adhesion of monocytes to vascular endothelial cells) [13] through MEK/extracellular regulated kinase (ERK) 1/2 cascade. Such a difference has been suggested to depend on the physical and chemical properties of oxysterol molecules, which influence their distribution inside the cell, their interaction with receptors, and/or their ability to modify certain cellular structures, mainly those playing key roles in signal transduction such as lipid rafts [14,15].

Epidemiological studies have shown that a high intake of tomato and tomato-derived products, rich in lycopene, is associated with a decreased risk for atherosclerosis and other cardiovascular diseases [16], but the mechanisms of such a benefit have not been clarified. We speculate that lycopene may affect the immune function, possibly because of its ability to modulate cellular redox environment and cell-to-cell interactions and/or to regulate anti-inflammatory transcription factors, such as peroxisome proliferator-activated receptor γ (PPARγ). The increasingly recognized role of immune/inflammatory processes in atherogenesis suggests that the intake of potentially immunomodulating and anti-inflammatory compounds might inhibit the initial damage to the arterial wall, largely by lowering the production of cytokines [17,18]. In agreement with this hypothesis, high intakes of lycopene have been found to be inversely correlated with the severity of atherosclerosis, as evaluated by intima/media thickness [19]. Moreover, a down-regulation of cytokine production by lycopene or tomato and tomato products has been reported in cell cultures as well as in human subjects. In addition, it is known that NF-κB-mediated expression of cellular adhesion molecules and pro-inflammatory cytokines are implicated in arterial wall thickening [20]. Interestingly, lycopene inhibits NF-κB activation [21], as shown, for example, in dendritic cells [22]. The hypothesis of an inhibition of cytokine production by lycopene though a redox mechanism is supported by previous observations that lycopene possesses redox properties in vitro as well as in vivo in many biological systems [23,24], although conflicting results have been also reported [25]. Lycopene, because of its high number of conjugated double bonds, has been reported to exhibit higher singlet oxygen quenching ability compared to β-carotene or α-tocopherol [26] and to act as a potent antioxidant, preventing the oxidative damage of critical biomolecules including lipids, LDL, proteins, and DNA [27]. Moreover, recently, the carotenoid has been shown to modulate redox sensitive molecular pathways involved in cell proliferation and apoptosis [28].

The aim of this study was to determine whether lycopene was able to prevent the effects of two different oxysterols, 7-KC and 25-OHC, both present in high amounts in the atheromasic plaque, on pro-inflammatory cytokine secretion and expression in human macrophages and to investigate potential mechanism(s) implicated in such a prevention. To this purpose, human THP-1 macrophages were treated with 7-KC or 25-OHC, alone and in combination with lycopene, in a range of carotenoid concentrations achievable in vivo after supplementation (0.5–2 μM) [29] and the levels of tumor necrosis factor α (TNFα), IL-1β, IL-6 and IL-8 were studied. Moreover, to test possible mechanism(s) by which lycopene may affect oxysterol-induced cytokine production, reactive oxygen species (ROS) formation, NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase), MAP kinase expression, NF-κB binding activity and PPARγ expression were also evaluated.

2. Materials and methods

2.1. Cell culture

THP-1 (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI Dutch Modified (Sigma, Milan, Italy) without antibiotics and supplemented with 10% fetal calf serum, non essential aminoacids, 2 mM glutamine, and 1 mM sodium piruvate. Cells were maintained in log phase by seeding twice a week at density of 3×10^8 cells/L at 37°C under 5% CO_2/air atmosphere. 7-KC, 25-OHC (Sigma, Milan, Italy) and lycopene (LycoRed Natural Products Industries Ltd, Beer Sheva, Israel) were delivered to the cells (10^9 cells/L) using tetrahydrofuran (THF) as a solvent. To avoid the formation of peroxides, the solvent contained 0.025% butylated hydroxytoluene (BHT). The stock solutions of lycopene were prepared immediately before each experiment. From the stock solutions, aliquots of lycopene were rapidly added to the culture medium to give the final concentrations indicated. Control cultures were treated with THF+BHT. The amount of THF added to the cells was not greater than 0.5% (v/v), and it was the same in control cells as well as in treated ones. No differences were found between cells treated with THF and untreated cells in terms of cell number, viability and ROS production. After the addition of lycopene, the medium was not further replaced throughout the experiments. Experiments were routinely carried out on triplicate cultures. At the times indicated, cells were harvested and quadruplicate haemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

2.2. Chemiluminescence immunometric assay of cytokines

TNFα and IL-6 were measured by a commercial Enzyme-labeled, chemiluminescence sequential immunometric assay (DRG International, Mountainside, NJ, USA) in cell culture media. The intra-assay and inter-assay coefficient of variation (CV) for TNFα was 4.5% and 9 % respectively; the detection limit was <3 pg/ml. The intra and inter-assay CV for IL-6 was 4.2% and 5.4%, respectively. The detection limit was <2 pg/ml. IL-8 and IL-1β were measured by a solid-phase, two-site chemiluminescence immunometric assay (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). The intra and interassay CV for IL-8 and IL-1β was <3.8%, 6.7%, 4.8% and 6.5%, respectively. The detection limit was 2 pg/ml for IL-8 and 1.5 pg/ml for IL-1β.

2.3. Real-time polymerase-chain reaction (RT-PCR) of IL-8 and PPARγ

Total RNA was extracted from cells samples using Trizol according to manufacturer's protocols (Invitrogen Life Technologies, Paisley, UK); the RNA was eluted in diethyl pyrocarbonate (DEPC) treated water (0.01% DEPC) and stored at −80° until RT-PCR analysis. Nucleic acid concentrations were measured by spectrophotometry (Hewlett-Packard HP UV/VIS spectrophotometer 8450).

RT-PCR assay was performed using the two-step method. For the first-step of reverse transcription, we used QuantiTect Reverse Trancription kit (Qiagen, Hilden, Germany) with 500 ηg of total RNA as template RNA, following the manufacturer's procedure.

For the second step of real-time polymerase chain reactions (PCR), we employed QuantiTect SYBR Green Kits (Qiagen) and QuantiTect Primer Assays (Qiagen) for human β-actin, IL-8 and PPARγ, according to manufacturer's protocol described for the real-time thermal cycler LightCycler (Roche). PCR data obtained by the LightCycler software were automatically analysed by the Relative Quantification Software (Roche) and expressed as target/reference ratio. Our approach was based on the calibrator-normalized relative quantification including correction for PCR efficiency.

2.4. Measurement of ROS

Cells treated with varying concentrations of lycopene were harvested to evaluate ROS production using the di (acetoxymethyl ester) analog (C-2938) of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes, Eugene, OR, USA) as described in [30]. Before the addition of the fluorescent probes, 2×10^6 cells were washed to eliminate the amount of lycopene not cell associated. Fluorescent units were measured in each well after 30-min incubation with DCF (10 μM) by use of a Cytofluor 2300/2350 Fluorescence Measurement System (Millipore, Bedford, MA, USA). Lycopene did not alter the basal fluorescence of DCF.

2.5. Western blot analysis of NOX-4, p38 and p-p38, ERK1/2, pERK1/2, JNK, p-JNK expression

Cells (10×10^6) were harvested, washed once with ice-cold phosphate-buffered saline (PBS), and gently lysed for 30 min in ice-cold lysis buffer (1 mM MgCl2, 350 mM NaCl, 20 mM Hepes, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM Na4P2O7, 1 mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 1 mM Na3VO4, 20% glycerol, 1% NP40). Cell lysates were centrifuged for 10 min at 4°C (10,000×g) to obtain the supernatants, which were used for Western blot analysis. The anti-NOX-4 (clone N-15, Cat. No. sc-21860), anti-p38 (clone C-20, Cat. No. SC-535), anti p-p38 (clone D-8, Cat. No. 7973), anti-ERK1/2 (clone K-23, Cat. No. SC-94), anti p-ERK1/2 (clone E-4, Cat. No. SC-7383), anti-Jun N-terminal kinase (JNK) (clone C-17, Cat. No. SC-474) and anti-p-JNK (clone G-7, Cat. No. SC-6254) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were washed and exposed to horseradish peroxidase-labeled secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) for 45 min at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantified by densitometric scanning.

2.6. Nuclear extracts and electrophoretic mobility-shift assay

Frozen cell pellets were processed to obtain nuclear extracts. Briefly, 12×10^6 cells were collected, washed twice and pelletted by 200×*g* centrifugation for 10 min. The pellet was resuspended in 440 μl cold buffer A (20 mmol/L HEPES, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L $Na_4P_2O_7$, 20 mmol/L NaF and 1 mmol/L polymethylsulfonyl fluoride, 1.5 mmol/L aprotinin) by gentle pipetting. The cells were allowed to swell on ice for 15 min, then 25 μL 10% NP40 was added and the tube was vigorously vortexed for 10 s. The homogenate was centrifuged for 30 s at 20,000×*g*. The nuclear pellet was resuspended in 50 μl ice-cold buffer B (20 mmol/L HEPES, 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L $Na_4P_2O_7$, 20 mmol/L NaF and 1 mmol/L polymethylsulfonyl fluoride, 1.5 mmol/L aprotinin, 20% v/v glycerol), and the tube was vigorously rocked for 15 min at 4°C on a shaking platform. The nuclear extract was centrifuged for 5 min at 20,000×*g* at 4°C.

Binding reactions containing 5 μg nuclear extracts, 10 mmol/L Tris–HCl (pH 7.6), 5 % glycerol, 1 mmol/L EDTA, 1 mmol/L DTT, 50 mmol/L NaCl and 3 mg poly(dI-dC) were incubated for 30 min with 5000 cpm of ^{32}P-end-labeled double-stranded oligonucleotide in a total volume of 20 μl. The probe was 5′-AGTTGAGGGGACTTTCC-CAGGC3′. Labeling of the probe was obtained by incubating 5 pmol of oligonucleotide with 10 pmol [α-^{32}P]ATP and 3 UT4 polynucleotide kinase for 30 min at 37°C. The probe was then purified with MicroBIO-Spin P-30 columns. Complexes were separated on 60 g/L polyacrylamide gels with 45 mmol/L Tris-borate, 1 mmol/L EDTA, pH 8 buffer. After fixation and drying, gels were exposed on phosphor screens which were then analyzed by phosphor/fluorescence imager STORM 840 (Molecular and Dynamics, Sunnyvale, CA, USA). The intensity of the revealed bands was directly quantified by Image QuaNT software (Molecular Dynamics).

2.7. Analysis of p65 protein

Nuclear extracts, 25–30 μg of protein, were separated by sodium dodecyl sulfate-polyacrilamide gel electrophoresis with the use of 40-120 g/L Bis-Tris gels (NOVEX, San Diego, CA, USA) and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) with the use of a semidry system. Immunoblots were blocked overnight at 4°C in 50 g/L dried milk in PBS, pH 7.4 plus 0.05% Tween 20. Blots were incubated with polyclonal primary antibodies to p65 (C20, sc 372, lot # J250, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS plus 0.05% Tween 20 for 1–2 h at room temperature. The blots were washed and exposed to a horseradish peroxidase-labeled secondary antibody (Amersham Pharmacia Biotech) for 45 min at room temperature. Following incubation with secondary antibodies, the immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantified by densitometric scanning.

2.8. Coimmunoprecipitation

THP-1 cells were washed twice in ice-cold PBS, collected, resuspended in 1 ml of ice-cold lysis buffer (10 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 0.15 M NaCl and 1 mM EDTA) and incubated for 30 min on ice. After centrifugation, supernatants were used as whole-cell extracts for immunoprecipitation with antibodies against p65 (Santa Cruz Biotechnology), followed by Western blotting with antibodies specific for human PPARγ (H-100; Santa Cruz Biotechnology).

2.9. Statistical analysis

Three separate cultures per treatment were utilized for analysis in each experiment. Values were presented as means±S.E.M. Multifactorial two-way analysis of variance (ANOVA) was adopted to assess any differences among the treatments and the times (Figs. 1A–D, 5A). When significant values were found ($P<.05$), post hoc comparisons of means were made using Honestly significant differences test. One-way ANOVA was used to determine differences between different concentrations or treatments in Figs. 2A–D, 3A–C, 4A, B, 6B, D, F, 7D, 8A, B). When significant values were found ($P<.05$), post hoc comparisons of means were made using Fisher's test. Differences were analyzed using Minitab Software (Minitab, State College, PA, USA).

3. Results

3.1. Effects of lycopene on oxysterol-induced cytokine secretion and mRNA expression

Because monocytes and macrophages are thought to be a source of pro-inflammatory molecules, we first examined the ability of THP-1 cells to produce cytokines in the presence of oxysterols. In particular, we analysed the effects of varying concentrations of 7-KC and 25-OHC in stimulating TNFα (Fig. 1A), IL-1β (Fig. 1B), IL-6 (Fig. 1C) and IL-8 (Fig. 1D), which have been well characterized as pro-inflammatory cytokines, in cells treated for 6 and 24 h. The oxysterols were tested in a range of concentrations which did not

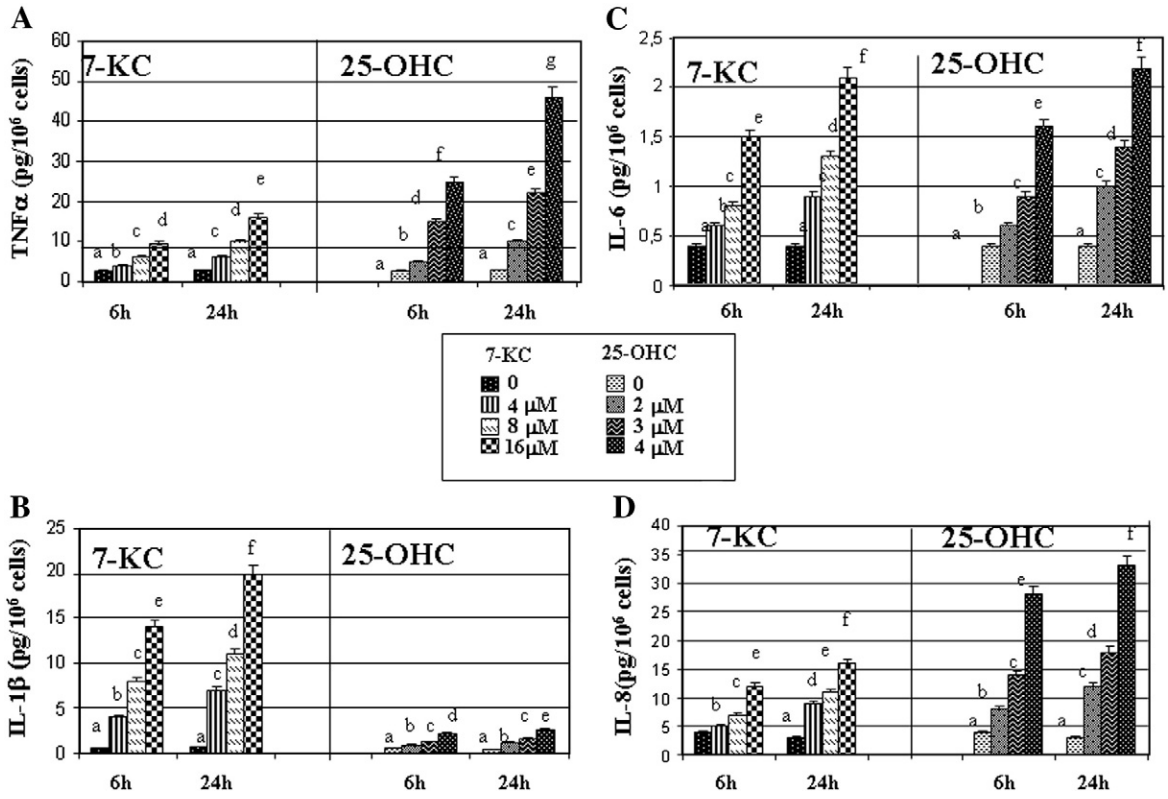

Fig. 1. Effects of varying oxysterol concentrations on TNFα (A), IL-1β (B), IL-6 (C) and IL-8 (D) secretion in culture medium of THP-1 cells treated for 6 and 24 h. Values were the means±S.E.M. of five experiments. Values not sharing the same letter were significantly different ($P<.005$, Tukey's test).

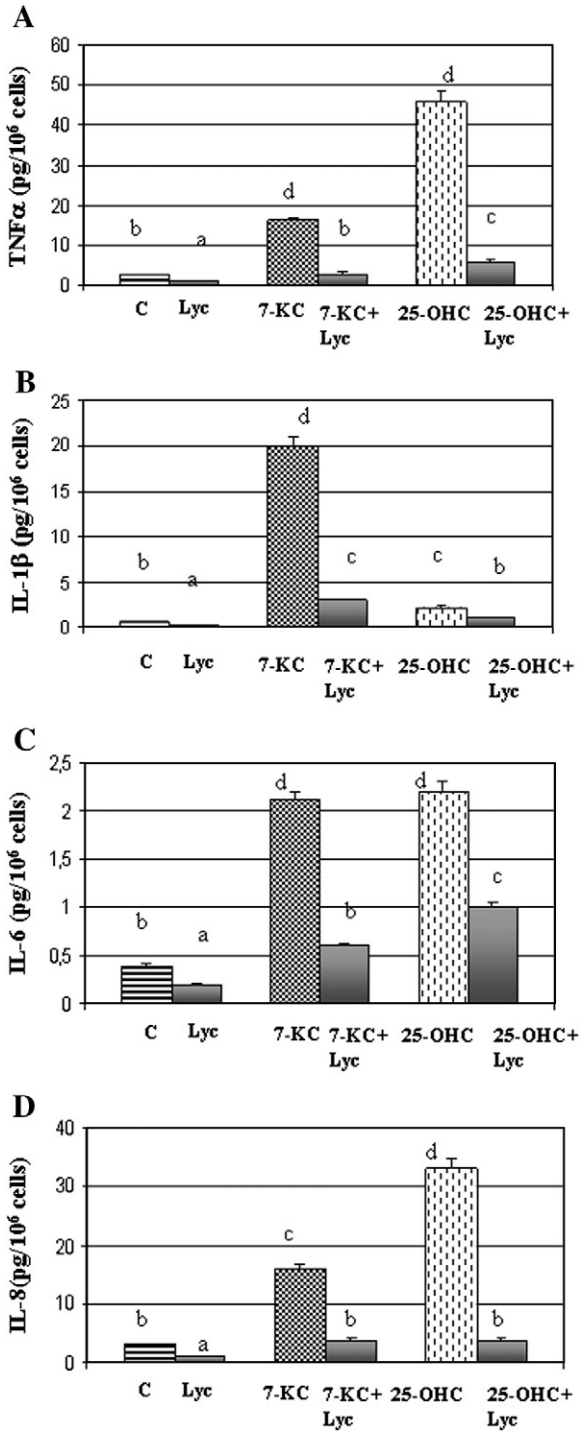

Fig. 2. Effects of lycopene on TNFα (A), IL-1β (B), IL-6 (C) and IL-8 (D) secretion in the absence and in the presence of oxysterols in culture medium of THP-1 cells treated for 24 h. The carotenoid was added at the concentration of 2 μM, 7-KC at 16 μM and 25-OHC at 4 μM. Values were the means±S.E.M. of five experiments. Values not sharing the same letter were significantly different ($P<.05$, Fisher's test).

alter the percentage of viable cells, measured by trypan blue dye exclusion method. 25-OHC caused a progressive loss of cell viability at concentrations greater than 4 μM and 7-KC at concentrations greater than 16 μM (data not shown), suggesting that 25-OHC is more biologically active than 7-KC in inducing cell death in THP-1 cells. Both the oxysterols induced a dose-dependent increase in the secretion of all the proinflammatory cytokines tested in the culture medium, although at different extent, depending on the kind of the oxysterol. At the concentrations tested, 25-OHC was more potent in stimulating TNFα and IL-8 secretion than 7-KC, whereas 7-KC was more effective in inducing IL-1β production. A similar weak increase of IL-6 was caused by both the oxysterols. Cytokine secretion was greater at 24 than at 6 h. We did not observe any cytokine release at 3 h of oxysterol treatment (data not shown).

A 24-h treatment of cells with lycopene (2 μM) inhibited significantly the basal secretion of TNFα (Fig. 2A), IL-1β (Fig. 2B), IL-6 (Fig. 2C) and IL-8 (Fig. 2D) as well as those induced by both 7-KC (16 μM) or 25-OHC (4 μM).

When THP-1 cells were treated with different concentrations of lycopene (0.5–2 μM), alone (Fig. 3A) and in combination with 7-KC (Fig. 3B) or 25-OHC (Fig. 3C) for 24 h and the secretion of IL-8 was measured, a dose-dependent inhibition of oxysterol-induced IL-8 production by the carotenoid was observed. The secretion of oxysterol-induced TNFα, IL-1β, IL-6 was also inhibited by lycopene, at concentrations ranging from 0.5 to 2 μM, in a dose-dependent manner (data not shown).

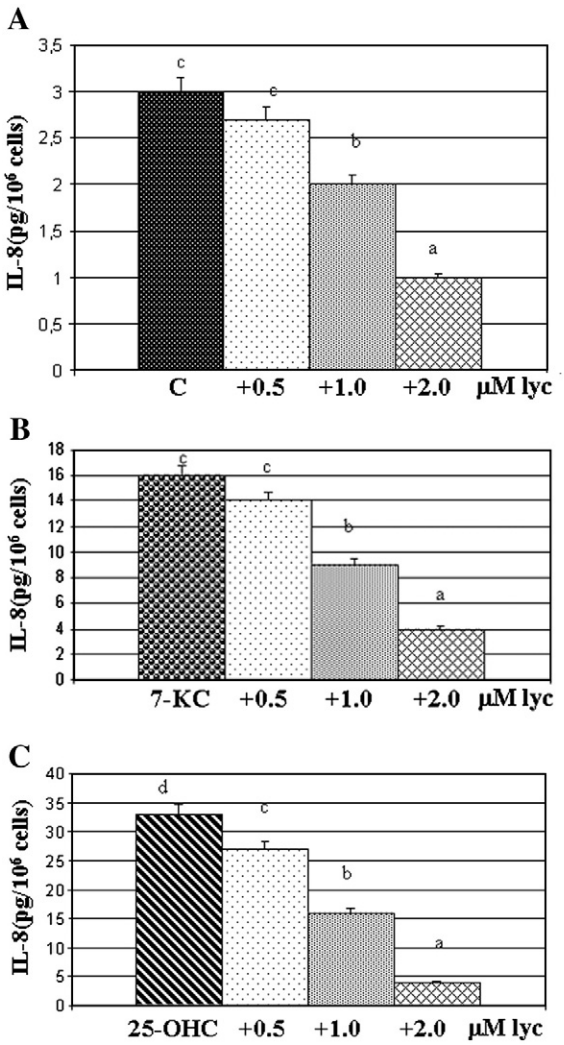

Fig. 3. Effects of varying lycopene concentrations on IL-8 secretion in the absence (panel A) and in the presence of 7-KC (panel B) or of 25-OHC (panel C) in culture medium of THP-1 cells treated for 24 h. 7-KC was added at the concentration of 16 μM and 25-OHC at the concentration of 4 μM. Values were the means±S.E.M. of three experiments. Values not sharing the same letter were significantly different ($P<.05$, Fisher's test).

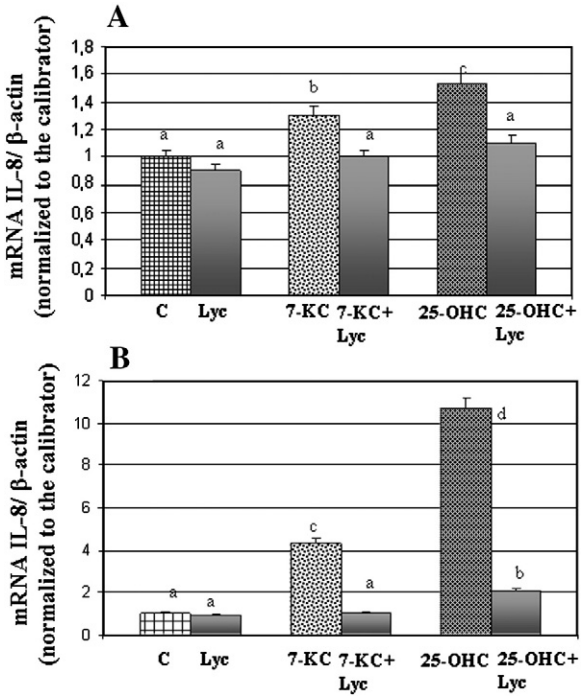

Fig. 4. IL-8 mRNA expression in THP-1 cells. We incubated THP-1 cells in the absence (control) and in the presence of 7-KC (16 μM) or 25-OHC 4 μM), alone and in combination with lycopene (2 μM) for 6 (A) and for 24 h (B). Then, we harvested the cells, isolated the RNA, and measured IL-8 mRNA level by reverse transcription polymerase chain reaction. The values were the means±S.E.M. of three independent experiments. Values not sharing the same letter were significantly different ($P<.05$, Fisher's Exact test).

The effects of 7-KC and 25-OHC on the IL-8 mRNA expression were quantified by real-time RT-PCR (Fig. 4). Data from three independent experiments show an increase of IL-8 mRNA levels in both 7-KC- and 25-OHC-treated macrophages. Such an increase was greater at 24 h than at 6 h (Fig. 4A, B). To explore whether the reduced levels of IL-8 protein observed in cells treated with lycopene could be attributed to a reduced gene transcription, we analysed the expression of IL-8 in cells treated with a combination of oxysterols and lycopene. The carotenoid reduced 7-KC and 25-OH-induced IL-8 mRNA levels at both the time points indicated.

3.2. Effects of lycopene on redox-sensitive pathways involved in oxysterol-induced cytokine production

Since it has been suggested that oxysterols may increase the levels of cytokines by modulating redox-sensitive pathways, we first measured the oxidative status of THP-1 cells treated with oxysterols, alone and in combination with lycopene. To quantify the level of cellular oxidative stress generated by oxysterols, we used a fluorimetric assay to measure ROS production. The results are shown inFig. 5A. The effects of 25-OHC (4 μM) on ROS production were similar to those found for 7-KC (16 μM) in our previous study [8]; therefore, they were brought here only for comparison. Both the oxysterols induced a significant increase in ROS production compared with the control. The increase in ROS production was more precocious than cytokine release, since it was observed at 3 h of oxysterol treatment. A further increase of ROS production was observed at 6 h. Lycopene at the concentration of 2 μM strongly reduced oxysterol-induced ROS production. A weak inhibition of ROS production by lycopene in the absence of oxysterols was also observed, but the values were not significant.

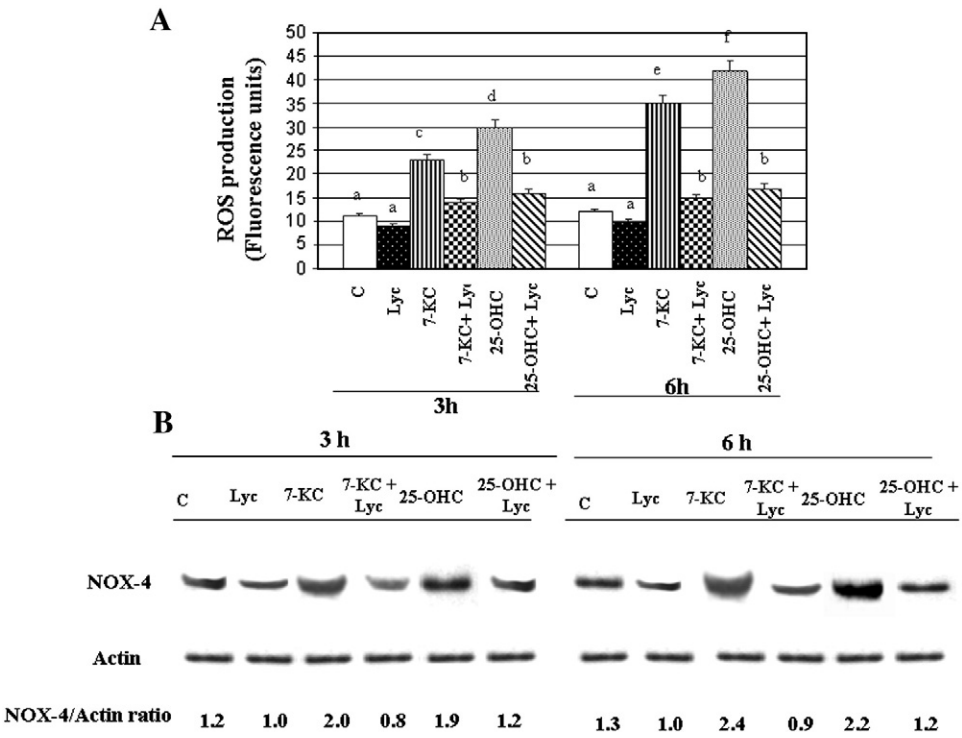

Fig. 5. Effects of lycopene, alone and in combination with 7-KC or 25-OHC, on ROS production (A) and NOX-4 expression (B) in THP-1 cells treated for 3 and 6 h. The carotenoid was added at the concentration of 2 μM, 7-KC at 16 μM and 25-OHC at 4 μM. In panel A, values were the means±S.E.M. of five experiments. Values not sharing the same letter were significantly different ($P<.005$, Tukey's test).

Since the increase in ROS levels by 7-KC has been reported to involve modulation of NOX-4 [8], we also measured the expression of this enzyme following treatment with 25-OHC (Fig. 5B). Similar to 7-KC (16 μM), 25-OHC (4 μM), induced a robust up-regulation (about twofold from the basal level) of NOX-4 at 3 h of incubation, which was further enhanced by a 6-h treatment. This effect was completely prevented by the concomitant addition of lycopene (2 μM).

We next investigated intracellular signal transduction triggered by the oxysterols, with the aim of identifying a mechanism of gene regulation common to the two oxysterols (Fig. 6). For this purpose, the possible modulation of the mitogen-activated protein kinase (MAPK) cascade was analyzed in macrophages treated with oxysterols alone and in combination with lycopene. These kinases have been reported to be activated by various stress stimuli, including treatments with 7-KC [8], and they have been also implicated in oxysterol-induced cytokine secretion and apoptosis. Similar to 7-KC (16 μM), 25-OHC (4 μM) induced a remarkable increase in the level of the phosphorylated forms of JNK (p-JNK) (Fig. 6A, B), and ERK1/2 (p-ERK1/2) (Fig. 6C, D) at 3 h of incubation. A slight increase in p-p38 was also detectable (Fig. 6E, F). Such increases were all prevented by the addition of lycopene (2 μM).

In view of recent studies suggesting that NF-κB plays an important role in up-regulating cytokine production, we investigated the involvement of this redox-sensitive transcription factor in THP-1 macrophages treated with 7-KC or 25-OHC. Following THP-1 cell incubation in the presence of 7-KC (16 μM) or 25-OHC (4 μM), net enhancement of NF-κB DNA binding was evident at 3-h treatment (Fig. 7A). This effect was completely prevented by the addition of lycopene (2 μM). Similar results were found when nuclear extracts were prepared from macrophages treated with oxysterols, alone and in combination with lycopene and nuclear translocation of the NF-κB p65 subunit was detected by Western blotting (Fig. 7B). Both the oxysterols induced the nuclear translocation of the NF-κB subunit p65 within 3 h. Such an effect was inhibited by the addition of lycopene (2 μM). Similarly, the specific NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) completely prevented the DNA binding of NF-κB induced by oxysterols (Fig. 7C) as well as IL-8 protein secretion (Fig. 7D). In these experiments, THP-1 cells were pre-incubated with PDTC (10 μM) for 30 min, followed by incubation in the absence or presence of oxysterols for 3 h.

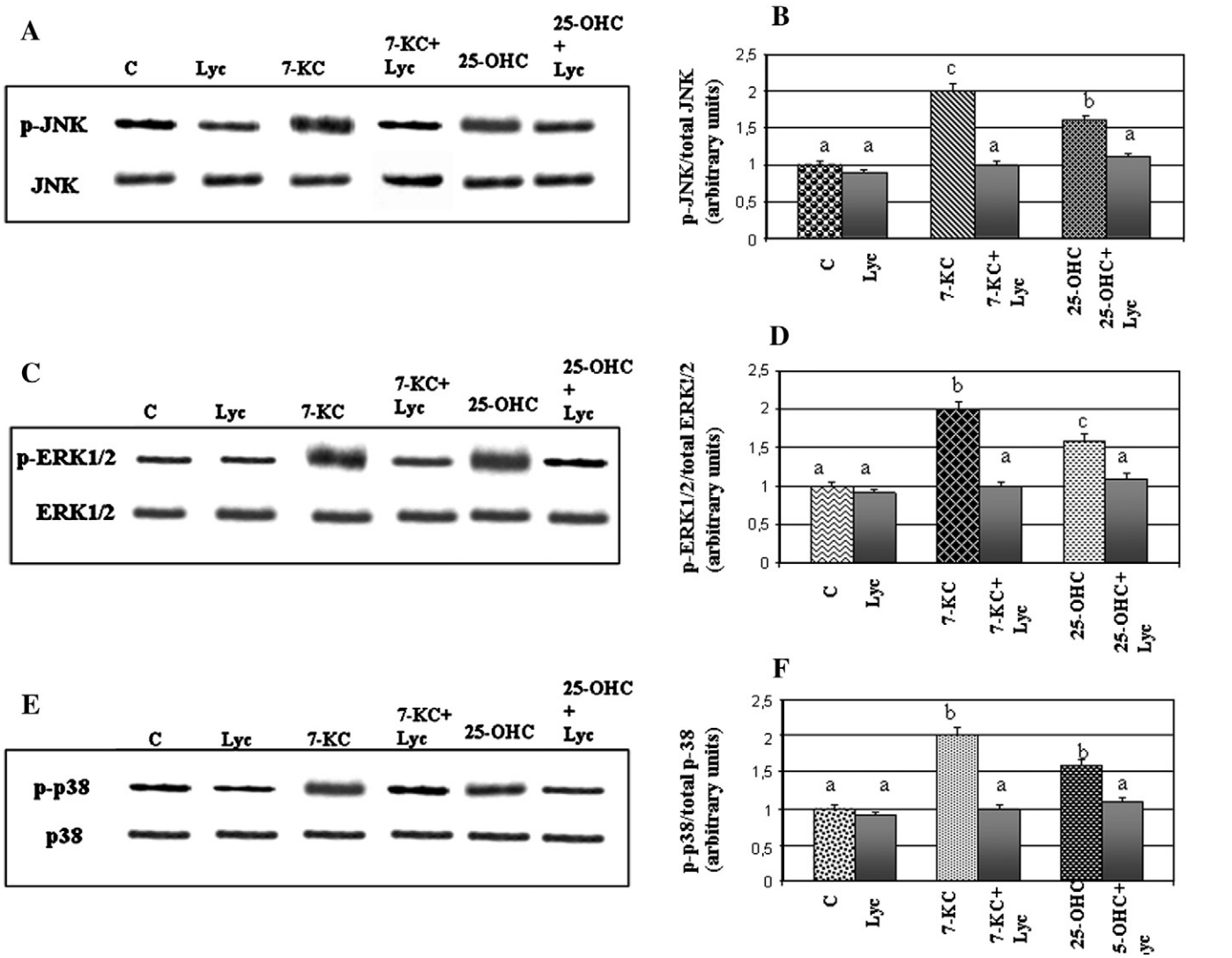

Fig. 6. Effects of lycopene, alone and in combination with 7-KC or 25-OHC, on JNK (panels A, B) ERK1/2 (panels C, D) and p38 (panels E, F) expression in THP-1 cells treated for 3 h. (A, C and E) representative Western blotting analyses. (B, D and F) densitometric analyses. The carotenoid was added at the concentration of 2 μM, 7-KC at 16 μM and 25-OHC at 4 μM. In panels B, D and F, values were the means±S.E.M. of five experiments. Values not sharing the same letter were significantly different ($P<.05$, Fisher's Exact test).

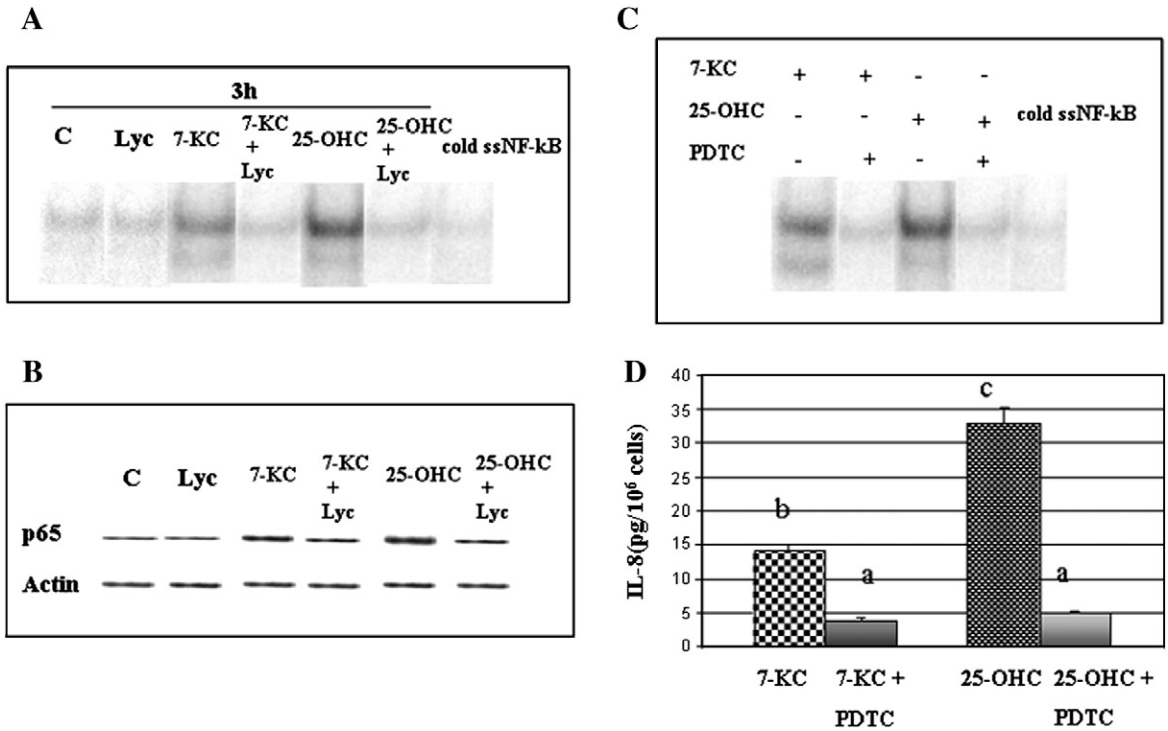

Fig. 7. Effects of lycopene on the induction of NF-κB DNA-binding activity upon a 3h-oxysterol exposure of THP-1 cells. The specificity was demonstrated by using excess unlabeled NF-κB oligonucleotides (=cold ssNF-κB) which competed away binding. (A) The carotenoid was added to the cells at the concentration of 2 μM, 7-KC at 16 μM and 25-OHC at 4 μM. (B) Western blot analysis of NF-κB (p65)-associated proteins in THP-1 cells treated with 2 μM lycopene, 16 μM 7-KC or 4 μM 25-OHC. (C) NF-κB DNA binding activity of oxysterols after addition of PDTC. One representative of three experiments was shown for each panel. (D) IL-8 protein secretion after addition of PDTC. THP-1 cells were pre-incubated with PDTC (10 μM) for 30 min, followed by incubation in the absence or presence of oxysterols for 3 h. In panel D, values were the means±S.E.M. of three experiments. Values not sharing the same letter were significantly different ($P<.05$, Fisher's Exact test).

3.3. Effect of lycopene on PPARγ: interaction with NF-κB

PPARγ activation has been shown to inhibit cytokine production by preventing activation and translocation of NF-κB. Therefore, we measured the levels of PPARγ in THP-1 cells treated in the absence (Fig. 8A) and in the presence of 7-KC (16 μM) or 25-OHC (4 μM) (Fig. 8B), alone and in combination with lycopene. A 3-h treatment with the carotenoid alone increased PPARγ mRNA. The combined treatment with the carotenoid and the oxysterols increased PPARγ levels in a greater extent than the treatment with lycopene or oxysterols alone. The interaction between NF-κB and PPARγ in macrophages following treatment with oxysterols and/or lycopene is demonstrated in Fig. 8C. In these experiments, macrophages were pre-treated with oxysterols for 3 h and then, with lycopene for other 3 h. The extracts were prepared and the complex NF-κB-PPARγ was immunoprecipitated with antibodies against p65, followed by Western blotting with antibodies specific for human PPARγ. The results show that the highest levels of PPARγ were observed following treatment with a combination of oxysterols and lycopene, suggesting that NF-κB induced by oxysterols is associated with PPARγ.

4. Discussion

Several evidences suggest that oxysterols may play key roles in various chronic inflammation-related diseases, including atherosclerosis. In fact, it is generally accepted that vascular areas of atherosclerotic progression are in a state of persistent inflammation, and the association of hypercholesterolemia with atherosclerosis is well established. Given that numerous oxidative events are associated with the development of atherosclerotic plaque, it is now accepted that oxidized LDL (oxLDL) plays a major role in the initiation and promotion of fatty streaks and fibrotic plaques and that oxysterols represent some of the most attractive oxidative and pro-inflammatory agents present in oxLDL. Indeed, 7-KC and 25-OHC, which are major components of oxLDL, are known to contribute to the genesis of atherosclerosis [31] and are found at increased levels in atherosclerotic lesions, and in the plasma of patients with cardiovascular diseases [32]. In fact, the levels of 7-KC and 25-OHC found in plasma in healthy subjects are from 0.022 to 2.0 μM [33,34], while the amount of oxysterols, including 7-KC and 25-OHC, in the aterosclerotic plaque of hypercholesterolemic patients are about 45-fold higher [35].

Our data evidence that both 7-KC and 25-OHC, at concentrations which can be reached in the plaque of hypercholesterolemic subjects, acted as proinflammatory agents by increasing the secretion of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα and by enhancing their mRNA levels (i.e., IL-8). In our experimental conditions, 25-OHC was more potent than 7-KC in stimulating IL-8 production, even though 25-OHC was used at lower concentrations than 7-KC (4 vs. 16 μM). These data are in agreement with previous investigations showing that 25-OHC is extremely potent in stimulating IL-8 production in several cells [13]. On the other hand, in our study, 7-KC seems to act as a potent activator of IL-1β secretion. This observation is in agreement with previous findings, showing that, oxysterols oxidized at C7 play a key role in the stimulation of IL-1β secretion [12]. However, it should be pointed out that increased levels of IL-1β have been also found following 25-OHC treatment, alone and in combination with LPS [36,37]. In U937 and THP-1 cells, stimulation of the secretion of various chemokines involved in the recruitment of immunocompetent cells at the subendothelial level, namely MCP-1, MIP-1β, TNFα, IL-1β and IL-8, was also reported, mainly in the presence of 7β-hydroxycholesterol and 25-hydroxycholesterol [38]. Currently, little is known about the mechanisms involved in oxysterol-induced inflammation at molecular level. Various modes

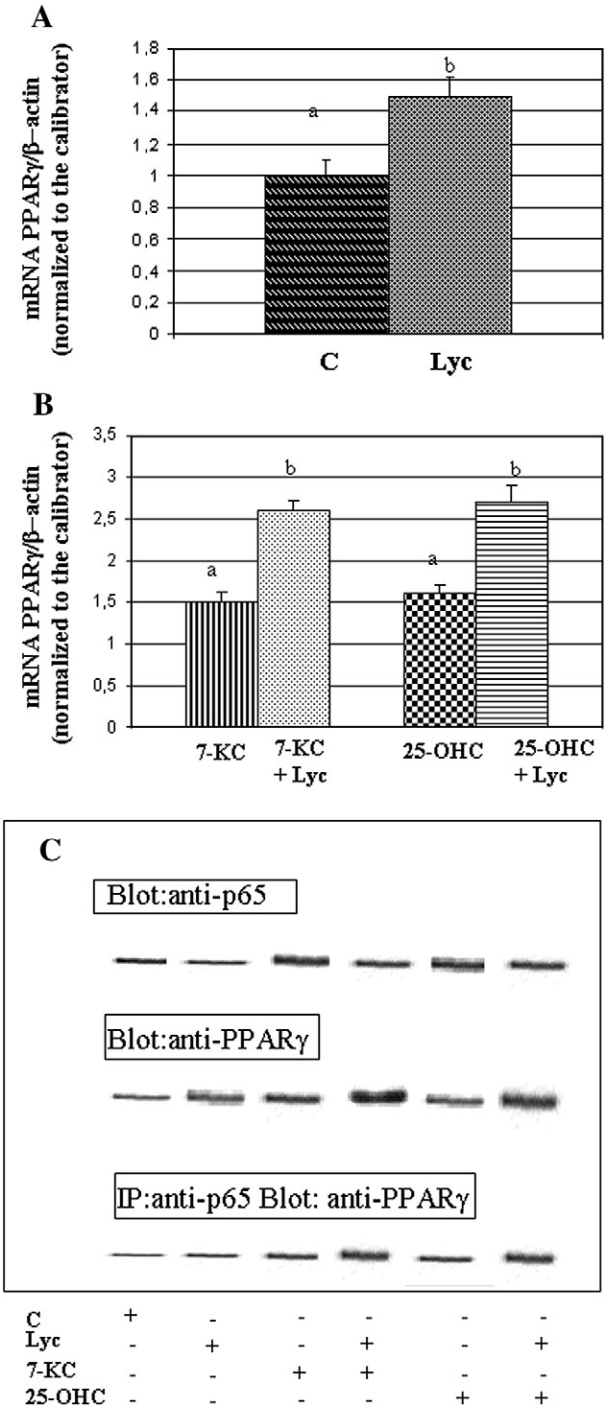

Fig. 8. Effects of lycopene on PPARγ mRNA in THP-1 cells in the absence (panel A) and in the presence of 7-KC or of 25-OHC (panel B). The carotenoid was added at the concentration of 2 μM, 7-KC at 16 μM and 25-OHC at 4 μM for 3 h. The interaction between NF-κB and PPARγ in macrophages following treatment with oxysterols and/or lycopene is demonstrated in panel C. In these experiments, macrophages were pre-treated with oxysterols for 3 h and then with lycopene for another 3 h. The extracts were prepared and the complex NF-κB–PPARγ was immunoprecipitated with antibodies against p65, followed by Western blotting with antibodies specific for human PPARγ. In panels A and B, values were the means±S.E.M. of five experiments. Values not sharing the same letter were significantly different ($P<.05$, Fisher's Exact test).

of regulation of gene expression are probably involved since the activation of liver X receptor (LXR) receptors can only be envisaged with 25-hydroxycholesterol, but not with 7-KC, which is a poor ligand of both LXRα and LXRβ [39]. It has been suggested that oxysterols may increase the levels of ROS, which, at moderate concentrations, may act as second messengers in signal transduction, by modulating redox sensitive MAP kinases and by affecting the activation of redox-sensitive transcription factors such NF-κB. Interestingly, It has been recently shown that IL-8 secretion by oxysterols is associated with activation of the MEK/ERK1/2 signaling pathway [40] and NF-κB has been recently involved as a mediator of oxysterol-induced chemokine production in U937 cells [38].

The efficiency of 7-KC in enhancing ROS production through increase in NOX-4 expression and in activating ERK1/2, p38, JNK has been recently reported in human macrophages [8]. Moreover, other studies show that both 7-KC and 25-OHC, acted as stressor agents, by modulating NADPH oxidase levels [11,41,42]. In our study, 25-OHC was more potent than 7-KC as a stressor agent. However, it is interesting to note that MAPK activation was much greater in cells treated with 7-KC than in cells treated with 25-OHC. This observation suggests that other pathways are implicated in 7-KC-induced MAPK activation. In particular, it has been reported that 7-KC induced ERK1/2 phosphorylation through a calcium-dependent tyrosine kinase [43]. Moreover, in our study, we evidenced that both the oxysterols were able of enhancing the nuclear binding activity of NF-κB.

In this study, we demonstrated for the first time that lycopene was able to inhibit oxysterol-induced proinflammatory cytokine production, at both protein and mRNA levels.

The potential ability of lycopene to influence cytokine levels may be at least in part explained by the fact that it is lipophilic compound able to situate in or within the cell membrane, where surface molecules modulate the primary immune response and where the carotenoid could moderate ROS production and the activity of redox-sensitive kinases and transcription factors, such as NF-κB.

The reduction of pro-inflammatory cytokines by lycopene and/or tomato products has been reported previously in several in vitro [44–46] and in vivo studies [47–53], although IL-1β and TNFα levels were unaffected or even stimulated by the carotenoid in two recent in vitro studies [54,55], and the consumption of tomato products has been reported to be unable in modulating immune functions in elderly humans [56].

According to the hypothesis that proinflammatory cytokine release may be induced by changes in intracellular redox status, we observed that, in human macrophages treated with 7-KC or 25-OHC, lycopene was able to inhibit ROS production as well as to suppress both redox-based MAPK phosphorylation and NF-κB activation. This finding agrees with recent studies showing that lycopene blocked NF-κB signalling [22,57]. These observations suggest that ROS play an important role in NF-κB activation and inflammatory gene expression. In agreement with this suggestion lycopene acted as a powerful antioxidant in our experimental conditions, by preventing the increase in intracellular ROS production induced by the oxysterols. The antioxidant properties of lycopene are well known. Lycopene was found to inactivate several kinds of free radicals, including hydrogen peroxide, nitrogen dioxide [58,59] thiyl and sulphonyl radicals [60]. Moreover, lycopene and tomato products have been reported to inhibit lipid and DNA oxidation [61].

The role of NF-κB activation in oxysterol-induced cytokine stimulation was confirmed further by incubating oxysterol- treated THP-1 cells with PDTC, a chemical that stabilizes the NF-κB/IkB-α complex [62] and inhibits the nuclear translocation of activated NF-κB. The incubation of THP-1 cells with PDTC inhibited the DNA-binding activity of NF-κB induced by both 7-KCHO and 25-OHC and prevented cytokine stimulation. These data clearly indicate the importance of NF-κB in oxysterol-induced cytokine expression in THP-1 cells, although the involvement of other transcription factor(s) cannot be ruled out.

The anti-inflammatory effects of PPARγ activators are well known as well as well as the interactions between PPARγ and NF-κB [63].

PPARγ activation has been shown to inhibit cytokine production by preventing activation and translocation of NF-κB. In our study, lycopene was able to increase PPARγ levels. In accordance with this, a modulation of this transcription factor by the carotenoid has been previously reported [64]. It should be noted that, in our study, an enhancement of PPARγ was also observed in oxysterol-treated cells. This finding is not surprising in view of previous observations, showing that oxLDL induces PPARα and PPARγ activation [65]. Since the interactions between PPARγ and NF-κB [63], it is presumable to think that the enhanced levels of PPARγ induced by oxysterols were not able to adequately counteract the increased levels of NF-κB observed in oxysterol-treated cells. The nuclear interactions between NF-κB and PPARγ in macrophages following treatment with oxysterols and lycopene is shown by coimmunoprecipitation experiments. The results show that the highest levels of PPARγ were observed following treatment with a combination of oxysterols and lycopene, suggesting that NF-κB induced by oxysterols is associated with PPARγ and that PPARγ prevents the activation of NF-κB.

In conclusion, the data reported here support a potential role for lycopene in preventing oxysterol-induced pro-inflammatory cytokine production. We suggest that lycopene exerted such a prevention through a mechanism involving an inhibition of NADPH oxidase, which, in turn, affected ROS production, and redox-sensitive MAPK expression and, consequently, NF-κB activation. Moreover, the carotenoid could also reduce cytokine levels through an induction of PPARγ which further inhibited NF-κB activation. This finding may indicate novel immunosuppressive properties of lycopene, which may be therapeutically useful in controlling chronic immune and/or inflammatory diseases, such as atherosclerosis, pointing out new insight into the molecular mechanisms by which the carotenoid may control atherogenic processes.

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