Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 34–46
Journal of Nutritional Biochemistry

Lycopene prevents 7-ketocholesterol-induced oxidative stress, cell cycle arrest and apoptosis in human macrophages☆

Paola Palozza[a,*], Rossella Simone[a], Assunta Catalano[a], Alma Boninsegna[a], Volker Böhm[b], Kati Fröhlich[b], Maria Cristina Mele[c], Giovanni Monego[d], Franco O. Ranelletti[e]

[a]Institute of General Pathology, Catholic University School of Medicine, 1 00168 Rome, Italy
[b]Institute of Nutrition, Friedrich-Schiller-Universität, 07743 Jena, Germany
[c]Institute of Biochemistry and Clinical Biochemistry, Catholic University School of Medicine, 1 00168 Rome, Italy
[d]Institute of Anatomy, Catholic University School of Medicine, 1 00168 Rome, Italy
[e]Institute of Histology, Catholic University School of Medicine, 1 00168 Rome, Italy

Received 15 May 2008; received in revised form 12 September 2008; accepted 2 October 2008

Abstract

The present study was undertaken to examine whether lycopene is able to counteract 7-ketocholesterol (7-KC)-induced oxidative stress and apoptosis in human macrophages. Human THP-1 macrophages were exposed to 7-KC (10–25 μM) alone and in combination with lycopene (0.5–2 μM), and we monitored changes in cell oxidative status [reactive oxygen species (ROS) production, NOX-4, hsp70 and hsp90 expressions, 8-OHdG formation] and in cell proliferation and apoptosis. After 24 h of treatment, lycopene significantly reduced the increase in ROS production and in 8-OHdG formation induced by the oxysterol in a dose-dependent manner. Moreover, the carotenoid strongly prevented the increase of NOX-4, hsp70 and hsp90 expressions as well as the phosphorylation of the redox-sensitive p38, JNK and ERK1/2 induced by the oxysterol. The attenuation of 7-KC-induced oxidative stress by lycopene coincided with a normalization of cell growth in human macrophages. Lycopene prevented the arrest in G0/G1 phase of cell cycle induced by the oxysterol and counteracted the increased expression of p53 and p21. Concomitantly, it inhibited 7-KC-induced apoptosis, by limiting caspase-3 activation and the modulatory effects of 7-KC on AKT, Bcl-2, Bcl-xL and Bax. Comparing the effects of lycopene, β-carotene and (5*Z*)-lycopene on ROS production, cell growth and apoptosis show that lycopene and its isomer were more effective than β-carotene in counteracting the dangerous effects of 7-KC in human macrophages. Our study suggests that lycopene may act as a potential antiatherogenic agent by preventing 7-KC-induced oxidative stress and apoptosis in human macrophages.

Keywords: Lycopene; 7-Ketocholesterol; Oxidative stress; Cell cycle; Apoptosis

1. Introduction

Oxysterols constitute an important family of molecules resulting from the autooxidation of cholesterol in air, enzymatic transformation of cholesterol in various cell species [1] or low-density lipoprotein (LDL) cholesterol peroxidation [2]. Oxysterols accumulate in the subendothelial level of the arterial wall during the atheromatous process [3,4] and are believed to mediate the development of atherosclerosis [5]. At the present time, a large number of studies have suggested that oxysterols are involved in the initiation and progression of atherosclerosis [6], according to their wide range of biological activities. Indeed, oxysterols are frequently present at high levels in the atherosclerotic plaque [7] and they have been reported to induce IL-1β secretion in vascular endothelial cells and, consequently, the expression of adhesion molecules necessary for the recruitment of monocytes and T lymphocytes found in atherosclerotic plaques [8]. Therefore, potential roles of these molecules in the initiation of atherogenic process have been suggested [9]. Moreover, oxysterols have been reported to be

☆ Supported by the European Community's Sixth Framework Programme (IP 016213).
* Corresponding author. Institute of General Pathology, Catholic University School of Medicine, 1 00168 Rome, Italy. Tel.: +39 06 3016619; fax: +39 06 3386446.
E-mail address: p.palozza@rm.unicatt.it (P. Palozza).

0955-2863/$ – see front matter.
doi:10.1016/j.jnutbio.2008.10.002

strongly toxic to a number of tumoral and normal cell species, including those of the vascular wall [10]. Among the oxysterols of pathophysiological interest, there is 7-ketocholesterol (7-KC), which is oxidized at position 7. This compound has been reported to be present in large amounts in atheromatous plaques from hypercholesterolemic subjects at different stages of development [11]. Moreover, it has been shown to act as a potent inducer of apoptosis in vascular cells, suggesting its critical role in atherosclerosis [12,13].

Some epidemiological studies have shown an inverse correlation between lycopene consumption (notably through tomatoes) and incidence of atherosclerosis [14,15]. These results, together with an understanding of the contribution of excessive free-radical generation in the onset of the disease, led to the hypothesis that high intake of tomato-derived carotenoids, such as lycopene, might play a preventive role in the development of atherosclerotic disease [16]. Lycopene, the acyclic form of β-carotene, is one of the major carotenoids in the Western diet. It accounts for about 50% of carotenoids in human serum [17]. The antioxidant protective effect of lycopene and tomatoes has been shown in vitro as well as in vivo [18–22], although negative results have been also reported [23,24]. 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 [25] and to act as a potent antioxidant, preventing the oxidative damage of critical biomolecules including lipids, LDLs, proteins and DNA [26]. Moreover, recently, the carotenoid has been shown to modulate redox-sensitive molecular pathways involved in cell proliferation and apoptosis [27].

In this study, we speculate that lycopene may counteract oxidative and apoptotic damages triggered by 7-KC, affecting the growth of vascular cells and, presumably, preventing the formation and the development of atheromatous plaque. To this purpose, human THP-1 macrophages were treated with 7-KC alone and in combination with lycopene, in a range of carotenoid concentrations achievable in vivo after supplementation (0.5–2 μM). It is well recognized that macrophages play important roles in the atherosclerotic process since increased concentrations of oxysterols in macrophage foam cells, resulting from the phagocytosis of oxidized LDL (OxLDL), may lead to an arrest of cell proliferation, to an induction of cell death and, consequently, to plaque rupture [28,29]. In these cells, the effects of lycopene on 7-KC-induced oxidative stress, cell cycle arrest and apoptosis were 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, nonessential amino acids, 2 mM glutamine and 1 mM sodium pyruvate. Cells were maintained in log phase by seeding twice a week at a density of 3×10^8 cells/L at 37°C under 5% CO_2/air atmosphere. 7-KC and lycopene (LycoRed Natural Products Industries Ltd., Beer Sheva, Israel) were delivered to the cells (10^9 cells/L) using THF[4] as a solvent. The solvent used in lycopene experiments contained 0.025% butylated hydroxytoluene to avoid the formation of peroxides. 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. The amount of THF added to the cells was not greater than 0.5% (v/v). Control cultures received an amount of solvent (THF) equal to that present in 7-KC- and lycopene-treated ones. No differences were found between cells treated with THF and untreated cells in terms of cell number, viability and reactive oxygen species (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 hemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

2.2. 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, Inc., Eugene, OR) as described in Ref. [30]. Before the addition of the fluorescent probes, 2×10^6 cells were washed to eliminate the amount of lycopene that is 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 Corp., Bedford, MA). Lycopene did not alter the basal fluorescence of DCF.

2.3. Assays for 8-OHdG

Cytospin samples were prepared as follows: cells were diluted in sucrose buffer (0.25 M sucrose, 1.8 mM $CaCl_2$, 25 mM KCl and 50 mM Tris, pH 7.5) at a density of about 3.5×10^9 cells/L. A total of 50 μl was added to carbowax–ethanol buffer (carbowax stock: 77 ml of PEG 1000 in 50 ml of water, 1 ml of stock in 74 ml of 70% ethanol) (Sigma Italia, Milan, Italy) and mixed. Aliquots of 150 μl were placed into cytospin funnels and centrifuged at 300 rpm for 5 min on slides coated with aminopropyltriethoxysilane (Kindler, Freiburg, Germany). Samples were air dried for 10–30 min, fixed in 95% cold ethanol (−20°C) for 10 min and stored at −20°C. Detection of 8-OHdG by immunohistochemistry coupled with DAB (Vector, Burlingame, CA) was carried out essentially as described by Yarborough et al. [31]. 1F7 monoclonal antibody for 8-OHdG was kindly provided by Dr. R.M. Santella, Columbia School of Public Health, New York. Semiquantitative evaluation of the staining was carried

out with the use of an optical microscope (ECLIPSE E600, Nikon, at ×400) connected to an Image-Pro Plus Version 4.1 (Media Cybernetics, USA). Nuclear staining was evaluated in approximately 100 cells of randomly chosen images by operators who were blind to the status of cell treatment, as recommended in Ref. [26]. Negative and positive controls (untreated and 0.5 mM H_2O_2-treated cells, respectively) were included within each batch of slides. Data are reported as units of optical density. Detection of 8-OHdG by an HPLC-ECD method [32] validated the results obtained by immunohistochemical analysis.

2.4. Cell cycle analysis

Cell cycle stage was analyzed by flow cytometry. Aliquots of 10^6 cells were harvested by centrifugation, washed in phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol and treated with 1 g/L RNase for 30 min. Propidium iodide was added to a final concentration of 50 g/L. Data were collected, stored and analyzed using MultiCycle software (MultiCycle for Windows, Phoenix, Flow Systems, San Diego, CA).

2.5. Apoptosis detection

The percentage of apoptotic cells was determined by TUNEL [33]. Briefly, cells were centrifuged, fixed with acetone and incubated for 5 min with the hybridization buffer (Boehringer-Mannheim, Germany). Then, 2.5 units of terminal deoxynucleotidyltransferase (Tdt) and 100 pmol biotin-dUTP in hybridization buffer were added and incubated for 1 h at 37°C. Thereafter, the cells were incubated with streptavidin–biotin–peroxidase complex for 30 min at room temperature. The sites of peroxidase binding were detected with diaminobenzidine. The percentage of TUNEL-positive apoptotic cells (labeling index, LI%) was counted at ×400 magnification. In the absence of Tdt, no unspecific staining was observed. For each slide, three randomly selected microscopic fields were observed and at least 100 cells per field were evaluated.

The activity of caspase-3 was measured by the fluorimetric assay as described in Ref. [33]. Briefly, cells were incubated for the indicated times and then harvested. Cells (2×10^6) were lysed in 50 mM Tris–HCl buffer, pH 7.5, containing 0.5 mM EDTA, 0.5% IGEPAL and 150 mM NaCl. Cell lysates were incubated with 50 μM fluorigenic substrate, Ac-DEVD-AMC (caspase-3; Alexis Biochemicals), in a reaction buffer (10 mM Hepes, pH 7.5, containing 50 mM NaCl and 2.5 mM DTT) for 120 min at 37°C. The release of AMC was measured with excitation at 380 nm and emission at 460 nm using a fluorescence spectrophotometer.

2.6. Western blot analysis of NOX-4, hsp70, hsp90, p53, p21, AKT, Bax, Bcl-xL, Bcl-2, p38 and p-p38, ERK1/2, pERK1/2, JNK and p-JNK expression

Cells (10×10^6) were harvested, washed once with ice-cold PBS and gently lysed for 30 min in ice-cold lysis buffer (1 mM $MgCl_2$, 350 mM NaCl, 20 mM Hepes, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM $Na_4P_2O_7$, 1 mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 1 mM Na_3VO_4, 20% glycerol and 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-p53 (clone DO-1, Cat. No. SC-126), anti p-21 (clone F-5, Cat. No. SC-6246), anti-AKT (clone B-1, Cat. No.5298), anti-Bax (clone P-19, Cat. No. SC-526), anti-Bcl-2 (clone Bcl-2/100/D5), anti-Bcl-xL S/L (clone L-19, Cat. No. SC-1041), 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-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), as well as anti-hsp70 (clone K-20, Cat No. SC-1060) and anti-hsp90 (clone C-20, Cat. No. 8262). The blots were washed and exposed to horseradish-peroxidase-labeled secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL) for 45 min at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantified by densitometric scanning.

2.7. Extraction and analysis of 7-KC

7-KC was extracted with 1 vol methanol and 3 vol hexane from 10×10^6 cells after a 24-h treatment with 25 μM 7-KC. The compound was analyzed by HPLC, by using a cyano-bonded column (25 cm×4.6 mm, 5 μm) (Supelco, Bellefonte, PA) with a 2-cm precolumn (Supelco) and hexane-2-propanol (95:5, v/v) at a flow rate of 1.0 ml/min. 7-KC was detected by UV spectrophotometry at 234 nm. The concentration of the compound in cells was calculated from a calibration curve generated from peak height of 7-KC in calibration samples, as described in Ref. [34].

2.8. Isolation of (5Z)-lycopene

(5*Z*)-Lycopene was isolated by using preparative C_{30} chromatography and was bought at CaroteNature (Lupsingen, Switzerland). The (5*Z*)-lycopene was isolated at 16±1°C using an HPLC pump model L-7100 (Merck, Darmstadt, Germany), detector model Lambda 1000 (Bischoff, Leonberg, Germany) and integrator model Chromatopac C-R6A (Shimadzu, Duisburg, Germany). For separation, a preparative C_{30} (300×10.0 mm, 5 μm) column (YMC Europe, Schermbeck, Germany), preceded by a C_{18} ProntoSil 120-5-C18 H (10×4.0 mm, 5 μm) column (Bischoff), was used. A mixture of methanol and methyl *tert*-butyl ether (MTBE) (6:4, v/v) constituted a mobile phase at a flow rate of 4.0 ml/min; the detection wavelength was 450 nm. Due to the limited stability of the isolated isomer, all steps had to be completed rapidly and under subdued light. The mixture of lycopene isomers (prepared by iodine isomerization, see Ref. [35]) was dissolved in methanol/MTBE (6:4, v/v). The separation was carried out with a mobile phase consisting of

a mixture of methanol and MTBE (6:4, v/v). The eluate containing the (5*Z*)-lycopene was concentrated under vacuum at room temperature in a rotary evaporator. The residue of the solvent was dried under a nitrogen flow at room temperature. The purity of the separated isomer was checked by means of an analytical C_{30} HPLC-DAD [35] and found to range between 95% and 100%.

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. 1B, 3B and 7). When significant values were found ($P<.05$), post hoc comparisons of means were made using Honestly Significant Difference test. One-way ANOVA was used to determine differences between different concentrations and treatments in Figs. 1A, C and E; 2; 3A, C and D; 4B and D; 5B and D–F; and 6B, D and F and Table 1. 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, Inc., State College, PA).

3. Results

3.1. Cellular incorporation of 7-KC in the absence and in the presence of lycopene

The content of 7-KC in THP-1 macrophages was found to be 4.10±0.40 μg/10^6 cells after a 24-h treatment with 7-KC (25 μM). The combined addition of lycopene (2 μM) and 7-KC (25 μM) to the cells did not significantly modify the incorporation of the oxysterol, which resulted to 4.07±0.40 μg/10^6 cells after a 24-h treatment.

3.2. Increased oxidative stress by 7-KC and prevention by lycopene

3.2.1. ROS production and NOX-4 expression

The incubation of THP-1 with 7-KC induced a significant increase of intracellular ROS production, as shown by the

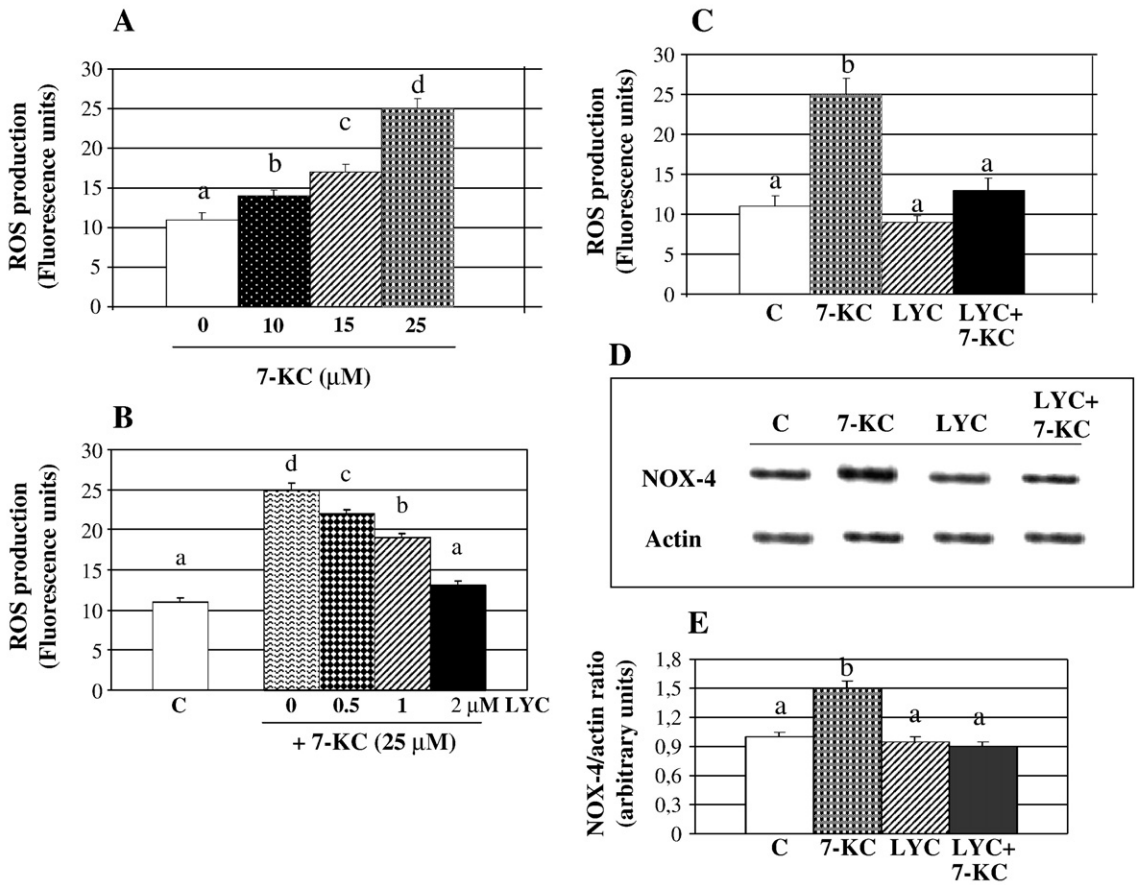

Fig. 1. ROS production (A–C) and NOX-4 expression (D and E) in THP-1 macrophages treated with 7-KC alone and in combination with lycopene for 24 h. Panel A shows the effects of different concentrations of the oxysterol; Panel B shows the effects of different concentrations of lycopene in the presence of 25 μM 7-KC; Panel C shows the effects of 7-KC (25 μM) and lycopene (2 μM), alone and in combination; Panel D shows a representative Western blot analysis; and Panel E shows the densitometric analyses of five different immunoblots of NOX-4 in cells treated with 7-KC (25 μM) and/or lycopene (2 μM). Values were the means±S.E.M., $n=5$. Values not sharing the same letter were significantly different ($P<.05$, Fisher's test) (A, C and E). In Panel B, the treatment/concentration interaction was significant ($P<.05$). Values not sharing the same letter were significantly different ($P<.002$, Tukey's test).

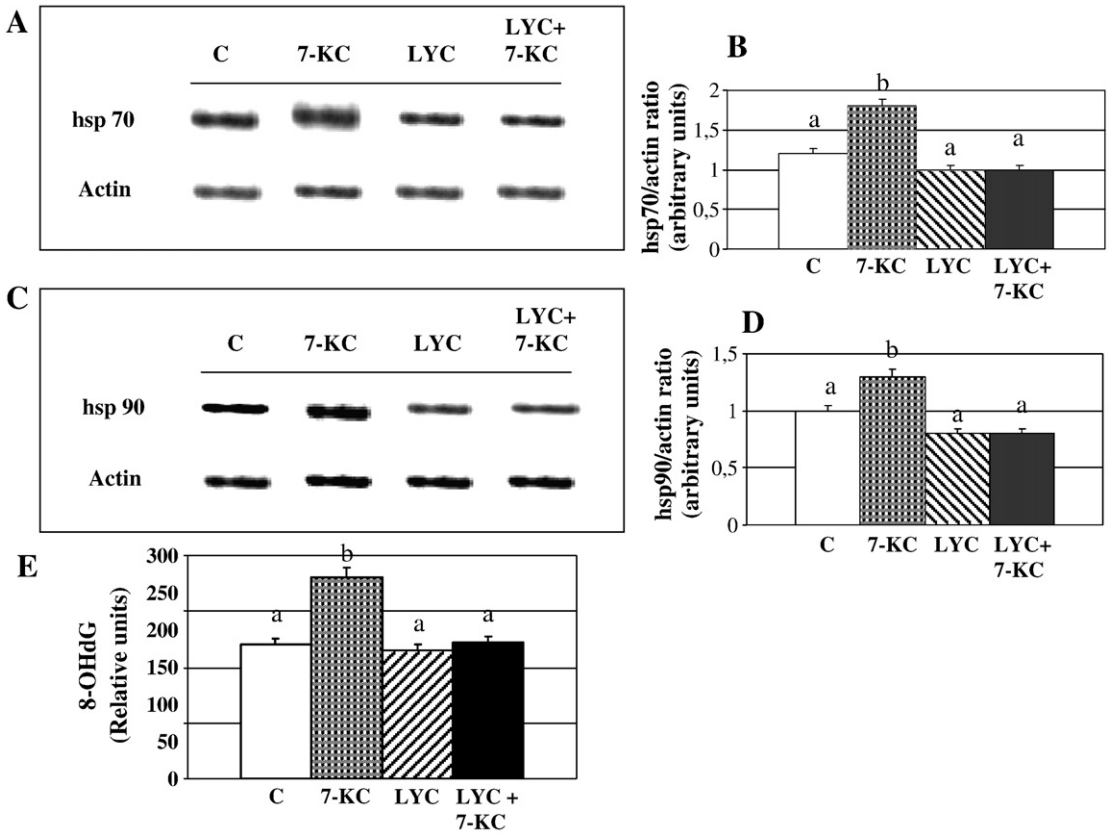

Fig. 2. Effects of 7-KC (25 μM) and lycopene (2 μM) on the expression of hsp70 (A and B), hsp90 (C and D) and 8-OHdG formation (E) in THP-1 macrophages treated for 24 h. (A and C) Representative Western blot analyses. (B and D) Densitometric analyses. The values were the means±S.E.M., n=5. In Panel A, values not sharing the same letter were significantly different (P<.05, Fisher's test).

increased fluorescence obtained by DCF with respect to internal control (i.e., macrophages incubated in the presence of the vehicle alone). Such an effect was dose dependent and it is shown after 24 h of incubation (Fig. 1A), but it can be observed precociously, starting from 3 h of treatment (data not shown). The addition of lycopene to cells treated with 7-KC at the concentration of 25 μM prevented intracellular ROS production after 24 h of treatment in a dose-dependent manner (Fig. 1B). This prevention was complete following the addition of lycopene at the concentration of 2 μM (Fig. 1C). On the other hand, cells treated with lycopene alone (2 μM) only showed a slight but not significant decrease in DCF fluorescence (Fig. 1C).

We also measured the expression of NOX-4 (Fig. 1D and E), one of the homologues sharing with NOX-2 putative NAD(P)H and flavin-binding sites, as well as functional oxidase activity that produces the superoxide anion and which is expressed in a large variety of cell types. 7-KC induced a robust up-regulation (twofold from the basal level) of NOX-4. This effect was prevented by the concomitant addition of lycopene (2 μM).

3.2.2. hsp expression and oxidative DNA damage

Due to the pathogenic features of oxysterols, and because the inducible form of hsp70 and hsp90 can be viewed as markers of cells responding to toxic stimuli [36], we evaluated whether 7-KC could increase the expression of these two proteins and whether lycopene could counteract such an effect in human macrophages (Fig. 2A–D). THP-1 cells were incubated with 7-KC alone, at the concentration of 25 μM, and in combination with lycopene, at the concentration of 2 μM, for 24 h. In agreement with the previous results, the carotenoid completely prevented the increase in both hsp70 and hsp90 induced by the oxysterol. Such an effect was dependent on the dose of carotenoid (data not shown).

In humans, the presence of 8-OHdG has also been identified in aorta fragments from patients suffering from severe atherosclerotic lesions and in lymphocytes of patients with high atherosclerotic risks [37], but factors that induce oxidative stress and DNA damage have not yet been identified. Therefore, we asked whether or not 7-KC could have side effects leading to the formation of 8-OHdG, which is considered to be a premutagenic DNA lesion [38] and whether lycopene could be able to prevent such a dangerous effect (Fig. 2E). The oxidative DNA damage was revealed by the presence of 8-OHdG. THP-1 cells were incubated with 7-KC alone, at the concentration of 25 μM, and in combination with lycopene, at the concentration of 2 μM, for 24 h. As it can be observed, the formation of 8-OHdG was significantly

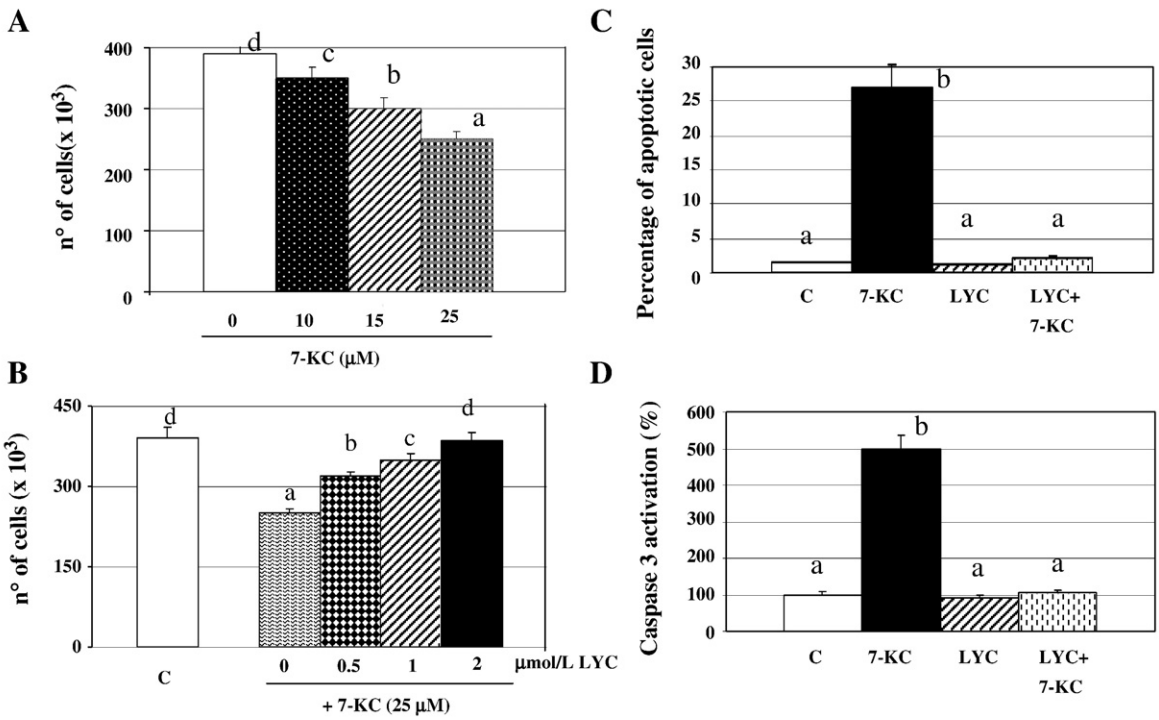

Fig. 3. Cell growth (A and B) and apoptosis (C and D) of THP-1 macrophages treated with 7-KC and/or lycopene for 24 h. Panel A shows the effects of different concentrations of the oxysterol; Panel B shows the effects of different concentrations of lycopene in the presence of 25 μM 7-KC; Panels C and D show the effects of 7-KC (25 μM) and lycopene (2 μM), alone and in combination, on apoptosis by TUNEL method and caspase-3 activation, respectively. Values were the means±S.E.M., n=5. Values not sharing the same letter were significantly different (P<.05, Fisher's test) (A, C and D). In Panel B, the treatment/concentration interaction was significant (P<.05). Values not sharing the same letter were significantly different (P<.001, Tukey's test).

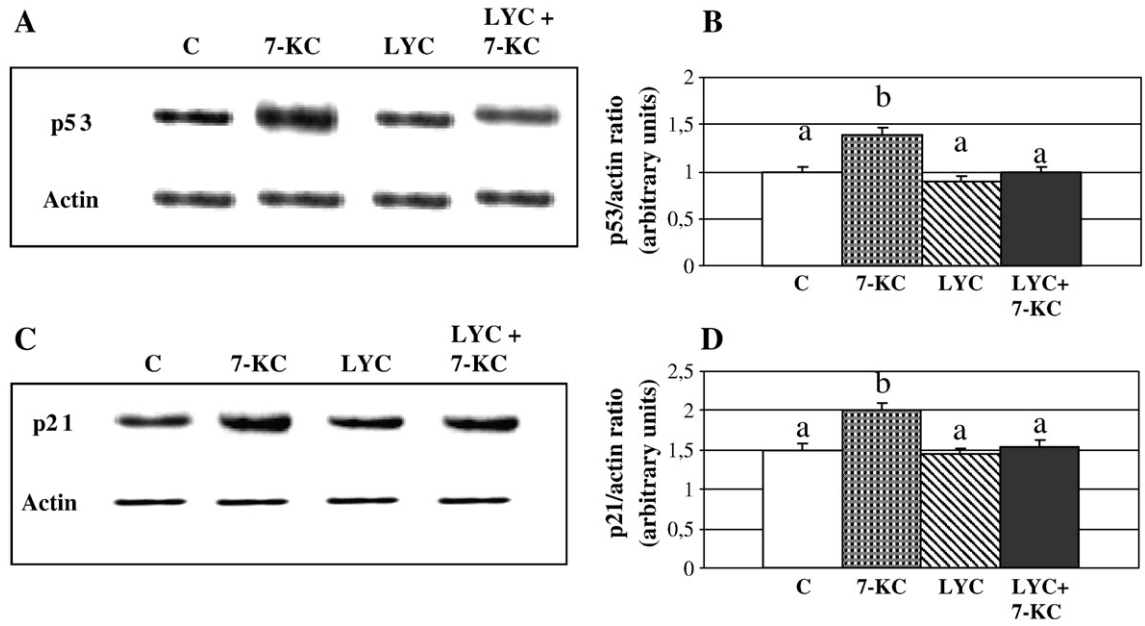

Fig. 4. Effects of 7-KC (25 μM) and lycopene (2 μM) on the expression of p53 (A and B) and p21 (C and D) in THP-1 macrophages treated for 24 h. (A and C) Representative Western blot analyses. (B and D) Densitometric analyses. The values were the means±S.E.M., n=5. In Panels B and D, values not sharing the same letter were significantly different (P<.05, Fisher's test).

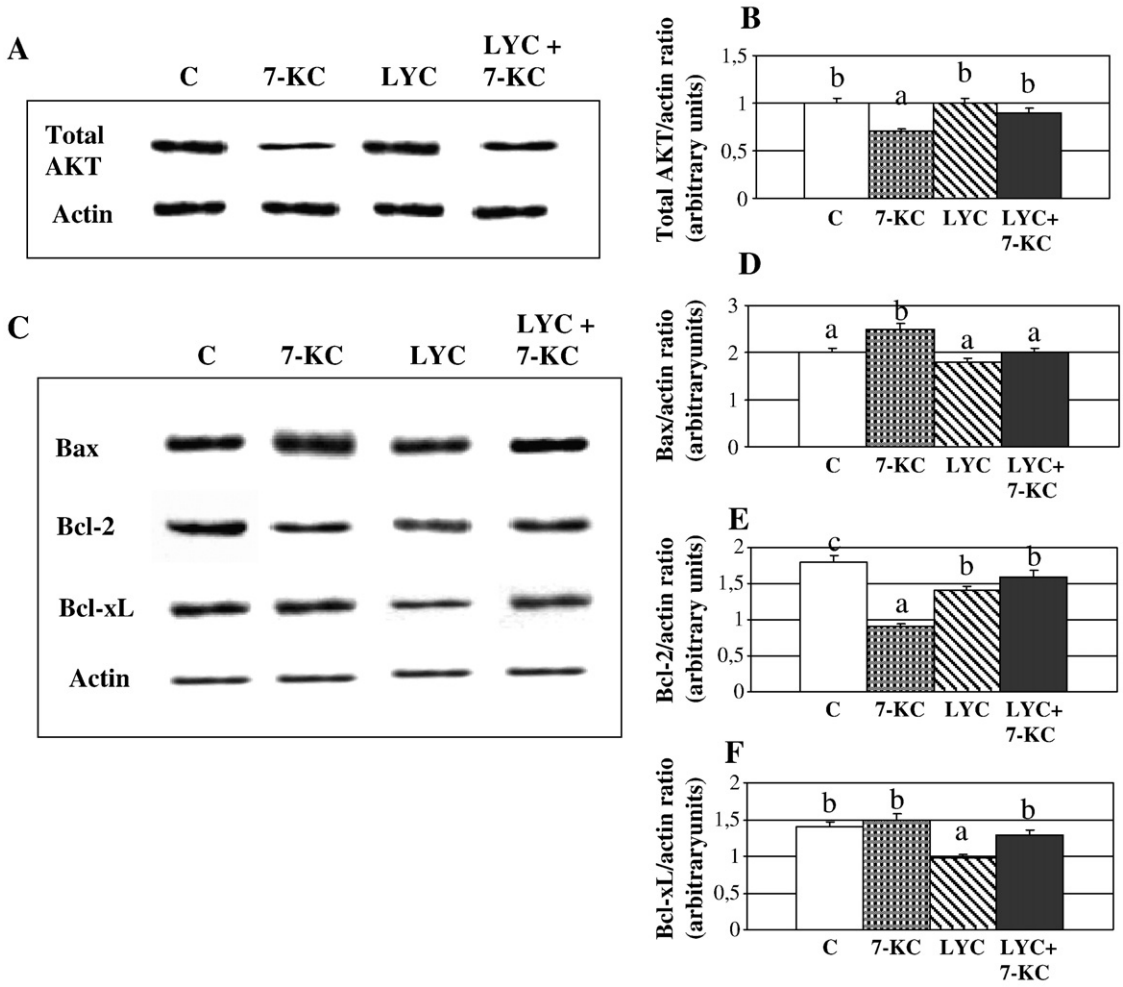

Fig. 5. Effects of 7-KC (25 μM) and lycopene (2 μM) on AKT, Bax, Bcl-2 and Bcl-xL expression, in THP-1 macrophages treated for 24 h. (A and C) Representative Western blot analysis. (B and D–F) Densitometric analyses. Values were the means±S.E.M., n=5. Values not sharing the same letter were significantly different (P<.05, Fisher's test).

higher in 7-KC-treated cells than in untreated cells. However, lycopene, at this concentration, was completely able to counteract the effect of the oxysterol on oxidative DNA damage.

3.3. Effects of 7-KC on cell growth and modulatory effects by lycopene

3.3.1. Cell cycle progression and apoptosis

The characterization of the effects of 7-KC on the growth of THP-1 cells was performed at different concentrations (0–25 μM) (Fig. 3A) after 24 h of incubation. At this time, cell number was decreased in a dose-dependent manner in cells treated with 7-KC, as compared with the control cells (Fig. 3A). When THP-1 cells were simultaneously incubated with 7-KC (25 μM) and lycopene for 24 h in a range of carotenoid concentrations, which is achievable in vivo after supplementation (0.5–2 μM) (Fig. 3B), we observed that the carotenoid counteracted cell growth inhibition induced by 7-KC in a dose-dependent manner. The inhibitory effect of 7-KC on cell growth was completely prevented by the addition of the carotenoid at 2 μM. On the other hand, the carotenoid alone, in a range of concentrations that goes from 0.5 to 2 μM, was ineffective in modifying the growth of THP-1 cells (data not shown).

To elucidate the mechanism(s) responsible for the reduction of cell number by 7-KC and the concomitant prevention exerted by the carotenoid on this effect, we first examined possible changes in cell cycle progression. Table 1 shows the distribution of THP-1 cells in the different cell cycle phases after 24 h of 7-KC (25 μM) and lycopene (2 μM) treatment, alone and in association, with respect to vehicle-control-treated cells. In the presence of 7-KC, a significant increase in the percentage of cells in the G0/G1 phase was observed. Such an effect was accompanied by a concomitant decrease in the percentage of cells in the S phase. Interestingly, the carotenoid alone, at least at this concentration, was unable to modify cell cycle progression of THP-1 with respect to control cells. However, it completely prevented the arrest in cell cycle progression exerted by the oxysterol.

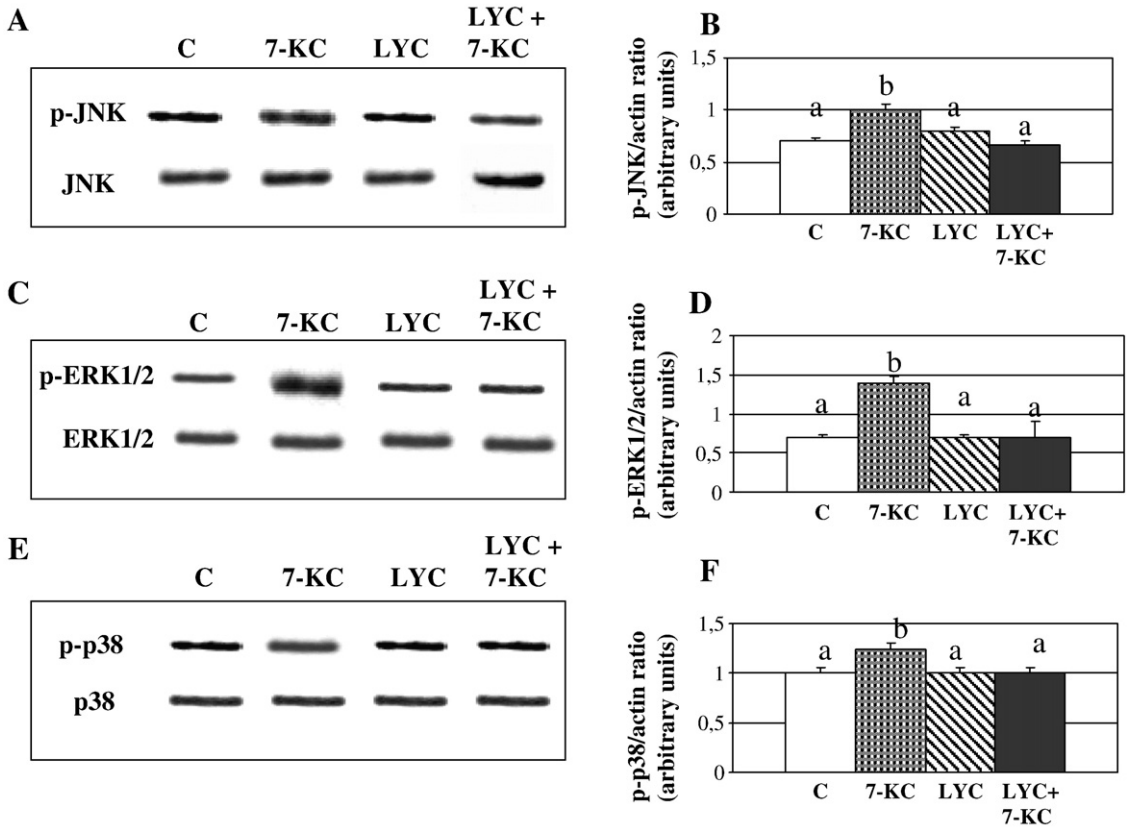

Fig. 6. Effects of 7-KC (25 μM) and lycopene (2 μM) on the expression of phosphorylated JNK (A and B), ERK1/2 (C and D) and p38 (E and F) in THP-1 macrophages treated for 24 h. (A, C and E) Representative Western blot analyses. (B, D and F) Densitometric analyses. The values were the means±S.E.M., n=5. In Panels B, D and F, values not sharing the same letter were significantly different (P<.05, Fisher's test).

We also measured apoptosis induction following treatment with 7-KC and lycopene, alone and in association, in THP-1 cells. A proapoptotic action of 7-KC (25 μM) was found in THP-1 cells treated with the oxysterol for 24 h. Exposure to 7-KC led to a significant increase in apoptosis induction, as shown by both the percentage of apoptotic cells measured by TUNEL method (Fig. 3C) and the activation of caspase-3 (Fig. 3D). On the other hand, the concomitant addition of lycopene at the concentration of 2 μM to cells treated with the oxysterol under the same experimental conditions exerted a complete protection on apoptosis induction.

Table 1
Effects of 7-KC and lycopene (LYC) on cell cycle progression in THP-1 macrophages

Treatment	Cell cycle (24 h)		
	G0/G1	S	G2
C	57.9±2.0[a]	25.0±1.4[b]	17.1±1.5[a]
LYC*	58.1±2.3[a]	24.6±1.2[b]	17.3±1.0[a]
7-KC[#]	68.1±2.5[b]	13.9±1.3[a]	18.0±1.9[a]
LYC*+7-KC[#]	57.7±2.1[a]	25.2±1.1[b]	17.1±0.5[a]

The values were the means±S.E.M. of three experiments. [#]7-kC=25 μM; *LYC=2 μM. Within the same column, values not sharing the same letter were significantly different (P<.05, Fisher's test).

3.3.2. Cell-cycle- and apoptosis-related proteins

In an attempt to explore the effects of 7-KC and lycopene on cell-cycle-regulating proteins, we measured the expression of p53 and p21 in THP-1 cells, following a 24-h exposure to the oxysterol (25 μM) and to the carotenoid (2 μM), alone and in combination (Fig. 4A–D). Lycopene alone did not significantly modify the expression of both p53 and p21 with respect to control cells, whereas 7-KC alone remarkably increased both of them. However, such an effect was almost completely prevented by the combined addition of the carotenoid to the cells.

AKT has been well characterized as an antiapoptotic kinase that transduces cellular survival signals in many cell types. AKT is believed to play a critical role in the survival of macrophages. We therefore examined the effect of a 24-h treatment on the expression of AKT in THP-1 cells (Fig. 5A and B). The results clearly indicate that AKT was down-regulated in THP-1 cells in response to treatment with 7-KC for 24 h. Interestingly, such an effect was completely reverted by the addition of lycopene at 2 μM. In an effort to investigate molecular pathways involved in apoptosis induction by 7-KC and the concomitant protection exerted by lycopene on this process, we also examined the effects of these two compounds on the expression of the apoptosis promoter protein Bax and on the expression of the apoptosis-

blocking proteins Bcl-2 and Bcl-xL (Fig. 5C–F). Bax expression was significantly increased, whereas Bcl-2 and Bcl-xL levels were remarkably decreased by a 24-h treatment with 7-KC in THP-1 cells. Such effects were completely counteracted by the concomitant addition of lycopene.

3.3.3. p38, JNK and ERK1/2

We also examined the expression of the p38, JNK and ERK1/2 mitogen-activated protein (MAP) kinases (Fig. 6). These kinases have been reported to be activated by various stress stimuli, including an overproduction of ROS, and they have been also implicated in apoptosis induction. 7-KC (25 μM) induced a remarkable increase in the level of the phosphorylated forms of p38 (p-p38), JNK (p-JNK) and ERK1/2 (p-ERK1/2) after 24 h of incubation. Such increases were all prevented by the addition of lycopene (2 μM).

3.3.4. Comparison of the effects of lycopene, β-carotene and lycopene (5Z)-isomer on ROS production and cell growth in THP-1 cells exposed to 7-KC

We also compared the ability of lycopene with those of (5*Z*)-lycopene and β-carotene in modulating ROS production (Fig. 7A), cell growth (Fig. 7B) and apoptosis (Fig. 7C) in THP-1 macrophages. 7-KC (25 μM) was added to macrophages in the absence or in the presence of different concentrations of carotenoids (0.5–2 μM) for 24 h. Lycopene and its isomer were more effective than β-carotene, added at the same concentration, in preventing 7-KC-induced ROS production, cell growth inhibition and apoptosis induction.

4. Discussion

It is now well accepted that oxysterols, including 7-KC, play important roles in atherosclerosis [9]. Indeed, 7-KC has been found to be abnormally elevated in the plasma and atherosclerotic plaques of hypercholesterolemic patients [8,39,40] and increased plasma levels of oxysterols were recently associated with an increased risk of atherosclerosis in humans [41,42]. Moreover, the ability of some oxysterols to trigger prooxidative reactions can be considered an important event in vascular dysfunction and atherogenesis [43,44].

In the present study, we reported that 7-KC is able to induce a strong oxidative stress in human macrophages, by increasing ROS production through NOX-4 up-regulation,

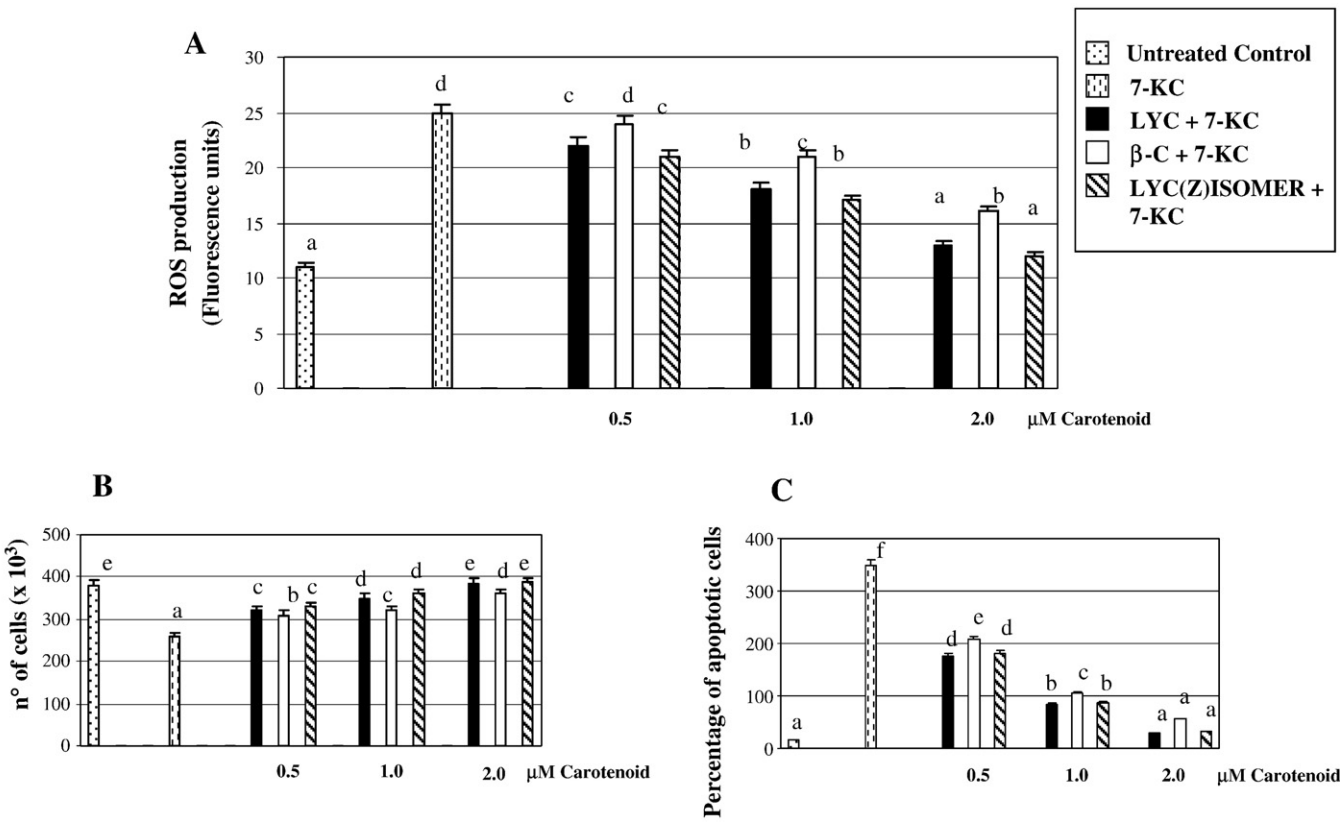

Fig. 7. Effects of lycopene, β-carotene and lycopene (5*Z*)-isomer on ROS production (A), cell growth (B) and apoptosis (C) in THP-1 macrophages treated with 7-KC. 7-KC (25 μM) was added to macrophages in the absence or in the presence of different concentrations of carotenoids (0.5–2 μM) for 24 h. Values were the means±S.E.M., n=4. The treatment/concentration interaction was significant (P<.05). Values not sharing the same letter were significantly different (A: P<.003; B: P<.001, C: P<.002, Tukey's test).

by enhancing the expression of heat shock proteins and oxidative DNA damage and by altering redox-sensitive MAP kinases.

The specific effects of 7-KC on ROS production have been recently reported by us and by other authors [12,45], and they seem to be mediated by a robust up-regulation of NADPH oxidase [12]. In fact, we recently observed that the selective inhibitor of NADPH oxidase diphenyleneiodonium or the NOX-4 siRNA was able to completely prevent 7-KC-dependent ROS increase [12].

De novo synthesis of stress proteins is generally regarded as a defense response triggered by a number of toxic stimuli [36,44]. Among stress proteins, hsp70 and hsp90 are highly inducible and are thought to represent an early response of stressed cells to adverse conditions [46,47]. In particular, hsp70 has been detected in areas of atherosclerotic lesions [48], suggesting that sites of increased hsp expression during plaque evolution may represent areas of the arterial wall undergoing cytotoxic stress [49]. It has been reported that OxLDLs induce the expression of hsp70 in cultured human endothelial cells [49] and vascular smooth muscle cells [50], and it has been hypothesized that this event may represent a cytoprotective response to OxLDL cytotoxicity. Since oxysterols account for most of OxLDL cytotoxicity [50,51], it is reasonable to speculate that they are responsible for the damage to vascular cells. This hypothesis is strongly supported by our data, showing that 7-KC was responsible for a remarkable increase in hsp70 and hsp90 expressions in human macrophages.

Considering the high overproduction of ROS occurring in the presence of 7-KC, it was also of interest to identify the effects of these compounds on oxidative DNA damage, which is a chemical modification frequently identified in atherosclerotic plaques [52]. On the basis of the present study, 7-KC was able to induce oxidative DNA damage, as revealed by the formation of 8-OHdG in human macrophages. These results are in agreement with previous observations that oxysterols, and in particular 7-β-hydroxycholesterol and 7-KC, are mutagenic to Chinese hamster lung fibroblasts [53] since they induce the formation of 8-OHdG, which is considered to be a premutagenic DNA lesion [38]. In the present work, the detection of 8-OHdG thus constitutes a link between the overproduction of ROS and the mutagenicity triggered by 7-KC. In addition, the detection of 8-OHdG opens new fields of investigation between oxidative DNA damage and apoptosis [37].

Many apoptosis-inducing factors have been also shown to elicit oxidative stress; hence, it may be that oxidative stress is deeply involved in the apoptotic process. Increased concentrations of 7-KC in THP-1 cells have been linked to cell death and plaque rupture occurring in the atherosclerotic process. The proapoptotic effects of 7-KC observed in our experimental conditions have already been demonstrated by us and other laboratories [12,13,54,55]. The death of apoptotic macrophage foam cells induced by 7-KC has been shown to modulate the cellularity of the plaque and is believed to play important roles in plaque growth [56]. The results presented in this study are consistent with the hypothesis that an important regulatory event in the induction of apoptosis by the oxysterol is the decreased expression of AKT [12]. It has been recently suggested that the degradation of AKT plays a key role in apoptotic signal transduction pathways [57]. However, it should be considered that 7-KC has been reported to activate apoptosis in fibroblasts through a signal transduction pathway that requires phosphorylation of STAT-1 at Ser-727 [58]. This is noteworthy because some signal transduction pathways to STAT-1 serine phosphorylation precede through AKT [59]. However, some reports that have implicated other kinases in STAT-1 serine phosphorylation, including protein kinase C and p38 MAP kinase, have appeared [60,61]. According to this hypothesis, in our study, 7-KC was able to induce a significant increase in phosphorylated MAP kinases. The responses of the various Bcl-2 family members studied in this work seem to be strictly related to the loss of AKT. Sensitization to apoptotic induction by down-regulation of Bcl-xL and Bcl-2 should lead to activation of Bax.

We also observed that 7-KC can induce arrest in cell cycle progression in the G0/G1 phase, through a mechanism probably involving DNA oxidative damage. A consensus that p53 responds to the types of stress signals that cause DNA damage has emerged. Activation of p53 by this signal inhibits cell growth, by arresting proliferation through increased expression of p21 and/or inducing apoptosis through Bax increase. In our work, a clear relationship appeared to exist between the expression of p53 and that of p21. Cells exposed to 7-KC significantly increased their p53 and p21 content and such effects were maintained for the entire incubation period.

In this study, we demonstrated for the first time that lycopene, at concentrations that can be achievable in vivo in human plasma, may counteract the oxidative processes triggered by 7-KC in human macrophages. In fact, the carotenoid was able to inhibit 7-KC-induced ROS production, directly by its antioxidant properties or indirectly by its ability in inhibiting the expression of NADPH oxidase. Moreover, it decreased the levels of hsp70 and hsp90 and the oxidative DNA damage induced by the oxysterol. These results are in agreement with several studies showing that lycopene is an effective antioxidant and free-radical scavenger. The carotenoid has been shown to act as a potent antioxidant, preventing the oxidative damage of critical biomolecules including lipids, LDLs, proteins and DNA [26,62,63]. In in vitro systems, lycopene was found to inactivate several kinds of free radicals, including hydrogen peroxide, nitrogen dioxide [64,65] thiyl (RSz) and sulphonyl (RSO2z) radicals [66]. Moreover, lycopene was shown to be the most effective antioxidant in protecting 2,2′-azobis(2,4-dimethylvaleronitrile)-induced lipid peroxidation of the liposomal membrane [67]. In addition, the carotenoid was able to inhibit the oxidation of LDL in vitro [68], and it was also shown to significantly reduce the levels of OxLDL in

subjects consuming tomato sauce, tomato juice and lycopene oleoresin capsules as sources of lycopene [14]. Moreover, tomato products or purified lycopene supplementation have been previously shown to decrease oxidative damage in cellular DNA in healthy volunteers [62,69–74]. Finally, several studies showed that consumption of tomato and tomato juice leads to decreased lymphocyte DNA damage [62,74]. Although these findings demonstrated that lycopene may prevent oxidative damage in several experimental models, its efficacy presumably depends on the concentration and the experimental model. In fact, It has been suggested that the ability of carotenoids to protect cells against oxidative damage may be rapidly lost under certain circumstances and that these molecules may exhibit prooxidant effects [75,76]. Accordingly with this, recently, an increased level of oxidative DNA damage product in LNCaP human prostate cancer cell treated with lycopene (5 μM) was described [77]. Moreover, Yeh et al. [78] showed that lycopene enhances UVA-induced oxidative stress in C3H cells, suggesting that under UVA irradiation, the carotenoid may produce oxidative products that are responsible for the prooxidant effects.

Our data show that a redox mechanism seems implicated in the preventive effects of the carotenoid on the arrest of cell cycle progression and on apoptosis induction provoked by 7-KC. In fact, 7-KC increased ROS production and, consequently, intracellular oxidative DNA damage through changes in NOX-4 expression. Such events were responsible for an increase in p53 expression, which, in turn, enhanced the levels of p21, responsible for an arrest of cell cycle progression, and Bax, responsible for apoptosis induction. In our model, lycopene prevented the increase in ROS production and, consequently, the oxidative DNA damage induced by 7-KC, normalizing the levels of NOX-4, p53, p21 and Bax and the growth in human macrophages, strongly suggesting that the changes in protein levels were accompanied by changes in cell homeostasis and functions. In fact, the carotenoid prevented the decrease in AKT expression by limiting the production of 7-KC-induced ROS, as suggested by Rusinol et al. [57]. According to this, H_2O_2- and 15-deoxyprostaglandin J2-induced apoptosis are accompanied by degradation of AKT and both are examples of apoptosis with ROS as second messengers. Moreover, lycopene normalized to the control the expression of Bcl-2, Bcl-xL and Bax induced by the oxysterol. The modulatory effects of the carotenoid on the levels of the Bcl-2 family proteins seem to be particularly interesting in light of the data supporting a role for Bcl-2 in an antioxidant pathway, whereby this protein prevents programmed cell death by decreasing formation of ROS and lipid peroxidation products [79]. In addition, lycopene prevented the increased expression of p53, responsible for the consequent increased levels of both p21 and Bax, presumably through a reduction of oxidative DNA damage. Finally, the carotenoid limited 7-KC-induced phosphorylation of the p38, JNK and ERK1/2 MAP kinases, which are known to be modulated by different oxidative stimuli, including oxidative stress.

It is noteworthy that the carotenoid alone was ineffective in modifying intracellular ROS production, cell cycle progression and apoptosis in human macrophages. This finding is not surprising in view of several reports suggesting that lycopene may act as a powerful growth-inhibitory agent in tumor but not in normal cells [80].

We also compared the ability of lycopene with those of its (5*Z*)-lycopene isomer and β-carotene in inhibiting ROS production, cell growth and apoptosis in THP-1 macrophages. Interestingly, lycopene and its (5*Z*)-lycopene isomer were more effective than β-carotene in preventing cell damage induced by 7-KC. Such results are not surprising in view of previous observations showing that lycopene, because of its high number of conjugated double bonds, has been shown to exhibit higher antioxidant activity compared to β-carotene [25]. On the other hand, no remarkable differences were found in our model between (all-*E*)-lycopene and (5*Z*)-lycopene, although a greater antioxidant capacity of (5*Z*)-lycopene isomers has been reported in more simple models than in cells.

In conclusion, the data reported here support a potential role for lycopene in preventing the 7-KC-induced oxidative stress and apoptosis in human macrophages. This finding may have important implications in the prevention of atherosclerosis. It should be pointed out that although the amount of 7-KC found in plasma from healthy subjects (0.022–2.0 μM) [81,82] or in cooked beef or pork meat (0.09–4.32 μM) [83] was much lower than the amount of 7-KC used in this study, in the atherosclerotic plaque of hypercholesterolemic patients, the amount of oxysterols, including 7-KC, is about 40-fold higher. In such tissues, lycopene could strongly prevent the detrimental effects of the oxysterols by reducing such oxidative stress and apoptosis.

Acknowledgments

We are very much grateful to Dr. Yoav Sharoni and LycoRed Natural Products Industries Ltd. for lycopene supply.

References

[1] Gerry AB, Satchell L, Leake DS. A novel method for production of lipid hydroperoxide- or oxysterol-rich low-density lipoprotein. Atherosclerosis 2008;197:579–87.
[2] Dzeletovic S, Babiker A, Lund E, Diczfalusy U. Time course of oxysterol formation during in vitro oxidation of low density lipoprotein. Chem Phys Lipids 1995;17:119–28.
[3] Leake DS. Oxidised low density lipoproteins and atherogenesis. Br Heart J 1993;69:476–8.
[4] Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med 1996;20:707–27.
[5] Steinberg D, Witztum JL. Is the oxidative modification hypothesis relevant to human atherosclerosis? Circulation 2002;105:2107–11.
[6] Brown AJ, Jessup W. Oxysterols and atherosclerosis. Atherosclerosis 1999;142:1–28 [Review].

[7] Garcia-Cruset S, Carpenter KL, Guardiola F, Mitchinson MJ. Oxysterols in cap and core of human advanced atherosclerotic lesions. Free Radic Res 1999;30:341–50.
[8] Lemaire S, Lizard G, Monier S, Miguet C, Gueldry S, Volot F, Gambert P, Néel D. Different patterns of IL-1beta secretion, adhesion molecule expression and apoptosis induction in human endothelial cells treated with 7alpha-, 7beta-hydroxycholesterol, or 7-ketocholesterol. FEBS Lett 1998;440:434–9.
[9] Colles SM, Maxson JM, Carlson SG, Chisolm GM. Oxidized LDL-induced injury and apoptosis in atherosclerosis. Potential roles for oxysterols. Trends Cardiovasc Med 2001;11:131–8.
[10] Yin J, Chaufour X, McLachlan C, McGuire M, White G, King N, Hambly B. Apoptosis of vascular smooth muscle cells induced by cholesterol and its oxides in vitro and in vivo. Atherosclerosis 2000; 148:365–74.
[11] Björkhem I, Andersson O, Diczfalusy U, Sevastik B, Xiu RJ, Duan C, Lund E. Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. Proc Natl Acad Sci USA 1994;91:8592–6.
[12] Palozza P, Serini S, Verdecchia S, Ameruso M, Trombino S, Picci N, Monego G, Ranelletti FO. Redox regulation of 7-ketocholesterol-induced apoptosis by beta-carotene in human macrophages. Free Radic Biol Med 2007;42:1579–90.
[13] Luthra S, Dong J, Gramajo AL, Chwa M, Kim DW, Neekhra A, Kuppermann BD, Kenney MC. 7-Ketocholesterol activates caspases-3/7, -8, and -12 in human microvascular endothelial cells in vitro. Microvasc Res 2008;75:343–50.
[14] Rao AV. Lycopene, tomatoes, and the prevention of coronary heart disease. Exp Biol Med (Maywood) 2002;227:908–13 [Review].
[15] Rissanen TH, Voutilainen S, Nyyssönen K, Salonen R, Kaplan GA, Salonen JT. Serum lycopene concentrations and carotid atherosclerosis: the Kuopio Ischaemic Heart Disease Risk Factor Study. Am J Clin Nutr 2003;77:133–8.
[16] Visioli F, Riso P, Grande S, Galli C, Porrini M. Protective activity of tomato products on in vivo markers of lipid oxidation. Eur J Nutr 2003; 42:201–6.
[17] Gerster H. The potential role of lycopene for human health. J Am Coll Nutr 1997;16:109–26.
[18] Willcox JK, Catignani GL, Lazarus S. Tomatoes and cardiovascular health. Crit Rev Food Sci Nutr 2003;43:1–18.
[19] Rao AV, Agarwal S. Bioavailability and in vivo antioxidant properties of lycopene from tomato products and their possible role in the prevention of cancer. Nutr Cancer 1998;31:199–203.
[20] Suganuma H, Inakuma T. Protective effect of dietary tomato against endothelial dysfunction in hypercholesterolemic mice. Biosci Biotechnol Biochem 1999;63:78–82.
[21] Breinholt V, Lauridsen ST, Daneshvar B, Jakobsen J. Dose–response effects of lycopene on selected drug-metabolizing and antioxidant enzymes in the rat. Cancer Lett 2000;154:201–10.
[22] Hadley CW, Clinton SK, Schwartz SJ. The consumption of processed tomato products enhances plasma lycopene concentrations in association with a reduced lipoprotein sensitivity to oxidative damage. J Nutr 2003;133:727–32.
[23] Shaish A, Daugherty A, O'Sullivan F, Schonfeld G, Heinecke JW. Beta-carotene inhibits atherosclerosis in hypercholesterolemic rabbits. J Clin Invest 1995;96:2075–82.
[24] Briviba K, Schnäbele K, Rechkemmer G, Bub A. Supplementation of a diet low in carotenoids with tomato or carrot juice does not affect lipid peroxidation in plasma and feces of healthy men. J Nutr 2004;134: 1081–3.
[25] Di Mascio P, Kaiser S, Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 1989;274:532–8.
[26] Southon S. Increased fruit and vegetable consumption: potential health benefits. Nutr Metab Cardiovasc Dis 2001;11:78–81.
[27] Palozza P, Sheriff A, Serini S, Boninsegna A, Maggiano N, Ranelletti FO, Calviello G, Cittadini A. Lycopene induces apoptosis in immortalized fibroblasts exposed to tobacco smoke condensate through arresting cell cycle and down-regulating cyclin D1, pAKT and pBad. Apoptosis 2005;10:1445–56.
[28] Mitchinson MJ, Hardwick SJ, Bennett MR. Cell death in atherosclerotic plaques. Curr Opin Lipidol 1994;7:324–9.
[29] Ball RY, Stowers EC, Burton JH, Cary NR, Skepper JN, Mitchinson MJ. Evidence that the death of macrophage foam cells contributes to the lipid core of atheroma. Atherosclerosis 1995;114:45–54.
[30] Palozza P, Piccioni E, Avanzi L, Vertuani S, Calviello G, Manfredini S. Design, synthesis, and antioxidant activity of FeAOX-6, a novel agent deriving from a molecular combination of the chromanyl and polyisoprenyl moieties. Free Radic Biol Med 2002;33:1724–35.
[31] Yarborough A, Zhang YJ, Hsu TM, Santella RM. Immunoperoxidase detection of 8-hydroxydeoxyguanosine in aflatoxin B1-treated rat liver and human oral mucosal cells. Cancer Res 1996;56:683–8.
[32] Laws GM, Adams SP. Measurement of 8-OHdG in DNA by HPLC/ECD: the importance of DNA purity. Biotechniques 1996;20:36–8.
[33] Palozza P, Serini S, Torsello A, Di Nicuolo F, Maggiano N, Ranelletti FO, Wolf FI, Calviello G. Mechanism of activation of caspase cascade during β-carotene-induced apoptosis in human tumor cells. Nutr Cancer 2003;47:76–87.
[34] Chen BH, Chen YC. Evaluation of the analysis of cholesterol oxides by liquid chromatography. J Chromatogr A 1994;661:127–36.
[35] Fröhlich K, Conrad J, Schmid A, Breithaupt DE, Böhm V. Isolation and structural elucidation of different geometrical isomers of lycopene. Int J Vitam Nutr Res 2007;77:369–75.
[36] Hightower LE. Heat shock, stress proteins, chaperones, and proteotoxicity. Cell 1991;66:191–7.
[37] Martinet W, Knaapen MW, De Meyer GR, Herman AG, Kockx MM. Elevated levels of oxidative DNA damage and DNA repair enzymes in human atherosclerotic plaques. Circulation 2002;106:927–93.
[38] Rozalski R, Gackowski D, Roszkowski K, Foksinski M, Olinski R. The level of 8-hydroxyguanine, a possible repair product of oxidative DNA damage, is higher in urine of cancer patients than in control subjects. Cancer Epidemiol Biomarkers Prev 2002;11:1072–5.
[39] Iuliano L, Micheletta F, Natoli S, Ginanni Corradini S, Iappelli M, Elisei W, Giovannelli L, Violi F, Diczfalusy U. Measurement of oxysterols and alpha-tocopherol in plasma and tissue samples as indices of oxidant stress status. Anal Biochem 2003;312:217–23.
[40] Vaya J, Aviram M, Mahmood S, Hayek T, Grenadir E, Hoffman A, Milo S. Selective distribution of oxysterols in atherosclerotic lesions and human plasma lipoproteins. Free Radic Res 2001;34:485–97.
[41] van Reyk DM, Brown AJ, Hult'en LM, Dean RT, Jessup W. Oxysterols in biological systems: sources, metabolism and pathophysiological relevance. Redox Rep 2006;11:255–62 Review.
[42] Yasunobu Y, Hayashi K, Shingu T, Yamagata T, Kajiyama G, Kambe M. Coronary atherosclerosis and oxidative stress as reflected by autoantibodies against oxidized low-density lipoprotein and oxysterols. Atherosclerosis 2001;155:445–53.
[43] Salonen JT, Salonen R, Seppänen K, Kantola M, Suntioinen S, Korpela H. Interactions of serum copper, selenium, and low density lipoprotein cholesterol in atherogenesis. BMJ 1991;302:756–60.
[44] Hansson GK. Inflammation and immune response in atherosclerosis. Curr Atheroscler Rep 1999;1:150–5 [Review].
[45] Zhou L, Shi M, Guo Z, Brisbon W, Hoover R, Yang H. Different cytotoxic injuries induced by lysophosphatidylcholine and 7-ketocholesterol in mouse endothelial cells. Endothelium 2006;13:213–26.
[46] Welch WJ. In: Morimoto RI, Tissières A, Geogopoulos C, editors. Stress proteins in biology and medicine. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1990. p. 223–78.
[47] Feige U, Polla BS. Experientia. Hsp70-a multi-gene, multi-structure, multi-function family with potential clinical applications. Experientia 1994;50:979–86.
[48] Johnson AD, Berberian PA, Tytell M, Bond MG. Differential distribution of 70-kD heat shock protein in atherosclerosis. Its potential role in arterial SMC survival. Arterioscler Thromb Vasc Biol 1995;15:27–36.

[49] Zhu W, Roma P, Pellegatta F, Catapano AL. Oxidized-LDL induce the expression of heat shock protein 70 in human endothelial cells. Biochem Biophys Res Commun 1994;200:389–94.
[50] Pirillo A, Jacoviello C, Longoni C, Radaelli A, Catapano AL. Simvastatin modulates the heat shock response and cytotoxicity mediated by oxidized LDL in cultured human endothelial smooth muscle cells. Biochem Biophys Res Commun 1997;231:437–41.
[51] Rodriguez IR, Alam S, Lee JW. Cytotoxicity of oxidized low-density lipoprotein in cultured RPE cells is dependent on the formation of 7-ketocholesterol. Invest Ophthalmol Vis Sci 2004;45:2830–7.
[52] Martinet W, Knaapen MW, De Meyer GR, Herman AG, Kockx MM. Oxidative DNA damage and repair in experimental atherosclerosis are reversed by dietary lipid lowering. Circ Res 2001;88:733–9.
[53] Sevanian A, Peterson AR. The cytotoxic and mutagenic properties of cholesterol oxidation products. Food Chem Toxicol 1986;24:1103–10.
[54] Lizard G, Monier S, Cordelet C, Gesquiere L, Deckert V, Gueldry S, Lagrost L, Gambert P. Characterization and comparison of the mode of cell death, apoptosis versus necrosis, induced by 7β-hydroxycholesterol and 7-ketocholesterol in the cells of the vascular wall. Arterioscler Thromb Vasc Biol 1999;19:1190–200.
[55] Miguet C, Monier S, Bettaieb A, Athias A, Bessede G, Laubriet A, Lemaire S, Neel D, Gambert P, Lizard G. Ceramide generation occurring during 7-β-hydroxycholesterol and 7-ketocholesterol-induced apoptosis is caspase independent and is not required to trigger cell death. Cell Death Differ 2001;8:83–99.
[56] Biasi F, Leonarduzzi G, Vizio B, Zanetti D, Sevanian A, Sottero B, Verde V, Zingaro B, Chiarpotto E, Poli G. Oxysterol mixtures prevent proapoptotic effects of 7-ketocholesterol in macrophages: implications for proatherogenic gene modulation. FASEB J 2004;18:693–5.
[57] Rusinol AE, Thewke D, Liu J, Freeman N, Panini SR, Sinensky MS. AKT/protein kinase B Regulation of BCL family members during oxysterol-induced apoptosis. J Biol Chem 2004;279:1392–9.
[58] Rhee SH, Jones BW, Toshchakov V, Vogel SN, Fenton MJ. Toll-like receptors 2 and 4 activate STAT1 serine phosphorylation by distinct mechanisms in macrophages. J Biol Chem 2003;278:22506–12.
[59] Agrawal S, Agrawal ML, Chatterjee-Kishore M, Stark GR, Chisolm GM. Stat1-dependent, p53-independent expression of p21(waf1) modulates oxysterol-induced apoptosis. Mol Cell Biol 2002;22: 1981–92.
[60] Nguyen H, Ramana CV, Bayes J, Stark GR. Roles of phosphatidylinositol 3-kinase in interferon-gamma-dependent phosphorylation of STAT1 on serine 727 and activation of gene expression. J Biol Chem 2001;276:33361–8.
[61] Song JH, So EY, Lee CE. Increased serine phosphorylation and activation of STAT1 by oncogenic Ras transfection. Mol Cells 2002; 13:322–6.
[62] Pool-Zobel BL, Bub A, Müller H, Wollowski I, Rechkemmer G. Consumption of vegetables reduces genetic damage in humans: first results of a human intervention trial with carotenoid-rich foods. Carcinogenesis 1997;18:1847–50.
[63] Agarwal S, Rao AV. Tomato lycopene and low density lipoprotein oxidation: a human dietary intervention study. Lipids 1998;33:981–4.
[64] Böhm F, Tinkler JH, Truscott TG. Carotenoids protect against cell membrane damage by the nitrogen dioxide radical. Nat Med 1995;1: 98–9.
[65] Böhm F, Edge R, Burke M, Truscott TG. Dietary uptake of lycopene protects human cells from singlet oxygen and nitrogen dioxide—ROS components from cigarette smoke. J Photochem Photobiol B 2001;64: 176–8.
[66] Mortensen A, Skibsted LH, Sampson J, Rice-Evans C, Everett SA. Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants. FEBS Lett 1997;418:91–7.
[67] Woodall AA, Britton G, Jackson MJ. Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: relationship between carotenoid structure and protective ability. Biochim Biophys Acta 1997;1336:575–86.
[68] Fuhrman B, Volkova N, Rosenblat M, Aviram M. Lycopene synergistically inhibits LDL oxidation in combination with vitamin E, glabridin, rosmarinic acid, carnosic acid, or garlic. Antioxid Redox Signal 2000;2:491–506.
[69] Riso P, Pinder A, Santangelo A, Porrini M. Does tomato consumption effectively increase the resistance of lymphocyte DNA to oxidative damage? Am J Clin Nutr 1999;69:712–8.
[70] Porrini M, Riso P. Lymphocyte lycopene concentration and DNA protection from oxidative damage is increased in women after a short period of tomato consumption. J Nutr 2000;130:189–92.
[71] Porrini M, Riso P, Oriani G. Spinach and tomato consumption increases lymphocyte DNA resistance to oxidative stress but this is not related to cell carotenoid concentrations. Eur J Nutr 2002;41: 95–100.
[72] Riso P, Visioli F, Erba D, Testolin G, Porrini M. Lycopene and vitamin C concentrations increase in plasma and lymphocytes after tomato intake. Effects on cellular antioxidant protection. Eur J Clin Nutr 2004; 58:1350–8.
[73] Zhao X, Aldini G, Johnson EJ, Rasmussen H, Kraemer K, Woolf H, Musaeus N, Krinsky NI, Russell RM, Yeum KJ. Modification of lymphocyte DNA damage by carotenoid supplementation in post-menopausal women. Am J Clin Nutr 2006;83:163–9.
[74] Rehman A, Bourne LC, Halliwell B, Rice-Evans CA. Tomato consumption modulates oxidative DNA damage in humans. Biochem Biophys Res Commun 1999;262:828–31.
[75] Palozza P. Prooxidant actions of carotenoids in biologic systems. Nutr Rev 1998;56:257–65 [Review].
[76] Young AJ, Lowe GM. Antioxidant and prooxidant properties of carotenoids. Arch Biochem Biophys 2001;385:20–7 [Review].
[77] Hwang ES, Bowen PE. Effects of lycopene and tomato paste extracts on DNA and lipid oxidation in LNCaP human prostate cancer cells. Biofactors 2005;23:97–105.
[78] Yeh SL, Huang CS, Hu ML. Lycopene enhances UVA induced DNA damage and expression of heme oxygenase 1 in cultured mouse embryo fibroblasts. Eur J Nutr 2005;44:365–70.
[79] Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, Bredesen DE. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science 1993;262:1274–7.
[80] Palozza P. Carotenoids and modulation of cancer: molecular targets. Curr Pharmacogenomics 2004;2:35–45.
[81] Schroepfer GJ. Oxysterols: modulators of cholesterol metabolism and other processes. Physiol Rev 2000;80:361–554.
[82] Dzeletovic S, Breuer O, Lund E, Diczfalusy U. Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. Anal Biochem 1995;225:73–80.
[83] Raith K, Brenner C, Farwanah H, Muller G, Eder K, Neubert RH. A new LC/APCI-MS method for the determination of cholesterol oxidation products in food. J Chromatogr A 2005;1067:20711.