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Phytochemical indicaxanthin suppresses 7-ketocholesterol-induced THP-1 cell apoptosis by preventing cytosolic Ca^{2+} increase and oxidative stress

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Abstract

7-Ketocholesterol (7-KC)-induced apoptosis of macrophages is considered a key event in the development of human atheromas. In the present study, the effect of indicaxanthin (Ind), a bioactive pigment from cactus pear fruit, on 7-KC-induced apoptosis of human monocyte/macrophage THP-1 cells was investigated. A pathophysiological condition was simulated by using amounts of 7-KC that can be reached in human atheromatous plaque. Ind was assayed within a micromolar concentration range, consistent with its plasma level after dietary supplementation with cactus pear fruit. Pro-apoptotic effects of 7-KC were assessed by cell cycle arrest, exposure of phosphatidylserine at the plasma membrane, variation of nuclear morphology, decrease of mitochondrial trans-membrane potential, activation of Bcl-2 antagonist of cell death and poly(ADP-ribose) polymerase-1 cleavage. Kinetic measurements within 24 h showed early formation of intracellular reactive oxygen species over basal levels, preceding NADPH oxidase-4 (NOX-4) over-expression and elevation of cytosolic Ca^{2+}, with progressive depletion of total thiols. 7-KC-dependent activation of the redox-sensitive NF-κB was observed. Co-incubation of 2·5 μM of Ind completely prevented 7-KC-induced pro-apoptotic events. The effects of Ind may be ascribed to inhibition of NOX-4 basal activity and over-expression, inhibition of NF-κB activation, maintaining cell redox balance and Ca homeostasis, with prevention of mitochondrial damage and consequently apoptosis. The findings suggest that Ind, a highly bioavailable dietary phytochemical, may exert protective effects against atherogenetic toxicity of 7-KC at a concentration of nutritional interest.

Key words: Indicaxanthin: Apoptosis: 7-Ketocholesterol: Macrophages: NADPH oxidase-4

Discovering the activity of dietary phytochemicals at the level of intracellular signal transduction pathways is now considered the basis to suggest their eventual health effects. The bioactivity of these molecules is generally ascribed to redox and antioxidant properties, with a growing body of evidence indicating that most compounds are to be considered for their roles as modulators of redox-mediated signalling cascades, including those relevant to either survival or cell death[1]. Moreover, whatever their properties, bioavailability has to be ascertained so that the impact on human health and nutritional importance of dietary phytochemicals can be assessed. Betalains are nitrogen-containing pigments occurring in the Cariophyllales order of plants, including beetroot and cactus pear, and in some fungal genera[2,3]. Indicaxanthin (Ind, Fig. 1), the yellow betalain characterising the edible fruit of the cactus *Opuntia ficus indica*, has recently emerged as a radical scavenger and antioxidant[4,5] with peculiar physico-chemical characteristics, allowing the molecule to interact with and locate in membranes[6,7] and the potential to act at the level of body cells and tissues. Anti-inflammatory and protective effects of Ind have been shown *in vitro*, in endothelial cell cultures, where it can inhibit the cytokine-induced redox state alteration and modulate the expression of adhesion molecules[8], and in either healthy or pathological erythrocytes[9,10]. Other studies showed modulatory activity of Ind on the contractility of isolated mouse ileal muscle[11,12]. Different from the majority of dietary phytochemicals, Ind has appeared to be quite stable in absorptive gastrointestinal conditions[13], is not metabolised by human enterocytes[14] or hepatocytes[15] and is bioavailable, reaching plasma micromolar concentrations after a dietary supplementation with cactus pear fruits[16]. In the present study, we investigated the activity of Ind in THP-1 cells, a human monocyte–macrophage cell line, against the cytotoxicity of 7-ketocholesterol (7-KC). Oxysterols are a family of bioactive lipids generated in the body through either enzymatic or non-enzymatic oxidation of cholesterol or absorbed from the diet[17]. These compounds are involved in physiological processes[18–21]; however, there is evidence that some of them have deleterious effects[22,23]. The potential of oxysterols

Abbreviations: 7-KC, 7-ketocholesterol; BAD, Bcl-2 antagonist of cell death; Iκ-Bα, NF-κB with its inhibitor κ Bα; Ind, indicaxanthin; NOX-4, NADPH oxidase-4; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species.

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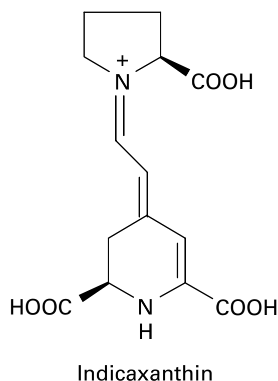

Fig. 1. Molecular structure of indicaxanthin.

to trigger pro-oxidative, pro-inflammatory and cytotoxic reactions, widely documented in a number of cells of the vascular wall, including human artery smooth muscle[24,25] and endothelial cells[26–28] as well as in immunocompetent cells such as monocyte/macrophages[29,30], led to the consideration that these compounds and 7-KC, in particular[22], are important contributors to the progression of vascular dysfunction[31,32] and development of atheromas. The level of 7-KC in plasma of healthy subjects is from 0·001 to 4·7 μM[22], whereas the amount measured in atherosclerotic plaque may be more than 40-fold higher[33,34]. In the present study we simulated a pathophysiological condition by challenging human monocyte/macrophage THP-1 cells with 16 μM-7-KC, a concentration that can be reached in atherosclerotic plaque of hypercholesterolaemic subjects. Here, we report that Ind, when co-incubated at a concentration comparable with that measured in human plasma after a dietary supplementation with cactus pear fruits, totally prevents the 7-KC-induced apoptosis of THP-1 cells by interfering with molecular mechanisms known to be involved in the cytotoxicity of this oxysterol.

Experimental methods

Unless stated otherwise, all reagents and materials were from Sigma Chemical Company and solvents were of the highest purity or HPLC grade.

Indicaxanthin preparation

Ind was isolated from cactus pear (*O. ficus indica*) fruits (yellow cultivar). The phytochemical was separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25[4]. Fractions containing the pigment were submitted to cryodesiccation and purified according to Stintzing *et al.*[35]. Briefly, the desiccated material was re-suspended in 1% acetic acid in water and submitted to semi-preparative HPLC using a Varian Pursuit C18 column (250 × 10 mm inner diameter; 5 mm; Varian), eluted with a 20 min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with a flow of 3 ml/min. Spectrophotometric revelation was at 482 nm. The elution volumes relevant to Ind were collected. Samples after cryodesiccation were re-suspended in 5 mM-PBS (pH 7·4) at a suitable concentration and used immediately or stored at −80°C. Concentration of the samples was evaluated spectrophotometrically in a DU-640 Beckman spectrophotometer by using a molar coefficient at 482 nm of 42 800[36]. Ind was filtered through a Millex HV 0·2 μm filter (Millipore) immediately before use.

Cell culture

THP-1 cells (American Type Culture Collection) were grown in Roswell Park Memorial Institute medium supplemented with 2 mM-L-glutamine, 10% fetal bovine serum, 100 U (60 μg)/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml gentamicin. Cells were maintained in log phase by seeding twice a week at a density of 3×10^8 cells/l in humidified 5% CO_2 atmosphere at 37°C. In all experiments, THP-1 cells were seeded in triplicate in twenty-four-well culture plates at a density of $1·25 \times 10^5$ cells/cm^2 and made quiescent through overnight incubation. Then, cells were treated with 7-KC at a final concentration of 16 μM, alone or with Ind, and incubation times were as indicated in the text. 7-KC was delivered to the cells using tetrahydrofuran as a solvent, at a final concentration of 0·1% (v/v). Untreated cells incubated with 0·1% tetrahydrofuran were used as control. No differences were found between cells treated with tetrahydrofuran and untreated cells in terms of cell number, viability and reactive oxygen species (ROS) production. 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.

Measurement of 7-ketocholesterol

7-KC was extracted with four volumes of a methanol–hexane mixture (1:3, v/v) from 3×10^6 cells treated with 16 μM-oxysterol for 12 h. 7-KC was analysed by HPLC using a cyano-bonded column (Luna 5 μm, 250 × 4·6 mm; Phenomenex) equipped with a CN-guard cartridge (2·0 × 4·0 mm; Phenomenex) and hexane at a flow rate of 1 ml/min. 7-KC was detected spectrophotometrically at 234 nm. Quantification was by reference to standard curves constructed with 5–100 ng of the purified compound, and by relating the amount of the compound under analysis to the peak area.

Cell cycle analysis

Cell cycle stage was analysed by flow cytometry. Aliquots of 1×10^6 cells were harvested by centrifugation, washed with PBS and incubated in the dark in a PBS solution containing 20 μg/ml propidium iodide and 200 μg/ml RNase, for 30 min, at room temperature. Then, samples were immediately subjected to fluorescence-activated cell sorting analysis by Epics XL™ flow cytometer using Expo32 software (Beckman Coulter). At least 1×10^4 cells were analysed for each sample.

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Acridine orange and ethidium bromide morphological fluorescence dye staining

Acridine orange stains DNA bright green, allowing visualisation of the nuclear chromatin pattern. Apoptotic cells have condensed chromatin that is uniformly stained. Ethidium bromide stains DNA orange, but is excluded by viable cells. Dual staining allows separate enumeration of populations of viable non-apoptotic, viable (early) apoptotic, non-viable (late) apoptotic and necrotic cells. After treatment, the medium was discarded. Cells were washed with PBS first and then incubated with 100 μl of PBS containing 100 μg/ml of ethidium bromide plus 100 μg/ml of acridine orange. After 20 s, ethidium bromide/acridine orange solution was discarded and cells were immediately visualised by means of a fluorescent microscope equipped with an automatic photomicrograph system (Leica). Multiple photographs were taken at randomly selected areas of the well to ensure that the data obtained are representative.

Measurement of phosphatidylserine exposure

Flow cytometry by double staining with Annexin V/propidium iodide was used to detect externalisation of phosphatidylserine to the cell surface. Cells were adjusted at 1×10^6 cells/ml with combining buffer. Cell suspension (100 μl) was added to a new tube, and incubated with 5 μl Annexin V and 10 μl of 20 μg/ml propidium iodide solution at room temperature, in the dark or 15 min. Then, samples of at least 1×10^4 cells were subjected to fluorescence-activated cell sorting analysis by appropriate two-dimensional gating method.

Measurement of mitochondrial transmembrane potential

Mitochondrial transmembrane potential ($\Delta\Psi_m$) was assayed by flow cytofluorometry, using the cationic lipophilic dye 3,3′-dihexyloxacarbocyanine iodide[3] (Molecular Probes, Inc.) which accumulates in the mitochondrial matrix. Changes in mitochondrial membrane potential are indicated by a reduction in the 3,3′-dihexyloxacarbocyanine iodide-induced fluorescence intensity. Cells were incubated with 3,3′-dihexyloxacarbocyanine iodide[3] at a 40 nmol/l final concentration, for 15 min at 37°C. After centrifugation, cells were washed with PBS and suspended in 500 μl PBS. Fluorescent intensities were analysed in at least 1×10^4 cells for each sample.

Measurement of intracellular reactive oxygen species

ROS level was monitored by measuring fluorescence changes that resulted from intracellular oxidation of dichlorodihydrofluorescein diacetate. Dichlorodihydrofluorescein diacetate, at 10 μM final concentration, was added to the cell medium 30 min before the end of the treatment. The cells were collected by centrifugation for 5 min at 2000 rpm at 4°C, washed, suspended in PBS and immediately subjected to fluorescence-activated cell sorting analysis. At least 1×10^4 cells were analysed for each sample.

Measurement of cellular thiols

After treatment, cells were collected by centrifugation, washed twice with cold PBS containing 0·025 % butylated hydroxytoluene and lysed by sonication. Cell lysates were mixed with 10 % SDS and 30 μM of 5,5′-dithiobis-(2-nitrobenzoic acid) and incubated with shaking at room temperature for 30 min. The total amount of reduced thiols, including both protein thiols and glutathione, was measured spectrophotometrically at 412 nm.

Measurement of cytosolic calcium

Intracellular Ca^{2+} concentration in a single cell was measured using fluo-3/AM as a fluorescent Ca^{2+} probe, whose intensity is directly representative of cellular concentration of the ion. Fluo-3/AM, at 2 μM final concentration, was added into the cell medium 40 min before the end of the treatment. After centrifugation, cells were washed with PBS and suspended in 500 μl PBS. The fluorescent intensities were analysed by fluorescence-activated cell sorting analysis in at least 1×10^4 cells for each sample.

Western blot analysis

After treatment, cells were collected by centrifugation, washed twice with cold PBS and gently lysed for 60 min in ice-cold lysis buffer (10 mM-HEPES, 1·5 mM-$MgCl_2$, 10 mM-KCl, 0·5 mM-phenylmethylsulphonyl fluoride (PMSF), 1·5 μg/ml soyabean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0·1 mM-benzamidine and 0·5 mM-dithiothreitol (DTT)). The lysates were centrifuged at 13 000 ***g*** for 5 min and supernatants (cytosolic fraction) were immediately portioned and stored at −80°C up to 2 weeks. The nuclear pellet was resuspended in 60 μl of high-salt extraction buffer (20 mM-HEPES (pH 7·9), 420 mM-NaCl, 1·5 mM-$MgCl_2$, 0·2 mM-EDTA, 25 % (v/v) glycerol, 0·5 mM-PMSF, 1·5 μg/ml soyabean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0·1 mM-benzamidine and 0·5 mM-DTT) and incubated with shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at 13 000 ***g*** and the supernatant was portioned and stored at −80°C. The protein concentration of each sample was determined by using the Bradford protein assay reagent (Bio-Rad). Protein samples (80 μg/line) were separated on 12 % SDS-PAGE and transferred to nitrocellulose membrane. The immunoblot was incubated overnight at 4°C with blocking solution (5 % skimmed milk), followed by incubation with anti-poly(ADP-ribose) polymerase (PARP) monoclonal antibody (clone D-1, catalogue no. SC-365315; Santa Cruz Biotechnology), anti-phospho-Bad polyclonal affinity-purified antibody (clone Ser 136, catalogue no. SC-7999; Santa Cruz Biotechnology), anti-NF-κB with its inhibitor κ Bα (Iκ-Bα) monoclonal antibody (clone 417208, catalogue no. MAB4299; R&D Systems), anti-phospho-Iκ-Bα polyclonal antibody (S32/S36, catalogue no. AF4809; R&D Systems), anti-NF-κB p65 monoclonal antibody (clone 532 301 catalogue No. MAB 5078; R&D Systems) or anti-NADPH oxidase-4 (NOX-4) (clone N-15, catalogue no. SC -21 860; Santa Cruz Biotechnology) for 1 h at room temperature. Blots were washed two

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times with Tween 20/Tris-buffered saline and incubated with a 1:2000 dilution of horseradish peroxidase-conjugated anti-IgG antibody (Dako Denmark) for 1 h at room temperature. Blots were again washed five times with Tween 20/Tris-buffered saline and then developed by enhanced chemiluminescence (Amersham Life Science). Immunoreactions were also performed using β-actin antibody as loading controls.

Statistics

Results are given as means and standard deviations. Three independent observations were carried out for each experiment, replicated three times. Statistical comparisons were made using a one-way ANOVA, followed by Fisher's test. $P<0.05$ was considered statistically significant.

Results

Protective effect of indicaxanthin on 7-ketocholesterol-induced cell growth arrest and apoptosis

In comparison with control cells (vehicle alone), a 24 h incubation of human monocyte/macrophage THP-1 cells with 16 μM-7-KC caused a significant decrease in cell growth, whereas co-incubation with 7-KC and 0·1–2·5 μM-Ind resulted in a dose-dependent cell protection (Fig. 2(A)). Ind at a concentration of 2·5 μM completely restored the growth of cells exposed to 7-KC for at least 72 h (Fig. 2(B)). Ind alone did not modify THP-1 cell growth at 2·5 μM, nor at 25 μM (not shown). The distribution of THP-1 cells in the cell cycle phases after 24 h treatment with 7-KC, either alone or in association with Ind, is shown in Fig. 2(C). A significant percentage increase of

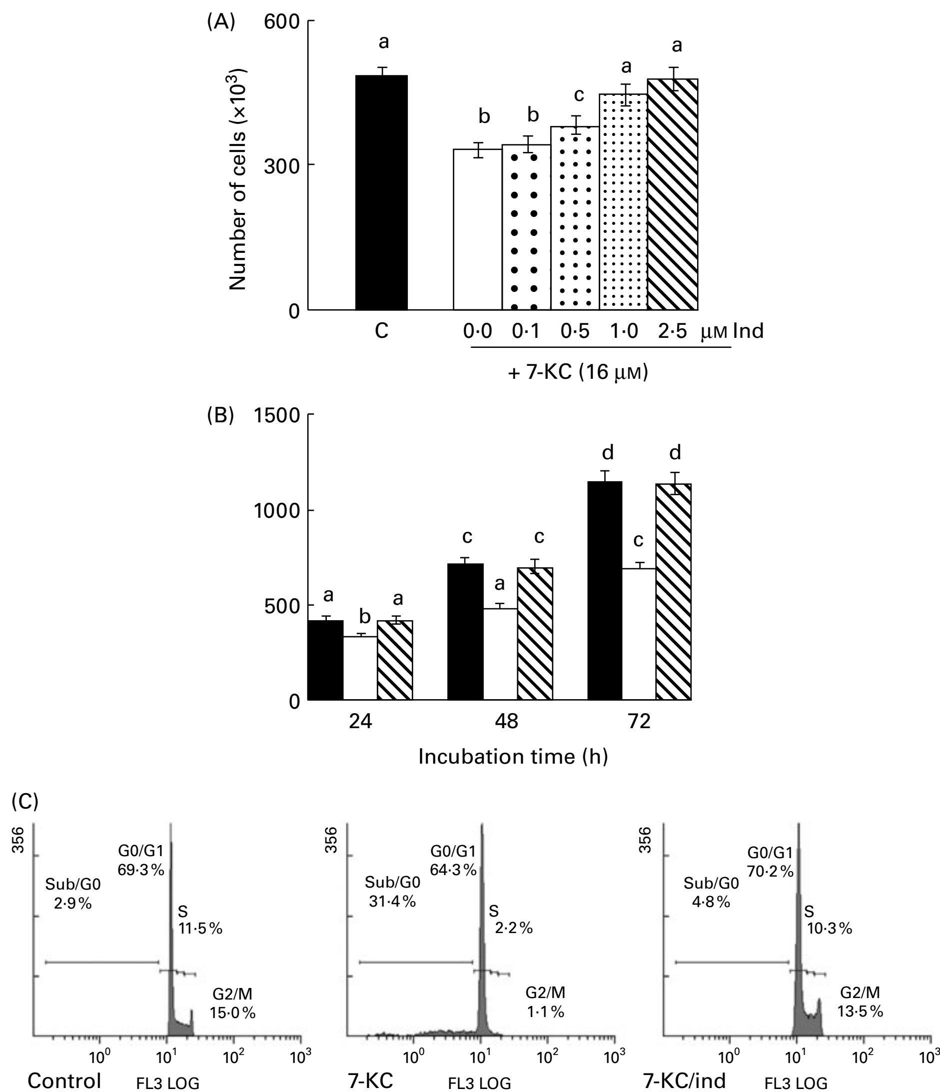

Fig. 2. Effect of indicaxanthin (Ind) on 7-ketocholesterol (7-KC)-induced growth and cell cycle arrest in THP-1 cells. (A) Dose-dependent effect of Ind in the presence of 7-KC (16 μM) after 24 h incubation; (B) time-dependent effects of 2·5 μM-Ind in the presence of 7-KC (16 μM); (C) percentage of propidium iodide-stained THP-1 cells in different phases of the cell cycle, as determined by flow cytometry: control (C, ■), cells treated with vehicle; 7-KC (□), cells treated for 24 h with 7-KC (16 μM); 7-KC/Ind (▨), cells treated with 7-KC in the presence of Ind (2·5 μM). Values are means and standard deviation: of three experiments carried out in triplicate represented by vertical bars. [a,b,c,d]Mean values with unlike letters were significantly different ($P<0.05$; Fisher's test). (C) Representative of three experiments with comparable results.

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cells in the G0/G1 phase, accompanied by a concomitant decrease in the percentage of cells in the S and G2/M phases and induction of a subG1-cell population, were observed in the presence of 7-KC. Co-incubation with 2·5 μM-Ind completely prevented the arrest of cell cycle progression caused by oxysterol (Fig. 2(C)).

The incorporation of 7-KC in THP-1 cells was measured either in the absence or in the presence of Ind. HPLC analysis of cell extracts after a 12 h incubation with 7-KC revealed 1·46 (SD 0·6) μg of 7-KC/10^6 cells (*n* 6), which non-significantly varied upon addition of 2·5 μM-Ind (1·53 (SD 0·5) μg/10^6 cells, *n* 6).

It has widely been reported that oxysterol-induced cell toxicity is associated with pro-apoptotic events. Externalisation of plasma membrane phosphatidylserine, changes of mitochondrial transmembrane potential, variation of nuclear morphology and PARP-1 cleavage were evaluated in THP-1 cells treated with 7-KC. Flow cytometry analysis of Annexin V-fluorescein isothiocyanate and propidium iodide-stained cells after 12 h treatment with 7-KC indicated a high percentage of cells in early apoptosis, with externalised phosphatidylserine (Fig. 3(A)). Loss of mitochondrial transmembrane potential was indicated by decreased mitochondrial 3,3′-dihexyloxacarbocyanine iodide-red fluorescence (Fig. 3(B)). Treatment with 7-KC for 24 h caused changes to cell morphology, with an increase in the number of cells permeable to ethidium bromide, with fragmented and/or condensed nuclei and appearance of membrane blebbing (Fig. 3(C)). One of the key executioners of apoptotic cell death is caspase 3, responsible for proteolytic cleavage of many key proteins, including PARP-1. In comparison with control cells, high levels of the 89 kDa cleaved product from PARP-1, with a decrease of the 116 kDa native protein, were observed in 7-KC-treated THP-1 cells (Fig. 3(D)). Co-incubation with 7-KC and Ind resulted in a total prevention of 7-KC-dependent cell death induction, as shown by the absence of any apoptotic feature (Fig. 3(A)–(D)).

Prevention by indicaxanthin of 7-ketocholesterol-induced oxidative stress and NF-κB activation

Because of their peculiar NOX-4 activity, macrophages generate basal levels of intracellular ROS[37]. The pro-apoptotic activity of 7-KC has been associated with NOX-4 induction, ROS overproduction and variation of the redox status of cells[38–40]. The intracellular ROS level, as well as the level of total thiols, were monitored from 30 min to 24 h in THP-1 cells incubated with 7-KC, either in the absence or in the presence of Ind. In the absence of Ind, we observed a biphasic ROS production, with a significant peak after 1 h treatment and a sustained progressive increase between 3 and 24 h of observation (Fig. 4(A)). Cell total thiols progressively decreased along 24 h of exposure to 7-KC (Fig. 4(B)). Co-incubation of cells with 7-KC and 2·5 μM-Ind completely prevented the intracellular increase of ROS and thiol loss (Fig. 4(A) and (B)). The dose-dependent effect of Ind on ROS production, monitored after 24 h incubation in the presence of 7-KC, is shown in the inset. Notably, the level of ROS measured in the presence of 7-KC and Ind was significantly ($P<0·05$) lower than control cells along the entire incubation time interval, which was not observed in cells incubated with Ind alone, neither at 2·5 μM (Fig. 4(A)) nor at 10 μM-Ind (data not shown).

The expression of NOX-4 in THP-1 cells after 1 and 3 h of incubation with either 7-KC or 7-KC and Ind was measured by Western blotting analysis (Fig. 4(C)). With respect to control, densitometric analysis showed no variation of NOX-4 protein after a 1 h treatment of THP-1 cells with 7-KC, whereas a 2-fold enhanced level was evident after 3 h. Up-regulation of NOX-4 protein was prevented by
co-incubation of cells with 7-KC and Ind.

As a redox-sensitive transcription factor, NF-κB has recently been found to be involved in the early response of monocytes–macrophages to 7-KC injury[38,41]. In quiescent cells, NF-κB combines with its inhibitor κ Bα (Iκ-Bα) in cytosol. Upon cell activation, Iκ-Bα undergoes phosphorylation and ubiquitination-dependent degradation, thus leading to nuclear translocation of NF-κB. A Western blotting analysis of THP-1 cells treated with 7-KC for 12 h showed a net decrease of the cytosolic Iκ-Bα with increase of its phosphorylated form (phospho-Iκ-Bα), accompanied by nuclear translocation of the NF-κB p65 subunit. NF-κB activation was completely prevented by co-incubating 7-KC and Ind (Fig. 5).

Inhibitory effect of indicaxanthin on 7-ketocholesterol-induced intracellular Ca^{2+} elevation

A rise in cytosolic-free Ca $(Ca^{2+})_i$ is considered an early step in the signalling cascade induced by oxysterols. A Fluo-3/AM staining, followed by flow cytometry analysis, was performed to measure the Ca^{2+} content in THP-1 cells after treatment with 7-KC, either in the absence or in the presence of Ind, during the time interval between 30 min and 6 h. In comparison with control cells, a 7-KC-dependent $(Ca^{2+})_i$ elevation was not evident at 30 min, whereas the Ca^{2+}-associated fluorescence clearly increased at 1 h of treatment and high levels were maintained for the following 5 h (Fig. 6(A)). THP-1 cells co-incubated with 7-KC and Ind showed a staining intensity quite comparable with control, with total prevention of the oxysterol-induced increase of cytosolic Ca^{2+} along the entire incubation time (Fig. 6(A)). The dose-dependent effect of Ind on the intracellular Ca elevation, monitored after 6 h incubation in the presence of 7-KC, is shown in the inset.

7-KC-dependent $(Ca_2^+)_i$ elevation is associated with the activation of the phosphatase calcineurin, one target of which is the pro-apoptotic protein Bcl-2 antagonist of cell death (BAD)[42]. Phosphorylated BAD in its serine 75 and/or 99 is bound to the 14-3-3 protein in the cytoplasm[43]. Once dephosphorylated, BAD translocates to the mitochondria, where it allows cytochrome *c* release following heterodimerisation[44] with the anti-apoptotic proteins Bcl-2 and Bcl-X_L[45]. Western blot analyses performed with phospho-BAD ser 99-specific antibody showed that treatment of THP-1 cells with 7-KC decreased the phosphorylated BAD levels by more than 95 % compared with control cells, whereas co-treatment with Ind prevented this effect, thus inhibiting BAD activation (Fig. 6(B)).

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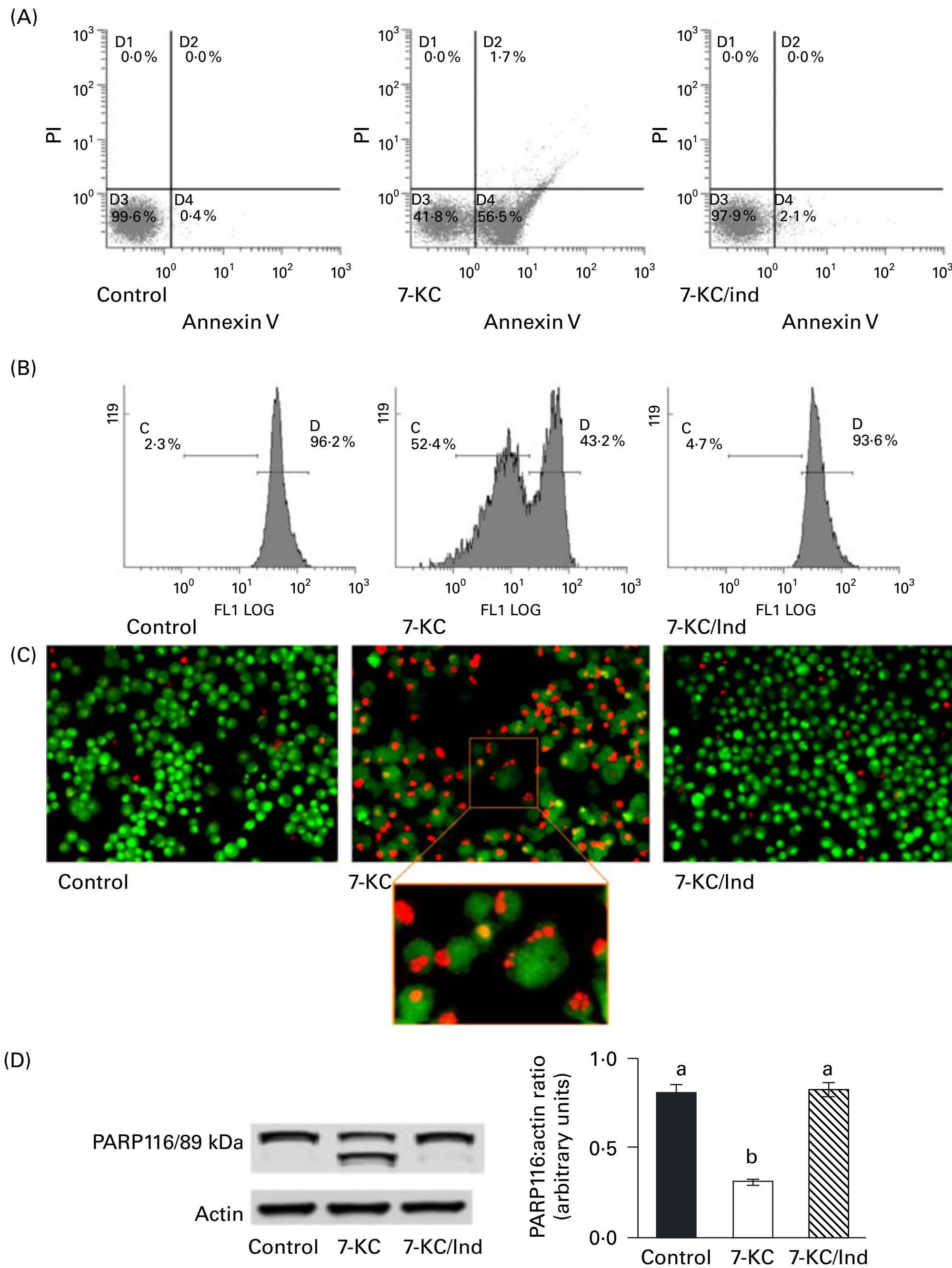

Fig. 3. Effect of indicaxanthin (Ind) on cell apoptosis induced by 7-ketocholesterol (7-KC) in THP-1 cells. (A) Percentage of Annexin V/propidium iodide (PI) double-stained THP-1 cells, as determined by flow cytometry. (B) Fluorescence intensity of 3,3′-dihexyloxacarbocyanine iodide-treated cells, as determined by flow cytometry. (C) Fluorescence micrographs of ethidium bromide/acridine orange double-stained THP-1 cells in 200 × magnification. Inset shows 400 × magnification. (D) Poly(ADP-ribose) polymerase cleavage by immunoblotting with densitometric analysis of the immunoblots. Control, cells treated with vehicle; 7-KC, cells treated for 12 h (A, B) or 24 h (C, D) with 7-KC (16 μM); 7-KC/Ind, cells treated with 7-KC in the presence of Ind (2·5 μM). Representative images of three experiments with comparable results. Data of the densitometric analysis are means and standard deviations. [a,b] Mean values with unlike letters were significantly different ($P<0·05$; Fisher's test). (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

Discussion

Cytotoxic oxysterols such as 7-KC have been implicated in the pathophysiology of atherosclerosis, where they induce apoptosis in cells of the vascular wall and in monocytes/macrophages[39,46–48], thus contributing to the development of atheromatous plaque[49]. In the present study, evidence is provided that the phytochemical Ind, at a concentration achievable in human subjects after dietary supplementation with cactus pear fruit[16], prevents 7-KC-induced apoptosis of human monocyte/macrophage THP-1 cells by preventing alteration of the cell oxidative balance and dysregulation of intracellular Ca homeostasis. The 7-KC-induced arrest of cell cycle progression and other pro-apoptotic events, such as nuclear morphological changes, externalisation of

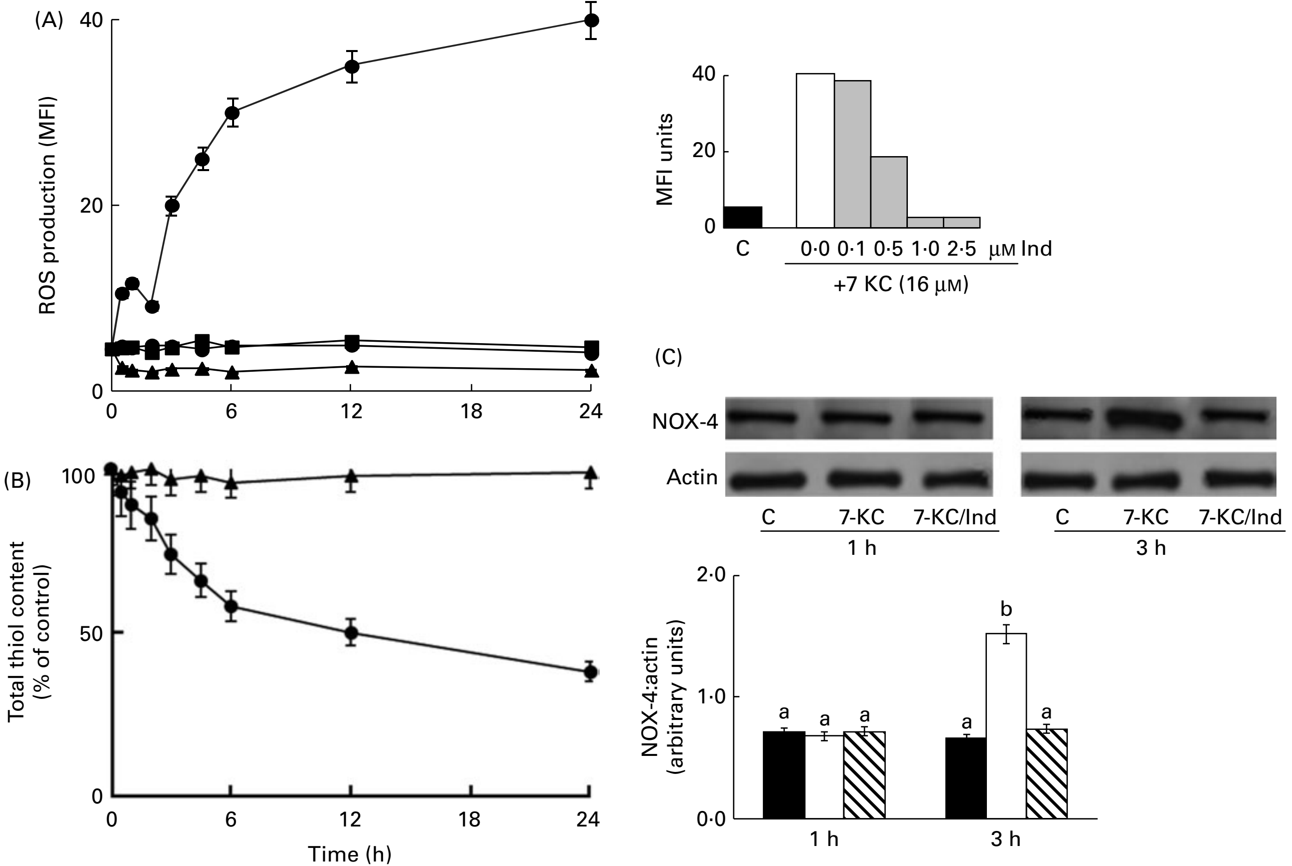

Fig. 4. Effect of indicaxanthin (Ind) on oxidative stress induced by 7-ketocholesterol (7-KC) in THP-1 cells. (A) Reactive oxygen species (ROS) production. The inset shows dose-dependent effects of Ind in the presence of 7-KC (16 μM) after 24 h incubation. (B) Thiol depletion. (C) NADPH oxidase-4 (NOX-4) expression in THP-1 cells treated with 7-KC (16 μM) alone or in combination with Ind (2·5 μM) at different time intervals. Control (C, ■), cells treated with vehicle; 7-KC (●), cells treated with 7-KC (16 μM); 7-KC/Ind (○), cells treated with 7-KC in the presence of Ind (2·5 μM). ▲, Ind. (A) and (B) Cellular ROS and total thiol were assayed using flow cytometry (dichlorodihydrofluorescein diacetate staining) and by 5,5′-dithiobis-2-nitrobenzoic acid reaction, respectively, as reported in the Experimental methods section. Data are means and standard deviations of three separate experiments carried out in triplicate. (C) NOX-4 by immunoblotting with densitometric analysis of the immunoblots. ■, Control; □, 7-KC; ▨, 7-KC/Ind. Representative images of three experiments with similar results. Data of the densitometric analysis are means and standard deviations. [a,b] Mean values with unlike letters were significantly different ($P<0·05$; Fisher's test). MFI, mean fluorescence intensity.

phosphatidylserine in the plasma membrane, depolarisation of mitochondrial membrane and PARP cleavage, were completely inhibited by the co-incubated phytochemicals. As Ind did not affect the incorporation of 7-KC in the cells, its action was investigated taking into account oxysterol activities known to be associated with cell toxicity. Oxysterols affect cell processes by biochemical and membrane biophysical mechanisms[50–52]. As far as 7-KC is concerned, it has been suggested that it modulates cell processes possibly through effects within the plasma membrane[42]. 7-KC transfers spontaneously between membranes, partitions in the less densely packed L_d domains and has a tendency to destabilise membrane rafts[53,54], which may affect raft-associated functional proteins, including Ca channels[42], and possibly perturb the activity of signalling enzymes including NADPH oxidase[55,56]. Indeed, two events have been reported as pivotal to stimulate an apoptotic process after challenging THP-1 cells with 7-KC, influx of free Ca into the cytosol and intracellular overproduction of ROS[40,42].

NOX-4, the constitutively active homologue of the NOX family of membrane NADPH oxidase[57], is considered the source of ROS following stimulation of macrophages with oxidised lipids[38,39,47,58]. The enzyme, which is known to continuously produce low ROS levels with a signalling function[37,59,60], under physiological conditions does not affect macrophage viability. Increased levels of the enzyme activity are considered to depend on the protein expression[61]. In macrophages, NOX-4 can be over-expressed after certain stimuli such as oxLDL[58] and 7-KC[38,39], leading to a high and sustained ROS production. In THP-1 cells, in particular, up-regulation of NOX-4 has been shown to be required for 7-KC-induced apoptosis[39,58]. Searching for the effect of Ind on the redox status of THP-1 cells after 7-KC treatment, the intracellular levels of ROS and thiols were monitored for 24 h from the addition of the oxysterol. A very early and slowly increasing ROS production was observed, followed by a sustained gradual rise due to over-expression of NOX-4, with a parallel depletion of total thiols, indicating a cell redox environment irreversibly compromised. Under these conditions, impairment of mitochondrial function is expected, with induction of an apoptotic sequence[62,63]. In accordance with our observations, a time- and peroxide-dependent decrease of reduced glutathione has been shown to be substantially implicated in

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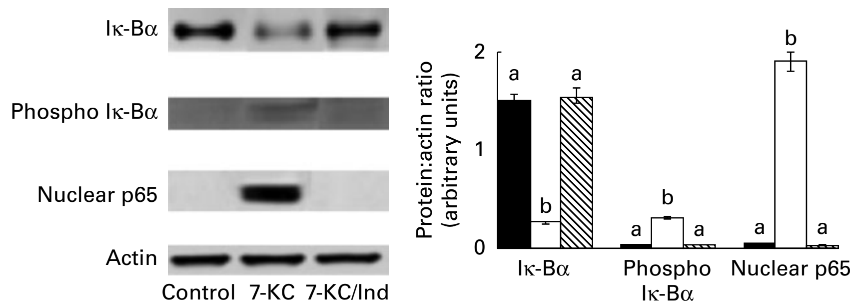

Fig. 5. Effect of indicaxanthin (Ind) on 7-ketocholesterol (7-KC)-induced Iκ-Bα (NF-κB with its inhibitor κ Bα) degradation and p65 nuclear translocation in THP-1 cells. Cellular (phospho Iκ-Bα and Iκ-Bα) and nuclear (p65) lysates were obtained as reported in the Experimental methods section. Representative image of three separate Western blotting analysis with comparable results and densitometric analyses of immunoblot. Data of the densitometric analysis are means and standard deviations. [a,b] Mean values with unlike letters were significantly different ($P < 0.05$; Fisher's test). Control (■), cells treated with vehicle; 7-KC (□), cells treated for 12 h with 7-KC (16 μM); 7-KC/Ind (▧), cells treated with 7-KC in the presence of Ind (2·5 μM).

the control of the 7-KC-induced apoptosis in U937 monocytes[48]. The finding that co-incubated Ind completely prevented early oxidative events, as well as up-regulation of NOX-4, and maintained the cell redox balance suggested that downstream events sensitive to cell redox changes, including those contributing to apoptotic death, were affected by the phytochemicals. The observed early formation of ROS in a time preceding NOX-4 over-expression suggests some acute enzyme regulation by 7-KC. Molecular mechanisms of NOX-4 activation are still unknown; however, acute regulation of the NOX-4 activity has been reported, e.g. in adipose cells after stimulation with insulin[64,65], in human aortic endothelial cells by oxidised phospholipids through rac1 translocation[66] and in cardiac myocytes, where a local regulatory role of Ca^{2+} on the enzyme activity has been shown[67]. As far as immune-competent cells and oxysterols are concerned, a prompt overproduction of ROS has also been observed in murine macrophages after 7-KC stimulation[40].

Specific molecular targets of oxidation mediating cellular responses to the NADPH oxidase may be different in different cells[68]; ROS, however, serve as a common intracellular messenger for NF-κB activation[69,70]. In the present as well as other studies with human monocyte cell lines[38,41], NF-κB activation has been found to be associated with 7-KC pro-inflammatory and pro-apoptotic effects. In line with the protection of the redox state of the cell environment, our data show that Ind inhibited the 7-KC-stimulated ROS-dependent activation of NF-κB.

Increase of cytoplasmic Ca^{2+} concentration is one of the most rapid cellular response upon exposure to various oxysterol congeners, including 7-KC, and precedes events of the apoptotic cascade[71,72], i.e. activation of the Ca-dependent

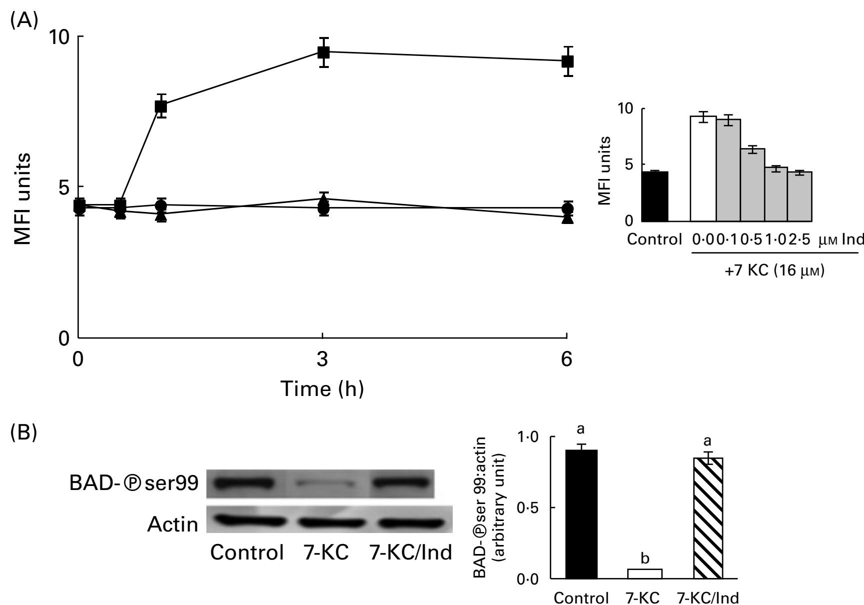

Fig. 6. 7-Ketocholesterol (7-KC)-induced (A) increase of free intracellular Ca^{2+} (B) and decrease of phospho-Bcl-2 antagonist of cell death ser99 levels with densitometric analysis of the immunoblots in THP-1 and effect of indicaxanthin (Ind) cells. (A) Ca^{2+} levels were assayed after cell loading with fluo-3/AM followed by flow cytometry analysis. Data are means and standard deviations of three separate experiments carried out in duplicate. The inset shows dose-dependent effects of Ind in the presence of 7-KC (16 μM) after 6 h incubation. (B) Representative image of three separate Western blotting analyses with comparable results. [a,b] Mean values with unlike letters were significantly different ($P < 0.05$; Fisher's test). Control, cells treated with vehicle; 7-KC, cells treated for 12 h with 7-KC (16 μM); 7-KC/Ind, cells treated with 7-KC in the presence of Ind (2·5 μM). MFI, mean fluorescence intensity.

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phosphatase calcineurin, dephosphorylation, activation and translocation to mitochondria of the pro-apoptotic protein BAD, release of cytochrome *c* in the cytosol and caspase activation[44,45]. Ind totally prevented the 7-KC-induced intracellular Ca elevation, as well as the downstream pro-apoptotic events such as dephosphorylation of BAD, mitochondrial depolarisation and caspase 3-dependent PARP cleavage, which provides a rationale for the cytoprotective effect through interference with the mitochondria-mediated apoptotic process[62,73]. Finally, under the conditions applied in the present study, the increase of intracellular ROS after treatment with 7-KC preceded Ca elevation. 7-KC has been reported to promote the influx of Ca^{2+} across the THP-1 plasma membrane through distinct mechanisms, including perturbation of the membrane lipid rafts followed by translocation of Ca^{2+} channels into the rafts[42] and/or interaction with subunits of Ca channels, leading to modulation of their gating or electrophysiological properties[74]. Ca ion channels may be redox regulated[75,76]. The present results may suggest a role of ROS in promoting the 7-KC-stimulated Ca influx in our THP-1 cell system.

The protective activity of redox-active and antioxidant natural compounds against oxysterol-induced apoptosis of macrophages has been the object of some research[38,39,48]. It has been shown that the ability of antioxidants to counteract 7-KC-induced apoptosis does not only depend, and cannot simply be explained, by their capability to inhibit production of ROS[77]. In this context, the present study shows that the anti-apoptotic effect of Ind is associated not only with maintaining of the cell redox balance, but also with the inhibition of the 7-KC-dependent elevation of Ca^{2+} in the cell cytosol. A mechanism explaining, at a molecular level, the activity of Ind cannot definitely be assessed at this time; however, previous studies and present findings allow some speculation to be made. In aqueous systems Ind can partition into either saturated or unsaturated phosphatidylcholine liposomes, where it locates at the so-called palisade domain, between the core hydrophobic region and the polar head groups[5–7]. Such a location would allow Ind to react with radicals generated and/or adsorbed onto the membrane surface[5], or possibly modulate membrane processes by biophysical mechanisms. We observed that Ind alone did not scavenge ROS formed by the basal activity of NOX-4; however, the enzyme appeared to be regulated negatively by the concurrent action of Ind and 7-KC. Biophysical variations of the membrane, in particular at the raft microdomain, are known to modulate NOX-4[55,56]. As destruction of membrane rafts is a consequence of 7-KC treatment[50,53], it is tempting to speculate that Ind and 7-KC interacting at the THP-1 cell membrane could bring about some stabilising effect, leading to modulate NOX-4 enzyme activity and possibly prevent opening of Ca channels. It is important, however, that no effect was elicited by Ind alone on the cell redox status, as in contrast with abnormal cells, e.g. the tumour ones, interference of redox-active compounds with the redox environment of normal cells might interfere with certain physiological cellular and organ functions, and would thus eventually be detrimental rather than beneficial.

Previous studies have shown that the regular consumption of cactus pear fruits positively affects the body's redox balance, decreases oxidative damage to lipids and improves antioxidant status in healthy human subjects[78]. The present findings demonstrate that Ind, a highly bioavailable phytochemical[16], characteristic of these fruits, can prevent the 7-KC-induced apoptosis of human macrophages when assayed at nutritionally relevant concentrations. Though these seasonal fruits are not common, fruit derivatives, such as juices or concentrated extracts to be added to other food preparations (yogurt, other fruit juices, etc), can easily be prepared by industrial food manufacturers. The stability of Ind[79] may allow such treatments. Given the importance of oxidised cholesterol derivatives in the pathogenesis of atherosclerosis, the present findings providing new information on the bioactive potential of Ind further highlight the contribution of *O. ficus indica* fruits to human health.

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