Research paper

Evidence of cell damage induced by major components of a diet-compatible mixture of oxysterols in human colon cancer CaCo-2 cell line

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ABSTRACT

Cholesterol oxidation products, termed oxysterols, have been shown to be more reactive than unoxidized cholesterol, possessing marked pro-inflammatory and cytotoxic effects in a number of cells and tissues. Oxysterols, absorbed with the diet as products of cholesterol auto-oxidation, have recently been suggested to potentially interfere with homeostasis of the mucosal intestinal epithelium, by promoting and sustaining irreversible damage.

However, the treatment of colon cancer cells with a diet-compatible mixture of oxysterols does not elicit the same responses than individual components added to the cells at the same concentrations at which they are present in the mixture. Sixty μM oxysterol mixture showed a slight pro-apoptotic effect on human colon cancer CaCo-2 cell line, evaluated in terms of caspase-3 and caspase-7 activation; conversely, 7α-hydroxycholesterol, 7β-hydroxycholesterol and 5α,6α-epoxycholesterol were identified to be able to induce a significant pro-apoptotic effect if added to cell culture singly; 7β-hydroxycholesterol had stronger action than other compounds. The enhanced production of reactive oxygen species through up-regulation of the colonic NADPH-oxidase isoform NOX1 appeared to be the key event in oxysterol-induced apoptosis in these colon cancer cells. As regards pro-inflammatory effects of oxysterols, IL-8 and MCP-1 were evaluated for their chemotactic activity. Only MCP-1 production was significantly induced by 7β-hydroxycholesterol, as well as by cholesterol and oxysterol mixture. However, oxysterol-induced inflammation appeared to be NOX1-independent, suggesting a secondary role of this enzyme in inducing inflammation in colon cancer cells.

A selective cell death induced by specific oxysterols against colon cancer cells, mainly exploiting their ability to activate NOX1 in generating oxidative reactions, might represent a promising field of investigation in colorectal cancer, and might bring new insights on strategies in anticancer therapy.

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1. Introduction

Cholesterol oxidation products, termed oxysterols, may either originate endogenously, through enzymatic or non-enzymatic reactions, or may derive from the diet. As regards exogenous sources of oxysterols, foods containing cholesterol are susceptible to oxidation: oxidative reactions occur during food processing, mainly on exposure to heat treatment and during long-term storage.

The oxysterols most commonly found in processed and/or stored cholesterol-rich food are 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6α-epoxycholesterol, and 5β,6β-epoxycholesterol [1,2]. Oxysterols are now thought to be involved in the initiation and progression of major chronic diseases, including

atherosclerosis, neurodegenerative processes, diabetes, and ethanol intoxication [3]. Oxysterols are more polar and more readily diffusible through cell membranes, and have consistently been shown to be more reactive than unoxidized cholesterol, possessing marked pro-inflammatory and cytotoxic effects in a number of cells and tissues [4,5].

The intestinal mucosa is constantly exposed to environmental insults, including food-derived lipid oxidation compounds. Regulation of the mucosal response to dietary antigens is controlled via the activation of innate and adaptive immunity, as well as through inflammatory reactions. Recent studies point to control pathogen-associated molecular patterns being influenced by changes in cellular redox status [6]; dysregulation of these molecular events could be a pathogenic mechanism in human chronic intestinal diseases, particularly in inflammatory bowel diseases (IBD), as well as in the development of colorectal cancer [7,8].

In previous work, we found that challenging CaCo-2 cells with a representative mixture of the oxysterols most commonly found in cholesterol-rich foodstuffs may contribute to oxidative unbalance by inducing the generation of reactive oxygen species (ROS) through up-regulation of the colonic NADPH-oxidase isoform NOX1 [9,10]. Interestingly, oxysterols exert their biological action in ways that differ depending on the degree of cell differentiation (i.e. normal enterocyte-like CaCo-2 cells, or the undifferentiated CaCo-2 cell line) [9].

Oxysterols are always present in dietary lipids as a mixture; however, the contributions of individual oxysterol components to inducing intestinal damage must be taken into account. Different oxysterols have been found to interact in producing their effect on other cell types; specific competing oxysterols that induce cell death in U937 monocytic cell line have been detected [11]. Certain oxysterols exert their cytotoxicity through NADPH oxidase activation in macrophages and vascular cells [12,13]; the same oxysterols applied as a mixture to endothelial cells or macrophages do not elicit the same response [14,15]. Although oxysterols showed lower cytotoxicity when applied to vascular cells as a mixture rather than as individual compounds, a mixture of oxysterols, like that detectable in hypercholesterolemic subjects, increased the expression of several inflammatory molecules in phagocytic cell lines [16,17]. It has recently been suggested that the cytotoxicity of selected oxysterols against cancer cells could be exploited as an anticancer agent [18]. Even assuming that a mixture of oxysterols has more biological relevance than individual components also in the intestinal tract, it is essential to assess how individual oxysterols contribute to cell damage, and how they may influence colon cancer cell survival.

Because of the significant impact that oxysterols might exert on the homeostasis of the human digestive tract, it appeared to be of primary interest to investigate the potential selective toxic effects of the principal components of a well-defined dietary oxysterol mixture on human colon carcinoma CaCo-2 cell line.

2. Materials and methods

2.1. Chemicals

Unless otherwise specified, reagents and chemicals were obtained from Sigma−Aldrich (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I (4500 mg/L glucose and 110 mg/L sodium pyruvate) and foetal bovine serum (FBS) were from Invitrogen, Life Technologies Italia s.r.l. (S. Giuliano Milanese, Italy). Ethanol and Triton X-100 were from VWR International s.r.l. (Milan, Italy). Bio-Rad protein assay dye reagent was from Bio-Rad (Milan, Italy). Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was from Alexis, Vinci-Biochem (Vinci, Florence, Italy). Protein A-Sepharose resin, Hybond ECL nitrocellulose membranes and ECL-plus kit were from GE Healthcare (Milan,

Italy). Mouse anti-Noxa1 was from Abcam (Cambridge, MA, USA). Goat anti-Nox1 primary antibody, donkey anti-goat and mouse anti-rabbit HRP-conjugated secondary antibodies were from Santa Cruz, Tebu-Bio s.r.l. (Magenta, Milan, Italy). Mowiol 4-88 was from Calbiochem, Inalco S.p.A. (Milan, Italy). Glass base dishes were from Bibby Sterilin Italia (Milan, Italy). PBS and microscope cover-glasses were from Bio-Optica (Milan, Italy). 25 cm^2 plastic flasks and 96 multi-well plates were from Falcon, Becton Dickinson Labware Europe (Meylan Cedex, France). Oxysterols were from Steraloids Inc. (Newport, USA). DuoSet ELISA (Enzyme Linked ImmunoSorbent Assay) kit for human IL-8 was from R&D System (Space Import Export, Milan, Italy). MCP-1 Instant ELISA kit was from Bender MedSystems GmbH, Vienna (Prodotti Gianni, Milan, Italy).

2.2. Cell line culture and treatments

Human colon adenocarcinoma CaCo-2 cell line (Cell Bank Interlab Cell Line Collection, Genoa, Italy) (passage number: 18-20), was cultured in DMEM-GlutaMAX™-I supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amfotericin B) at 37 °C in a 5% CO$_2$ humidified atmosphere.

CaCo-2 cells were plated at 2.5×10^5/ml; after 2 days the cells reached 70% confluence with 1×10^6/ml density and were used for the treatments. Before each treatment, CaCo-2 cells were made quiescent through overnight incubation in serum-free medium. Cells were then placed in DMEM with 2% FBS and challenged with cholesterol, individual oxysterols, or the oxysterol mixture, at 37 °C, for the incubation times indicated in the figure legends.

Cholesterol and the oxysterol mixture were diluted in ethanol (0.1% v/v) and added to cells to reach different final concentrations (30 μM, 60 μM or 90 μM). The percentage composition of the oxysterol mixture used was: 7-ketocholesterol (7K) (42.96%), 5α,6α-epoxycholesterol (α-epox) (32.3%), 5β,6β-epoxycholesterol (β-epox) (5.76%), 7α-hydroxycholesterol (7α-OH) (4.26%), 7β-hydroxycholesterol (7β-OH) (14.71%). The oxysterol mixture concentration was calculated using an average molecular weight of 403 g/mol.

The concentrations used for oxysterol treatments, when individual oxysterols were applied to the cells, were the same at which they were present in the mixture at the 60 μM concentration: 25.8 μM 7K, 19.4 μM α-epox, 3.4 μM β-epox, 2.6 μM 7α-OH, 8.8 μM 7β-OH. In some experiments, cells were pre-treated for 30 min at 37 °C with 5 μM NADPH oxidase inhibitor diphenylene iodonium (DPI); the solvent used for diluting DPI was dimethyl sulfoxide.

In the control groups, cells were incubated with the same amount of solvent used to dilute the various substances employed for treatments.

2.3. Evaluation of cell proliferation and viability

Cell counting and viability was routinely checked by the Trypan Blue exclusion method using a Bürker chambre: number of cells were counted in 20 different fields using a light microscope (20×/ 0.5 objective lens), and cell viability was calculated as the percentage of viable cells present/field.

Cell proliferation was evaluated using MTT test: 100 μl cell suspensions (in concentrations of 5,000, 10,000 and 20,000 cells/ well) were seeded in 96-well plates and incubated for 24 h in presence of different concentrations of oxysterol mixture. Formazan formation was evaluated following the procedure by Alley and colleagues [19], recorded at 540 nm wavelength using a microplate reader (Model 680 Microplate Reader, Bio-Rad, Milan, Italy), and expressed as percentage of the control group.

Extracellular release of lactate dehydrogenase (LDH) was checked as parameter of necrosis. LDH was evaluated spectrophotometrically

at a wavelength of 340 nm, and expressed as a percentage, taking as 100% the amount of enzyme released into the medium when 0.5% Triton X-100 was added to a culture flask containing the same cell density as the test specimen.

2.4. Evaluation of caspases-3 and caspase-7 activities

After 24 h treatment, cells were trypsinized and harvested by centrifugation at 80 g for 5 min, washed with cold 0.1 M PBS and suspended in 600 μl ice-cold lysis buffer (25 mM HEPES, 5 mM MgCl$_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride) for 15 min; the suspension was frozen/thawed ten times and then sonicated for 15 s at 5% output power (Sonicator Ultrasonic Liquid Processor Model W-385, Heat Systems-Ultrasonics Inc., NY, USA), then centrifuged at 10,000g for 30 min at 4 °C. Cell supernatant was collected and diluted to achieve a protein concentration of 0.5 mg/ml and incubated for 60 min at 37 °C with 65 μM (final concentration) fluorogenic substrate Ac-DEVD-AMC. Caspase-3 and caspase-7 activities were evaluated as the release of fluorescent 7-amino-4-methylcoumarin (AMC), using a spectrofluorimeter (Kontron SFM 25, USA) set to 380 nm excitation and 510 nm emission, and calibrated with 1 mM AMC as standard solution. Proteins were evaluated with a Bio-Rad protein assay dye reagent [20].

2.5. Evaluation of activated NADPH oxidase complex by immunoblotting

To evaluate activation of the NADPH oxidase colonic isoform (NOX1), the binding of the cytoplasmic subunit Noxa1 to the membrane enzyme component Nox1 was evaluated through Western Blotting, by immunoprecipitation for Nox activator 1 (NoxA1) and by immunoblotting for Nox organizer 1 (Nox1). After 1 h treatment, cells were processed as described for caspase-3 evaluation, using a different incubation buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA, 200 mM sucrose). After centrifugation at 10,000g for 30 min at 4 °C, supernatant was used for the isolation of cell membranes: cell supernatant was centrifuged at 100,000g × 75 min, and the pellet collected. The membrane fraction was suspended in cold lysis buffer [10 mM Tris–HCl pH 7.4, 1 mM EDTA, Triton X-100 0.5% (v/v)] for 20 min. Immunoprecipitation and immunoblotting were performed as described by Biasi et al. [9]. Proteins were evaluated with the Bio-Rad protein assay dye reagent, following the Bradford method [20].

2.6. Measurement of intracellular production of reactive oxygen species

Intracellular reactive oxygen species (ROS) production was visualized by laser scanning confocal microscopy, using the 2′,7′-dichlorofluorescein (DCF) and dihydroethidium (DHE) fluorescent probes. Cells were grown on glass base dishes.

DCF detection: after 1 h treatment with an oxysterol or the mixture, cells were incubated for 10 min with 10 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA). Once cleaved by cell membranes, DCFH permeates cells and becomes oxidized by ROS to become the DCF fluorescent molecule. DCF fluorescence was directly monitored on cell culture plates using a laser scanning confocal microscope equipped with an inverted microscope, plan neofluar lens 40×/0.75 (microscope Zeiss LSM 510, Carl Zeiss S.p.A., Arese, Milan, Italy). The instrument was set to 488 nm exciting laser band, with a 515 nm long-pass emission filter.

DHE detection: after 1 h treatment with an oxysterol or the mixture, cells were incubated for 30 min with 10 μM DHE and fluorescence was immediately detected on glass base dishes in confocal microscopy (plan neofluar lens 40×/0.75) by setting the

exciting laser band to 543 nm, and using a 560–615 nm band-pass emission filter.

All images were processed using LSM 510 Image Examiner software (Zeiss S.p.A.).

2.7. Evaluation of IL-8 and MCP-1 protein levels by ELISA

After 24 h treatment, cell culture medium was collected, centrifuged at 14,000g for 10 min and the supernatant was stored for ELISA detection. IL-8 levels were quantified using the DuoSet ELISA kit (R&D System) and MCP-1 was evaluated using the Instant ELISA kit from Bender MedSystems GmbH following the manufacturer's instructions. The plates were read at 450 nm with wavelength correction of 550 nm in a microplate reader (Model 680 Microplate Reader, Bio-Rad, Milan, Italy) and the data were analysed using SlideWrite Plus software (Advanced Graphics Software). The cytokine evaluation of each sample was performed in triplicate.

2.8. Statistical analysis

Results are expressed as means ± S.D. The statistical significance of parametric differences among sets of experimental data was evaluated by the one-way ANOVA test, associated with either Bonferroni's multiple comparison or Student Newman–Keuls post tests, as indicated in the figure legends.

3. Results

3.1. Effects of different concentrations of a representative dietary oxysterol mixture on cell viability and proliferation

With the aim of investigating whether dietary oxysterol mixture exerts a necrogenic effect on colon cancer CaCo-2 cells, cell growth and viability was investigated using the selective Trypan Blue dye exclusion staining, and MTT test was used for cell proliferation and viability. Necrosis was also detected as percentage of lactate dehydrogenase release in the cell medium. The most representative dietary oxysterols, i.e. 7K, α-epox, β-epox, 7α-OH, and 7β-OH, were mixed in the proportions resulting from the complete autoxidation of cholesterol [1]. CaCo-2 cells were treated with increasing concentrations of the oxysterol mixture (30, 60 or 90 μM) or with the same concentrations of unoxidized cholesterol.

Oxysterol mixture concentrations of 30 and 60 μM did not exert any necrogenic effect, at least not within the time of experimental observation. At 24 h treatment, viability was still good, similar to that observed for cholesterol, ranging from 95 to 99%, with correspondingly poor LDH cell release (2–4%). As regards cell proliferation, cell count did not show any significant cell number decrease; only MTT-formazan production decreased at 60 μM concentration ($p < 0.01$).

Conversely, treatment with 90 μM oxysterol significantly decreased MTT-formazan formation and percentage of viable cells (85%), and increased the release of necrosis parameter LDH (10%); these two last were also significantly different from cholesterol group ($p < 0.001$) (Table 1). Based on this data, to exclude any necrosis and to achieve the maximum biological effects, all subsequent experiments used 60 μM concentration of oxysterols, added individually or as a mixture.

3.2. Induction of apoptosis in colon cancer CaCo-2 cells by the oxysterol mixture compared to its individual components

Fig. 1 shows the induction of apoptosis in terms of caspase-3/-7 activities, in cells treated with 60 μM oxysterol mixture or its individual components. Each oxysterol was added singly to CaCo-2

Table 1
Effect of different concentrations of the oxysterol mixture on CaCo-2 cell proliferation and viability.

	Trypan blue (% viable cells)	Cell count 10^3 cells/ml	MTT (% control)	LDH (% release)
Control	98 ± 2	1064 ± 171	100	2 ± 2
30 μM cholesterol	98 ± 3	1115 ± 261	98.1 ± 5.7	3 ± 2
30 μM oxysterol mixture	98 ± 2	1073 ± 209	96.7 ± 5.2	2 ± 2
Control	99 ± 1	1195 ± 254	100	2 ± 2
60 μM cholesterol	97 ± 3	1048 ± 275	95.3 ± 5.1	2 ± 2
60 μM oxysterol mixture	95 ± 4	1154 ± 256	87.2 ± 4.9[a]	3 ± 2
Control	97 ± 3	1043 ± 257	100	2 ± 1
90 μM cholesterol	95 ± 4	1096 ± 100	90.7 ± 8.2	4 ± 4
90 μM oxysterol mixture	85 ± 3[b]	1070 ± 152	89.7 ± 5.3[a]	10 ± 3[b]

Cells were treated for 24 h with cholesterol or oxysterol mixture. The % of viable cells was counted in 20 different fields per sample, using Trypan blue staining. LDH was evaluated as the percentage of enzyme released in the medium compared to that present in the medium of cells cultured at the same density and treated with 0.5% Triton X-100 (taken as 100%). Data on Trypan blue staining, cell counting and LDH are means ± SD of ten different experiments. Data on MTT test are means ± SD of four experiments. Statistical difference within experimental groups was calculated using ANOVA associated with the Bonferroni post test.
  [a] Significantly different from control ($p < 0.01$).
  [b] Significantly different from control and cholesterol experimental groups ($p < 0.001$).

cells at the same concentrations as when applied in the mixture, i.e. 25.8 μM 7K, 19.4 μM α-epox, 3.4 μM β-epox, 2.6 μM 7α-OH, and 8.8 μM 7β-OH. Incubation was for 24 h.

Unoxidized cholesterol was unable to induce caspase-3 activity, while only a slight significant enzyme increase was observed in oxysterol mixture group compared to controls. Conversely, when administered singly, α-epox and 7α-OH values were significantly higher than controls. Caspase-3/7 activation in β-OH-treated cells

was significantly increased compared to both controls and oxysterol mixture.

3.3. Activation of NOX1 and ROS generation by oxysterols added singly or as a mixture to colon cancer CaCo-2 cells

The ability of the different components of the oxysterol mixture to induce activation of the colonic NADPH oxidase NOX1 was investigated in CaCo-2 cells treated for 1 h with the 60 μM oxysterol mixture, or with its individual components. NOX1 percentage activation was compared to controls (addition of solvent alone) taken as 100%.

CaCo-2 cells incubated with cholesterol did not show any significant NOX1 increase, while cells challenged with the oxysterol mixture showed a slight but significant difference compared to controls. As regards cell treatment with individual components, similarly to findings for caspases' activity, α-epox and 7β-OH were significantly more efficient than the oxysterol mixture in inducing NOX1 activity. In particular, NOX1 activation by 7β-OH was significantly elevated compared to controls, and also compared to the oxysterol mixture (Fig. 2). Consequent on the stimulation exerted on NOX1 activity, a burst in ROS production by CaCo-2 cells was observed, the trend being similar to that detected for NOX1 (Fig. 3).

3.4. Evidence of NOX1 involvement in oxysterol-dependent apoptosis activation

Activation of caspase-3/7 and NOX1 induction were demonstrated to be causally related, by adding the NADPH oxidase inhibitor DPI to the incubation medium 30 min before cell treatment. DPI was found to prevent the activation of NADPH oxidase as induced by oxysterols, and completely prevented the activity of caspase-3 and caspase-7 in CaCo-2 cells (Fig. 4).
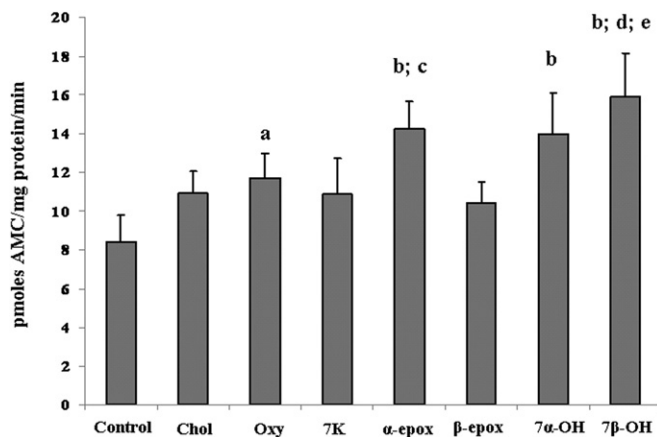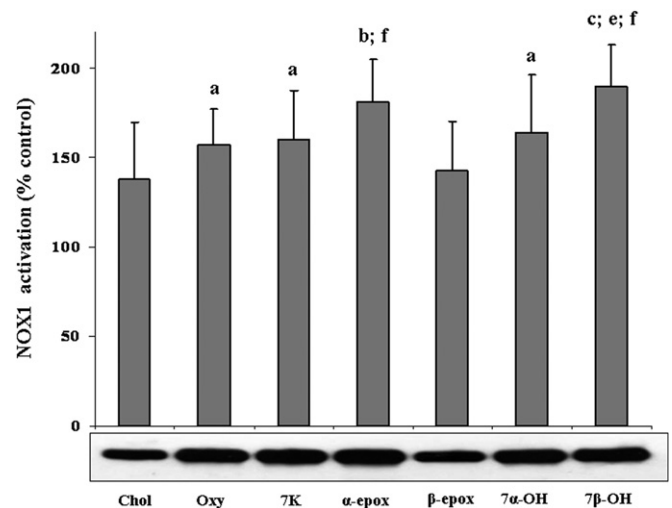

Fig. 1. Different response to apoptosis induced by 60 μM oxysterol mixture or by single oxysterol mixture components in colon cancer CaCo-2 cells. CaCo-2 cells were treated for 24 h with 60 μM cholesterol, oxysterol mixture, individual oxysterols or solvent alone. Caspase-3/-7 specific activity was expressed as pmoles of fluorescent 7-amino-4-methylcumarin (AMC) released/mg cell protein/min. C: control (solvent); Chol: 60 μM cholesterol; Oxy: 60 μM oxysterol mixture; 7K: 25.8 μM 7-ketocholesterol; α-epox: 19.4 μM 5α,6α-epoxycholesterol; β-epox: 3.4 μM 5β,6β-epoxycholesterol; 7α-OH: 2.6 μM 7α-hydroxycholesterol; 7β-OH: 8.8 μM 7β-hydroxycholesterol. Data are means ± S.D. of four experiments. Statistical difference within experimental groups was calculated using ANOVA associated with the Bonferroni post test: significantly different versus control ([a]$p < 0.05$; [b]$p < 0.001$); significantly different versus cholesterol ([c]$p < 0.05$; [d]$p < 0.001$); significantly different versus oxysterol mixture ([e]$p < 0.01$).

Fig. 2. Activation of the colonic NADPH oxidase isoform (NOX1) by oxysterols added singly or as a mixture to colon cancer CaCo-2 cells. CaCo-2 cells were treated for 1 h with 60 μM cholesterol, oxysterol mixture, individual oxysterols or solvent alone. Activated NOX1 was detected as described in the Material and methods section. Protein levels were evaluated by densitometry and were reported as NOX1 percentage activation compared to controls taken as 100%. Chol: 60 μM cholesterol; Oxy: 60 μM oxysterol mixture; 7K: 25.8 μM 7-ketocholesterol; α-epox: 19.4 μM 5α,6α-epoxycholesterol; β-epox: 3.4 μM 5β,6β-epoxycholesterol; 7α-OH: 2.6 μM 7α-hydroxycholesterol; 7β-OH: 8.8 μM 7β-hydroxycholesterol. Data are means ± S.D. of four experiments. Statistical difference within experimental groups was calculated using ANOVA associated with the Bonferroni post test ([a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$); significantly different versus cholesterol ([e]$p < 0.05$); significantly different versus oxysterol mixture ([f]$p < 0.05$).
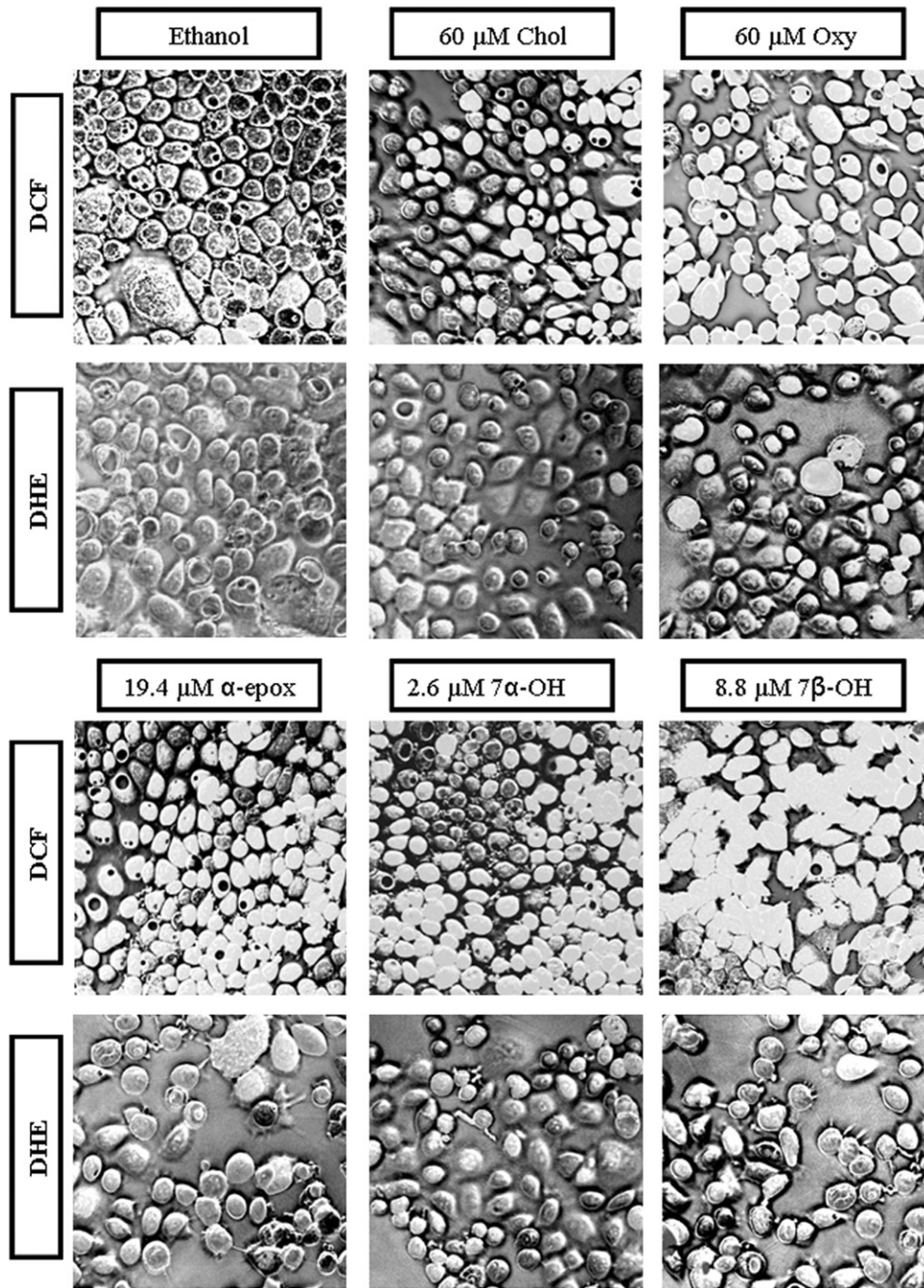
Fig. 3. Reactive oxygen species (ROS) production in colon cancer CaCo-2 cells treated with oxysterols mainly involved in NOX1 activation. After 1 h treatment with different sterols, cells were incubated with DCF-DA or DHE. ROS production was detected as intracellular DCF or DHE fluorescence by laser scanning confocal microscopy as described in the Material and methods section. The images were elaborated by a LSCM 510 Image Examiner Program, and are visualized in grey scale (white corresponds to high fluorescence). Here are shown images related to ROS generation by cholesterol and oxysterol mixture, compared to the three oxysterol components that showed ability to induce NOX1 activation.

3.5. Effect on IL-8 and MCP-1 production displayed by oxysterols added singularly or as a mixture to colon cancer CaCo-2 cells

With the aim of investigating whether oxysterols exert a pro-inflammatory effect on colon cancer cells, IL-8 synthesis was investigated after CaCo-2 cell treatment with the oxysterol mixture or its different components. Fig. 5 shows the levels of IL-8 excreted in the cell medium. The only slight increase in IL-8 was observed in the oxysterol mixture treatment group, but neither 7β-OH, nor α-epox, nor 7α-OH enhanced cytokine synthesis. Interestingly, pre-treatment of cells with DPI only caused 0–20% inhibition of IL-8 production.

Unlike IL-8, cell treatment with 7β-OH significantly induced MCP-1, which was also increased by cholesterol and oxysterol mixture. However, DPI pre-treatment wasn't able to reduce MCP-1 induction to control values in all experimental groups (Fig. 6).

4. Discussion

The findings reported here point to a selective role of certain components of a representative mixture of oxysterols, as found in cholesterol-rich foodstuffs, in inducing death of colon cancer cells. It is now generally accepted that cholesterol and oxysterols are
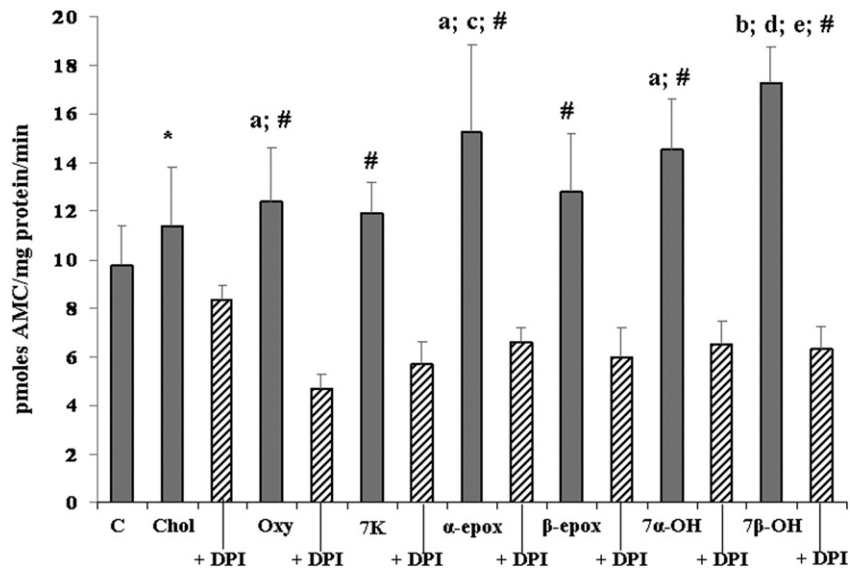
Fig. 4. Evidence of NOX1 involvement in apoptosis induced by oxysterols in CaCo-2 cancer cells: inhibition of caspase-3/-7 activity by DPI. Cell pre-treatment with 5 μM DPI (30 min) was made in order to inhibit NOX1 activation; CaCo-2 cells were further incubated (still in the presence of the chemical inhibitor) for 24 h with 60 μM cholesterol, oxysterol mixture, individual oxysterols or solvent alone. C: control (solvent); Chol: 60 μM cholesterol; Oxy: 60 μM oxysterol mixture; 7K: 25.8 μM 7-ketocholesterol; α-epox: 19.4 μM 5α,6α-epoxycholesterol; β-epox: 3.4 μM 5β,6β-epoxycholesterol; 7α-OH: 2.6 μM 7α-hydroxycholesterol; 7β-OH: 8.8 μM 7β-hydroxycholesterol; +DPI: different experimental groups pre-treated with DPI. Caspase-3/-7 activity was evaluated as shown in Fig. 1. Data are means ± S.D. of four experiments. Statistical difference within experimental groups was calculated using ANOVA associated with the Student Newman−Keuls post test: significantly different versus control ([a]$p < 0.01$; [b]$p < 0.001$); significantly different versus cholesterol ([c]$p < 0.05$; [d]$p < 0.01$); significantly different versus oxysterol mixture ([e]$p < 0.01$); significantly different versus sterol treatments in presence of DPI (*$p < 0.05$; #$p < 0.001$).

involved in the development of different vascular degenerative disorders, due to their pro-oxidant and pro-inflammatory properties [3]. These compounds have recently been suggested to be associated with the pathogenesis of IBD, and with an increased risk of developing colorectal cancer. They may be absorbed in the diet as products of cholesterol auto-oxidation. Excessive dietary intake of cholesterol, as well as of oxidized lipids, in the form of animal fats, may sustain inflammatory and apoptotic reactions, reducing the normal intestinal barrier function, and predisposing the colonic mucosa to cancer [10,21].

Conversely, colon cancer cells behave differently than normal cells, becoming more resistant against pro-inflammatory and pro-apoptotic molecules so as to safeguard their survival.

We determined the necrogenic effect in colon cancer CaCo-2 cells of a representative dietary mixture of oxysterols, which has very recently been shown to potentially interfere with the homeostasis of the intestinal epithelium [9,10]. The oxysterol mixture was used in concentrations corresponding to low (30 μM), moderate (60 μM) and high (90 μM) daily dietary intake. Our results on LDH release and Trypan Blue staining show that, when oxysterols are present as a mixture, only the 90 μM concentration appeared to be necrogenic. Thus, 60 μM was used in these experiments as being the highest concentration that induces biological effects without any necrosis.

In the here reported experiments, 60 μM oxysterol mixture exerted a slight apoptotic effect compared to controls and to
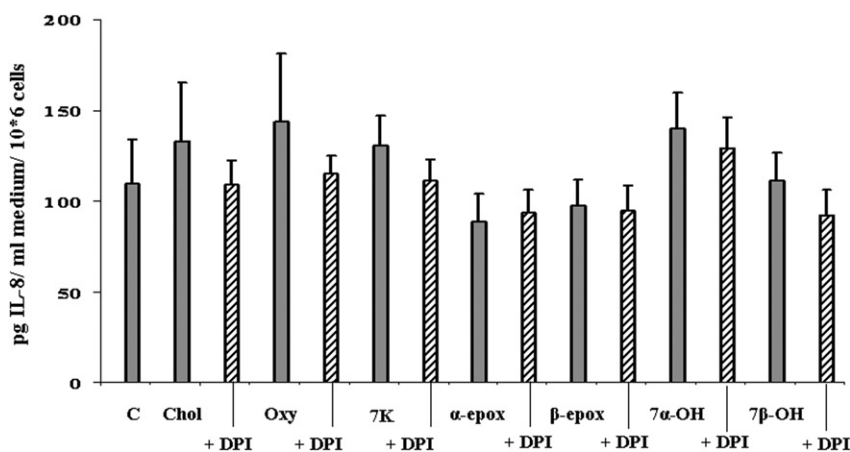

Fig. 5. Evaluation of IL-8 production in colon cancer CaCo-2 cells treated with oxysterols added as a mixture or singly. The production of IL-8 was analysed by ELISA in the culture medium of CaCo-2 cells pre-treated or not for 30 min with 5 μM DPI and then challenged for 24 h with 60 μM cholesterol, oxysterol mixture, individual oxysterols or solvent alone. C: control (solvent); Chol: 60 μM cholesterol; Oxy: 60 μM oxysterol mixture; 7K: 25.8 μM 7-ketocholesterol; α-epox: 19.4 μM 5α,6α-epoxycholesterol; β-epox: 3.4 μM 5β,6β-epoxycholesterol; 7α-OH: 2.6 μM 7α-hydroxycholesterol; 7β-OH: 8.8 μM 7β-hydroxycholesterol; +DPI: different experimental groups pre-treated with DPI. The cytokine concentration was expressed as pg of IL-8 released/10^6 cells. Data are shown as means ± S.D. of five experiments in triplicate. No statistical difference within experimental groups was found (calculated using ANOVA associated with the Student Newman−Keuls post test).
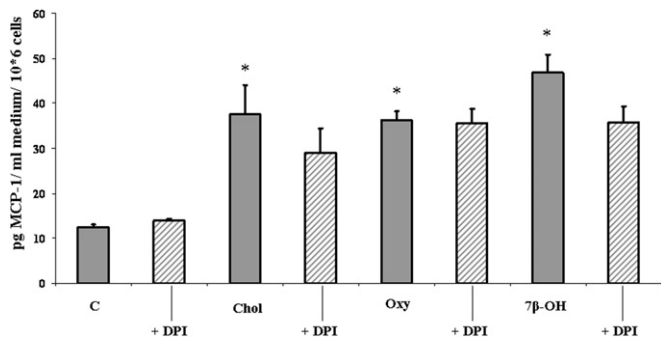
Fig. 6. Evaluation of MCP-1 production in colon cancer CaCo-2 cells treated with oxysterol mixture or 7β-hydroxycholesterol. The production of MCP-1 was analysed by ELISA in the culture medium of CaCo-2 cells pre-treated or not for 30 min with 5 μM DPI and then challenged for 24 h with 60 μM cholesterol, 60 μM oxysterol mixture, 8.8 μM 7β-hydroxycholesterol or solvent alone. C: control (solvent); Chol: 60 μM cholesterol; Oxy: 60 μM oxysterol mixture; 7β-OH: 8.8 μM 7β-hydroxycholesterol; +DPI: different experimental groups pre-treated with DPI. The cytokine concentration was expressed as pg of MCP-1 released/10^6 cells. Data are shown as means ± S.D. of 3 experiments in duplicate. Statistical difference within experimental groups was calculated using ANOVA associated with the Student Newman–Keuls post test: significantly different versus control (*$p < 0.001$).

unoxidized cholesterol. These findings are in agreement with our previous results showing no necrogenic action, and a moderate induction of apoptosis by 30 μM concentration of the same oxysterol mixture, in undifferentiated CaCo-2 cancer cells, compared to differentiated enterocyte-like CaCo-2 cells, which were more sensitive to the sterol challenge [9].

MTT assay showed a decrease on cell viability and proliferation following incubation with increasing concentrations of oxysterol mixture (60 μM/90 μM) (Table 1). Yellow tetrazolium salt MTT has the property of acting as hydrogen acceptor to be reduced by NADP(H)-dependent oxido-reductase enzymes, such as mitochondrial reductase enzyme, into its purple formazan product [22]. The observed formazan decrease might reflect a mitochondrial dysfunction, in agreement with many studies, which have reported a role of oxysterols in the induction of alteration of mitochondrial integrity, decrease of mitochondrial transmembrane potential and release of cytochrome *c* [23].

Oxysterols exert their damage in CaCo-2 colon cancer cells differently when they are administered singly. Studies from O'Sullivan and colleagues [24] have shown that 25-hydroxycholesterol, α-epoxy, β-epoxy or 7β-OH added individually to undifferentiated CaCo-2 cells determined a reduction in cell viability. The cytotoxicity exerted on undifferentiated CaCo-2 cells by 24 h treatment was assessed by the neutral red uptake assay and showed IC50 values with increasing concentrations of β-epoxy and 7β-OH (0–50 μM) [24]. Similar results were obtained in Caco-2 cells treated with 30 μM 7β-OH that inhibited cell growth by 50% at 32 h incubation and induced apoptosis, but in this case caspase-3 activity remained within control range during the whole observation time (72 h) [25]. In another study 30 μM of individual oxysterol components in the diet has shown that all α-epox, β-epox, 7K, and 7β-OH significantly reduced U937 viability and increased caspase activities and DNA fragmentation [26]. Very high concentrations of 7K (up to 120 μM) altered mitochondrial permeability and the relative distribution of total RNA in G1 and G2 phases of the cell cycle in 6 days plated CaCo-2 cells [27].

The influence of each component in inducing apoptosis in cancer cells, which were unresponsive to the oxysterol mixture used for our experiments, has been investigated: 7K, α-epox, β-epox, 7α-OH and 7β-OH were administered to cells individually, at the same concentration at which they are present in the 60 μM

mixture. Among these components only α-epox (19.4 μM), 7α-OH (2.6 μM) and 7β-OH (88.8 μM) induced an enhancement of caspase-3/-7 activity; with stronger effect of 7β-OH than oxysterol mixture. This suggests that oxysterols have a much lower effect when given to colonic cells as a mixture rather than as individual compounds. This effect has been confirmed in other cell types treated with other oxysterols, mainly involving 7β-OH or 7K: addition of 25-hydroxycholesterol to 7β-OH or 7K significantly reduced the amount of oxysterol-induced apoptosis, which, on the contrary, was enhanced by 20–40 μM 7β-OH or 7K in human U937 pro-monocytic cells [11,28]. Experimental studies on vascular cells reported that equimolar amounts of 7β-OH and 7K decreased the degree of apoptosis caused by 7K alone endothelial cells and in murine J774.A1 macrophages [14,15]. Conversely, synergistic cytotoxicity was found between 7K and 7β-OH, administered to monocytes in proportions similar to that found in atherosclerotic lesions [11].

In the experiments with colon cancer cells reported here, 7β-OH was found to be pro-apoptotic, through caspase-3/7 activation, and to be more effective than other oxysterols. However, other results suggest different pathways for 7β-OH inducing CaCo-2 cell death, rather than caspase-3 activation [25]. The same research group further challenged undifferentiated CaCo-2 cells 12 and 24 h in the presence of 30 μM 7β-OH, showing a decrease of cell viability and mitochondrial membrane potential, as well as the release of cytochrome *c*. However, 7β-OH did not affect the expression of the two members of Bcl-2 family proteins Bcl-2 and Bax, which are known to regulate mitochondrial membrane permeabilization. They showed that this oxysterol was also able to induce lysosomal membrane permeabilization, and suggested that cytotoxic effect of 7β-OH could be explained by a direct action on membrane integrity of both mitochondria and lysosomes [29]. The observed different effect of 7β-OH on the same CaCo-2 cell type may be only explained by the different concentration we used in our experiments (8.8 μM). Notably, low concentrations of 7K and α-epox (2.5 μM) have been found to induce the proliferation and migration of smooth muscle cells [30].

The oxysterols-dependent apoptosis in CaCo-2 cancer cells was mediated most likely through a strong early generation of ROS upon activation of the colonic NADPH oxidase complex NOX1. Indirect confirmation of these results comes from the significant protection against pro-apoptotic events afforded by cell pre-treatment with specific NADPH oxidase inhibitor. These data confirm the causative association of the pro-oxidant and pro-apoptotic effect observed in aortic smooth muscle cells and J774A.1 macrophages [12,15] and emphasise the major involvement of the NOX1 isoform in oxysterol-dependent apoptosis in CaCo-2 cancer cells, as already proposed for differentiated colonic cells [9].

As regards oxysterol-induced inflammation, several studies have reported their ability to induce expression and synthesis of various pro-inflammatory cytokines and chemokines. Evidence has been found in different cell types, using oxysterols either as individual compounds [31,32] or in a mixture [16,17], in which the single components were found to interact synergistically to induce the over-expression of specific pro-inflammatory genes. Similar results were also found in enterocyte-like CaCo-2 cells: the oxysterol mixture induced the over-expression of significant inflammatory molecules. Of the various oxysterols, 7β-OH markedly enhanced the expression of the frankly pro-inflammatory cytokines IL-1α, IL-6, IL-8, MCP-1 and IL-23 in normal intestinal cells more efficiently than oxysterol mixture [10].

In this study the production of Il-8 and MCP-1, the two well-known pro-inflammatory cytokines with chemoattractant property for neutrophils and macrophages, respectively, have been investigated. Contrary to other studies, IL-8 was not significantly

induced by either the oxysterol mixture or the individual oxysterols. These results agree with findings regarding the production of IL-8 by certain oxysterols in CaCo-2 cells pre-treated with IL-1β. Also in this case, 7β-OH had a slight effect on IL-1β-induced IL-8 mRNA expression [33].

Conversely, MCP-1 was highly produced by 7β-OH, and to a lesser extent by oxysterol mixture and the parent unoxidized cholesterol. MCP-1 has a crucial role in intestinal chronic inflammation characterized by a macrophage infiltration; its protein expression was augmented in the mucosa of patients with IBD [34]. Contradictory results come from other studies on human monocytic U937 and THP-1 that reported the high capability of 20 μM 7β-OH to induce IL-8 secretion, but not MCP-1 [35,36]. These data agree with our previous results on differentiated CaCo-2 cells that reported a significant up-regulation of both expression and synthesis of IL-8 by 7β-OH; however undifferentiated intestinal cells behave differently. Notably, the partial or even absent inhibition of MCP-1 by DPI suggests a secondary role of NADPH oxidase in the induction of inflammation by oxysterols in this colon cancer cytotype. Other mechanisms are probably involved in the induction of inflammatory chemokines in undifferentiated CaCo-2 cells; the inflammatory microenvironment in colorectal cancer is quite distinct from the surrounding normal tissue, allowing cancer cells to grow. Other cytokines mainly involved in immune response, such as interferon-γ and IL-17, have been found very low in cultured cells from human colorectal tissue [37].

The proven capacity of some oxysterols to induce cell death, suggest these compounds as potential anticancer agents. Different oxysterols isolated from *Selaginella tamariscina* plant, have been considered as promising candidates as anticancer compounds for their cytotoxic effects against tumour cells [38]. *In vitro* experiments in different cancer cell lines (U937, K562 and HepG2) have shown that treatments with 7β-OH reinforced the sensitivity of tumour cells to chemotherapy drugs or gamma radiation [39]. The presence of OH group substituent at ring B of sterols, as in 7-hydroxycholesterols, was shown to be essential for the destabilization of the cellular membrane and for inducing cytotoxicity. Similarly, epoxycholesterols can act as pro-drugs with increased cell death of cancer cells *in vivo* because of the decreased extracellular pH in solid tumours. In particular, α-epox showed a greater increase in cytotoxicity than β-epox [18].

5. Conclusions

The findings reported here emphasise that colon cancer cells are susceptible to the selective apoptotis induced by specific oxysterols, mainly through NOX1-dependent ROS generation. Conversely the partial response of these cancer cells to inflammatory stimulus induced by oxysterols, principally 7β-OH, is still unclear and appears to be NOX1-independent. The complex interaction between adaptive and innate immunity in inducing high or moderate inflammation in the intestinal mucosa makes colon cancer cell response to oxysterols different from other cytotypes.

Based on the evidence of the potential activity of 7-hydroxycholesterols and α-epoxycholesterol in inducing cell death, these oxysterols might be taken into account to assess new anticancer therapeutic strategies. The selective cytotoxicity of these compounds against colon cancer cells, exploiting their ability to activate NADPH oxidase in generating oxidative reactions, might represent a promising field of investigation in colorectal cancer.

Acknowledgements

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