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Qualitative and quantitative comparison of the cytotoxic and apoptotic potential of phytosterol oxidation products with their corresponding cholesterol oxidation products

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Phytosterols contain an unsaturated ring structure and therefore are susceptible to oxidation under certain conditions. Whilst the cytotoxicity of the analogous cholesterol oxidation products (COP) has been well documented, the biological effects of phytosterol oxidation products (POP) have not yet been fully ascertained. The objective of the present study was to examine the cytotoxicity of β-sitosterol oxides and their corresponding COP in a human monocytic cell line (U937), a colonic adenocarcinoma cell line (CaCo-2) and a hepatoma liver cell line (HepG2). 7β-Hydroxysitosterol, 7-ketositosterol, sitosterol-3β,5α,6β-triol and a sitosterol-5α,6α-epoxide–sitosterol-5β,6β-epoxide (6:1) mixture were found to be cytotoxic to all three cell lines employed; the mode of cell death was by apoptosis in the U937 cell line and necrosis in the CaCo-2 and HepG2 cells. 7β-Hydroxysitosterol was the only β-sitosterol oxide to cause depletion in glutathione, indicating that POP-induced apoptosis may not be dependent on the generation of an oxidative stress. A further objective of this study was to assess the ability of the antioxidants α-tocopherol, γ-tocopherol and β-carotene to modulate POP-induced cytotoxicity in U937 cells. Whilst α/γ-tocopherol protected against 7β-hydroxycholesterol-induced apoptosis, they did not confer protection against 7β-hydroxysitosterol- or 7-ketositosterol-induced toxicity, indicating that perhaps COP provoke different apoptotic pathways than POP. β-Carotene did not protect against COP- or POP-induced toxicity. In general, results indicate that POP have qualitatively similar toxic effects to COP. However, higher concentrations of POP are required to elicit comparable levels of toxicity.

Phytosterol oxides: Cholesterol oxides: Cytotoxicity: Apoptosis

Plant sterols (phytosterols) are membrane constituents of all plants with a structure analogous to that of cholesterol (zoosterols). Dietary intakes are estimated to be between 160 and 400 mg/d among different populations and are largely derived from vegetable oils, nuts, seeds and grains (Ostlund, 2002). During the last decade there has been an unprecedented escalation of interest in the potential health benefits of phytosterols, in particular as efficacious cholesterol-lowering agents. Moreover, phytosterols may possess anticarcinogenic, anti-atherosclerotic, anti-inflammatory and anti-oxidative activities (De Jong *et al.* 2003; Berger *et al.* 2004). Consequently, esters of phytosterols are being incorporated into a growing spectrum of functional foods such as margarine and salad dressings in a number of countries (Moreau *et al.* 2002). Dietary intakes are projected to increase accordingly. De Jong *et al.* (2004) reported that if liberal phytosterol fortification is allowed, the average daily intake might exceed 8·6 g.

Given their structural similarity to cholesterol, it is reasonable to assume that phytosterols are also susceptible to oxidation. The mechanisms of formation of cholesterol oxidation products (COP) are well documented (Tai *et al.* 1999). Furthermore, evidence exists that several COP may be linked to a series of human diseases, including atherogenesis, cytotoxicity, mutagenesis and carcinogenesis (Brown & Jessup, 1999). However, data on the mechanism of phytosterol oxidation are very limited, as are data on the level of phytosterol oxidation products (POP) present in food.

Some studies have noted modest amounts of POP in French fries, potato chips and vegetable oils (Lee *et al.* 1985; Dutta, 1997; Dutta & Appelqvist, 1997), wheat flour (Nourooz-Zadeh & Appelqvist, 1992), coffee (Turchetto *et al.* 1993) and commercial margarine (Grandgirard, 2002). Limited evidence suggests that some POP can pass the intestinal barrier, albeit at a low level (Grandgirard *et al.* 1999; Tomoyori *et al.* 2004). Recently, Grandgirard *et al.* (2004) identified appreciable quantities of oxidised phytosterols in plasma samples of healthy individuals. Two possibilities exist concerning the origin of these compounds in plasma. First, they may stem from absorption of the small amount of POP in food. However, second, their *in vivo* transformation into phytosterol oxides cannot be excluded. Plat *et al.* (2001) detected elevated levels of POP in serum of phytosterolaemic patients. In this latter study, phytosterol oxide concentrations in the serum of healthy control samples were below the limit of detection. In an earlier study, Aringer *et al.* (1976) observed a 27-hydroxylation of campesterol and sitosterol and a 29-hydroxylation of sitosterol in rat liver mitochondria.

Data on biological effects of POP are scarce. Abortifacient effects were described in mice (Pakrashi & Basak, 1976).

Abbreviations: COP, cholesterol oxidation products; FCS, fetal calf serum; POP, phytosterol oxidation products.

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Meyer & Spitellier (1997) described an increase in POP during ageing of photoautotrophic cell cultures, as well as cytotoxicity of these compounds in mealworms (Meyer *et al.* 1998). Adcox *et al.* (2001) demonstrated that a mixture of POP caused cellular damage in cultured macrophage-derived cells similar to that caused by COP. More recently, Maguire *et al.* (2003) reported that thermally oxidised derivatives of phytosterols demonstrate similar patterns of toxicity towards a human monocytic cell line (U937) as the COP 7β-hydroxycholesterol, but at higher concentrations. The authors describe the evaluation of epoxide prepared by the oxidation of commercial β-sitosterol (approximately 60% purity). In view of the projected increase in the consumption of foods enriched with phytosterols, which are known to be liable to oxidation, together with the undesirable health effects attributed to the related COP, additional information on the toxicological significance of POP is warranted.

β-Sitosterol represents the most abundant phytosterol in nature. Individual oxides of β-sitosterol were prepared for us by colleagues in the Department of Chemistry, University College Cork. The objective of the present study was to investigate the cytotoxicity and apoptotic potential of individual β-sitosterol oxides towards a human monocytic blood cell line (U937), a colonic adenocarcinoma cell line (CaCo-2) and a human hepatoma cell line (HepG2). These cells were employed as U937 cells have previously been shown to undergo apoptosis when treated with certain COP. CaCo-2 cells were used because the gut is the initial site of exposure, absorption and metabolism of dietary components. HepG2 cells retain many functions of the normal liver cell and have been used in many studies evaluating the cytotoxic potential of compounds. For comparison, the cytotoxic effects of the corresponding COP were also assessed in each cell line. The effect of individual POP on cellular glutathione levels was assessed in U937 cells as an index of oxidative stress. A further objective was to assess the ability of some antioxidants commonly found in human diets, i.e. α- and γ-tocopherol and β-carotene, to modulate POP-induced cytotoxicity.

Materials and methods

Materials

All chemicals and cell culture reagents were obtained from Sigma Chemical Co. (Poole, UK) unless otherwise stated. Tissue culture plastics were supplied by Costar (Cambridge, UK). Cell lines were obtained from the European Collection of Animal Cell Cultures (Sailsbury, UK). Information on the purity of the cholesterol oxides (purity $>95\%$) was obtained from Sigma.

Cell maintenance

Human monocytic U937 cells were grown in suspension in RPMI 1640 medium supplemented with 2 mM-L-glutamine and 10 % (v/v) fetal calf serum (FCS). Human colonic adenocarcinoma CaCo-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) FCS, 2 mM-L-glutamine and 1 % non-essential amino-acids. HepG2, a human hepatoma cell line, was grown in Williams E medium supplemented with 2 mM-L-glutamine and 10 % (v/v) FCS. The cells were grown at 37°C and 5 % (v/v) CO_2 in a humidified incubator. Cells were screened for mycoplasm contamination by the Hoechst staining method (Mowels, 1990) and were cultured in the absence of antibiotics. Exponentially growing cells were used throughout the experiments.

Production of β-sitosterol oxides

Individual POP are not commercially available and therefore were synthesised, purified and characterised by the Analytical and Biological Chemistry Research Facility (ABCRF) Department of Chemistry, University College Cork (McCarthy *et al.* 2005). The target compounds were sitosterol-5α,6α-epoxide (α-epoxysitosterol), synthesised as previously described by Maguire *et al.* (2003) sitosterol-5β,6β-epoxide (β-epoxysitosterol), 7β-hydroxysitosterol, 7-ketositosterol and sitosterol-3β,5α,6β-triol (triolsitosterol). The first objective was to obtain β-sitosterol with the best possible purity, in order to prepare unambiguous phytosterol oxides. Pure β-sitosterol (approximately 98·9 % purity) was synthesised from stigmasterol of approximately 95 % purity from Sigma-Aldrich. The strategy for the synthesis of β-sitosterol from stigmasterol involved the protection of the double bond in the β-ring followed by hydrogenation of the side-chain double bond. Ethanol was employed as the solvent and Pd–C as the catalyst at 345 kPa pressure. This was then followed by deprotection of the methyl ether to give β-sitosterol of approximately 98·9 % purity as defined by GC–MS and NMR spectroscopy. The second objective was to synthesise the target oxidation products from this pure β-sitosterol. The procedures followed in this work were very similar to those employed by Li & Wilson (1999) for the synthesis of COP.

Cell treatment with β-sitosterol oxides

For experiments, U937 cells, CaCo-2 cells or HepG2 cells were adjusted to a density of 2×10^5 cells/ml supplemented with reduced serum media, 2·5 % (v/v) FCS. Oxides of β-sitosterol were added to the tissue culture medium at the concentration of 30 μM, 60 μM or 120 μM. For comparison, cells were also incubated with 30 μM of the corresponding cholesterol oxides, namely, cholesterol-5α,6α-epoxide (α-epoxycholesterol), cholesterol-5β,6β-epoxide (β-epoxycholesterol), 7β-hydroxycholesterol, 7-ketocholesterol and cholestane-3β,5α,6β-triol (triolcholesterol). All compounds were dissolved in ethanol and the final concentration in the cultures did not exceed 0·4 % (v/v). Equivalent quantities of ethanol were added to control cells and samples were incubated for 24 h at 37°C and 5 % (v/v) CO_2. A concentration of 30 μM COP was selected for this study as it approximates the upper physiological concentration found in human plasma. Emanuel *et al.* (1991) reported a concentration as high as 37 μM in human plasma, following the ingestion of a test meal of spray-dried powdered eggs. Since this study is a comparative study of the cytotoxic and apoptotic potential of POP with their corresponding COP, the phytosterol oxides were used at concentrations as high as 120 μM as this is the concentration that showed similar toxic effects to the COP. The concentration of POP used in our *in vitro* models is higher than those encountered physiologically. Plat *et al.* (2001) reported 7β-hydroxysitosterol concentrations about 2 μM and slightly higher concentrations for 7-ketositosterol in serum from phytosterolaemic patients.

Treatment of U937 cells with β-sitosterol oxides in the presence of α/γ-tocopherol or β-carotene

U937 cells were adjusted to a density of 2×10^5 cells/ml supplemented with reduced serum media, 2·5 % (v/v) FCS. Cells were treated with 10 μM of α-tocopherol or γ-tocopherol or 2 μM-β-carotene for 1 h at 37°C and 5 % (v/v) CO_2. Subsequently, cells were treated with 30 μM-7β-hydroxycholesterol or 120 μM-7β-hydroxysitosterol or 7-ketositosterol. All compounds were dissolved (with the exception of β-carotene) in ethanol and the final concentration in the cultures did not exceed 0·4 % (v/v). β-Carotene was dissolved in hexane and the final concentration in the cultures did not exceed 0·4 % (v/v). Equivalent quantities of ethanol/hexane were added to the control cells. To determine if the tocopherols or β-carotene had any effect on toxicity, the cells were also incubated with these compounds alone. Samples were incubated for 24 h at 37°C and 5 % (v/v) CO_2.

Cell viability

The viability of the U937 cells was assessed after 24 h by the flurochrome-mediated viability assay as previously described by Strauss (1991). Briefly, cells were mixed 1:1 (v/v) with a solution of fluorescein diacetate and ethidium bromide, then incubated at 37°C for 5 min before being layered onto a microscope slide. Under these conditions, viable cells fluoresce green, whereas non-viable cells fluoresce red. Samples were examined at × 200 magnification on a Nikon fluorescence microscope (The micron optical Co. Ltd., Enriscorthy, Co. Wexford, Ireland) using blue light (450–490 nm). Two hundred cells were scored from each slide and cell viability was expressed as the percentage of viable (green) cells.

The viability of the CaCo-2 and HepG2 cells was assessed after 24 h by the Neutral Red uptake assay as described by Borenfreund & Puerner (1984). The Neutral Red uptake assay was chosen for the CaCo-2 and HepG2 cells as both are adherent cell lines and this is the most suitable viability assay for adherent cells. Whilst the fluorescein diacetate/ethidium bromide assay can be employed as a viability assay for the CaCo-2 and HepG2 cells, it would have involved scraping the cells and we felt this step might result in over- or underestimating the ratio of viable to non-viable cells. Following treatments, medium was removed from the cells. Neutral Red dye (50 μg/ml complete medium) was added to each well of the six-well plate and incubated for 2 h at 37°C and 5 % (v/v) CO_2 to allow for uptake of the dye into the lysosomes of viable uninjured cells. The medium was removed and cells were washed with Krebs buffer. The cells were subsequently treated with 2 ml of 1 % glacial acetic acid/50 % ethanol to burst cells open and release the dye. After agitation for a few minutes on a plate shaker, the samples were transferred to microcuvettes and absorbance was determined spectrophotometrically (540 nm).

Morphological analysis of cell nuclei

Nuclear morphology of control and oxysterol-treated cells was assessed by fluorescence microscopy after staining with Hoechst 33 342. Approximately 4×10^5 cells were centrifuged at 200 ***g*** for 10 min to form a pellet. Hoechst 33 342 stain (200 μl, 5 μg/ml) was added and the samples were incubated at 37°C and 5 % (v/v) CO_2 for 1 h. Stained samples were placed on a microscope slide and examined under UV light (Nikon Labophot fluorescence microscope, × 400 magnification; The Micron Optical Co. Ltd.). A total of 300 cells per sample were analysed and the percentage of fragmented and condensed nuclei was calculated. Apoptotic cells were characterised by nuclear condensation of chromatin and/or nuclear fragmentation (Dubrez *et al.* 1996).

DNA fragmentation assay

Detection of small DNA fragments was conducted as described in O'Callaghan *et al.* (1999). Briefly, 2×10^6 cells were harvested and the pellets were lysed, RNAse A (0·25 mg/ml) was added and the samples incubated at 50°C for 1 h. Proteinase K (5 mg/ml) was added and the samples were incubated at 50°C for a further 1 h before being loaded into the wells of a 1·5 % (w/v) agarose gel. A 100–1500 bp DNA standard (Promega; Medical Supply Co. Ltd, Dublin, Ireland) was used to assess DNA fragmentation. Electrophoresis was carried out in agarose gels prepared in tri(hydroxymethyl)aminomethane–borate–EDTA buffer at 0·3 V/mm. DNA was visualised under UV light on a transilluminator (312 nm) after ethidium bromide staining and photographed using a digital camera (Kodak; Anacherm Ltd, Dublin, Ireland).

Determination of cellular glutathione levels

The cellular level of glutathione was measured according to the method of Hissin & Hilf (1976). Briefly 2×10^5 cells were centrifuged at 100 000 ***g*** for 25 min. Supernatant (100 μl) was diluted in 1·8 ml phosphate–EDTA buffer (0·1 mol Na_2PO_3/l, 0·005 mol EDTA/l, pH 8) and mixed with 100 μl *o*-phthaldaldehyde (1 μg/μl). Samples were incubated at 25°C for 15 min and the fluorescence was detected at 420 nm following activation at 350 nm. The glutathione concentration of the samples was determined from a standard curve. Results were adjusted for protein content (Smith *et al.* 1985) and expressed as a percentage of the control.

Statistical analysis

All data points are the mean values of at least three independent experiments. Data were analysed by ANOVA followed by Dunnett's test. The software employed for statistical analysis was Prism (Hearne Scientific Software, Dublin, Ireland).

Results

Effect of β-sitosterol oxides on cell viability

Viability was assessed in U937 cells by the fluorescein diacetate/ethidium bromide method following exposure to β-sitosterol oxides for 24 h. Viability in the control samples was above 90 % for all experiments. The phytosterol oxides caused no observable change in cell viability at the lower concentration of 30 μM (Table 1). In contrast, 30 μM-β-epoxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol and triolcholesterol caused a significant reduction ($P < 0{\cdot}001$) in viable cells to 73, 77, 67 and 13 %, respectively. Exposure to 60 μM of 7β-hydroxysitosterol resulted in a significant decrease ($P < 0{\cdot}001$) in cell viability to 83 %. All other phytosterol oxides had no significant effect on cell viability at this concentration. At 120 μM, both 7β-hydroxysitosterol and 7-ketositosterol significantly reduced ($P < 0{\cdot}001$) cell viability to

Table 1. Percentage of viable cells following exposure for 24 h to 30, 60 or 120 μM α-epoxysitosterol (α-epox), β-epoxysitosterol (β-epox), α-epoxysitosterol–β-epoxysitosterol (6:1) mixture (α/β-epox), 7β-hydroxysitosterol (7β-OH), 7-ketositosterol (7-keto) or triolsitosterol (triol). Cells were also treated with 30 μM of the corresponding cholesterol oxides (chol). All compounds were dissolved in ethanol and equivalent quantities of ethanol (EtOH) were added to control cells. Viability of the U937 cells was assessed by the fluorescein diacetate/ethidium bromide assay; viability of the CaCo-2 and HepG2 cells was assessed via the Neutral Red uptake assay

(Values are means with their standard errors of at least three independent experiments)

	% Cell viability					
	U937 cells		CaCo-2 cells		HepG2 cells	
Compound	Mean	SE	Mean	SE	Mean	SE
Control (EtOH)	98·2	0·3	100·0	0·0	100·0	0·0
30 μM-α-epox	94·0	0·9	102·6	1·2	110·6	3·9
60 μM-α-epox	94·3	1·2	101·1	2·2	107·4	4·6
120 μM-α-epox	95·4	1·1	101·1	4·4	107·3	4·5
30 μM-α-epox chol	89·0	4·5	98·9	3·6	98·2	2·2
Control (EtOH)	97·6	0·7	100·0	0·0	100·0	0·0
30 μM-β-epox	97·0	0·9	111·7	3·5	100·2	2·6
60 μM-β-epox	94·3	1·3	110·2	4·7	101·5	4·8
120 μM-β-epox	91·0*	0·7	109·0	0·6	99·2	1·8
30 μM-β-epox chol	72·8**	1·4	63·9*	7·0	57·6*	7·0
Control (EtOH)	93·8	2·0	100·0	0·0	100·0	0·0
30 μM-α/β-epox	90·7	3·6	103·8	2·6	94·9	3·0
60 μM-α/β-epox	96·4	0·9	96·6	3·2	89·9	2·5
120 μM-α/β-epox	77·4	10·2	76·7*	5·6	73·2**	8·4
Control (EtOH)	98·2	0·3	100·0	0·0	100·0	0·0
30 μM-7β-OH	93·3	1·7	91·6	11·7	76·9	13·2
60 μM-7β-OH	82·7**	1·2	61·9*	9·1	13·1**	2·1
120 μM-7β-OH	64·7**	6·2	40·8**	4·0	4·6**	0·7
30 μM-7β-OH chol	76·7**	2·1	88·8*	0·3	87·3	3·3
Control (EtOH)	98·2	0·3	100·0	0·0	100·0	0·0
30 μM-7-keto	94·7	0·7	93·0	4·7	84·7	7·7
60 μM-7-keto	88·5	2·4	64·4**	7·0	26·7**	15·1
120 μM-7-keto	64·7**	6·2	23·3**	4·2	7·1**	3·1
30 μM-7-keto chol	67·2**	7·3	82·9*	6·1	86·5	4·5
Control (EtOH)	95·5	1·2	100·0	0·0	100·0	0·0
30 μM-triol	92·5	4·1	97·1	0·9	90·3	7·7
60 μM-triol	94·5	1·1	78·6	9·2	81·6	5·1
120 μM-triol	59·6*	7·6	54·8**	13·6	65·5**	9·8
30 μM-triol chol	12·9**	2·5	3·0**	1·7	10·6**	5·2

Mean values were significantly different from those of the control group (ANOVA followed by Dunnett's test): *$P<0·05$, **$P<0·001$.

65 %. Exposure to 120 μM-β-epoxysitosterol or 120 μM-triolsitosterol caused a significant decrease ($P<0·05$) in viable cells to 91 and 60 %, respectively. The α-epoxysitosterol was not toxic to the U937 cells at any of the concentrations tested.

Viability in the CaCo-2 cells was assessed by the Neutral Red uptake assay following exposure to the oxides for 24 h. Viability in the control samples was set at 100 %. The phytosterol oxides caused no observable change in cell viability at the lower concentration of 30 μM (Table 1). In contrast, 30 μM-β-epoxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol caused a significant reduction ($P<0·05$) in viable cells to 64, 89 and 83 %, respectively. Triolcholesterol, at 30 μM, caused a considerable reduction ($P<0·001$) in cell viability to 3 %. Exposure to 60 μM 7β-hydroxysitosterol resulted in a significant decrease ($P<0·05$) in cell viability to 62 %, whilst 60 μM-7-ketositsoterol significantly reduced ($P<0·001$) cell viability to 64 %. All other phytosterol oxides had no significant effect on cell viability at this concentration. At 120 μM, 7β-hydroxysitosterol, 7-ketositosterol and triolsitosterol significantly reduced ($P<0·001$) cell viability to 41, 23 and 55 %, respectively. The α-epoxysitosterol–β-epoxysitosterol mixture (6:1), at 120 μM, also caused a significant decrease ($P<0·05$) in viable cells to 77 %. At all concentrations tested, β-epoxysitosterol and α-epoxysitosterol were not toxic to the CaCo-2 cells.

Viability in the HepG2 cells was also assessed by the Neutral Red uptake assay following exposure to the oxides for 24 h. Viability in the control samples was set at 100 %. The phytosterol oxides caused no observable change in cell viability at the lower concentration of 30 μM. In contrast, 30 μM-β-epoxycholesterol caused a significant reduction ($P<0·05$) in viable cells to 58 % whilst triolcholesterol, at 30 μM, caused a considerable reduction ($P<0·001$) in cell viability to 11 %. Exposure to 60 μM-7β-hydroxsitosterol or 60 μM-7-ketositosterol resulted in a significant decrease ($P<0·001$) in cell viability to 13 and 27 %, respectively. All other phytosterol oxides had no significant effect on cell viability at this concentration. At 120 μM, 7β-hydroxysitosterol and 7-ketositosterol significantly reduced ($P<0·001$) cell viability to 5 and 7 %. Triolsitosterol and α-epoxysitosterol–β-epoxysitosterol, at 120 μM, also caused a significant decrease ($P<0·001$) in viable cells to 66 and 73 %, respectively. As with the CaCo-2 cells, β-epoxysitosterol and α-epoxysitosterol were not toxic to the HepG2 cells.

Effect of β-sitosterol oxides on induction of apoptosis

Condensed and fragmented nuclei were determined by morphological examination following staining with Hoechst 33 342. Nuclei, which were condensed or fragmented, were identified as apoptotic cells. The apoptotic cells did not exceed 3·1 % in the control cells (Table 2). The phytosterol oxides caused no observable change in the percentage of apoptotic nuclei at the lower concentration of 30 μM. In contrast, exposure to 30 μM-7β-hydroxycholesterol, 7-ketocholesterol, β-epoxycholesterol and triolcholesterol resulted in a significant increase in apoptotic nuclei to 11 % ($P<0·001$), 11 % ($P<0·05$), 12 % ($P<0·001$) and 15 % ($P<0·001$), respectively. Following incubation with 60 μM-7β-hydroxysitosterol, there was a significant increase ($P<0·001$) in apoptotic nuclei to 12 %, whilst 60 μM-7-ketositosterol significantly increased ($P<0·05$) apoptotic nuclei to 17 %. All other phytosterol oxides did not induce apoptosis at this concentration. At 120 μM, both 7β-hydroxysitosterol and 7-ketositosterol significantly increased ($P<0·001$) apoptotic nuclei to 19 and 40 %, respectively, and produced a ladder-like pattern on agarose gels (Fig. 1). Exposure to 120 μM α-epoxysitosterol–β-epoxysitosterol or 120 μM triolsitosterol caused a significant increase ($P<0·05$) in apoptotic nuclei to 21 and 10 %, respectively. For the latter treatment groups, DNA ladders were evident on the agarose gel (Fig. 1). Whilst β-epoxysitosterol caused a significant increase in apoptotic nuclei to 7 %, it failed to produce a ladder-like pattern on agarose (Fig. 1). At all concentrations tested, α-epoxysitosterol did not induce apoptosis nor was any DNA laddering evident (Fig. 1). There was no evidence of apoptosis in either the CaCo-2 or the HepG2 cell line under any of the experimental conditions in the present study (data not shown).

Ability of antioxidants to modulate phytosterol oxidation product-induced toxicity

To assess the ability of the antioxidants α-tocopherol, γ-tocopherol and β-carotene to modulate 7β-hydroxysitosterol- or

Table 2. Percentage of condensed or fragmented nuclei in U937 cells following exposure for 24 h to 30, 60 or 120 μM α-epoxysitosterol (α-epox), β-epoxysitosterol (β-epox), α-epoxysitosterol–β-epoxysitosterol (6:1) mixture (α/β-epox), 7β-hydroxysitosterol (7β-OH), 7-ketositosterol (7-keto) or triolsitosterol (triol). Cells were also treated with 30 μM of the corresponding cholesterol oxides (chol). All compounds were dissolved in ethanol and equivalent quantities of ethanol (EtOH) were added to control cells. The morphology of the nuclei was assessed using Hoechst 33 342 stain (see p. 445), and the number of apoptotic nuclei expressed as a percentage of the total number

(Values are means with their standard errors of at least three independent experiments)

	% Apoptotic nuclei in U937 cells									
	Control (EtOH)		30 μM		60 μM		120 μM		30 μM chol	
Compound	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
α-epox	1·6	0·3	3·1	0·5	2·9	1·2	2·4	0·7	3·4	1·2
β-epox	3·1	0·4	2·1	0·8	4·0	0·8	7·0*	0·6	12·2**	0·6
α/β-epox	1·6	0·3	1·8	0·4	5·6	2·2	20·9**	5·0	–	–
7β-OH	1·9	0·1	5·9	2·1	12·3**	2·2	19·4**	1·9	11·3**	0·9
7-keto	1·9	0·1	3·0	0·2	17·1*	4·9	39·7**	3·8	11·1*	1·2
triol	0·6	0·4	0·8	0·4	2·1	0·7	9·5*	2·3	14·8**	2·8

Mean values were significantly different from those of the control group (ANOVA followed by Dunnett's test): *$P<0·05$, **$P<0·001$.

7-ketositosterol-induced toxicity, U937 cells were first treated with the antioxidants for 1 h and subsequently treated with the oxides for 24 h. Viability was assessed via the fluorescein diacetate/ethidium bromide assay whilst condensed and fragmented nuclei were determined by staining with Hoechst 33 342. Viability in the control samples was above 90 % for all experiments (Table 3). Apoptotic nuclei did not exceed 4 % in the controls. Cell treatment with tocopherols or β-carotene alone had no effect on cell viability or apoptotic nuclei. Exposure of U937 cells to 30 μM-7β-hydroxycholesterol significantly decreased ($P<0·001$) viable cells to 71 % and significantly increased ($P<0·001$) the condensed and fragmented nuclei to 15 %. When 7β-hydroxycholesterol and the tocopherols were co-incubated (30 μM/10 μM), both α-tocopherol and γ-tocopherol inhibited the decrease in viable cells and the increase in apoptotic nuclei induced by the COP. Treatment of U937 cells with 120 μM of

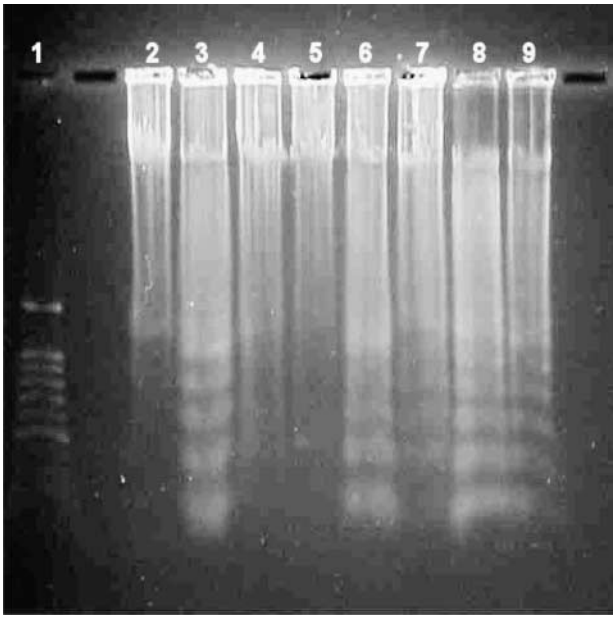


Fig. 1. Induction of DNA fragmentation following incubation for 24 h with 30 μM 7β-hydroxycholesterol (7β-OH chol) or 120 μM α-epoxysitosterol (α-epox), β-epoxysitosterol (β-epox), α-epoxysitosterol–β-epoxysitosterol (6:1) mixture (α/β-epox), triolsitosterol (triol), 7-ketositosterol (7-keto) or 7β-hydroxysitosterol (7β-OH). DNA fragmentation was assessed by agarose gel electrophoresis. Lane 1, molecular weight marker; lane 2, solvent control; lane 3, 7β-OH chol; lane 4, α-epox; lane 5, β-epox; lane 6, α/β-epox; lane 7, triol; lane 8, 7-keto; lane 9, 7β-OH.

Table 3. Effect of α-tocopherol (α-TOC), γ-tocopherol (γ-TOC) and β-carotene (β-Car) on 7β-hydroxycholesterol (7β-OH chol)-, 7β-hydroxysitosterol (7β-OH)- and 7-ketositosterol (7-keto)-induced toxicity. U937 cells were treated with α-TOC (10 μM), γ-TOC (10 μM) or β-Car (2 μM) for 1 h. Subsequently, the cells were treated with 7β-OH chol (30 μM), 7β-OH (120 μM) or 7-keto (120 μM) for 24 h. Samples were processed either for cell viability or stained with Hoechst 33 342 and analysed by fluorescence microscopy (see p. 445)

(Values are means with their standard errors of at least three independent experiments)

	% Viable cells		% Apoptotic nuclei	
Treatment	Mean	SE	Mean	SE
Control (ethanol)	97·4	1·3	3·0	0·1
Control (hexane)	91·4	3·2	3·9	1·2
α-TOC	98·4	0·7	2·2	0·2
β-TOC	97·5	0·4	2·9	0·2
β-Car	97·3	1·0	3·1	0·5
30 μm-7β-OH chol	70·5**	2·7	15·2**	1·3
7β-OH chol/α-TOC	89·8	0·9	4·0	0·5
7β-OH chol/γ-TOC	88·5	4·4	6·6	2·3
7β-OH chol/β-Car	77·9**	6·1	13·1**	1·6
120 μm-7β-OH	54·9**	2·6	24·5**	2·5
7β-OH/β-TOC	60·4**	4·4	21·2**	3·1
7-OH/γ-TOC	59·1**	4·5	25·0**	3·9
7β-OH/β-Car	62·4**	2·4	20·1**	1·0
120 μm-7-keto	73·9**	2·6	26·1**	1·5
7-keto/α-TOC	84·8*	2·5	20·6**	2·0
7-keto/γ-TOC	79·5**	0·3	24·0**	1·5
7-keto/β-Car	75·7**	2·4	22·8**	1·5

Mean values were significantly different from those of the control group (ANOVA followed by Dunnett's test): *$P<0·05$, **$P<0·001$.

7β-hydroxysitosterol or 7-ketositosterol significantly decreased ($P<0{\cdot}001$) the viable cells to 55 and 74 % and significantly increased ($P<0{\cdot}001$) apoptotic nuclei to 25 and 26 %, respectively. Neither α-tocopherol nor γ-tocopherol offered protection against the toxicity induced by either of these oxides. Furthermore, increasing the concentration of the tocopherols to 100 μM did not confer protection against the POP (data not shown). β-Carotene (2 μM) did not protect against COP- or POP-induced toxicity.

Effect of β-sitosterol oxides on the antioxidant status of U937 cells

To determine the antioxidant status of U937 cells, the cellular glutathione levels were measured. A significant decrease ($P<0{\cdot}05$) in glutathione content was evident following incubation with 120 μM 7β-hydroxysitosterol for 24 h (Table 4). At 30 μM, 7β-hydroxycholesterol and triolcholesterol caused a significant reduction in glutathione content to 67 % ($P<0{\cdot}001$) and 57 % ($P<0{\cdot}05$) of control, respectively. None of the other compounds affected the glutathione status of the cell.

Discussion

A limited number of studies have compared the biological effects of COP and POP. Kakis *et al.* (1977) showed that 7-ketocholesterol inhibited cholesterogenesis by 20–30 %, whereas 7-ketositosterol had no effect. Oxides of cholesterol and a β-sitosterol–campesterol mixture exhibited similar patterns of toxicity as indicated by lactate dehydrogenase leakage, cell viability and mitochondria dehydrogenase activity in a cultured macrophage cell line (C57BL/6; Adcox *et al.* 2001). In a more recent study, Maguire *et al.* (2003) concluded that the cytotoxic effects of a mixture of POP in U937 cells closely resemble those of the COP 7β-hydroxycholesterol, although higher concentrations of the POP mixture were required. However, it should be noted that practically all of the toxicity studies carried out to date have involved the use of mixtures of phytosterol oxides. To establish the hierarchy of toxicity of these oxides, it is critical to investigate the biological activity of single purified POP. Until recently, a barrier to progress in this area was the lack of availability of pure phytosterol oxides. In the present study the cytotoxicity of pure β-sitosterol oxides, synthesised as outlined (p. 444), was investigated. Results indicate that individual oxides of β-sitosterol exhibit similar patterns of toxicity as the corresponding COP in the three cell lines employed.

The β-sitosterol oxides 7-ketositosterol and 7β-hydroxysitosterol, oxidised at the C-7 position, were found to be the most cytotoxic to the U937, CaCo-2 and HepG2 cell lines (Table 1). Several *in vitro* studies have reported that 7-ketocholesterol and 7β-hydroxycholesterol are potent inducers of apoptosis in human vascular cells, i.e. artery smooth muscle cells, endothelial cells and monocyte-macrophages (Leonarduzzi *et al.* 2002) and thus may have a putative role in the pathogenesis of atherosclerosis. In the present study, 7-ketositosterol and 7β-hydroxysitosterol, albeit at higher concentrations compared with the corresponding COP, induced apoptosis in U937 cells (a monocytic cell line) as determined by Hoechst staining and DNA ladders.

Cholestane-3β,5α,6β-triol (triolcholesterol) is reported to be one of the most cytotoxic oxysterols towards rabbit aortic smooth muscle cells (Peng *et al.* 1979), mouse L cells (Higley & Taylor, 1984) and Chinese hamster V79 lung fibroblasts (Sevanian & Peterson, 1986) and its involvement in atherosclerosis has been investigated (Jacobson *et al.* 1985; Matthias *et al.* 1987). We report similar findings. Triolcholesterol was the most toxic oxide towards the three cell lines under investigation. The triol derivative of sitosterol was also found to be cytotoxic to the U937, CaCo-2 and HepG2 cells and induced apoptosis in the U937 cells. However, triolcholesterol was found to be more toxic than the triolsitosterol. Meyer & Spitellier (1997) observed an increase in phytosterol epoxides and triols in photoautotrophic cell cultures during ageing. Using cholesterol epoxides and triolcholesterol as standards of known bioactivity, the same authors studied the cytotoxicity of 5,6-epoxides of sitosterol and stigmasterol and derived 3,5,6-trihydroxyphytosteranes in mealworms (Meyer *et al.* 1998). They reported that all compounds were toxic, in particular, the 3β,5α,6β-trihydroxyphytosterols. However, the phytosterol derivatives were found to be less active than the corresponding COP.

Results of the present study indicate that α-epoxysitosterol, an isomer of β-epoxysitosterol, was not toxic to the cell lines employed in this study. It had no effect on cell viability or induction of apoptosis, nor was any DNA laddering evident. Similarly, Maguire *et al.* (2003) reported that the α-epoxide derivative of sitosterol failed to induce apoptosis and was not cytotoxic to U937 cells. While the α-epoxysterol was synthesised from the parent compound β-sitosterol (60% purity) and all other compounds employed in the press study were derived from β-sitosterol (99% purity), our findings are consistent with results reported by O'Callaghan *et al.* (2001) for the corresponding cholesterol oxide. In contrast, the present study found the α-epoxysitosterol–β-epoxysitosterol mixture (6:1) to be cytotoxic to the CaCo-2 and HepG2 cells and induced apoptosis in the U937 cells as indicated by Hoechst staining and DNA ladders. The β-epoxide isomer of sitosterol induced cytotoxicity and apoptosis in the U937 cells; however, the level of apoptosis was not great enough to produce a ladder-like pattern on agarose gel (Fig. 1).

Table 4. Glutathione concentration in U937 cells, expressed as a percentage of control, following exposure for 24 h to 120 μM α-epoxysitosterol (α-epox), β-epoxysitosterol (β-epox), α-epoxysitosterol–β-epoxysitosterol (6:1) mixture (α/β-epox), 7β-hydroxysitosterol (7β-OH), 7-ketositosterol (7-keto) or triolsitosterol (triol). Cells were also treated with 30 μM of the corresponding cholesterol oxides (chol)

(Values are means with their standard errors of at least three independent experiments)

	Glutathione content (% of control) in U937 cells			
	120 μM		30 μM chol	
Compound	Mean	SE	Mean	SE
α-epox	101·5	3·0	94·5	2·8
β-epox	89·4	9·3	89·9	5·3
α/β –epox	143·2	8·5	–	–
7β-OH	65·0*	5·3	66·8**	1·5
7-keto	74·9	2·3	90·5	2·9
Triol	94·7	6·6	57·2*	11·0

Mean values were significantly different from those of the control group (ANOVA followed by Dunnett's test): *$P<0{\cdot}05$, **$P<0{\cdot}001$.

It has been proposed that the β-epoxide isomer of cholesterol is metabolised to triolcholesterol twice as rapidly as the α-epoxide isomer, which may explain the difference in toxicity between these two isomeric forms (Peterson *et al.* 1988). A similar situation may pertain to the corresponding α- and β-epoxysitosterol isomers.

Whilst 7β-hydroxysitosterol, 7-ketositosterol and triolsitosterol were found to be cytotoxic to CaCo-2 and HepG2 cells, these compounds failed to induce apoptosis in both cell lines. Similarly, O'Callaghan *et al.* (2002) reported that 7β-hydroxycholesterol induced apoptosis in U937 cells but not in HepG-2 cells as indicated by morphological examination, flow cytometry and DNA laddering techniques. Therefore, it can be concluded that the cells died by necrosis rather than apoptosis. Lizard *et al.* (1999) also observed a cell-specific effect for 7β-hydroxycholesterol and 7-ketocholesterol, which were shown to induce apoptosis in vascular endothelial and vascular smooth muscles cells but necrosis in human fibroblasts.

It has been reported that glutathione depletion precedes and may be one of the earliest events in oxysterol-induced apoptosis (Lizard *et al.* 1998). In the present study, a reduction in cellular glutathione was observed following incubation for 24 h with 120 μM 7β-hydroxysitosterol and 30 μM of the corresponding COP (Table 4). Not all oxides that induced apoptosis in the present study affected glutathione status, indicating that POP- or COP-induced apoptosis may not be dependent on the generation of an oxidative stress. Similarly, Therond *et al.* (2000) reported that oxysterols may have a cytotoxic effect independent of the glutathione status of the cell.

Vitamin E, in particular α-tocopherol, has been shown to protect against lipid peroxidation (Buttriss & Diplock, 1988) and may also protect against oxysterol-induced cytotoxicty *in vitro* (Lizard *et al.* 2000; Lyons *et al.* 2001; Rosenblat & Aviram, 2002). In the present study, α- and γ-tocopherol inhibited 7β-hydroxycholesterol-induced cytotoxicity and apoptosis in U937 cells. Lyons *et al.* (2001) report that, despite a greater incorporation of γ-tocopherol into U937 cells, α-tocopherol, but not γ-tocopherol was more effective at inhibiting 7β-hydroxycholesterol-induced apoptosis in the U937 cell culture model. In the latter study, cells were exposed to treatment for 48 h while the incubation period used in the present study was 24 h. It is possible that the protection seen by γ-tocopherol in the present study is lost after 48 h. Although γ-tocopherol is rapidly taken up by cells in culture, it also disappears more quickly than α-tocopherol and is present at much lower levels in plasma (Tran & Chan, 1992). Similarly, Uemura *et al.* (2002) found that apoptosis induced by 7β-hydroxycholesterol and 7-ketocholesterol in vascular endothelial cells was prevented by α-tocopherol. These authors suggest that α-tocopherol was protective not only by scavenging reactive oxygen species but also by inhibiting caspase activity. In contrast, results from the present study indicate that α/γ-tocopherol did not confer protection against 7β-hydroxysitosterol- or 7-ketositosterol-induced cytotoxicity and apoptosis in U937 cells (Table 3). It may be that different apoptotic pathways are provoked by COP *v.* POP in spite of their structural similarity. We have, for example, previously shown that different COP (7β-hydroxycholesterol *v.* β-epoxycholesterol) provoked apoptosis by different signalling pathways (Ryan *et al.* 2004*a*). Trolox (a water-soluble synthetic analogue of α-tocopherol) protected against 7β-hydroxycholesterol-induced apoptosis but did not protect against cell death induced by β-epoxycholesterol (Ryan *et al.* 2004*a*). Clearly, more research is required to further elucidate this interesting difference between COP- and POP-induced apoptosis.

Carotenoids have been implicated as important dietary nutrients having antioxidant potential and their beneficial effects in human disease prevention have been widely reported (Tapiero *et al.* 2004). It is known that certain COP, e.g. 7β-hydroxycholesterol, are able to induce oxidative stress in cells (Miguet-Alfonsi *et al.* 2002; Ryan *et al.* 2004*b*). Therefore, we examined whether the carotenoid β-carotene would protect against COP- and POP-induced cellular toxicities. In contrast to the tocopherols, β-carotene did not confer any protection against either COP- or POP-induced cellular damage (Table 3). Studies to date suggest that the antioxidant activity of carotenoids most likely involves the scavenging of two reactive oxygen species, singlet molecular oxygen and peroxyl radicals. On the other hand, oxidative stress induced by 7β-hydroxycholesterol has been associated with alterations in superoxide dismutase activity and glutathione concentration (Ryan *et al.* 2004*b*) and stimulation of superoxide anion production (Miguet-Alfonsi *et al.* 2002).

In conclusion, our data represent the first direct comparison of COP and POP with respect to cytotoxicity and effects on apoptosis. POP have qualitatively similar toxic effects to COP. However, from a quantitative perspective, higher concentrations of POP are required to elicit comparable degrees of cytotoxicity and modulatory effects on apoptosis. In addition, there may be slight differences in the apoptotic pathways provoked by these oxides. In light of the increasing intakes of phytosterols due to the proliferation of phytosterol-enriched foods, further investigation of the potential toxicity of POP is warranted.

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