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Author(s)	Lordan, Sinéad; O'Neill, Cora; O'Brien, Nora M.
Publication date	2008-08
Original citation	Lordan, S., O' Neill, C. and O' Brien, N.M. (2008) Effects of apigenin, lycopene and astaxanthin on 7-beta-hydroxycholesterol-induced apoptosis and Akt phosphorylation in U937 cells. British Journal of Nutrition, 100: 287-296
Type of publication	Article (peer-reviewed)
Link to publisher's version	http://dx.doi.org/10.1017/S0007114507898643 Access to the full text of the published version may require a subscription.
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British Journal of Nutrition (2008), **100**, 287–296 doi:10.1017/S0007114507898643
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Effects of apigenin, lycopene and astaxanthin on 7β-hydroxycholesterol-induced apoptosis and Akt phosphorylation in U937 cells

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(Received 19 June 2007 – Revised 26 October 2007 – Accepted 19 November 2007 – First published online 11 January 2008)

Oxysterols arise from the enzymic or non-enzymic oxidation of cholesterol and have been shown to be cytotoxic to certain cell lines. In particular, apoptosis induced by the oxysterol 7β-hydroxycholesterol (7β-OH) has been associated with the generation of oxidative stress, cytochrome *c* release and caspase activation. Due to the fundamental importance of apoptosis in pathological processes, the identification of substances capable of modulating this form of cell death is now actively researched. The objective of the present study was to investigate if apigenin, lycopene and astaxanthin could inhibit 7β-OH-induced apoptosis in U937 cells. Pretreatment with 0·1 μM-astaxanthin protected against apoptosis, while lycopene did not oppose the adverse effects of 7β-OH. At low concentrations, apigenin did not protect against oxysterol-induced apoptosis; however, at higher concentrations it intensified cell death. Additionally, we investigated the effect of 7β-OH, apigenin and astaxanthin on the activation of the serine threonine kinase Akt (phosphorylated Akt:Akt ratio) to determine whether the effect on cell viability and growth was linked to the Akt signalling pathway. Akt activation was decreased in the oxysterol-treated cells compared with control cells; however, this did not attain significance. Interestingly, activation of Akt was significantly reduced compared with control cells following incubation with apigenin and astaxanthin both in the absence and in the presence of 7β-OH. Our data suggest that apigenin, lycopene and astaxanthin failed to protect against 7β-OH-induced apoptosis, and the decrease in cell viability and the increase in apoptotic nuclei induced by the antioxidants appear to be associated with down regulation of Akt activity.

Akt: Antioxidants: Cytotoxicity: 7β-Hydroxycholesterol

Oxysterols are the oxidation products of cholesterol and have been shown to possess many potent and diverse biological activities *in vitro*. Consequently, oxysterols have been implicated as possible causes of atherosclerotic lesions as well as a variety of other diseases. The accumulation of oxysterols can occur in several different ways, the major ones being dietary intake (nutritional oxysterols) and internal chemical and enzymic oxidation[1]. Non-enzymic oxidation occurs primarily on the sterol nucleus, especially at the 7-position and at the 5,6-double bond. These oxysterols, such as 7β-hydroxycholesterol (7β-OH), which predominates in early and advanced atherosclerotic lesions[2], are strongly cytotoxic against the cells of the vascular wall and are capable of stimulating superoxide anion production[3]. In monocytes/macrophages, oxysterols induce apoptosis by mechanisms which include a sustained rise in cytosolic free Ca[4–6], activation of Ca-dependent apoptotic and survival pathways[7,8], and induction of oxidative stress[9,10]. Therefore, the inhibition of these processes by supplementation with antioxidants may be an attractive therapeutic strategy to prevent and possibly treat atherosclerosis and related diseases[11].

Flavonoids have diverse pharmacological properties, including antioxidant, cytoprotective and anti-inflammatory activities[12]. Apigenin is a non-mutagenic flavone found in a variety of fruits and vegetables including parsley, onions, wheat sprouts, chamomile, seasonings, tea and orange[13]. Apigenin has gained attention due to its health benefits and the ability of this compound to chelate metal ions, thereby preventing the formation of reactive oxygen species such as the hydroxyl radical; this is suggested as one possible mechanism of cytoprotection[14].

Carotenoids are phytochemicals that are considered to be beneficial in the prevention of a variety of major diseases. Lycopene is the most predominant carotenoid in human plasma and several epidemiological studies have suggested that high consumption of lycopene-rich foods may protect against CVD[15,16]. Tomatoes and tomato products (juice, ketchup, soup, sauce) are the major contributors of lycopene in the diet[17], while other rich sources include watermelon, pink grapefruit, pink guava and papaya. The antioxidant activity of lycopene is highlighted by its singlet oxygen-quenching property and its ability to trap peroxyl radicals[18]. Another carotenoid, astaxanthin, is commonly found in

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 7β-OH, 7β-hydroxycholesterol; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.
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marine animals and is responsible for the pink/red colouration of crustaceans[19]. Within the cell, astaxanthin can effectively scavenge lipid radicals and destroy peroxide chain reactions, thereby protecting fatty acids and biological membranes from oxidative damage. The antioxidant activity of astaxanthin is claimed to be sevenfold greater than that of α-tocopherol[20].

The aim of the present study was to investigate the effects of apigenin, lycopene and astaxanthin on 7β-OH-induced apoptosis in a human monocytic cell line (U937). While there is considerable evidence that these compounds have antioxidant potential, there are studies suggesting that carotenoids and phenolic phytochemicals with particular structures exhibit pro-oxidant activity under certain conditions[21,22]. In the present study, α-tocopherol was included as a positive control as it has previously been shown to protect against 7β-OH-induced cell death in U937 cells[23]. U937 cells are regularly employed as a macrophage reference model in studies investigating oxysterol-induced cell death[24,25]. In addition, apigenin and astaxanthin in the absence and presence of 7β-OH on the activation of the serine threonine kinase Akt was examined using Western blot analysis of the phosphorylated Akt:Akt ratio. Akt, also known as protein kinase B, regulates essential cellular functions such as migration, proliferation, differentiation, apoptosis and metabolism[26]. The activation of the Akt pathway provides cells with a survival signal that allows them to withstand apoptotic stimuli[27]. For instance, it has recently been reported that carotenoids suppress tissue factor activity (which plays a key role in vascular thrombosis) by enhancing phosphorylation of Akt in endothelial cells[28], while Munteanu *et al.* [29] found that oxidised LDL-stimulated Akt phosphorylation in THP-1 monocytes was inhibited by α-tocopherol. Hence, the purpose of investigating Akt activation in the present study was two-fold; initially to ascertain whether it was affected by 7β-OH-induced apoptosis and second, in view of the importance of Akt in controlling cell survival, to determine whether the antioxidants in the presence of 7β-OH modulate the Akt pathway.

Materials and methods

Materials

All chemicals and cell-culture reagents were obtained from the Sigma Chemical Co. (Dublin, Republic of Ireland) unless otherwise stated. Tissue-culture plastics were supplied by Greiner Bio (Frickenhausen, Germany). Information on the purity of the 7β-OH (purity > 95 %) was obtained from Sigma. Western blotting reagents were supplied by Bio-Rad (Hemel Hempstead, Herts, UK). Cell lines were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wilts, UK).

Maintenance of cell lines

Human monocytic U937 cells were grown in suspension in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10 % (v/v) heat-inactivated fetal bovine serum supplied by Gibco (Paisley, Renfrewshire, UK). The cells were grown at 37°C in 5 % CO_2 in a humidified incubator. The cells were screened for mycoplasma contamination by the Hoechst staining method[30] and were cultured in the absence of antibiotics. Exponentially growing cells were used throughout.

Treatment of cells with 7β-hydroxycholesterol, α-tocopherol, apigenin, lycopene and astaxanthin

U937 cells were adjusted to a density of 2×10^5 cells/ml in RPMI-1640 medium supplemented with fetal bovine serum (25 ml/l). Cells were intially pretreated with various concentrations of α-tocopherol (10 μM), apigenin (0·5–50 μM), lycopene or astaxanthin (0·1–1 μM) for 1 h followed by treatment with 30 μM-7β-OH. Samples were then incubated for 24 h at 37°C in 5 % CO_2. Overall, α-tocopherol, apigenin, lycopene and astaxanthin were in contact with the cells for 25 h. 7β-OH was dissolved in ethanol for delivery to cells and the final concentration of ethanol in the cultures did not exceed 0·3 % (v/v). α-tocopherol, apigenin, lycopene and astaxanthin were dissolved in ethanol, dimethyl sulfoxide, hexane and methylethylketon respectively, where the final concentration of the solvent in the culture medium was < 0·5 %. Equivalent quantities of solvent were added to control cells and samples were incubated for 24 h. For subsequent assays, three concentrations of apigenin (5, 10, 50 μM) were selected.

Cell viability

Following 24 h incubation, 25 μl of cells were removed for assessment of cell viability. Viability was monitored using a modification of the fluorochrome-mediated viability assay as described by Strauss[31]. Briefly, cells were mixed 1:1 (v/v) with a solution of fluorescein diacetate and ethidium bromide, and then incubated at 37°C for 2–5 min before being layered onto a microscope slide. Under these conditions, live cells fluoresce green, whereas dead cells fluoresce red. Dying cells have a green cytoplasm and red nucleus. Samples were examined at 200 × magnification on a Nikon fluorescence microscope using blue light (450–490 nm). Cells (200) were scored from each slide and cell viability was expressed as the percentage of viable (green) cells.

Morphological analysis of cell nuclei

Nuclear morphology of control and treated cells was assessed by fluorescence microscopy after staining with Hoechst 33342. Approximately 4×10^5 cells were centrifuged at 200 ***g*** for 10 min to form a pellet. Hoechst 33342 stain (200 μl; 5 μg/ml) was added and the samples incubated at 37°C in 5 % CO_2 for 1 h. Stained samples (25 μl) were placed on a microscope slide and examined under UV light (Nikon Labophot fluorescence microscope, 400 × magnification). A total number 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[32].

DNA fragmentation assay

Detection of small DNA fragments was conducted as described in O'Callaghan *et al.* [33]. Briefly 2×10^5 cells were harvested and the pellets were lysed, RNAse A (0·25 mg/ml) was added and the samples were incubated at

50°C for 1 h. The condensate was spun down and proteinase K (5 mg/ml) was added. The samples were incubated at 50°C for a further 1 h before being loaded into the wells of a 1·5 % agarose gel. A 100–1500 bp DNA standard (Promega, Madison, WI, USA) was used to assess DNA fragmentation. Electrophoresis was carried out in 1·5 % agarose gels prepared in 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)–borate–EDTA buffer at 3 V/cm. DNA was visualised under UV light on a transilluminator (312 nm) following ethidium bromide staining and photographed using a Bio Imaging System (GeneGenius, Cambridge, Cambs, UK).

Cell proliferation

Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using Cell Proliferation kit I (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, 1×10^5 cells were treated with 7β-OH, α-tocopherol, apigenin, lycopene and astaxanthin in the wells of a ninety-six-well costar plate for 24, 48 and 72 h. At the end of the treatment, 10 µl MTT reagent was added. After 4 h incubation at 37°C, 100 µl of the solubilisation solution was placed in each well to dissolve the tetrazolium crystals. Following overnight incubation the absorbance at 570 nm was recorded using a plate reader.

Western blot analysis

Akt and phosphorylated Akt (Ser473) levels were investigated by Western blot analysis of cell lysates which had been preincubated with apigenin and astaxanthin and then additionally exposed to 30 µM-7β-OH for 24 h. Cells (5×10^6) were harvested at the end of treatment, washed twice in PBS, and suspended in radioimmunoprecipitation assay (RIPA) buffer (150 mM-NaCl, 50 mM-Tris-HCl, 0·1 % SDS, 0·5 % sodium deoxycholate, 1 mM-Na_3VO_4, 1 mM-NaF) supplemented with 5 % protease inhibitor cocktail (Calbiochem, Nottingham, Notts, UK). After 30 min incubation at 4°C in the lysis buffer (RIPA) the samples were sonicated. Cell debris was eliminated by centrifugation at 10 000 ***g*** for 30 min and the supernatant fraction was collected. Protein concentrations were determined by the bicinchonic assay method. Protein (20 µg) was incubated in loading buffer (0·5 M-Tris-HCl, pH 6·8, 10 % SDS, 10 % glycerol, 0·05 % β-mercaptoethanol and 0·05 % bromophenol blue) and heated at 100°C for 2 min. Samples were separated by SDS-PAGE and electroblotted onto a polyvinylidine difluoride membrane. Membranes were stained with 0·1 % ponceau S solution to ensure protein loading and transfer was equal. Non-specific binding sites were blocked by placing the membrane in 5 % non-fat dry milk in Tris-buffered saline–Tween (0·1 % Tween 20 in Tris-buffered saline) for 1 h. The membranes were incubated overnight at 4°C with goat monoclonal antibody directed against Akt 1/2, diluted 1/500 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or a rabbit monoclonal antibody directed against phosphorylated Akt (Ser473) (Cell Signaling Technology, Inc., Danvers, MA, USA) diluted 1/1000; all antibodies were diluted in Tris-buffered saline–Tween. After three washes in Tris-buffered saline–Tween the membranes were incubated with appropriate horseradish peroxidase-conjugated donkey anti-goat (Santa Cruz Biotechnology) or anti-rabbit (Cell Signaling Technology) for 1 h at room temperature. Following washing, immunoreactive bands were visualised using an enhanced chemiluminescence detection kit (Perkin Elmer, Waltham, MA, USA) and subsequent exposure to autoradiography film. The bands were identified according to their migration value. Quantification of results was performed by densitometry (GeneGenius gel documentation and analysis system; Syngene, Cambridge, Cambs, UK), quantifying the density of identically sized areas (corresponding to immunoreactive bands) and results analysed as total integrated densitometric volume values (arbitrary units). Results are reported as mean values with their standard errors. Both Akt and phosphorylated Akt (Ser473) antibody immunoreactivities were tested to ensure that immunoreactivity measured was linear with increasing protein concentration.

Statistics

All data points are the mean values with their standard errors of at least three independent experiments. Where appropriate, data were analysed by one-way ANOVA. The software employed for statistical analysis was GraphPad Prism (version 4; GraphPad Software, San Diego, CA, USA).

Results

Effect of apigenin, lycopene and astaxanthin on 7β-hydroxycholesterol-induced toxicity

U937 cells were exposed simultaneously to 30 µM-7β-OH and increasing concentrations of apigenin, lycopene and astaxanthin (Tables 1–3). Following 24 h incubation, samples were counted and stained with either fluorescein diacetate or ethidium bromide to assess membrane integrity or Hoechst 33342 to examine nuclear morphology. Viability in the control samples was above 95 % in all experiments. Treatment of

Table 1. Effect of apigenin on 7β-hydroxycholesterol (7β-OH)-induced cytotoxicity†

(Mean values with their standard errors of three independent experiments)

Treatment	% Viable cells		% Apoptotic nuclei	
	Mean	SE	Mean	SE
Control	97·5***	0·5	3·8	1·0
30 µM-7β-OH	70·3	2·2	18·0	1·3
5 µM-Apigenin	98·8***	0·4	5·5	1·1
10 µM-Apigenin	94·3***	1·1	10·7	1·5
50 µM-Apigenin	11·7***	1·8	74·4***	3·7
30 µM-7β-OH + 10 µM-α-tocopherol	83·9*	2·1	11·7	1·3
30 µM-7β-OH + 0·5 µM-apigenin	65·8	4·9	15·9	1·5
30 µM-7β-OH + 2 µM-apigenin	79·7	4·2	15·6	1·6
30 µM-7β-OH + 5 µM-apigenin	82·2	2·3	15·0	2·2
30 µM-7β-OH + 10 µM-apigenin	69·8	0·9	24·9	5·8
30 µM-7β-OH + 50 µM-apigenin	27·8***	4·1	57·1***	11·2

Mean value was significantly different from that for the 30 µM-7β-OH treatment: *$P < 0·05$, ***$P < 0·001$ (ANOVA followed by the Bonferroni test).

† U937 cells were treated with 0·5, 2, 5, 10 and 50 µM-apigenin, over 24 h, in the presence or absence of 30 µM-7β-OH. Samples were processed for either cell viability or stained with Hoechst 33342 and analysed by fluorescence microscopy.

U937 cells with 7β-OH decreased viability to approximately 68 % and increased the number of apoptotic nuclei. With the exception of 50 µM-apigenin (Table 1) and 1 µM-astaxanthin (Table 3), none of the compounds tested was found to be toxic to the cells. The antioxidant α-tocopherol has previously been shown to decrease the toxicity of 7β-OH to U937 cells[23]. Similarly, in the present study, 10 µM-α-tocopherol significantly ($P<0{\cdot}05$) increased the viability and decreased the apoptotic death of cells exposed to the oxysterol. In the presence of 7β-OH, the lower concentrations of apigenin did not significantly alter cell viability; however, the flavonoid at 50 µM potentiated cell death (Table 1). Exposure to lycopene for 24 h did not oppose the adverse effects of 7β-OH in U937 cells (Table 2). Pretreatment with 0·1 µM-astaxanthin significantly ($P<0{\cdot}01$) protected against 7β-OH-induced apoptosis while the higher concentration (1 µM) appeared to potentiate the toxicity of the oxysterol (Table 3). Cell death by apoptosis was confirmed by the DNA fragmentation assay (Fig. 1). A ladder-like pattern was observed following 24 h exposure to apigenin, lycopene and astaxanthin in the presence of 30 µM-7β-OH. Treatment with α-tocopherol visibly reduced DNA laddering produced by the oxysterol, confirming a protective effect.

Cell proliferation

7β-OH-induced cytotoxicity was further investigated by the measurement of cell proliferation over 72 h. The absorbance of formazan crystals liberated over time from MTT labelling reagent was used to assess U937 cell growth. Following 24, 48 and 72 h incubation, 30 µM-7β-OH caused a significant ($P<0{\cdot}001$) delay in cell proliferation relative to the control. Pretreatment with α-tocopherol did not protect against the decrease in cell proliferation. As shown in Fig. 2, apigenin did not provide any protection against the decline in cell growth over 72 h. In addition, 10 µM-apigenin alone significantly ($P<0{\cdot}001$) inhibited U937 cell proliferation; however, this was suppressed even further in the presence of the oxysterol. As expected, 50 µM-apigenin significantly ($P<0{\cdot}001$) decreased cell growth both in the absence and in the presence of 7β-OH.

In the absence of the oxysterol, lycopene had no effect on cell proliferation and it did not protect against the suppressed cell growth induced by 7β-OH (Fig. 3). At the 48 ($P<0{\cdot}05$) and 72 h ($P<0{\cdot}01$) time points, 1 µM-astaxanthin significantly inhibited proliferation of U937 cells but not to the same extent as 7β-OH (Fig. 4). Similar to lycopene, astaxanthin did not protect against the oxysterol-induced decrease in cell growth at any of the concentrations tested.

Table 2. Effect of lycopene on 7β-hydroxycholesterol (7β-OH)-induced cytotoxicity†

(Mean values with their standard errors of three independent experiments)

	% Viable cells		% Apoptotic nuclei	
Treatment	Mean	SE	Mean	SE
Control	96·5***	0·8	4·4**	0·3
30 µM-7β-OH	67·2	2·7	12·5	1·6
0·1 µM-Lycopene	97·2***	0·8	6·5	1·2
0·5 µM-Lycopene	94·1***	2·0	7·5	0·8
1 µM-Lycopene	95·5***	2·0	8·4	0·7
30 µM-7β-OH + 10 µM-α-tocopherol	85·7*	4·6	7·4	1·3
30 µM-7β-OH + 0·1 µM-lycopene	72·3	2·9	12·5	1·4
30 µM-7β-OH + 0·5 µM-lycopene	64·7	6·4	13·0	0·5
30 µM-7β-OH + 1 µM-lycopene	65·9	3·3	15·6	2·0

Mean value was significantly different from that for the 30 µM-7β-OH treatment: *$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$ (ANOVA followed by the Bonferroni test).

† U937 cells were treated with 0·1, 0·5 and 1 µM-lycopene, over 24 h, in the presence or absence of 30 µM-7β-OH. Samples were processed for either cell viability or stained with Hoechst 33342 and analysed by fluorescence microscopy.

Table 3. Effect of astaxanthin on 7β-hydroxycholesterol (7β-OH)-induced cytotoxicity†

(Mean values with their standard errors of three independent experiments)

	% Viable cells		% Apoptotic nuclei	
Treatment	Mean	SE	Mean	SE
Control	96·2**	0·9	4·9***	2·1
30 µM-7β-OH	67·9	6·8	22·0	2·9
0·1 µM-Astaxanthin	92·6*	2·0	4·9***	0·8
0·5 µM-Astaxanthin	89·8*	4·6	7·0***	1·2
1 µM-Astaxanthin	74·8	5·4	15·0	2·2
30 µM-7β-OH + 10 µM-α-tocopherol	92·3*	1·7	8·3***	0·5
30 µM-7β-OH + 0·1 µM-astaxanthin	88·2	0·3	9·9**	2·4
30 µM-7β-OH + 0·5 µM-astaxanthin	79·2	2·2	15·9	0·2
30 µM-7β-OH + 1 µM-astaxanthin	50·8	7·4	48·6***	1·7

Mean value was significantly different from that for the 30 µM-7β-OH treatment: *$P<0{\cdot}05$, **$P<0{\cdot}01$, ***$P<0{\cdot}001$ (ANOVA followed by the Bonferroni test).

† U937 cells were treated with 0·1, 0·5 and 1 µM-astaxanthin, over 24 h, in the presence or absence of 30 µM-7β-OH. Samples were processed for either cell viability or stained with Hoechst 33342 and analysed by fluorescence microscopy.

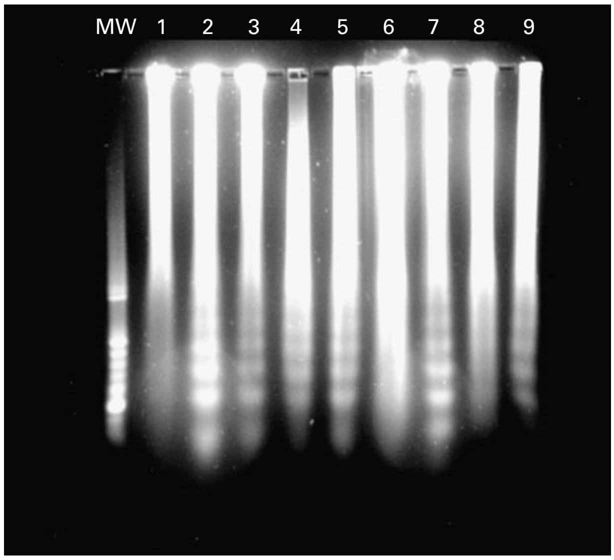

Fig. 1. Induction of DNA fragmentation in U937 cells at 24 h. DNA was isolated and electrophoresed in 1·5 % gels (3 V/cm) as described in Materials and methods. After staining with ethidium bromide, gels were visualised under UV light on a transilluminator (312 nm) and photographed using a Bio Imaging System (GeneGenius, Cambridge, Cambs, UK). Lane 1, control; lane 2, 30 µM-7β-OH; lane 3, 30 µM-7β-OH + 10 µM-α-tocopherol; lane 4, 50 µM-apigenin; lane 5, 30 µM-7β-OH + 50 µM-apigenin; lane 6, 1 µM-lycopene; lane 7, 30 µM-7β-OH + 1 µM-lycopene; lane 8, 1 µM-astaxanthin; lane 9, 30 µM-7β-OH + 1 µM-astaxanthin. MW, molecular weight marker.

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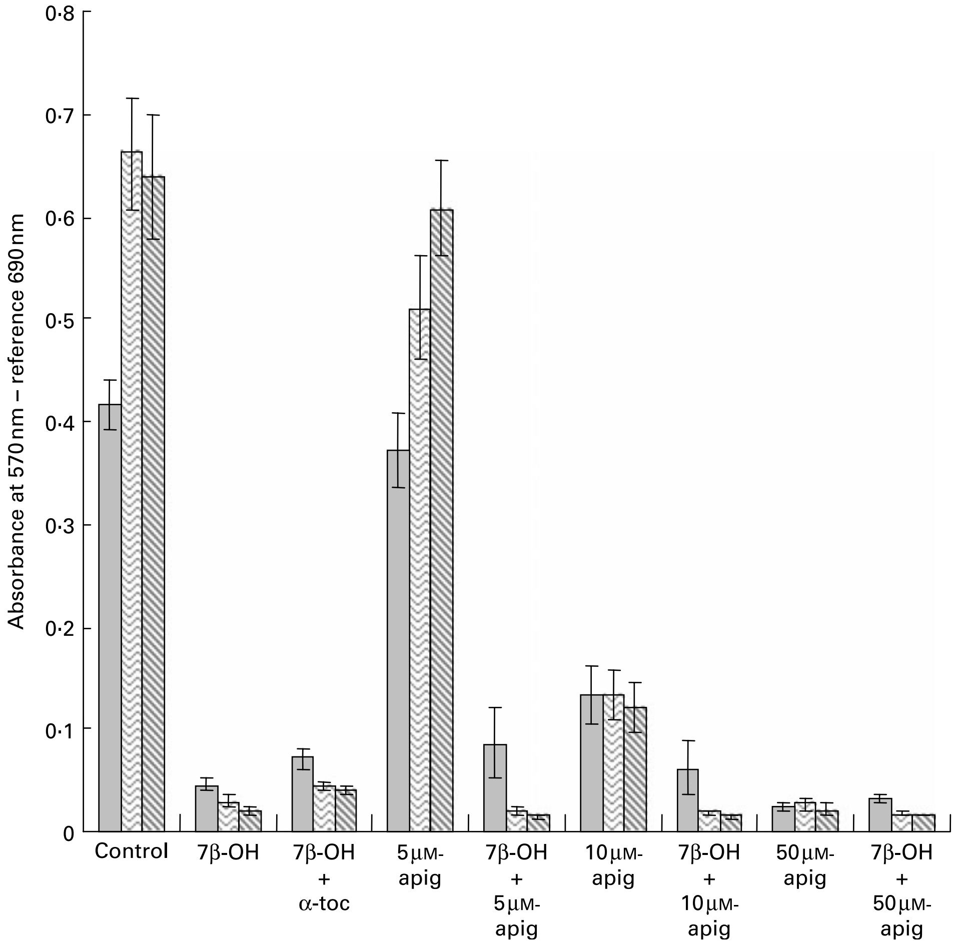

Fig. 2. Effect of apigenin (apig) on cell proliferation. U937 cells were treated with 5, 10 and 50 μM-apig in the presence or absence of 30 μM-7β-OH, for 24 (▒), 48 (▒) and 72 (▒) h. Values are means of three independent experiments, with their standard errors represented by vertical bars. toc, Tocopherol.

Akt levels and Akt activation

Western blotting was employed to investigate the effect of 7β-OH, apigenin and astaxanthin on Akt activation and to determine whether the effects of these agents on cell viability and growth was linked mechanistically to this signalling pathway. Akt activation is commonly measured using the ratio of phosphorylated active Akt:total Akt[34]. In the oxysterol-treated cells the ratio of phosphorylated Akt:total Akt was reduced, thus Akt activity was reduced; however, this did not attain significance (Fig. 5). Interestingly, activation of Akt was significantly reduced compared with control and 7β-OH-treated levels following incubation with apigenin (10 μM) and astaxanthin (1 μM). Moreover, this reduction in Akt activation was also observed when the cells were treated with these concentrations of apigenin and astaxanthin alone.

Discussion

The oxysterol 7β-OH has been shown to induce oxidative stress in U937 cells, leading to apoptotic cell death[9,10,35,36]. Due to the fundamental importance of apoptosis in pathological processes, the identification of substances capable of modulating this form of cell death is now actively researched. On the basis of experimental evidence that flavonoids and carotenoids are antioxidants and have the ability to scavenge free radicals, we examined the effects of apigenin, lycopene and astaxanthin on 7β-OH-induced cytotoxicity.

The data presented in the present report confirm that 7β-OH induces apoptosis in human monocytic U937 cells. In addition, our findings show that apigenin, lycopene and astaxanthin provide little or no protection against 7β-OH-induced apoptosis. Following 24 h incubation with 7β-OH, lower concentrations of apigenin did not protect against the decrease in cell viability or proliferation, while at 50 μM, it intensified cell death. These results are comparable with a study conducted by Wang *et al.* [37] in which the effects of four structurally related flavonoids on HL-60 cell viability were investigated. Following 12 h treatment, apigenin appeared to be more potent, with a half-maximal inhibitory concentration (IC_{50}) of 50 μM and dose-dependent inhibition of cell viability. Also, DNA fragmentation only became apparent with between 20 and 40 μM-apigenin treatment, and the induction of caspase-3 activity by apigenin paralleled this pattern.

Carotenoids have been suggested to be protective against coronary vascular disease. Before the present study, the effects of lycopene and astaxanthin on oxysterol-induced apoptosis

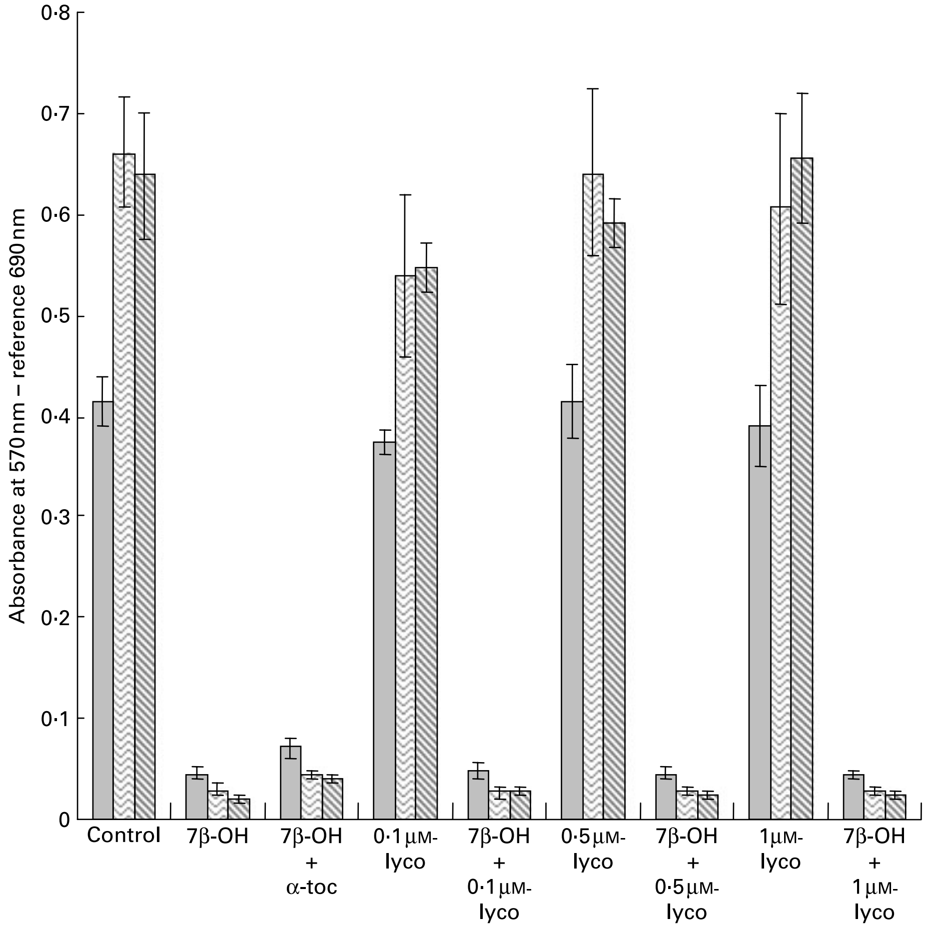

Fig. 3. Effect of lycopene (lyco) on cell proliferation. U937 cells were treated with 0·1, 0·5 and 1 μM-lyco in the presence or absence of 30 μM-7β-OH, for 24 (▯), 48 (▨) and 72 (▧) h. Values are means of three independent experiments, with their standard errors represented by vertical bars. toc, Tocopherol.

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had not been investigated. Lycopene is one of the most potent antioxidants among the dietary carotenoids and may help lower the risk of chronic diseases including cancer and heart disease[18]. However, none of the concentrations of lycopene employed in the present study protected against the increase in apoptotic nuclei or the decrease in cell growth in 7β-OH-treated cells. The present results also showed that pretreatment with 0·1 μM-astaxanthin significantly ($P<0·01$) protected against 7β-OH-induced apoptosis whereas the carotenoid at 1 μM exaggerated the toxicity of the oxysterol. A study involving Watanabe heritable hyperlipidaemic rabbits evaluating the possible anti-atherosclerotic effect of α-tocopherol and astaxanthin found that both antioxidants improved plaque stability and significantly diminished apoptosis[38]. Overall, astaxanthin may possess some protective properties relative to oxysterol-induced apoptosis but only at low concentrations.

The generation of oxidative stress has been implicated in the earlier stages of apoptosis induced by certain oxysterols. Ryan *et al.* [9] demonstrated that in 7β-OH-induced apoptosis, in U937 cells, glutathione levels began to decrease after 6 h coupled with an increase in superoxide dismutase activity. However, no evidence of apoptosis was reported after 6 h. The reduced levels of glutathione within the cell did not appear to alter between the 6 h and the 24 h time point and the superoxide dismutase activity was not significantly increased following 24 h treatment with the oxysterol. In addition, O'Callaghan *et al.* [39] observed that there was no significant change in the activity of the catalase enzyme in U937 cells following a 12 h incubation in the presence of 7β-OH. The results observed in the present study, along with numerous other studies[40–44], have shown that several compounds with proven antioxidant properties do not protect against oxysterol-induced cell death. Taken together, these results suggest that although oxidative stress may be an initiator of 7β-OH-induced apoptosis, it is not completely responsible for the induction of cell death.

7β-OH is present at enhanced levels in atherosclerotic plaques and is believed to play a key role in atherogenesis. As apoptosis of vascular cells occurring in atherosclerotic plaques is believed to promote thrombotic events and plaque rupture it is of interest to determine the mechanisms of cell death triggered by these oxysterols[7]. Akt has been well characterised as an anti-apoptotic kinase that transduces cellular survival signals in many cell types[45]. A previous study demonstrated that, in the murine macrophage-like cell line P388D1, oxysterols (25-hydroxycholesterol and 7-ketocholesterol) induced the degradation of Akt[46]. To determine whether 7β-OH-induced apoptosis could also induce Akt degradation or effect Akt activation, we examined the levels of both total Akt and active

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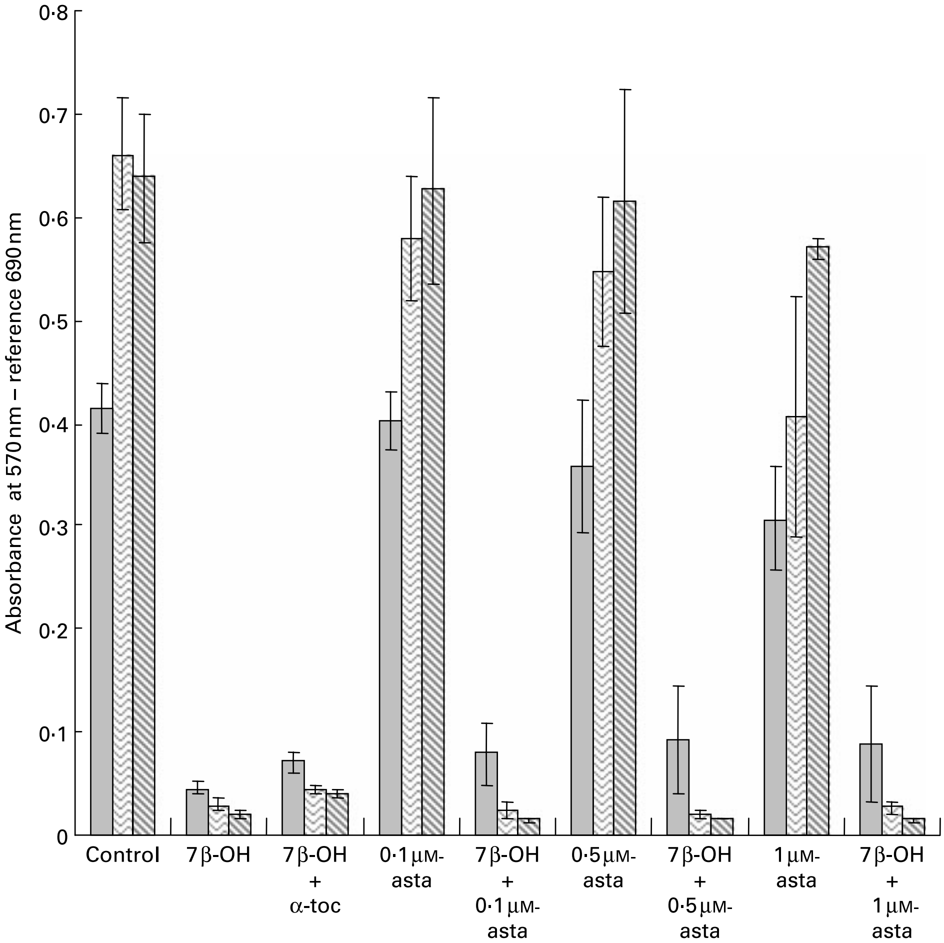

Fig. 4. Effect of astaxanthin (asta) on cell proliferation. U937 cells were treated with 0·1, 0·5 and 1 μM-asta in the presence or absence of 30 μM-7β-OH, for 24 (▥), 48 (▨) and 72 (▧) h. Values are means of three independent experiments, with their standard errors represented by vertical bars. toc, Tocopherol.

phosphorylated Ser473 Akt in U937 cells. The ratio of phosphorylated Akt:total Akt was reduced in the oxysterol-treated cells. However, this did not attain significance and, unlike the previous study[46], total Akt levels were unchanged in the presence of 7β-OH. In addition, it was found that concentrations of apigenin (10 μM) and astaxanthin (1 μM) that decrease cell proliferation and viability also significantly decreased Akt activation without significantly affecting Akt levels, with this effect remaining the same when using the combination of the antioxidant plus the oxysterol. These results suggest that the anti-proliferative effects of these antioxidants may be mechanistically linked to the inhibition of Akt, and occur in the absence and presence of the oxysterol. In agreement with this Way *et al.* [47] have reported that apigenin inhibits Akt phosphorylation at serine 473 in three breast cancer cell lines. They found that apigenin directly inhibited PI3K activity, an upstream mediator of Akt, and indirectly caused an inhibitory effect on Akt kinase activity. Following on from this study, it was found that apigenin induced apoptosis through cytochrome *c* release with subsequent activation of caspase-3[48]. Therefore it appears that the antioxidant, alone or together with the oxysterol, but not the oxysterol alone, greatly inhibits Akt signalling. Palozza *et al.* [49] have recently found that Akt was down regulated in THP-1 cells in response to treatment with 7-ketocholesterol for 24 h and this effect was completely reverted by the addition of β-carotene. The authors maintain that the carotenoid may be altering redox-sensitive molecular pathways involved in the control of cell proliferation and apoptosis and, consequently, by acting as a possible anti-atherogenic agent. In contrast, our data suggest that apigenin and astaxanthin at the concentrations tested provide no benefit in the treatment of atherosclerosis as their induction of apoptosis is significantly involved in the down regulation of the Akt pathway.

Although the present study focuses on the protective potential of antioxidants against oxysterol-induced apoptosis and atherosclerosis, the negative effects observed following treatment with high concentrations of apigenin and astaxanthin could also be looked at from a chemotherapeutic perspective. Some dietary components modulate cell signalling pathways, among other mechanisms, which activate cell death signals and induce apoptosis in precancerous or cancer cells, resulting in the inhibition of cancer development and/or progression[50]. Numerous studies have reported that apigenin may serve as a chemotherapeutic agent because of its ability to inhibit cell proliferation and induce apoptosis in cancer cells[37,47,48,51–53]. Additionally, Tanaka *et al.* [54–56] have shown that oral administration of astaxanthin inhibits

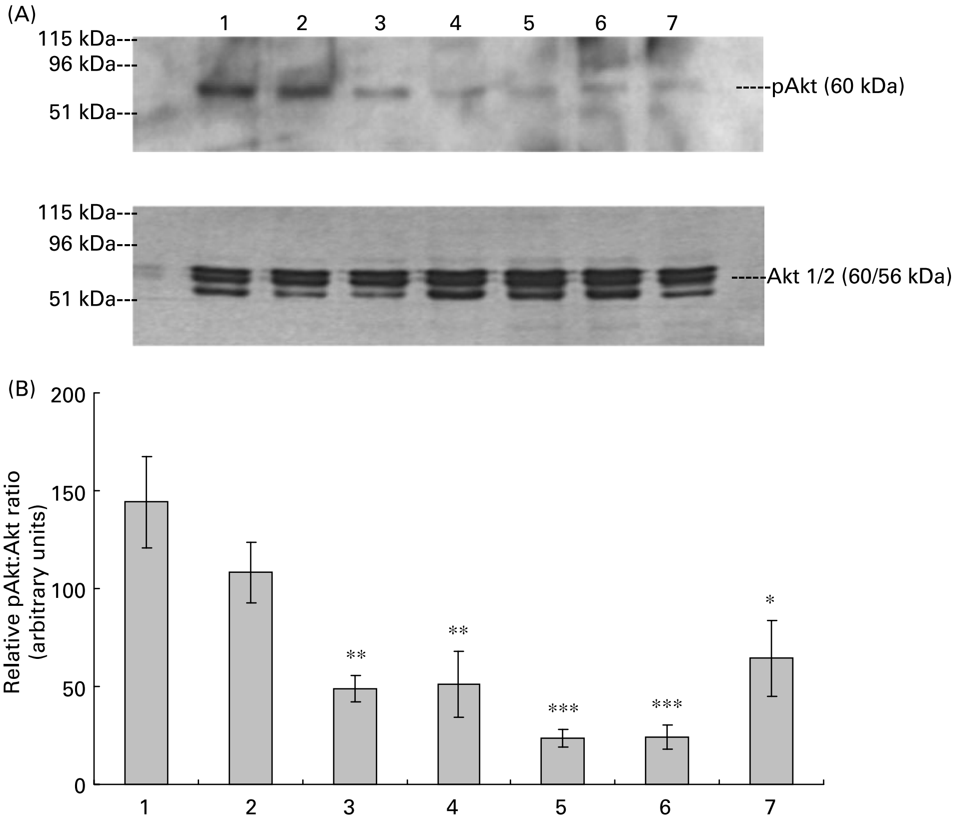

Fig. 5. Effect of 7β-OH on phosphorylation of Akt in U937 cells. Akt expression was visualised at 24 h. Lane 1, control; lane 2, 30 μM-7β-OH; lane 3, 30 μM-7β-OH + 10 μM-α-tocopherol; lane 4, 10 μM-apigenin; lane 5, 30 μM-7β-OH + 10 μM-apigenin; lane 6, 1 μM-astaxanthin; lane 7, 30 μM-7β-OH + 1 μM-astaxanthin. (A) Western blot. The data represent one of three independent experiments yielding similar results. (B) Relative phosphorylated Akt (pAkt):Akt ratio. Values are means of three independent experiments, with their standard errors represented by vertical bars. Mean value was significantly different from that of control: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (ANOVA followed by Tukey's test).

carcinogenesis in mice urinary bladder, in the oral cavity and rat colon. This effect has been partially attributed to suppression of cell proliferation. Moreover, epidemiological data provide evidence concerning the health benefits of these compounds in the development of cancer. De Stefani *et al.* [57] analysed dietary data from 541 individuals with lung cancer and 540 hospitalised controls and reported significant inverse associations with increasing carotenoid, glutathione, flavonoid and vitamin E intake. Overall, the present results demonstrate the pro-apoptotic properties of apigenin and astaxanthin that could support their potential use as chemopreventive and therapeutic agents against carcinogenic disease.

In conclusion, as apigenin, lycopene and high concentrations of astaxanthin failed to protect against 7β-OH-induced apoptosis we present evidence that the initial oxidative stress generated by the oxysterol may concede to other apoptotic events. Studies have shown that the apoptotic process induced by 7β-OH is associated with generation of reactive oxygen species, followed by permeabilisation of lysosomal and mitochondrial membranes, Bcl-x_L degradation, activation of caspase-9 and caspase-3, ultimately leading to degradation of poly(ADP-ribose) polymerase[3,9,35,39]. We have also shown that, while 7β-OH does not have a considerable effect on Akt, however, apigenin or astaxanthin alone or combining them with the oxysterol inhibits Akt activation in U937 cells.

Acknowledgements

The present study was funded by the Higher Education Authority (Dublin, Republic of Ireland). There is no conflict of interest that we should disclose. Each of the three authors contributed equally to the manuscript.

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