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α-Tocopherol, but not γ-Tocopherol Inhibits 7β-Hydroxycholesterol-induced Apoptosis in Human U937 Cells

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Oxysterols, particularly those oxidised at position 7, are toxic to cells in culture and have been shown to induce apoptosis in cell types such as vascular endothelial cells, smooth muscle cells and monocytes. The precise mechanism by which oxysterols induce apoptosis is unknown but may involve the generation of oxidative stress. In the present study we examined the ability of α-TOC, α-TOC acetate (α-TOCA) and γ-TOC to protect against 7β-hydroxycholesterol (7β-OHC)-induced apoptosis of human monocytic U937 cells. 7β-OHC is one of the most commonly detected oxysterols in foods and its level in plasma has been positively associated with an increased risk of atherosclerosis.

The present study demonstrates a significant decrease in cell membrane integrity and cellular glutathione levels when U937 cells were treated with 30 μM 7β-OHC. DNA fragmentation also occurred, as measured by agarose gel electrophoresis, and the number of apoptotic cells increased as assessed by nuclear morphology. Analysis by HPLC showed that there was a greater incorporation of γ-TOC into U937 cells after a 48 h incubation, than either α-TOC or α-TOCA. However, despite the increased uptake of γ-TOC, only α-TOC, and not γ-TOC or α-TOCA was effective at inhibiting 7β-OHC-induced apoptosis in U937 cells.

Keywords: Oxysterol, 7β-hydroxycholesterol, apoptosis, α-tocopherol, γ-tocopherol, U937 cells

INTRODUCTION

Cholesterol is found ubiquitously in mammalian tissues as an integral component of cell membranes[1] and, in its pure form, exhibits no apparent cytotoxic activity *in vivo* or *in vitro*.[2,3] However, in recent years attention has focused on the oxygenated derivatives of cholesterol (oxysterols), in particular their cytotoxicity and their possible involvement in the initiation and/or development of atherosclerosis and cardiovascular disease.

Although the oxysterol content of fresh products is extremely low, oxysterols can form during processing and storage of foods. Significant amounts are associated with cholesterol-rich processed foods such as commercially dried

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whole egg and milk products, freeze-dried meat products, infant milk formulae, frozen ready meals and other foods of animal origin. In addition, cholesterol present in dairy spreads based on milk fat and vegetable oils is more susceptible to oxidation, which may be significant when considering the proliferation of these products as replacements for butter. The increasing use of γ-irradiation as a method for food preservation may also lead to the generation of oxysterols such as 7-ketocholesterol and 5,6-epoxides.[4–6] The prevalence of cytotoxic oxysterols in foods has prompted suggestions that they may pose a risk to human health.

There is evidence that oxysterols are absorbed and incorporated into chylomicrons.[7] Moreover appreciable amounts of oxysterols have been detected in plasma and aortic tissue of rabbits fed cholesterol-rich diets.[8] Oxysterols have also been identified in human plasma, oxidised low density lipoproteins (LDL) and atherosclerotic plaque.[9–13] Oxysterols can also be formed endogenously. Cholesterol catabolism results in the production of some of the most common oxysterols found *in vivo*.[14] It is possible therefore, that oxysterols from both exogenous and endogenous sources may be available to participate in the development of vascular disease in humans.

In cell culture, oxysterols have been shown to be toxic to a variety of human and animal cell types including smooth muscle cells, fibroblasts, vascular endothelial cells, macrophages and lymphocytes. It is not known whether oxysterols exert the same effects *in vivo*, but damage to these cell types would assist in the development of atherosclerosis.[14–18] At least 60 oxysterols have been identified, of which around 28 have been shown to be cytotoxic *in vitro*.[1,4]

The precise mechanism by which oxysterols induce toxicity in cell cultures is unknown but may involve the regulation of cholesterol biosynthesis or altering membrane fluidity, permeability, stability, and activity of membrane bound enzymes. Other reported activities include the stimulation of platelet aggregation, alteration of gap junction communication and modulation of intracellular calcium levels.[3,19–23] Recent studies have demonstrated that in the presence of oxysterols, certain cell types preferentially die by apoptosis.[16,17,24,25] However little is known about the mechanism by which oxysterols activate the apoptotic pathway. Lizard *et al.*[10] postulated a mechanism involving the participation of reactive oxygen species (ROS) and found that 7-ketocholesterol-induced death of human U937 cells was prevented by the antioxidants glutathione and *N*-acetylcysteine, and also by high concentrations (100 μM) of vitamin E.[26] Other workers have also suggested that generation of an oxidative stress may play a key role in 7-ketocholesterol cell toxicity and have protected cultured cells from the effects of this oxysterol by using a commercial mixture of tocopherols (Covi-ox).[27]

In the present study we examined the ability of tocopherols to protect against 7β-hydroxycholesterol (7β-OHC)-induced apoptosis of human monocytic U937 cells. 7β-OHC is thought to be formed by the auto-oxidation of cholesterol and is one of the most commonly detected oxysterols in foods. Two recent human epidemiological studies indicate that raised plasma levels of 7β-OHC may be associated with an increased risk of atherosclerosis.[9,28] Vitamin E is the major antioxidant in the cell membrane, and its presence within the low density lipoprotein (LDL) particle suggests it may be a potential anti-atherogenic compound due to its ability to inhibit lipid oxidation. Although α-tocopherol (α-TOC) is the most biologically active form of vitamin E and is found at high levels in plasma,[29] γ-tocopherol (γ-TOC) has also been found to be a powerful antioxidant *in vitro*[30] and constitutes up to 70% of the dietary intake of tocopherols.[31] Recent findings have shown that low plasma levels of γ-TOC and a high α-TOC:γ-TOC ratio may be associated with coronary heart disease.[32] These reports, together with other studies suggest that there may be a positive protective role for the γ-form of tocopherol in cardiovascular disease.

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In the present study we report that, despite a greater incorporation of γ-TOC into U937 cells, α-TOC, but not γ-TOC was more effective at inhibiting 7β-OHC-induced apoptosis in the U937 cell culture model. To help understand the mode of action of 7β-OHC, α-tocopherol acetate (α-TOCA) was also included in the study as esterification of the tocopherol molecule reduces its ability to react with lipid radicals in liposomes and cell culture models.[33]

MATERIALS AND METHODS

Materials

Tissue culture reagents and chemicals were purchased from Sigma-Aldrich Ireland Ltd. (Dublin, Republic Of Ireland) unless stated otherwise. Tissue culture plastics were obtained from Costar (Cambridge, MA). Human monocytic U937 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Information on the purity of 7β-OHC (> 95% purity), α-TOC, γ-TOC and α-TOCA (> 95% purity) was supplied by Sigma-Aldrich. Solvents used were of high performance liquid chromatography grade. Compounds were dissolved in ethanol for delivery to the cells. The final concentration of ethanol in the cell cultures did not exceed 0.2% (v/v).

Cell Maintenance and Treatment

Human monocytic U937 cells were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum (FCS). Cells were grown in the absence of antibiotics in a humidified atmosphere of 5% CO_2:95% air, 37 °C. Cultures were screened for the presence of mycoplasma using the Hoechst staining method of Mowles[34] and were found to be free of contamination.

Cells were seeded at a density of 1×10^5 cells/ml in 25 cm^2 flasks for all experimental conditions. Prior to treatment with the test compounds, the serum concentration was reduced to 2.5%. Both 7β-OHC and the antioxidants (α-TOC, α-TOCA, γ-TOC) were added to the culture medium immediately prior to addition of the cells. 7β-OHC and the antioxidants were added to give a final concentration of either 30 μM or 10 μM respectively in the growth media, and equivalent volumes of ethanol were added to control samples. All incubations were for 48 h at 37 °C/5% CO_2. Manipulations involving the tocopherols, and all lipid and tocopherol extractions were performed in an amber-lit laboratory. Glass vessels were blown through with N_2(g) before use, and samples were handled under N_2(g).

Cell Viability and Staining with Hoechst 33342

Cell proliferation was assessed using a haemocytometer and viability was monitored using a fluorescein diacetate (FDA)/ethidium bromide (EtBr) assay as previously described.[35] Briefly, 25 μl of cell sample was incubated with an equal volume of FDA/EtBr solution (3–5 min, 37 °C) and analysed under blue fluorescence light (Nikon, 200 × magnification).

Nuclear morphology was assessed according to Dubrez *et al.*[36] Approximately 1×10^6 cells were harvested (200 × g, 10 min) and the pellet re-suspended in 200 μl Hoechst 33342 stain (5 μg/ml in PBS) for 45–60 min (37 °C/5% CO_2). Samples were applied to microscope slides and viewed under UV light (Nikon, 400 × magnification). A total of 300 randomly selected cells were examined per slide and the number of fragmented and/or condensed nuclei was expressed as a percentage of the control. Apoptotic nuclei were characterised as smaller in size, more irregularly shaped and more brightly stained than healthy nuclei.

Determination of Cellular Glutathione Levels

The cellular level of glutathione was measured according to the method of Hissin and Hilf.[37]

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Briefly 4×10^6 were centrifuged at $100{,}000 \times g$ for 15 min. The supernatant was diluted in phosphate-EDTA buffer (pH 8) and mixed with 1 μg/μl solution of *o*-phthalaldehyde. After incubation at room temperature for 15 min, the fluorescence at 420 nm was detected after activation at 350 nm. Protein was determined by the bicinchoninic acid (BCA) method.[38]

DNA Gel Electrophoresis Assay

Oligonucleosomal DNA fragments were detected as described by Swat *et al.*[39] Cells (2×10^6) were harvested ($200 \times g$, 10 min) and DNA extracted by lysing the pellet with 20 μl of a solution containing 50 mM Tris, 10 mM EDTA and 0.5% (w/v) sodium lauryl sarcosinate. Samples were incubated with RNAse A (0.25 mg/ml) for 1 h at 50 °C. After centrifugation, samples were incubated with proteinase K (5 mg/ml, 1 h, 50 °C). A 1.8% agarose gel was prepared in TBE buffer (0.45 M Tris, 0.45 M boric acid, 2 mM EDTA, pH 8) and samples were electrophoresed at 3 V/cm for 4 h and for a further 0.5 h at 5.5 V/cm. Gels were stained with EtBr and examined using a UV transilluminator. Photographs were taken using a digital camera (Kodak).

HPLC Analysis

Cellular uptake of tocopherol was measured using a modification of the method of Lang *et al.*[40] Briefly, after exposure to either 10 μM α-TOC, α-TOCA or γ-TOC for 48 h, 8×10^6 cells were washed with PBS (3×5 min washes), and harvested by centrifugation. An ethanolic solution containing 25 mM recovery standard (either α-TOCA or δ-tocopherol) and 0.05% butylated hydroxytoluene (BHT) was added to the cell pellets, and the samples were extracted three times in hexane following saponification. The hexane fractions were pooled and dried under a gentle stream of nitrogen, reconstituted in dichloromethane : acetronitrile : methanol (1:7:2) and analysed by HPLC as previously described.[41] Samples were injected onto a Shimadzu SCL-10A model HPLC, equipped with a SIL-10A autoinjector, and two 150 mm × 4.6 mm C18 columns enclosed in a water jacket maintained at 25 °C. Samples were eluted using a mobile phase of acetonitrile : methanol : dichloromethane (15:4:1) containing 10 mM ammonium acetate, 4.5 mM BHT and 3.6 mM triethylamine at 1.5 ml/min. Peaks were detected at 325 nm using a Shimadzu SPD-10AV UV-visible detector. Results were collected and analysed using Millenium Chromatography Manager data collection software (Waters Corporation, Milford, MA).

Capillary Gas Chromatography

Incorporation of 7β-OHC into U937 cells was quantified by a modification of the method of Folch *et al.*[42] Briefly, 5×10^6 cells were sonicated and mixed with 4.75 ml chloroform : methanol (2:1), vortexed and filtered. Following centrifugation, the lower phase was dried under nitrogen, reconstituted in pyridine (100 μl) and bis (trimethyl-silyl) trifluoro-acetamide (BSTFA; 50 μl) and allowed to stand for 30 min in the dark at room temperature. Samples were reconstituted in ethyl acetate for analysis by capillary gas chromatography (Shimadzu, Model 14A) and injected onto a DB5-MS capillary column (30 m × 0.25 mm) with split injection of 12 ml/min. Nitrogen was the carrier gas used. The initial temperature of the injector port was 190 °C and this was increased to a final temperature of 255 °C in a stepwise fashion. Data was collected and analysed using Millenium 2:1 software.

Statistical Analysis

Where appropriate, data were analysed using one way analysis of variance (ANOVA) followed by Dunnett's test. Unless otherwise stated, results are presented as the mean ± standard deviation (SD) of duplicate cultures from 3 to 5 separate experiments.

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RESULTS

7β-OHC-Induced Toxicity

U937 cells were exposed to increasing concentrations of 7β-OHC (10–30 µM; 0.4–12.5 µg/ml) for 48 h (37 °C, 5% CO_2). Samples were removed at different times during the incubation period, counted and stained with either FDA/EtBr to assess membrane integrity (Figure 1A) or Hoechst 33342 to examine nuclear morphology (Figure 1B). A significant ($p < .01$) decrease in membrane integrity as determined by an increase in the number of EtBr permeable cells was noted following treatment with 7β-OHC. The percentage of cells exhibiting condensed and/or fragmented nuclei was also significantly higher ($p < .01$) in the oxysterol-treated cells. However, significant cytotoxicity occurred only after a 48 h incubation with the highest concentration (30 µM) of 7β-OHC (Figure 1). Cells treated with 30 µM 7β-OHC were analysed for oxysterol content by gas chromatography (GC). Despite taking great care when handling samples for lipid extraction, there was variation in the results obtained from GC analysis. However, no 7β-OHC was detected in any of the control samples, whereas the cells treated with 30 µM 7β-OHC incorporated 114 ± 104 ng oxysterol/10^6 cells after a 48 h treatment. The level of cholesterol in the control samples was found to be 92 ± 52 ng/10^6 cells, and 50 ± 35 ng/10^6 cells in the samples treated with 7β-OHC.

Levels of intracellular glutathione were measured at 12 h and 20 h post-treatment with 7β-OHC. After 12 h incubation, there was no significant decrease in glutathione levels (48.1 ± 4.3 nmol/mg protein and 37.2 ± 2.3 nmol/mg protein for the control and treated samples respectively). However there was a significant decrease ($p < .01$) after 20 h treatment with 7β-OHC (Figure 2). The depletion of cellular glutathione was seen before the 7β-OHC-induced changes in cell membrane integrity and condensed and/or fragmented nuclei were evident.

Incorporation of Tocopherol into U937 Cells

Either α-TOC, γ-TOC or α-TOCA was added to U937 cells to give a final concentration in the growth media of 10 µM. After incubating the cells for 48 h, samples were removed and the tocopherol content of the cells measured by HPLC. Control samples were found to have an α-TOC content of 5.2 ng/10^6 cells, whereas γ-TOC was not detected. In samples supplemented with α-TOC, the cellular level was increased approximately 10 fold to 49.6 ± 8.1 ng/10^6 cells. Again, γ-TOC was not detected in these samples. Incubation with α-TOCA resulted in a 13-fold increase in α-TOC content to 66.1 ± 29.0 ng/10^6 cells, γ-TOC was also detected at a level of 1.9 ± 0.9 ng/10^6 cells. Finally, when samples were treated with γ-TOC, the cellular content was increased to 170.2 ± 33.6 ng/10^6 cells. The level of α-TOC detected in these samples was 2.9 ± 0.2 ng/10^6 cells. Thus there was an increased incorporation (3-fold increase) of γ-TOC into U937 cells compared to α-TOC.

Effect of Tocopherol on 7β-OHC-induced Toxicity

Having established the conditions for 7β-OHC-induced cytotoxicity in U937 cells and determined whether or not the oxysterol and the tocopherols were being incorporated into the cells, we next investigated what effect the different tocopherols had on 7β-OHC-induced cell death. U937 cells were exposed simultaneously to 30 µM 7β-OHC and 10 µM of either α-TOC, α-TOCA or γ-TOC for 48 h (37 °C, 5% CO_2). Samples were then counted and stained with either FDA/EtBr to assess membrane integrity or Hoechst 33342 to examine nuclear morphology (Table I).

Treatment of U937 cells with 30 µM 7β-OHC significantly decreased ($p < .01$) the percentage of cells that were able to exclude EtBr. The percentage of condensed and fragmented nuclei as assessed by staining with Hoechst 33342 was

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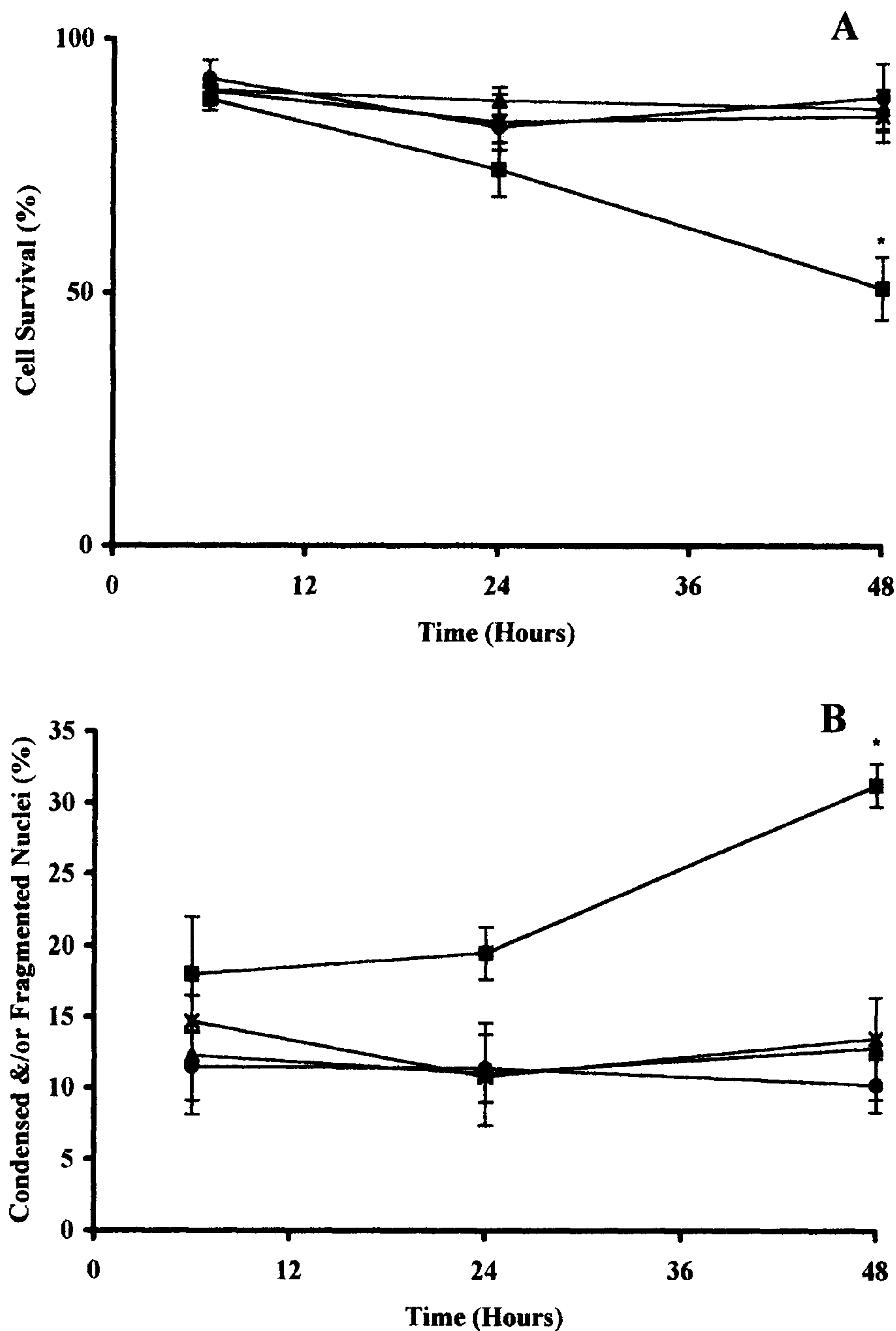

FIGURE 1 Time course of 7β-hydroxycholesterol (7β-OHC)-induced cell death. U937 cells (1×10^5 cells/ml) were treated with increasing concentrations of oxysterol for 48 h. Samples were removed at the indicated times and processed for either cell viability (A), or stained with Hoechst 33342; (B) to determine the percentage of condensed and/or fragmented nuclei as outlined in the materials and methods section. Circles: control; Triangles: 1 µM 7β-OHC; Crosses: 10 µM 7β-OHC; Squares: 30 µM 7β-OHC. Results represent the mean ± SE of duplicate cultures from 3 to 4 separate experiments. *Significantly different from control ($p < .01$) as assessed by ANOVA followed by Dunnett's test.

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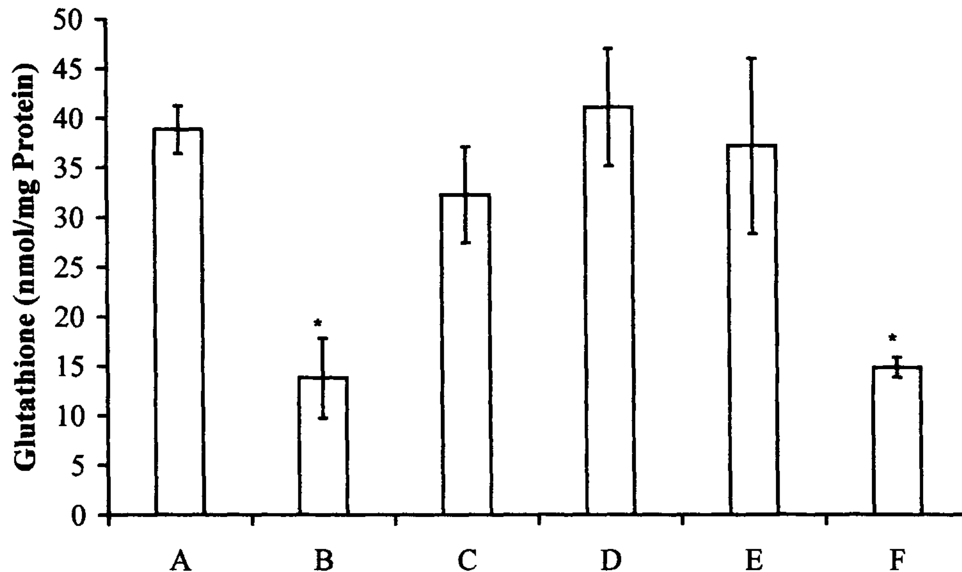

FIGURE 2 Cellular content of glutathione. U937 cells were seeded at a density of 1×10^5 cells/ml in $25\,cm^2$ flasks in the presence or absence of 30 µM 7β-hydroxycholesterol (7β-OHC). α-Tocopherol (α-TOC), or γ-tocopherol (γ-TOC) were added to give a final concentration of 10 µM in the growth medium. After a 20 h incubation (37 °C, 5% CO_2), samples were removed and analysed for glutathione content using o-phthalaldehyde as described in the materials and methods section. (A) Control; (B) 7β-OHC; (C) α-TOC; (D) α-TOC and 7β-OHC; (E) γ-TOC; (F) γ-TOC; and 7β-OHC. Results are the mean values ± SE of cultures from 2/4 separate experiments. *Significantly different from control ($p < .01$) as assessed by ANOVA followed by Dunnett's

TABLE I Effect of either α-tocopherol (α-TOC), α-tocopherol acetate (α-TOCA), or γ-tocopherol (γ-TOC) on 7β-hydroxycholesterol (7β-OHC)-induced cytotoxicity (U937 cells (1×10^5 cells/ml) were treated with 7β-OHC:tocopherol (30 µM:10 µM), tocopherol alone or 7β-OHC alone for 48 h. Samples were processed for either cell viability, or stained with Hoescht 33342 and analysed by fluorescence microscopy as outlined in the materials and methods section)

Treatment	% Viable cells[a]	% Apoptotic nuclei[a]
Control	93 ± 3	10 ± 2
30 µM 7β-OHC	68 ± 5[b]	22 ± 7[b]
10 µM α-TOC	94 ± 2	10 ± 1
30 µM 7β-OHC and 10 µM α-TOC	86 ± 5[c]	12 ± 3
Control	97 ± 1	8 ± 2
30 µM 7β-OHC	75 ± 6[b]	24 ± 3[b]
10 µM α-TOCA	95 ± 1	11 ± 1
30 µM 7β-OHC and 10 µM α-TOCA	74 ± 6[b]	22 ± 3[b]
Control	97 ± 1	7 ± 2
30 µM 7β-OHC	50 ± 10[b]	34 ± 8[b]
10 µM γ-TOC	97 ± 1	7 ± 1
30 µM 7β-OHC and 10 µM γ-TOC	44 ± 6[b]	32 ± 7[b]

[a] Results represent the mean ± SE of duplicate cultures from three separate experiments.
[b] Significantly different from control ($p < .01$).
[c] Significantly different from control ($p < .05$), as assessed by ANOVA followed by Dunnett's test.

also significantly increased in the presence of the oxysterol (Table I). When 7β-OHC and tocopherol were added simultaneously to the medium in a ratio of 3:1, only α-TOC was able to prevent the decrease in membrane integrity and increase in apoptotic nuclei induced by the oxysterol (Table I). Raising the concentration of α-TOCA and γ-TOC to 100 µM in the growth medium also did

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not prevent oxysterol-induced cytotoxicity (data not shown). Neither α-TOC, α-TOCA nor γ-TOC had any toxic effects when added on their own to U937 cells (Table I). Finally, depletion of cellular glutathione, which constituted an early event in 7β-OHC-induced apoptosis, did not occur when U937 cells were co-incubated with α-TOC, but did occur when γ-TOC was the antioxidant used (Figure 2).

Effect of α-TOC, γ-TOC and α-TOCA on 7β-OHC-Induced DNA Fragmentation

Cell samples were treated with 30 μM 7β-OHC in the presence or absence of either 10 μM α-TOC, γ-TOC or α-TOCA and processed for gel electrophoresis as described in the materials and methods. Treatment with 10 μM α-TOC visibly reduced DNA laddering produced by 30 μM 7β-OHC (Figure 3).

FIGURE 3 Effect of either α-tocopherol (α-TOC), γ-tocopherol (γ-TOC) or α-tocopherol acetate (α-TOCA), on 7β-hydroxycholesterol (7β-OHC)-induced DNA fragmentation. DNA fragments were detected as described in the materials and methods section. Cells (2×10^6) were harvested and DNA was extracted and electrophoresed on an agarose gel. Gels were stained with EtBr and examined using a UV transilluminator. Photographs were taken using a digital camera (Kodak). Lane 1: DNA marker (Promega); Lane 2: Negative control; Lane 3: 7β-OHC; Lane 4: 7β-OHC and α-TOC; Lane 5: 7β-OHC and α-TOCA; Lane 6: 7β-OHC and γ-TOC; Lane 7: Negative control.

DISCUSSION

Oxysterols have been postulated to be, in part, responsible for the cytotoxicity of oxidised LDL to a variety of cell types. Although there is as yet, no conclusive evidence to show that oxysterols are involved in the initiation and/or development of atherosclerosis in humans, oxidised LDL, and oxysterols isolated from LDL, including 7β-OHC, have been shown to be cytotoxic to cells in culture. In the present study, the major membrane and LDL antioxidant (vitamin E) was investigated for its ability to prevent 7β-OHC-induced killing of U937 cells.

Incubation for 48 h with 7β-OHC (30 μM) induced significant loss of membrane integrity in human U937 cells. Changes consistent with apoptotic cell death were also observed, including an increased number of condensed and fragmented nuclei (Figure 1), and internucleosomal cleavage of DNA as determined by agarose gel electrophoresis (Figure 3). The level of cellular glutathione was also significantly decreased prior to the onset of apoptosis induced by 7β-OHC (Figure 2). It has been reported that glutathione depletion precedes and may be an early indicator of apoptotic cell death,[10] however it may not be an obligatory pathway in oxysterol-mediated cytotoxicity.[43] Finally, we have previously shown that treatment of U937 cells with 30 μM 7β-OHC for 48 h results in an increase in the number of hypodiploid cells, which is generally considered to be an indication that apoptosis has occurred.[25] No significant toxic effects were noted when lower concentrations (1–10 μM) of oxysterol were used, or when the incubation time was less than 48 h. We were not able to demonstrate any increase in lipid

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peroxidation in the presence of 7β-OHC, measured either by TBARS or commercial kit (Calbiochem, data not shown). Other workers have also not detected an increase in lipid oxidation using TBARS in the presence of oxysterols, but have detected an increase in antioxidant enzyme activity.[27]

Supplementation of U937 cells with either 10 μM α-TOC, α-TOCA or γ-TOC resulted in an increase in tocopherol content of 9.5 fold, 12.7 fold or 32.7 fold respectively over the baseline, unsupplemented level. Tran and Chan[31] have suggested that this preferential incorporation of γ-TOC into human cells could be due to the absence of the methyl group at position 5 on the chromanol ring, making γ-TOC more water soluble. However, the cellular content of γ-TOC gives no information on the orientation within the membrane interior, or the subcellular distribution of the molecule. Despite the increased uptake of γ-TOC into the cells, only simultaneous incubation with α-TOC prevented the toxic effects of the oxysterol (Table I). This inhibitory effect was not seen with either α-TOCA or γ-TOC. Both these molecules are known to have less antioxidant activity under certain conditions; the former due to esterification of the −OH group at position 6, and the latter as a result of the smaller number of methyl groups on the chromanol ring.[33]

Although γ-TOC is rapidly taken up by cells in culture, it also disappears more quickly than α-TOC. It is also present at much lower levels in plasma.[31] The ability of tocopherols to protect against the cytotoxicity of oxidised LDL towards cells in culture has been previously reported, and the efficacies of α-TOC and γ-TOC has been different in these systems. de Nigris *et al.*[44] found that α-TOC was more effective than γ-TOC at inhibiting the induction of apoptosis by oxidised LDL in human smooth muscle cells. γ-TOC at a concentration of 10 μM has essentially no effect on the reduction of apoptosis, however concentrations of 50 μM were effective. In contrast, Li *et al.*[45] found lower concentrations of γ-TOC to be effective at inhibiting oxidised LDL-induced apoptosis of human coronary artery endothelial cells. Christen *et al.*[30] reported that γ-TOC was extremely effective in removing peroxynitrite-derived nitrating species by a non-antioxidant mechanism.

It has been suggested that oxysterols could exert their cytotoxic effects through a mechanism involving oxidative stress.[10,27] However other mechanisms are also involved, such as the effects on cholesterol biosynthesis, membrane fluidity, permeability and stability. We were unable to detect any increase in lipid peroxidation in the presence of 7β-OHC, nor did we determine whether the decrease in GSH was due to oxidative consumption or extrusion into the culture media. Therefore we cannot discount the possibility that the protective effects of α-TOC were due to some other effect on the cell.

In conclusion, the present study demonstrates the killing of U937 cells by 7β-OHC is by apoptosis. α-TOC but not α-TOCA or γ-TOC (tocopherol:oxysterol, 1:3) was much more effective at preventing 7β-OHC-induced cell death of U937 cells, despite the greater cellular incorporation of γ-TOC into the cells. We are currently working to understand the mechanism by which α-TOC is more protective in U937 cells.

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