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The role of the mitochondria in apoptosis induced by 7β-hydroxycholesterol and cholesterol-5β,6β-epoxide

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Oxysterols are oxygenated derivatives of cholesterol that may be formed endogenously or absorbed from the diet. Significant amounts of oxysterols have frequently been identified in foods of animal origin, in particular highly processed foods. To date, oxysterols have been shown to possess diverse biological activities; however, recent attention has focused on their potential role in the development of atherosclerosis. Oxysterols have been reported to induce apoptosis in cells of the arterial wall, a primary process in the development of atheroma. The aim of the present study was to identify the role of the mitochondria in the apoptotic pathways induced by the oxysterols 7β-hydroxycholesterol (7β-OH) and cholesterol-5β,6β-epoxide (β-epoxide) in U937 cells. To this end, we investigated the effects of these oxysterols on mitochondrial membrane potential, caspase-8 activity, the mitochondrial permeability transition pore and cytochrome *c* release. 7β-OH-induced apoptosis was associated with a loss in mitochondrial membrane potential after 2 h, accompanied by cytochrome *c* release from the mitochondria into the cytosol after 16 h. Pre-treatment with a range of inhibitors of the mitochondrial permeability transition pore protected against 7β-OH-induced cell death. In contrast, β-epoxide induced a slight increase in caspase-8 activity but had no effect on mitochondrial membrane potential or cytochrome *c* release. The present results confirm that 7β-OH-induced apoptosis occurs via the mitochondrial pathway and highlights differences in the apoptotic pathways induced by 7β-OH and β-epoxide in U937 cells.

Apoptosis: Mitochondria: 7β-Hydroxycholesterol: Cholesterol-5β,6β-epoxide: U937 cells

Oxidised LDL (oxLDL) contains many potentially pro-atherogenic molecules, including oxysterols. Numerous studies, to date, have implicated oxLDL in the initiation and propagation of atherosclerosis (Kinscherf *et al.* 1998; Maziere *et al.* 2000; Napoli *et al.* 2000; Colles *et al.* 2001). OxLDL has been shown to induce apoptosis in cells of the arterial wall, a primary process in the development of atheroma (Uemura *et al.* 2002; Chen *et al.* 2004). The ability of oxLDL to induce apoptotic cell death has been attributed to its oxysterol component. The cholesterol oxidation products in oxLDL include 7β-hydroperoxycholesterol, 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol, 25-hydroxycholesterol, cholesterol-5α,6α-epoxide (α-epoxide) and cholesterol-5β,6β-epoxide (β-epoxide) (Schroepfer, 2000). Oxysterols have previously been shown to induce apoptosis in cells of the arterial wall (Lizard *et al.* 1999), although the exact mechanism remains to be elucidated.

Apoptosis is a highly conserved process characterised by nuclear and cytoplasmic condensation, membrane budding, formation of discrete membrane-bound vesicles known as apoptotic bodies and DNA fragmentation. There are two main apoptotic pathways; the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway. Oxysterols are known to have the potential to induce apoptosis through both pathways (Panini & Sinensky, 2001). Most studies, to date, have focused on examining the ability of oxysterols to induce apoptosis through the mitochondrial pathway with particular attention on 7-ketocholesterol and 7β-OH, known to be potent inducers of apoptosis (Lizard *et al.* 2000; O'Callaghan *et al.* 2002; Miguet-Alfonsi *et al.* 2002; Ryan *et al.* 2004*a*; Seye *et al.* 2004). Both of these oxysterols have been shown to induce apoptosis via the generation of an oxidative stress, release of cytochrome *c* from the mitochondria and caspase-9 activation, which in turn activates caspase-3 in a caspase cascade culminating in DNA fragmentation and cell death. Few studies have focused on examining the exact mechanism of apoptosis induced by other oxysterols present in oxLDL.

Previous studies from our laboratory highlighted differences between the apoptotic pathways induced by two oxysterols; 7β-OH and β-epoxide (Ryan *et al.* 2004*b*). β-Epoxide-induced apoptosis, in contrast to apoptosis induced by 7β-OH, was not associated with a depletion of the intracellular antioxidant, glutathione. Pre-treatment of U937 cells with an inhibitor of cytochrome *c* release prevented apoptosis in cells treated with 7β-OH but did not protect against β-epoxide-induced cell death. The purpose of the present study was to further investigate potential differences, at a mitochondrial level, in the signal transduction pathways induced by 7β-OH and β-epoxide. To this end, we examined the effects of the oxysterols on mitochondrial membrane potential, caspase-8 activation, the mitochondrial permeability transition pore (MPTP) and cytochrome *c* release, in U937 cells.

Abbreviations: BA, bongkrekic acid; CATR, carboxyatractyloside; CsA, cyclosporin A; α-epoxide, cholesterol-5α,6α-epoxide; β-epoxide, cholesterol-5β,6β-epoxide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide; MPTP, mitochondrial permeability transition pore; 7β-OH, 7β-hydroxycholesterol; oxLDL, oxidised LDL.

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Materials and methods

Materials

All chemicals and cell culture reagents were obtained from the Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated. Tissue culture plastics were supplied by Costar (Cambridge, UK). Information on the purity of the oxysterols (purity >95 %) was obtained from Sigma. Mitochondrial inhibitors, bongkrekic acid (BA), cyclosporin A (CsA) and carboxyatractyloside (CATR), were obtained from Calbiochem (Nottingham, UK). The ADP analogue used in the present study was a cell permeable caged ADP-K^+ purchased from Calbiochem as used by Burzik *et al.* (2003). The cytochrome *c* immunoassay and caspase-8 fluorometric assay kit were obtained from R&D systems (Abingdon, UK). Cell lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK).

Maintenance of cell lines

Human monocytic U937 cells were grown in suspension in RPMI-1640 medium supplemented with 10 % (v/v) fetal bovine serum. The cells were grown at 37°C and 5 % CO_2 in a humidified incubator. The cells were screened for mycoplasma contamination by the Hoechst staining method (O'Callaghan *et al.* 1999) and were cultured in the absence of antibiotics. Exponentially growing cells were used throughout.

Measurement of mitochondrial membrane potential

Changes in mitochondrial membrane potential were assessed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide (JC-1), a lipophilic cationic dye. In healthy cells, the dye stains the mitochondria bright red. In apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria. U937 cells were adjusted to a density of 2×10^5 cells/ml in RPMI-1640, supplemented with 2·5 % fetal bovine serum. 60 μM-7β-OH, 60 μM-β-epoxide and 10 μM-etoposide were added to the tissue culture medium. Before incubation with JC-1, samples were exposed to oxysterols dissolved in ethanol and the final concentration of ethanol in the cultures did not exceed 0·3 % (v/v). The samples were incubated for 0, 2, 4 and 6 h. Following incubation, cells were centrifuged for 5 min at 400 ***g***. The supernatant fraction was removed and cells were re-suspended in 400 μl PBS and 10 μl JC-1 dye (5 μg/ml). Cells were incubated at 37°C and 5 % CO_2 for 15 min followed by centrifugation for 5 min at 400 ***g***. The cells were re-suspended in fresh PBS and 200 μl/well was dispensed into a ninety-six-well microtitre plate. The red fluorescence (excitation 550 nm, emission 600 nm) and green fluorescence (excitation 485 nm, emission 535 nm) were detected using a fluorescence platereader (spectrafluorplus). The red fluorescence:green fluorescence ratio was determined.

Measurement of caspase-8 activity

Caspase-8 activity was measured using the commercially available caspase-8 fluorometric assay kit from R&D systems (Abingdon, UK). U937 cells were incubated with 30 μM-7β-OH, 30 μM-β-epoxide and 10 μM-etoposide for 6 and 9 h. Following incubation, 1×10^6 cells were collected and the caspase-8 assay was performed according to instructions in the assay kit. Briefly, supernatant fractions from lysed and centrifuged cells were transferred to a flat-bottomed microtitre plate to which reaction buffer and substrate were added. After 90 min incubation at 37°C, fluorescence (excitation 400 nm, emission 505 nm) was measured using a fluorescence platereader (spectrafluorplus). The protein content of the samples was assessed using the BCA method (Smith *et al.* 1985) and caspase-8 activity per mg protein was calculated. Results were expressed as fold increase relative to the control.

Treatment of cells with inhibitors of the mitochondrial permeability transition pore

U937 cells were adjusted to a density of 2×10^5 cells/ml in RPMI-1640, supplemented with 2·5 % fetal bovine serum and pre-treated for 1 h with inhibitors of the MPTP; 5 μM-BA, 1 μM-CsA, 15 μM-CATR or 5 mM-ADP. Following pre-incubation, cells were treated with 30 μM-7β-OH, 30 μM-β-epoxide or 10 μM-etoposide. Samples were incubated for a further 24 h at 37°C and 5 % CO_2. 7β-OH, β-epoxide and CsA were dissolved in ethanol for delivery to cells and the final concentration of ethanol in the cultures did not exceed 0·3 % (v/v). The rest of the inhibitors were dissolved in distilled water and etoposide was dissolved in dimethylsulfoxide. Control cells were treated with an equivalent amount of solvent.

Cell viability

Following a 24 h incubation, 25 μl cells were removed for assessment of cell viability. Viability was monitored using a modification of the fluorochrome-mediated viability assay as 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 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 etoposide-, 7β-OH- and β-epoxide-treated cells was assessed by fluorescence microscopy following staining with Hoechst 33 342 (O'Callaghan *et al.* 1999). 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 incubated at 37°C and 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 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).

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Investigation of cytochrome c *release by enzyme-linked immunosorbent assay*

To assess the release of cytochrome *c* into the cytosol, following 9, 12 and 16 h treatment with 30 μM-7β-OH, 30 μM-β-epoxide or 10 μM-etoposide, U937 cells were separated into cytosolic and mitochondrial subfractions using a cytosol/fractionation kit obtained from Calbiochem (Nottingham, UK). Briefly 5×10^7 cells were collected by centrifugation at 600 ***g*** for 5 min. Cells were washed in ice-cold PBS and re-suspended in an extraction buffer containing diothiothreitol and protease inhibitors. Following incubation on ice for 10 min, cells were homogenised in an ice-cold dounce tissue grinder. Homogenisation was accomplished by douncing 35–40 times with a Griffiths tube homogeniser. Efficiency of homogenisation was confirmed by examining the homogenised sample under a microscope. The homogenate was transferred to a fresh microcentrifuge tube and centrifuged at 700 ***g*** for 10 min. The supernatant fraction was centrifuged at 14 000 ***g*** for 1 h to obtain the cytosolic fraction. Following dilution the samples were added to a ninety-six-well microplate coated with a monoclonal antibody against cytochrome *c* and incubated for 2 h at room temperature. At the end of the incubation period the cells were washed four times. Cytochrome *c* conjugate was added to each well and the cells were incubated for a further 2 h. The microwells were washed four times and the enzymic activity was determined by the addition of 200 μl tetramethylbenzidine containing H_2O_2 (0·002 %). After 30 min of incubation at room temperature, the reaction was stopped by the addition of 50 μl sulfuric acid (1 M). The absorbance was read on a Model 450 Microplate Reader (Bio-Rad) at 450 nm. Results were expressed as a percentage of the control (100 %).

Statistics

All data points are the mean values with their standard errors of at least three independent experiments (unless otherwise stated). Where appropriate, data were analysed by one-way ANOVA followed by Dunnett's test. The software employed for statistical analysis was Prism (GraphPad Inc., San Diego, CA, USA).

Results

Mitochondrial membrane potential

Depolarisation of the inner mitochondrial membrane is reported to be an early key event for initiation of the apoptotic cascade in cells undergoing apoptosis via the intrinsic pathway. The loss of mitochondrial membrane potential was assessed by measuring the generation of red fluorescence of JC-1 dye using a fluorescence platereader (red fluorescence, excitation 550 nm, emission 600 nm; green fluorescence, excitation 485 nm, emission 535 nm). When mitochondrial membrane potential is intact, the JC-1 dye accumulates and stains the mitochondria bright red. If the mitochondrial membrane potential collapses, the JC-1 cannot accumulate within the mitochondria. In these cells JC-1 remains in the cytoplasm in a green fluorescent monomeric form. The red:green fluorescence ratio is decreased in cells undergoing a loss in mitochondrial membrane potential. Following a 2 h incubation, the red:green fluorescence ratio in U937 cells treated with 7β-OH decreased to 58 % (Fig. 1). Etoposide also induced a loss in potential across the mitochondrial membrane

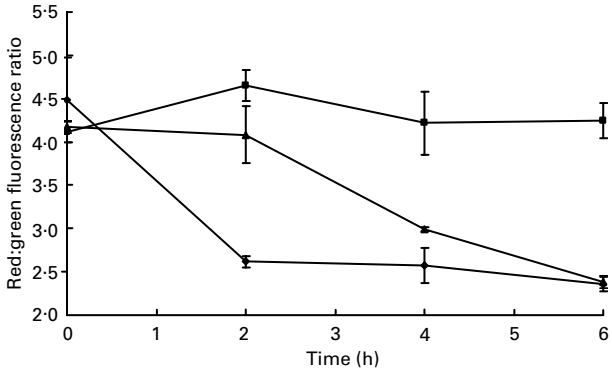

Fig. 1. Mitochondrial membrane potential measurements of U937 cells following treatment with 60 μM-7β-hydroxycholesterol (–◆–), 60 μM-cholesterol-5β,6β-epoxide (–■–) or 10 μM-etoposide (–▲–) for 0, 2, 4 and 6 h. Changes in mitochondrial membrane potential were assessed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide (JC-1) dye. In healthy cells the dye accumulates as aggregates in the mitochondria, which stain bright red. In apoptotic cells JC-1 exists in monomeric form and stains the cytosol green. The red:green fluorescence ratio is decreased in cells undergoing apoptosis with loss of mitochondrial transmembrane potential. Data are means of three independent experiments, with standard errors represented by vertical bars.

after 4 h (71 %), which was further decreased after 6 h (57 %). There was no loss of mitochondrial membrane potential in U937 cells treated with β-epoxide.

Caspase-8 activity

Caspase-8 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. Activation of caspase-8 may lead to the direct activation of caspase-3, thought to be the key enzyme in apoptosis control. The activity of caspase-8 was assessed in U937 cells treated with 30 μM-7β-OH, 30 μM-epoxide or 10 μM-etoposide for 6 and 9 h. There was a significant ($P<0·05$) increase in caspase-8 activity after 6 h in cells treated with etoposide (Fig. 2). In cells treated with β-epoxide, there was a 1·5-fold increase in caspase-8 activity, relative to the control, after 9 h but this was not significant (Fig. 2). 7β-OH did not significantly increase caspase-8 activity in U937 cells.

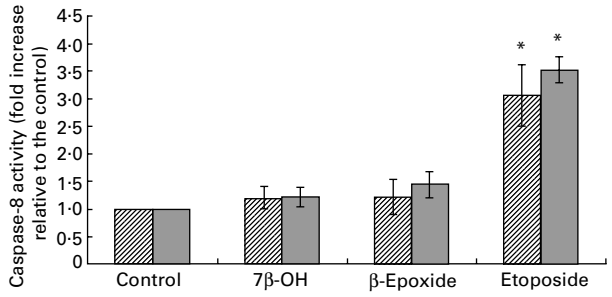

Fig. 2. Caspase-8 activity, expressed relative to protein content, in U937 cells treated with 30 μM-7β-hydroxycholesterol (7β-OH), 30 μM-cholesterol-5β,6β-epoxide (β-epoxide) or 10 μM-etoposide for 6 h (■) and 9 h (■). Results are expressed as fold increase relative to the control. Data are means of four independent experiments, with standard errors represented by vertical bars * Mean value was significantly different from control ($P<0·05$; ANOVA followed by Dunnett's test).

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Ability of inhibitors of the mitochondrial transition pore to protect against oxysterol-induced cytotoxicity and cell death

Viability was assessed in U937 cells by the fluorescein diacetate–ethidium bromide assay and apoptotic nuclei were quantified following staining with Hoechst 33 342. Cell samples were treated with 30 μM-7β-OH, 30 μM-β-epoxide or 10 μM-etoposide for 24 h in the presence or absence of 5 μM-BA, 1 μM-CsA, 15 μM-CATR or 5 mM-ADP. BA, CsA and ADP significantly ($P<0·05$) protected against the decrease in cell viability and the increase in apoptotic nuclei induced by 7β-OH but had no observed effect on the cells exposed to β-epoxide (Tables 1 and 2). There was an increase in viability in U937 cells pre-treated with CsA before incubation with etoposide but pre-treatment did not restore cell viability to control values (Table 1). CATR did not significantly protect against oxysterol-induced or etoposide-induced apoptosis.

Cytochrome c *release*

The intrinsic cell death pathway involves the initiation of apoptosis as a result of a disturbance of intracellular homeostasis. In this pathway cytochrome *c*, normally localised in the intermembrane space of the mitochondria, is released into the cytosol initiating a cascade of events, which culminates in apoptotic cell death. The release of cytochrome *c* in U937 cells treated with 30 μM-7β-OH, 30 μM-β-epoxide or 10 μM-etoposide was measured using an ELISA assay. There was no increase in cytochrome *c* after 9 or 12 h (results not shown). After 16 h cytochrome *c* levels were increased in the cytosol of 7β-OH and etoposide-treated cells to 155 and 132 % of the control respectively (Fig. 3). There was no increase in the percentage of cytochrome *c* in the cytosol of β-epoxide-treated cells.

Discussion

Oxysterols, also known as cholesterol oxidation products, may be produced endogenously or absorbed from the diet and are particularly found in highly processed foods of animal origin. The most common oxysterols found in foodstuffs include 7β-OH and β-epoxide as well as 7-ketocholesterol, α-epoxide, 20-hydroxycholesterol, 19-hydroxycholesterol and 25-hydroxycholesterol. Dietary sources of oxysterols are limited to foods of animal origin since only these contain cholesterol in appreciable amounts. Among foods and food products rich in cholesterol are eggs and egg products, meat, meat products and animal fats, fish and fish oil, milk and milk products and deep fried foods (Bosinger *et al.* 1993; PaniangvaIt *et al.* 1995; Leonarduzzi *et al.* 2002). Various studies have shown that the total amount of oxysterols in food can reach between 1 and 10 % of total cholesterol (Paniangvait *et al.* 1995). In normal human plasma, average oxysterol concentrations are approximately 1 μM. In disease states, however, such as in hypercholesterolaemia, concentrations may be elevated to as high as 20–30 μM (Schroepfer, 2000). Oxysterols have been consistently detected in atherosclerotic plaque from human patients (Brown *et al.* 1997). Accumulating evidence suggests certain oxysterols may play a role in the pathogenesis and progression of atherosclerosis (Leonarduzzi *et al.* 2004). Because of the strong pro-apoptotic activity of certain oxysterols and their potential involvement in atherosclerosis, an understanding of the mechanism of their biochemical effects is required.

The objective of the present study was to investigate the role of the mitochondria in apoptosis induced by 7β-OH and β-epoxide, in U937 cells. Both oxysterols occur in significant quantities in oxLDL and are potentially pro-atherogenic. The mitochondria play a pivotal role during the initial stages of the intrinsic pathway of cell death; hence this pathway is also referred to as the mitochondrial pathway of apoptosis. Etoposide has been extensively studied and was used in the present study as a positive control for the mitochondrial pathway of apoptosis (Custodio *et al.* 2002). Initially we examined the effect of the oxysterols on mitochondrial membrane potential using JC-1 dye. Depolarisation of the inner mitochondrial membrane is thought to be an early key event in cells undergoing apoptosis via the mitochondrial pathway (Bernardi *et al.* 1999). Certain oxysterols have previously been shown to induce apoptosis associated with loss of mitochondrial membrane potential (Miguet *et al.* 2001; Miguet-Alfonsi *et al.* 2002; Monier *et al.* 2003; Seye *et al.* 2004). After 2 h treatment with 7β-OH and etoposide there was a decrease in potential across the mitochondrial membrane to 58 and 71 % respectively. Initiation of the intrinsic pathway is thought to occur as a result of disturbance of the mitochondria due to certain factors, such as loss of mitochondrial membrane potential or the presence of an oxidative stress (Ashe & Berry, 2003). Previous work from our laboratory illustrated that β-epoxide-induced apoptosis did not involve the generation of an oxidative stress (Ryan *et al.* 2004*b*). In the present study we found that, in cells treated with β-epoxide, there was no change in the mitochondrial membrane potential. These results indicate that certain characteristic features of the earlier stages of the mitochondrial pathway are not present in β-epoxide-treated cells.

Table 1. Percentage viable U937 cells following incubation with 30 μM-7β-hydroxycholesterol (7β-OH), 30 μM-β-epoxide or 10 μM-etoposide, over 24 h, in the presence or absence of 5 μM-bongkrekic acid (BA), 1 μM-cyclosporin A (CsA), 15 μM-carboxyatractyloside (CATR) or 5 mM-adenosine 5′-diphosphate (three independent experiments)

(Mean values with their standard errors)

	BA				CsA				CATR				ADP			
	Control		Treated		Control		Treated		Control		Treated		Control		Treated	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	98·8	0·2	98·8	0·4	99·2	0·2	99·2	0·3	98·7	0·6	98·8	0·2	98·5	0·6	98·7	0·3
7β-OH	67·7	5·7	90·3	1·8	72·1	4·6	93·2	1·0	68·5	2·2	68·3*	2·7	67·5	3·2	90·1	0·6
β-Epoxide	55·7	3·8	57·7*	4·9	66·3	3·2	61·2*	2·7	64·7	2·0	58·2*	1·4	65·1	2·1	67·3*	1·2
Etoposide	35·3	1·2	53·1*	1·7	39·8	4·5	81·3*	2·0	39·3	6·3	37·7*	3·2	39·3	6·3	60·3*	5·3

*Mean value was significantly different from control ($P<0·05$) (ANOVA followed by Dunnett's test).

Table 2. Percentage apoptotic nuclei as assessed by Hoechst staining in U937 cells exposed to 30 μM-7β-hydroxycholesterol (7β-OH), 30 μM-β-epoxide or 10 μM-etoposide, over 24 h, in the presence or absence of 5 μM-bongkrekic acid (BA), 1 μM-cyclosporin A (CsA), 15 μM-carboxyatractyloside (CATR) or 5 mM-adenosine 5′-diphosphate (three independent experiments)

(Mean values with their standard errors)

	BA				CsA				CATR				ADP			
	Control		Treated		Control		Treated		Control		Treated		Control		Treated	
	Mean	SE	Mean	SE‘	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	1·3	0·2	1·8	0·6	2·1	0·3	1·8	0·4	2·2	0·1	2·9	0·8	2·2	0·1	3·0	0·6
7β-OH	25·5	4·3	7·4	0·7	27·3	1·8	9·6	1·4	27·7	1·0	23·1*	1·7	28·1	0·7	7·9	2·7
β-Epoxide	28·3	1·4	29·8*	2·6	28·7	1·8	26·7*	1·2	23·1	2·6	21·3*	1·8	23·3	2·6	22·2*	2·3
Etoposide	54·7	0·9	35·1*	1·0	58·1	3·5	55·1*	4·5	58·7	3·3	53·34*	2·4	63·8	1·8	58·1*	1·2

*Mean value was significantly different from control ($P<0·05$) (ANOVA followed by Dunnett's test).

To further probe the importance of the mitochondria in 7β-OH and β-epoxide-induced apoptosis we examined the role of the MPTP, also known as the mitochondrial megachannel, in oxysterol-induced apoptosis. The MPTP is a non-specific pore that opens in the inner mitochondrial membrane under conditions such as oxidative stress or loss in mitochondrial membrane potential (Green & Kroemer, 1998). We investigated the effect of a range of inhibitors of the MPTP; BA, a potent inhibitor of the MPTP and also known to prevent the breakdown of the inner mitochondrial transmembrane potential; CsA, which acts as an inhibitor of MPTP opening by preventing cyclophilin D binding to the adenine nucleotide translocase, ADP, an inhibitor of the opening of the MPTP and of cytochrome *c* release from the mitochondria (Crompton, 1999; Halestrap *et al.* 2002); CATR, a selective inhibitor of the cytosolic side-specific ADP–ATP carrier. CsA is a known potent inhibitor of MPTP (Halestrap *et al.* 2004; Piret *et al.* 2004; Hans *et al.* 2005); however, the complex of CsA with cytosolic cyclophilin A may also have an effect on the Ca-activated protein phosphatase, calcineurin. BA, CsA and ADP protected against 7β-OH-induced apoptosis, illustrating the importance of the MPTP in 7β-OH-induced cell death. The inability of CATR to protect U937 cells against 7β-OH-induced apoptosis suggests that the cytosolic ADP–ATP carrier does not play a critical role in 7β-OH-induced cell death. The present results indicate that β-epoxide-induced cell death is not dependent on the opening of the MPTP as none of the inhibitors protected against the increase in apoptotic nuclei in cells treated with β-epoxide.

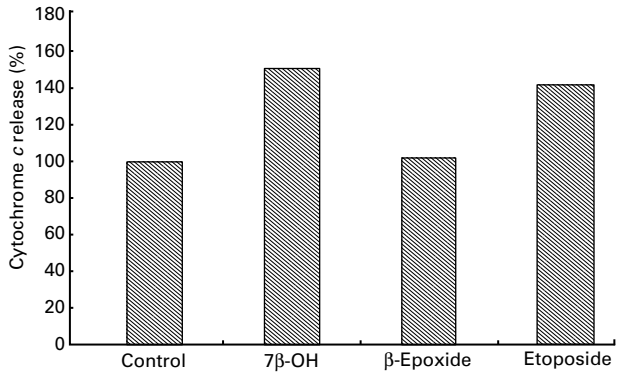

Fig. 3. The percentage increase in cytochrome *c* release, as assessed by ELISA, relative to control (100 %), in U937 cells following a 16 h incubation with 30 μM-7β-hydroxycholesterol (7β-OH), 30 μM-cholesterol-5β,6β-epoxide (β-epoxide) or 10 μM-etoposide. Data are means of two independent experiments, with standard deviations represented by vertical bars

Perturbation of the mitochondria may lead to the release of various pro-apoptotic molecules. A number of studies have shown that 7-ketocholesterol- and 7β-OH-induced apoptosis is associated with the release of cytochrome *c* from the mitochondria, where it is normally localised, into the cytosol where it may initiate the intrinsic pathway (Miguet *et al.* 2001; Biasi *et al.* 2004; Seye *et al.* 2004). Mitochondrial inhibitors BA and CsA have been used routinely to examine their ability to block MPTP (Halestrap *et al.* 2004; Piret *et al.* 2004; Hans *et al.* 2005). It is our assumption that because these reagents prevent MPTP opening they would also prevent release of cytochrome *c*. Previous work from our laboratory found that an inhibitor of cytochrome *c* release protected against 7β-OH-induced apoptosis but not β-epoxide-induced cell death (Ryan *et al.* 2004*b*). We examined the mitochondrial release of cytochrome *c* into the cytosol via ELISA following 9, 12 and 16 h treatment with the oxysterols. In U937 cells treated with 7β-OH there was an increase in release of cytochrome *c* into the cytosol, after 16 h, to approximately 155 % of the control. While the increase in cytochrome *c* release in the present study was lower than that recently observed, our data cannot be compared quantitatively with that of Berthier *et al.* (2004) and Vindis *et al.* (2005), as their experimental conditions differed significantly from ours. There was no increase of cytochrome *c* in the cytosol of cells treated with β-epoxide, confirming that β-epoxide-induced apoptosis is independent of cytochrome *c* release.

In order to examine the possible involvement of the extrinsic pathway in β-epoxide-induced cell death we measured the activity of caspase-8 after 6 and 9 h treatment with the oxysterols. Caspase-8 is an initiator of the extrinsic pathway of apoptosis and may either directly activate downstream effectors, such as caspase-3, or cleave the BH3-only protein Bid, which is then translocated to the mitochondria to activate the intrinsic mitochondrial pathway. Etoposide has previously been shown to increase both caspase-8 and caspase-9 activity in U937 cells (Martinsson *et al.* 2001), activating both death receptor-mediated and mitochondria-mediated cell death pathways. In the present study there was a 3·5-fold increase in caspase-8 activity following treatment with etoposide for 9 h. In cells treated with β-epoxide for 9 h, the slight (1·5-fold) increase in caspase-8

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activity was not statistically significant ($P<0{\cdot}05$) compared with control cells.

The present study confirms that 7β-OH-induced apoptosis occurs via the mitochondrial pathway. In contrast, β-epoxide-induced apoptosis, in U937 cells, proceeds independent of changes in mitochondrial membrane potential, opening of the MPTP and release of cytochrome *c*. The slight increase in caspase-8 activity, in cells treated with β-epoxide, is not sufficient to implicate caspase-8 as the sole initiator of β-epoxide-induced apoptosis. Preliminary data from our laboratory, with Ca channel blockers verapamil and nifedipine, have indicated that Ca may have a role in oxysterol-induced apoptosis. Based on the present results it would not be possible to state the exact mechanism through which β-epoxide exerts its effects and further work must be carried out in this area. However, our findings have eliminated certain potential effectors typically activated during the mitochondrial pathway of apoptosis.

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