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Modulation of cholestan-3β,5α,6β-triol toxicity by butylated hydroxytoluene, α-tocopherol and β-carotene in newborn rat kidney cells *in vitro*

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Cholesterol oxidation products (COP) have been reported to influence vital cellular processes such as cell growth, cell proliferation, membrane function and *de novo* sterol biosynthesis. The objectives of the present study were: (1) to develop an *in vitro* model using newborn rat kidney (NRK) cells to investigate the actions of COP; (2) to investigate the effect of COP on cell viability, endogenous antioxidant enzymes activities, i.e. superoxide dismutase (*EC* 1.15.1.1; SOD) and catalase (*EC* 1.11.1.6; CAT), and the extent of lipid peroxidation in this model; (3) to determine whether the addition of 100–1000 nM-α-tocopherol, β-carotene or butylated hydroxytoluene (BHT) could protect against COP-induced cytotoxicity. NRK cells were cultured in the presence of various concentrations (5–50 μM) of cholesterol or cholestan-3β,5α,6β-triol (cholestantriol) for a period of 24 h. Cholesterol over the range 5–50 μM did not induce cytotoxicity as indicated by the neutral-red-uptake assay or the lactate dehydrogenase (*EC* 1.1.1.27)-release assay. However, cell viability was compromised by the addition of > 10 μM-cholestantriol ($P < 0.05$). The addition of β-carotene (100–1000 nM) did not increase cell viability significantly in cholestantriol-supplemented cells. However, the addition of α-tocopherol (1000 nM) and BHT (1000 nM) significantly increased percentage cell viability above that of the cholestantriol-supplemented cells but not back to control levels. SOD and CAT activities in NRK cells significantly decreased ($P < 0.05$) following incubation with cholestantriol. The addition of > 750 nM-α-tocopherol, β-carotene or BHT returned SOD and CAT activities to that of the control. Lipid peroxidation was significantly induced ($P < 0.05$) in the presence of cholestantriol. Supplementation of the cells with α-tocopherol (250, 500 or 1000 nM) or BHT (750 or 1000 nM) resulted in a reduction in the extent of lipid peroxidation ($P < 0.05$). The addition of β-carotene over the concentration range of 250–1000 nM did not reduce lipid peroxidation significantly compared with cells exposed to cholestantriol alone. These findings suggest that addition of exogenous antioxidants may be beneficial in the prevention of COP-induced toxicity *in vitro*.

Cholesterol: Cholestan-3β,5α,6β-triol: Kidney: Antioxidants

Cholesterol (cholestan-5-en-3β-ol) is an unsaturated lipid which is ubiquitously found in mammalian tissues. The presence of unsaturated bonds in the cholesterol molecule readily allows it to undergo autoxidation by diverse oxygen species, yielding cholesterol oxidation products (COP). The non-enzymic oxidation of cholesterol can be instigated by ground-state dioxygen, or reactive oxygen species such as superoxide, H_2O_2 and hydroxyl radical (Smith, 1981; Gumulka *et al.* 1982; Kumar & Singhal, 1991). The free-radical-mediated reactions produce COP via two distinct processes either by the formation of sterol hydroperoxides in the β-ring or on the side chain, or by the dehydrogenation of the 3β-alcohol

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group (Smith, 1981; Schroepfer, 1982). The previously mentioned reactive oxygen species can stimulate lipid peroxidation resulting in damage to cellular components. However, antioxidants delay significantly or prevent formation of reactive oxygen species. Antioxidants include the naturally-occurring compounds α-tocopherol and β-carotene, and the synthetic compound butylated hydroxytoluene (BHT). α-Tocopherol is a major chain-breaking antioxidant (Riemersma *et al.* 1990) and an effective scavenger of lipid radicals (McCay, 1985). β-Carotene functions as an antioxidant by trapping the chain-propagating peroxy radicals (Burton & Ingold, 1984). BHT, a monohydric phenolic antioxidant, is particularly effective in suppressing oxidation of animal fats (Shahidi & Wanasundara, 1992). In addition, endogenous antioxidant enzymes are present in cells. These enzymes include superoxide dismutase (*EC* 1.15.1.1; SOD) and catalase (*EC* 1.11.1.6; CAT) which catalyse the decomposition of superoxide radicals and H_2O_2 respectively.

COP have been reported as having many biological activities including cytotoxicity (Smith, 1981; Parish *et al.* 1986; Peng *et al.* 1991, 1992). However, cholesterol has been reported to show no toxic effects *in vitro* or *in vivo* (Peng *et al.* 1979). At cellular level, COP are reported to influence such vital functions as cell growth and proliferation (Smith & Johnson, 1989). Guardiola *et al.* (1996) have attributed the ability of COP to alter cell function to two main factors: first, their ability to inhibit 3-hydroxy-3-methylglutaryl-CoA reductase (*EC* 1.1.1.34) activity, resulting in a reduction in endogenous cholesterol synthesis; and second, the capability of COP to replace cholesterol molecules in membranes, thereby perturbating membrane properties. *In vivo*, COP toxicities are associated with carcinogenicity, mutagenicity, atherogenicity and other physiological changes including organ atrophy (Smith & Johnson, 1989; Kumar & Singhal, 1991; Peng *et al.* 1992). However, there is increasing evidence that certain antioxidants reduce the level of COP *in vivo* (Carew *et al.* 1987; Bjorkhem *et al.* 1991; Hodis *et al.* 1992) and consequently are potentially anti-atherogenic. These findings suggest that an increased antioxidant consumption may be beneficial in the prevention of COP-induced toxicities *in vivo*.

COP may be present in various foodstuffs of animal origin as only these contain appreciable amounts of cholesterol (Homberg & Bielefeld, 1985; Pie *et al.* 1991). COP are found at low levels, of the order of mg/kg to μg/kg, in cholesterol-rich processed foods (Park & Addis, 1987; Sarantinos *et al.* 1993). COP are also found at similar levels in freshly-drawn blood (Smith & Johnson, 1989), therefore the ingestion of dietary COP, even at low levels, may pose an avoidable toxic burden to human health. The objectives of the present study were, in the first instance, to develop an *in vitro* cell model system for the investigation of COP; second, to establish the effect of COP on endogenous antioxidant enzyme activities; and finally to evaluate whether the addition of exogenous antioxidants (natural or synthetic) would protect against COP-induced toxicities, including the modulation of endogenous antioxidant enzyme activities.

MATERIALS AND METHODS

Materials

Tissue culture materials, including Dulbecco's Modified Eagle's Medium (with 25 mM-HEPES, 4·5 g glucose/l and without sodium pyruvate), gentamycin (10 mg/ml), newborn calf serum, penicillin and streptomycin (500 IU/ml and 500 mg/ml respectively) and trypsin (*EC* 3.4.21.4; 2·5 g/l), were purchased from Gibco BRL, Life Technologies (Paisley, Strathclyde). DL-α-Tocopherol, CAT (from bovine liver), cholesterol, cholestan-

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3β,5α,6β-triol(cholestantriol), cytochrome C (type III, from horse heart), neutral red (practical grade), β-NADPH (type I), β-NADH (grade III, from yeast), pyruvic acid (type II), and SOD (from bovine liver) were all purchased from Sigma Chemical Co. (Poole, Dorset). $FeSO_4.7H_2O$, H_2O_2 of low mineral content, tetrahydrafuran (THF), thiobarbituric acid and TCA were purchased from BDH (Poole, Dorset). Bio-Rad dye reagent concentrate was purchased from Bio-Rad (Munich, Germany). Titanium oxysulfate was purchased from Riedel-de-Haen (Seelze, Germany). β-Carotene (99–100% pure) was obtained from F. Hoffmann-La Roche, Basel, Switzerland. All reagents were reagent grade, unless otherwise stated.

Newborn rat kidney (NRK) cells

NRK cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, Wilts.) at passage number 2. The cells were cultured in a humidified incubator (Forma Scientific, Marietta, OH, USA) in an atmosphere of CO_2–air (5 : 95, v/v; O_2 partial pressure of 150 torr) at 37° in Dulbecco's Modified Eagle's Medium supplemented with penicillin and streptomycin (15 ml/l), gentamycin (20 ml/l) and newborn calf serum (50 ml/l). When the cells reached 80–90% confluency, they were collected by trypsin treatment, followed by centrifugation (2000 ***g*** for 10 min) and resuspended in supplemented growth medium. Plastic petri dishes (100 mm diameter) were seeded with 2×10^6 cells in 9 ml culture medium per dish. After 3–4 d of culture, the culture medium was removed and replaced by the same volume of medium with or without cholesterol or cholestantriol in the presence or absence of the various antioxidants.

*Incubation with test compounds, neutral-red-uptake assay and lactate dehydrogenase (*EC *1.1.1.27; LDH)-release assay*

A stock solution of cholesterol or cholestantriol was prepared in THF and vortexed for 30 s. The final concentration of THF in the culture medium was < 5 ml/l. A control group containing similar amounts of THF was included in the experiments. This concentration of THF was found not to alter significantly the cell viability, endogenous antioxidant enzyme activities or the extent of lipid peroxidation (data not shown). β-Carotene was obtained from F. Hoffmann-La Roche in sealed ampoules under N_2 and stored at −70°. Before use, the purity of β-carotene was determined by HPLC analysis and was found to be > 99%. A stock solution of β-carotene was prepared in absolute alcohol in a light-proofed vessel and vortexed for 30 min. The concentration of the prepared solution was measured spectrophotometrically at 452 nm before further dilution (mmolar extinction coefficient = 140·8; De Ritter & Purcell, 1981). β-Carotene was then added at the desired concentrations to the culture medium. α-Tocopherol was dissolved in absolute alcohol and the absorbance of this solution was measured spectrophotometrically at 292 nm before further dilution (mmolar extinction coefficient 3·27; Bieri *et al.* 1979). BHT was dissolved in absolute alcohol to a concentration of 1 mM before further dilution. The final concentration of absolute alcohol in the culture medium was < 1%. A control group containing absolute alcohol was included and no differences were seen between the absolute alcohol control and a control without absolute alcohol. In all experiments, the incubation period with cholesterol or cholestantriol in the presence or absence of the various antioxidants was 24 h at 37°. Neutral-red-uptake assay was performed as an index of cell injury by the method of Hunter *et al.* (1987). Cytotoxicity, as determined by the neutral-red-uptake assay, was expressed as a percentage of cell viability relative to that of

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non-supplemented cells. LDH release was determined as an alternative index of cell viability by the method of Vassault (1983). LDH release was expressed as a percentage of the total LDH released from cells treated with Triton X-100 (100 ml/l).

Cell sonicates, superoxide dismutase and catalase assays

The cell sonicates were obtained for enzyme analysis by rapidly aspirating the incubation medium followed by the addition of 0·5 ml of the appropriate buffer at 0° to the cells. The cells were removed from the culture dishes by scraping and then placed on ice. The cells were disrupted by pulse sonication using a MSE Soniprep (Model 150; MSE Scientific, Manor Royal, Crawley, Sussex) at 13 amp/s. All sonications were performed for 20 s with cells at 4°. The cell sonicates were centrifuged for 10 min at 100000 ***g*** (Beckman XL-90 centrifuge, Ti90 rotor head; Beckman, Palo Alto, CA, USA) at 4°. CAT activity in the cell sonicates was determined on the same day as harvesting, using the method of Baudhuin *et al.* (1964). The sonicates for determination of SOD activity were stored at −80°. Total SOD activity in the NRK cells was determined by the method of McCord & Fridovich (1969). All enzyme activities were expressed as units of enzyme activity/mg protein. The enzyme assays are used routinely in our laboratory and typically have a CV of less than 5%. Details of the enzymic assays used have been described previously (Lawlor & O'Brien, 1994).

Thiobarbituric acid-reactive substances (TBARS) assay and protein assay

TBARS were measured as an index of lipid peroxidation by a modification of the method of Kornburst & Mavis (1980). Cells were cultured in petri dishes (100 mm) with the culture medium containing cholesterol or cholestantriol in the presence or absence of the various antioxidants for 24 h at 37°. Following the period of exposure and the removal of the culture media, the cells were scraped into a solution of KCl (11·5 g/l). The cells were then pulse-sonicated as outlined previously. Lipid peroxidation was induced in the samples by the addition of $FeSO_4$ (5 mM) and ascorbic acid (2 mM) in a 80 mM Tris maleate buffer (pH 7·4). A solution of thiobarbituric acid (3·75 g/l), TCA (150 g/l) and HCl (250 mM) was added to the samples followed by vortexing and boiling for a period of 15 min. Lipid peroxides react with this solution resulting in the production of a pink fluorochrome (malondialdehyde; MDA) which is measured spectrophotometrically. Following cooling and centrifugation, the supernatant fraction was removed and the absorbance of the sample determined at 535 nm. The MDA concentrations of the samples were calculated using an extinction coefficient of $1{\cdot}56 \times 10^5$/mol per cm (Beuge & Aust, 1978). TBARS are expressed as nmol MDA/mg protein. Total protein was determined in the various sonicates using the Bio-Rad Microassay (Bradford, 1976), using bovine serum albumin as a standard.

Statistical analysis

Results are presented as mean values with their standard errors of the means. Data were analysed by one-way ANOVA followed by least significant difference (LSD). The level of statistical significance was taken as $P < 0{\cdot}05$.

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RESULTS

Effect of cholestan-3β,5α,6β-triol on cell viability, endogenous antioxidant enzyme activities and lipid peroxidation

NRK cells were incubated with 5–50 μM-cholesterol or cholestantriol for 24 h to determine the effects on cell viability, antioxidant enzyme activities (SOD and CAT) and lipid peroxidation. Cholesterol over this concentration range was not cytotoxic to the cells (Fig. 1) as determined by the neutral-red-uptake assay. The addition of low concentrations of cholestantriol (< 10 μM), also, did not influence cell viability. Supplementation with concentrations of 15 μM-cholestantriol, or higher, significantly reduced cell viability relative to that of non-supplemented cells ($P < 0{\cdot}05$; Fig. 1). At 50 μM-cholestantriol, the percentage cell viability was as low as 4·6.

LDH release was measured as an alternative index of cytotoxicity. Supplementation of cells with cholesterol (5–50 μM) did not influence cell viability, as measured by this assay (results not shown). Three concentrations of cholestantriol, i.e. 5, 12 and 15 μM were tested (Fig. 2). These concentrations corresponded to approximately 100, 75 and 50% cell viability, as determined from the neutral-red-uptake assay (Fig. 1). The addition of 12 or 15 μM-cholestantriol induced a significant release of LDH (5·81 (SE 0·83) and 8·68 (SE 0·01) respectively; $P < 0{\cdot}05$; Fig. 2) relative to the control untreated cells. A concentration of 15 μM-cholestantriol which induced a significant level of cytotoxicity in this cell-line was selected for use in all subsequent experiments.

NRK cells were incubated in the presence of various concentrations (100–1000 nM) of α-tocopherol, β-carotene or BHT for a period of 24 h (Table 1). Following incubation, the effect of these compounds on percentage cell viability was assessed using the neutral-red-

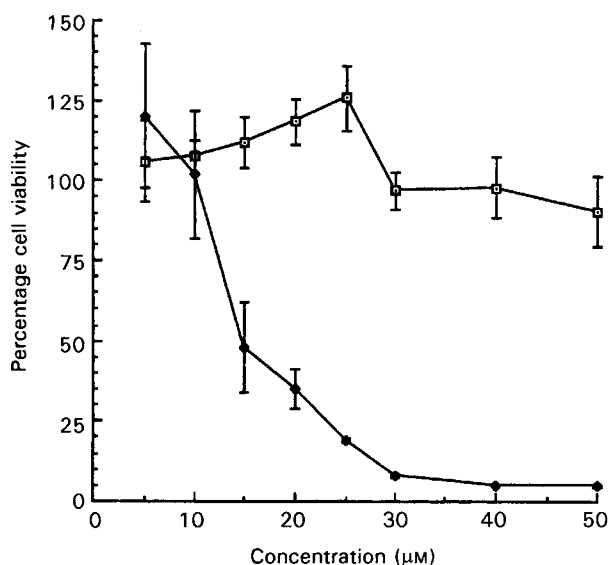

Fig. 1. Effect of cholesterol and cholestan-3β,5α,6β-triol on the percentage cell viability of newborn rat kidney (NRK) cells. NRK cells were incubated with cholesterol (□) or cholestan-3β,5α,6β-triol (◆) (5–50 μM). Percentage cell viability was determined using the neutral-red-uptake assay. Results are expressed as the percentage cell viability relative to that of cells incubated without cholesterol or cholestan-3β,5α,6β-triol. Values are means with their standard errors represented by vertical bars for six culture dishes incubated simultaneously.

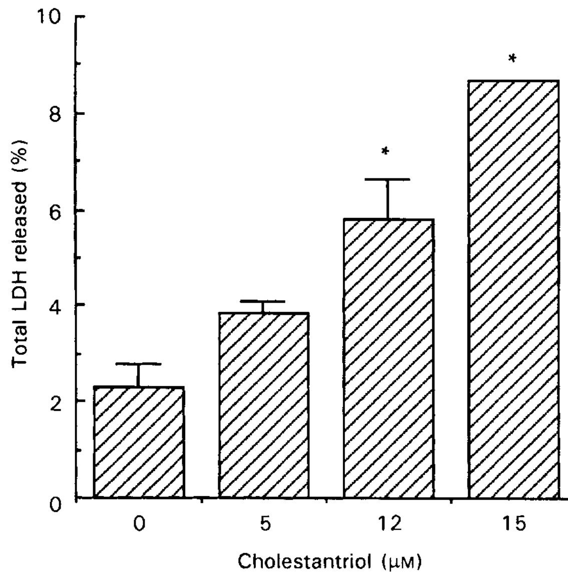

Fig. 2. Effect of cholestan-3β,5α,6β-triol on the lactate dehydrogenase (*EC* 1.1.1.27; LDH) release (total % release) in newborn rat kidney (NRK) cells. NRK cells were incubated with or without cholestan-3β,5α,6β-triol (5–15 μm) for 24 h. LDH in the medium was measured and expressed as a percentage of total LDH released from NRK treated with Triton X (100 ml/l). Values are means with their standard errors represented by vertical bars for six culture dishes incubated simultaneously. Mean values were significantly different from those for NRK cells with no cholestan-3β,5α,6β-triol (ANOVA, LSD): $*P < 0.05$.

Table 1. *The effect of α-tocopherol, β-carotene and butylated hydroxytoluene (BHT) on percentage cell viability* in newborn rat kidney cells*†

(Mean values with their standard errors for six culture dishes incubated simultaneously)

Concentration (nM)	α-Tocopherol (% cell viability)		β-Carotene (% cell viability)		BHT (% cell viability)	
	Mean	SE	Mean	SE	Mean	SE
0‡	100·0	3·2	100·0	3·2	100·0	3·2
100	96·1	2·7	96·2	2·0	100·8	6·2
250	110·2	4·4	101·7	2·1	116·9	4·2
500	104·2	4·8	106·8	1·6	103·6	5·0
750	96·8	7·5	99·1	6·4	94·3	6·3
1000	98·9	4·6	99·8	6·2	94·3	0·7
LSD ($P < 0·05$)§	NS		NS		NS	

LSD, least significant difference.

* Percentage cell viability was determined using the neutral-red-uptake assay. Results are expressed as the percentage cell viability relative to that of cells incubated without α-tocopherol, β-carotene or BHT.

† For details of procedures, see pp. 480–482.

‡ Control cells containing no α-tocopherol, β-carotene or BHT.

§ Statistical analysis of data was done by one-way ANOVA, followed by LSD.

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Table 2. *The effect of α-tocopherol, β-carotene and butylated hydroxytoluene (BHT) on percentage cell viability‡ in cholestan-3β,5α,6β-triol (cholestantriol)-supplemented newborn rat kidney cells§*

(Mean values with their standard errors for six culture dishes incubated simultaneously)

Cholestantriol (μM)	Concentration (nM)	α-Tocopherol (% cell viability)		β-Carotene (% cell viability)		BHT (% cell viability)	
		Mean	SE	Mean	SE	Mean	SE
0	0‖	100·0	3·2	100·0	3·2	100·0	3·2
15	0	43·6*	1·1	43·6*	1·1	43·6*	1·1
15	1000	47·2*†	0·7	43·1*	1·3	47·6*†	0·8
LSD ($P < 0·05$)¶		3·1		NS		3·2	

LSD, least significant difference.
* Mean values were significantly different from those for the control ($P < 0·05$).
† Mean values were significantly different from those for cholestantriol-supplemented cells ($P < 0·05$).
‡ Percentage cell viability was determined using the neutral-red-uptake assay. Results are expressed as the percentage cell viability relative to that of cells incubated without α-tocopherol, β-carotene or BHT.
§ For details of procedures, see pp. 480–482.
‖ Control cells containing no cholestantriol, α-tocopherol, β-carotene or BHT.
¶ Statistical analysis of data was done by one-way ANOVA, followed by LSD.

uptake assay. Over these concentration ranges, none of the antioxidants were found to be cytotoxic (Table 1).

The potential protective effect of α-tocopherol, β-carotene or BHT against cholestantriol (15 μM)-induced cytotoxicity was also investigated (Table 2). β-Carotene (1000 nM) failed to have any effect on the viability (as measured by the neutral-red-uptake assay) of the cholestantriol-supplemented cells. However, 1000 nM-α-tocopherol or -BHT significantly increased cell viability above that of the cholestantriol-supplemented cells but not back to control levels (Table 2).

NRK cells were incubated with 15 μM-cholesterol or cholestantriol for 24 h to determine effects on antioxidant enzymes SOD and CAT and lipid peroxidation. The results indicated that following exposure to cholesterol, SOD and CAT activities were not affected (results not shown). However, cholestantriol significantly decreased SOD activity ($P < 0·05$) relative to that of the control (Tables 3, 4 and 5). Similarly, CAT activity in cells exposed to cholestantriol was significantly ($P < 0·05$) lower than that of cells cultured in the absence of cholestantriol (Tables 3, 4 and 5). Supplementation with cholesterol (5–50 μM) did not result in any significant changes in the extent of lipid peroxidation compared with that of non-supplemented cells (results not shown). However, the presence of 15 μM-cholestantriol in the incubation media significantly ($P < 0·05$) increased lipid peroxidation relative to that of the control, as measured by the TBARS assay (Tables 3, 4 and 5).

Effect of BHT, α-tocopherol and β-carotene on cholestan-3β,5α,6β-triol-induced modulation of antioxidant enzymes and lipid peroxidation

The potential of various antioxidants to protect against cholestantriol-induced modulation of antioxidant enzymes was also investigated. NRK cells were incubated with 15 μM-cholestantriol in the presence or absence of increasing concentrations of BHT, α-tocopherol or β-carotene (250–1000 nM) for 24 h. The cholestantriol-induced reduction in SOD

Table 3. *The effect of butylated hydroxytoluene (BHT) on cholestan-3β,5α,6β-triol (cholestantriol)-induced modulation of the antioxidant enzymes superoxide dismutase* (EC *1.15.1.1; SOD) and catalase* (EC *1.11.1.6; CAT) (units† /mg protein) and lipid peroxidation in newborn rat kidney cells‡*

(Mean values with their standard errors for six culture dishes incubated simultaneously)

Cholestantriol (μM)	BHT (nM)	SOD (Units/mg protein)		CAT (Units/mg protein)		TBARS (nmol MDA/mg protein)	
		Mean	SE	Mean	SE	Mean	SE
0	0§	5·69	0·54	11·23	0·36	1·24	0·13
15	0	1·80*	0·03	3·80*	1·44	3·75*	0·39
15	250	3·10*	0·19	6·39*	1·68	4·40*	0·20
15	500	5·34	0·69	7·03*	0·74	3·38*	1·07
15	750	5·25	0·68	8·36	0·01	1·41	0·21
15	1000	4·94	0·52	6·68	0·11	2·19	0·22
LSD ‖		2·47		3·55		1·66	

TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; LSD, least significant difference.
* Mean values were significantly different from those for the control ($P < 0{\cdot}05$).
† For SOD activity, one unit is defined as the amount of SOD required to inhibit the maximum rate of cytochrome C reduction by 50%. For CAT activity, one unit is defined as 1 μmol H_2O_2 removed per min.
‡ For details of procedures, see pp. 480–482.
§ Control cells containing no cholestantriol or BHT.
‖ Statistical analysis of difference from control values was done by one-way ANOVA, followed by LSD.

Table 4. *The effect of α-tocopherol on cholestan-3β,5α,6β-triol (cholestantriol)-induced modulation of the antioxidant enzymes superoxide dismutase* (EC *1.15.1.1; SOD) and catalase* (EC *1.11.1.6; CAT) (units† /mg protein) and lipid peroxidation§ in newborn rat kidney cells‡*

(Mean values with their standard errors for six culture dishes incubated simultaneously)

Cholestantriol (μM)	α-Tocopherol (nM)	SOD (Units/mg protein)		CAT (Units/mg protein)		TBARS (nmol MDA/mg protein)	
		Mean	SE	Mean	SE	Mean	SE
0	0§	5·69	0·54	11·23	0·36	1·24	0·13
15	0	1·80*	0·03	3·80*	1·44	3·75*	0·39
15	250	3·55*	0·69	6·12*	1·07	2·30	0·71
15	500	3·63*	0·31	9·07	1·23	2·04	0·20
15	750	4·80	0·82	8·47	0·41	2·62*	0·06
15	1000	2·99	0·52	8·21	0·32	2·10	0·22
LSD ‖		1·72		3·78		1·12	

TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; LSD, least significant difference.
* Mean values were significantly different from those for the control ($P < 0{\cdot}05$).
† For SOD activity, one unit is defined as the amount of SOD required to inhibit the maximum rate of cytochrome C reduction by 50%. For CAT activity, one unit is defined as 1 μmol H_2O_2 removed per min.
‡ For details of procedures, see pp. 480–482.
§ Control cells containing no cholestantriol or α-tocopherol.
‖ Statistical analysis of data was done by one-way ANOVA, followed by LSD.

activity was partially restored by the addition of 250 nM-BHT (Table 3). At levels of 500, 750 and 1000 nM-BHT, SOD activity was not significantly different ($P < 0{\cdot}05$) from the control value. BHT (250–1000 nM) also partially restored the COP-induced reduction in CAT activity. However, only at 750 nM-BHT, was CAT activity not significantly different from that of the control. Addition of cholestantriol to the cells induced an increase in

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Table 5. *The effect of β-carotene on cholestan-3β,5α,6β-triol (cholestantriol)-induced modulation of the antioxidant enzyme superoxide dismutase* (EC *1.15.1.1; SOD) and catalase* (EC *1.11.1.6; CAT) (units † /mg protein) and lipid peroxidation in newborn rat kidney cells‡*

(Mean values with their standard errors for six culture dishes incubated simultaneously)

Cholestantriol (μM)	β-Carotene (nM)	SOD (Units/mg protein)		CAT (Units/mg protein)		TBARS (nmol MDA/mg protein)	
		Mean	SE	Mean	SE	Mean	SE
0	0§	5·69	0·54	11·23	0·36	1·24	0·13
15	0	1·80*	0·03	3·80*	1·44	3·75*	0·39
15	250	3·09*	0·19	5·17*	0·79	4·96*	1·12
15	500	5·34	0·69	5·67*	0·59	4·23*	0·26
15	750	5·25	0·68	7·46*	0·21	4·57*	0·47
15	1000	4·94	0·52	10·35	1·17	3·25*	0·16
LSD ‖		2·19		2·58		1·27	

TBARS, thiobarbituric acid-reactive substances; MDA, malonyldialdehyde; LSD, least significant difference.

* Mean values were significantly different from those for the control.

† For SOD activity, one unit is defined as the amount of SOD required to inhibit the maximum rate of cytochrome C reduction by 50%. For CAT activity, one unit is defined as 1 μmol H_2O_2 removed per min.

‡ For details of procedures, see pp. 480–482.

§ Control cells containing no cholestantriol or β-carotene.

‖ Statistical analysis of data was done by one-way ANOVA, followed by LSD.

TBARS ($P < 0{\cdot}05$). The inclusion of 750 and 1000 nM-BHT resulted in a significant ($P < 0{\cdot}05$) reduction in the extent of lipid peroxidation, returning TBARS levels to those of control cells.

Addition of 250–1000 nM-α-tocopherol restored the cholestantriol-induced reduction in SOD activity (Table 4). However, only the inclusion of 750 nM-α-tocopherol resulted in a level of SOD activity which was not significantly different ($P < 0{\cdot}05$) from that of the control cells. Addition of the higher levels of α-tocopherol tested (500, 750 and 1000 nM) prevented the significant ($P < 0{\cdot}05$) cholestantriol-induced reduction in CAT activity. The inclusion of α-tocopherol (250, 500 and 1000 nM) also led to a significant reduction ($P < 0{\cdot}05$) in the extent of lipid peroxidation.

β-Carotene, at levels of 500, 750 or 1000 nM, prevented the cholestantriol-induced reduction in SOD activity. The enzyme activity at these concentrations of β-carotene was not significantly different from that of the control cells (Table 5). β-Carotene supplementation also restored the cholestantriol-induced reduction in CAT activity of the cells, but this was only significant ($P < 0{\cdot}05$) at 1000 nM-β-carotene. The addition of β-carotene over the concentration range 250–1000 nM did not influence significantly the extent of lipid peroxidation compared with cells exposed to cholestantriol alone.

DISCUSSION

The presence and accumulation of COP in mammalian tissues has been well documented (Smith & Johnson, 1989; Kucuk *et al.* 1992). COP toxicities have been implicated in the aetiologies of certain chronic human diseases, for example atherosclerosis and cancer (Cook & McDougall, 1967; Morin *et al.* 1992). COP have been reported as having a variety of biological activities, including altering cell growth, cell proliferation, the rate of DNA synthesis and cytotoxicity (Kandutsch & Chen, 1977; Smith & Johnson, 1989; Peng *et al.* 1992). The cytotoxic effects of COP *in vitro* appear to vary depending on the cell-line

under investigation, the composition of the incubation medium, the dose and solubility of the compound to be tested and the duration of exposure time (Peng *et al.* 1992). There are obvious difficulties in extrapolating results obtained *in vitro* to the situation *in vivo*, but the effects of COP have been demonstrated consistently in a wide variety of model systems. For example, cholestantriol has been shown to be cytotoxic in Chinese hamster ovary (CHO) cells, Syrian hamster embryo cells, porcine vascular smooth muscle cells and Chinese hamster V79 lung fibroblasts (Chan & Chan, 1980; Kelsey & Pienta, 1981; Baranowski *et al.* 1982; Sevanian & Peterson, 1986). In addition, cytotoxic and atherogenic effects of cholestantriol have been observed *in vivo* in squirrel monkeys (*Saimiri sciureus*), New Zealand White rabbits and Wistar rats (Taylor *et al.* 1983; Peng *et al.* 1985; Matthias *et al.* 1987). Such examples illustrate clearly the benefits of objective interpretation of model systems on specific issues in human health. Mortality induced by atherosclerosis has been reported to be higher among the Indian community in London compared with the rest of the population (Jacobson, 1987). A suggested risk factor in the Indian community was the consumption of clarified butter (ghee) which contains significant amounts of COP (Prasad & Subramanian, 1992). Hodis *et al.* (1992) demonstrated that the antioxidant probucol (4,4′-(isopropylenedithio)bis(2,6,-di,butyl-phenol)) was capable of reducing the concentration of COP in the plasma and aortic wall of cholesterol-fed rabbits with hypercholesterolaemia. Dietary antioxidants, therefore, may exert a modulatory role on COP-induced toxicity *in vivo*. In the present study, the NRK cell-line was used as an *in vitro* model to investigate COP-induced toxicity and its modulation by antioxidants.

The concentrations 5–50 μM-cholesterol and -cholestantriol were chosen as these ranges have been used previously in *in vitro* studies investigating the cytotoxicity of cholesterol and COP and their effect on cell membrane function (Sevanian & Peterson, 1984; Hennig & Boissonneault, 1987; Boissonneault *et al.* 1991). Cholestantriol was demonstrated, using the neutral-red-uptake assay, to be toxic to NRK cells over the concentration range 10–50 μM in a dose-dependent manner. LDH release was also used as a determinant of cell injury. A marked release of intracellular LDH into the culture media was observed after 24 h exposure to cholestantriol (>10 μM); this release is indicative of membrane damage. In addition, cholesterol over the same concentration range (5–50 μM) was not cytotoxic to this cell-line. This supports the findings of Peng *et al.* (1979) who reported that cholesterol appeared to show no toxic effects *in vitro*. Guardiola *et al.* (1996) reported that COP can be incorporated into membranes, substituting for cholesterol, and as a result can affect membrane stability. The mechanism of toxicity of cholestantriol, therefore, may be partly due to its direct incorporation into the cell membrane and the reduction in the cholesterol content.

Three antioxidants were selected, the naturally-occurring α-tocopherol and β-carotene and the synthetic antioxidant BHT. α-Tocopherol was chosen as it is the principal lipophilic antioxidant in cell membranes (Burton *et al.* 1983; Machlin & Bendich, 1987; Packer, 1991). This compound acts by suppressing free-radical-initiated oxidation and is an efficient quencher of singlet oxygen (Bradley & Min, 1992). β-Carotene was selected as it is the most abundant carotenoid in nature (Bonorden & Pariza, 1994). It acts as a multifunctional lipid-soluble antioxidant capable of physically quenching singlet oxygen and inhibiting free-radical chain reactions (Foote & Denny, 1968). BHT is a synthetic phenolic compound used by the food industry as an additive and in packaging materials and as such is found in human diets. This compound can act as either a chain-breaking antioxidant or as a quencher of singlet oxygen (Bonorden & Pariza, 1994). It was selected for the present study to compare

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the effectiveness of naturally-occurring and synthetic antioxidants. Concentrations of 1000 nM-α-tocopherol and -β-carotene were chosen as these had previously been demonstrated to protect endogenous antioxidant enzymes against oxidative stress *in vitro* (Lawlor & O'Brien, 1994, 1995). A similar concentration (1000 nM) of BHT was used to standardize the level of antioxidant under investigation. The addition of β-carotene at 1000 nM failed to significantly increase the viability of the NRK cells cultured in the presence of cholestantriol-supplemented media. Supplementation of the media with 1000 nM-α-tocopherol or BHT increased the percentage cell viability of cholestantriol-exposed cells by 3·6 and 4% respectively. Although this increase was significant, it did not return percentage cell viability to the level of the control. Zhou *et al.* (1995) also demonstrated that α-tocopherol could not maintain cell viability at control levels following supplementation with cholestantriol. In addition, Zhou *et al.* (1995) observed that BHT did not reduce cell death induced by 26-hydroxycholesterol.

Exposure of NRK cells to cholestantriol (15 μM) resulted in an impairment of the enzymic antioxidant systems (SOD and CAT). Mantha *et al.* (1993) demonstrated a reduction in the SOD activities *in vivo* following administration of a hypercholesterolaemic diet to rabbits. A reduction in SOD activity may result in a flux of superoxide anion, which has the potential to inhibit the actions of catalase (Kono & Fridovich, 1982). Cholestantriol supplementation also leads to an increase in lipid peroxidation in the NRK cells. This increase may in part be due to the reduction in SOD and CAT activities which may lead to a potentiation of the lipid peroxidation reactions (Punnonen *et al.* 1991). Decreases in endogenous antioxidant systems and a concomitant increase in lipid peroxidation have also been reported following exposure to u.v. irradiation (Maeda *et al.* 1991; Morliere *et al.* 1991; Punnonen *et al.* 1991).

Addition of higher levels (> 750 nM) of α-tocopherol, β-carotene or BHT to the cholestantriol-supplemented cells prevented a decrease in SOD and CAT activities. All three compounds behaved similarly in this regard (Tables 3, 4 and 5), although their mechanism of protection may be different. Further studies are required to investigate whether the antioxidants have different mechanisms of action. The presence of α-tocopherol and BHT also resulted in a reduction in the extent of lipid peroxidation (Tables 3 and 4). However, β-carotene did not significantly protect against the triol-induced increase in lipid peroxidation (Table 5). Inhibitory effects of α-tocopherol and BHT on lipid peroxidation have been well documented in the literature (Pelle *et al.* 1989; Shahidi & Wanasundara, 1992; Princen *et al.* 1995; Viana *et al.* 1996). Our results suggest that β-carotene is not as effective as α-tocopherol or BHT in protecting cells against COP-induced increases in lipid peroxidation. These results support the findings of Princen *et al.* (1992), Reaven *et al.* (1994) and Shaish *et al.* (1995), who found that β-carotene failed to protect against lipid peroxidation. However, at higher O_2 partial pressures such as that of atmospheric air, β-carotene has a depleted antioxidant capacity (Lawlor & O'Brien, 1997). Thus further work is required to determine whether β-carotene at the lower partial pressures experienced *in vivo* may be more effective in protecting the cells against these COP-induced increases in lipid peroxidation.

In conclusion, our findings indicate that the NRK cell-line is a useful model in which to study the modulatory effects of antioxidants on COP-induced toxicity *in vitro*.

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REFERENCES

Baranowski, A., Adams, C. W. M., Bayliss High, O. B. & Bowyer, D. B. (1982). Connective tissue responses to oxysterols. *Atherosclerosis* **35,** 1–10.

Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R., Jacques, P. & de Duve, C. (1964). Tissue fractionation studies. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, D-aminoacid oxidase and catalase in rat liver tissue. *Biochemical Journal* **92,** 179–184.

Beuge, J. A. & Aust, S. D. (1978). Microsomal lipid peroxidation. In *Methods in Enzymology*, vol. 52, pp. 302–310 [S. Fleischer and L. Packer, editors]. New York: Academic Press.

Bieri, J. G., Tolliver, T. J. & Catignani, G. L. (1979). Simultaneous determination of α-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *American Journal of Clinical Nutrition* **32,** 2143–2149.

Bjorkhem, I., Henriksson-Freyschuss, A., Breuer, O., Diczfalusy, U., Berglund, L. & Henriksson, P. (1991). The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arteriosclerosis and Thrombosis* **11,** 15–22.

Boissonneault, G. A., Hennig, B. & Ouyang, C. M. (1991). Oxysterols, cholesterol biosynthesis, vascular endothelial cell monolayer barrier function. *Proceedings of the Society for Experimental Biology and Medicine* **196,** 338–343.

Bonorden, W. R. & Pariza, M. W. (1994). Antioxidant nutrients and protection from free radicals. In *Nutritional Toxicology*, pp. 19–48 [F. N. Kotsonis, M. Mackey and J. Hjelle, editors]. New York: Raven Press Ltd.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248–254.

Bradley, D. G. & Min, D. B. (1992). Singlet oxygen oxidation in food. *Critical Reviews in Food Science and Nutrition* **31,** 211–236.

Burton, G. W. & Ingold, K. U. (1984). Beta-carotene: an unusual type of lipid antioxidant. *Science* **224,** 569–576.

Burton, G. W., Joyce, A. & Ingold, K. U. (1983). Is vitamin E the only lipid soluble chain breaking antioxidant in human blood plasma and erythrocyte membranes? *Archives of Biochemistry and Biophysics* **221,** 281–290.

Carew, T. E., Schwenke, D. C. & Steinberg, D. (1987). Antiatherogenic effect of probucol unrelated to its hypocholestemic effect: evidence that antioxidants *in vivo* can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proceedings of the National Academy of Sciences USA* **84,** 7725–7729.

Chan, J. T. & Chan, J. C. (1980). Toxic effects of cholesterol derived photoproducts on Chinese hamster embryo cells. *Photochemistry and Photobiophysics* **1,** 113–117.

Cook, R. P. & McDougall, J. D. B. (1967). Experimental atherosclerosis in rabbits after feeding cholestanetriol. *British Journal of Experimental Pathology* **49,** 265–271.

De Ritter, E. & Purcell, A. E. (1981). Carotenoid analytical methods. In *Carotenoids as Colorants and Vitamin A Precursors*, pp. 883–923 [J. C. Bauernfeind, editor]. London: Academic Press.

Foote, C. S. & Denny, R. W. (1968). Chemistry of singlet oxygen: VII. Quenching by β-carotene. *Journal of the American Chemical Society* **90,** 6233–6235.

Guardiola, F., Codony, R., Addis, P. B., Rafecas, M. & Boatella, J. (1996). Biological effects of oxysterols: current status. *Food Chemistry and Toxicology* **34,** 193–211.

Gumulka, J., Pyrek, J. S. & Smith, L. L. (1982). Interception of oxygen species in aqueous media by cholesterol: formation of cholesterol epoxides and secosterols. *Lipids* **17,** 197–203.

Hennig, B. & Boissonneault, G. A. (1987). Cholestan-3β,5α,6β-triol decreases barrier function of cultured endothelial cell monolayers. *Atherosclerosis* **68,** 255–261.

Hodis, H. N., Chauhan, A., Hashimoto, S., Crawford, D. W. & Sevanian, A. (1992). Probucol reduces plasma and aortic wall oxysterol levels in cholesterol fed rabbits independently of its plasma cholesterol lowering effect. *Atherosclerosis* **96,** 125–134.

Homberg, E. & Bielefeld, B. (1985). Freie und gebundene Sterine in rohen und raffinierten Palmoelen. III. Verhalten der sterinhaltigen Lipoproteine bei der Raffination. (Free and bound sterols in raw and refined palm oils. III. Behaviour of sterol-containing lipoproteins during refining.) *Fette Seifen Anstriche* **87,** 333–336.

Hunter, S. M., Chrzanowski, C., Barnett, C. R., Brand, H. N. & Sawell, J. K. (1987). A comparison of *in vitro* cytotoxicity assays and their application to water samples. *Alternative Laboratory Techniques to Animals* **15,** 20–29.

Jacobson, M. S. (1987). Cholesterol oxides in Indian ghee: possible cause of unexplained high risk of atherosclerosis in Indian immigrant populations. *Lancet* **ii,** 656–658.

Kandutsch, A. A. & Chen, H. W. (1977). Consequences of blocked sterol synthesis in cultured cells, DNA synthesis and membrane composition. *Journal of Biological Chemistry* **252,** 409–415.

Kelsey, M. I. & Pienta, R. J. (1981). Transformation of hamster embryo cells by neutral sterols and bile acids. *Toxicological Letters* **9,** 177–182.

Kono, Y. & Fridovich, I. (1982). Superoxide radical inhibits catalase. *Journal of Biological Chemistry* **257,** 5751–5754.

https://doi.org/10.1079/BJN19970165 Published online by Cambridge University Press

Kornburst, D. J. & Mavis, R. D. (1980). Relative susceptibility of microsomes from lung, heart, liver, kidney, brain and testes to lipid peroxidation: correlation with vitamin E content. *Lipids* **15,** 315–322.

Kucuk, O., Lis, L. J., Dey, T., Mata, R., Westerman, M. P., Yachnin, S., Szostek, R., Tracy, D., Kauffman, J. W., Gage, D. A. & Sweeley, C. C. (1992). The effects of cholesterol oxidation products in sickle and normal red blood cell membranes. *Biochimica et Biophysica Acta* **1103,** 296–302.

Kumar, N. & Singhal, O. P. (1991). Cholesterol oxides and atherosclerosis: a review. *Journal of the Science of Food and Agriculture* **55,** 497–510.

Lawlor, S. M. & O'Brien, N. M. (1994). Development of an *in vitro* cell culture model to investigate the induction and quantification of oxidative stress and its inhibition by α-tocopherol. *Toxicology In Vitro* **8,** 67–73.

Lawlor, S. M. & O'Brien, N. M. (1995). Astaxanthine: antioxidant effects in chicken embryo fibroblasts. *Nutrition Research* **15,** 1695–1704.

Lawlor, S. M. & O'Brien, N. M. (1997). Antioxidant activity of β-carotene at low oxygen partial pressure in chicken embryo fibroblasts. *British Journal of Nutrition* **77,** 133–140.

McCay, P. B. (1985). Vitamin E: interactions with free radicals and ascorbate. *Annual Reviews in Nutrition* **5,** 323–340.

McCord, J. M. & Fridovich, I. (1969). Superoxide dismutase. An enzyme function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry* **244,** 6049–6055.

Machlin, L. J. & Bendich, A. (1987). Free radical tissue damage: protective role of antioxidant nutrients. *FASEB Journal* **1,** 441–445.

Maeda, K., Naganuma, M. & Fukuda, M. (1991). Effects of chronic exposure to ultraviolet-A including 2% ultraviolet-B on free radical reduction systems in hairless mice. *Photochemistry and Photobiology* **54,** 737–740.

Mantha, S. V., Prasad, M., Kalra, J. & Prasad, K. (1993). Antioxidant enzymes in hypercholesterolemia and effects of Vitamin E in rabbits. *Atherosclerosis* **101,** 135–144.

Matthias, D., Becker, C. H., Godicke, W., Schmidt, R. & Ponsold, K. (1987). Action of cholestane-3β,5α,6β-triol on rats with particular reference to the aorta. *Atherosclerosis* **63,** 115–124.

Morin, R. J., Hu, B., Peng, S. K. & Sevanian, A. (1992). Cholesterol oxidation and cancer. In *Biological Effects of Cholesterol Oxides*, pp. 191–202 [S. K. Peng and R. J. Morin, editors]. London: CRC Press.

Morliere, P., Moysan, A., Santus, R., Huppe, G., Maziere, J. C. & Dubertret, L. (1991). UVA-induced lipid peroxidation in cultured human fibroblasts. *Biochimica et Biophysica Acta* **1084,** 261–268.

Packer, L. (1991). Protective role of vitamin E in biological systems. *American Journal of Clinical Nutrition* **53,** 1050S–1055S.

Parish, E. J., Nanduri, B. B., Kohl, H. H. & Taylor, F. R. (1986). Oxysterols: chemical synthesis, biosynthesis and biological activities. *Lipids* **21,** 27–30.

Park, S. W. & Addis, P. B. (1987). Cholesterol oxidation products in some muscle foods. *Journal of Food Science* **52,** 1500–1503.

Pelle, E., Maes, D., Padulo, A., Kim, E. K. & Smith, W. P. (1989). *In vitro* model to assess alpha-tocopherol efficacy. *Annals of New York Academy of Sciences* **570,** 491–494.

Peng, S. K., Hu, B. & Morin, R. J. (1991). Angiotoxicity and atherogenicity of cholesterol oxides. *Journal of Clinical Laboratory Analysis* **5,** 144–152.

Peng, S. K., Sevanian, A. & Morin, R. J. (1992). Cytotoxicity of cholesterol oxides. In *Biological Effects of Cholesterol Oxides*, pp. 147–166 [S. K. Peng and R. J. Morin, editors]. London: CRC Press.

Peng, S. K., Taylor, C. B., Hill, J. & Safarik, J. (1985). Cholesterol oxidation derivatives and arterial endothelial damage. *Atherosclerosis* **54,** 121–133.

Peng, S. K., Tham, P., Taylor, C. B. & Mikkelson, B. (1979). Cytotoxicity of oxidation derivatives of cholesterol on cultured aortic smooth muscle cells and their effects on cholesterol biosynthesis. *American Journal of Clinical Nutrition* **32,** 1033–1042.

Pie, J. E., Spahis, K. & Seillan, C. (1991). Cholesterol oxidation in meat products during cooking and frozen storage. *Journal of Agricultural and Food Chemistry* **39,** 250–254.

Prasad, C. R. & Subramanian, R. (1992). Qualitative and comparative studies of cholesterol oxides in commercial and home-made Indian ghees. *Food Chemistry* **45,** 71–73.

Princen, H. M. G., van Duyvenvoorde, W., Buytenhek, R., van der Laarse, A., Poppel, G., Leuven, J. A. G. & van Hinsbergh, V. W. M. (1995). Supplementation with low doses of Vitamin E protects LDL from lipid peroxidation in men and women. *Arteriosclerosis, Thrombosis and Vascular Biology* **15,** 325–333.

Princen, H. M. G., van Poppel, G., Vogelezang, C., Buytenhek, R. & Kok, F. J. (1992). Supplementation with vitamin E but not β-carotene *in vivo* protects low density lipoprotein from lipid peroxidation *in vitro*: effect of cigarette smoking. *Arteriosclerosis and Thrombosis* **12,** 554–562.

Punnonen, K., Puntala, A., Jansen, C. T. & Ahotupa, M. (1991). UVB irradiation induces lipid peroxidation and reduced antioxidant enzyme activities in human keratinocytes *in vitro*. *Acta Dermatovenerologica* **71,** 239–273.

https://doi.org/10.1079/BJN19970165 Published online by Cambridge University Press

Reaven, P. D., Ferguson, E., Navab, M. & Powell, F. L. (1994). Susceptibility of human LDL to oxidative modification: effects of variations in β-carotene concentration and oxygen tension. *Arteriosclerosis and Thrombosis* **14,** 1162–1169.

Riemersma, R. A., Oliver, M., Elton, R. A., Alfthan, G., Vartiainen, E., Salo, M., Rubba, P., Mancini, M., Georgi, H., Vuilleumier, J. P. & Gey, K. F. (1990). Plasma antioxidants and coronary heart disease: vitamins C and E, and selenium. *European Journal of Clinical Nutrition* **44,** 143–150.

Sarantinos, J., O'Dea, K. & Sinclair, A. J. (1993). Cholesterol oxides in Australian foods: identification and quantification. *Food Australia* **45,** 485–490.

Schroepfer, G. J. (1982). Sterol biosynthesis. *Annual Review of Biochemistry* **51,** 555–585.

Sevanian, A. & Peterson, A. R. (1984). Cholesterol epoxide is a direct-acting mutagen. *Proceedings of the National Academy of Sciences USA* **81,** 4198–4202.

Sevanian, A. & Peterson, A. R. (1986). The cytotoxic and mutagenic properties of cholesterol oxidation products. *Food Chemistry and Toxicology* **24,** 1103–1110.

Shahidi, F. & Wanasundara, P. K. J. P. D. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition* **32,** 67–103.

Shaish, A., Daugherty, A., O'Sullivan, F., Schonfeld, G. & Heinecke, J. W. (1995). Beta-carotene inhibits atherosclerosis in hypercholesterolemic rabbits. *Journal of Clinical Investigation* **96,** 2075–2082.

Smith, L. L. (1981). The autoxidation of cholesterol. In *Autoxidation in Food and Biological Systems*, pp. 119–131 [M. G. Simic and M. Karel, editors]. New York: Plenum Press.

Smith, L. L. & Johnson, B. H. (1989). Biological activities of oxysterols. *Free Radicals in Biology and Medicine* **7,** 285–332.

Taylor, C. B., Peng, S. K., Safarik, J., Kapuscinska, B., Bochenek, W. & Hill, J. (1983). Experimental atherosclerosis in squirrel monkeys by feeding with cholestane-3β,5α,6β-triol. *Federation Proceedings* **42,** 789 Abstr.

Vassault, A. (1983). Lactate dehydrogenase. In *Methods of Enzymatic Analysis*, vol. 3, pp. 118–126 [H. U. Bergmeyer, editor]. Weinheim, Germany: Verlag Chemie.

Viana, M., Barbas, C., Bonet, B., Bonet, M. V., Castro, M., Fraile, V. & Herrera, E. (1996). *In vitro* effects of a flavonoid-rich extract on LDL oxidation. *Atherosclerosis* **123,** 83–91.

Zhou, Q., Wasowicz, E. & Kummerow, F. A. (1995). Failure of vitamin E to protect cultured human arterial smooth muscle cells against oxysterol-induced cytotoxicity. *Journal of the American College of Nutrition* **14,** 169–175.

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