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Pal, Sebely; Thomson, Andrew M.; Bottema, Cynthia Denise Klemme; Roach, Paul D.
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Nutritional Journal, 2003; 2:3

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Monday, 28 October 2013

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Research

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α -Tocopherol modulates the low density lipoprotein receptor of human HepG2 cells

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Published: 12 May 2003

Received: 19 December 2002

Nutrition Journal 2003, 2:3

Accepted: 12 May 2003

This article is available from: <http://www.nutritionj.com/content/2/1/3>

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Abstract

The aim of this study was to determine the effects of vitamin E (α -tocopherol) on the low density lipoprotein (LDL) receptor, a cell surface protein which plays an important role in controlling blood cholesterol. Human HepG2 hepatoma cells were incubated for 24 hours with increasing amounts of α , δ , or γ -tocopherol. The LDL receptor binding activity, protein and mRNA, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase mRNA, cell cholesterol and cell lathosterol were measured. The effect of α -tocopherol was biphasic. Up to a concentration of 50 μ M, α -tocopherol progressively increased LDL receptor binding activity, protein and mRNA to maximum levels 2, 4 and 6-fold higher than control, respectively. The HMG-CoA reductase mRNA and the cell lathosterol concentration, indices of cholesterol synthesis, were also increased by 40% over control by treatment with 50 μ M α -tocopherol. The cell cholesterol concentration was decreased by 20% compared to control at 50 μ M α -tocopherol. However, at α -tocopherol concentrations higher than 50 μ M, the LDL receptor binding activity, protein and mRNA, the HMG-CoA reductase mRNA and the cell lathosterol and cholesterol concentrations all returned to control levels. The biphasic effect on the LDL receptor was specific for α -tocopherol in that δ and γ -tocopherol suppressed LDL receptor binding activity, protein and mRNA at all concentrations tested despite the cells incorporating similar amounts of the three homologues. In conclusion, α -tocopherol, exhibits a specific, concentration-dependent and biphasic "up then down" effect on the LDL receptor of HepG2 cells which appears to be at the level of gene transcription. Cholesterol synthesis appears to be similarly affected and the cell cholesterol concentration may mediate these effects.

Introduction

It has been known for over 60 years that the vitamin E (α -

tocopherol) status of rabbits can affect their plasma cholesterol concentration. In 1936, Morgulis and Spencer [1]

reported that the plasma cholesterol was twofold higher than normal in rabbits made deficient in vitamin E and that dietary replenishment of the vitamin normalised the cholesterol concentration. This effect was later confirmed by others in the rat [2–4] as well as in the rabbit [5–7]. In animal models of diet-induced hypercholesterolaemia, where the animals are not deficient in vitamin E, α -tocopherol supplementation also often decreases plasma cholesterol [8–12]. This is not always the case however; in some studies either no change [13–15] or even an increase [16] in plasma cholesterol was observed. In the rat however, a concomitant deficiency in selenium may be more relevant to increases in plasma cholesterol than the induced deficiency in vitamin E [17]

Changes in the plasma cholesterol concentration may result from effects the vitamin has on liver cholesterol metabolism. Hepatic cholesterol synthesis has been found to be increased in vitamin E-deficient rabbits [5] and the conversion of cholesterol into bile acids was observed to be decreased [5,6]. Such an increase in cholesterolgenesis and a decrease in cholesterol catabolism is consistent with the increase in liver cholesterol concentration found in the vitamin E-deficient rat [3,4].

There is however no data on the effects of α -tocopherol, the biologically active homologue of vitamin E, [18] on the hepatic low density lipoprotein (LDL) receptor which is well known to play a major role in the control of plasma cholesterol [19,20]. The importance of the LDL receptor is most clearly seen in the human genetic disorder called familial hypercholesterolaemia where a deficiency in the receptor causes high levels of plasma cholesterol which lead to the premature development of atherosclerosis [20]. The LDL receptor is also highly regulated in that various dietary and pharmaceutical agents can affect its expression [19,20]

The aim of the present study was therefore to determine whether vitamin E could regulate the LDL receptor. Cultured human HepG2 hepatoma cells, highly differentiated hepatocytes known to express lipoprotein receptors, [21–23] were grown in the absence of added vitamin E. Three naturally occurring vitamin E homologues, α , δ and γ -tocopherol [18] were tested for their effects on the HepG2 cell LDL receptor mRNA, protein and LDL-binding activity. The effect of α -tocopherol on the mRNA of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, and on the cellular concentration of lathosterol, an index of cholesterol synthesis, was also determined. The cell's cholesterol concentration was also measured.

Methods and materials

Cell culture

The HepG2 cells were grown under 5% CO₂ at 37°C in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 12 µg/ml penicillin, 16 µg/ml gentamicin, 20 mM HEPES buffer, 10 mM NaOH, 2 mM L-glutamine and 10% (v/v) fetal calf serum (FCS) (Commonwealth Serum Laboratories, Melbourne, Australia) as previously described [21–23]. For enrichment experiments, cells were grown to 80–90% confluency, and varying amounts of α , δ or γ -tocopherol (Purity 95%; Sigma-Aldrich, Castle Hill, Australia) in ethanol were added to supplemented DMEM and the cells were incubated in the media for 24 h. The cells were then extensively washed in phosphate buffered saline (PBS: 10 mM phosphate, 154 mM NaCl, pH 7) before being scraped from the flasks and resuspended in PBS. Cell viability was assessed using the trypan blue dye exclusion test. Cellular protein was determined using the method of Lowry et al [24].

Cellular Tocopherol Content

The tocopherol content of the cells was measured using the method of Yang and Lee [25]. Briefly, 1.0 ml of 1% ascorbic acid in 100% ethanol added to 1.0 ml of cell suspension (Alpha-tocopherol acetate was used as an internal standard) and heated at 70°C for 2 min; then 0.3 ml of saturated KOH was added and incubated for 30 min in a 70°C water bath. After cooling on ice, 1.0 ml distilled water and 4.0 ml hexane were added and shaken vigorously for 2 min; then the phases were separated by centrifugation at room temperature, 3000 × g for 10 min. An aliquot of hexane phase (3.0 ml) was pipetted and dried under a stream of N₂ [2] and redissolved in 0.2 ml methanol. The aliquots (20 ml each) were injected to high performance liquid chromatography (Waters, Milford, MA, USA) for analysis on a C 18 column (5 mm 3 4.6 mm 3 25 cm) with the mobile phase of methanol-water (95:5) and detected by a fluorometer set at excitation 205 and emission 340 nm. The coefficient of variation over two assessments was less than 5%.

LDL receptor binding assay

Human LDL, 1.025 > d > 1.050 g/ml, was isolated from 2–4 days-old blood (Red Cross, Adelaide, Australia) by sequential ultracentrifugation [26] and conjugated to colloidal gold (LDL-gold) as described. [27,28] Freshly collected and intact HepG2 cells (100 µg of protein) were incubated for 1 h at room temperature with LDL-gold (20 µg protein/ml) and buffer (60 mM Tris-HCL, pH 8.0, and 20 mg/ml BSA) in a total of 300 µl either in the presence of 2 mM Ca(NO₃)₂ to measure total binding or 20 mM EDTA to measure calcium-independent binding. Cells were then centrifuged at 400 × g for 10 min, resuspended and washed in 300 µl of 2 mM Ca(NO₃)₂ for total binding or 300 µl of 20 mM EDTA (pH 8.0) for nonspecific

binding. After centrifugation at $400 \times g$ for 10 min, the cells were resuspended in 120 μ l of 4% (w/v) gum arabic and the cell-bound LDL-gold was quantified using a silver enhancement solution (IntenSE BL kit, Amersham, Sydney, Australia) and a Cobas Bio autoanalyser (Roche Diagnostica, Nutley, NJ). The HepG2 cell LDL receptor binding activity was taken to be the total binding minus the calcium-independent binding and expressed as ng LDL protein bound per mg cell protein (ng LDL/mg cell). The binding of LDL-gold to the LDL receptor has been shown to be indistinguishable from the binding of native or 125 I-LDL and the method has been found to be more sensitive than the 125 I-LDL technique [27,28]. The coefficient of variation for measurement of LDL receptor binding activity is 10%.

LDL receptor protein mass assay

The HepG2 cells were solubilized by incubation for 12 h in a solution of 1.5% (w/v) Triton X-100 containing 50 mM Tris-maleate (pH 6), 2 mM CaCl_2 , 1 mM phenylmethylsulphonyl fluoride (PMSF) and 10 mM n-ethylmaleamide. Solubilized cell protein (100 μ g) and rainbow molecular weight-markers (Pharmacia LKB, Uppsala, Sweden) were separated by electrophoresis on 2–15% sodium dodecyl sulphate (SDS)-polyacrylamide gradient gels at 30 mA for 5 h. Separated proteins were electrotransferred at 45 V for 12 h onto 0.45 μ m nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and the membranes were blocked for one hour at room temperature in 10 mM Tris-HCL buffer, pH 7.4, containing 154 mM NaCl and 10% (w/v) skim milk powder.

After washing in 10 mM Tris-HCL buffer, pH 7.4, containing 154 mM NaCl and 1% (w/v) skim milk powder, the membranes were incubated with a polyclonal anti-LDL receptor antibody (3.7 μ g protein/ml in 10 mM Tris-HCL buffer, pH 7.4, containing 154 mM NaCl and 1% (w/v) skim milk powder). The antibody was raised in rabbits against the LDL receptor purified from bovine adrenal cortex and recognises the LDL receptor of other species [29,30]. The membranes were then incubated with anti-rabbit IgG linked to horseradish peroxidase (Amersham, North Ryde, Australia), diluted 1:5000 in 10 mM Tris-HCL buffer, pH 7.4 containing 154 mM NaCl and 1% (w/v) skim milk powder and subsequently washed twice with 10 mM Tris-HCL buffer, pH 7.4, containing 154 mM NaCl and 2 mM CaCl_2 . The membranes were then soaked in enhanced chemiluminescence substrate solution for horseradish peroxidase (ECL detection kit, Amersham, North Ryde, Australia) and exposed to hyper-film ECL (Amersham, North Ryde, Australia) for 1 to 5 min. The films were then scanned to determine the intensity of the LDL receptor protein bands using an LKB Ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnolo-

gy, North Ryde, Australia) and the measurements in arbitrary absorbance units were taken as the mass of LDL receptor protein in the HepG2 cells. The assay was optimised to give a linear response in the range of LDL receptor protein expressed by the HepG2 cells. The coefficient of variation for measurement of LDL receptor protein mass is 10%.

LDL receptor mRNA assay

Cellular RNA was isolated from the HepG2 cells using the procedure of Chomczynski and Sacchi [31] and the LDL receptor mRNA was measured using reverse transcription and the polymerase chain reaction (PCR) as modified from the method of Powell and Kroon [32].

The RNA was reversed transcribed into cDNA along with a synthetic piece of cRNA, AW109 (Perkin-Elmer Cetus Instruments, Norwalk, CT) which was used as an internal standard because it contains primer site sequences unique to the LDL receptor. The reaction mixture (11.94 μ l) contained 1 μ l cell total RNA (120 ng/ μ l), 1 μ l of AW109 cRNA (4×10^4 copies/ μ l), 1 μ l PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCL), 2 μ l of 25 mM MgCl_2 , 0.5 μ l of RNasin (20 U/ μ l, Perkin-Elmer Cetus Instruments, Norwalk, CT), 0.5 μ l of random hexanucleotide primers (50 μ M, Perkin-Elmer Cetus Instruments, Norwalk, CT), 1.5 μ l each of 10 mM dGTP, 10 mM dATP and 10 mM dCTP, 0.94 μ l of 10 mM dTTP (Perkin-Elmer Cetus Instruments, Norwalk, CT) and 0.5 μ l of Moloney Murine Leukemia Virus reverse transcriptase (C50 U/ μ l, Perkin-Elmer Cetus Instruments, Norwalk, CT). It was then heated to 23°C for 10 min, 45°C for 15 min, 95°C for 5 min in a thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT) and finally chilled on ice.

The LDL receptor cDNA was then amplified using the polymerase chain reaction (PCR) to incorporate in its primer-specific sequence a digoxigenin (DIG)-labelled dUT. The PCR mixture (20 μ l) contained 5 μ l of the reverse transcription reaction mixture, 0.5 μ l of 1 mM digoxigenin-11-dUTP, 2 μ l of PCR buffer (100 mM Tris HCl, pH 8.3, 500 mM KCL), 0.25 μ l of AmpliTaq DNA Polymerase (5 U/ μ l, Perkin-Elmer Cetus Instruments, Norwalk, CT), 0.60 μ l of the LDL receptor downstream primer AW125 (25 μ M, Perkin Elmer Cetus, Norwalk, CT), 0.60 μ l of the LDL receptor upstream primer AW126 (25 μ M, Perkin Elmer Cetus, Norwalk, CT) and 11.05 μ l deionised H_2O . The mixture was overlaid with mineral oil and the amplification was done with a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT) using the following conditions for 27 cycles: denaturation at 95°C for 1 min followed by primer annealing at 55°C for 1 min and then extension at 72°C for 1 min. At the end of the 27th cycle, a final extension period of 10 min at 72°C was done.

Each PCR reaction mixture (10 μ l) was size fractionated by electrophoresis for 90 min at 90 V in 3% (w/v) agarose gels with 0.8 mM Tris acetate, pH 8.5, and 0.04 mM EDTA as running buffer. The DNA was then transferred onto positively charged nylon membranes (Boehringer Mannheim, Rose Park, Australia) by blotting for 4 hours in 0.15 M Na₃Citrate, pH 7.6 and 1.5 M NaCl. The nylon membranes were then baked for 1 h at 100°C and rinsed in 30 mM Na₃Citrate, pH 7.6, and 0.3 M NaCl. The membranes were subsequently incubated in 0.1 mM Tris-HCL, pH 7.5, and 0.1 M NaCl for 5 min at room temperature and blocked for 30 min at room temperature in 0.1 mM Tris-HCL, pH 7.5, 0.1 M NaCl and 10% (w/v) skim milk powder. The membranes were then incubated for 30 min with an anti-digoxigenin-IgG antibody, conjugated to alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in 0.1 mM Tris-HCL, pH 7.5, 0.1 M NaCl and 1% (w/v) skim milk powder. The membranes were subsequently washed 3 times for 20 min in 0.1 mM Tris-HCL, pH 7.5, 0.1 M NaCl, incubated in 0.1 M Tris-HCL, pH 9.5, 0.1 M NaCl and 50 mM MgCl₂ for 5 min and then soaked for 5 min in ECL alkaline phosphatase substrate solution consisting of 100 μ g/ml CSPD (disodium 3-(4-methoxy-spiro{1,2-dioxetane-3,2-(5-chloro) tricyclo [3.3.1.1]decan}-4-y)phenyl phosphate) (Boehringer Mannheim, Rose Park, Australia) in 0.1 M Tris-HCL, pH 9.5, 0.1 M NaCl and 50 mM MgCl₂. They were then blotted dried, sealed in plastic, incubated at 37°C for 20 min and finally exposed to hyper-film ECL (Amersham, North Ryde, Australia) for 5 to 30 min. The films were scanned using the LKB Ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, North Ryde, Australia) to determine the intensity of the two bands corresponding to 1) cellular LDL receptor mRNA at 258 bp and 2) synthetic AW109 internal standard RNA at 301 bp. The amount of LDL receptor mRNA in the HepG2 cells was calculated relative to the intensity of the band for the known amount of AW109 RNA added as internal standard and was expressed per μ g of cellular total RNA. The assay was optimised to give a linear response in the range of LDL receptor mRNA expressed by the HepG2 cells. The coefficient of variation for measurement of LDL receptor mRNA is less than 7 %.

HMG-CoA reductase mRNA assay

The HMG-CoA reductase mRNA of HepG2 cells was measured using reverse transcription and the polymerase chain reaction, as described above for the LDL receptor mRNA [31,32]. The AW109 cRNA was also used as the internal standard in this assay because it contains coding sequences of the HMG-CoA reductase gene. The same PCR reaction mixture was used except that HMG-CoA reductase-specific primers were included, namely AW102 and AW104 (Perkin Elmer Cetus, Norfolk, CT) as downstream and upstream primers, respectively. The coefficient of var-

iation for measurement of HMG-CoA reductase mRNA is 8%.

Cholesterol and lathosterol measurements

Cells were frozen at -80°C for at least 24 h and slowly thawed for sterol analysis. Thawed cells were centrifuged for 5 min at 400 \times g. They were then homogenised by re-suspending in 1 ml of SDS buffer (0.1% SDS, 1 mM EDTA and 0.1 M Tris Base, pH 7.4) and taken up in a syringe with an 18 gauge needle 4–8 times. Cholesterol and lathosterol were then extracted using hexane, subjected to saponification, derivatised using Trisil-TBT (Power Sil-Prep Kit, Alltech, Deerfield, IL) and measured by gas chromatography (GC) as described by Wolthers et al [33]. The sterol concentrations were expressed relative to the cellular protein as measured using the method of Lowry et al [24]. Lathosterol is a precursor in the cholesterol biosynthetic pathway and has been used as an index of cholesterol synthesis [34]. The cholesterol in the media was measured in the same way. The coefficient of variation for measurement of cholesterol and lathosterol in our laboratory is 6%.

Results

Effects of α -tocopherol on the LDL Receptor

After incubation for 24 h in media containing 0 to 100 μ M α -tocopherol, cultured HepG2 cells and their media were analysed for their vitamin E content. The amount of α -tocopherol in the cells was found to increase linearly relative to the concentration added to the media at the start of the 24 h incubation (Fig. 1). The HepG2 cells therefore effectively incorporated α -tocopherol at all concentrations. No α -tocopherol was detected in cells or in the media of cells incubated in the absence of added α -tocopherol.

The LDL receptor binding activity of the HepG2 cells incubated for 24 h in media containing 0 to 100 μ M α -tocopherol was measured as the calcium-dependent binding of colloidal gold-LDL. The effect of α -tocopherol on this LDL receptor binding activity was found to be biphasic (Fig. 2A). In the first phase, the binding activity progressively increased to 120% of control with increasing concentrations of α -tocopherol up to 50 μ M but, in a second phase, it progressively decreased from this level to control values with higher concentrations of the vitamin.

Since changes in LDL receptor binding activity usually reflect changes in the number of receptors [20], measured the relative amounts of LDL receptor protein present in the HepG2 cells incubated for 24 h in media containing 0 to 100 μ M α -tocopherol. Using a polyclonal antibody against the LDL receptor, a single band was visualized which corresponded to a protein with the molecular mass of the LDL receptor, 130 kDa [20,27]. The effect of α -tocopherol on the LDL receptor protein was also found to be

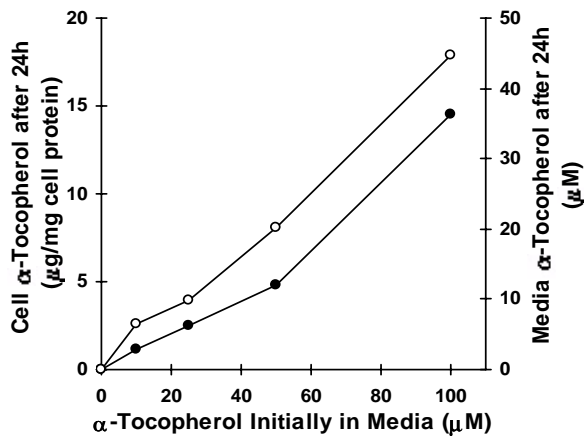


Figure 1
The enrichment of HepG2 cells with α -tocopherol. Cells were incubated for 24 h at 37°C in media containing the indicated initial concentrations of α -tocopherol. After 24 h, the vitamin content of the cells (●, left y-axis scale) and the concentration of α -tocopherol remaining in the media (○, right y-axis scale) were measured by HPLC as described in Methods and materials. Values are means of duplicate determinations.

biphasic (Fig. 2B). The intensity of the LDL receptor band progressively increased up to 4.5-fold above control with increasing concentrations of α -tocopherol up to 50 μM but then decreased from this level with higher concentrations of the vitamin. The biphasic changes observed in the binding of LDL-gold to the HepG2 cells can therefore be attributed to biphasic changes in the amount of LDL receptors present in the cells.

Since changes in both LDL receptor protein and binding activity usually reflect changes in gene transcription [20], we measured the relative amounts of LDL receptor mRNA present in the HepG2 cells incubated for 24 h in media containing 0 to 100 μM α -tocopherol. The effect of α -tocopherol on the LDL receptor mRNA was also found to be biphasic (Fig. 2C). The amount of receptor mRNA progressively increased up to 6.5-fold above control with increasing concentrations of α -tocopherol up to 50 μM but then decreased from this level at higher concentrations of the vitamin. The biphasic changes observed in the binding of LDL-gold to the HepG2 cells and the amount of LDL receptor protein present in the cells can therefore be attributed to biphasic changes in the amount of LDL receptor mRNA. Furthermore, this suggests a biphasic effect on gene transcription.

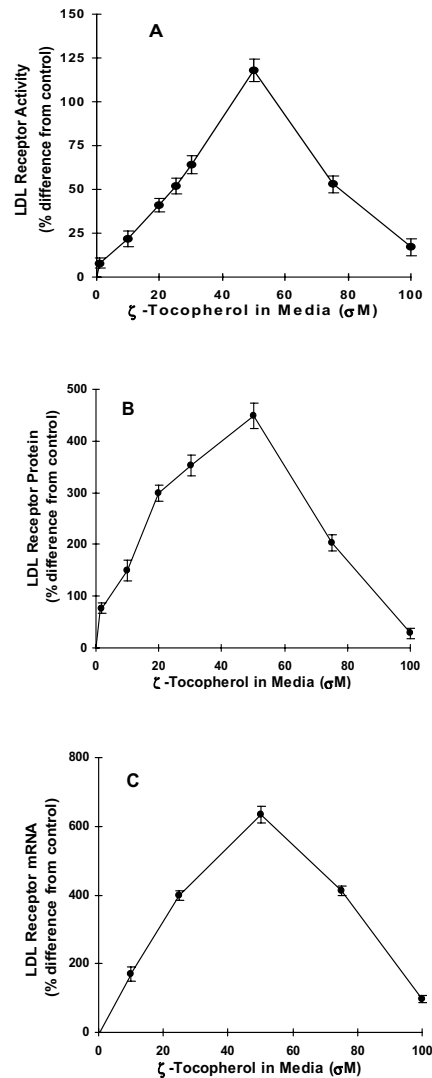


Figure 2
The effect of α -tocopherol on LDL receptor binding activity, protein and mRNA of HepG2 cells. Cells were incubated for 24 h at 37°C in media containing the indicated concentrations of α -tocopherol. The LDL receptor binding activity (A) was measured in triplicate using colloidal-gold LDL, the LDL receptor protein (B) was measured by western blotting and the LDL receptor mRNA (C) was measured using a PCR and western blotting technique as described in Methods and materials and the data was expressed as the percent difference (mean \pm SEM of three experiments) from the values obtained with control cells not pretreated with α -tocopherol. The LDL receptor activity of control cells averaged 30 ± 4.4 ng LDL/mg cell protein, the LDL receptor protein in control cells averaged 0.2 ± 0.09 absorbance units and the amount of LDL receptor mRNA in control cells averaged $4.0 \pm 0.5 \times 10^5$ copies/ μg RNA.

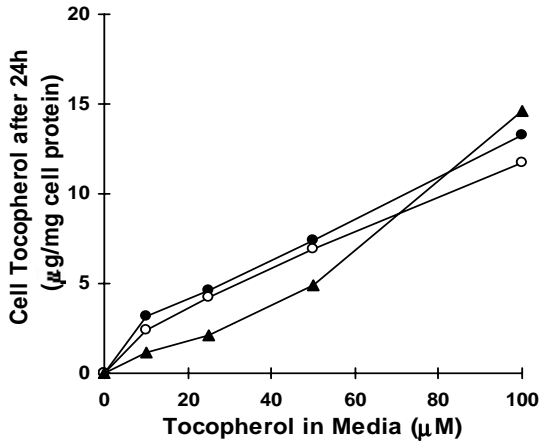


Figure 3
The enrichment of HepG2 cells with different tocopherols. Cells were incubated for 24 h at 37°C in media containing the indicated concentrations of either α-tocopherol (●), δ-tocopherol (○) or γ-tocopherol (▲). After 24 h, the tocopherols in the cells were measured by HPLC as described in Methods and materials. Values are means of duplicate determinations.

Effects of δ- and γ-tocopherol on the LDL Receptor

There are four naturally occurring tocopherols: α-, β-, δ- and γ-tocopherol [18]. To investigate whether the biphasic regulation of the LDL receptor observed with α-tocopherol was a property of other tocopherols, HepG2 cells were incubated for 24 h in media containing either 0 to 100 µM α-tocopherol, δ-tocopherol or γ-tocopherol. All three tocopherols were similarly incorporated by the cells (Fig. 3) with the cellular content of each tocopherol increasing linearly relative to the concentration of the homologue present at the start of the 24 h incubation.

The LDL receptor binding activity (Fig. 4A), protein (Fig. 4B) and mRNA (Fig. 4C) were increased 2, 4.5 and 7-fold over control, respectively, at 50 µM α-tocopherol but were reduced close to control levels at 100 µM α-tocopherol. In contrast, LDL receptor binding activity (Fig. 4A), protein (Fig. 4B) and mRNA (Fig. 4C) were reduced compared to control at all concentrations of δ- and γ-tocopherol tested. Therefore, like the biphasic regulation seen with α-tocopherol, the downregulation observed with δ- and γ-tocopherol appeared to be at the level of gene transcription.

The tocopherols, at all concentrations, had no effect on the growth of the HepG2 cells as judged by total cellular

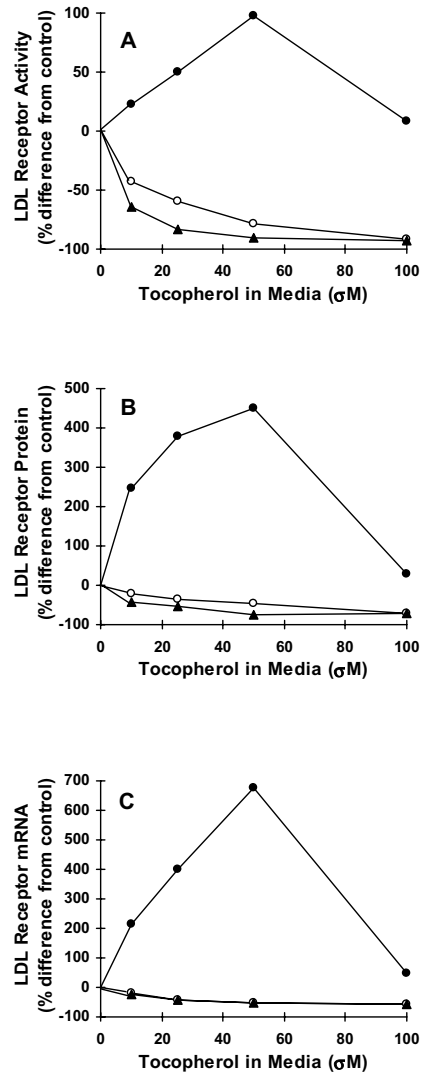
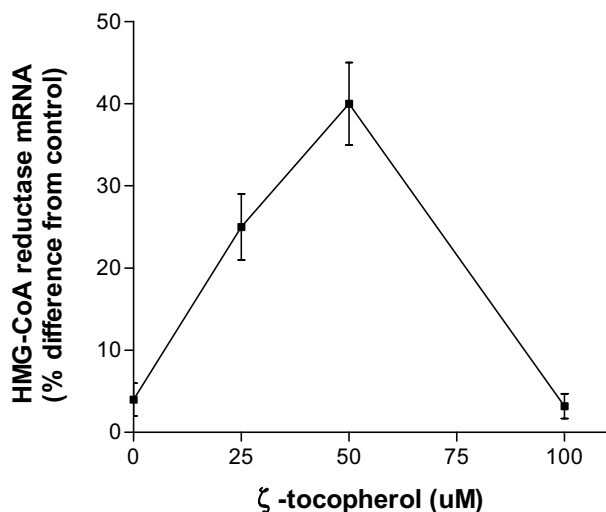


Figure 4
The effects of different tocopherols on the LDL receptor binding activity, protein and mRNA of HepG2 cells. Cells were incubated for 24 h at 37°C in media containing the indicated concentrations of either α-tocopherol (●), δ-tocopherol (○) or γ-tocopherol (▲). The LDL receptor binding activity (A) was measured in triplicate using colloidal-gold LDL. The LDL receptor protein (B) was measured by western blotting and the LDL receptor mRNA (C) was measured using a PCR and western blotting technique as described in Methods and materials and the data was expressed as the percent difference from the values obtained with control cells not pretreated with α-tocopherol. The LDL receptor activity in control cells averaged 40 ± 5 ng LDL/mg cell protein, the LDL receptor protein in control cells averaged 0.85 ± 0.39 absorbance units and the amount of LDL receptor mRNA in control cells averaged 4.0 ± 0.5 × 10⁻⁵ copies/µg RNA.

**Figure 5**

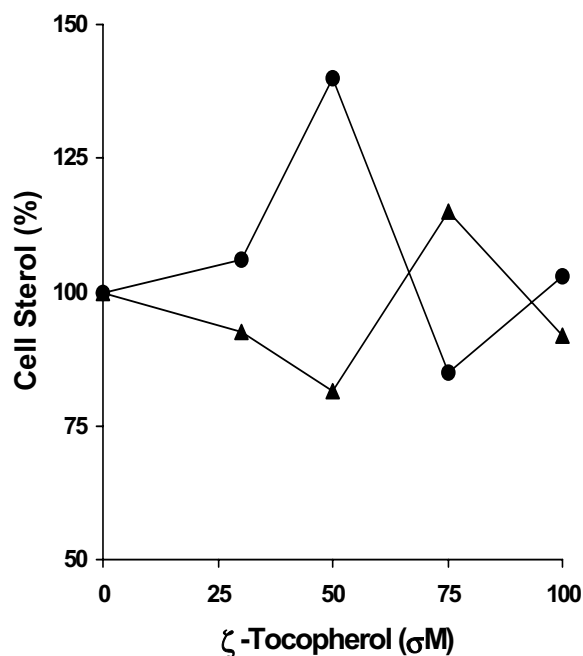
The effects of different tocopherols on HMG-CoA mRNA of HepG2 cells. Cells were incubated for 24 h at 37°C in media containing the indicated concentrations of α -tocopherol. HMG-CoA mRNA was measured using a PCR and western blotting technique as described in Methods and materials and the data was expressed as the percent difference from the values obtained with control cells not pre-treated with α -tocopherol. The HMG-CoA mRNA in control cells averaged $4.2 \pm 0.8 \times 10^5$ copies/ μg RNA.

protein nor on cell viability as judged by the trypan blue dye exclusion test.

Effects of α -tocopherol on the HMG-CoA reductase mRNA and on cell sterols

The HMG-CoA reductase reaction is the rate limiting step in the de novo biosynthesis of cholesterol and regulation of the enzyme is often in parallel to that of the LDL receptor [20]. Vitamin E had the same biphasic effect on the HMG-CoA reductase mRNA as it did on LDL receptor binding activity, protein and mRNA. At 25 μM α -tocopherol, the HMG CoA reductase mRNA was increased by $25 \pm 2.5\%$ over control, at 50 μM it was increased by $40 \pm 3.5\%$ but at 100 μM it was close to control levels, only $3.2 \pm 2.0\%$ higher. The amount of HMG-CoA reductase mRNA in control cells averaged $4.2 \pm 0.8 \times 10^5$ copies/ μg RNA (Figure 5).

Consistent with the effects of vitamin E on the HMG-CoA mRNA, the concentration of lathosterol in the cells, an indicator of cholesterol synthesis, was also increased by 40% over control at 50 μM α -tocopherol but it was close to control levels at 75 and 100 μM α -tocopherol (Fig. 6).

**Figure 6**

The effect of α -tocopherol on the cellular concentration of cholesterol and lathosterol of HepG2 cells. Cells were incubated for 24 h at 37°C in media containing the indicated concentrations of α -tocopherol. Cellular cholesterol (●) and lathosterol (▲) were measured in duplicates by GC as described in Methods and materials and the data was expressed relative to the values of control cells not pre-treated with α -tocopherol set at 100%. The cholesterol concentration in control cells was 9 mg/mg cell protein and the lathosterol concentration was 13 μg /mg cell protein.

In contrast, the effects of vitamin E on the concentration of cholesterol in the cells was inversely related to its effects on cellular lathosterol (Fig. 6). At 50 μM α -tocopherol, the cell cholesterol concentration was reduced by 20% compared to control but it was close to control values at 75 and 100 μM α -tocopherol. Vitamin E however had little effect on the cholesterol concentration in the media; the values were 92%, 92%, 105% and 94% of control at 30, 50, 75 and 100 μM α -tocopherol, respectively. The cholesterol concentration in the media of the control cells was 25 μM .

Discussion

α -Tocopherol consistently modulated the expression of the LDL receptor of HepG2 cells in a biphasic manner. The receptor was progressively upregulated when the cells were incubated with concentrations up to 50 μM α -tocopherol but it was downregulated towards control levels at higher concentrations. The biphasic effect was observed

whether LDL receptor binding activity, protein or mRNA was measured. The upregulation phase was specific for α -tocopherol in that only downregulation of the LDL receptor was observed with δ - and γ -tocopherol at comparable concentrations. The α -tocopherol also had the same biphasic effect on the mRNA of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis [20] and on the cellular lathosterol concentration, another index of cholesterol synthesis. [33,34] The cell cholesterol concentration may have mediated the biphasic "up-then-down" effects on the LDL receptor and on cholesterol synthesis because δ -tocopherol had an inverse "down-then up" effect on the cell cholesterol concentration.

The parallel biphasic modulation by α -tocopherol of LDL receptor binding activity, protein and mRNA suggests that the effect was at the level of gene transcription. For the same reason, the downregulation observed with δ - and γ -tocopherol also appeared to be at the level of gene transcription. This is consistent with what is known about the regulation of the LDL receptor [20], it is well documented to be at the level of gene transcription whether it is dependent [35] or independent [36] of sterols. The concomitant "up-then-down" biphasic change in the HMG-CoA reductase mRNA observed with α -tocopherol also suggests that the transcription of both the LDL receptor and the HMG-CoA reductase genes was coordinately up-regulated. This parallel regulation fits very well with the "down-then-up" effects of vitamin E on the cellular cholesterol concentration as both genes are known to respond to the same sterol feedback regulatory system [35,37]. Furthermore, the same "up-then-down" effect on the cellular lathosterol concentration indicates that the changes in HMG-CoA reductase mRNA were translated into parallel changes in cholesterol synthesis.

The upregulation of the LDL receptor by concentrations of α -tocopherol up to 50 μ M is consistent with a report on the effect of γ -tocotrienol, a natural farnesylated analogue of the tocopherols [18] on the LDL receptor. In that study, 10 μ M γ -tocotrienol, the only concentration tested, increased the amount of LDL receptor protein in HepG2 cells by 75% over control [38]. The tocotrienol also slightly decreased the HMG-CoA reductase mRNA and inhibited cholesterol synthesis by further inhibiting HMG CoA reductase activity at a post-transcriptional level [38]. In contrast, in the present study, there was an increase in cellular lathosterol concentration, an index of cholesterol synthesis [33,34]. The results herein are also not consistent with the evidence that vitamin E decreases cholesterol synthesis in vitamin E-deficient rabbits [5].

The "up then down" regulation seen with α -tocopherol is a novel observation for the LDL receptor and cholesterol synthesis. However, the vitamin E homologue has

previously been found to have concentration-dependent biphasic "up then down" effects on phospholipase A₂ activity [39] and on the synthesis of prostaglandins [40] and prostacyclins [41]. There have also been reports that prostaglandins can upregulate the LDL receptor, an effect that appears to be mediated through camp [42,43]. However, in contrast to the present findings, the effects on prostaglandin synthesis were not specific to α -tocopherol in that the β , δ and γ tocopherols also had biphasic "up then down" effects [40]. Vitamin E is also known to have effects on other cellular regulatory systems including the protein kinase C (PKC) signalling pathway [44] which is also involved in the regulation of the LDL receptor [36,45]. Clearly, there are a number of regulatory pathways through which α -tocopherol could have effects on the LDL receptor. The present observations are therefore generally consistent with what is already known about the effects of vitamin E on cellular metabolism.

The observed upregulation of the LDL receptor at concentrations from 0 to 50 μ M α -tocopherol offers an explanation for the high plasma cholesterol seen in vitamin E-deficient animals and its lowering by replenishment with the vitamin [1-7]. Tocopherols were undetectable when the HepG2 cells were grown in the absence of added tocopherols; these cells therefore essentially mimic liver cells in vitamin E-deficient animals. If replenishment with α -tocopherol in deficient animals upregulates the hepatic LDL receptor as it does in the vitamin E-free HepG2 cells, then the clearance of LDL and other lipoproteins from the circulation should increase [19,20] and contribute to the lowering of the high plasma cholesterol seen in vitamin E-deficient animals.

The biphasic "up then down" nature of the LDL receptor response to the increasing α -tocopherol concentrations in the present study may also explain the variable effects that dietary supplementation with vitamin E can have on plasma cholesterol when animals are not deficient in the vitamin. In such animals made hypercholesterolaemic by diet, α -tocopherol supplementation can result in 1) a decrease [8,12], 2) an increase [16] or 3) no change [13-15] in plasma cholesterol. The present results would suggest that the vitamin E level prior to supplementation could have been 1) lower than optimal, 2) optimal or 3) higher than optimal for maximal LDL receptor activity, respectively and thus result in 1) an increase, 2) a decrease or 3) no change in LDL receptor activity, respectively, upon supplementation.

In humans, vitamin E deficiency is very rare and plasma levels of the vitamin are generally higher than in animals. Nonetheless, in a recent placebo controlled, cross-over human trial, doses of vitamin E (73.5 mg /day for 6 weeks) was observed to significantly decrease plasma cho-

lesterol by 5.3% and plasma triglycerides by 18.3% while increasing the plasma α -tocopherol from 26.8 to 32.2 μ M [46], concentrations within the range where the increase in the LDL receptor was observed in the present study. In the same study, 500 mg/day of vitamin C also decreased plasma cholesterol by 6.2% and triglycerides by 8.5%.

However, other recent human intervention studies with generally higher vitamin E doses have mostly shown no change or an increase in plasma cholesterol [47–50]. Importantly, the increase in plasma cholesterol, when seen, is due mainly to an increase in high density lipoprotein (HDL) cholesterol, a lipoprotein which is considered to be anti-atherogenic [50]. Why HDL cholesterol is increased rather than LDL cholesterol, as might be expected if the LDL receptor is decreased, is unclear. However, the LDL receptor is only one of the factors which can affect plasma cholesterol, and α -tocopherol may have effects on other aspects of cholesterol and lipoprotein metabolism.

In conclusion, α -tocopherol has been shown to have a concentration-dependent biphasic "up then down" effect on the LDL receptor of HepG2 cells. The effect appears to be at the level of gene transcription and is specific for α -tocopherol in that δ - and γ -tocopherol only had down-regulatory effects. The α -tocopherol also had the same effects on HMG-CoA reductase mRNA levels and the cellular lathosterol concentration, indices of cholesterol synthesis. These vitamin E effects may have been mediated through the observed "down-then-up" effect on the cellular cholesterol concentration. These results may explain the hypercholesterolaemia observed in animal models of vitamin E deficiency and may be relevant to the variable effect α -tocopherol supplementation has on plasma cholesterol in animals and humans not deficient in the vitamin.

Acknowledgments

We would like to thank Dr. Paul Kroon and Dr. Elizabeth Powell, (Dept. of Biochemistry, University of Queensland), for teaching us the measurement of mRNA by quantitative PCR with a nonradioactive label. Special thanks are due to Calliope Triantafyllidis (CSIRO) for all her expert technical assistance and thanks to Dr. Mavis Abbey (CSIRO), Dr. Paul Nestel (CSIRO) and Dr. Andrew Thomson (Adelaide Uni.) for their support and advice.

References

- Morgulis S and Spencer HC **Studies on the blood and tissues in nutritional muscular dystrophy** *J Nutr* 1936, **12**:173-190
- Chen LH, Liao S and Packett LV **Interaction of dietary vitamin E and protein level or lipid source with serum cholesterol level in rats** *J Nutr* 1972, **102**:729-732
- Kaseki H, Kim EY, Whisler RL and Cornwell DG **Effect of an oral dose of vitamin E on the vitamin E and cholesterol content of tissues of the vitamin E-deficient rat** *J Nutr* 1986, **116**:1631-1639
- Yasuda M, Fujita T and Mizunoya Y **Liver and plasma lipids in vitamin E-deficient rats** *Chem Pharm Bull* 1979, **27**:447-451
- Eskelson CD, Jacobi HP and Fitch DM **Some effects of vitamin E deficiency on *in vitro* cholesterol metabolism** *Physiol Chem Phys* 1973, **5**:319-329
- Chupukcharoen N, Komaratat P and Wilairat P **Effects of vitamin E deficiency on the distribution of cholesterol in plasma lipoproteins and the activity of cholesterol 7 α -hydroxylase in rabbit liver** *J Nutr* 1985, **115**:468-472
- Oriani G, Salvatori G, Maiorano G, Belisario MA, Pastinese A, Manchisi A and Pizzuti G **Vitamin E nutritional status and serum lipid pattern in normal weanling rabbits** *J Animal Sci* 1997, **75**:402-408
- Wilson RB, Middleton CC and Sun GY **Vitamin E, antioxidants and lipid peroxidation in experimental atherosclerosis of rabbits** *J Nutr* 1978, **108**:1858-1867
- Westrope KL, Miller RA and Wilson RB **Vitamin E in a rabbit model of endogenous hypercholesterolemia and atherosclerosis** *Nutr Reports Internat* 1982, **25**:83-88
- Phonpanichrasamee C, Komaratat P and Wilairat P **Hypocholesterolemic effect of vitamin E on cholesterol-fed rabbit** *Internat J Vit Nutr Res* 1990, **60**:240-244
- Wójcicki J, Rózewicka L, Barcew-Wiszniewska B, Samochowiec L, Juzwiak S, Kadlubowska D, Tustanowski S and Juzyszyn Z **Effect of selenium and vitamin E on the development of experimental atherosclerosis in rabbits** *Atherosclerosis* 1991, **87**:9-16
- Ozer NK, Sirikci O, Taha S, San T, Moser U and Azzi A **Effect of vitamin E and probucol on dietary cholesterol-induced atherosclerosis in rabbits** *Free Rad Biol Med* 1998, **24**:226-233
- Morel DW, de la Llera-Moya M and Friday KE **Treatment of cholesterol-fed rabbits with dietary vitamins E and C inhibits lipoprotein oxidation but not development of atherosclerosis** *J Nutr* 1994, **124**:2123-2130
- Parker RA, Sabrah T, Cap M and Gill BT **Relation of vascular oxidative stress, α -tocopherol, and hypercholesterolemia to early atherosclerosis in hamsters** *Arterioscler Thromb Vasc Biol* 1995, **15**:349-358
- Sulli KC, Sun J, Giraud DW, Moxley RA and Driskell JA **Effects of β -carotene and α -tocopherol on the levels of tissue cholesterol and triglyceride in hypercholesterolemic rabbits** *J Nutr Biochem* 1998, **9**:344-350
- Prasad K and Kalra J **Oxygen free radicals and hypercholesterolemic atherosclerosis: effect of vitamin E** *Am Heart J* 1993, **125**:958-973
- Stone WL **The effects of cholesterol supplementation on plasma lipoprotein-cholesterol levels in rats fed diets deficient in vitamin E and/or selenium** *Nutr Res* 1988, **8**:1061-1071
- Burton GVV **Vitamin E: molecular and biological function** *Proc Nutr Soc* 1994, **53**:251-262
- Dietschy JM, Turley SD and Spady DK **Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans** *J Lipid Res* 1993, **34**:1637-1659
- Brown MS and Goldstein JL **A receptor-mediated pathway for cholesterol homeostasis** *Science* 1986, **232**:34-47
- Havekes LM, De Wit ECM and Princen HMG **Cellular free cholesterol in Hep G2 cells is only partially available for down-regulation of low-density-lipoprotein receptor activity** *Biochem J* 1987, **247**:739-746
- Kambouris AM, Roach PD and Nestel PJ **Demonstration of a high density lipoprotein (HDL)-binding protein in Hep G2 cells using colloidal gold-HDL conjugates** *FEBS Lett* 1988, **230**:176-180
- Kambouris AM, Roach PD, Calvert GD and Nestel PJ **Retroendocytosis of high density lipoproteins by the human hepatoma cell line, HepG2** *Arteriosclerosis* 1990, **10**:582-590
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ **Protein measurement with the Folin phenol reagent** *J Biol Chem* 1951, **193**:265-275
- Yang CS and Lee M-J **Methodology of plasma retinol, tocopherol and carotenoid assays in cancer prevention studies** *J Nutr Growth Cancer* 1987, **4**:19-27
- Havel RJ, Eder HA and Bragdon JH **The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum** *J Clin Invest* 1955, **34**:1345-1353
- Roach PD, Zollinger M and Noël S-P **Detection of the low density lipoprotein (LDL) receptor on nitrocellulose paper with colloidal gold-LDL conjugates** *J Lipid Res* 1987, **28**:1515-1521
- Roach PD, Hosking J, Clifton PM, Bais R, Kusenic B, Coyle P, Wight MB, Thomas DW and Nestel PJ **The effects of hypercholesterolemia, simvastatin and dietary fat on the low density**

- lipoprotein receptor of unstimulated mononuclear cells** *Atherosclerosis* 1993, **103**:245-254
29. Roach PD, Balasubramaniam S, Hirata F, Abbey M, Szanto A, Simons LA and Nestel PJ **The low-density lipoprotein receptor and cholesterol synthesis are affected differently by dietary cholesterol in the rat** *Biochim Biophys Acta* 1993, **1170**:165-172
 30. Balasubramaniam S, Szanto A and Roach PD **Circadian rhythm in hepatic low-density-lipoprotein (LDL)-receptor expression and plasma LDL levels** *Biochem J* 1994, **298**:39-43
 31. Chomczynski P and Sacchi N **Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction** *Anal Biochem* 1987, **162**:156-159
 32. Powell EE and Kroon PA **Measurement of mRNA by quantitative PCR with a nonradioactive label** *J Lipid Res* 1992, **33**:609-614
 33. Wolthers BG, Walrecht HT, vander Molen JC, Nagel GT, Van Doormaal JJ and Wijnandts PN **Use of determinants of 7-lathosterol (5 α -cholest-7-en-3 β -ol) and other cholesterol precursors in serum in the study and treatment of disturbances of sterol metabolism, particularly cerebrotendinous xanthomatosis** *J Lipid Res* 1991, **32**:603-612
 34. Kempen HJM, Glatz JFC, Gevers Leuven JA, van der Voort HA and Katan MB **Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans** *J Lipid Res* 1988, **29**:1149-1155
 35. Wang X, Sato R, Brown MS, Hua X and Goldstein JL **SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis** *Cell* 1994, **77**:53-62
 36. Makar RSJ, Lipsky PE and Cuthbert JA **Sterol-independent, sterol response element-dependent, regulation of low density lipoprotein receptor gene expression** *J Lipid Res* 1998, **39**:1647-1654
 37. Millinder Vallett S, Sanchez HB, Rosenfeld JM and Osborne TF **A direct role for sterol regulatory element binding protein in activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene** *J Biol Chem* 1996, **271**:12247-12253
 38. Parker RA, Pearce BC, Clark RW, Gordon DA and Wright JJK **Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase** *J Biol Chem* 1993, **268**:11230-11238
 39. Tran K, Wong JT, Lee E, Chan AC and Choy PC **Vitamin E potentiates arachidonate release and phospholipase A₂ activity in rat heart myoblastic cells** *Biochem J* 1996, **319**:385-391
 40. Diplock AT, Xu G-L, Yeow C-L and Okikiola M **Relationship of tocopherol structure to biological activity, tissue uptake, and prostaglandin biosynthesis** *Ann NY Acad Sci* 1989, **570**:72-84
 41. Weimann BJ, Steffen H and Weiser H **Effects of α - and γ -tocopherol (α -T, γ -T) and α -tocotrienol (α -TT) on the spontaneous and induced prostacyclin (PGI₂) synthesis from cultured human endothelial cells (HEC) and rat aorta segments *in vivo*** *Ann NY Acad Sci* 1989, **570**:530-532
 42. Nield H and Middleton B **Transient elevation of cAMP by prostaglandins triggers subsequent up-regulation of LDL receptor activity in cultured human cells** *Biochem Soc Trans* 1994, **22**:210S
 43. Nield H and Middleton B **Prostaglandins which elevate cyclic AMP increase low density lipoprotein receptor mRNA and activity in human extra-hepatic cells** *Biochem Soc Trans* 1995, **23**:5S
 44. Azzi A, Boscoboinik D, Marilley D, Özer NK, Stäubli B and Tasinato A **Vitamin E: a sensor and an information transducer of the cell oxidation state** *Am J Clin Nutr* 1995, **62**:1337S-1346S
 45. Kumar A, Chambers TC, Cloud-Helfin BA and Mehta KD **Phorbol ester-induced low density lipoprotein receptor gene expression in HepG2 cells involves protein kinase C-mediated p42/44 MAP kinase activation** *J Lipid Res* 1997, **38**:2240-2248
 46. Hamilton IMJ, Gilmore WS, Benzie IFF, Mulholland CW and Strain JJ **Interactions between vitamins C and E in human subjects** *Brit J Nutr* 2000, **84**:261-267
 47. Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ and Brown MJ **Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS)** *Lancet* 1996, **347**:781-786
 48. Princen HMG, van Duyvenvoorde W, Buytenhek R, van der Laarse A, van Poppel G, Gervers Leuven JA and van Hinsberg VWM **Supplementation with low doses of vitamin E protects LDL from lipid peroxidation in men and women** *Arterioscler Tromb Vasc Biol* 1995, **15**:325-333
 49. Jialal I, Fuller CJ and Huet BA **The effect of α -tocopherol supplementation on LDL oxidation: A dose-response study** *Arterioscler Thromb Vasc Biol* 1995, **15**:190-198
 50. Muckle TJ and Nazir DJ **Variation in human blood high density lipoprotein response to oral vitamin E megadosage** *Am J Clin Pathol* 1989, **91**:165-171

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