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Regulation of cell signalling by vitamin E

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Vitamin E, the most important lipid-soluble antioxidant, was discovered at the University of California at Berkeley in 1922. Since its discovery, studies of the constituent tocopherols and tocotrienols have focused mainly on their antioxidant properties. In 1991 Angelo Azzi's group (Boscoboinik *et al*. 1991*a*,*b*) first described non-antioxidant cell signalling functions for α-tocopherol, demonstrating that vitamin E regulates protein kinase C activity in smooth muscle cells. At the transcriptional level, α-tocopherol modulates the expression of the hepatic α-tocopherol transfer protein, as well as the expression of liver collagen alpha1 gene, collagenase gene and α-tropomyosin gene. Recently, a tocopherol-dependent transcription factor (tocopherol-associated protein) has been discovered. In cultured cells it has been demonstrated that vitamin E inhibits inflammation, cell adhesion, platelet aggregation and smooth muscle cell proliferation. Recent advances in molecular biology and genomic techniques have led to the discovery of novel vitamin E-sensitive genes and signal transduction pathways.

Résumé

La vitamine E, l'antioxydant liposoluble le plus important, fut découverte à l'Université de Californie à Berkeley en 1922. Depuis sa découverte, les études sur les tocophérols et les tocotrienols que constitue cette vitamine, ont été centrées pour la plupart sur leurs propriétés antioxydantes. En 1991, le groupe de Angelo Azzi (Boscoboinik *et al*. 1991*a*,*b*) fut le premier à décrire les fonctions autres que les antioxydantes et de transmission de signaux de l'α-tocophérol, en démontrant la régulation par la vitamine E de l'activité de la protéine kinase C dans les cellules de muscle lisse. Au niveau de la transcription, l'α-tocophérol module l'expression de la protéine de transfert hépatique de l'α-tocophérol, ainsi que l'expression du gène alpha1 du collagène du foie, du gène de la collagénase et du gène de l'α-tropomyosine. Récemment, un facteur de transcription dépendant du tocophérol (la protéine associée au tocophérol) a été découvert. Il a été démontré sur des cellules cultivées que la vitamine E inhibe l'inflammation, l'adhésion cellulaire, l'agrégation des plaquettes et la prolifération des cellules de muscle lisse. Les avancées récentes de la biologie moléculaire et des techniques génomiques ont conduit à la découverte de nouveaux gènes et des mécanismes de transduction des signaux sensibles à la vitamine E.

Antioxidants: Vitamin E: Gene expression: Cell signalling: Cardiovascular disease

Chemistry and antioxidant properties of vitamin E

Vitamin E consists of a mixture of tocopherols and tocotrienols that are synthesised by plants from homogenestic acid. All are derivatives of 6-chromanol with an aliphatic side-chain. The four tocopherol homologues (α-, β-, γ- and δ-) have a fully saturated C_{16} phytol side-chain, whereas tocotrienols have a similar isoprenoid chain containing three double bonds. Individual tocopherols are named according to the position and number of the methyl groups on the

Abbreviations: COX, cyclo-oxygenase; ICAM-1, intracellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor kappa B; PKC, protein kinase C; PUFA, polyunsaturated fatty acids; α-TTP, α-tocopherol transfer protein; VCAM-1, vascular cell adhesion molecule-1.

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	R_1	R_2	R_3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3

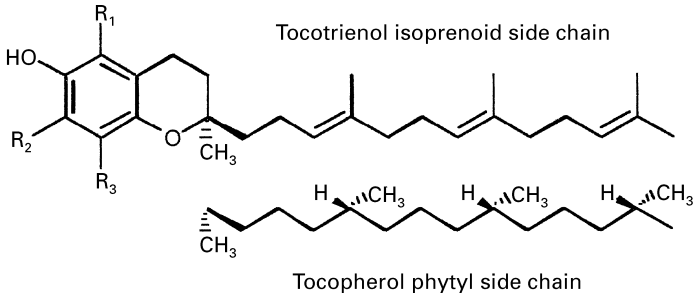

Fig. 1. Molecular structure of vitamin E stereoisomers.

phenol ring, with the α-, β-, γ- and δ-tocopherols containing three, two, two and one methyl groups respectively (Fig. 1). These structural differences determine biological activity, α-homologues being the most biologically active.

The majority of the functionality of vitamin E is through its role as an antioxidant, maintaining the structural integrity of virtually all cells in the body. Its antioxidant function is mediated through the reduction of free radicals, thus protecting the body against the deleterious effects of such highly-reactive oxygen and nitrogen species, which have been implicated in ageing and a number of chronic diseases such as atherosclerosis, cancers and rheumatoid arthritis (Halliwell, 1996; Parthasarathy *et al*. 1999; Malins *et al*. 2001). The reactive oxygen species which include H_2O_2, the superoxide radical ($O_2^{\bullet -}$), and the highly-reactive hydroxyl radical ($OH^{\bullet}$), are by-products of normal aerobic metabolism formed during the respiratory and phagocytic processes, and during microsomal cytochrome P-450 metabolism. The reactive nitrogen species include $NO^{\bullet}$ and peroxynitrite, formed by the reaction of $NO^{\bullet}$ and $O_2^{\bullet -}$.

The polyunsaturated fatty acids (PUFA) of biological membranes are particularly susceptible to free radical attack due to their high degree of unsaturation. In brief, the process is initiated by a free radical such as $OH^{\bullet -}$, which extracts H from PUFA resulting in a $PUFA^{\bullet}$ radical. Following molecular rearrangement to form a conjugated diene the molecule is susceptible to attack by O_2 resulting in a peroxyl radical ($PUFAOO^{\bullet}$). $PUFAOO^{\bullet}$ are capable of extracting H from adjacent PUFA, thus propagating a chain reaction. Such auto-oxidation continues, severely affecting the functionality of the tissue, unless the free radicals are scavenged. Due to its abundance, lipid solubility and efficacy with respect to radical quenching, vitamin E is considered to be the most important antioxidant in cell membranes (Ingold *et al*. 1987; Halliwell, 1996; Brigelius-Flohe & Traber, 1999).

The antioxidant property of vitamin E is exerted through the phenolic hydroxyl group, which readily donates its H to the $PUFAOO^{\bullet}$ radical, resulting in the formation of a stable lipid species. In donating the H, vitamin E becomes a relatively unreactive free radical, as the unpaired electron becomes delocalised into the aromatic ring. The efficiency of this protection depends on two factors: first the mobility of the molecule in membranes, which is determined by the aliphatic tail; second the number of methyl species on the chromanol ring, with each methyl group conferring additional antioxidant capacity. In addition, the proximity of the methyl species to the hydroxyl group is an important factor. Thus, α-homologues, which have the greatest number of methyl species, and in which these methyl groups flank the hydroxyl group, are thought to be more effective than the other homologues.

α-Tocotrienol has been shown to be more effective in protecting against lipid peroxidation than α-tocopherol (Serbinova *et al*. 1991; Suzuki *et al*. 1993). A reason suggested for this greater effectiveness is the nature of the aliphatic tail. The isoprenoid chain of α-tocotrienol has a stronger disordering effect on membranes than α-tocopherol. This property leads to a greater mobility and more uniform distribution within the membrane. NMR studies have also shown that the chromanol ring of α-tocotrienol is situated closer to the membrane surface. These factors contribute to a greater ability of tocotrienols to interact with radicals and allow for quicker recycling of the molecule to its active reduced form (Serbinova *et al*. 1991; Suzuki *et al*. 1993).

Although vitamin E plays a unique role within membranes it does not function in isolation. Protecting the cell from the deleterious effect of oxidative stress involves an array of other membrane and water-soluble antioxidants and antioxidant enzymes, which together form the 'antioxidant defence system' (Fig. 2). In this multifactorial system the cytosolic metalloenzymes serve in the prevention of free radical formation. Superoxide dismutase serves to convert $O_2^{\bullet -}$ to H_2O_2, whereas glutathione peroxidase and catalase further reduce H_2O_2, thus preventing the formation of the highly-reactive $OH^{\bullet}$. The water-soluble antioxidants can act as cofactors for the antioxidant enzymes, can serve as independent antioxidants or can function in the recycling of vitamin E (Packer *et al*. 2001). Vitamin E exists in membranes at a concentration of one molecule per 2000–3000 phospholipids and, therefore, would become rapidly depleted unless it was regenerated to its active form. *In vitro* evidence suggests that ascorbate helps regenerate membrane-bound vitamin E, converting the tocopheroxyl radical to its native form and resulting in the formation of an ascorbyl radical (Kagan & Tyurina, 1998). However, *in vivo* evidence is currently lacking.

Absorption and transport

To date the majority of information available on vitamin E absorption and transport is based on tocopherol (Cohn *et al*. 1992; Kayden & Traber, 1993; Herrera & Barbas, 2001). In

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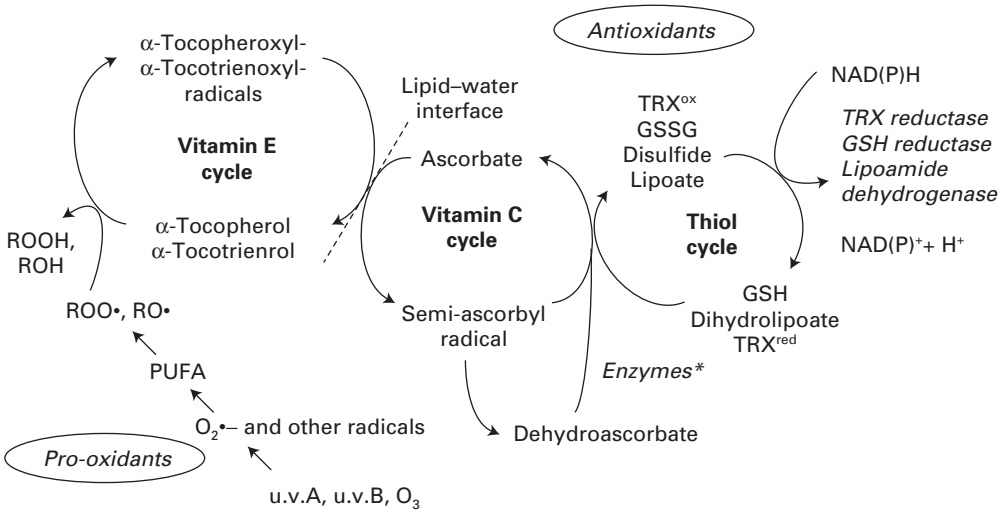

Fig. 2. The antioxidant network showing the interaction among vitamin E, vitamin C and thiol redox cycles. PUFA, polyunsaturated fatty acids; GSH, glutathione; TRX, thioredoxin; TRX^{ox}, TRX^{red}, oxidised and reduced forms of TRX respectively. * Thiol transferase (glutaredoxin), GSH-dependent dehydroascorbate reductase, protein disulfide isomerase, TRX reductase. (From Packet *et al*. 2001.)

the small intestine, tocopheryl esters hydrolysed to free vitamin E species are incorporated into mixed micelles due to the action of bile salts and pancreatic juices. Lack of these gastric secretions, as occurs in individuals with conditions such as pancreatitis, cystic fibrosis or choleostatic liver disease, leads to vitamin E malabsorption and resultant nutrient deficiency symptoms. The micelles enter the enterocyte via passive diffusion and the tocopherols are packaged into chylomicrons along with the phospholipid, cholesterol, triacylglycerol and apolipoprotein moieties. On entry into the circulation via the lymphatic system the chylomicrons are sequentially hydrolysed, due to the action by lipoprotein lipase attached to the capillary endothelium in the target tissue such as muscle and adipose tissue; a proportion of the tocopherol is released and taken up by the endothelial cells. The resulting chylomicron remnants are taken up by the liver by receptor-mediated endocytotic processes.

In contrast to vitamins A and D, there does not appear to be a specific carrier protein for vitamin E in the circulation. Instead, vitamin E is incorporated into lipoprotein particles in a non-specific manner. In hepatic cells tocopherol from chylomicron remnants binds to cytosolic α-tocopherol transfer protein (α-TTP; Catignani & Bieri, 1977; Hosomi *et al*. 1997), which mediates its transfer to the site of VLDL synthesis (rough endoplasmic reticulum and Golgi apparatus). This 32 kDa protein is expressed almost exclusively in the liver, and recent evidence from animal studies suggests that dietary α-tocopherol modulates hepatic α-TTP mRNA levels (Fechner *et al*. 1998). Unlike tocopherol absorption, which is thought to be non-selective with respect to isomer, α-TTP displays stereoisomer specificity, with almost exclusive incorporation of α-tocopherol into the nascent VLDL particle. Relative affinities of tocopherol analogues for α-TTP, calculated from competition studies, are as follows (%): α-tocopherol 100, β-tocopherol 38, γ-tocopherol 9, δ-tocopherol 2 (Hosomi *et al*. 1997). The majority of non-α isomers are excreted via the bile (Traber & Kayden, 1989). α-TTP is now recognised to be the primary determinant of plasma tocopherol levels. Mutations of the α-TTP gene lead to reduced plasma and tissue α-tocopherol, which may ultimately lead to a severe condition known as ataxia with vitamin E deficiency, with associated neuronal and retinal damage (Traber *et al*. 1990; Ben Hamida *et al*. 1993). In a recent study α-TTP knockout mice (Ttpa+/– and Ttpa–/–) were used as a model to examine the association between vitamin E deficiency and atherosclerosis (Terasawa *et al*. 2000). Plasma and tissue α-tocopherol were reduced in a stepwise manner from controls through Ttpa+/– to Ttpa–/–, with an absence of liver α-TTP in liver homogenates from Ttpa–/– and a 50 % reduction in protein level in the Ttpa+/– animals. The vitamin E deficiency was associated with increased lesions in the proximal aorta and increased rates of lipid peroxidation. These findings further demonstrate the role of this transfer protein in tocopherol metabolism and, ultimately, in CHD risk.

Approximately 50–70 % of the total secreted VLDL is hydrolysed to LDL, with associated transfer of tocopherols into the LDL fraction (Welty *et al*. 2000). In the circulation tocopherol exchanges rapidly between the lipoprotein particles, although > 90 % is contained within the LDL and HDL fractions (Behrens *et al*. 1982). The 75 kDa plasma phospholipid transfer protein facilitates tocopherol exchange between HDL and LDL (Lagrost *et al*. 1998).

The mechanisms of peripheral cellular uptake of vitamin E are poorly understood, although simultaneous uptake of tocopherol via receptor-mediated lipoprotein endocytosis, or via fatty acid-binding proteins, may be involved. However, recent evidence suggests that specific membrane tocopherol-binding proteins may also mediate tocopherol uptake (Dutta-Roy, 1999).

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Information on intracellular tocopherol transport is currently lacking. Due to its strong hydrophobicity, transfer to cellular sites requires a specific transfer protein. However, it is still unclear how many other α-tocopherol-binding proteins exist and which mechanisms regulate tocopherol transfer within peripheral cells. Recently, a novel binding protein, tocopherol-associated protein, has been identified (Stocker *et al*. 1999; Zimmer *et al*. 2000; Blatt *et al*. 2001; Yamauchi *et al*. 2001). This 46 kDa protein, which displays substantial sequence homology to α-TTP, is ubiquitously expressed, although the highest levels have been observed in the liver, brain and prostate (Zimmer *et al*. 2000). It is suggested that this protein plays an important general role in intracellular tocopherol metabolism. Structural analysis of tocopherol-associated protein suggested that it is a member of the widespread SEC14-like protein family, which plays a role in phospholipid exchange in the cell. Recent ligand competition studies indicate that tocopherol-associated protein binds to α-tocopherol but not other tocopherol isomers (Blatt *et al*. 2001). Although research is at an early stage, it is likely that tocopherol-associated protein will prove an important molecule with respect to cellular tocopherol events.

Cell signalling

Protein kinase C and protein phosphatase 2A activity

Since the discovery of the tocopherols and tocotrienols, it is their antioxidant properties that have received most attention. It is now clear, however, that the role of vitamin E goes beyond its antioxidant function. The first observation of a cell signalling role for vitamin E was the finding by Angelo Azzi's group (Boscoboinik *et al*. 1991*a*,*b*) that smooth muscle cell proliferation and protein kinase C (PKC) activity are inhibited by α-tocopherol (see Table 1). The inhibition of smooth muscle cell proliferation was specific to α-tocopherol; Trolox, phytol, β-tocopherol and α-tocopheryl esters had no effect. As α-tocopherol and β-tocopherol have very similar free radical-scavenging activities, the mechanism by which α-tocopherol acts on PKC is not thought to be related to its antioxidant properties. Subsequent studies have shown that PKC is inhibited in a number of other cell types, including monocytes (Devaraj *et al*. 1996), neutrophils (Kanno *et al*. 1995), fibroblasts (Hehenberger & Hansson, 1997) and mesangial cells (Tada *et al*. 1997). Most importantly, this inhibition of PKC by α-tocopherol occurs at concentrations close to those measured in human plasma (Azzi *et al*. 2001). Anti-proliferative effects of vitamin E were not seen for HeLA cells, suggesting that there are different cell-specific pathways of cellular proliferation in which vitamin E can act (Fazzio *et al*. 1997). In addition, the inhibition of PKC was not related to a direct interaction of α-tocopherol with the enzyme or with a diminution of its expression. Instead, PKC inhibition by α-tocopherol is linked to the activation of a protein phosphatase 2A, which in turn dephosphorylates PKC-α and thereby inhibits its activity (Clement *et al*. 1997; Ricciarelli *et al*. 1998). An inhibitory effect of α-tocopherol on PKC may be seen only at the cellular level and is not evident with recombinant PKC.

Cyclo-oxygenase

Cyclo-oxygenase (COX) has two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cells, whereas COX-2 is regulated by growth factors, tumour promoters, cytokines, glucocorticoids and lipopolysaccharides. COX converts arachidonic acid into prostaglandin E_2, the precursor of thromboxane and eicosanoid synthesis. High levels of COX-2 in epithelial cells are associated with

Table 1. Important findings in experimental vitamin E research

The early history	
1922	The existence of vitamin E was recognised by Evans and Bishop when it became clear that this fat-soluble factor (named factor X) prevented fetal death in animals fed a diet containing rancid lard
1924	Sure gave factor X the name vitamin E as the 5th serial alphabetical designation for vitamins
1925	Evans proposed the word tocopherol from the Greek 'tos' for childbirth and 'phero' meaning to bring forth and 'ol' for the alcohol portion of the molecule
The early years of research (description of structural and functional features)	
1930	Characterization of vitamin E deficiency symptoms (testicular atrophy, fetal resorption, encephalomalacia, paralysis associated with dystrophic muscle) in various animals
1938	Fernholz elucidates the structure of vitamin E
1938	Synthesis of vitamin E by Karrer
1955	Revelation by Gordon and colleagues that mature infants had low levels of blood tocopherol and abnormal haemolysis of erythrocytes, incubated in the presence of H_2O_2
1967	Study by Bunyan and colleagues on the antioxidant impact of vitamin E on polyunsaturated fatty acids
1968	Vitamin E was recognised formally as an essential nutrient for man by inclusion in the recommended dietary allowances table of the US Food and Nutrition Board.
Recent vitamin E research (non antioxidant function and influence of vitamin E on gene expression)	
1991	Evidence by Boscoboinik *et al*. (1991*a*,*b*) that smooth muscle cell proliferation is inhibited by α-tocopherol through protein kinase C modulation
1998	Discovery by Fechner *et al*. (1998) that the expression of α-tocopherol transfer protein in the liver is induced by α- and ß-tocopherol
1998	Modulation of liver collagen α1 gene transcription by α-tocopherol (Chojkier *et al*. 1998)
1999	Evidence by Aratri *et al*. (1999) that increased transcription level of α-tropomyosin is caused by α-tocopherol
2001	Discovery of α-tocopherol as a transcriptional regulator of gene expression via association with a transcription factor tocopherol-associated protein (Yamauchi *et al*. 2001)

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the inhibition of apoptosis, and overexpression of COX-2 has been implicated in the pathogenesis of neoplastic diseases. An up-regulation of COX-2 transcription has been shown in most human colo-rectal cancers (Fosslien, 2001). Interestingly changes in arachidonic acid metabolism stimulate cell proliferation via activation of PKC, indicating that PKC might be one of the primary signalling pathways through which certain tumours are initiated or maintained. In recent years, a role for COX-2 in atherogenesis has been identified. Immunocytochemical studies using anti-COX-2 have shown that COX-2 is localised to macrophages in atherosclerotic lesions of patients with coronary artery disease (Baker *et al*. 1999).

In monocytes derived from aged mice it has been shown that a vitamin E-induced decrease in prostaglandin E_2 production is mediated via decreased COX activity (Wu *et al*. 2001). However, vitamin E has no effect on COX mRNA and protein levels, indicating a post-translational regulation of the COX enzyme. Non-α-tocopherol homologues were like β-tocopherol, also effective in inhibiting COX activity, but the extent of inhibition varied in proportion to their antioxidant capacity, suggesting that an antioxidant mechanism may be involved.

It has been shown in lipopolysaccharide-stimulated RAW264.7 macrophages and interleukin 1β-treated A549 human epithelial cells that γ-tocopherols inhibited the production of prostaglandin E_2 due to a direct inhibition of COX-2 (Jiang *et al*. 2000). Furthermore, the major metabolite of dietary γ-tocopherol also exhibited an inhibitory effect in these cells. In contrast, α-tocopherol at 50 μM slightly reduced (25 %) prostaglandin E_2 formation in macrophages, but had no effect in epithelial cells. Similar to the previously mentioned study, the inhibitory effect of γ-tocopherol and 2,7,8-trimethyl-2-(beta-carboxyethyl)-6-hydroxychroman stemmed from their inhibition of COX-2 activity, rather than from affecting protein expression or substrate availability, and appeared to be independent of their antioxidant activity.

Nuclear factor kappa B

The transcription factors of the nuclear factor kappa B (NF-κB)/Rel family control the expression of a spectrum of different genes involved in inflammatory and proliferative responses. The typical NF-κB dimer is composed of the p50 and p65 subunits, and it is present in an inactive form in the cytosol bound to the inhibitory proteins, NF-κB inhibitory unit. Following activation by various stimuli, including inflammatory or hyperproliferative cytokines, reactive oxygen species and bacterial wall components, the phosphorylation and proteolytic removal of NF-κB inhibitory unit from the complex occurs. Activated NF-κB then immediately enters the nucleus where it interacts with regulatory kappa B elements in the promoter and enhancer regions, thereby controlling the transcription of inducible genes (Baeuerle & Baltimore, 1996; Baeuerle & Henkel, 1996; Saliou *et al*. 2001). Importantly, activated NF-κB has been identified *in situ* in human atherosclerotic plaques, but not in cells of normal vessels devoid of atherosclerosis (Brand *et al*. 1996), as well as in an arterial injury model (Lindner & Collins, 1996). Furthermore, NF-κB is activated by an atherogenic diet (Liao *et al*. 1993), and by oxidised LDL (Brand *et al*. 1997) and advanced glycation endproducts (Yan *et al*. 1994). Cumulatively, these observations suggest a key role for NF-κB in atherogenesis.

A spectrum of key genes known to be involved in the development of atherosclerosis have been shown to be regulated by NF-κB, including the genes encoding for tumour necrosis factor α, interleukin 1, the macrophage or granulocyte colony-stimulating factor, monocyte chemoattractant protein-1 (MCP-1), c-myc and the adhesion molecules vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1; Rimbach *et al*. 2000; Collins & Cybulski, 2001). In the early stages of an atherosclerotic lesion different types of cells (macrophages, smooth muscle cells and endothelial cells) interact, causing a loss of homeostasis and a self-propagating system leading to dysfunction and lesion development in the artery wall (Rimbach *et al*. 2001). Fig. 3 shows a sketch of the regulation of NF-κB activation; some of the major genes involved in atherogenesis are also listed.

Several lines of evidence, including the inhibition caused by various antioxidants, suggest that NF-κB is subject to redox regulation. Since it has a pivotal role in the inflammatory response, a major effort has focused on developing therapeutic agents that regulate NF-κB activity. In this scenario vitamin E may play an important role, either by directly affecting key steps in the activation pathway of NF-κB, or by modulating the intracellular redox status which is, in turn, one of the major determinants of NF-κB activation. Consistent experimental data is accumulating to suggest that the anti-inflammatory properties of vitamin E are in part due to its ability to down regulate NF-κB. Suzuki & Packer (1993) examined the effect of vitamin E derivatives on tumour necrosis factor α-induced NF-κB activation. Incubation of human Jurkat T-cells with tocopheryl acetate or α-tocopheryl succinate exhibited a concentration-dependent inhibition of NF-κB activation. Similarly, gel-shift studies with the macrophage cell line THP-1 pretreated with α-tocopheryl succinate and then activated with lipopolysaccharide showed an inhibition of NF-κB activity by 43 % at 50 μM α-tocopheryl succinate *v*. the α-tocopheryl succinate-untreated group (Nakamura *et al*. 1998). However, α-tocopherol had no effect on NF-κB activity. Vitamin E transport was analysed in this study by simultaneous determination of vitamin E and its derivatives using HPLC. The vitamin E recovered from culture pellets showed that approximately the same amounts of α-tocopherol and α-tocopheryl succinate had been transferred, and both vitamin derivatives were recovered in the unchanged form. These observations indicate that unchanged α-tocopheryl succinate may itself inhibit NF-κB activation and/or translocation to the nucleus.

α-Tropomyosin, cell adhesion proteins, chemokines and scavenger receptors

An involvement of tropomyosin in the progression of restenosis has been suggested (Kocher *et al*. 1991). Early after balloon injury smooth muscle cells that have migrated into the intima contain decreased amounts of tropomyosin, whereas late after balloon injury tropomyosin returns

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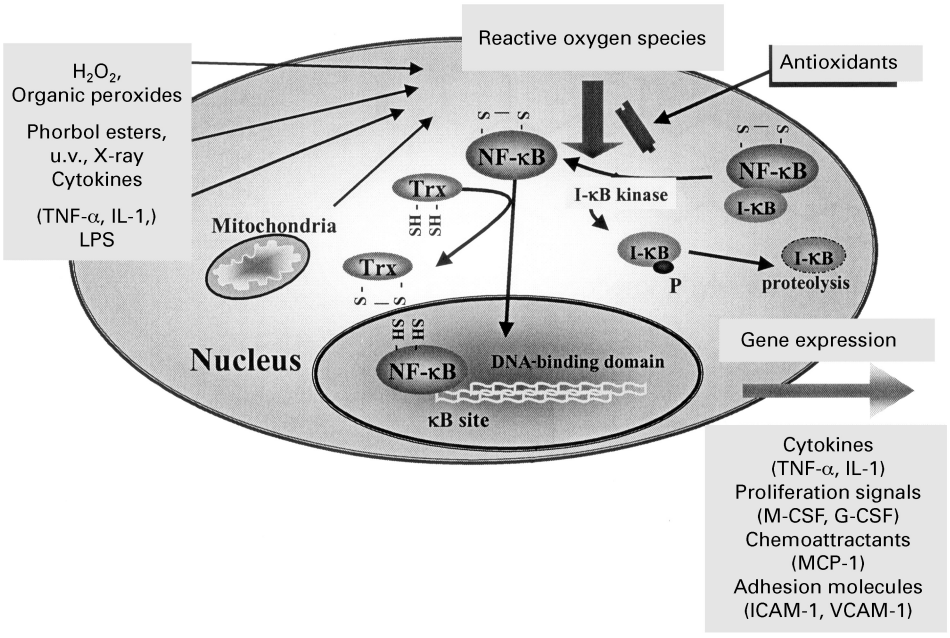

Fig. 3. Regulation of nuclear factor kappa B (NF-κB) activity. TNF-α, tumour necrosis factor α; IL-1, interleukin 1; M-CSF, G-CSF, macrophage and granulocyte colony-stimulating factor respectively; MCP-1 monocyte chemoattractant protein-1; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; TRX, thioredoxin; I-κB, inhibitory unit of NF-κB; κB, kappa B.

towards normal values. Aratri *et al*. (1999) reported induction of α-tropomyosin expression in rat vascular smooth muscle cells by α-tocopherol using differential display techniques. No significant changes in mRNA levels were observed when ß-tocopherol was used. The authors suggest that the overexpression of tropomyosin induced by α-tocopherol may decrease the contractility of smooth muscle cells, and hence form the molecular basis for the hypotensive effect of vitamin E.

Activation of endothelial cells results in release of vascular cytokines such as interleukin 1 and tumour necrosis factor α. These cytokines in turn induce the expression of cell surface adhesion molecules such as VCAM-1 and ICAM-1, which are centrally involved in the endothelial recruitment of neutrophils (Cybulski & Gimbrone, 1991). Focal expression of ICAM-1 and VACM-1 has been reported in arterial endothelium overlying early foam cell lesions in both dietary and genetic models of atherosclerosis in rabbits (Thiery *et al*. 1996). This expression, together with the activation of MCP-1, leads to infiltration of mononuclear cells into the wall and it is widely supposed to result in an increase in the oxidation and scavenging of LDL, formation of lipid-laden foam cells and development or progression of atherosclerotic plaques (Rubanyi, 1993).

As mentioned earlier, transcription of ICAM-1, VCAM-1 and MCP-1 is dependent, at least in part, on the activation of NF-κB. Cell culture studies have shown that treatment of endothelial cells with oxidised LDL increases expression of mRNA and proteins levels of ICAM-1 and VCAM-1 (Yoshida *et al*. 2000). However, pretreatment with α-tocopherol reduced cell adhesion protein expression in a dose-dependent manner. Consistent with this finding, adherence of polymorphonuclear leucocytes or mononuclear leucocytes to endothelial cells activated by oxidised LDL (which is much higher than adherence to unstimulated endothelial cells) was reduced by supplementation of the endothelial cells with α-tocopherol. Furthermore, interleukin 1β-induced production of MCP-1 was dose-dependently suppressed by enrichment of human endothelial cells with vitamin E (Zapolska-Downar *et al*. 2000). From this and other studies it is suggested that the putative anti-atherogenic effect of α-tocopherol may in part be due to a down-regulation of cell adhesion proteins and chemokines. Despite evidence that vitamin E down regulates cell adhesion proteins *in vitro*, *in vivo* evidence is currently lacking.

Ricciarelli *et al*. (2000) have recently demonstrated that the CD36 scavenger receptor, which transports oxidised LDL into the cytosol, is expressed in human smooth muscle cells. Interestingly, α-tocopherol inhibited the uptake of oxidised LDL by a mechanism involving down-regulation of CD36 mRNA and protein expression. It is hypothesised that beneficial cardiovascular effects of α-tocopherol are at least in part mediated by lowering the uptake of oxidised LDL, which subsequently results in a reduction in foam cell formation.

Nitric oxide and platelet aggregation

NO produced by the endothelial NO synthase is a pivotal molecule in the regulation of vascular tone. Additionally, its production suppresses expression of pro-inflammatory cytokines, adhesion molecules (De Caerina *et al*. 1995) and MCP-1 (Busse & Fleming, 1995). It also inhibits platelet

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adhesion to the endothelium (De Graaf *et al*. 1992), can modify the permeability of the arterial wall (Cardonna-Sanclemente & Born, 1995), suppresses vascular smooth muscle cell proliferation and migration (Garg & Hassid, 1989), and can act as an antioxidant (Patel *et al*. 2000). The major risk factors for atherosclerosis (age, Matz *et al*. 2000; hypercholesterolaemia, Stroes *et al*. 1995; diabetes, Williams *et al*. 1996; hypertension, Panza *et al*. 1995; smoking, Celermajer *et al*. 1993; low birth weight, Leeson *et al*. 1997), are all associated with impaired NO activity, often before appreciable disease develops. In rabbits inhibition of NO production accelerates experimental atherosclerosis (Naruse *et al*. 1994), whilst increases in NO synthesis reduce it (Cooke *et al*. 1992). Importantly, NO inhibits NF-κB (Matthews *et al*. 1996). This effect may account for its influence on the transcription of genes for adhesion proteins, MCP-1 and others. The postulation of key roles for both NO and NF-κB is therefore not self-contradictory.

There is evidence from studies in rabbits that vitamin E reverses the well-established deleterious effects of hypercholesterolaemia on NO activity. Stewart-Lee *et al*. (1994) found that relaxation in response to acetylcholine, an NO-dependent phenomenon, in the carotid artery was reduced after 4 weeks of diet-induced hypercholesterolaemia, but was restored by the addition of 2 g α-tocopheryl acetate/kg to the diet. Andersson *et al*. (1994) obtained a similar result for the coronary circulation. The mechanisms underlying this effect are a matter of controversy. It has been suggested that inactivation of NO by reactive oxygen species is increased during hypercholesterolaemia and reduced by vitamin E (Andersson *et al*. 1994; Stewart-Lee *et al*. 1994). However, Böger *et al*. (1998) found that vitamin E did not reduce reactive oxygen species release by aortic tissue from cholesterol-fed rabbits; instead, they suggested its protective effect on the NO pathway was related to its inhibition of LDL oxidation. Since PKC inhibits NO (Davda *et al*. 1994), another possible mechanism arises from the observation that hypercholesterolaemia increases PKC levels in rabbit aortic smooth muscle, and this effect is reduced by vitamin E (Özer & Azzi, 2000).

Whatever the mechanism, protective effects of vitamin E on NO function might be expected to reduce atherosclerosis in hypercholesterolaemic rabbits. Although many studies have found such an effect (for example, see Williams *et al*. 1992; Böger *et al*. 1998; Schwenke & Behr, 1998), other studies have not (for example, see Freubis *et al*. 1995) and some studies have shown an increase in the extent and severity of lesions (Godfried *et al*. 1989). Keaney *et al*. (1994) obtained an interesting result which may in part account for these discrepancies; while 1 g α-tocopherol/kg diet protected against the inhibitory effect of hypercholesterolaemia on the NO pathway, 10 g α-tocopherol/kg diet markedly increased it, and also increased the severity of lesions, despite the fact that the oxidisability of LDL was still reduced. Possible mechanisms include pro-oxidant effects of α-tocopherol, or reactions of α-tocopherol with NO to give the tocopheroxyl radical (Keaney *et al*. 1994).

Li *et al*. (2001) studied the effect of different isoforms of vitamin E on NO activity and platelet aggregation in human platelet-rich plasma. All three isoforms tested (α-, β- or δ-tocopherol) markedly decreased ADP-induced platelet aggregation and increased NO release in a dose-dependent manner. The isoforms did not affect constitutively-expressed NO synthase protein expression, but increased constitutively-expressed NO synthase phosphorylation. Furthermore, it has been demonstrated in human subjects that oral supplementation with α-tocopherol (400–1200 mg/d) resulted in an increase in platelet tocopherol concentration that correlated with marked inhibition of polymorphonuclear leucocyte-mediated platelet aggregation (Freedman *et al*. 1996). Platelets derived from these subjects also demonstrated apparent complete inhibition of PKC. These findings represent another potential mechanism by which tocopherol could prevent the development of coronary artery disease.

Differential gene expression

Microarray technology enables us to investigate genes differentially expressed in response to an antioxidant treatment, thereby offering the possibility of greater insight into the biological properties of antioxidants (Watanabe *et al.* 2001). To examine the molecular events associated with Se and vitamin E deficiency in rats, cDNA array technology has been applied to define the transcriptional response in rat liver after 7 weeks on a Se- and/or vitamin E-deficient diet (Fischer *et al.* 2001). Atlas™ Rat cDNA Toxicology Array II from Clontech (Oxford, UK) was used to monitor simultaneously the expression of 465 genes (Table 2); a change of ≥2-fold was considered significant ($P<0{\cdot}05$). Vitamin E deficiency alone did not significantly affect any of the genes monitored. Of course, other genes not present on the cDNA membrane could have been differentially regulated by vitamin E. Additionally, tissues other than liver might be more susceptible to vitamin E-induced changes in differential gene expression.

In addition to a 13·9-fold down-regulation of the cellular glutathione peroxidase gene, Se deficiency alone was accompanied with an increase in the expression of UDP-glucuronosyltransferase 1 and bilirubin UDP-glucuronosyltransferase isoenzyme 2. These two enzymes are known to have an important function in the detoxification of xenobiotics in liver. Similarly, rat liver cytochrome P450 4B1, which is also involved in xenobiotic metabolism and inducible by glucocorticoids, was induced 2·3-fold. The mRNA levels of arachidonate 12-lipoxygenase were 2·4-fold higher in Se-deficient animals than in controls. It has been shown that arachidonate 12-lipoxygenase and phospholipid hydroperoxide glutathione peroxidase are opposing enzymes balancing the intracellular concentration of hydroperoxy lipids; an inhibition of phospholipid hydroperoxide glutathione peroxidase activity increases the enzymic catalysis of arachidonate 12-lipoxygenase (Chen *et al.* 2000).

In combined Se and vitamin E deficiency, 5 % of all genes monitored were differentially expressed. The double deficiency was characterised by down-regulation of genes that inhibit programmed cell death, including defender-against-cell-death 1 protein, inhibitor of apoptosis protein 1 and Bcl2-L1. Furthermore, the expression level of early-growth-response protein 1, known as a suppressor of growth and transformation and an inducer of apoptosis, was

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Table 2. Selection of selenium and vitamin E deficiency-related changes (Δ–Se–E) in gene expression in rat liver

GenBank accession	Δ–Se–E (fold)	Gene	Function
Apoptosis or cell cycle:			
Y13336	↓ **2.0**	Defender against cell death 1 protein	Protection against apoptosis
AF081503	↓ **2.6**	Inhibitor of apoptosis protein 1	Protection against apoptosis
U72350	↓ **3.2**	Bcl2-L1	Protection against apoptosis
M22413	↓ **2.0**	Carbonic anhydrase III	Antioxidant, protection against apoptosis
D90345	↓ **2.2**	T-complex protein 1 alpha subunit	Chaperone, folding of proteins
X82021	↓ **2.2**	HSC70-interacting protein	Stabilisation of the chaperone HSC70
J03969	↓ **2.9**	Nucleophosmin	Stimulation of normal cell growth
D14014	↓ **3.1**	G1/S-specific cyclin D1	Initiation of cell cycle, oncogene
J04154	↑ **2.1**	Early growth response protein 1	Suppression of growth and induction of apoptosis
U77129	↑ **2.0***	SPS1/Ste20 homologue KHS1	Transducer of signals in Mitogen-activated protein-kinase pathway
Antioxidant defense or stress response or inflammation:			
X12367	↓ **18.8**	Cellular glutathione peroxidase I	Peroxide detoxification
J05181	↓ **3.4**	γ-Glutamylcysteine synthetase	Glutathione synthesis
U22424	↓ **2.2**	11-β-Hydroxysteroid dehydrogenase 2	Conversion of corticosterone into 11-dehydrocorticosterone
L49379	↓ **2.3**	Multispecific organic anion exporter	Detoxification, export of leukotriene C_4
J02608	↑ **15.3***	DT-diaphorase	Xenobiotic metabolism
D00753	↑ **2.1**	Serine protease inhibitor-3	Acute-phase protein
J00696	↑ **2.3**	α-1 Acid glycoprotein	Acute-phase protein
J00734	↑ **2.3**	Fibrinogen γ chain	Acute-phase protein
S65838	↑ **3.6**	Metallothionein 1	Acute-phase protein, antioxidant

↓down-regulation; ↑, up-regulation.
*Gene signal at background level in one array.

increased 2-fold. Carbonic anhydrase III, which was reported recently to play a role as an antioxidant preventing H_2O_2-inducible apoptosis (Raisanen *et al.* 1999), was down regulated 2-fold. A stronger tendency towards negative cell cycle progression in livers of double-deficient rats was further suggested by the down-regulation of nucleophosmin and G1/S-specific cyclin D1, which has been characterised as an important signal in anti-apoptotic mechanisms.

Combined Se and vitamin E deficiency also resulted in an induction of acute-phase proteins (metallothionein, DT-diaphorase, alpha-1 acid glycoprotein) and serine proteinase inhibitor-3. A further indication of pro-inflammatory responses in rats fed diets deficient in Se plus vitamin E is that they exhibited higher expression of the fibrinogen γ chain, which has been shown to be up regulated in the rat liver during inflammation. The induction of pro-inflammatory genes was accompanied by a concerted depression of the anti-inflammatory enzyme 11-β-hydroxy-steroid dehydrogenase 2, which converts the glucocorticoid corticosterone to its inactive 11-dehydro form in the rat, thereby controlling glucocorticoid access to receptors.

Analysis of differential gene expression in the endothelium is critical to our understanding of the sequence of events leading to the formation of atherosclerotic lesions. In order to address this question and gain a more comprehensive overview of the molecular mechanisms involved in the contribution of oxidised LDL to the pathophysiology of atherosclerosis, we determined a global gene expression profile in primary human endothelial cells in the presence and absence of oxidised LDL using high-density cDNA membranes. Gene expression analysis again focused on mRNA that showed >2-fold change in their expression level. Employing this criterion seventy-eight of 588 genes were differentially expressed. Oxidised LDL altered the expression of genes encoding for transcription factors (e.g. GATA-2), cell receptors (e.g. advanced glycation endproduct-related receptor precursor), adhesion molecules (e.g. P-selectin), extracellular matrix proteins (e.g. matrix metalloproteinase 9) and enzymes involved in cholesterol metabolism (e.g. farnesyltransferase β). Interestingly, in primary human endothelial cells some of the genes, which were up regulated by oxidised LDL, were down regulated by vitamin E. The experimental strategy identified several novel oxidised LDL- and vitamin E-sensitive genes. Cardiovascular specific DNA arrays are an important platform for obtaining a global genetic portrait and understanding the complex molecular events leading to atherosclerosis.

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