OXIDANTS, ANTIOXIDANTS AND CARDIOVASCULAR DISEASE

G. G. DUTHIE, K. W. J. WAHLE AND W. P. T. JAMES
Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB

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INTRODUCTION

Cardiovascular disease (CVD) remains the major cause of death in the United States and Western Europe and occlusion of the coronary arteries by the atheromatous plaque accounts for most of these deaths (Ross, 1986; Slattery & Randall, 1988). Demographic differences in the incidence of CVD have been interpreted as implicating diet and cholesterol in the pathogenesis of the disease, but results of intervention trials to reduce serum cholesterol by dietary or pharmacological means are equivocal (Grundy, 1986; Reckless, 1987; Rifkind, 1987; Tyroler, 1987; James & Ralph, 1988; McCormick & Strabanek, 1988; Waterlow, 1988). Gey (1986) estimated that the major recognized risk factors for CVD, namely smoking, hypercholesterolaemia and hypertension, only account for 50–60% of the variance in the occurrence of CVD. Consequently, there is scope for the inclusion of other dependent and independent risk factors which may contribute to the apparent multifactorial nature of the disease. The purpose of the present article is to review recent evidence implicating reactive free radicals in the development of CVD.

BIOLOGICAL IMPLICATIONS OF FREE RADICALS

Free radicals are molecules or molecular fragments with an unpaired electron. The presence of the unpaired electron can convey considerable reactivity to the free radical which by

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hydrogen abstraction can damage a wide range of biological material, including DNA, nucleotide co-enzymes, proteins and lipids (Slater, 1984). Polyunsaturated fatty acids are particularly susceptible to free-radical-mediated peroxidation leading to disturbances in membrane structure and function (Slater *et al.* 1987). Free radicals have been implicated in the aetiology of many diseases, including tumour formation, toxic liver injury, neuromuscular disorders, arthritis, iron overload and gastrointestinal and liver disorders (Halliwell & Grootveld, 1987). However, indications of free radical activity do not necessarily implicate free radicals as the prime cause of the disease. Tissue injury is liable to increase free radical reactions and consequently the presence of free radicals may arise as only a secondary consequence of the original disease state (Halliwell, 1987).

Endogenous production of free radicals occurs during normal aerobic metabolism. Activated oxygen intermediates are formed by stepwise reduction of O_2 to water and by secondary reactions with protons and transition metals such as Fe and Cu (Byczkowski & Gessner, 1988). The superoxide anion (O_2^-) is produced by many cell redox systems including ischaemia-derived xanthine oxidase (*EC* 1.1.3.22), aldehyde oxidase (*EC* 1.2.3.1) and membrane-associated NADPH oxidases (Fridovich, 1983). About 1–4% of the total O_2 uptake by mitochondria may be used for O_2^- production and about 20% of O_2^- produced intramitochondrially may be ejected into the cell (Forman & Boveris, 1982). In addition, phagocytic cells, including macrophages and monocytes, increase their O_2 uptake when stimulated and release large amounts of O_2^- into the extracellular fluid through the action of NADPH oxidase (Klebanoff, 1982). Although O_2^- is not particularly reactive, having a low second-order rate constant with biomolecules, it is capable of diffusing through relatively large distances through the cell where, in the presence of Fe and Cu, a metal-catalysed Haber–Weiss reaction is thought to occur resulting in the formation of the highly reactive hydroxyl radical ($OH^\cdot$) (Slater, 1984).

$$O_2^- \xrightarrow{\text{diffusion}} O_2^- \xrightarrow[\text{dismutase (EC 1.15.1.1)}]{\text{superoxide}} H_2O_2 \xrightarrow{Fe^{2+} \text{ or } Cu^{2+}} OH^\cdot \longrightarrow \text{damage}$$

Although Fe and Cu are transported and stored in specific proteins which minimize their reaction with reduced O_2 metabolites, under appropriate conditions such metal complexes are likely catalysts for in vivo $OH^\cdot$ production (Koster & Slee, 1986). $OH^\cdot$ will react, in the immediate environment where it is produced, with most biological material (Slater, 1984) and these radicals are the major protagonists in the superoxide theory of O_2 toxicity (Fridovich, 1983). This suggests that while O_2 is essential for life, aerobic life forms persist despite their requirement for O_2 (Bast, 1986). As well as being derived from O_2 metabolism, potentially injurious free radicals are also present in pollutants, halogenated anaesthetics, and are generated by ionizing radiation (Halliwell & Gutteridge, 1985). For instance, each puff of a cigarette contains 10^{14} free radicals in the gas phase and 10^{15} in the tar phase (Church & Pryor, 1985), indicating that smokers are under a high and sustained free radical load which may cause tissue damage (Duthie *et al.* 1989).

Due to their short half-lives, relatively low concentrations and the ethical and logistical considerations in obtaining samples, the direct detection of free radicals in human tissue by electron-spin-resonance techniques is not usually feasible (Slater *et al.* 1987). Consequently, the presence of by-products of free radical-mediated damage to biomolecules in biological fluids is often used as an indirect measure of free radical stress. In particular, free radical-mediated peroxidation of polyunsaturated fatty acids yields a wide range of products (Slater, 1984). Many of these products, such as lipid hydroperoxides, conjugated dienes, malonaldehyde, fluorescent products, ethane and pentane are used to assess susceptibility to free radical activity. However, these indices can be non-specific and their production

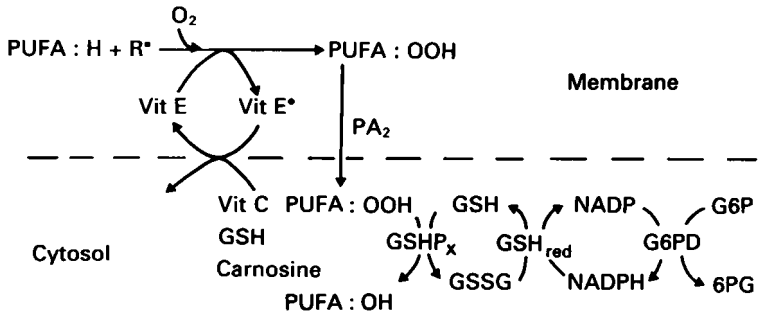

Fig. 1. Some of the major antioxidant defence mechanisms within the cell. PUFA:H, polyunsaturated fatty acid; PUFA:OOH, fatty acid hydroperoxide; PUFA:OH, fatty acid hydroxide; GSH, reduced glutathione; GSSG, oxidized glutathione; $GSHP_x$, glutathione peroxidase (*EC* 1.11.1.9); PA_2, phospholipase A_2 (*EC* 3.1.1.4); GSH_{red}, glutathione reductase (*EC* 1.6.4.2); G6PD, glucose-6-phosphate dehydrogenase (*EC* 1.1.1.49); 6PG, 6-phosphogluconoate; R•, free radical.

does not necessarily relate directly to lipid peroxidation (Halliwell & Gutteridge, 1985). For example, conjugated dienes may arise from dietary sources unrelated to free radical-mediated lipid peroxidation (Iversen *et al.* 1985) and the frequently used thiobarbituric acid test for malonaldehyde can react with other substances (Wade & van Rij, 1988). Expired ethane and pentane, measures of lipid peroxidation of ω-3 and ω-6 fatty acids in vivo, may also arise through processes not related to free radical-mediated tissue damage, such as production by intestinal organisms and flushing from adipose tissue, and pentane may be metabolized by the liver before its detection in the expired breath (Duthie *et al.* 1987). As yet, no ideal method exists to assess free radical activity in humans, and results need to be interpreted with caution. New methods involving detection of by-products of free radical oxidation of uric acid and DNA in body fluids may offer alternative and more specific assessment of oxidant stress (Halliwell & Grootveld, 1987).

ANTIOXIDANT DEFENCE MECHANISMS

Living organisms possess a range of mechanisms to protect themselves from the potentially injurious effects of free radicals. Vitamin E is the major lipid-soluble antioxidant breaking the chain of lipid peroxidation in cell membranes and preventing the formation of lipid hydroperoxides (Halliwell, 1987). If formed, potentially toxic lipid hydroperoxides are released from the cell membrane by phospholipase A_2 (*EC* 3.1.1.4) (van Kuijk *et al.* 1987) and degraded by selenium-dependent glutathione peroxidase (*EC* 1.11.1.9). Hydrogen peroxide formed from O_2^- by superoxide dismutase is prevented from forming $OH^•$ by intervention of glutathione peroxidase and catalase (*EC* 1·11·1·6). β-Carotene has been recently suggested to have an antioxidant function (Willson, 1987). Vitamin C in addition to directly scavenging free radicals in the cytoplasm may also participate in the regeneration of vitamin E and the antioxidant peptide, glutathione (Willson, 1987). In plasma, uric acid and proteins have pronounced antioxidant potential (Wayner *et al.* 1987) and peptides such as carnosine and homocarnosine (Kohen *et al.* 1988) may be important antioxidants in muscle tissue. A representation of the antioxidant defence mechanisms of the cell is shown in Fig. 1.

The effectiveness of the antioxidant defence system is dependent on adequate dietary intake of foods containing antioxidants such as vitamins E and C and the metal cofactors required for antioxidant enzymes. The antioxidant status of the body can be considerably

influenced by diet (Slater *et al.* 1987), and should the normal defence mechanisms be weakened by nutritional deficiencies then pathognomonic consequences may occur. Recent evidence suggests that a relative deficiency in antioxidants in conjunction with relatively high concentrations of peroxidizable substrate may potentially have an important role to play in the development of the atheromatous plaque.

EXPERIMENTAL EVIDENCE IMPLICATING FREE RADICALS IN ATHEROSCLEROSIS

LIPID HYDROPEROXIDES

It is generally accepted that damage to the arterial endothelium is a prerequisite for subsequent development of the occlusive atheromatous plaque but the cause of the initial lesion is not known (Dodson & Horton, 1987). Lipid hydroperoxides derived from free radical-mediated oxidation of polyunsaturated fatty acids are capable of causing such damage. Monolayers of porcine pulmonary artery endothelial cells are irreversibly damaged by linoleic acid hydroperoxides (18:2-OOH) as judged by enhanced albumin transfer across the monolayer (Hennig *et al.* 1986). Such damage is inhibited by previous incubation of the cells with vitamin E (Hennig *et al.* 1987). In addition, when serum lipid hydroperoxide levels of rabbits are elevated by injection of 18:2-OOH into the bloodstream, marked damage and denudation of aortic endothelial cells is observed (Yagi, 1987). Aggregation of platelets around the sites of injury was also observed. Platelets may participate in the early stages of the atheroma (Betteridge, 1987). As prostacyclin production, which normally impairs platelet aggregation and adherence, is inhibited by lipid hydroperoxides (Moncada *et al.* 1976) conditions appropriate for initiating atherogenesis may arise. It is possible that a relatively low antioxidant status could lead to raised serum lipid hydroperoxides. Formation of lesions and platelet adherence may then take place. Interestingly, serum lipid hydroperoxide concentrations in males increase with age, achieving a plateau between 41 and 50 years (Yagi, 1987). Women, who have a much lower incidence of CVD, do not have such a marked age-related increase in serum hydroperoxides (Yagi, 1987). High lipid hydroperoxide concentrations in serum are also found in diabetics, a high CVD risk group (Yagi, 1987). Although suggestive, such observations do not prove a causal relationship between lipid hydroperoxides and atheroma. Similarly, reported increases in lipid hydroperoxides in patients with diagnosed atherosclerosis (Miki quoted by Yagi, 1987) do not necessarily mean that lipid hydroperoxides have caused the disease. Such increases may have arisen as a secondary consequence of the disease state.

There is much interest in the prophylactic effect of polyunsaturated fatty acids on CVD (Bang, 1988), particularly in relation to ω-3 fatty acids in fish oils. However, the relative ease of fatty acid peroxidation both by endogenous free radical activity and through oxidation in the gastrointestinal tract may be detrimental in subjects of marginal and low antioxidant status due to the formation of lipid hydroperoxides. Polyunsaturated fatty acids may be atherogenic (Diplock, 1987).

OXIDATION OF LOW-DENSITY LIPOPROTEINS

The appearance of lipid-laden foam cells at the site of injury in the blood vessel wall contributes to the growth of the atheromatous plaque. Such cells are derived from monocyte–macrophages and arterial smooth muscle cells (Mitchison & Ball, 1987; Quinn *et al.* 1987). The processes involved in the formation of foam cells are not completely

understood but uptake of oxidized low-density lipoproteins (LDL) is a major factor. LDL have been implicated in atherosclerosis for many years (Rudel *et al.* 1986). LDL, in addition to being a major carrier of cholesterol in the bloodstream (Brown & Goldstein, 1986) is also a major carrier of vitamin E. Despite the presence of the antioxidant, LDL is susceptible to oxidation, presumably because it contains large amounts of unsaturated fatty acids (Heinecke, 1987). The content of vitamin E is markedly decreased in oxidized LDL (Jurgens *et al.* 1987). Compared to native LDL, oxidized LDL exhibits a number of properties which are potentially atherogenic. These include recognition and preferential uptake by scavenger receptors of macrophages, chemotactic responses with respect to other monocytes–macrophages and regulation of a platelet-derived growth factor produced by endothelial cells (Jurgens *et al.* 1987). Monocytes may be attracted to the site of injury on the artery wall by mechanisms as yet unclear (Quinn *et al.* 1987). Uptake of oxidized LDL by monocytes promotes chemotactic attraction of monocytes–macrophages in vitro. However, on phenotypic transformation of monocytes to macrophages, oxidized LDL appears to inhibit macrophage motility, thus potentially reducing their ability to migrate away from the arterial wall. The enhanced rate of uptake of oxidized LDL may then convert macrophages into foam cells (Steinbrecher *et al.* 1984; Heinecke, 1987; Quinn *et al.* 1987; Hennig & Chow, 1988). Because the macrophage itself can oxidatively modify native LDL, autocatalytic progression may lead to the continuous growth of the atheroma (Quinn *et al.* 1987). Lipid hydroperoxides also appear to enhance the incorporation of oxidized LDL into cultures of endothelial cells and macrophages (Hennig & Chow, 1988), thus potentially contributing to plaque formation. In addition, oxidized LDL is capable of damaging endothelial cells directly, thus possibly further exacerbating growth of plaques (Heinecke, 1987).

The mechanisms by which LDL is oxidized are not precisely defined. Although LDL can be modified by lipoxygenases and phospholipase A_2 (Sparrow *et al.* 1988), major contenders for LDL oxidation in vivo are oxygen radicals. Sources of these oxygen radicals are likely to be macrophages and endothelial cells. Hiramatsu *et al.* (1987) showed that human monocytes stimulated to produce O_2^- readily oxidized LDL. O_2^- appeared to be the initiator of oxidation as the reaction was inhibited by the early addition of superoxide dismutase. However, once initiated, oxidation was O_2^- independent, suggesting that other unknown factors were involved in the subsequent propagation process. Oxidation was also dependent on the presence of transition metals, implicating $OH^{\cdot}$ in the propagation process.

Cultures of endothelial and smooth muscle cells are also capable of oxidizing LDL by free radical-mediated mechanisms (Steinbrecher *et al.* 1984). LDL modification is directly proportional to the rate of O_2^- production (Heinecke *et al.* 1986). The biochemical bases for O_2^- production and LDL oxidation by endothelial and smooth muscle cells are unclear but appear to be dependent on the presence of L-cystine (Heinecke, 1987). Other possible sources of O_2^- are through the activity of xanthine oxidase and the NADPH cytochrome P-450 reductase-dependent reactions in microsomes (Hennig *et al.* 1987).

Whether or not oxidized LDL contributes to atherogenesis remains a matter of speculation. However, oxidized lipids have been detected in atherosclerotic plaques (Glavind *et al.* 1952), suggesting that some of the in vitro observations described previously may occur in vivo. The possibility that a low nutritional antioxidant status predisposes the individual to develop atherosclerosis due to enhanced oxidation of LDL requires further study. However, the scheme in Fig. 2 summarizes a potential mechanism for the development of the atheroma.

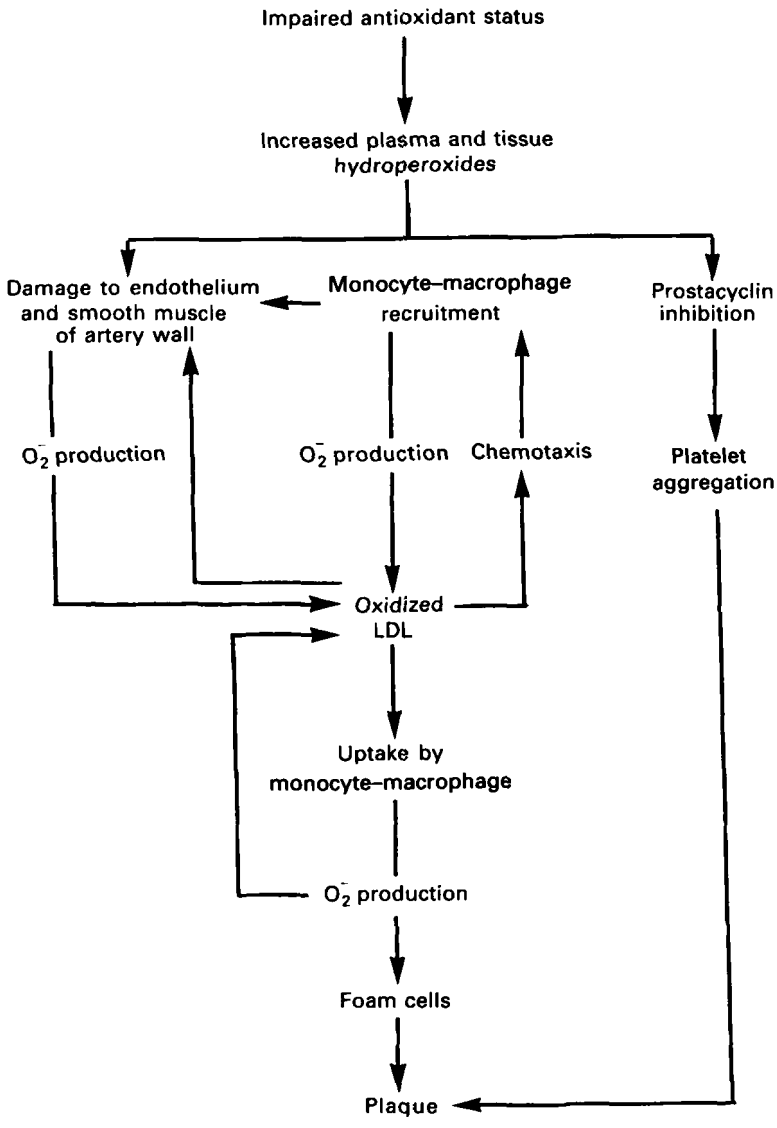

Fig. 2. Flow diagram of possible interactions contributing to the development of the atheromatous plaque. For detailed explanation see p. 55. LDL, low-density lipoprotein; O_2^-, superoxide anion.

EPIDEMIOLOGICAL EVIDENCE FOR FREE RADICAL INVOLVEMENT IN CARDIOVASCULAR DISEASE

Epidemiological studies may not provide mechanisms for the development of disease but can indicate correlations which may be worthy of causal investigation. Interpretation of epidemiological results may also lead to contention. McCormick & Strabanek (1988) point out that to date over 246 risk factors have been identified for CVD, many of which are presumably spurious. Bearing this in mind, recent epidemiological evidence suggests that

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inverse correlations between antioxidants and mortality from CVD do exist. Standardized mortality from CVD is statistically related to consumption of fruit and green vegetables from which vitamin C intake can be interpolated (Palgi, 1981; Acheson & Williams, 1983) and to plasma vitamin C (Gey *et al.* 1987*b*). Gey (1986) and Gey *et al.* (1987*a*) in cross-cultural investigations found that the cumulative 'antioxidant index',

$$\frac{\text{cholesterol}}{\text{vitamin A} \times \text{vitamin E (stand)} \times \text{vitamin C} \times \beta\text{-cartotene} \times \text{Se}'}$$

derived from actual measurements of the nutrients in plasma was strongly and negatively correlated with age-standardized mortality for CVD. Recent provisional information involving the determination of antioxidants in plasma of middle-aged men from twelve European populations reveals significant correlations between age-specific mortality and lipid-standardized vitamin E and vitamin A (r^2 0·49 and 0·33 respectively; Gey & Puska, 1988). On the basis of such observations, Diplock (1987) has suggested that the recommended daily allowances for vitamin E and vitamin C should be increased 3–5-fold and 2-fold respectively. Intervention trials involving supplementation with antioxidant vitamins would appear to be warranted. However, it should be noted that cohort studies, which may be of greater value than correlational studies, have found no association between vitamin E and CVD (Salonen *et al.* 1985; Kok *et al.* 1987).

ANTIOXIDANTS AND CARDIOVASCULAR DISEASE: INDIRECT EVIDENCE

Evidence that mega-dose supplementation with antioxidants provides effective treatment for atherosclerosis is anecdotal, ill-controlled and lacking in scientific consistency (Walquist & Flint, 1983; Diplock, 1987). However, some indicative experimental results are worth mentioning.

VITAMIN E

Wilson *et al.* (1978) found that addition of 10 g vitamin E/kg to the diets caused a marked reduction in aortic and coronary atherosclerosis in rabbits. Similar effects were not apparent when diets were supplemented with antioxidants such as butylated hydroxytoluene suggesting that the effect of vitamin E was not related to its function as an antioxidant.

Smokers, a CVD high-risk group in Northern latitudes, show a response to vitamin E supplements. Smokers' alveolar fluid is deficient in vitamin E (Pacht *et al.* 1986). A potential consequence of an impaired antioxidant content of the lungs is enhanced peroxidation of erythrocyte membranes in transit through the alveolar capillaries. Duthie *et al.* (1989) found that erythrocytes of smokers were more susceptible to hydrogen peroxide-induced peroxidation in vitro compared with those of non-smokers. This effect was abolished when smokers were supplemented with 1 g vitamin E/d for 2 weeks. Similarly, the increased pentane expiration observed in smokers is reduced by vitamin E supplements (Shariff *et al.* 1988). Plasma indices of lipid peroxidation are also increased in smokers (Duthie *et al.* 1989). Smokers appear to be particularly susceptible to free radical activity which may lead to oxidation of LDL and plaque development. Further, although a strong correlation exists between number of cigarettes smoked and incidence of CVD in Northern Europe, such a relationship is less marked in Southern Europe and Japan (Waterlow, 1988). This may reflect differences in consumption of food containing antioxidants such as vitamin E.

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Table 1. *Epidemiological studies: cardiovascular disease (CVD) and selenium*

Reference	Type of study	Country	Comments
Salonen *et al.* (1982)	Case-control within prospective follow up	Finland	2–3-fold increase in CVD mortality when serum Se < 45 μg/l
Miettinen *et al.* (1983)	Case-control within prospective follow up	Finland	No clear associations between Se and CVD.
Virtamo *et al.* (1985)	Cohort	Finland	Possible relationship between low Se and stroke
Salonen *et al.* (1985)	Case-control within prospective follow up	Finland	No clear associations between Se and CVD
Kok *et al.* (1987)	Case-control within prospective follow up	Netherlands	No clear associations between Se and CVD
Ringstad *et al.* (1987)	Case-control	Norway	Low Se not associated with excess risk of CVD
Robinson & Thomson (1983)	Correlational	New Zealand	No association
Ellis *et al.* (1984)	Correlational	England	No association

VITAMIN C

Cigarette smokers have lower plasma vitamin C concentrations (Pelletier, 1968; Duthie *et al.* 1989) and possibly abnormal ascorbate turnover (Kallner *et al.* 1981) than non-smokers. However, vitamin C has many biochemical functions (Levine, 1986). Whether or not the lower ascorbate concentrations in smokers reflect increased oxidant stress is not certain. In guinea-pigs, marginal vitamin C deficiency is associated with the appearance of intimal plaques (Sulkin & Sulkin, 1975). Further, leucocyte ascorbic acid is significantly lower in patients with atherosclerosis compared with controls (Ramirez & Flowers, 1980). Vitamin C concentrations are also low in aortic tissue from patients with atherosclerotic occlusive disease (Dubick *et al.* 1987). Such observations, although indicative, do not directly implicate vitamin C as an aetiological factor in CVD, nor do they necessarily invoke its function as a biological antioxidant.

SELENIUM

Although not in itself an antioxidant, Se is the essential metal cofactor for the activity of the antioxidant enzyme, glutathione peroxidase (Rotruck *et al.* 1973). Therefore, a low Se status could theoretically lead to increased intimal damage by lipid hydroperoxides and increased susceptibility of LDL to oxidation. In addition, Se deficiency may also lead to impaired prostacyclin synthesis (Schoene *et al.* 1986), thus promoting platelet aggregation. However, findings from studies relating blood Se levels to CVD are equivocal (Ringstad, 1988). For example, Salonen *et al.* (1982) found an inverse association between serum Se and risk of death from CVD, whereas no such association was observed by Miettinen *et al.* (1983) and Kok *et al.* (1987). In addition, no differences in tissue Se were apparent in patients who died with, or without, myocardial infarction (Ringdal *et al.* 1986). References of studies relating Se and CVD are given in Table 1. Low Se does not appear to be an independent risk factor in countries with intermediate Se intake. Moreover, the Finnish findings suggest that, even for low Se areas, the available evidence is inconsistent. Consequently, although Se deficiency has long been known to cause cardiomyopathies in animals (Burk, 1978) and is associated with Keshan disease in humans (Yang *et al.* 1984), evidence for its involvement in CVD is minimal. In addition, whereas vitamin E is well tolerated in large doses (Bendich & Machlin, 1988), Se is potentially toxic and therapeutic supplementation with Se as a preventative measure is not warranted (Diplock, 1987).

β-CAROTENE

Also known as provitamin A, β-carotene has antioxidant capability. In vitro, small amounts retard oxidation of methyl linoleate. It is particularly effective at low partial pressures of O_2 at which its antioxidant capacity exceeds that of vitamin E (Burton, 1989).

Consequently, β-carotene could assume importance during the ischaemia and reperfusion events that occur during a heart attack. In addition, β-carotene scavenges superoxide in cell cultures (Krinsky, 1989). As the provitamin is transported in plasma mainly in the LDL (Parker, 1989), potentially it could play a protective role in limiting LDL oxidation. As yet there is little evidence available to assess whether increasing the dietary intake of β-carotene by supplementation or by increasing consumption of fruit and vegetables reduces the risk of CVD. However, observations that serum concentrations of β-carotene are greater in women than in men (Parker, 1989) and that the carotenoid intakes of male smokers are only 70–80% those of non-smokers (Ziegler, 1989) suggest that further study of the relationship between carotenoids and CVD is worthwhile.

CONCLUSION

The field of research into CVD is immense, risk factors abound and controversy continues unabated as to the causes of the disease. Therefore, it is with some trepidation that we suggest that free radicals are implicated in the pathogenesis of CVD. Nevertheless, the recent experimental evidence relating to LDL oxidation and the epidemiological correlations between plasma antioxidant concentrations and incidence of CVD indicate that biochemical reactions involving oxidant stress may provide a much needed mechanistic basis for the disease. The possibility that nutritional intervention to increase intake of antioxidants may reduce mortality from CVD requires investigation.

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