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Symposium on 'Diet and CVD'

Antioxidants and CVD

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The involvement of free radicals and reactive oxygen and nitrogen species in the pathology of inflammatory and degenerative disease has been widely accepted, although the centrality of these species to the outcome of these diseases is still a matter for debate. In the case of CVD, and particularly the development of the atherosclerotic plaque, the oxidation of LDL is of particular importance and appears to explain many of the events that occur during the life history of the plaque. The corollary of this situation is that antioxidants must be a benign force to protect the population from the modern scourge of heart disease. In fact, recent evidence from intervention studies with large doses of the antioxidant vitamins and other antioxidants in foods has been very disappointing. Here, the background for the belief that antioxidants ought to be beneficial is examined and an attempt made to explain why the results of these intervention studies have been unsuccessful. It is agreed that a diet rich in fruit and vegetables is protective for both CVD and cancer, but the explanation for this effect may not necessarily lie with the presence of antioxidants.

Atherosclerosis: CVD: Dietary antioxidants: Antioxidant vitamins

Since it was first proposed that free radical production is associated with ageing and the degenerative diseases there has been the idea that dietary antioxidants may make an important contribution to slowing down these processes. During the 1960s these ideas became rather fanciful and extraordinary claims were made for the antioxidant vitamins, particularly vitamin E, that had little scientific merit. Nevertheless, it was not unusual to find small shops in US cities that were devoted to the sale of vitamin E.

This notion took some time to catch on in the UK, but now specialist 'health food' shops, major pharmacy chains and also the major supermarkets are selling such products as part of a multi-billion dollar industry in supplements or nutriceuticals.

It was perhaps not until the discovery of the enzyme superoxide dismutase by Fridovich[1] and the realisation that substantial amounts of free radicals and reactive oxygen species are produced as by-products of metabolism that the scientific standing of the field was restored. The realisation that the free radical NO plays a central role in the normal physiology of the vasculature[2], leading to the award of three Nobel prizes in 1998, undoubtedly firmly fixed this story on the map.

Discoveries leading to an understanding of the role of oxidative processes in the development of atherosclerosis

During the 1970s the main emphasis of research was connected with cholesterol metabolism, and the discovery of the LDL receptor led to the award of Nobel prizes to Brown and Goldstein. Three disparate strands of research probably led to the conclusion that oxidative processes are also involved. An Austrian chemist, the late Hermann Esterbauer, with a particular interest in aldehyde chemistry discovered that LDL, the main villain in terms of cholesterol deposition in the artery walls, can be oxidised readily in the presence of transition metal ions (particularly of Fe and Cu) and that this process involves the breakdown of LDL-PUFA by free radical attack to form aldehydes[3]. The formation of Schiff's bases with the ε-amino groups of

Abbreviation: HSP, heat-shock protein.

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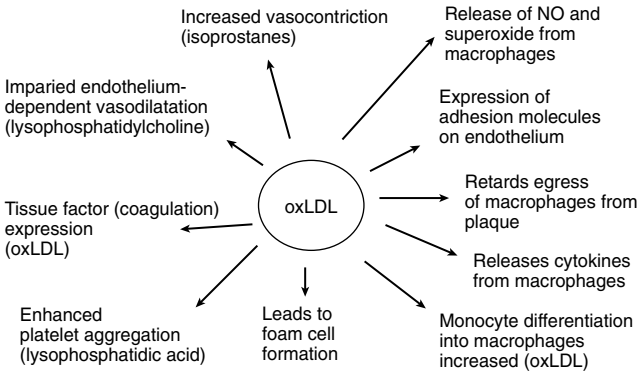

Fig. 1. The diverse actions of products resulting from the oxidation of LDL (oxLDL).

lysine residues then modifies the LDL-apoB and changes its recognition by LDL receptors. Around the same time Daniel Steinberg and his coworkers found that incubation of LDL with cells such as macrophages or other cells in culture leads to a change in the LDL so that it is no longer recognised by its normal receptor[4]. The LDL become oxidised, more electronegative and are recognised by the scavenger receptor on macrophages. This scavenger receptor is now seen as the vehicle by which damaged and electronegative proteins, apoptotic cells and cell debris are removed from the tissues by macrophages. Furthermore, the oxidised LDL is cytotoxic and causes many of the effects that are associated with the development of atherosclerosis through it diverse oxidation products (Fig. 1), including the formation of the large lipid-laden foam cells, which are bloated macrophages full of lipid. These findings link the role of LDL and oxidation to the development of atherosclerosis.

In this same period epidemiological studies had also advanced in relation to the link between diet and CVD. Up to this point the main emphasis had been on the role of dietary lipids, but it was then shown that there is an independent inverse link between the consumption of fruit and vegetables and mortality from CVD (see later). This finding allowed many researchers to make the assumption that this relationship is a result of the presence of antioxidants in these foods, although there are many other virtues to this diet that may also explain the link.

LDL is the major physiological transporter of lipid-soluble antioxidants (particularly α-tocopherol), as well as the PUFA, to tissues, in addition to its role as a cholesterol transporter. It has been found that if α-tocopherol (but not β-carotene) is added exogenously to the LDL or oral doses are given to the donor of the plasma from which the LDL is prepared these particles became much more resistant to oxidation[3]. Indeed, the first endogenous material to become oxidised in the presence of Cu^{3+}, before the lipids oxidise, is α-tocopherol. It was largely ignored at the time that the susceptibility of the LDL to oxidation is independent of the amount of endogenous α-tocopherol in the LDL isolated from a cohort of donors (the oral dose of tocopherol was well in excess of the dietary norm, even for a very healthy diet). It was found by other researchers that ascorbate can also increase the resistance to oxidation because the electrons are passed from the α-tocopherol in the LDL to ascorbate, thus preventing oxidation of PUFA[5].

Experimental evidence obtained from *in vitro* studies (in cultured cells) and from studies in which animals were given large doses has shown that α-tocopherol has a number of inhibitory effects on processes that lead to the formation of atherosclerotic plaque but it was not clear whether these effects were all associated with the antioxidant function of α-tocopherol. α-Tocopherol has even been found in healthy individuals to affect platelet function, which is attenuated by the vitamin even at oral doses of 50 mg/d[6] (much less than had been used in many trials, but still well above the intake from non-supplemented foods). There is also inhibition of LDL oxidation at these doses. These findings suggested that antioxidants, at least α-tocopherol, may have an effect on thrombosis, which is a key concomitant to myocardial infarction. In reality, the effects are minor compared with the actions of aspirin on platelet function, which reduces the risk of infarction by about 20%.

The existence of antibodies to oxidised LDL in the plasma seems to add supportive evidence to the oxidation hypothesis. The association between the concentration of these antibodies and the extent of atherosclerosis in patients with CVD is, however, weak[7]. Indeed, it has been suggested that the formation of these antibodies may be a protective response, and recent data show that healthy diets may increase the amount of circulating antibody[8].

Epidemiological studies on antioxidants and CVD

The experimental studies described earlier and the established inverse relationship between the consumption of fruit and vegetables and CVD have led to a number of new studies on patients and populations that, for the most part, seem to reinforce the central role of antioxidants as protective nutrients. CHD rates are known to be higher in areas in which fruit and vegetable consumption is lowest[9], and lower in countries in which the consumption of fruit and vegetables is higher[10,11]. Furthermore, vegetarians have lower rates of CHD[12]. A comparison of the diet of >75 000 nurses and approximately 39 000 male health professionals has shown a 31% reduction in the risk of stroke in the highest quintile of fruit and vegetable consumption compared with the lowest quintile[13]. In the Lyon Diet Heart Study of patients who had had a myocardial infarction the patients who followed a 'Mediterranean' diet had a reduced re-occurrence of myocardial infarction after 4 years[14]. It has also been shown that higher fruit and vegetable intake lowers CVD risk factors such as blood cholesterol levels and blood pressure[15,16].

These and other findings have led researchers to investigate whether the active factor in the fruit and vegetables could be their antioxidant content. The pioneering work of the WHO MONItoring of trends and determinants in CArdiovascular diseases Study has shown a north–south

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gradient in CVD risk across Europe that is inversely related to the plasma concentrations of vitamin E. Other studies have found similar relationships with Se and with flavonoids. These findings are supported by data from case–control studies[17,18]. Further studies have demonstrated a lower risk of CVD with a higher dietary intake of antioxidant nutrients[19,20]. The European Prospective Investigation of Cancer Study has found a relationship between high levels of ascorbate and reduced risk of CVD[21].

Prospective studies have investigated the contribution of vitamin supplements. In the Nurses' Health Study women in the highest quintile of α-tocopherol consumption, many of whom used supplements of vitamin E, were found to have a 44% lower risk of CHD compared with those in the lowest quintile[19]. Women in the fourth (next to highest) quintile for dietary intake of vitamin E (dietary vitamin rather than supplements) were also found to have a lower risk (26% lower than the lowest quintile). In a cohort of men the risk was also found to be lower with higher tocopherol intakes[20]. In the Iowa Women's Health Study 34 000 post-menopausal women were reported to show an inverse association between dietary vitamin E intake and deaths from CHD and stroke[22,23]; vitamin E supplements were not found to be associated with protection from CVD.

Intake of antioxidant flavonoids has been shown to be inversely associated with CHD risk in several studies. The Iowa Women's Study has found that increased intake of flavonoids is associated with a decreased risk of death from CHD[24]. A recent study from within the Iowa cohort has shown a strong inverse association between CHD and intake of some types of catechins.

Se is important to the activity of certain antioxidant enzymes, particularly glutathione peroxidise, and a micronutrient for which a substantial proportion of the population of the UK and some other countries have a marginal deficiency[25]. Patients who have had a myocardial infarction have low plasma Se concentrations[26], but not all studies have shown an inverse relationship between plasma Se concentrations and CVD.

In other studies the intake of fruit and vegetables has been shown to be associated with changes in markers for CVD or in measurable physiological changes in arterial function. Many of these studies have investigated the relationship between these markers and the level of antioxidants in the diet or the plasma. Plasma C-reactive protein, an index of inflammation, has also been shown to be strongly correlated with the incidence of and mortality from CVD[27]. In a prospective population study of 3258 men aged 60–79 years the plasma C-reactive protein levels were also found to be correlated inversely with plasma ascorbate concentrations and dietary vitamin C, even after adjustment for other confounding factors[28]. Furthermore, another marker for endothelial dysfunction, the tissue plasminogen activator-1 (which inhibits fibrinolysis), was also shown to be inversely correlated with these two variables.

The early observation that LDL and oxidised LDL can inhibit endothelium-dependent relaxation of arteries, a process mediated by the generation of NO, has led to a series of studies in which the effects of antioxidants on this process have been determined. The original observations in an experimental protocol using rabbit aortic rings were that ascorbate reverses the actions of high concentrations of LDL but not that of already-oxidised LDL[29]. It has also been demonstrated in cell culture that increasing concentrations of ascorbate within the physiological range can enhance the synthesis of NO[30]. Direct measurement of arterial dilatation in response to acetylcholine using angiographic procedures has demonstrated improved endothelial responses in the human coronary artery after pretreatment with antioxidants. On the other hand, measurement of relaxations of the brachial artery using plethysmography or ultrasound techniques has shown that very large oral doses of vitamin C (2 g) or direct intravenous infusion of the vitamin can improve these responses if they have been impaired by atherosclerosis or hypercholesterolaemia, both of which damage the arterial endothelium[31]. Since endothelial dysfunction is widely held to be an early stage in the development of the atherosclerotic plaque this approach appears to be extremely promising and supports the epidemiological data. In animal and human studies α-tocopherol has also been found to have some beneficial effects[32].

Intervention trials

The stage was set for impatient members of the investigating community to determine by intervention studies whether antioxidants could have important therapeutic value alongside the well-established statins. Steinberg, one of the architects of the oxidation hypothesis, warned that the rush to begin intervention trials was premature. He pleaded that not enough was known about the detailed molecular events in the oxidative process to be able to establish either the appropriate antioxidants to intervene with or the stage in the atherosclerotic process that might be affected. In particular, it was not clear which oxidants were responsible for the oxidation process. Different oxidants have differing actions and require appropriate antioxidants to stop the process.

Another warning was unheeded, in that an earlier trial with the antioxidant drug probucol (Probucol Quantitative Regression Swedish Trial) on femoral vascular disease had shown that the drug is ineffective in halting arterial thickening[33]. This outcome was contrary to earlier evidence that it strongly reduces the extent of atherosclerosis in a rabbit model of the disease and is very effective as an inhibitor of LDL oxidation as it is powerful compared with most dietary antioxidants.

In recent years a number of intervention studies have been completed; some of them very expensive to run. They were of different design, some investigating a single antioxidant, some a combination of two or more antioxidants and some were a matrix design in which the effects of antioxidants were investigated together with a statin or fish oils. In most cases the antioxidants were in quantities many-fold greater than those found in any diet and at a level at which their action would be considered pharmacological rather than fulfilling any nutritional requirement.

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The Cambridge Heart Antioxidant Study was a 2-year controlled trial of 2002 patients with angiographically-proven coronary atherosclerosis who received either α-tocopherol or an inactive placebo[34]. Tocopherol treatment was found to lower the risk of coronary events, mainly through a reduction in non-fatal myocardial infarction; CVD deaths were not found to be significantly altered. Another study has found that α-tocopherol supplementation suppresses restenosis in surgically-induced atherosclerosis[35], although this outcome has not been found in all studies.

The other larger secondary-prevention studies, which have included many thousands of patients, have shown no protective effect of either α-tocopherol alone or in combination with fish oils, e.g. the Heart Outcomes Prevention Evaluation Study[36], and the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto-Prevenzione Study[37]. In the Alpha-Tocopherol Beta Carotene Prevention Study, in which men were supplemented for ≤6 years with β-carotene or vitamin E supplementation[38], no benefit was found in relation to aortic aneurysms[39]. β-Carotene supplements have been shown to increase cerebral haemorrhage[40] and increase deaths from myocardial infarction in male smokers[38].

Antioxidant therapy has been found to have particularly negative effects on smokers. The Beta Carotene and Retinol Efficacy Trial in which current smokers received a combination of 30 mg β-carotene and 7·5 mg vitamin A has shown a 28% increased risk of lung cancer[41]. In contrast, a study of β-carotene supplementation of US physicians has found no effects on smokers[42]. Meta-analyses of three randomised trials of β-carotene supplementation involving 70 000 healthy individuals and of vitamin E supplementation of 29 000 patients at high-risk of CVD in five large-scale trials has failed to confirm any protective effect of these vitamins for cancer or CVD[43].

The Medical Research Council/British Heart Foundation Heart Protection Study was a large trial that examined the effects of a cocktail of antioxidant vitamins (600 mg vitamin E, 250 mg vitamin C and 20 mg β-carotene) or placebo over 5 years in 20 536 UK adults (aged 40–80 years) with coronary disease, other occlusive arterial disease or diabetes mellitus[44]. The supplements were found to increase the blood levels of antioxidant vitamins but without any significant reduction in mortality from vascular disease or cancer. The protection given by treatment with a cholesterol-lowering statin was reported to be very evident, in contrast with the ineffectiveness of the antioxidant supplements. The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto-Prevenzione Trial examined both the effects of vitamin E and dietary fish oils. The latter were found to reduce the risk of death, non-fatal myocardial infarction or stroke but vitamin E supplementation (300 mg daily for 3·5 years) was not found to provide any benefit[37]. Further meta-analyses have shown that supplementation with antioxidants simply does not provide protection[45].

The results of these studies suggest that it is extremely unlikely that large-scale trials on the therapeutic use of antioxidants will be run again in the next few years unless overwhelming evidence is presented that there is indeed a role for antioxidants and that there is a reasoned explanation for the lack of effect in the previous trials. The cost of these lengthy investigations on such large numbers of patients is simply too high. However, there is still confidence in the concept of oxidative stress in atherosclerosis and work continues.

What could be the explanation?

It has been argued that perhaps the earlier observational studies were indeed misleading[46]. An analysis of a cohort of women aged between 60–79 years who were selected for the British Women's Heart and Health Study has been carried out in which the plasma α-tocopherol and ascorbate concentrations were measured. These measurements were found to show a strong association with socio-economic status indicators, so that the lower the socio-economic status, the lower the plasma concentration of these vitamins. It was argued that a more thorough analysis of socio-economic status would show its importance in the determination of antioxidant levels. It is well known that socio-economic status is strongly related to the incidence of CVD for a variety of reasons. Tunstall-Pedoe and colleagues have made a particular plea recently that deprivation is a factor that is often neglected, using the examples they have found in their studies in Glasgow[47]. They have also exposed very low levels of plasma vitamin C among those in the lowest socio-economic groups[48]. As is well known, low vitamin C is particularly associated with smoking, which may affect food choice. These issues do open the question of whether the major intervention trials did include only a small proportion of this deprived group and that they may specifically benefit from antioxidant therapy or nutritional advice to increase their plasma antioxidant levels. Such low levels of ascorbate have also been found in a study of the elderly, again predicting risk from CVD and all causes, although this association was not indicated for other antioxidants[49]. Dietary ascorbate from FFQ was not found to be correlated with risk of CVD.

These findings lead to the question of whether antioxidants are the key factor in a diet rich in fruit and vegetables or are other nutrients at work. Clearly, an increase in these foods is likely to lead to a decrease in other food constituents, particularly saturated fats, which would be beneficial. There would be an increase in dietary fibre that may also lower blood cholesterol a small amount and improve glucose tolerance, both important risk factors for CVD, and the salt content may be lower. There may also be an increase in the intake of PUFA alongside the increase in fat-soluble vitamins, particularly the tocopherols. Undoubtedly, these outcomes would be accompanied by an increased intake of the polyphenol antioxidants.

One interesting proposal has come from the Linus Pauling Institute, normally known for its advocacy of the benefits of vitamin C. Many groups have observed the effects of specific antioxidants on human subjects by measurement in plasma of the total antioxidant capacity or similar measurements. The ferric-reducing antioxidant potential has been shown to be increased by eating apples,

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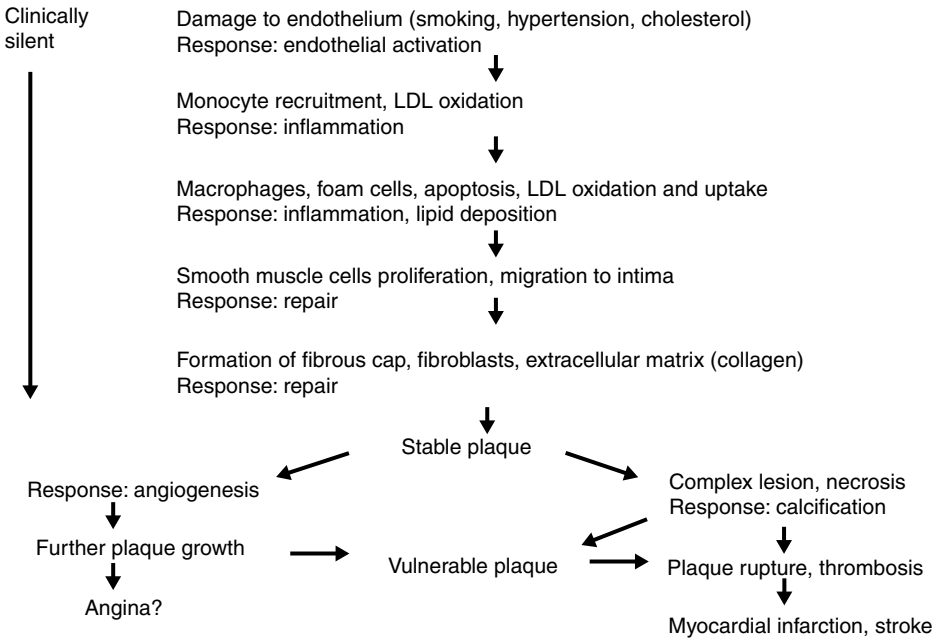

Fig. 2. The evolution of atherosclerosis showing initiation, inflammation, repair processes, angiogenesis and fissure.

Table 1. Possible explanations for the lack of action of dietary antioxidants in the prevention of CVD in randomised trials

1. Only a minority primary population has a low intake of vitamins or some may need more for saturation
2. Not all antioxidants act with equal activity against different free radicals
3. Oxidation process is initiated at an early stage in atherosclerosis. Antioxidants are less able to influence later stages
4. Access of antioxidants to the necrotic core of unstable plaques. Larger doses needed
5. Where free transition metal ions or delocalised, antioxidants have pro-oxidant properties
6. Primary initiating factor is either high levels of blood cholesterol and/or smoking. Oxidation processes play a role in the development of atherogenesis, but not a central one
7. Consumption of fruit and vegetables means that less of the dietary constituents likely to raise blood cholesterol are being consumed

for example, compared with bagels, but much of this increase can be attributed to the presence of fructose in the fruit[50]. The rapid metabolism (phosphorylation) of fructose and utilisation of ATP leads to increased purine catabolism and the formation of uric acid, with a rise in plasma urate levels. Urate is a good endogenous antioxidant and so increases the antioxidant capacity of the blood. The authors point out that the literature shows increases in the total antioxidant capacity of plasma when flavonoid-rich foods and drinks, including red wine and green tea, are consumed, although the mechanism is not clear. They provide evidence that urate levels are increased by these foods and suggest that this factor may explain the increase in plasma antioxidant capacity associated with flavonoid intake.

Another consideration is the actual events that occur within the artery wall during the evolution of the atherosclerotic plaque. This process involves a series of events beginning with endothelial injury, an inflammatory response that includes oxidation, but also reparative events such as the formation of the fibrous plaque, which is analogous to wound healing but also stimulated by the release of inflammatory materials from macrophages (see Fig. 2). Other repair events include angiogenesis (vasculogenesis), which increases the access of nutrients and O_2 to the thickened intima media and also permits the formation of new vessels through blocked areas. To this event may be added the process of apoptosis, which permits the orderly destruction of tissues in an affected region, non-occurrence of which will lead to necrosis. Only a minority of plaques ever develop to become life-threatening structures that fissure and release materials that initiate the formation of a thrombus. The effects of dietary antioxidants on all these stages are not completely understood.

The actions of oxidised LDL have been well established at a cellular level and explain many of the events that occur in atherosclerosis and to some extent in thrombosis. So, why do the antioxidants not work? A list of proposals is given in Table 1, although a number of these points have been alluded to earlier. Undoubtedly, it is the case that in the intervention studies the subjects chosen were likely to have had pre-existing atherosclerosis because of their age, and it may be argued that lifelong supplementation may have been necessary. Of course, no one would fund such a

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study nor would many researchers wish to take it on. It appears that restoration of endothelial function (measured as relaxation to specific agonists) by antioxidant therapy, considered to be the first step in atherosclerosis, does not reverse the subsequent stages of atherosclerosis or even arrest them.

One of the most important findings has been that the antioxidant content of the artery wall does not change significantly during the evolution of the atherosclerotic plaque. When plaques are graded according to stages agreed by pathologists only at the advanced stage is there some lowering in the amount of tocopherol present[51]. Homogenates made from the plaques and indeed the plaque lipoproteins contain α-tocopherol (and ascorbate) alongside the lipid oxidation products such as cholesteryl oxides and other oxidation products. Either the antioxidants do not function or they become radicalised in this environment. Antioxidants can become pro-oxidants, especially in the presence of metal ions, as indicated earlier (p. 215). The antioxidant may be cyclically depleted and repleted through its radical forms.

The realisation that two electron oxidants, particularly hypochlorite and peroxynitrite, may be the key oxidants that cause the main damage in atherosclerosis occurred after the main intervention studies had begun. The footprints of these oxidants are found not only in atherosclerotic plaque but also in other inflammatory diseases, such as rheumatoid arthritis, Alzheimer's disease and diabetes, in the form of protein modification resulting from the nitration or chlorination of protein tyrosine residues. Specific antibodies against the nitrotyrosine and chlorotyrosine are used to demonstrate their presence by immunohistochemistry. It has been proposed that these oxidants may be formed by the action of the enzyme myeloperoxidase found in macrophages as well as in neutrophils[52]. There is evidence that γ-tocopherol is a more effective antioxidant against peroxynitrite than α-tocopherol, and the flavonoid epigallocatechin and its gallate are yet more effective. These catechins are found in large amounts in green tea, chocolate, red wine and fruit such as apples.

Under experimental conditions epigallocatechin gallate at concentrations as low as 2 μM (it is water soluble) inhibits the protein nitration that occurs during the activation of blood platelets (M Sabetkar and KR Bruckdorfer, unpublished results). The concentration is important since, although large amounts of these compounds may be consumed, levels in the plasma are low. The maximum concentration for this compound that is found in the plasma is about 1 μM, although less in known about intracellular concentrations. The catechins are also metabolised and eliminated in the form of glucuronides, but some of these products also have antioxidant activity. No major intervention studies have been attempted using these compounds, despite the current enthusiasm for them. Certainly, the issue of biological availability is an important one. However, a mix of these compounds may have important collective antioxidant actions.

There are other illustrations of the actions of antioxidants that show that they do not all act in an identical manner and these diverse actions may include their so-called non-antioxidant actions. One of the key roles of the free radical NO released from the endothelium is to prevent the proliferation of smooth muscle cells and to maintain them in their contractile state, inhibiting differentiation into a fibroblastic phenotype. This fibroblastic phenotype biosynthesises collagen, a protein found in large amounts in fibrous plaque. Ascorbate is as an essential vitamin for the biosynthesis of collagen, specifically required for the hydroxylation of proline. It has been shown in skin fibroblasts that ascorbate opposes the inhibitory action of NO on collagen biosynthesis[53]. These studies have now been extended to show that epigallocatechin gallate has the opposite effect, enhancing the actions of NO. Since α-tocopherol is also believed to be anti-proliferative, its actions also differ from that of ascorbate in this aspect.

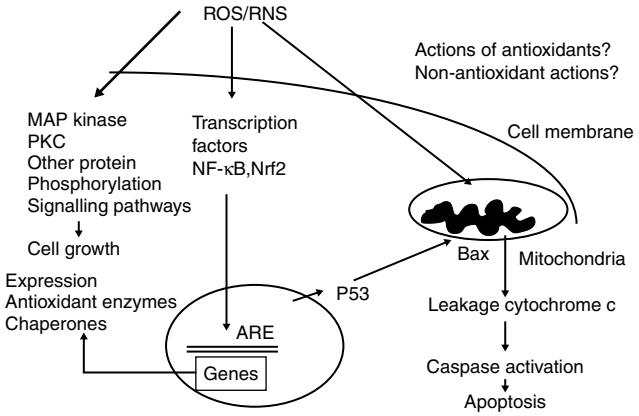

Fig. 3. The cellular responses to oxidative stress: the expression of protective enzymes and proteins or apoptosis. ROS, reactive oxygen species; RNS, reactive nitrogen species; MAP kinase, mitogen-activating protein kinase; PKC, protein kinase C; P53, a protein that initiates apoptosis; ARE, antioxidant response element; Bax, a pro-apoptotic protein.

Finally, the actions of antioxidants on the normal endogenous cellular responses to oxidative stress must be considered, especially if consumed in pharmacological doses. Cells respond in a number of ways to protect themselves. Excessive exposure of the cell may lead to death and necrosis where these responses are overwhelmed. The process of apoptosis leads to an orderly death of the cell and their removal by macrophages, and therefore increased expression of proteins such as P53, which activate this process. As a result, changes to mitochondrial structure and that of DNA can be observed. The cellular responses to oxidative stress are outlined in Fig. 3. For the most part it consists of the enhanced expression of genes, which leads to the increased synthesis of proteins that are protective for the cell. These proteins include antioxidant enzymes such as haemoxygenase-I, which not only forms the antioxidant bilirubin but also the protein itself is a chaperone (a class of protein that protects the integrity of other proteins during folding; many of which are termed heat-shock proteins (HSP) since they are also formed by raising the temperature of cells). These actions on genes are mediated through nuclear transcription factors, some of which are sensitive to the redox status of the cell, which can be changed by oxidative stress. These proteins are

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also regulated to an extent by NO by its interactions with sulphydryl groups on the transcription factors, which are themselves proteins, e.g. NF-κB, Nrf2 and hypoxia-inducible factor-1. These transcription factors operate through antioxidant response elements on the chromosome near regions where the antioxidant proteins are expressed. It is not thought that antioxidants themselves act on these antioxidant response element sites, but work at the transcription factor level. The mechanisms by which the polyphenols and phyto-oestrogens may augment gene expression for antioxidant enzymes and chaperones and increases in NO biosynthesis through NF-κB and Nrf2 have been reviewed and their action proposed to be cardio-protective[54].

In a different scenario, but still investigating oxidative stress, the action of antioxidant vitamins on the expression of genes following muscular exercise has been studied[55], a process in which it is known that oxidative stress occurs when reactive oxygen species are formed as a result of high levels of oxidative metabolism. Changes in the proteins of lymphocytes and skeletal muscle of untrained human subjects were studied with and without supplementation with ascorbate (0·5 g/d for 8 weeks). An increase in lymphocyte superoxide dismutase and catalase activities and the cellular content of the chaperone proteins HSP60 and HSP70 was found in response to a low concentration of H_2O_2 without ascorbate supplementation of the subjects. After supplementation the basal activity or content of the cellular proteins was shown to be slightly increased, with the cells giving an attenuated response to the H_2O_2. In muscle post exercise an increase in HSP60 and HSP70 was found following exercise, which for HSP60 was found to be diminished by supplementation with ascorbate, although the expression of HSP70 was raised. Clearly, ascorbate supplementation at this level may have both beneficial and deleterious effects on the response to oxidative stress.

Perhaps more directly concerned with atherosclerosis is the measurement of a protein termed tissue factor, a pro-coagulant protein that increases many-fold in the intima media of atherosclerotic plaque but is not expressed in normal tissue. In some preliminary work in a macrophage cell line it has been found that most of the antioxidants decrease the expression of tissue factor, which is normally increased following exposure to oxidised LDL or bacterial lipoploysaccharide if the antioxidants are put in the medium before or at the same time as the activator. If they are added only 20 min after the activator, then the opposite happens and the expression of tissue factor mRNA, measured quantitatively by competitive RT–PCR, increases, particularly for α-tocopherol and for folic acid (AO Oke and KR Bruckdorfer, unpublished results). It may be a far stretch of the imagination to compare these results with those from supplementation of patients with pre-existing CVD in the intervention trial. Nevertheless, it is clear that the antioxidants can produce some unexpected effects in different experimental scenarios. It may explain how under specific conditions, e.g. smoking, the antioxidants may behave in ways that could be deleterious and produce the unexpected data obtained in some of the human trials.

Conclusions

A survey of the progress over approximately 30 years of research into oxidative stress, free radicals and the role of dietary antioxidants as therapeutic agents has its high points and some very distinctive low points. It is clear that there is very much more to do in order to investigate the complex effects of dietary antioxidants and how they influence the complex signalling mechanisms that respond to oxidative stress. There needs to be a more subtle understanding of the fact that not all free radicals and other reactive species interact with the multitude of dietary antioxidants. The simple 'free radicals bad–antioxidants good' mantra is simply not adequate to comprehend how they may affect a lifelong process such as the development of atherosclerosis. It is clear that a role of oxidative stress is of some importance to these inflammatory conditions. It is not at all certain that a cocktail in pharmacological doses of two or three antioxidants is ever going to stop these pathological changes. The only near certainty is that a diet rich in fruit and vegetables is beneficial and family resources are better spent on that than on expensive supplements.

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