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Plant-Derived Compounds as Antioxidants for Health – Are They all Really Antioxidants?

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ABSTRACT

Numerous epidemiological studies have demonstrated that high consumption of fruits and vegetables correlates with good health and lower incidence of diseases like cancer and cardiovascular disease. It has been widely believed for over 20 years that this can be explained by "antioxidants". These phytochemicals were thought to "mop up" potentially harmful free radicals when they were produced in excess in the body, as a result of disease or environmental stress. The implication generally drawn from this "antioxidant hypothesis" is that the higher the antioxidant content of the plant foods we eat, the more antioxidants will be in our bloodstream and the healthier and longer lived we will be. Unfortunately, numerous studies carried out to test this hypothesis have produced contradictory evidence and it remains unproven. It appears, however, that both free radicals and phytochemicals can act as signalling molecules and that they can interact with our cellular systems at the molecular level and regulate genes and various biological pathways. Other health benefits attributed to antioxidants may result from regulation of processes that lead to cancer, cardiovascular and other diseases. Thus there is increasing evidence that the numerous potential health benefits of phytochemicals, many of which are currently labelled as "antioxidants", arise from mechanisms other than antioxidant capacity. We are only now starting to understand their mechanisms of action, which are much more complex than previously realised. We investigate the latest developments in this increasingly controversial area of research, to find out if science is any closer to understanding this area.

Keywords: phytochemical, oxidative stress, Vitamin C, Vitamin E
Abbreviations: **ARE**, antioxidant response element gene; **CVD**, cardiovascular disease; **CAT**, catalase; **ETC**, (mitochondrial) electron transport chain; **F&V**, fruits and vegetables; **FRAP**, Ferric Reducing Antioxidant Power; **GPX**, glutathione peroxidase; **MDA**, malondialdehyde; **ORAC**, Oxygen Radical Absorbance Capacity; **RONS**, Reactive oxygen and nitrogen species; **SIRT**, silent information regulator gene; **SOD**, Superoxide dismutase; **TAC**, Total Antioxidant Capacity

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INTRODUCTION

The traditional view of the health benefits from plant antioxidants, such as vitamins C and E, and β-carotene, is that they have a positive role in preventing or moderating diseases where damage from reactive oxygen and nitrogen species (RONS) is implicated. In the 22 years since this concept was initiated for coronary heart disease by Gey (1986, 1995), there have been enormous advances in our understanding of the importance of redox balance for health, and a clear realisation that not all RONS should be considered harmful. In fact, RONS play an important role as

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molecular signals for the control of redox balance and can initiate many of the adaptive cellular responses required for homeostasis (Droge 2002). How we measure the antioxidant effect that a plant-derived compound may have on humans *in vivo* is presently controversial and poorly defined. Popular perceptions of the health benefits of antioxidants have been strongly influenced by marketing, such as the phenomenon of "super fruits", promoted for their high ORAC values and by misinterpretation of the original "antioxidant hypothesis" (Gey 1986, 1995).

In this review we examine the relevance of food antioxidant capacity as a concept for determining potential antioxidant capacity *in vivo* in humans. This includes discussion of compounds that have significant antioxidant effects *in vivo* following metabolism, and yet other compounds such as polyphenolics and flavonoids that may have predominantly indirect antioxidant effects. In the context of health we regard a plant-based antioxidant as any plant-derived compound that either directly or indirectly contributes to *in vivo* redox balance in humans. This embraces the concept that oxidative protection and redox balance in humans is much more complex than just ingesting commonly recognised antioxidants, and while much of the purported antioxidant effect from plants is likely to be highly beneficial, it is occurring in complex ways that we are only just beginning to understand.

"ANTIOXIDANT" HEALTH BENEFITS OF FRUITS AND VEGETABLES

For human health it is well understood that antioxidants such as vitamin C, vitamin E and carotenoids are readily absorbed following ingestion and that dietary sources are essential for good nutrition (Harrison 2005; Rigotti 2007). Vitamin C appears to be absorbed by both glucose transporters (Arrigoni and De Tullio 2002) and a specific transporter (Tsukaguchi *et al.* 1999), and the lipophilic species by similar mechanisms to fats. There is also a range of other important compounds, often also referred to as antioxidants, including trace minerals and other vitamins, which in reality have a positive, but indirect effect on *in vivo* antioxidant protection (Chan 1993). Vitamin C is the only major antioxidant with a known specific biological function in humans; it is an electron donor for eight different enzymes (Levine *et al.* 2000; Arrigoni and De Tullio 2002). The most important of these is proline hydroxylase, the enzyme responsible for cross-linking proteins such as collagen (Chan 1993). In the absence of dietary vitamin C, the resulting deactivation of this enzyme results in scurvy after 4-6 weeks (Chan 1993). Most mammals can biosynthesise vitamin C from glucose, but humans are among the few that cannot, because of a defective gene for a key enzyme in the biosynthetic pathway (Harris 1996; Arrigoni and De Tullio 2002). Vitamin E is the collective name for eight "tocopherols" and "tocotrienols", isomeric mono-phenolic compounds with a lipid side chain (Sen *et al.* 2007). Naturally occurring vitamin E contains different proportions of these compounds, but synthetic material in supplements and fortified foods is primarily α-tocopherol. These compounds have low water solubility, but high fat (and therefore membrane) solubility and are thought to be the most important lipophilic antioxidants that protect lipids from peroxidation (Herrera and Barbas 2001). They are also one of the essential vitamins in the diet (Chan 1993). The third major dietary antioxidant group is the carotenoids. These are highly lipophilic terpenoids, usually highly coloured (yellow, orange or red), with no known specific biological function in human metabolism (Humphrey and Beale 2006). The most common carotenoid in plants and the one most used in intervention trials and supplements is β-carotene. Carotenoids have an indirect biological function in that some of them can be converted into vitamin A, an important component of the visual system.

Enzyme cofactors that are sometimes called antioxidants

There are several other micronutrients such as selenium, copper and zinc that are also essential for humans to maintain an effective antioxidant system. Selenium is an essential co-factor for glutathione peroxidase (an important antioxidant enzyme) and deficiency is associated with poor health, with studies associating low levels with cardiovascular disease (CVD). Copper and zinc are essential co-factors for superoxide dismutase, another antioxidant enzyme.

THE ANTIOXIDANT HYPOTHESIS

The health benefits of dietary antioxidants have been strongly promoted since the "antioxidant hypothesis" was first proposed by Gey (1986, 1995), in conjunction with the very influential suggestions by Pauling, who promoted very high doses of vitamin C (Pauling 1986). The central theme of the hypothesis was that suboptimal status of essential antioxidants or "poor antioxidant potential" is a major risk factor for CVD (Gey 1986, 1995). The essential antioxidants referred to are vitamins C and E, and β-carotene, with delivery via dietary intake of fruits and vegetables (F&V). This hypothesis was enthusiastically interpreted by many that, if more is good, much more must be better, and resulted in the proliferation of antioxidant supplements. However, as clearly indicated in the original study by Gey (1986), it was near-deficiency levels of plasma antioxidants and a low intake of F&V that are *associated with* increased incidence of CVD and associated mortality and by implication, possibly other diseases. Even moderate plasma levels of these antioxidants greatly decreased incidence of CVD.

RECENT OPINIONS ON THE LINK BETWEEN ANTIOXIDANTS AND HEALTH

A recent study investigating the link between fruit and vegetable intake and plasma concentrations of vitamins C and E, and β-carotene supports the link between F&V consumption and increased plasma concentrations of these antioxidants (Dauchet *et al.* 2008). However, experiments based on the use of antioxidant supplements to elevate plasma antioxidant concentrations and reduce CVD and mortality have been very disappointing. The technique of meta analysis is often used to combine results from many similar studies to look for overall trends. A recent and controversial meta analysis of 67 trials involving β-carotene, vitamin A, vitamin C, vitamin E, and selenium supplements found no evidence of a decrease in mortality or other benefits apart from a possible benefit from selenium (Bjelakovic *et al.* 2008). The controversy has been sparked by the conclusion that β-carotene, vitamin A and vitamin E significantly increased mortality.

Very recent studies not included in the above meta analysis only serve to confirm its conclusions. Recently, Clarke and colleagues concluded that α-tocopherol supplements had no effect, or were possibly negatively associated with all-cause mortality (Clarke *et al.* 2008). Similarly, there is little evidence to support antioxidant supplements to ameliorate age-related eye conditions (Klein *et al.* 2008). A study using high doses of vitamin C and α-tocopherol also failed to diminish oxidative stress in the elderly (Radak *et al.* 2008). Two reviews have discussed why antioxidants have largely failed in clinical trials (Steinhubl 2008; Willcox *et al.* 2008). Other recent studies that report no significant benefits and possible increases in mortality due to antioxidant supplements, except for possible benefits to the elderly and those with insufficient dietary intake (Bleys *et al.* 2007; Bardia *et al.* 2008; Bleys *et al.* 2008; Buijsse *et al.* 2008; Messerer *et al.* 2008).

An earlier meta analysis, specific to trials of vitamin E, similarly found no detectable benefits and an increase in mortality (Miller *et al.* 2005). Even if the mortality increase were not real, there is a mass of evidence that, whereas antioxidant intake from F&V may be associated with their un-

doubted health benefits, antioxidant supplements are beneficial in only a few situations. Apart from dietary deficiency and possible increased requirements arising from chronic illness and ageing, antioxidant supplementation appears potentially detrimental and largely useless. Similar conclusions were reached in two recent reviews (Stanner *et al.* 2004; Vina *et al.* 2007). Although a negative outcome from an intervention trial is not conclusive evidence that the intervention is of no value, the available evidence largely discredits the simplistic, but widely accepted beliefs that high dietary antioxidant intake is the panacea to prevent ageing and degenerative diseases and the higher the intake, the better the protection. The evidence is very compelling that modest intakes, obtainable from a reasonably healthy diet in the accepted sense, are both absolutely essential and highly beneficial for avoidance of deficiency and the associated health risks. On the other hand, higher intakes show no additional health benefits.

A new perspective is rapidly gaining momentum, which is that an alternative explanation for the observations is that small-molecule, plant-derived plasma antioxidants are simply biomarkers of F&V consumption and some factor(s) other than antioxidants are operating (Stanner *et al.* 2004; Bjelakovic and Gluud 2007; Vina *et al.* 2007). One caveat to this conclusion is that supplementary antioxidants and other vitamins are much more likely to be of benefit to the majority of third world populations who exist near the poverty line and may rarely get even the minimum recommended daily intake (RDI) of dietary antioxidants (Vina *et al.* 2007). Low dose supplements, fortified foods, or foods with naturally high levels of antioxidant vitamins would probably be very beneficial to the majority of the world's population.

REACTIVE OXYGEN AND NITROGEN SPECIES (RONS)

The body is subject to a wide range of physiological oxidants, and they often have dual roles which can be either beneficial or harmful, depending on where they are produced and their concentration. Some of these oxidants are free radicals with one or more unpaired electrons, while others are not radicals, but are reactive oxygen or nitrogen species that readily form free radicals (Droge 2002). Reactive oxygen and nitrogen species (RONS) is the term normally used to describe these oxidants collectively. Antioxidants are defined as molecules which have the property of inhibiting or slowing oxidative reactions and which act to maintain a reducing environment, In a biological context, both plants and animals have both non-enzymatic (i.e., small-molecule) and enzymatic antioxidants. Antioxidants have the primary ability to quench free-radical chain reactions, often initiated by RONS such as peroxide or superoxide. Historically, the focus on the actions of RONS was predominantly negative, i.e., they cause oxidative stress and damage vital molecules such as DNA, proteins or lipids. The modern understanding is one of "balance", where RONS are clearly recognised as often being important cellular signalling molecules (Droge 2002). RONS vary widely in their ability to cause damage and in their suitability as signalling molecules (Winterbourn 2008).

CELLULAR REDOX SIGNALLING AND REDOX BALANCE: NEW PERSPECTIVES ON RONS – ARE THEY REALLY ALL BAD?

Redox signalling and redox balance are perhaps the most important concepts in an understanding of the biological importance of RONS and antioxidants for cellular homeostasis, and hence the role that plant antioxidants potentially can play in human health (**Fig. 1**). RONS and redox balance have been discussed in detail in two recent reviews (Droge 2002; Winterbourn 2008).

Well before the antioxidant hypothesis was formulated, it was thought that mitochondrial respiration unavoidably generated free radicals (i.e., RONS) and these caused damage to macromolecules, leading to ageing and associated conditions such as cardiovascular disease and cancer (Harman 1956). This would imply that high intake of antioxidants should scavenge the free radicals and slow ageing. No research has contradicted the idea of RONS generation and associated damage since then, but the role of antioxidants has been seriously questioned. It now appears that RONS have many signalling effects that up-regulate endogenous defences and repair mechanisms against RONS. It also appears that there are relatively straightforward ways to modify mitochondrial metabolism adaptively, to increase its efficiency and to reduce leakage of RONS. These concepts are discussed in more detail below. Antioxidants appear, in some circumstances, to interfere with these adaptive processes. The relationships between RONS and antioxidants appear to be much more complex than was apparent in the past; indeed, the use of term "oxidative stress" needs to be better defined and perhaps relegated to being only a laboratory-based hypothesis (Azzi *et al.* 2004).

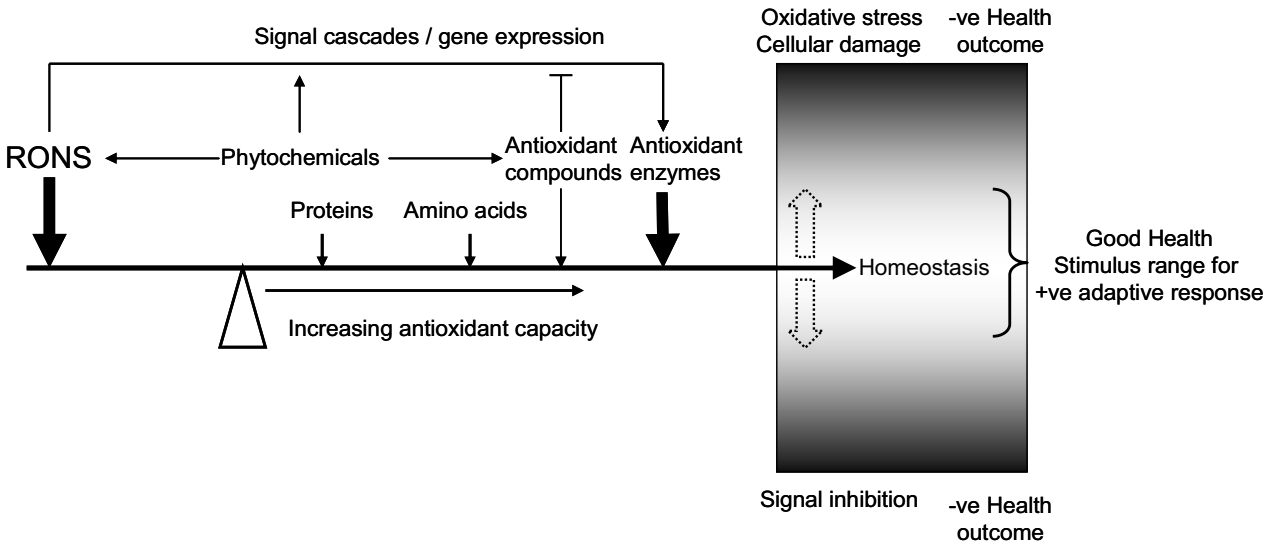

Fig. 1 The concept of redox balance, where reactive oxygen and nitrogen species (RONS) act both negatively, causing potential cellular damage, but conversely also act positively, as a signalling mechanism to up-regulate antioxidant defences and promote a reducing environment. Phytochemicals are increasingly recognised as playing multiple roles: as potential producers of RONS, as signalling molecules for adaptive responses and as direct antioxidant compounds *in vivo*. Tight regulation of this system is required for good health, with the universally recognised negative effect on oxidative stress and the more recently recognised need for minimal levels of RONS to stimulate adaptive responses.

SMALL-MOLECULE ANTIOXIDANTS ARE RELATIVELY INEFFECTIVE COMPARED WITH ENDOGENOUS DEFENCES

A recent review proposes that most dietary antioxidants actually have relatively limited capacity to protect cells from damage by RONS (Winterbourn 2008). Consideration of the relative rates of reaction and relative concentrations of oxidisable groups on macromolecules and small-molecule antioxidants leads to the conclusion that the latter are rarely concentrated enough and most are not reactive enough, to provide more than partial protection from oxidation. In addition, the quenching of a radical by an antioxidant generates another (antioxidant) radical, which has potential to be a pro-oxidant and cause similar damage to the original radical. This is only beneficial if the antioxidant is quickly regenerated, by some means. For example (see below), vitamin E radicals can be regenerated by ascorbate and the latter can be regenerated by an enzyme system, or two ascorbyl radicals can "disproportionate" to form one ascorbate molecule and one of dehydroascorbate (a relatively stable, non-radical species). The primary defence against antioxidants must, therefore, be antioxidant enzymes, which can react orders of magnitude faster than small-molecule antioxidants (Winterbourn 2008). The relative ineffectiveness of antioxidants *in vivo* is compounded by highly localised generation of RONS, primarily in mitochondria and by immune cells at sites of inflammation. Small-molecule antioxidants, however, are spread evenly throughout tissues and rarely present where they are apparently most needed at anything like the concentrations required to be effective (Shen *et al.* 2007). Consideration of "antioxidant" chemistry suggests that, under many physiological conditions, they are more likely to be pro-oxidants, i.e., compounds that generate free radicals (Rietjens *et al.* 2002)). This is not necessarily a bad thing in moderation, but suggests that the balance between pro- and anti-oxidative processes is far more complicated than commonly believed. It also raises the question of what constitutes a physiologically relevant antioxidant.

There have been many animal trials aimed at better defining the relationships between oxidative stress, ageing and antioxidant defences (Muller *et al.* 2007). In general it has been found that large reductions in the expression of antioxidant enzymes, produced by genetic manipulation, were needed before oxidative damage was increased and lifespan shortened. Increasing expression above normal levels appeared to have little or no benefit and was harmful in some cases. The exception to this is the mitochondrial form of SOD (Manganese-SOD, or Mn-SOD). The critical importance of Mn-SOD has been demonstrated by studies showing that mice that over-express the enzyme have a modestly extended lifetime (Hu *et al.* 2007), whereas MnSOD-knockout mice die within a few days of birth (Li *et al.* 1995).

It may be that mammals with normal levels of antioxidant enzymes and antioxidant intake have an optimal redox balance which cannot be significantly improved by any intervention. Interventions that normalise any deficiency of critical antioxidant enzymes, however, are likely to be beneficial to health.

The main small-molecule antioxidants in plasma are vitamin C, urate, bilirubin, glutathione and vitamin E. There are also protein functional groups that can act as small-molecule antioxidants, such as thiols. In plasma incubated with a free radical generator, only vitamin C provided complete protection against oxidative damage (Polidori *et al.* 2004). This concept was reinforced by Azzi and colleagues (Azzi *et al.* 2004; Azzi 2007) who questioned the value of a generic definition of antioxidants and pointed out that the term "antioxidant" is not experimentally constructive unless it is associated with a particular oxidant.

IN VIVO REGENERATION OF SMALL-MOLECULE ANTIOXIDANTS

There is no doubt that vitamins C and E are essential in the diet, but they also appear to be needed in relatively small amounts. The explanation for this appears to be that mammalian cells, even those that cannot make ascorbic acid (e.g., all human cells), can recycle it via the enzyme ascorbate reductase and in turn, use it to recycle tocopherols (Chan 1993; **Fig. 2**). Recycling of ascorbate has been demonstrated in human erythrocytes (Mendiratta *et al.* 1998), vascular endothelial cells (May *et al.* 2003) and mitochondria (May *et al.* 2007). Regeneration of vitamin E by ascorbate has also been demonstrated in erythrocytes (May *et al.* 1998).

Although the recycling system appears to be efficient, it is also not perfect. For example, dehydroascorbate undergoes an irreversible, apparently non-enzymic rearrangement to diketogulonic acid and is thus lost to the metabolism (Arrigoni and De Tullio 2002). It appears that dietary intake of vitamins C and E may only be necessary to replace the material lost by degradative side-reactions, or via urinary excretion. It has been observed (see below) that high antioxidant intake can lead to pro-oxidant effects. It is possible

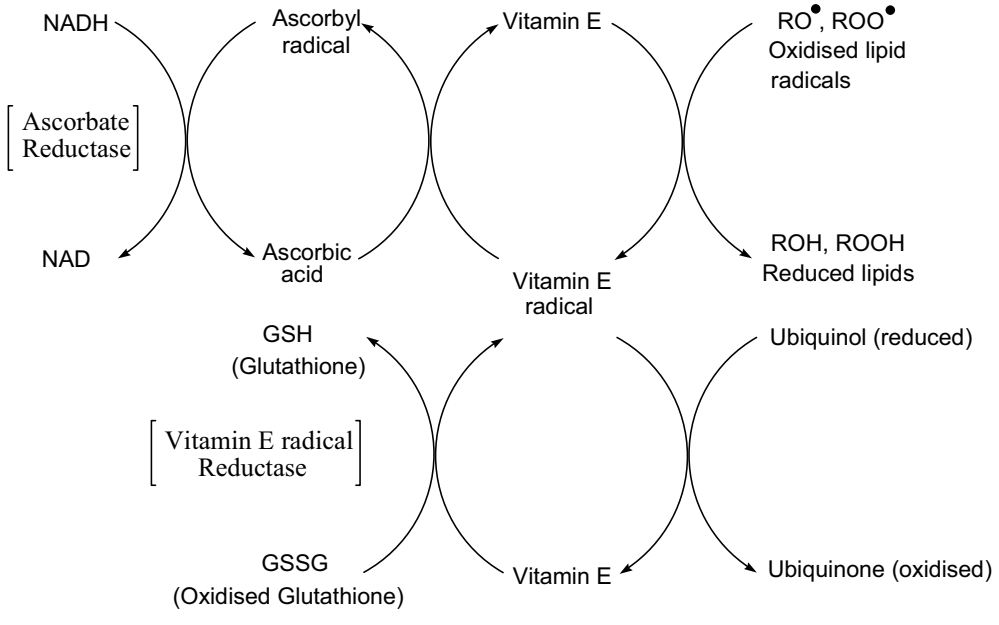

Fig. 2 Pathways for regeneration of vitamins C and E, adapted from Chan (1993). The interactions between vitamin E and ascorbic acid or lipids are non-enzyme catalysed reactions. These pathways are thought to reduce the dietary requirement for these two antioxidant vitamins greatly.

that the abnormally high concentration of antioxidant forms abnormally high concentrations of antioxidant radicals which transiently overwhelm the regeneration pathways.

MEASUREMENT OF TOTAL ANTIOXIDANT CAPACITY (TAC) – DOES IT HAVE ANY REAL MEANING?

Modern interest in foods and supplements high in antioxidants has in part been fuelled by misinterpretation of the antioxidant hypothesis, but also by the concept that the "total antioxidant capacity" (TAC) of plants and associated plant-derived supplements can be expressed as a single integrated value. In fact, this style of evaluating the antioxidant capacity of a food has become almost standard. While there is no universally accepted measure, popular TAC assays include Oxygen Radical Absorbance Capacity (ORAC) (Ou *et al.* 2001) and Ferric Reducing Antioxidant Power (FRAP) (Benzie and Strain 1999). The problem in considering health benefits to humans is that it has been widely assumed that a high TAC of a food is both readily transferable to humans and also provides health benefits. The concept of TAC has also been extrapolated to human plasma but it erroneously ignores the influence of enzymatic antioxidants, which in living systems dominate antioxidant protective capacity (Sies 2007; Winterbourn 2008). The major known antioxidant enzymes are superoxide dismutase (SOD; disproportionates superoxide into hydrogen peroxide and molecular oxygen) and catalase (reduces hydrogen peroxide to molecular oxygen and water) (Arrigoni and De Tullio 2002). Catalase has a very high turnover number, but a low affinity for hydrogen peroxide, so it rapidly reduces high concentrations, but leaves a residue. The residue is in turn removed by the high-affinity but relatively slow acting enzymes, glutathione peroxidase (catalyses the antioxidant action of glutathione, an endogenous antioxidant) and ascorbate peroxidase (Arrigoni and De Tullio 2002). A number of other antioxidant enzymes have been discovered relatively recently (Muller *et al.* 2007).

Significant flaws in the application of TAC to plasma have been demonstrated in a human study where subjects consumed apples with a high polyphenolic content and a large increase in plasma TAC was measured (Lotito and Frei 2004). The changes in plasma TAC, however, were not related to the polyphenolics, but to the high fructose content of the apples, which is known to be metabolised *in vivo* into uric acid. The physiological relevance of uric acid for health is contentious and high levels are related to increased mortality (Niskanen *et al.* 2004; Baker *et al.* 2007; Meisinger *et al.* 2008). Uric acid is a significant component of any TAC measure and contributes 7-62% of the value, depending on the assay used (Cao and Prior 1998), but the health benefit of a TAC increase arising from uric acid is questionable. Probenecid, the standard treatment for the urate-related condition, gout, lowered plasma urate and TAC in healthy exercising subjects compared with controls, but no other oxidative stress marker was changed (McAnulty *et al.* 2007), suggesting that urate has no effect on oxidative stress. Even if TAC were important to health, a high intake of antioxidants only appears to cause a transient increase at best. Vegetarian subjects, whilst having significantly higher concentrations of plasma F&V-derived antioxidants than omnivores, still had similar plasma TAC (Haldar *et al.* 2007). Further support for this conclusion comes from the study by Levine and colleagues (Levine *et al.* 1996) in which supplemental oral vitamin C intakes of more than 200 mg/day produced no further increase in plasma levels, the excess being rapidly excreted. It appears that TAC and concentrations of individual small-molecule antioxidants are tightly regulated and consumption of amounts greater than required to replace any losses from the recycling system are unnecessary and probably futile.

POLYPHENOLICS: PLANT-DERIVED COMPOUNDS WITH ASSUMED ANTIOXIDANT PROPERTIES *IN VIVO*

Polyphenolics are moderately hydrophobic poly-hydroxy aromatic compounds, a class made up of thousands of different structures and thought to be made by plants for many purposes, including feeding deterrence against mammals and insects and shielding from damaging solar UV radiation (Gould and Lister 2006; Stevenson and Hurst 2007). The best known are the anthocyanins that impart red or purple colours to, for example, apple skin, strawberries or blueberries. The main small-molecule antioxidant in plants is ascorbic acid, but polyphenolics are also thought to contribute (Gould and Lister 2006) and they generally have high antioxidant capacity as measured by *in vitro* assays, such as ORAC and FRAP (Clifford 2004). This high antioxidant capacity has been assumed to be responsible for the health benefits of F&V until relatively recently. Health benefits of polyphenolics, however, may be related to other molecular effects that have an overall benefit to the organism.

EPIDEMIOLOGICAL ASSOCIATION BETWEEN FLAVONOID POLYPHENOLICS AND HEALTH BENEFITS

A number of trials that associated F&V intake with health benefits also found weak associations with polyphenolics, particularly the flavonoids. Associations were found between flavonoid intake and increased flow-mediated arterial dilation (indicative of improved vascular function) (Heiss *et al.* 2007); reduction in heart attack risk (Tavani *et al.* 2006); CVD mortality (Mink *et al.* 2007) and vascular function (Naissides *et al.* 2006) in postmenopausal women; CVD, stroke, diabetes and cancer (Bayard *et al.* 2007); vascular endothelial function and blood pressure (Hodgson and Croft 2006) and lung cancer (Neuhouser 2004). Flavonoids did not reduce cancer incidence in elderly men (Hertog *et al.* 1994) but appeared to reduce mortality from CVD (Hertog *et al.* 1993). There may be an association between reduced incidence of oral and throat cancer and flavonoids (Rossi *et al.* 2007). In an interesting contrast with carotenoids, dietary flavonoids were inversely associated with lung cancer incidence in smokers but there was no association in non-smokers (Cui *et al.* 2008). A recent meta analysis of 133 intervention trials involving the relationship between flavonoid-rich foods and cardiovascular risk (Hooper *et al.* 2008) and the related editorial comment (Geleijnse and Hollman 2008) concluded that there appear to be significant benefits from cocoa and soy flavonoids. Evidence for other flavonoids is inconclusive, however, and much more work is needed to link flavonoids themselves clearly to the benefits.

POTENTIAL MECHANISMS OF HEALTH BENEFITS ATTRIBUTED TO FLAVONOIDS

Numerous *in vitro* and animal studies have found a number of potential mechanisms, unrelated to antioxidant capacity, by which flavonoids could account for at least part of the health benefits of F&V consumption (Stevenson and Hurst 2007). It appears that flavonoids are actually pro-oxidants under normal physiological conditions, i.e., they *generate* free radicals in small quantities (Long *et al.* 2000; Nakagawa *et al.* 2004; Ferraresi *et al.* 2005; Nemeikaite-Ceniene *et al.* 2005; Maeta *et al.* 2007). This apparently harmful property, however, appears to up-regulate synthesis of antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, in what might be termed an "indirect" antioxidant effect (Niering *et al.* 2005; Lee-Hilz *et al.* 2006; Myhrstad *et al.* 2006; Zhan and Yang 2006). The major mechanism of CVD prevention by flavonoids appears to be through regulation of inflammation. Inflammation is now thought to be the initiating step in atherosclerosis, whereas cholesterol accumulation and generation of

free radicals are consequences, rather than causes (Ross 1999). Much is now understood about the immunoregulatory effects of polyphenolics (Gonzalez-Gallego *et al.* 2007; Stevenson and Hurst 2007). Cancer preventative effects are thought to be related to regulation of cell-proliferation and apoptosis. It is possible that all these effects were disruptive of normal physiological functioning at early stages of mammalian evolution, but evolutionary adaptation has made us partially dependent on these phytochemicals for maintenance of normal physiological function. A hypothesis has been proposed that some polyphenolics can modulate mammalian hormone action and influence the balance of mammalian gut bacterial species (Baker 1995; Adlercreutz 1998; Baker 2002). Mammalian evolution appears to have incorporated dietary phytochemicals as hormone-like substances (Baker 1995). Some flavonoid synthesis enzymes have sequence homology with mammalian steroid biosynthesis enzymes, suggesting that modulation of steroid hormone action by flavonoids may be mediated by enzyme regulation, as well as by direct interaction with steroid receptors.

Some recent literature has added to our understanding of these processes. Flavonoids are extensively conjugated as soon as they are absorbed into intestinal cells (Clifford 2004) and exist in the body mainly as conjugates. Conjugation was thought potentially to "deactivate" flavonoids' biological functions, but evidence is accumulating that these functions are often only modulated or changed (Stevenson and Hurst 2007). Glucuronidation of flavonoids, for example, only slightly moderates their cytoprotective capacity against hydrogen peroxide toxicity (Stevenson *et al.* 2008).

ANTIOXIDANT CAPACITY OF FLAVONOIDS IS UNREALISED *IN VIVO*

The assumption that the health benefits of flavonoids and other polyphenolics are related to their high *in vitro* antioxidant capacity has been largely discredited by combining data on their average dietary intake, pharmacokinetics and bioavailability. It has been calculated that even a diet relatively rich in flavonoids would only transiently increase plasma TAC by about 2% (Clifford 2004). Flavonoids may have a high antioxidant *capacity*, but this capacity is apparently not realised *in vivo* to any significant extent. Similar arguments have been advanced regarding the neuroprotective effects of polyphenolics (Singh *et al.* 2008). Although they show various interesting effects *in vitro* and these appear to go well beyond antioxidant capacity, it is far from clear that they can reach the brain in sufficient concentrations to realise any actual benefits.

POLYPHENOLICS APPEAR TO REGULATE GENES AT REALISTIC PHYSIOLOGICAL CONCENTRATIONS

Recently, it has been found that polyphenolics can influence gene expression, in addition to the many effects they apparently have on proteins. Three common polyphenolics (ferulic acid, quercetin and resveratrol) were incubated with cultured human venous endothelial cells at a concentration of 0.1 μM and resulted in an at least 2-fold up- or down-regulation of 500 genes in a microarray of 10,000 genes (Nicholson *et al.* 2008). This concentration is low in physiological terms, compared with maximum circulating concentrations of individual polyphenolics, thought to be up to ~2 μm (Manach *et al.* 2005) and total concentrations of different polyphenolics, which have been estimated to range from 0.1 to 10 μM (Kroon *et al.* 2004) and from 3 to 22 μM (Clifford 2004). The total plasma concentration of small-molecule antioxidants, including vitamin C, tocopherols, and urate, is in the order of 100s of μM (Stevenson and Hurst 2007). It therefore appears that real plasma polyphenolic concentrations are easily high enough to significantly influence gene expression, but well below levels needed for any realistic effect through direct antioxidant capacity.

POLYPHENOLICS MAY BE ABLE TO MIMIC THE EFFECTS OF CALORIC RESTRICTION AND EXERCISE AND DECREASE PRODUCTION OF ROS

Caloric restriction has been demonstrated to extend lifetime significantly in relatively short lived species such as worms or rodents and greatly increase the number of cell divisions possible in yeast (Guarente 2008). Caloric restriction is now thought to provide these benefits primarily by mitochondrial biogenesis (increase in number of mitochondria per cell) and by switching between their alternative electron transport pathways, resulting in increased efficiency and reduced leakage of ROS (Guarente 2006; Westphal *et al.* 2007; Guarente 2008). These and other processes are apparently regulated by "Sirtuins", a family of regulatory protein deacetylases, coded by SIRT genes. Mitochondrial biogenesis is thought to be regulated by SIRT1. It remains to be seen whether caloric restriction can extend human lifespan, but six months of caloric restriction in a human trial produced similar cellular changes to those observed in other species, i.e., mitochondrial biogenesis, up-regulation of many genes, including SIRT1 and reduced DNA damage (Civitarese *et al.* 2007). The cellular changes were all consistent with those expected to increase lifespan and optimise health. To put all this in context, however, it has long been known that mitochondrial biogenesis is one of the adaptations to exercise (Hoppeler *et al.* 1973; Holloszy and Coyle 1984; Baar *et al.* 2002). Moderate exercise may therefore, also provide many of the benefits of caloric restriction.

It has been demonstrated *in vitro* that polyphenolics, particularly resveratrol, can enhance the activity of the recombinant human sirtuin coded by SIRT1, apparently by a conformational change to the enzyme. Resveratrol at 10 μM also extended the lifespan of yeast from ~23 to ~37 generations (Howitz *et al.* 2003). The enzyme-activation results have been questioned by subsequent studies (Grubisha *et al.* 2005; Kaeberlein *et al.* 2005) on the grounds that resveratrol has so far only shown activity with SIRT1 (it is ineffective on SIRT2), required highly supra-physiological concentrations (~3 fold activation at 20 μM) and a non-physiological small-peptide substrate to have a measurable effect. Further doubt arises from the observations that resveratrol bioavailability from a realistic dose is in the nanomolar range and that it exists *in vivo* almost entirely as conjugates, rather than as free resveratrol (Goldberg *et al.* 2003).

An alternative hypothesis that may explain the above observations equally well involves direct actions of resveratrol and other polyphenolics on mitochondria. It has long been known that inhibitors of the mitochondrial electron transport chain (ETC) cause increased ROS generation (Cadenas and Boveris 1980). It has also been shown that flavonoids can inhibit specific parts of the ETC (Hodnick *et al.* 1986; Hodnick *et al.* 1987; Santos *et al.* 1998; Gledhill *et al.* 2007), or the overall generation rate of ATP (Dorta *et al.* 2005). This raises the possibility that phytochemicals, if they were able to access the mitochondria *in vivo*, could increase ROS generation and induce MB and other beneficial changes in a similar way to exercise or calorie restriction. In addition, *in vitro* cell-culture studies have been reported in which resveratrol was found to induce MB and upregulate antioxidant enzymes including mitochondrial Mn-SOD (Robb *et al.* 2008a, 2008b).

Whatever the real mechanism for these effects, support for their relevance *in vivo* has been provided by two trials in mice, which showed that very high doses of dietary resveratrol (400 and 20 mg/kg/day respectively) reversed all the harmful biological changes induced by a high calorie diet, apart from weight gain (Baur *et al.* 2006; Lagouge *et al.* 2006). In one trial (Baur *et al.* 2006) there was no increase in SIRT1 protein levels and it was proposed that the observed increase in de-acetylation of a physiological substrate, PCG-1α, could have resulted from enzyme activation, rather than up-regulation of the gene. Alternatively, upregu-

lation of the whole mitochondrial signalling pathway involving SIRT 1 may have occurred. A recent mouse trial involving a realistic dose of quercetin (12.5 or 25 mg/kg/day) found further support for *in vivo* effects on mitochondria (Davis *et al*. 2009). Mice showed increased mRNA for PCG-1α and SIRT1, increased cytochrome C (an ETC component), mitochondrial biogenesis in brain and muscle cells and increased treadmill endurance capacity. There appears to be real potential for dietary polyphenolics to reduce RONS production indirectly and to generate an apparent antioxidant effect. There is a significant gap, however, between the concentrations apparently required for an effect and concentrations attainable *in vivo* from even a high-polyphenolic "normal" diet. Much work remains to be done to determine the relevance, if any, of this effect *in vivo*.

HIGH DOSES OF ANTIOXIDANTS ARE NOT ALWAYS HARMLESS

Some studies have found evidence that antioxidants are not necessarily always beneficial. In a human study, 500 mg/day of vitamin C decreased plasma 8-oxoguanine, a commonly used marker for oxidative DNA damage. This apparent benefit, however, was offset by an increase in 8-oxoadenine, a less commonly used, but equally valid marker (Podmore *et al.* 1998). Exercise-induced oxidative stress appeared to stimulate an arterial antioxidant response and reduce atherosclerosis in a rat model; however, vitamin E supplementation had no effect and inhibited the antioxidant response to exercise (specifically induction of SOD and eNOS – endothelial nitric oxide synthase; which helps regulate vasodilation) (Meilhac *et al.* 2001). Paradoxically, in mouse asthma models a vitamin E *deficiency* appeared to moderate lung inflammatory responses (Lim *et al.* 2008). Similarly, prolonged α-tocopherol deficiency in the brain *reduced* oxidative stress and superoxide production in mice (Cuddihy *et al.* 2008). In an *in vitro* study, α-tocopherol down-regulated the nuclear receptors, peroxisome proliferator-activated receptor-γ (PPARγ) and liver X receptor-α (LXRα), and may thereby be counter-productive for controlling atherosclerosis, by interfering with transport of cholesterol from blood vessel walls (Rode *et al.* 2008). In a comparison between "Ironman" triathletes and untrained controls, the athletes had higher resting plasma levels of glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD), plus lower malondialdehyde (MDA, a marker of lipid peroxidation). Participation in the event lowered the athletes' antioxidant enzymes and raised MDA. Athletes who took antioxidant supplements had greater increases in MDA than those that did not (Knez *et al.* 2007). This suggests that training-level exercise up-regulates antioxidant defences, but competition-level exercise suppresses them. Antioxidant supplements may cause further suppression.

A mechanism has been proposed (Palozza *et al.* 2008) to explain the increase in cancer incidence (Goodman *et al.* 2004) associated with the combination of carotenoid supplements and smoking. This essentially involves oxidised β-carotene metabolites, formed by interaction with smoking-generated RONS, down-regulating the retinoid signalling pathway. This leads to down-regulation of RARβ, a tumour suppressor and up-regulation of AP-1, a tumour inducer (Rietjens *et al.* 2002; Palozza *et al.* 2008). Another potentially harmful side-effect of high vitamin E intake could be interference with drug metabolism. Vitamin E is metabolised by the same enzyme pathways as some drugs, so there is potential for a drug-drug type interaction, whereby vitamin E inhibits the metabolism of a drug and causes an overdose (Wu and Croft 2007).

EXERCISE, OXIDATIVE STRESS AND THE RELATIONSHIPS WITH ANTIOXIDANTS

Antioxidant supplementation in relation to exercise

Antioxidants are of interest to exercise researchers because RONS are thought to contribute to muscle fatigue and to increase concentrations of oxidative stress markers. Use of supplements such as vitamins C and E to counter these effects have generally been disappointing for improving exercise performance (Peake *et al.* 2007). However, high doses of the antioxidant N-acetylcysteine (NAC) have been effective at increasing time to exhaustion in endurance-type exercise, albeit from high intravenous doses. Thus, suppression of RONS via antioxidants for enhancing some types of exercise performance seems possible. This may appear to support the use of antioxidants to enhance performance, but it must be balanced against other studies suggesting that antioxidant supplementation may have drawbacks as well as benefits. In an extensive review of studies relating antioxidants with exercise, no consistent benefits of antioxidant supplementation were found and there was evidence that supplementation can interfere with some aspects of adaptation to exercise (Peake *et al.* 2007). This conclusion was supported by subsequent reports. RONS production was only damaging during exhaustive exercise and inhibition of RONS production not only inhibited antioxidant enzyme induction, but may inhibit other adaptive responses to training (Gomez-Cabrera *et al.* 2008b). High dose vitamin C supplementation inhibited adaptations to exercise, one of the most significant being mitochondrial biogenesis (i.e., an increase in numbers and overall respiratory capacity of mitochondria in cells) (Gomez-Cabrera *et al.* 2008a). The overall message from these studies appears to be that dietary antioxidants with direct antioxidant effects may be beneficial if given in a single dose to reduce the oxidative stress damage associated with competitive sporting event participation, but because of the potential for inhibition of normal exercise-induced adaptive responses, ongoing supplementation may be detrimental to training programmes and ultimate performance.

Antioxidant benefits that are similar to exercise

Antioxidant benefits from exercise are primarily derived from adaptive changes affecting endogenous antioxidant capacity, such as enzymes and heat shock proteins, and share some common effects to those observed with many polyphenolics and flavonoids. Thus, exercise is a complementary approach to diet for controlling oxidative stress and its associated damaging effects. Moderate regular exercise induces low levels of RONS, which up-regulates antioxidant/repair enzymes and consequently reduces RONS-associated diseases (heart disease, type 2 diabetes, rheumatic arthritis, Alzheimer's and Parkinson's diseases, and certain cancers) (Ji 2007; Radak *et al.* 2007; Jackson 2008; Ji 2008; Packer *et al.* 2008; Radak *et al.* 2008). Intense exercise raises inflammatory cytokines, whereas regular, moderate intensity exercise reduces them and induces antioxidant and anti-inflammatory mediators in blood vessel walls (Wilund 2007). These observations may at least partly explain the benefits of exercise in prevention of CVD. In a demonstration of the adaptive capacity of muscle cells to exercise-induced oxidative stress, acute, muscle-damaging exercise greatly increased oxidative stress markers, but a repeat 3 weeks later produced a much smaller response (Nikolaidis *et al.* 2007). This suggests that exercise induces endogenous defences against oxidative stress and also that the effects are relatively long-lasting.

"ANTIOXIDANTS" APPEAR TO HAVE MANY NON-ANTIOXIDANT PROPERTIES OF BENEFIT TO HEALTH

As discussed above, the *in vitro* antioxidant capacity of polyphenolics appears not to be realised *in vivo* because of low bioavailability, but evidence is accumulating that they have potentially significant health benefits through regulatory functions that are apparently unrelated to antioxidant capacity. Less extensive but similar evidence is now emerging in relation to the other antioxidants discussed above. The findings come from a combination of *in vitro*, animal and human trials and effects have been found on inflammatory processes, regulation of cell proliferation and direct interactions with genes.

Regulation of inflammation

Hospitalised patients receiving long-term enteral nutrition showed reduced nuclear factor-κB (NFκB) levels when supplemented with carotenoids (Vaisman *et al.* 2006). NFκB is a central mediator of the inflammatory response. Vitamin C supplementation reduced exercise-induced airway narrowing (an inflammatory response) in asthmatics (Tecklenburg *et al.* 2007). Cell cultures exposed to high glucose concentrations in a diabetes model system, generated both excessive RONS and inflammatory transcription factors, but addition of the carotenoid astaxanthin suppressed both ROS and transcription factor production (Manabe *et al.* 2008). In another cell study, catechin polyphenolics, α-tocopherol and ascorbic acid were able to attenuate the reduction in secretion of transthyretin (TTR) and retinol binding protein (RBP) resulting from Interleukin-6 (Il-6) stimulation (El-Saadany *et al.* 2008).

A number of other clinical trials have demonstrated various positive health outcomes on immune function from supplementation with tocopherols, ascorbic acid and carotenoids (Webb and Villamor 2007). In addition, molecular mechanisms have been proposed to explain the anti-inflammatory effects of tocopherols (Reiter *et al.* 2007b).

Potential anti-cancer effects

Both tocotrienols (Nesaretnam *et al.* 2008) and ascorbic acid (Lin *et al.* 2006) inhibited growth and induced apoptosis (programmed cell death) in cancer cells *in vitro*. These properties could help to reduce initiation and growth of tumours *in vivo*. α-Tocopherol enhanced the ability of the flavonoid apigenin to induce cell death *in vitro* (Miyoshi *et al.* 2007). Tocopherols and resveratrol individually reduced tumour cell proliferation *in vitro* and showed a synergistic effect when combined (Reiter *et al.* 2007a). In rats, tocopherol supplementation suppressed mammary tumour growth (Suh *et al.* 2007) and lycopene and lutein inhibited proliferation of prostate cancer cells (Gunasekera *et al.* 2007). Lycopene is known to inhibit tumour metastasis and tissue invasion. It was demonstrated that these effects may be explained by inhibition of matrix metalloproteinase-9 expression (which facilitates tissue invasion by tumour cells) and down-regulation of the binding activity of NFκB and stimulatory protein-1 (Huang *et al.* 2007). These effects were also demonstrated not to be related to antioxidant capacity. Inhibition of angiogenesis (blood capillary formation and growth) is known to inhibit tumour growth, by starving the cells of nutrients. Tocopherols and tocotrienols have demonstrated anti-angiogenic effects in both *in vivo* and *in vitro* studies (Nakagawa *et al.* 2007; Miyazawa *et al.* 2008).

Gene regulatory effects

Antioxidant enzyme genes are regulated by an antioxidant response element (ARE). Carotenoids modulate cholesterol metabolism in cholesterol-fed rats and up-regulate antioxidant enzyme synthesis (Shih *et al.* 2008). Carotenoids, especially lycopene, can activate the ARE and it appears that oxidised lycopene may be the active form (Ben-Dor *et al.* 2005). This mechanism of action is similar to that exhibited by flavonoids (Stevenson and Hurst 2007). A review of vitamin C studies found no consistent effect on oxidative DNA damage but did find recent evidence pointing to regulation of gene expression and cell differentiation (Duarte and Lunec 2005).

Other potential health benefits

Vitamin C promotes vascular endothelial cell growth and may promote repair and regeneration of endothelium damage (Ulrich-Merzenich *et al.* 2007). α-Tocopherol, as expected, reduced lipid oxidation but also attenuated insulin-signalling disorder in diabetic rats (Minamiyamai *et al.* 2008). Several recent reviews have discussed the non-antioxidant health effects of vitamin E (Azzi 2007; Nesaretnam *et al.* 2007; Atkinson *et al.* 2008) and others have discussed the benefits to CVD from modulation of signal transduction and gene expression by vitamins C and E (Brigelius-Flohe 2006; Munteanu and Zingg 2007; Villacorta *et al.* 2007). It has been suggested that oxidised forms of lycopene may be responsible for health benefits additional to ARE activation (Lindshield *et al.* 2007).

CONCLUSION

The emerging consensus of recent research is that the whole concept of antioxidants and health needs to be re-evaluated. It is clear, however, that the antioxidant hypothesis, as originally proposed, is still as relevant today as it was 20 years ago. Moderate dietary intakes of the plant biochemicals traditionally referred to as antioxidants, to support regenerative metabolic pathways, appear to be optimal for health, and whilst deficiency is clearly detrimental, high intakes from supplements or diet are unnecessary and under some circumstances detrimental. The benefits of even a modest intake of dietary antioxidant vitamins are multiplied greatly by their role as cofactors for antioxidant enzymes and their regeneration by metabolic pathways linked to respiration. What has changed is that we can now appreciate the complexity and efficiency of our natural antioxidant defences and that any attempt to augment them must be thought out extremely carefully in order to have any chance of success.

There appears to be value in individual measures of the plasma antioxidants, vitamins C and E, within an optimal range, but there is no evidence that measures such as TAC are valid to indicate *in vivo* antioxidant status or health outcomes. Stimuli for optimising redox balance to mitigate oxidative stress come from a wide range of sources, such as antioxidants (when acting as pro-oxidants), exercise and caloric restriction. In reality, these appear to be highly effective at both up-regulating endogenous defences against RONS and reducing their production, by mitochondria or inflammatory processes, to an optimal level which minimises oxidative damage, but maintains their essential signalling/defensive role. This highlights the point that endogenous defences against RONS and the damage they do appear to be far more effective than dietary antioxidants in the "conventional" sense.

This new paradigm of "antioxidants" does not at all imply that the compounds we generally refer to as antioxidants are not beneficial to health, however. They appear to have numerous, if moderate, pharmacological effects, such as regulation of gene transcription, inflammation, cell proliferation and other processes. These effects appear to be much more significant to good health than "conventional" antioxidant effects. The term "nutraceuticals" may be much more appropriate for carotenoids and polyphenolics, and only vitamins C and E probably deserve the label "antioxidant", in addition to some apparent nutraceutical effects. As discussed above, many other "antioxidants" are actually enzyme cofactors needed for optimal functioning of endogenous antioxidant defences. A very useful alternative way

of defining the term "antioxidant" was proposed as long ago as 1998 (Institute of Medicine 1998). A review panel proposed a purely functional definition: "A dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species or both, on normal physiological function in humans". This definition is still equally valid today, in encompassing *any mechanism* that reduces damage from RONS, *in vivo*, rather than *in vitro*. We now know a lot more about the "non-radical scavenging" mechanisms that were unknown or relatively newly discovered at the time this definition was formulated.

Hormesis is a concept which may help to explain the fundamental basis of the apparent non-antioxidant health benefits of polyphenolics and carotenoids (Calabrese 2008a, 2008b; Stark 2008; Zhang *et al.* 2008). According to this concept, most exogenous compounds to which mammals are exposed are toxic at high doses and harmless or beneficial in low doses. There are numerous examples of this phenomenon from studies of the biological effects of phytochemicals. The induction of antioxidant enzymes by polyphenolics and carotenoids, for example, would fit well into this theory, as would the toxic effects for smokers from high carotenoid doses, but apparent benefits to non-smokers from more modest dietary intakes. There may well be many other similar responses to "antioxidants" that remain to be discovered.

The significance of this new understanding of the effects of plant nutraceuticals on health is that the old paradigm is misleading for plant breeders, functional food manufacturers, nutritionists and consumers. A belief that "high antioxidant" plants are the key to health and long life leads to incorrect choices being made. Breeding fruits, for example, guided by simplistic concepts like maximising their *in vitro* TAC, as has been done in the past, is unlikely to optimise their health benefits. It should be better to select new cultivars on the basis of a balance of the various classes of phytochemical, provided this is consistent with other important factors such as taste, macronutrient content, storage life and high productivity of the plant. If cultivars are selected for high content of a particular nutraceutical, this should be based on sound evidence that the compound of interest has a specific and demonstrable health benefit. Similarly, for the consumer, reliance on one "high antioxidant super-crop" is likely to be much less beneficial to overall health than consumption of a variety of different fruits and vegetables, containing many different health-promoting compounds.

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LATEST UPDATES

A further study of the benefits of resveratrol on health was undertaken by feeding groups of genetically obese (Zucker) rats and lean normal rats the modest dose of 10 mg/kg/day of resveratrol (Rivera *et al.* 2009). There was relatively little effect on the lean rats, but the obese rats benefited from improvements in dyslipidemia, hyperinsulinemia, hyperleptinemia and hypertension.

The effects of antioxidant supplementation on exercise adaptation arising from a training program have been reported (Ristow *et al.* 2009). Un-supplemented subjects showed large changes in markers of exercise adaptation, such as insulin sensitivity and levels of RNA for PPARγ, SOD2 and PCG1-α, whereas those treated with vitamin C (1000 mg/day) and vitamin E (400 IU/day) showed little or no change. This result is further evidence that excessive antioxidant intake can inhibit the beneficial signalling roles of RONS, that appear to be essential for exercise adaptation.

A recent and comprehensive review of the interrelationship of mitochondria, oxidative stress and optimal health has underlined the critical importance of hormetic stimuli in maximising mitochondrial efficiency, preventing metabolic syndrome and even optimising insulin response (Nunn *et al.* 2009). The mild oxidative stress required to stimulate these beneficial changes was proposed to be generated by many possible hormetic signals, including the well-established exercise, calorie restriction and polyphenolic pro-oxidants, but also temperature extremes, oxidised polyunsaturated fats, alcohol and drugs such as metformin and statins.

These reports reinforce the message from numerous earlier studies. High intake of antioxidants may be beneficial to restore health in situations where they may be very deficient, such as many disease states, advanced age, or high levels of extreme endurance exercise. Any reasonably healthy diet, moderate exercise program, or low-dose supplement should provide plenty of *in vivo* antioxidant capacity for maintenance of good health