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Polyphenols as Adaptogens – The Real Mechanism of the Antioxidant Effect?

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1. Introduction

It is well-established from numerous population-based observational studies, that consumption of polyphenol-rich foods, principally fruits and vegetables is beneficial to health, reducing mortality rates and the incidence of the major diseases of modern civilisation, cancer and cardiovascular disease (Stevenson & Hurst, 2007). Until relatively recently, it was widely believed that these health benefits were mediated by free radical-scavenging antioxidants, i.e., vitamins C and E and polyphenols, all compounds with high antioxidant capacity when measured by in vitro chemical tests such as "ORAC". A large body of research, however, has not found a conclusive link between the apparent health benefits of polyphenols and their antioxidant capacity. In addition, supplementation with vitamins C and E, which are thought to operate in the body by radical scavenging, has been the subject of intensive research and large-scale intervention trials. The overall conclusion of this work is that there is no consistent evidence that supplementation of these vitamins above normal dietary intakes is of any benefit to health (Bjelakovic et al., 2008). This suggests that the health benefits of vitamins C and E and polyphenols are not related to their antioxidant capacity. More recent research is, nevertheless, linking polyphenols to other biological effects that have the same end-result as chemical antioxidants were thought to have, i.e., a sustained decrease in free radicals in the body, resulting from enhanced endogenous antioxidant defences and/or reduced production in the mitochondria, the main source of free radical generation. In subsequent sections, the evidence for this is discussed.

2. Relevance of mitochondria to antioxidant effects of polyphenols

Mitochondria are the major producers of free radicals or reactive oxygen species (ROS) in the body and some of the adaptive effects of polyphenols that modulate oxidative stress appear to act through the mitochondria. It is beyond the scope of this review to cover mitochondrial biology in depth, but there is an excellent and comprehensive book on the subject (Scheffler, 2008). For the purposes of this review, an appreciation of the essentials of mitochondrial function will be sufficient to allow interpretation of studies on how polyphenols interact with mitochondria.

2.1 Mitochondria generate metabolic energy and ROS

Mitochondria are responsible for the bulk of cellular energy production (Scheffler, 2008), with only a small proportion being accounted for by the glycolytic pathway. The "electron transport chain" (ETC – Figure 1) oxidises NADH, one output from the TCA acid cycle (Brookes, 2005). This generates electrons, which are transferred through the various components of the ETC, ultimately reducing oxygen to water. In the process, a membrane potential (or proton gradient) is generated by the five ETC complexes pumping protons across the mitochondrial membrane. The return flow of protons through ATP synthase drives ATP synthesis from ADP. The ATP produced during this process is the main energy source used by cells and tissues.

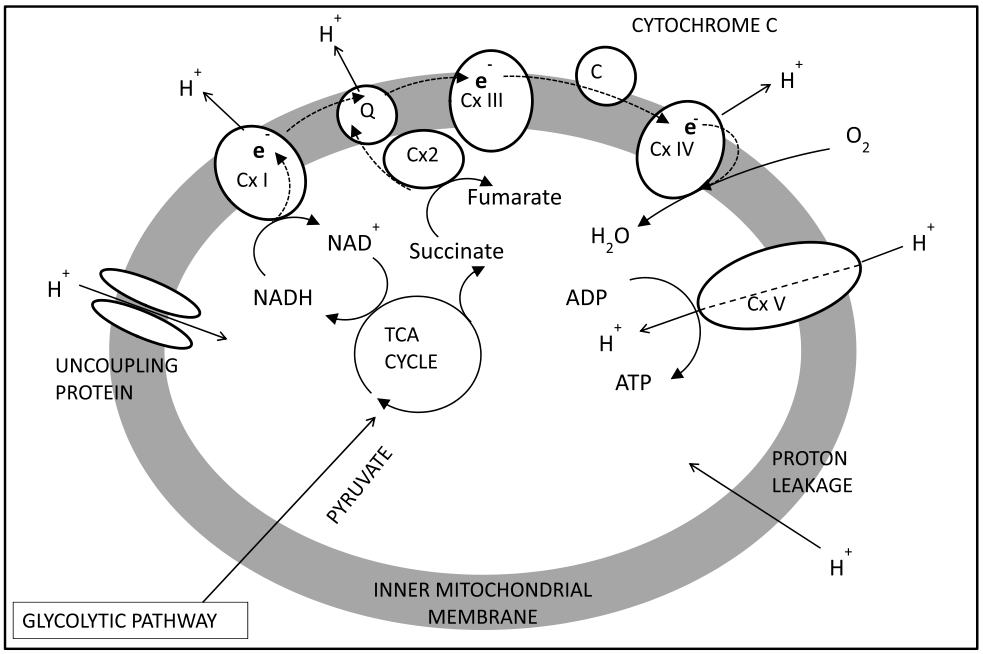

Fig. 1. Schematic summarising the main features of mitochondrial metabolism (Brookes, 2005). Electrons (e^-) from the NADH to NAD^+ transition are transferred between electron donors and acceptors, in the process generating energy to pump protons outside the inner membrane. The resulting proton gradient can leak back through the membrane or through uncoupling proteins, but most flow through Complex V (ATP synthase) and power ATP synthesis. Complex I (Cx I, NADH dehydrogenase) passes electrons to Complex III (Cx III, cytochrome bc1) via Q (ubiquinone). Complex II (Cx II, succinate dehydrogenase) delivers more electrons via Q, while Complex IV (Cx IV, cytochrome C oxidase) accepts electrons from Cx III, carried by cytochrome C and uses them to reduce oxygen to water.

The ETC is not 100% efficient and some oxygen molecules are incompletely reduced into the free radical (or reactive oxygen species – ROS) superoxide (Scheffler, 2008; Dorta et al., 2006). Superoxide can in turn generate other ROS species. The production of ROS by muscle cells is greatest during exercise, and the condition arising when ROS increase to damaging

levels, is termed "oxidative stress" (Powers & Jackson, 2008). Mitochondria are the major source (approx. 90%, the remainder coming from immune system action and the environment) of ROS in the body (Ristow et al., 2009), but in healthy and especially, physically fit individuals, the mitochondria are also well-equipped with antioxidant enzymes to inactivate ROS before they can do more than minor damage to DNA or other vital cellular components (Hu et al., 2007).

The mitochondrial form of the antioxidant enzyme superoxide dismutase (SOD), Manganese , or MnSOD, converts superoxide into hydrogen peroxide, which is not in itself a radical, but easily forms one (Hu et al., 2007). This is, in turn, reduced to water by a mitochondrial form of another enzyme, glutathione peroxidase (GPX) (Dorta et al., 2006). The cell cytosol has a different form of SOD (Copper Zinc, or CuZnSOD) and a different enzyme, catalase, is primarily responsible for removing hydrogen peroxide (Dinkova-Kostova & Talalay, 2008). Under most circumstances, mitochondria in healthy cells leak only a tiny proportion of ROS into the cytoplasm. ROS generation and leakage increase markedly in unhealthy cells during e.g., exhaustive exercise, because the ETC becomes much less efficient when working close to its maximum capacity.

2.2 Mitochondrial adaptation to oxidative stress

It is well established that exercise increases the mitochondrial content of muscle fibres (known as mitochondrial biogenesis or MB) and consequently their respiratory capacity (Holloszy & Coyle, 1984; Hood et al., 2006; Huang & Hood, 2009). More recently, the increase in ROS generation during exercise has been found to be the primary signal for this adaptive effect of exercise (Ji et al., 2008). The benefit to the organism of MB is greater energy generation capacity and reduced ROS generation for a given energy output. Although it might be expected that increases in mitochondrial/ETC density would lead to increased respiration and ROS production, the opposite actually appears to be the reality. ROS are primarily produced when the flow of electrons through the ETC is limited later in the chain (Barros et al., 2004; Kushnareva et al., 2002). Under these conditions, electrons back up and start to leak out of the complexes earlier in the chain, thereby generating ROS. Under conditions of rigorous exercise, for example, oxygen, the final electron acceptor, would be in limited supply, thus flow of electrons from Complex IV to oxygen would be limited and leakage would occur, primarily from Complex I (Brookes, 2005). There is strong supporting evidence for the link between limited electron flow and ROS generation. Compounds that inhibit any of the ETC complexes cause backing-up of electrons and increased ROS production (Cadenas & Boveris, 1980). The Complex III inhibitor antimycin A increased superoxide production in an in vitro cell model (Dairaku et al., 2004). Inhibition slows the flow of electrons through the ETC and increases the probability of incomplete reduction of oxygen, thereby making superoxide generation more likely.

Moderate regular exercise is reported to induce a low level of ROS, which is thought to up-regulate antioxidant/repair enzymes and consequently to reduce ROS-associated diseases (heart disease, type 2 diabetes, rheumatic arthritis, Alzheimer's and Parkinson's diseases, and certain cancers) (Radak et al., 2008; Ji et al., 2008; Jackson, 2008). Another complementary benefit is that increased mitochondrial respiration capacity and/or MB should reduce resting ROS generation and reduce the effects of aging (Lopez-Lluch et al., 2006). This comes about because ROS generation is highest when the flow of electrons

through the ETC is limited. Increased respiratory capacity permits faster electron flow and lowers basal ROS generation. High basal ROS generation is thought to be the main factor causing aging (Cadenas & Davies, 2000), through damage to mitochondrial DNA. Whereas nuclear DNA is heavily protected from damage by ROS, mitochondrial DNA is located in the inner mitochondrial membrane, close to the ETC complexes and very exposed to damage from ROS. It is thought that damage to mitochondrial DNA leads to synthesis of defective ETC protein subunits and thereby, defective ETCs, which generate less energy and more ROS. A negative feedback loop then results in further increase in ROS generation and DNA damage, to both mitochondrial and nuclear DNA (Huang & Hood, 2009; Droge, 2002).

2.3 Mitochondrial adaptation - a new mechanism of antioxidant action?

It is reasonable to assume that, if exercise-induced oxidative stress is the primary signal leading to mitochondrial adaptation and consequently, increased respiratory capacity and decreased basal ROS production, then other sources of oxidative stress could generate similar adaptations. This is an excellent example of the principle of hormesis (Calabrese, 2008), i.e., a non-linear, adaptive, dose response to a toxin. High doses of ROS are clearly harmful, but low doses appear to be essential to initiate the signalling pathways that lead to beneficial adaptive responses. A hypothesis has been proposed recently to explain how oxidative stress induces mitochondrial adaptation to improve efficiency of energy generation, thereby improving physical fitness and general health, ameliorating health issues such as metabolic syndrome and diabetes and above all, increasing life span (Nunn et al., 2009, 2010). Oxidative stressors that are proposed to induce mitochondrial adaptation include exercise, calorie restriction, ionising radiation and most relevant to this discussion, phytochemical "pro-oxidants" (Nunn et al., 2009; Ristow & Zarse, 2010). The ways in which exercise and polyphenols generate oxidative stress are discussed below. Ionising radiation generates ROS directly, from any molecule encountered (Harman, 1956), thereby causing oxidative stress. Calorie restriction stimulates increased respiration that also leads to oxidative stress (Guarente, 2008; Tapia, 2006).

2.4 Signalling pathways that control mitochondrial adaptation

It is thought that mitochondrial adaptation to exercise is primarily brought about by a complex signalling cascade, which is initiated by increased generation of ROS (Droge, 2002) generated by the mitochondria during exercise. The most important aspect of this adaptation is MB i.e., an increase in the number and mass of mitochondria and/or an increase in the density of ETC complexes (Hoppeler et al., 1973; Baar et al., 2002; Holloszy & Coyle, 1984). Most mitochondrial proteins, including those that make up the ETC, are actually encoded by nuclear genes (Scheffler, 2008) and these are regulated by nuclear respiratory factors (Nrf) including Nrf-1 and Nrf-2 (Hawley & Holloszy, 2009). These Nrfs activate genes that encode mitochondrial respiratory chain proteins. Nrf-1 also up-regulates expression of mitochondrial transcription factor A (TFAM), which is transported into the mitochondria and regulates transcription of the mitochondrial genome (Hawley & Holloszy, 2009). Expression of mitochondrial fatty acid oxidation enzymes is regulated by peroxisome-proliferator coactivator 1-α (PGC1-α) (Baar et al., 2002; Lopez-Lluch et al., 2006; Poderoso et al., 2000). Sirtuin1 (a regulatory protein deacetylase, is discussed further in Section 5.1) is thought to be involved in deacetylating and thus activating PGC-1α, which, in turn, co-activates peroxisome-proliferator activated receptor-γ (PPAR-γ).

PPAR-γ is primarily a receptor for fatty acids, which regulates fatty acid oxidation, but it can apparently also be activated by a number of natural products, including some polyphenols (Huang et al., 2005). The anti-diabetic drugs "glitazones" are known to activate PPAR-γ and have also been shown in vitro to induce MB and reduce mitochondrial ROS production (Fujisawa et al., 2009). Polyphenols isolated from red wine, particularly ellagic acid and epicatechin gallate (ECG), were able to activate PPARγ in vitro with similar affinity to the reference pharmaceutical compound rosiglitazone (Zoechling et al., 2011). A number of studies on resveratrol, another polyphenol found in red wine, suggest that many of its purported beneficial effects are mediated by its stimulation of PGC1-α signalling (Tan et al., 2008). Interactions with PPARγ therefore appear to be a possible means for polyphenols to influence mitochondrial adaptation. These interactions may be mediated through direct binding to and activation of PPARγ, or indirectly through its co-activator PGC1-α.

2.5 How do ROS interact with signalling pathways?

One uncertainty in our knowledge of the MB signalling pathway is how an increase in ROS generation initiates signalling. One possible mechanism involves oxidised lipids and the Electrophile (or Antioxidant) Response Element (ERE, or ARE). Mitochondria are primarily composed of membranes into which the ETC complexes are embedded. The lipids in these membranes should be highly susceptible to peroxidation by superoxide generated in their immediate vicinity. One species of breakdown products known to arise from lipid peroxidation is a number of conjugated aldehydes such as 4-hydroxy-2-nonenal (HNE) (Uchida et al., 1999). Conjugated aldehydes and ketones are also potent activators of the ERE (Dinkova-Kostova & Talalay, 2008). The ERE itself is a regulatory region in nuclear DNA that controls the expression of a series of cytoprotective proteins, including a number of antioxidant enzymes, cytochrome P-450 xenobiotic hydroxylases and xenobiotic conjugative enzymes (Dinkova-Kostova & Talalay, 2008). The transcription factor that controls the ERE is Nrf2, which is normally bound to the sensory protein Keap1. Binding of an inducer, such as HNE, or phytochemicals such as curcumin, some polyphenols or sulphoraphane releases Nrf2, which activates the ERE and the proteins it regulates (Dinkova-Kostova & Talalay, 2008). It is possible that the ERE and Nrfs are a link between lipid peroxidation by ROS and the signalling pathway for MB. It appears from this discussion that one of the potential mechanisms for the adaptive effects of polyphenols and other phytochemicals is direct interaction with the MB signalling pathway, through direct activation of either PPAR receptors or the ERE, through interaction with its regulatory protein Keap1.

3. Effect of exercise on mitochondria

This area of science has been subjected to intensive research for at least two decades and is now well understood. The mechanisms of action of exercise in mitochondrial adaptation would reasonably be expected to be very similar to those of other adaptogens, such as polyphenols, if they work through generation of oxidative stress. Evidence that polyphenols can have a hormetic effect through generation of oxidative stress is discussed below. Exercise science should, therefore, be a good source of insights into the mechanistic details of how polyphenols should interact with mitochondria, as well as providing validated assays to monitor these effects both in vitro and in vivo.

3.1 Macroscopic effects of exercise

The well-known benefits of exercise in weight management have been explored by a comparison of mitochondrial metabolism between trained athletes and sedentary individuals (Befroy et al., 2008). The athletes had a 53% higher resting TCA cycle flux, but the same ATP synthesis rate as the sedentary individuals. Essentially, the athletes appeared to have much higher mitochondrial respiration capacity, which "wasted" energy in the resting state, thereby raising their metabolic rate. As discussed above (Section 3.2), it would be reasonable to expect that the athletes' mitochondria would generate less ROS than the sedentary controls in a resting state, because of greater mitochondrial capacity and efficiency, in spite of their higher metabolic rate.

3.2 Antioxidants may inhibit exercise-induced mitochondrial adaptation

If the mechanism underlying the adaptive effects of exercise is initiated by increased oxidative stress, i.e., ROS production, then logically, radical-scavenging antioxidants may be expected to at best have no effect, or at worst hinder adaptations. A number of studies on the use of radical-scavenging antioxidants, i.e., vitamins C and E, appear to support this hypothesis. In a comparison between "Ironman" triathletes and untrained controls, the triathletes had higher resting plasma levels of glutathione peroxidase (GPX), catalase, and superoxide dismutase (SOD), plus lower malondialdehyde (MDA, a biomarker of lipid peroxidation). Participation in the Ironman event then lowered the athletes' antioxidant enzymes and raised MDA. Triathletes 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 taken during training may cause further suppression of endogenous antioxidant defences. Supplementation of both trained and untrained subjects during a one-month training programme revealed that the supplements suppressed several early biomarkers of mitochondrial adaptation, including expression of PGC1-α, PPAR-γ, SOD2 and GPX1 (Ristow et al., 2009). It appears, however, that there is no overall inhibition of exercise adaptation resulting from antioxidant supplementation because no effect on markers of oxidative stress or on increases in training-induced muscle performance was identified (Theodorou et al., 2011).

It appears reasonable to suggest that exercise is an antioxidant in itself, because it leads to significant up-regulation of antioxidant enzymes in humans (Gomez-Cabrera et al., 2006; Gomez-Cabrera et al., 2008). Similarly, SOD2, the specific mitochondrial form of the enzyme, was induced in rodents by exercise (Higuchi et al., 1985). Although antioxidant supplementation appears to be of no benefit to most sports training, it may be useful if applied in a well-planned and timely manner, to protect untrained individuals from the most damaging effects of high ROS production at the start of a fitness programme (Vincent et al., 2006). These findings are consistent with the hormetic hypothesis of polyphenol-stimulated mitochondrial adaptation. It seems reasonable to conclude that if radical-scavenging antioxidants inhibit the signalling of mitochondrial adaptive effects, but polyphenols promote them, then polyphenols are not primarily acting as radical-scavenging antioxidants in vivo.

4. Evidence that polyphenols can induce mitochondrial adaptation

4.1 Polyphenols may be able to regulate Sirtuins

"Sirtuins" are a family of regulatory protein deacetylases, coded by SIRT genes. Sirtuin1 is thought to play a role in regulation of MB (see above). SIRT1 activation by a synthetic activator was found to up-regulate lipid oxidation (a pathway located in the mitochondria), suggesting potential in treatment of obesity, diabetes and metabolic disorders (Feige et al., 2008). Mice over-expressing SIRT1 showed a phenotype resembling calorie restriction, supporting the involvement of the SIRT1 pathway (and by implication, MB) in adaptations to calorie restriction (Bordone et al., 2007).

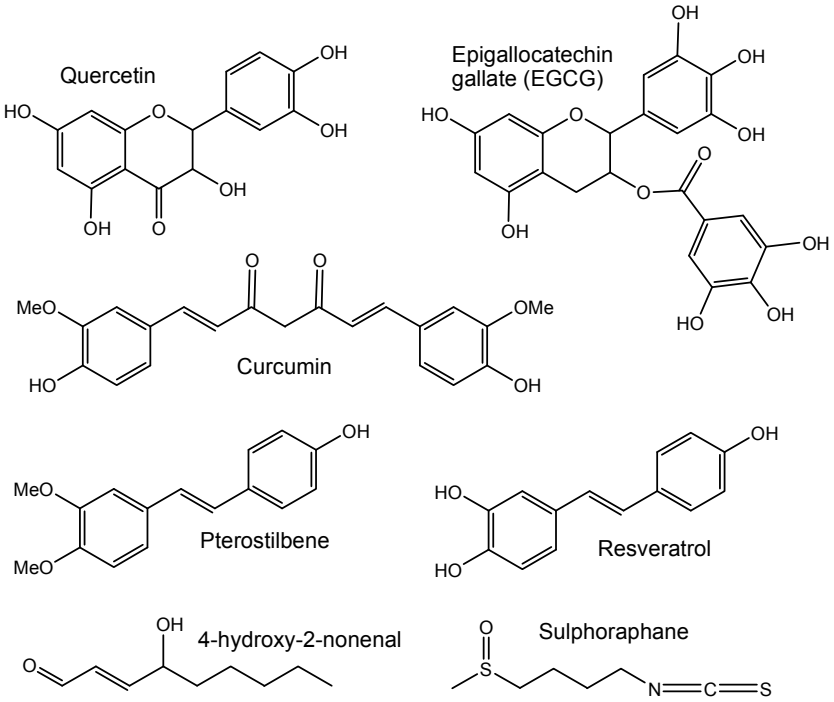

Fig. 2. Chemical structures of the compounds most often referred to in the text.

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). Chemical derivatives of resveratrol appear to be even more effective (Yang et al., 2007), suggesting that these compounds in some way decrease the DNA damage associated with aging. These enzyme-activation results have been questioned by subsequent studies (Grubisha et al., 2005; Kaeberlein et al., 2005) on the grounds that resveratrol required highly supra-physiological concentrations (a 3-fold activation at 20 μM) and a non-physiological substrate to have a measurable effect. Observations that the plasma concentration of resveratrol 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) cast further doubt on sirtuin activation as a key mechanism in vivo. It appears more likely that direct sirtuin activation is only a very minor mechanism of MB stimulation by polyphenols in vivo.

4.2 Inhibition of the ETC by polyphenols

It is well-established that inhibitors of the ETC increase ROS generation (Cadenas & Boveris, 1980). It has been shown, in vitro, that flavonoids can inhibit specific mitochondrial functions, including NADH oxidase (Hodnick et al., 1986), F1-ATPase (Gledhill et al., 2007) and the membrane permeability transition (Santos et al., 1998). Other in vitro studies found that polyphenols inhibited overall mitochondrial respiration (Hodnick et al., 1987) and the closely related rate of ATP generation (Dorta et al., 2005). The former study also detected a burst of ROS generation associated with the inhibition of respiration. A wide range of compounds was tested in these studies, to the extent that structure-activity relationships were established. The two best classes of compound appeared to be stilbenes (e.g., resveratrol) and flavonols (e.g., quercetin). These findings suggest that polyphenols, if they were able to access the mitochondria in vivo, could directly but transiently increase ROS generation, thereby inducing beneficial adaptations in a similar way to exercise.

4.3 Can polyphenols access mitochondria to exert biological effects?

The ability of a compound to inhibit mitochondrial metabolism strongly suggests, but does not prove, that it can access the interior of the mitochondria. Several in vitro studies, however, have provided indirect evidence to support the potential of polyphenols to access the interior of mitochondria in vivo. In a study of the effects of treatment with EGCG on rat neuronal cells in vitro, in which the cells were fractionated to isolate the mitochondria, 90-95% of the detectable EGCG was present in the mitochondrial fraction (Schroeder et al., 2009). Similarly, quercetin was rapidly and extensively absorbed by Jurkat cells and their isolated mitochondria, as well as by the mitochondria of preloaded cells (Fiorani et al., 2010). When isolated rat kidney mitochondria were treated with quercetin, various changes consistent with access of quercetin to the interior of the mitochondria were observed, including increased mitochondrial membrane permeability and oxygen consumption, but decreased membrane potential and oxidative phosphorylation (Ortega & Garcia, 2009). It thus appears that mitochondria would be easily able to absorb significant concentrations of polyphenols, provided the intracellular concentrations around them were high enough.

4.4 Bioavailability and access of polyphenols to mitochondria *in vivo*

Studies in vitro can only indicate potential for in vivo effects; to date there is very little direct evidence to support "mitochondrial bioavailability" in vivo. The many hundreds of polyphenol bioavailability studies done in vivo have only measured concentrations of polyphenols and their metabolites in plasma and/or urine; this proves only that the compound or compounds got as far as the circulatory system (Stevenson et al., 2008). A radioactive tracer approach is also unable to resolve the intracellular location of the radioactivity or the specific chemical compound involved. Studies on animals find radioactivity in all tissues in the body, but cannot distinguish between the original polyphenol, a conjugate or some much simpler breakdown product (Stevenson et al., 2008). This approach is also unable to resolve the intracellular location of the radioactivity.

Polyphenol feeding studies on pigs have found polyphenols at micromolar concentrations in a variety of tissues and organs (Bieger et al., 2008; Kalt et al., 2008; Kalt et al., 2007), but one concern with these studies is that entrained blood may not have been completely removed, thus casting doubt on the actual concentrations in the tissues. On balance, it seems probable that polyphenols can access tissues and therefore cells in vivo, but probably in the form of conjugated metabolites or breakdown products, rather than the unconjugated forms tested in vitro. We therefore have no solid evidence of the mitochondrial bioavailability in vivo of the polyphenols tested in vitro. A number of in vivo trials (discussed in later sections) have successfully detected physiological changes consistent with mitochondrial adaptation, linked to dosing with polyphenols, suggesting that polyphenols or their metabolites can in some way stimulate mitochondrial adaptation, but these trials give little information on how these effects could be mediated.

4.5 *In vitro* evidence for mitochondrial adaptation by polyphenols

The in vitro studies reported to date have nearly all been carried out on the polyphenols resveratrol and hydroxytyrosol. Resveratrol, at a supra-physiological concentration of 50 μM, was found to induce MB significantly and greatly up-regulate antioxidant enzyme synthesis including MnSOD in both mouse and human cell cultures (Robb et al., 2008). The critical importance of MnSOD to health and life itself has been demonstrated by several studies. Genetically modified mice that over-express this enzyme have a modestly extended lifetime (Hu et al., 2007), whereas MnSOD-knockout mice die within a few days of birth (Y. Li et al., 1995). Recombinant lactobacilli over-expressing the antioxidant enzymes SOD and CAT demonstrated greatly enhanced resistance to oxidative stress and significantly increased longevity compared with normal bacteria (An et al., 2011).

Resveratrol treatment of isolated human vascular endothelial cells up-regulated many biomarkers of mitochondrial adaptation, including PPAR-α, Nrf1, TFAM, mitochondrial DNA, ETC proteins and MB. Endothelial nitric oxide synthase (eNOS) was also up-regulated, but if NO synthesis was blocked, MB and the other adaptations were also blocked (Csiszar et al., 2009). These findings support the regulatory effects of eNOS/NO on mitochondria, at least in vascular cells. NO itself is well-established as an important factor in mitochondrial regulation (Cadenas et al., 2000).

Hydroxytyrosol (HT), a polyphenol found in olives and olive oil, at concentrations as low as 1 μM significantly stimulated MB and ETC complex synthesis, concomitant with up-regulation of the MB-signalling molecules PGC1-α and Nrf-1 and-2 (Hao et al., 2010). Very similar results were obtained from ARPE-19 cells, a human retinal pigment epithelial line, challenged with acrolein and in addition, HT increased expression of ERE-regulated phase II detoxifying enzymes (Lu et al., 2010). These in vitro studies provide considerable evidence that polyphenols could stimulate adaptive effects in vivo, provided they could accumulate in the cell or mitochondria at sufficient concentrations. Adaptations mediated by eNOS/NO would only require access to the vascular system, which is well-proven by numerous plasma bioavailability studies.

4.6 Other potential adaptogenic effects of polyphenols

Other adaptive effects, not necessarily related to mitochondria, have been associated with polyphenols. Quercetin glycosides stimulated glucose uptake in C2C12 mouse muscle cells

in vitro (Eid et al., 2010), an effect that should be beneficial in treating type 1 diabetes, a condition thought to involve mitochondrial dysfunction (Fujisawa et al., 2009). The glycolytic pathway for ATP generation in the cytosol converts glucose to lactate (Scheffler, 2008). Lactate is converted to pyruvate, which is a major input into the TCA cycle in the mitochondria (Figure 1). Treatment with elevated concentrations of pyruvate stimulates MB in both L6E9 myoblasts and C2C12 cells (Duguez et al., 2004; Wilson et al., 2007). Induction of MB by increased pyruvate supply in vitro suggests that there could be a similar effect in vivo. Stimulation of glucose uptake by muscle cells may increase glycolysis and indirectly stimulate MB and other mitochondrial adaptations. In other words, this may be an additional mechanism for stimulation of mitochondrial adaptation by polyphenols.

4.7 Animal trials linking polyphenols with mitochondrial adaptation

Support for the in vivo effects of polyphenolics being closely related to those of exercise on mitochondria has been provided by two trials in mice, which found that high doses of dietary resveratrol (400 and 20 mg/kg/day respectively) reversed all the harmful biological changes (in particular, a shortened lifespan) induced by a high calorie diet, apart from weight gain (Lagouge et al., 2006; Baur et al., 2006). Both trials found increased activity of the mitochondrial signalling molecules SIRT1 and PGC1-α, as well as increased insulin sensitivity. Although 400 mg/kg/day is far from a practical intake for humans, the absence of any ill-effects to the test animals strongly suggests that this compound should have no toxicity in humans at any realistic dietary intake. A subsequent trial found significant increases in the major antioxidant enzymes in mice dosed with resveratrol as part of a high fat diet (Robb et al., 2008). Of particular interest was a doubling of the activity of MnSOD in brain and kidney tissue, in agreement with the observations from in vitro trials of resveratrol.

Resveratrol and its methylated analogues were found to be effective in an in vitro cellular model of oxidative stress. One of these analogues, the naturally-occurring dimethylated derivative of resveratrol, pterostilbene, was subsequently found to reduce neuro-degeneration, a major contributor to age-related cognitive decline, in a long-term rat trial (Joseph et al., 2008). Mitochondrial dysfunction has been implicated in and may be a primary cause of most common neurodegenerative conditions, including those related to aging (Scheffler, 2008). Although these trials do not provide a definitive link between the observed health benefits and resveratrol-induced MB, the results are consistent with such a link and certainly lend support to the link between polyphenols and human health benefits.

4.8 *In vivo* evidence for mitochondrial adaptation by polyphenols

Highly significant augmentation of exercise performance resulted from dietary supplementation with quercetin in a mouse model (Davis et al., 2009). Groups of mice were fed 0, 12.5 or 25 mg/kg/day of quercetin (approximately equivalent to a realistic dose of 1 or 2 g/day for an 80 kg human) for 7 days. Increases (relative to placebo) were observed in gene expression of PCG-1α and SIRT1 by up to 2 fold in muscle and brain, whilst levels of cytochrome C (a marker of mitochondrial mass) increased by 23% in muscle and 18% in brain. Mitochondrial DNA also increased up to 2 fold (Davis et al., 2009). This suggests that the numbers of mitochondria approximately doubled and their overall respiration capacity increased by around 20%. The importance of these results is the clear link between quercetin

consumption, increases in biomarkers for mitochondrial adaptation, and MB itself. This trial is therefore, the first to demonstrate unequivocally mitochondrial adaptive effects of a common polyphenol, administered orally. Exercise trials were done on other, similarly dosed mice, in the same study. Forced treadmill running time to exhaustion increased by 37% at both doses of quercetin. Voluntary wheel running activity also increased, both in speed and in time spent. Total distance covered increased by ~45% by the end of the 7-day treatment period and was sustained for the following 7 days (Davis et al., 2009).

Although these performance improvements appear spectacular, they are not unexpected. Laboratory animals are over-fed, chronically under-exercised and lack social interaction and environmental stimulation, being confined to single cages for most of their lives (Cressey, 2010). Although such animals may be poor models of human responses in most cases, they would be expected to be excellent models of obese, unfit and depressed humans who could benefit most from exercise and a healthy, high-polyphenol diet. In this light, the physical performance improvements observed in the trial undertaken by Davis and colleagues would not be expected to be reproduced in humans; much smaller changes in human fitness would be expected from the same treatment. The salient point here is that the performance-enhancing effects of the quercetin are of minor relevance to human health, compared with the clear demonstration of its adaptive effects on mitochondria. In another study, resveratrol supplementation (0.2% w/w) for 12 weeks increased exercise performance in a mouse model of senescence, whereas performance declined in the control group (Murase et al., 2009). Polyphenols therefore, may have potential to ameliorate age-related physical decline in humans.

One aspect of the Davis et al. (2009) study that cannot be easily explained by mitochondrial adaptation is the large increase in voluntary wheel running. Davis and colleagues suggested that this resulted from an entirely different property of quercetin, namely that, in vitro, it is an adenosine A_1 receptor antagonist, as are caffeine and some other common flavonoids (Alexander, 2006). Caffeine is both psycho-stimulatory and ergogenic and this may explain the apparently increased motivation for the mice to exercise.

There is also solid evidence from human trials for adaptive effects of quercetin. A supplementary combination of quercetin (1 g), isoquercetin, EGCG and polyunsaturated fatty acids was tested on *trained* cyclists undergoing 3 hours of cycling on each of 3 days (Nieman et al., 2009). Significant decreases in inflammatory biomarkers were detected relative to the control (placebo), but no change was observed in performance, or any marker of exercise or mitochondrial adaptation. The latter finding is not unexpected in trained athletes, who would be expected to have minimal capacity for increased performance or additional exercise-induced adaptation. In a further trial, 1 g/day of quercetin for two weeks was tested on *untrained* male test subjects (Nieman et al., 2010). Distance travelled in 12 minutes on a treadmill was determined. Relative to placebo, a small but significant increase in distance was observed, accompanied by slight increases in RNA coding for the MB biomarkers PGC-1α, sirtuin 1, citrate synthase and cytochrome C oxidase (Complex IV) (Nieman et al., 2010). Similar results were obtained when Davis et al. (2009) undertook a human trial to examine whether they could replicate the effects previously observed in mice. Twelve untrained volunteers were given 500 mg of quercetin twice daily for 7 days, dissolved in a drink. After treatment, both VO_2max and ride time to fatigue on an exercise bike were determined. The observed increases in VO_2max and ride time to fatigue were

3.9% and 13.2%, respectively, compared with the control (placebo). Whilst no mitochondrial biomarkers were measured in this trial, the enhancement of endurance capacity observed, in the absence of any physical training, is entirely consistent with mitochondrial adaptation.

One should bear in mind that the physical performance aspects of polyphenol-stimulated mitochondrial adaptation are of minor importance in the context of human health. In this respect, mitochondrial dysfunction is of much higher importance, given that it is implicated in some way in both the major diseases of modern civilisation (CVD, cancer and neuro-degeneration) and the aging process (Huang & Hood, 2009). Mitochondrial adaptation, rather than antioxidant capacity, is emerging as the primary mode of action of the health benefits of dietary polyphenols. Whilst there is, to date, no evidence that polyphenol consumption can increase human lifespan, there is good evidence from animal trials (Baur et al., 2006; Lagouge et al., 2006). This suggests that humans may benefit similarly, even if only through reduction in the incidence of life-threatening diseases. It is unlikely that dietary polyphenols could have a major effect on the maximum life-span of humans, but they do appear to have great potential to increase the proportion of people who attain all or most of the maximum lifespan.

4.9 Polyphenolics, mitochondria and apoptosis

Mitochondria are the instigators of programmed cell death, or apoptosis (Dorta et al., 2006). If mitochondria are sufficiently damaged by oxidative stress and DNA damage to become dysfunctional and lose their capacity to adapt to oxidative stress, they initiate a signalling pathway leading to apoptosis, or programmed death, of the host cell and thus the demise of the defective mitochondria. This mechanism has been proposed to explain the "chemo-preventive" effects of polyphenolics (Roy et al., 2009; Juan et al., 2008), based on the observation that cancer cells in vitro are more sensitive to mitochondrial-induced apoptosis than normal cells. Therefore, polyphenols may promote apoptosis of cancerous cells in vivo. The ability to induce apoptosis was demonstrated in vitro for pterostilbene, a natural methylated derivative of resveratrol (Pan et al., 2007), resveratrol itself (Shakibaei et al., 2009), kaempherol, a flavonol similar to quercetin (Marfe et al., 2009), EGC (W. Li et al., 2009) and catechin (Iwasaki et al., 2009). This property of polyphenols may explain at least part of the putative anti-cancer effect of polyphenol-rich foods (see Introduction).

4.10 Does the antioxidant capacity of polyphenols have any role in health?

Polyphenol concentrations achieved in plasma from a realistic dietary intake are both transient and at most, ~2-4% of the small-molecule antioxidants normally present in the plasma; antioxidant enzymes contribute a large additional endogenous antioxidant capacity (Clifford, 2004; Stevenson & Lowe, 2009; Stevenson et al., 2009). In comparison, polyphenols are clearly insignificant in the overall context of resistance to oxidative stress. They could make a contribution at sites within the body where localised concentrations are much higher than the average. One example of this may be in the gastrointestinal (GI) tract, where polyphenol concentrations have been demonstrated to be many times that achieved in plasma (Stevenson et al., 2009), a consequence of the low proportions of most polyphenols that are absorbed from the GI tract. The cells of the GI tract are thus exposed to concentrations that should be more than sufficient for a significant radical-scavenging antioxidant effect. Another possible example is in cell membranes, where in vitro studies

found that up to 75% of polyphenols spiked into blood samples can end up bound to the membranes of the blood cells (Biasutto et al., 2010; Koren et al., 2010). This may allow them a significant and direct role in prevention of lipid peroxidation. If this membrane-binding phenomenon translates to the in vivo environment and is common to other cells (and to mitochondria) in the body, polyphenols may have a significant whole-body protective effect from lipid peroxidation.

5. The role of homeostasis in polyphenol action in the body

A good question to ask is why so many trials of antioxidants have failed to demonstrate any benefit and why polyphenol-induced mitochondrial adaptation appears only to be readily detected and manifested as an augmentation of exercise-induced adaptation. The answer may lie in the principle of homeostasis (van Ommen et al., 2009). Homeostasis is the normal state of a healthy cell or organism, where all biochemicals and enzymes are regulated to their optimal concentrations. When an organism is in homeostasis, dietary or pharmaceutical intervention has no particular benefit, because there is no "problem" to rectify. This may go some way towards explaining the apparent 'failure' of intervention trials with antioxidants. If the concentrations of endogenous antioxidants are tightly regulated, then addition of large amounts of exogenous antioxidants would result in down-regulation of endogenous synthesis to restore homeostasis. Van Ommen and colleagues propose that any search for biomarkers of the effects of interventions must be undertaken with simultaneous perturbation of homeostasis, so the intervention can assist its restoration. This is not a major consideration for pharmaceutical interventions, which are typically designed to treat disease, in which homeostasis has already been perturbed. Dietary interventions, in contrast, are usually aimed at optimisation of health, rather than treatment of disease. The search for health benefits of either dietary antioxidants or adaptogens will almost inevitably fail unless tested on subjects with pre-existing or applied oxidative stress. Oxidative stress may be applied to animals by treatment with toxins, enforced exercise, or the use of animal models of suitable disease states. For humans, the options are restricted to the use of test subjects with pre-existing conditions that elevate oxidative stress, such as metabolic syndrome, or the performance of endurance exercise by healthy subjects. If oxidative stress is applied during a trial, appropriate intervention reduces the magnitude of the perturbation (i.e., the stress) and accelerates the restoration of homeostasis.

6. Conclusion

A large body of evidence has now been accumulated to support the concept that polyphenols are primarily adaptogens rather than radical-scavenging antioxidants. This does not negate their potent capacity to reduce oxidative stress; rather it indicates that the mechanism is far more complex and subtle than previously realised. Several in vivo trials have clearly linked polyphenol interventions with actual mitochondrial adaptation, or macroscopic effects consistent with adaptation, specifically mitochondrial biogenesis (MB) and up-regulation of MB-signalling molecules and antioxidant enzymes as the main biomarkers established for detection of mitochondrial adaptation. In vitro trials have been entirely consistent in demonstrating up-regulation of the same biomarkers that provide clues to possible mechanisms of action for polyphenols. These are direct activation of the components of the MB signalling pathway (e.g., sirtuin 1 and PPARγ); direct activation of

the ERE via binding to its regulatory protein Keap1; stimulation of glycolysis and glucose uptake, which increases the supply of nutrients to the mitochondria; stimulation of NO synthesis, which is a known signal for MB; and generation of a mild oxidative stress in the mitochondria through inhibition of the ETC or other mechanisms.

Since mitochondrial dysfunction is implicated in aging and major diseases such as cancer, CVD and neuro-degeneration, any means of improving mitochondrial function, or inducing destruction of the most dysfunctional mitochondria, should be highly beneficial to healthy aging and maintenance of good health. Dietary polyphenols are almost certainly a good means of achieving these ends.

7. List of abbreviations and definitions

ORAC : Oxygen radical absorbance capacity; an in vitro measure of relative antioxidant power. **ROS**: Reactive oxygen species; another term for free radicals. **ETC**: Electron transport chain; a group of mitochondrial proteins that generates **ATP** (adenosine triphosphate) by reduction of oxygen to water. **NADH**: Nicotinamide adenine dinucleotide; a biochemical reducing agent. **MnSOD** or **SOD2**: Mitochondrial form of the antioxidant enzyme, manganese superoxide dismutase. **CuZnSOD** or **SOD1**: Cytosolic form of superoxide dismutase. **MB**: Mitochondrial biogenesis; the increase of mitochondrial numbers within cells. **Complex III**: One of the five components of the mitochondrial electron transport chain (ETC). **Nrf-1** and **Nrf-2**: Nuclear respiratory factors; transcription factors involved in control of adaptive responses. **TFAM**: Mitochondrial transcription factor A; transcription factor involved in control of adaptive responses. **PGC-1α**: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha. **PPAR-γ**: Peroxisome-proliferator activated receptor-γ; receptor involved in control of adaptive responses. **ERE/ARE**: Electrophile (or Antioxidant) Response Element; gene promoter controlling antioxidant enzyme gene expression. **HNE**: 4-hydroxy-2-nonenal; product of lipid peroxidation and likely activator of the ERE. **Keap1**: Kelch-like ECH-associated protein 1; a sensing protein linked to the ERE. **TCA cycle**: Tricarboxylic acid cycle; ATP-generating metabolic pathway in mitochondria. **eNOS**: Endothelial nitric oxide (**NO**) synthase enzyme. **GPX**: glutathione peroxidase; antioxidant enzyme. **MDA**: malondialdehyde; a commonly used plasma biomarker of lipid peroxidation. **SIRT**: silent mating type information regulation 2 homolog; gene coding for a sirtuin, or regulatory protein deacetylase. **EGCG**: epigallocatechin gallate; a flavonoid polyphenol mainly found in green tea.

8. References

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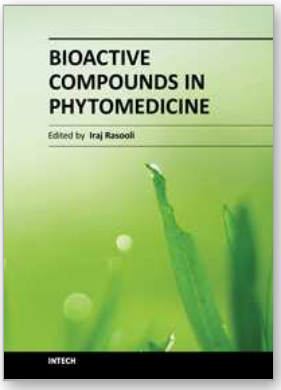

Bioactive Compounds in Phytomedicine

Edited by Prof. Iraj Rasooli

ISBN 978-953-307-805-2
Hard cover, 218 pages
Publisher InTech
Published online 18, January, 2012
Published in print edition January, 2012

There are significant concerns regarding the potential side effects from the chronic use of conventional drugs such as corticosteroids, especially in children. Herbal therapy is less expensive, more readily available, and increasingly becoming common practice all over the world. Such practices have both their benefits and risks. However, herbal self-therapy might have serious health consequences due to incorrect self-diagnosis, inappropriate choice of herbal remedy or adulterated herbal product. In addition, absence of clinical trials and other traditional safety mechanisms before medicines are introduced to the wider market results in questionable safe dosage ranges which may produce adverse and unexpected outcomes. Therefore, the use of herbal remedies requires sufficient knowledge about the efficacy, safety and proper use of such products. Hence, it is necessary to have baseline data regarding the use of herbal remedies and to educate future health professionals about various aspects of herbal remedies.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

David E. Stevenson (2012). Polyphenols as Adaptogens – The Real Mechanism of the Antioxidant Effect?, Bioactive Compounds in Phytomedicine, Prof. Iraj Rasooli (Ed.), ISBN: 978-953-307-805-2, InTech, Available from: http://www.intechopen.com/books/bioactive-compounds-in-phytomedicine/polyphenols-as-adaptogens-the-real-mechanism-of-the-antioxidant-effect-

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