Dietary Polyphenols: Antioxidants or not?

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Introduction

Our initial interest in the possible role of dietary components protecting against cardiovascular disease (CVD) was sparked by the pivotal human intervention studies in the early 1980's showing that a vegetarian diet could lower blood pressure¹. Population studies in the 1990's also started to show that dietary flavonoids were associated with reduced risk of CVD². During this time there was growing evidence that oxidative modification of lipoproteins, in particular low density lipoproteins (LDL), played an important role in atherogenesis³. Early reports that beverages such as red wine, that contained a high concentration of polyphenolic substances could inhibit LDL oxidation stimulated huge interest in this field⁴.

Over the past three decades our understanding of the role of diet derived polyphenolic compounds in disease prevention has grown. They were initially considered as simple antioxidants, but they and their *in vivo* metabolites, are now seen as signalling molecules. The work of Helmut Sies has been a great influence to those working in the field. His early review on strategies of antioxidant defence received over one thousand citations⁵. His ground breaking research on the mechanism of action of dietary flavanols has been of particular note. More than that, it is his clear thinking and deep knowledge of free radical chemistry that has helped to progress this field of research. This review will outline some developments in our thinking on dietary polyphenols with particular reference to their role in cardiovascular protection.

Polyphenols structure and function

Phenolic compounds are those that have at least one aromatic ring with one or more hydroxyl groups attached. There are many thousands of phenolic or polyphenolic compounds that occur as secondary plant metabolites and as such are found in plant derived foods and beverages. One of the major groups of polyphenols are the flavonoids, but there are many other classes of phenolics such as the phenolic acids commonly found in coffee beans and fruit (for examples of polyphenols see **figure 1**). There are a number of excellent reviews on dietary phenolics chemistry and biosynthesis which can be recommended to those who would like a comprehensive review of the field⁶.

Polyphenols as antioxidants

The antioxidant activity of polyphenols is characterised by the phenolic structure and those compounds with catechol like moieties and the ability to delocalise unpaired electrons have the strongest activity (ref). Given the role of oxidation in a number of disease pathways and the strong *in vitro* antioxidant activity of many phenolic compounds it was reasonable to assume that antioxidant activity explained the link between dietary and disease prevention. Substantial efforts were made to define simple assays that would measure antioxidant activity biological systems such as the lag time to low density lipoprotein (LDL) oxidation or total antioxidant capacity. While total antioxidant capacity may be useful in comparing different food items, it cannot be extrapolated into a health claim. Helmut Sies was able to clarify this in an excellent appraisal of the total antioxidant capacity concept⁷.

Many methods were developed to measure antioxidant capacity or radical trapping capacity in hope of having a simple assay to use in human testing. Unfortunately these assays don't take into account the important role of enzymatic antioxidant defences or specific molecular basis for the measurement. In plasma samples small molecules only account for part of the

trapping capacity while proteins play a major role. While some studies have suggested a transient increase in total antioxidant capacity after consumption of flavonoid or polyphenol rich food, this is more likely due to increases in uric acid following such foods⁸.

Consumption of flavonoid –rich foods is only likely to increase flavonoids in plasma to low (micromolar) concentrations at best⁹. In addition, metabolism of flavonoids is likely to significantly alter their antioxidant activity¹⁰.

Another and more valid approach to determine possible effects of dietary ‘antioxidants’ *in vivo* is to measure specific markers of peroxidative damage to biomolecules. An example of this would be the measurement of F₂-isoprostanes by validated mass spectrometry based assays¹¹. These prostaglandin-like compounds are formed by non-enzymatic oxidation of membrane arachidonic acid. Using this method a randomised cross-over trial led by Helmut Sies was able to show that flavanol-rich cocoa drink was able to significantly blunt the increase in F₂-isoprostanes caused by strenuous physical exercise¹². We also observed that red wine polyphenols (in the absence of alcohol) could reduce lipid peroxidative stress biomarkers in smoking subjects¹³. However, a number of other intervention trials have not shown a decrease in markers of oxidative damage in non-stressed subjects¹⁴.

Oxidative stress and cardiovascular disease

There is little evidence from prospective studies in humans with CVD and healthy controls that show a causative link between oxidative stress biomarkers and disease outcome. In a large nested case control study we saw no association between markers of lipid and protein oxidation and risk of coronary heart disease¹⁵. While the antioxidant activity of polyphenols has been well documented *in vitro*, well controlled intervention studies with validated markers of oxidative damage often failed to show any antioxidant action *in vivo*¹⁶. A position paper published by the ILSI Europe functional foods expert group, including Helmut Sies,

concluded that a direct antioxidant effect of polyphenols for cardiovascular health in humans is not established¹⁷.

Issues of bioavailability and metabolism need to be considered.

The bioavailability and metabolism of dietary polyphenols must be considered when considering the possible health benefits of these compounds. There is a large range in bioavailability between the different flavonoid classes with the isoflavones having the highest (33-100%), followed by the flavonols (12-41%), flavanones (11-16%) and the monomeric flavan-3-ols (2-8%). The anthocyanins also have very low bioavailability. The bioavailability of dietary polyphenols has been well reviewed and based on a large number of human studies⁹. Flavonoids that are not absorbed from the small intestine or stomach (entering the circulation in 1-3 hours) would be transported to the colon, where they are subjected to metabolism by the microbiota. Peak plasma levels for these flavonoids or their metabolites are usually reached after 4-6 hours. Flavonoids are broken down to a range of smaller phenolic acid metabolites¹⁸. Upon absorption polyphenols are readily metabolised to form glucuronide and sulphate conjugates as well as methylation of catechol groups. As a result mostly conjugated forms of polyphenols appear in the circulation and these forms can have profoundly altered bioactivity^{10, 19}. Most *in vitro* studies carried out in the past with aglycones do not address this issue.

While gut microflora play a key role in polyphenol metabolism, modifying polyphenol bioavailability and bioactivity, polyphenols may also beneficially regulate bacterial profiles²⁰, which may impact on CVD risk. The metabolism of dietary flavonoids by microbes in the gastrointestinal tract may be important for the potential health benefits of dietary polyphenols²¹. For example, quercetin glycosides can be metabolised by gastrointestinal microbes to simple phenolic acids such as 3,4-dihydroxyphenylacetic acid (**figure 2**). While a

relatively small % of flavonoid intake is absorbed as the intact flavonoid, a greater % is absorbed as the phenolic metabolites, primarily phenolic acids²¹. The importance of intestinal bacteria for polyphenol metabolism is highlighted by the fact that antibiotic treated or germ free animals no longer form phenolic acid metabolites from dietary flavonoids²². We have previously shown using a mass spectrometry targeted metabolomics approach that mixed dietary polyphenols (from grape seed extract, tea etc.) or from pure flavonoids give rise to specific phenolic acid metabolites²³⁻²⁵. Less is known about the bioactivity of these microbial metabolites²⁶. Understanding the role of the gut microbiome in health and disease and the influence of dietary polyphenols is likely to be a fruitful area for future research.

How do polyphenols reduce CVD risk as indicated in population studies

The data from human population studies makes it clear that increased fruit and vegetable consumption is linked to reduced risk of CVD and other chronic diseases²⁷. These protective properties are unlikely to be due to antioxidant effects of dietary polyphenols, however there is clear evidence from human intervention studies that certain polyphenols or polyphenol rich foods can reduce some CVD risk factors^{28, 29}. There is now consistent and convincing data indicating that particular dietary flavonoids can improve endothelial function acutely and with short-term regular intake³⁰. The primary proposed mechanism for this is the augmentation of NO production³⁰. There is also evidence that certain dietary polyphenols benefit BP³¹⁻³³.

Endothelial function

Endothelial dysfunction is an early event in the development of atherosclerosis. Measurement of flow-mediated dilatation of the brachial artery is used to assess endothelial function. A higher flow-mediated dilatation is associated with significantly lower risk of cardiovascular events³⁴. More than 25 randomised controlled trials have investigated effects of flavonoid-

rich foods and beverages on flow-mediated dilatation. These trials indicate that some flavonoids can improve endothelial function. Together, they indicate that flavonoid-rich foods can increase flow-mediated dilatation by an average of about 20-30% both acutely and with short-term ingestion. Some of the important studies in this area were conducted by Sies and colleagues, particularly the effects of flavanol-rich cocoa^{35, 36}.

Blood pressure

Effects of flavonoids to enhance nitric oxide production and improve endothelial function may contribute to lower BP. Results of population studies indicate that a long-term high flavonoid intake may result in lower systolic and diastolic BPs of between 2 and 10 mm Hg³⁰. In a recently completed 6 month randomised double-blind placebo-controlled trial we have shown that regular consumption of flavonoid-rich black tea resulted in significantly lower systolic and diastolic BPs of 2 to 3 mm Hg in men and women with normal to high-normal BPs³². In a population this would translate to approx. 10% lower risk of cardiovascular events. We also found that black tea resulted in significantly lower rate of BP variation, independent of effects on BP level³⁷. In addition, there is accumulating evidence that flavonoids from other food sources such as cocoa reduce BP³⁸.

Mechanistic insights: NO and NADPH oxidase

Helmut Sies has made a major contribution to our understanding of how dietary flavonoids improve vascular function. In an extremely interesting position paper published in Archives of Biochem Biophys in 2008³⁹ Sies and colleagues describe how dietary flavonoids such as epicatechin can increase nitric oxide (NO) bioavailability and that a methylated metabolite of the flavonol can inhibit endothelial NADPH oxidase. Hence they proposed that endothelial NO metabolism rather than general antioxidant activity is a major target of dietary flavonoids. This was followed by later work showing direct evidence that epicatechin

increases NO levels in human endothelial cells. We have also been able to demonstrate using reductive chemiluminescence detection to measure NO in human intervention studies that pure flavonoids such as quercetin and epicatechin or flavonoids –rich apples can increase NO levels in plasma^{31, 40}.

Mechanistic insights: AMPK and eNOS

We now know that dietary polyphenols can regulate vascular endothelial cell expression of genes important in cardiovascular health⁴¹. Adenosine monophosphate-activated protein kinase (AMPK) is an important sensor of cell energy status and can be activated by stressors such as oxidative stress, hypoxia and nutrient deprivation⁴². Certain dietary polyphenols such as resveratrol can also stimulate AMPK activity⁴³. Once activated AMPK switches on catabolic pathways that generate ATP while switching off ATP-consuming processes. Downstream targets of AMPK include enzymes of glucose and lipid metabolism, mitochondrial enzymes and eNOS, which is responsible for NO production. Activation of AMPK can prevent oxidative stress-induced vascular dysfunction via increased phosphorylation and activation of eNOS⁴⁴. We have also shown that quercetin and its *in vivo* metabolites can improve vessel function by inducing eNOS via phosphorylation of AMPK⁴⁵.

Mechanistic insights: Hmox-1

In addition to NO, heme oxygenase-1 (Hmox-1) is a key regulator of endothelial function. Under normal conditions, NO regulates the diameter of blood vessels and maintains an anti-proliferative and anti-inflammatory environment in the vessel wall⁴⁶. Endothelial dysfunction is thought to arise due to a decrease in the bioavailability/bioactivity of NO. Expression of Hmox-1 by endothelial cells may exert anti-inflammatory and antioxidant activity within the vasculature⁴⁷. Its induction may modulate endogenous cellular reactive oxygen species (ROS) generation and protect against ROS-induced oxidative damage⁴⁸.

Pharmacological inducers of Hmox-1, such as probucol, protect against vascular disease in several different animal models of atherosclerosis⁴⁹. This beneficial effect is associated with enhanced protection of arteries against endothelial dysfunction induced by oxidants such as hypochlorous acid (HOCl)⁵⁰. Moreover, the induction of Hmox-1 by antioxidants is independent of free radical scavenging. We have been able to confirm a critical role for Hmox-1 in the ability of dietary quercetin to protect against endothelial dysfunction and atherosclerosis in apolipoprotein E -/- mice fed a high fat diet⁵¹.

Nrf2 signalling

Since free radical scavenging mechanisms cannot be substantiated *in vivo* for dietary polyphenol antioxidants, then what can account for the beneficial effects of these compounds? Some polyphenols can induce protective enzymes *in vivo*, so that these compounds or their *in vivo* metabolites rather than acting as chemical antioxidants, generate signals for the production of protective enzymes. The example of dietary quercetin given above would be an example of such an effect. The developing concept of how nutritional ‘antioxidants’ work has been recently reviewed by Forman and colleagues⁵². This proposal for the mechanism of action of nutritional ‘antioxidants’, many of which are polyphenols, involves the somewhat paradoxical oxidative activation of the NF-E2-related factor 2 (Nrf2) signalling pathway which maintains protective enzymes against oxidant damage and repair. In this scenario low concentrations of phenolic compounds (or their metabolic products) and the quinones formed as part of redox cycling are electrophiles that can interact with Keap1 and lead to activation of Nrf2⁵².

Conclusions

Despite the fact that population studies continue to show that dietary flavonoids protect against CVD and total mortality⁵³, intervention studies with individual flavonoid compounds

have not always shown improvement to markers of cardiovascular health^{54, 55}. This raises a number of issues that remain to be resolved in bridging the gap between observational studies and intervention trials with pure flavonoids⁵⁶. These include the nature and form of the flavonoid to be tested, the duration of the trial and the type of participants to be included (healthy volunteers or those with disease risk factors). In addition the type of test or biomarker used to evaluate CVD risk may be important as well as the timing after flavonoid intake. With regards to vascular function, the time of peak plasma concentration or the time of metabolite appearance in plasma is likely to be important^{57, 58}. In the field of polyphenols and health the perspectives and advice of Helmut Sies are always keenly sort after. His recommendations to use the best analytical methods and study design will continue to play a critical role in the progress of this field. To take his advice, do not use the term ‘antioxidants’ for polyphenols in food but rather they should be considered as ‘bioactives’⁵⁸.

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Figure 1

Structure and names some specific examples of major flavonoid classes and phenolic acids.

**Flavonol
(quercetin)**

**Flavan-3-ol
(-)-epicatechin**

**Flavone
(luteolin)**

**Flavanone
(hesperetin)**

**Anthocyanidin
(cyanidin)**

**Proanthocyanidin
(B₂ dimer)**

**Isoflavone
(diadzein)**

**Phenolic acid
(chlorogenic acid)**

Figure 2

Proposed pathway for the colonic bacterial metabolism of quercetin glycosides in the human large intestine, resulting in the formation of phenolic acids such as 3,4-dihydroxyphenylacetic acid.

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