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Review

Polyphenolic phytochemicals – just antioxidants or much more?

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Abstract. Polyphenolic phytochemicals are ubiquitous in plants, in which they function in various protective roles. A 'recommended' human diet contains significant quantities of polyphenolics, as they have long been assumed to be 'antioxidants' that scavenge excessive, damaging, free radicals arising from normal metabolic processes. There is recent evidence that polyphenolics also have 'indirect' antioxidant effects through induction of endogenous protective enzymes. There is also increasing evidence for many potential benefits through polyphenolic-mediated regulation of cellular processes such as inflammation. Inductive or signalling effects may occur at concentrations much lower than required for effective radical scavenging. Over the last 2–3 years, there have been many exciting new developments in the elucidation of the *in vivo* mechanisms of the health benefits of polyphenolics. We summarise the current knowledge of the intake, bio-availability and metabolism of polyphenolics, their antioxidant effects, regulatory effects on signalling pathways, neuro-protective effects and regulatory effects on energy metabolism and gut health.

Keywords. Polyphenolic, flavonoid, phenolic acid, antioxidant, prooxidant, cancer, cardiovascular disease, inflammation, gene transcription, enzyme induction.

Introduction – what are polyphenolics?

Structural diversity

Polyphenolics (PPs) are a diverse class of plant secondary metabolites. They are characterised structurally by the presence of one or more six-carbon aromatic rings and two or more phenolic (i.e. linked directly to the aromatic ring) hydroxyl groups. Strictly speaking, mono-phenols such as *p*-coumaric acid are not PPs, but they share many of their properties and characteristics and are most usefully considered as 'functional PPs'. There are five major classes of PP (Fig. 1). As a broad generalisation, the major biosynthetic pathway starts with phenylalanine, which is converted into cinnamic acids and then elaborated into the various other classes of compounds, ending with the anthocyanins. There are numerous smaller classes of compounds arising from other biosynthetic pathways [1].

Functions in plants

PPs appear to have many diverse functions in plants, e.g. colour of leaves, flowers and fruit, anti-microbial function, anti-fungal function, insect feeding deterrence, screening from damage by solar UV radiation, chelation of toxic heavy metals and anti-oxidant protection from free radicals generated during the photosynthetic process [2]. Plants contain an enzyme, polyphenol oxidase, which functions to polymerise all available PPs, in the presence of oxygen at the site of an injury to the plant. These polymers serve to seal the

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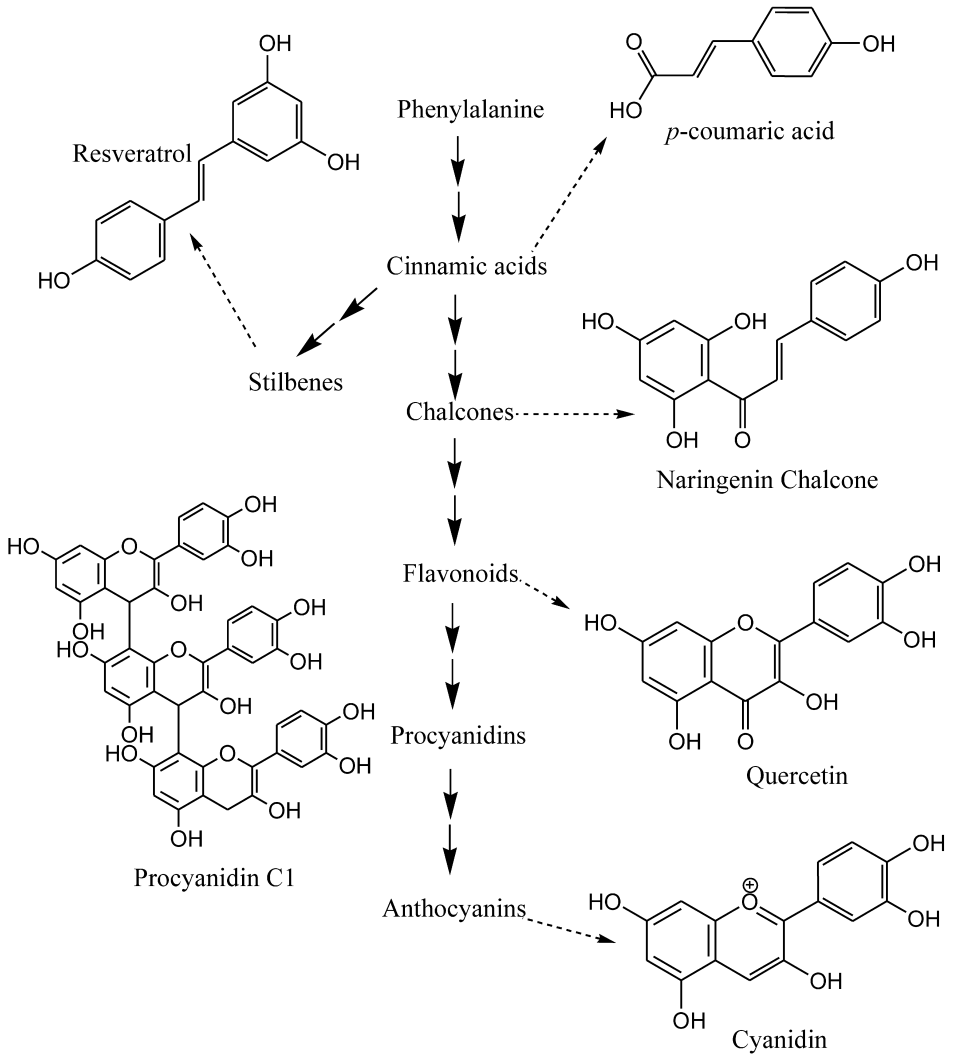

Figure 1. Summary of major polyphenolic biosynthetic pathway with examples of the structure of each major class of compounds.

damaged tissues and further deter insect feeding. The enzyme is a major problem for the food industry; left unchecked it can quickly turn cut fruit, vegetables or extracted juices brown and unpalatable [3]. PPs also appear to function as deterrents to feeding by higher animals. Some taste very bitter, e.g. naringin (a citrus flavonoid), polyphenol oxidase products and the lower-molecular-weight procyanidins (found in most plant materials) [4]. The higher-molecular-weight procyanidins (also known as tannins) precipitate proteins in the mammalian digestive system and impair digestion. Procyanidins have a particular affinity for proline-rich proteins which occur in saliva. It is thought that these salivary proteins evolved to allow mammals to detoxify the procyanidins before they enter the digestive system. Tree bark contains very high levels of procyanidins and animals that eat bark, such as deer, have saliva very high in proline-rich proteins [2].

Intake, bio-availability and metabolism of PPs

Dietary intake estimates

There have been various studies carried out to estimate the daily consumption of dietary PPs, e.g. those of Hertog et al. [5]. The figures obtained were obviously highly dependent on the accuracy of food compositional data, which have often been incomplete. It is only recently that more comprehensive data have become available, e.g. the USDA databases [6]. The NEODIET reviews [7] are the most comprehensive initiative so far to estimate accurately the PP composition of foods. Combining this review with diet-diary data has produced interesting results [8]. The main findings, from diet-diary studies in the UK are that mean dietary intake of all PPs is 780 and 1058 mg/day for females and males, respectively. Approximately half of the intake comprises hydroxycinnamates, whereas total flavonoids make up only

around 20–25% and anthocyanins only ~1%. Anthocyanin intakes may be significantly higher in populations consuming more berry fruit or red wine than are found in a typical British diet, but they are clearly not a major class of dietary PP.

Absorption from the gastrointestinal tract and colonic metabolism

PPs are apparently absorbed from the upper gastrointestinal tract by a number of mechanisms, which have not been fully characterised. Some glycosides, mainly glucosides, but not rutinosides, appear to interact with the active sugar transporter (SGLT1), lactase phloridzin hydrolase or cytosolic β-glucosidase [9]. The more hydrophobic aglycones appear to undergo passive diffusion. Only 5–10% of PP are absorbed in this manner, the remainder passing into the colon [8]. The colonic micro-flora appears able to break down flavonoids and procyanidins into simpler hydroxycinnamic, hydrocinnamic (phenylpropionic), phenylacetic and benzoic acid derivatives [10, 11]. These, in turn, can be absorbed via the colon and augment the phenolic acids directly absorbed from the diet. Concentrations of flavonoids in human faecal water (a good indication of intra-colonic concentrations) ranged from ~0.1–1 μM, whereas phenolic acids were found at micromolar concentrations of tens or even hundreds [10]. Two recent studies produced further evidence that colonic low-molecular-weight acid metabolites are both derived from PPs and bioavailable. Chocolate (high in flavonols and procyanidins) intake increased urinary excretion of phenolic acids in healthy test subjects [12]. Volunteers who consumed 1 g per day of procyanidin-rich grape seed extract showed a consistent increase in the excretion of 3-hydroxyphenylpropionic acid and some other putative colonic metabolites [13]. Cocoa procyanidins are stable during gastric transit and therefore available to reach the colon [14]. Intestinal cells can efficiently glucuronidate flavonoids and other absorbed PPs [15] and excrete the conjugates back into the lumen [16]. This phenomenon, combined with biliary excretion, further increases the proportion of PPs available to the colonic micro-flora. Procyanidins are particularly poorly absorbed into the circulation [17], with only the low-molecular-weight species reportedly detectable, at only trace concentrations [18]. It appears that dietary cinnamic acids and the phenolic acids produced by the colonic micro-flora have relatively high bio-availability. In a human trial involving urine analysis of subjects consuming a diet very high in PPs, around 98% of the PP metabolites found in urine samples were hydroxycinnamic acids (at least some of which would have come directly from the diet) and many colonic metabolites [19]. There has long been a strong emphasis on biological and mechanistic studies on flavonoids, but relatively little interest in the more prevalent phenolic acids. These are worthy of more intense study in the future.

Pharmacokinetics and metabolism

Both the liver and intestine contain high levels of phase I and II metabolic enzymes which hydroxylate and conjugate xenobiotic compounds, respectively. While hydroxylation of PPs has not been reported, PPs are extensively conjugated by glucuronidation, sulphation and methylation [20]. It has been estimated that 90–95% of absorbed PPs are converted to conjugates [8]. Conjugation has been found to modulate or even radically change biological activities of PPs in *in vitro* studies [20]. There have been numerous studies to estimate the maximum attained concentration of individual PPs in the circulation, the time taken to achieve it and their half-lives for disappearance from the circulation [18]. Time taken to reach maximal circulatory concentrations varies from ~1 to 6 h and the concentration attained can be as low as 0.03 μM for anthocyanins and ferulic acid and as high as 2.56 μM for genistein or 4 μM for gallic acid. Half-life ranges from 1.3 h for gallic acid up to 7.1 h for genistein and 19.9 h for rutin. A recent study supports these findings [21]. Consumption of 270 g of fried onions by human test subjects resulted in detection of quercetin glucuronides, sulphates and methyl ethers in the plasma. Peak concentrations of ~1 μM were attained ~30 min after consumption and decreased to near zero by ~6 h. In contrast, rats dosed with a modest 8 mg/kg of grape anthocyanins rapidly attained peak concentrations of ~0.3–0.5 μM in both plasma and brain [22]. These concentrations are significantly higher than those found in earlier studies, possibly because techniques of measuring anthocyanin metabolites are improving. Multi-drug-resistance (MDR) associated protein efflux pumps may limit the absorption of epigallocatechin gallate (EGCG, a tea catechin) and potentially other PPs by many cells [23]. This may mean that intracellular PP concentrations may be lower than those in the circulation, possibly further limiting their potential *in vivo* activity.

Maximum circulating concentrations of total PPs (both conjugated and unconjugated forms) have been estimated to range from 0.1 to 10 μM [24] and from 3 to 22 μM [8]. Given that these values are maxima and clearance appears to be at a similar rate to absorption, the maximum steady-state concentration of total PPs is unlikely to exceed ~1–5 μM, even with a diet exceptionally high in PPs. Any potential *in vivo* benefit from dietary PPs must be evaluated according to the principle that it is unlikely to be significant unless demonstrable at low-micromolar concentra-

tions of most of the conjugates, or nanomolar concentrations of residual unconjugated forms. The exception to this principle is the gastrointestinal tract, where cells may be exposed to concentrations many times higher than in the circulation. Higher concentrations of PP aglycones, e.g. 10, 50 or 100 μM, are often used in *in vitro* experiments. These do not necessarily demonstrate potential health benefits, but may provide valuable insights into mechanisms of action. *In vivo* administration in animal trials of therapeutic applications, by injection or infusion, circumvents the detoxification system and can readily achieve 50–100 μM concentrations of PP aglycones.

Effects of unnaturally high concentrations of PPs

Although there is no evidence that normal dietary intakes of PPs are in any way harmful, excessive amounts have shown toxicity. If detoxification systems are artificially circumvented by injection of high doses of phenolic aglycones, liver and kidney damage can occur in animal models [25–27]. An extremely high and prolonged dietary intake of caffeic acid can cause fore-stomach cancer in rats [28]. Intra-peritoneal administration of PPs to mice caused liver injury [29]. High doses of PPs can significantly alter the bioavailability and metabolism of some drugs, with potential for indirect harm, through causing over-dosing [30]. Phytoestrogens (principally the soy isoflavones, genistein and diadzein) are commonly taken as supplements by women as an alternative form of hormone replacement therapy. They also exhibit, however, genotoxicity *in vitro* [31], indicating some potential for the same effect *in vivo*. Male rats dosed with large amounts (75–150 mg/kg) of a kiwifruit (*Actinidia chinensis*) extract showed suppression of testosterone levels and sperm count [32]. Silymarin, a mixture of PPs from the milk thistle plant, is commonly used as a treatment for liver conditions such as cirrhosis. In a study of its effect on cancer, however, it mildly increased the number of mammary tumours induced in rats by 1-methyl-1-nitrosourea and stimulated the growth of MCF-7 breast cancer cells in culture [33]. As discussed above, PPs appear to be compounds that plants have evolved to be, among other purposes, toxic to organisms that feed on them. It has been suggested that PPs are simply toxins, to which we may have become relatively resistant, that induce endogenous protective mechanisms at moderate dietary intakes, and that high-dose fortified foods or dietary supplements are of unproven efficacy and possibly harmful [34, 35]. Flavonoids have shown many potential therapeutic effects, but very few have been developed into practical treatments [36]. It appears that dietary intake is both beneficial and harmless because we have evolved mechanisms to deal with any toxic effects of PPs, but therapeutic administration and very high dose supplements may do more harm than good. It is perhaps somewhat ironic that we humans find high consumption of plant foods, as commonly recommended by authorities such as the USDA [37], one of the keys to good health and wellbeing, when plants have evolved to become unpalatable and toxic to us!

Antioxidant effect of PPs

'Conventional' antioxidant capacity

PPs can function, *in vitro*, in a similar manner to other antioxidant compounds by inactivating harmful free radicals, such as lipid peroxides, and by chelation of divalent metal ions, thereby reducing their oxidative potential [4]. PPs at relatively high concentrations and in the presence of other antioxidants such as tocopherols and ascorbic acid undoubtedly have potent antioxidant capacity in the plant tissue from which they derive and in any food in which they are incorporated. They are thought to preserve the lipid component of foods from oxidative degradation. The current literature, however, suggests that the situation *in vivo* is probably very different.

The 'antioxidant hypothesis' was first proposed by Gey in 1987 [38]. In essence, the hypothesis states that dietary 'small-molecule' antioxidants (e.g. urate, ascorbate and tocopherols) act as antioxidants *in vivo* and consequently lower the incidence of some diseases. PPs have since been widely assumed to be included in the class of *in vivo* antioxidant compounds, a concept further inferred from their *in vitro* antioxidant properties. Many intervention trials have studied the effects of antioxidant supplementation, but although some trials showed health benefits, many showed no benefits, or even detrimental effects. Consequently, the value of supplementation with exogenous 'chemical' antioxidants is increasingly questioned [39]. A recent, admittedly controversial, meta-analysis of numerous antioxidant supplementation studies concluded that supplementation with β-carotene, vitamin A and vitamin E (a mono-phenol), but not vitamin C or selenium, significantly increased mortality [40]. PPs, in particular, do not appear to be present in the circulation at high enough concentrations to contribute significantly to total antioxidant capacity. The combined concentration of circulating ascorbate and simple phenols, for a normal individual, has been estimated to be in the range of 159–380 μM [8] and the concentration of urate, another endogenous antioxidant, has been reported to be of a similar order [41]. In the context of an estimated plasma concentration of total low-molecular-weight antiox-

idants totalling around 500 μM (combining the estimates above), even the maximum estimated concentration of PP of 10–20 μM represents only a transient 2–4% increase. The increase in plasma total antioxidant capacity (TAC) from apple consumption can be completely explained by a ~37% increase in urate concentration, as a consequence of fructose metabolism, with no detectable effect associated with the apple PPs [41, 42]. Furthermore, apple PPs do not alter TAC. The same authors have recently proposed that this phenomenon applies more generally to other plant-derived, 'high-antioxidant' foods [43].

The contribution of PPs to TAC is further limited by phase II metabolic conjugation. Flavonoid aglycones should be hydrophobic enough to incorporate into cell membranes and lipoprotein lipids and interfere with lipid peroxidation. Flavonoid aglycones have log *P* values of ~2 (similar to many synthetic drugs) and cinnamic acids values of ~1 [44]. Circulating PPs, however, are predominantly conjugated and the log *P* of the glucuronyl residue is −2.3 (calculated using Chemdraw), so metabolic conjugates of PPs would have log *P* values around zero or negative. Such conjugates are too polar [8] to interact significantly with lipids.

'Indirect' antioxidant capacity – activation of endogenous antioxidant mechanisms

Although PPs do not appear to have any significant *in vivo* antioxidant capacity in the conventional sense (discussed above), a number of studies have shown that they can protect cells from oxidative stress *in vitro*, at physiologically relevant concentrations. Recent studies indicate that PPs may induce the up-regulation of endogenous antioxidant enzymes *in vivo* and thereby exert an 'indirect' antioxidant effect. In a rat model of cerebral ischaemia (stroke), oral pre-treatment with isoliquiritigenin reduced the severity of reperfusion injury, in part by maintaining levels of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, which would normally decrease [45]. Glutathione peroxidase catalyses reduction of both hydrogen peroxide and lipid hydroperoxides, using glutathione as a source of reducing equivalents. The concentrations used in this study (1–100 μg/ml, ~3.5–350 μM catechin equivalent) are outside the range for circulating PPs, but probably more realistic for intestinal lumenal concentrations. PPs may have much greater effects on intestinal than on other cells and even a 'direct' antioxidant effect. Flavonoids induce electrophile-responsive element (EpRE)-mediated expression of enzymes, such as NAD(P)H-quinone oxidoreductase (NQO1, an antioxidant enzyme) and glutathione S-transferases (GSTs; phase II conjugative enzymes). The flavonoid quercetin and bilberry extract can up-regulate expression of two genes containing EpREs [46]. A correlation was found between the calculated prooxidant potential of flavonoids and the ability to induce these antioxidant defence enzymes in cell cultures [47]. Bilberry extract and quercetin were also able to induce EpRE-mediated expression of enzymes [48]. A pre-treatment with the flavonoid kaempherol (50 μM) was able to inhibit significantly the extensive DNA damage in rat hepatoma cells induced by 500 μM hydrogen peroxide. Kaempherol is rapidly taken up and glucuronidated by the cells, but in the absence of hydrogen peroxide halved cell viability itself [49]. Luteolin elevated catalase and superoxide dismutase concentrations and induced apoptosis in lung cancer cells, at relatively high concentrations, but did not increase reactive oxygen species (ROS) [50].

A study in our laboratory showed that when human Jurkat T cells are incubated under normal, control culture conditions for 18 h, ~80% survival is observed, but the presence of 50 μM hydrogen peroxide reduces survival to ~55%. PPs co-incubated with hydrogen peroxide reduce cell death in a dose-dependent manner, and the concentration required to halve cell death relative to control (EC_{50}) ranges from 0.15 μM for quercetin to 0.4 μM for caffeic acid and 2.6 μM for sinapic acid [51]. According to the bioavailability studies discussed above, these concentrations are not unrealistic *in vivo*, even for phenolic aglycones. At concentrations below 5 μM, no quercetin and ~3% of catechin were detectable at the end of the incubation (unpublished data). These results suggest that PPs may induce a protective effect early in the experiment, which persists after they have degraded, or that the degradation products themselves are responsible for the protection. In a study of telomerase inhibition by EGCG in human cancer cells, the inhibition markedly increased after the EGCG had apparently degraded or undergone structural modification [52]. This indicates that rearranged PPs may sometimes have much greater activity than the native forms. Enzymic glucuronidation of PPs, which does not necessarily generate the same isomeric glucuronides as would be found *in vivo*, modulates the EC_{50} range to ~1–5 μM (unpublished data). It appears that PPs can provide significant protection from oxidative stress at concentrations much lower than would be required for 'chemical' antioxidant protection. Conjugation with glucuronides weakens this protective capacity, but the range of effective concentration remains within the accepted *in vivo* concentration range. Chemically permethylated flavonoids had severely reduced antioxidant capacity in *in vitro* 'chemical' antioxidant assays, but the capacity to

protect Jurkat T cells from hydrogen-peroxide-induced oxidative stress was only moderately impaired [53]. This suggests that PPs may operate against oxidative stress by mechanisms other than radical scavenging. In another study, green tea PPs had no detectable effect on DNA repair in hydrogen-peroxide-treated Jurkat cells, making this an unlikely mechanism for the cytoprotective effect in this instance [54]. In contrast, however, apple PP extracts did reduce DNA damage in human Caco-2 colon cancer cells [55, 56]. Flavonoids from *Scutellaria baicalensis* inhibited protein nitration and lipid peroxidation by the hemin-nitrite-H_2O_2 system in liver cells, in a dose-dependent manner and may protect against some forms of liver damage [57].

What is the mechanism by which PPs can induce indirect protective processes against ROS? Ironically, the evidence suggests that PPs can mediate these protective mechanisms by virtue of their ability to act as prooxidants and *generate* ROS. The cytotoxic effects of green tea PPs on liver cells *in vitro* have been elucidated. The major cytotoxic mechanism found with hepatocytes was mitochondrial membrane potential collapse and ROS formation [29]. Treatment of cultured monoblast cells with 50 or 100 μM quercetin initially reduced hydrogen peroxide concentrations, effectively a 'chemical' antioxidant effect. After 2 h, however, glutathione concentrations decreased and superoxide increased [58]. This suggests that glutathione is required for the antioxidant activity of quercetin, and once glutathione is depleted, quercetin becomes a prooxidant. It is possible that glutathione is depleted by the activity of glutathione peroxidase. Another study found a correlation between the cytotoxicity and rates of single-electron oxidation of individual PPs, pointing to a leading role for ROS generation in their cytotoxicity [59].

Further evidence for the generation of ROS by PPs has come from other studies. PPs can generate hydrogen peroxide under physiological conditions of pH and temperature, in PP-rich beverages such as tea or coffee [60] and in cell culture media [61]. Tea catechins generate hydrogen peroxide in yeast growth media and induce nuclear localisation of the oxidative stress response transcription factor Yap 1 [62]. In Jurkat cell cultures, EGCG can reduce iron (III) to iron (II), which in turn generates hydroxyl radicals from hydrogen peroxide through the Fenton reaction, decreasing the viability of the cells and increasing caspase-3 activity [63].

Hydrogen peroxide is a potent signalling molecule in itself [64] and may be a candidate for mediation of some of the consequences of PP prooxidation. Hydrogen peroxide, at 3–15 μM causes 25–45% growth stimulation in cultured cells. At 120–150 μM, a temporary growth arrest for 4–6 h is observed and a 40-fold transient adaptive response in which genes for oxidant protection and damage repair are preferentially expressed, with the maximum response after 18 h. Hydrogen peroxide at 0.5–1.0 mM induces apoptosis and necrosis at 5.0–10.0 mM [65].

Oxidative stress leads to an intracellular redox imbalance and consequent oxidative DNA lesions. If PPs can reduce oxidative stress by induction of antioxidant enzymes, that may provide a mechanism to explain reduced cancer incidence. Such lesions are thought to be strongly implicated in cancer initiation [66]. Quercetin and luteolin at 10 μM were able to significantly suppress the formation of the DNA oxidative damage marker, 8-oxo-7,8-dihydrodeoxyguanosine, by ROS in cell cultures, suggesting an ability to limit genetic damage [67].

Attempts to reproduce these indirect antioxidant effects *in vivo* have produced relatively unconvincing results. A trial involving human subjects consuming 750 ml of cranberry juice or placebo daily for 2 weeks and monitored by numerous tests found no change in antioxidant status, antioxidant enzymes, biomarkers of lipid status pertinent to heart disease, or oxidative DNA damage [68]. A similar trial involving dried cranberry juice found no effect at 400 mg/day, while at 1200 mg/day, the only biomarker that changed was decreased serum concentrations of advanced protein oxidation products. Fruit and vegetable intake, however, has been correlated with antioxidant enzyme induction. In a 25-day human intervention trial, 600 g/day of fruit and vegetables induced increased glutathione peroxidase activity by ~15% and decreased plasma lipid oxidation rates, without significantly changing any of the many other measured markers of redox status [69].

Both DNA and RNA have multiple binding sites for green tea catechins [70] and binding constants for some flavonoids have been determined [71]. If DNA binding were applicable more generally to PPs, it may provide the basis for a further mechanism to explain the reported regulatory effects of PPs on antioxidant enzyme expression, but at the gene transcription level.

Human trials demonstrating benefits of dietary polyphenolics

A number of recent studies have strengthened the relationship between PPs and disease prevention. These trials, combined with earlier studies [72–76], indicate a strong link between PP intake and reduced incidence of cardiovascular disease (CVD) and cancer, the two most prevalent life-threatening diseases of the developed world. In a case-control study in Italy,

anthocyanin intake was inversely related to risk of acute myocardial infarction. No significant correlation, however, was observed for other flavonoids [77]. De-alcoholised red wine, but not normal red wine, was able to reduce arterial stiffness in hypercholesterolemic post-menopausal women [78]. This indicates that PPs are beneficial to this condition, but the benefit is negated by alcohol. Daily consumption of a high-flavonol cocoa drink for 7 days resulted in significant, dose-dependent and sustained flow-mediated arterial dilation in subjects with smoking-related endothelial dysfunction [79]. In a large US study (34 000 pos-menopausal women), food composition tables and diet questionnaires were combined to demonstrate a significant correlation between reduced incidence of death from CVD and coronary heart disease and intake of flavanones, anthocyanidins and certain flavonoid-rich foods [80]. A dietary supplement of superoxide dismutase (SOD) was found to potentially benefit CVD by reducing carotid artery intima media thickness (thickening of the artery, a marker for atherosclerosis) [81]. No change in TAC was detected, but a 34 % decrease in malondialdehyde, a marker for lipid peroxidation was observed. This indicates that any induction of SOD or other antioxidant enzymes by PPs should also be beneficial to CVD. A study from Panama revealed that an island sub-population with an exceptionally high cocoa flavanol intake had a ~ 10 times lower incidence of CVD and cancer and fourfold less diabetes than the general population, although other factors cannot be completely excluded [82].

A long-term diet questionnaire study in Italy showed a partial inverse correlation between plant food consumption and flavonoid intake with renal cell carcinoma, indicating that flavonoids may explain part of the reduction in cancer incidence [83]. Other studies revealed a similar relationship between intake of some flavonoids and both colorectal cancer [84] and breast cancer [85]. Intake of soy isoflavones correlated significantly with a lower incidence of prostate cancer in Japanese men over the age of 60 [86].

Numerous epidemiological studies have indicated that individuals who consume a diet containing high amounts of fruit and vegetables have a reduced incidence of age-associated diseases such as neurodegenerative diseases [87]. A study in young healthy adults showed that flavanol-rich cocoa can increase the cerebral blood flow to grey matter, suggesting a potential for treatment of vascular impairment and thus for maintaining central nervous system health [88]. A study over 10 years found an association between flavonoid intake and performance in cognitive tests. High flavonoid intake was associated with higher baseline scores and slower decline in scores over the course of the study [89].

Consumption of flavonol-rich dark chocolate resulted in reduced blood pressure and increased insulin sensitivity in healthy subjects [90]. An orally administered apple PP extract reduced exercise-induced fatigue in a trial involving 2-h periods on a bicycle ergometer. In contrast, vitamin C had no effect [91]. Kiwifruit consumption promoted laxation in the elderly and may improve bowel function [92]. Taken overall, therefore, recent trials support a role for PPs in disease prevention, especially CVD and, potentially, cancer. More detailed evaluation is required to substantiate these early findings, and future research should also be targeted at how PPs counter CVD and inhibit cancer proliferation.

Regulatory effects of PPs on signalling pathways

Inflammatory pathways

Inflammation is a normal protective response induced by tissue injury or infection and functions to combat foreign invaders in the body, e.g. bacteria, viruses and non-self cells, and to remove dead or damaged host cells. The affected tissues release inflammatory mediators (cytokines) including the proinflammatory cytokines tumour necrosis factor-α (TNF) and interleukin-1 (IL-1). In a complex signalling cascade, these mediators up-regulate and modulate other inflammatory cytokines and immunoglobulins from activated leukocytes, which in turn have actions to up-regulate cellular adhesion molecules in inflamed tissue [93]. Phagocytosis of bacteria by leucocyte neutrophils leads to the 'neutrophil burst' in which various ROS are generated in order to neutralise the invading organisms. There is also concomitant up-regulation of other enzyme systems which contribute to the protective and repair processes including phospholipase A_2, cycloxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), inducible nitric oxide synthase (iNOS) and the central regulator of the inflammatory process, nuclear factor-kappa B (NF-κB) [93]. PPs may act as inflammation modulatory agents by various mechanisms, including down-regulation of NF-κB, or the various enzymes involved (including those that generate ROS), by inhibition of the activity of those enzymes, or by increasing the cells, ability to scavenge ROS [93].

CVD as a predominantly inflammatory condition

CVD is a good example of an area of research that has received much recent attention as both an inflammatory condition but also as a potential target for PP therapy. Much effort has been applied to the study of the ability of PPs to inhibit oxidation of low-density lipoprotein (LDL), oxidised LDL being a major

constituent of atherogenic plaques, the basis of CVD. As discussed above, however, the quantitative significance of this capacity is doubtful. CVD arises from the formation of atherogenic plaques on arterial walls. This is thought to be initiated by adhesion of leucocytes to the walls and their subsequent migration into the sub-endothelial space [94]. This process is essentially an inflammatory one, being induced by TNF and IL-1β; the TNF response is mediated by NF-κB, and many relevant genes contain NF-κB binding sites, including those for the cell adhesion molecules vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein (MCP) [95]. The chronic phase of CVD, formation of atherogenic plaques, may be followed by the acute phase, i.e. thrombotic activity (clot formation) at the site of the lesions. Recent evidence suggests that PPs might help to prevent CVD. CVD is an excellent example of undesirable inflammation and PPs appear to have a considerable potential to regulate the inflammatory aspect of CVD. Prevention of LDL oxidation may be incidental to their benefits to CVD, as it appears that atherosclerosis is largely an inflammatory process in its early stages. Mice fed red wine extract or catechin with and without alcohol showed an increased area of atherosclerotic lesions with alcohol, but the wine extract or catechin reduced thrombotic activity [96]. Red wine consumption, however, did not show any detectable effect on any immune/inflammatory marker in healthy men [97]. Nobiletin, a polymethylated flavonoid, may reduce plasma cholesterol concentrations and reduce atherosclerosis at the level of the vascular wall by inhibiting macrophage foam cell formation [98]. Reducing blood pressure by use of PPs may also be of use in controlling CVD. Angiotensin is an oligopeptide in the blood that causes vasoconstriction and increased blood pressure. It is derived from the precursor molecule angiotensinogen by angiotensin-converting enzyme. It plays an important role in blood pressure regulation, via the renin-angiotensin system. Some low-molecular-weight procyanidins can inhibit the activity of angiotensin-converting enzyme *in vitro* with IC_{50} values in the low micromolar range [99]. This activity could potentially have a lowering effect on blood pressure. In a hypertensive rat model, oral quercetin treatment reduced blood pressure and normalised concentrations of glutathione, glutathione peroxidase and NO [100].

In vitro studies of PPs and inflammation

Recent *in vitro* studies have further highlighted the potential of PPs to modulate various parts of the inflammatory process. Silibinin can suppress the expression of inflammatory genes such as CD80 and MHC class I and inhibit the lipopolysaccharide (LPS)-induced activation of mitogen-activated protein (MAP) kinases (important in vascular gene regulation) and the nuclear translocation of the NF-κB p65 subunit in murine dendritic cells [101]. Tangeretin and nobiletin, polymethylated flavonoids from citrus peel, inhibit IL-1β-induced COX-2 expression in human lung carcinoma cells [102]. Bicalein and bicalin, flavonoids from *S. baicalensis*, inhibited LPS-induced COX-2 gene expression in cultured cells [103]. Resveratrol can protect cultured venous endothelial cells from oxidised-LDL-induced cytotoxicity (as opposed to inhibiting oxidation of LDL) and the resulting generation of ROS [104]. Some flavonoids can inhibit TNF-induced adhesion molecule expression in human aortic endothelial cells, the first stage of atherosclerosis [105]. Two flavonoids can inhibit induction of iNOS in cell cultures [106, 107]. Quercetin inhibits cytokine and iNOS expression through inhibition of the NF-κB pathway without modification of c-Jun N-terminal kinase activity in macrophages [108]. Quercetin and catechin inhibit the proinflammatory effect of advanced glycation end products in human monocytes, indicating a potential for amelioration of diabetic vascular complications [93]. Luteolin and other flavonoids interfere with LPS signalling pathways, reducing activation of several MAP kinase family members, and inhibit inflammatory mediator expression.

Periodontal disease is associated with infection-mediated gingiva inflammation. Periodontal tissue destruction, via bacterial secretion of LPS molecules, plays a key role in the disease development. The flavonoid luteolin appears to interfere with LPS signalling pathways in human gingival fibroblasts, reducing activation of several MAP kinase family members and inhibiting inflammatory mediator expression [109]. Similar effects were seen with an *A. polygama* extract in macrophages. This extract also reduced carrageenan-induced rat paw oedema, a model of inflammation [110]. With regard to allergic inflammation, apple procyanidins can exert an anti-allergenic effect by inhibition of histamine release from mast cells, via inhibition of the interaction between IgE and its high-affinity receptor, FcepsilonRI [111]. Asthma is an obstructive airway inflammation mediated by an allergic activation of the immune system. A clinical correlation has been shown between apple consumption and healthy lung function in humans [112]. Interestingly, anthocyannis [113] and blackcurrant and boysenberry PP extracts [our unpublished observations] have also been found to modulate allergy-mediated pathways in a mouse model of asthma and in lung epithelial cells, respectively. Moreover, in an animal model of asthma, *A. polygama*

inhibits cytokine levels with a crucial role in the pathology [114].

PPs in cancer-related regulatory roles

Many of the signalling molecules involved in inflammation, e.g. NFκB, activator protein-1 (AP-1) or MAP kinases, are also involved in more generic processes such as the regulation of cell proliferation and differentiation [115]. PPs may both modulate cell signalling pathways thereby inhibiting cancer development or progression and induce apoptosis in malignant cells. EGCG has been shown to block each stage of carcinogenesis by modulating signal transduction pathways involved in cell proliferation, transformation, inflammation, apoptosis, metastasis and invasion [116]. Resveratrol exhibited anti-proliferative effects in cancer cells through multiple mechanisms, i.e. inhibition of MAP kinase/extracellular signal-regulated kinase (MEK), extracellular signal-regulated protein kinase (ERK) signalling, down-regulation of c-Jun and suppression of AP-1 DNA-binding and promoter activity [117].

PPs may also inhibit angiogenesis and, thereby, tumour growth, via inhibition of vascular endothelial growth factor (VEGF) [115]. Some PPs can inhibit release of VEGF from MDA human breast cancer cells *in vitro*, at a concentration as low as 0.1 μM [118]. Quercetin at 100 μM inhibits angiogenesis via a mechanism involving both suppression of eNOS and early M phase cell cycle arrest [119].

PPs may inhibit cancer growth and proliferation by regulatory effects on pathways other than those already discussed. Silymarin can inhibit Akt serine/threonine kinase in leukaemia cells, thereby inactivating the Akt signalling pathway, inducing apoptosis and slowing cell growth [120]. Quercetin can significantly inhibit matrix-metalloproteases 2 and 9 in cultured prostate cancer cells at 50 μM. These enzymes are involved in facilitation of metastasis and tumour invasion [121]. Nobiletin has a similar effect in human colorectal cancer cells [122]. Propolis (a PP concentrate produced by honey bees) is mildly genotoxic at high concentrations, but inhibits doxorubicin mutagenesis at relatively low concentrations [123]. Resveratrol, as determined by gene microarrays, is able to affect expression of genes involved in apoptosis and may thereby inhibit tumour formation [124]. Up-regulation of gamma-glutamylcysteine synthetase can increase levels of the endogenous antioxidant glutathione, while up-regulation of Fos-related antigen-1 (Fra-1) can suppress activation of AP-1, a signalling pathway implicated in carcinogenesis [46, 48]. The thioredoxin system, composed of thioredoxin reductase (TrxR), thioredoxin (Trx) and NADPH, controls a wide range of cellular activities and its inhibition can induce cell death. The flavonoids quercetin and myricetin inhibited TrxR with IC_{50}s under 1 μM, potentially demonstrating another mechanism for cancer prevention by PPs [125]. Quercetin enhanced cisplatin-induced apoptosis in human lung cancer cells [126]. In this study, there was no increase in antioxidant enzyme expression, but other apoptosis-related genes may have been regulated. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor known to mediate the toxic and carcinogenic effects of polycyclic aromatic hydrocarbons (PAHs). EGCG is capable of antagonising AhR-mediated gene transcription, indicating potential as a chemopreventive agent [127]. A library of natural and chemically modified flavonoids was screened for anti-proliferative activity in a colon cancer cell line [128]. Compounds with activity also activated caspases, indicating that the anti-proliferative activity may be attributable to an apoptotic response. PPs can inhibit toxic nitrosation processes and carcinogenic nitrosamine formation within the acidic environment of the stomach [129].

MDR is a major obstacle in both cancer chemotherapy and accessibility of pharmaceutical approaches into the central nervous system. One of the mechanisms involved in the development of MDR is the over-expression of the drug transporter, P-glycoprotein (P-gp). Several PPs are inhibitors of P-gp action and may therefore have potential to reverse MDR and aid in the treatment of cancer and central nervous system diseases [130].

Neuro-protective effects of PPs

A consensus is developing that PPs are neuroprotective. The emerging hypothesis on the mechanism of neuro-protection by PPs is that they act by a combination of protection of neuronal cells from oxidative stress, by induction of antioxidant defences, modulation of signalling cascades, apoptotic processes or the synthesis/degradation of the amyloid β peptide [131, 132]. Dietary supplementation with blueberry extract significantly increased the survival of implanted dopamine neurons and ameliorated rotational behaviour asymmetries in aged rats. These neurons are thought to be particularly sensitive to oxidative damage [133].

Rats on a long term intake of grape juice showed oxotremorine enhancement of K^+-evoked release of dopamine from striatal slices and improvements in cognitive performance in a water maze and in motor function, suggesting that grape juice may be beneficial in ameliorating the neuro-degenerative effects of aging [134]. EGCG at 1 μM was able to reduce

neuronal cell death in serum-starved cells and to promote neurite outgrowth, suggesting a capacity to reduce or even reverse neuro-degeneration [135]. Citrus flavonoids stimulated CRE-dependent transcription and induced neurite outgrowth in PC12D cells, and oral administration of nobiletin rescued impaired memory in olfactory-bulbectomised mice [136]. Baicalien at concentrations up to 5 μM inhibited LPS-stimulated activation of microglial cells and associated NO, superoxide and TNF release in a dose-dependent manner, thus indicating potential for neuro-protection via an anti-inflammatory effect [137].

Radio-tracer studies demonstrated the existence of binding sites for PP in rat brain with nanomolar binding constants [138]. If these binding sites were mediators of neuro-protective effects, significant benefits may be expected from a normal dietary intake of PPs.

Formation of amyloid deposits in the brain is common to a number of neurodegenerative disorders, such as Alzheimer's disease. PPs can inhibit the formation of these fibrils *in vitro*, regardless of redox status, suggesting a direct interaction [139]. Mulberry extract exhibited a neuro-protective effect *in vivo* using a transient middle cerebral artery occlusion model of brain injury [140].

Regulatory effects of PPs on energy metabolism and gut health

Metabolic syndrome involves reduced tissue sensitivity to insulin, insulin over-production, and consequent defects in glucose metabolism. It is generally accepted to be the precursor of type II diabetes and a major contributor to obesity. As discussed above, the active glucose transporter SGLT1 appears to be one way that PP glucosides are absorbed from the intestine. Many such compounds also appear to inhibit transport of glucose, particularly the dihydrochalcone glucoside phloridzin, characteristic of apples and also found in strawberries, potentially lowering the effective glycaemic index (GI) of a meal [8]. PP glycosides can also inhibit digestive amylase and glycosidase enzymes, thereby potentially slowing glucose liberation from starch and other sugars. Significant reductions in dietary GI in human trials have been demonstrated with realistic intakes of PPs [8].

Recent reports have provided further evidence for effects of PPs on GI and revealed other potential mechanisms of benefit to metabolic syndrome. Black tea catechins can inhibit α-glucosidase in the gut and slow breakdown of maltose, thereby slowing the resulting rise in blood glucose [141]. Black tea and, more particularly, green tea can increase urinary excretion of citric acid cycle intermediates in humans [142]. This suggests modulation of oxidative energy metabolism or biosynthetic pathways. Green tea PP can inhibit glutamate dehydrogenase (GDH), some at nanomolar ED_{50}s [143]. This enzyme is involved in regulation of insulin secretion and the PPs can inhibit insulin secretion, except under high-energy conditions when the GDH is probably fully inhibited anyway. Under-expression of endothelial nitric oxide synthase (eNOS) is strongly implicated in metabolic syndrome. Red wine, a rich source of PPs, enhances eNOS function [144].

PPs can clearly regulate the absorption of nutrients from the gut, but also appear to benefit gut health directly. As discussed above, it is estimated that 90–95% of dietary PPs are not absorbed directly, and accumulate in the colon. Colonic metabolites of tea PPs inhibit the growth of pathogenic bacteria much more strongly than that of commensal bacteria [145]. Three studies showed beneficial effects on a trinitrobenzenesulphonic acid (TNBS) model of rat colitis, another inflammatory disease. *Turnera ulmifolia* is a plant from South America used in traditional medicine for different types of inflammatory diseases. Pre-treatment with *T. ulmifolia* extract, at 250 and 500 mg/kg, significantly attenuated colonic damage induced by TNBS [146]. Rutin, a poorly absorbed glycoside of quercetin, acted as a quercetin delivery system to the large intestine and appeared to have an anti-inflammatory effect through quercetin-mediated inhibition of TNF- induced NF-κB activation [147]. A similar effect was observed with another quercetin glycoside, quercitrin (quercetin rhamnoside) [108]. Rutin and quercitrin appear to be very poorly absorbed by the small intestine, presumably because of a lack of specific digestive glycosidases. Presumably the rat colonic micro-flora was responsible for liberating the much better absorbed quercetin aglycone from these glyosides.

Discussion

We have found that PPs are potentially far more than 'just antioxidants' (Table 1), but that they are probably insignificant players as 'conventional' antioxidants. They appear, under most circumstances, to be just the opposite, i.e., prooxidants, that nevertheless appear to contribute strongly to protection from oxidative stress by inducing cellular endogenous enzymic protective mechanisms. They appear able to regulate not only antioxidant enzyme gene transcription but also numerous aspects of intercellular signalling cascades involved in the regulation of cell growth, inflammation and many other processes. It has been

Table 1. Summary of main findings from the papers reviewed about PPs and their *potential* health benefits.

Finding
UK dietary intake ~ 1 g/day, about 50 % hydroxycinnamates and 25 % flavonoids.
~ 90 % of dietary PPs are not directly bio-available but are metabolised by the colonic micro-flora into bio-available phenolic acids.
Phase II conjugation of PPs is extensive *in vivo* and may radically change biological activity.
More PPs is not necessarily better; exceptionally high doses have demonstrated detrimental effects.
Estimated total concentrations of circulating PPs and conjugates are too low to make a significant contribution to plasma total antioxidant capacity.
In vitro, PPs can induce the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase.
PPs appear to be pro-oxidants in cell-based studies, but nevertheless appear to protect cultured cells from oxidative stress.
Human trials show correlations between PP (particularly flavonoid) intake and reduced incidence of cancer and cardiovascular disease (CVD), and slower neuro-degeneration.
In vitro regulation of inflammatory pathways by inhibition of signalling molecules such as TNF and by down-regulation of inflammatory genes may be the mechanism of beneficial effects on CVD.
PPs may be able to help regulate blood pressure.
PPs may be able to modulate immune responses in allergic conditions.
Regulation of cell proliferation and differentiation, angiogenesis and apoptosis; may explain anti-cancer effects.
Neuro-protection, by protection of neuronal cells from oxidative stress, induction of antioxidant defences and modulation of signalling cascades and apoptotic processes.
Regulation of metabolic syndrome, via inhibition of glucose uptake and regulation of some metabolic pathways.
Benefits to gut health by growth inhibition of pathogenic gut bacteria and modulation of inflammatory bowel conditions.

proposed recently [148] that many of the beneficial effects of PPs may occur at the genetic level, and genomic techniques may be the best way forward in understanding how they operate. The limited bio-availability of PPs, however, especially in the unconjugated form, makes the relevance of many *in vitro* studies questionable [149], since the vast majority of such studies have used PP aglycones. Resveratrol, for example, has been demonstrated *in vitro* to have many beneficial properties, but it is almost completely conjugated *in vivo* and the biological activity of the conjugates needs to be determined [150–152]. This applies to a greater or lesser extent to all PPs [24, 153]. There have been numerous reports on the metabolism, conjugation and pharmacokinetics of PPs [15, 21, 150, 154–168]. There has, however, been relatively little study of the biological effects of PP conjugates or colonic metabolites [169]. In contrast with the hundreds of studies on PP aglycones, reported studies on the bio-activity of glucuronides are few. These cover inhibition of: LDL oxidation in human serum [170], induction of ROS in mouse cells [171] and hypertrophy of cultured rat aortic smooth muscle cells [172] by quercetin; release of arachidonic acid from human colon cancer cells by EGCG [173] and oxidative-stress-induced cell death by epicatechin [174]. Glucuronides, at least, appear to have significant biological activities, but not necessarily the same potency or activity as the aglycones. Recent research has demonstrated many *potential* biological effects of PPs, but these are of questionable relevance *in vivo* unless repeated *in vitro* on conjugates. This is an area with great potential for progress in the future, by combining the extensive pharmacokinetic information on PPs with cell-based studies on known *in vivo* conjugates. Recent research has produced plenty of evidence for direct interaction of PPs with DNA and gene transcription. PPs may be just plant toxins, designed to discourage us from consuming them, but we and our mammalian ancestors have had millions of years of exposure to PPs, unlike man-made environmental toxins. It would be surprising if we had not adapted to PPs by developing high-affinity binding sites that regulate the genes and pathways responsible for protection from these toxins. It appears that PPs may have become an essential part of our cellular regulatory processes, for example, by maintaining up-regulated levels of our endogenous antioxidant and other defences, thereby minimising the response time for the defence against oxidative, toxic and other stresses. PP may have originally up-regulated antioxidant defences via their prooxidant effect and ROS such as hydrogen peroxide, but may have gradually assumed a concomitant, more direct and much more potent signalling role by direct interaction with the relevant genes. This might explain the protective effects from oxidative stress, discussed above, exhibited by both natural and synthetic polymethylated flavonoids. Methylation appears to mostly eliminate their direct antioxidant capacity and, presumably,

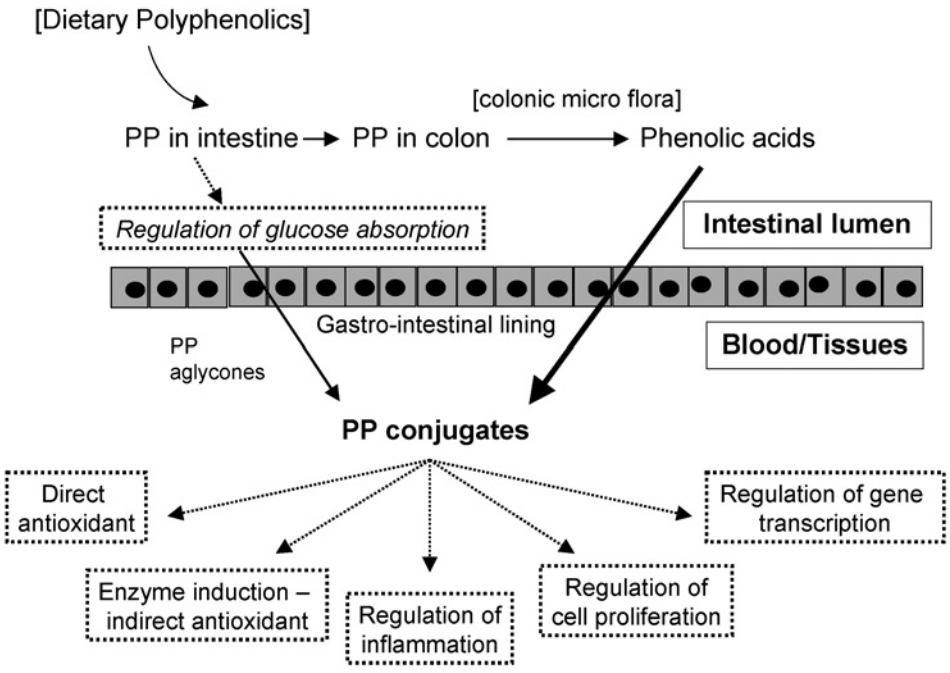

Figure 2. Schematic representation of the main processes by which PPs mediate health benefits. PPs act as direct antioxidants in the micromolar range but these effects are probably less important physiologically than the indirect adaptive antioxidant pathways that are initiated or the emerging anti-inflammatory actions recently revealed (which may occur in the nanomolar range). Furthermore, a host of other mechanisms is apparent.

prooxidant capacity, but may have less influence on their ability to act as signalling molecules.

Recent research has shown that PPs have potential as *in vivo* antioxidants, but they appear to function by mechanisms very different from, and much more subtle than previously believed. They are also potentially beneficial in numerous other ways, possibly at *in vivo* concentrations previously thought to be too low to provide useful health benefits (Fig. 2). Further research will be needed to reveal in more detail the true health potential of PPs.

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