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Review Article

Dietary flavonoids: Role of (−)-epicatechin and related procyanidins in cell signaling

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ABSTRACT

Plant polyphenols are among the most abundant phytochemicals present in human diets. Increasing evidence supports the health-promoting effects of certain polyphenols, including flavonoids. This review discusses current knowledge of the capacity of monomeric flavanols, i.e., (−)-epicatechin and (+)-catechin, and their derived procyanidins to modulate cell signaling and the associations of these actions with better health. Flavanols and procyanidins can regulate cell signaling through different mechanisms of action. Monomers and dimeric procyanidins can be transported inside cells and directly interact and modulate the activity of signaling proteins and/or prevent oxidation. Larger and nonabsorbable procyanidins can regulate cell signaling by interacting with cell membrane proteins and lipids, inducing changes in membrane biophysics, and by modulating oxidant production. All these actions would be limited by the bioavailability of flavanols at the target tissue. The protection from cardiac and vascular disease and from cancer that is associated with a high consumption of fruit and vegetables could be in part explained by the capacity of flavanols and related procyanidins to modulate proinflammatory and oncogenic signals.

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Contents

Introduction

Research in the past decades has promoted the understanding of how certain chemical species interact with cell components, triggering molecular events that lead to well-orchestrated cell responses. These responses range from changes in cell metabolism to altering major decisions on cell fate. Plant polyphenols are among the most abundant phytochemicals present in human diets, and increasing evidence points to important health-promoting effects of select polyphenols, e.g., flavonoids and stilbenes. The possibility that plant-derived compounds could interact with cells and affect cell

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signaling is getting extensive experimental support. This evidence supports the concept that a food is a vehicle to provide the body not only with essential nutrients but also with compounds that contribute to normal function and can help to prevent/ameliorate disease. Flavonoids are one example of these plant compounds, and flavanols are a subgroup of flavonoids that have been associated with human health benefits. This review discusses current knowledge of the capacity of the flavanols (−)-epicatechin and (+)-catechin, and their derived procyanidins, to promote health through the modulation of cell signaling.

Flavanols and procyanidins: chemical structure

Flavanols and procyanidins are polyphenols occurring in plants, in which they are secondary metabolites with various characterized functions [1,2]. Plant phenolics include several thousand chemical structures, from simple molecules such as phenolic acids (four carbons) to highly polymerized compounds, such as condensed tannins (MW>1000). Flavonoids are a chemically defined family of polyphenols (Fig. 1) that have a basic structure of two aromatic rings (A and B) linked through three carbons that usually form an oxygenated heterocycle (C ring). The chemical characteristics of the C ring define the various subfamilies of flavonoids by providing different arrangements of hydroxy, methoxy, and glycosidic groups, and the bonding with other monomers [1]. Flavanols are a subfamily of flavonoids in which the C ring is a saturated heterocycle with a hydroxyl group in position 4. They can have OH or OCH_3 groups in up to three positions in the B ring. Flavanols are present in plants as aglycones, as oligomers, or esterified with gallic acid. Most flavanols present in nature are stereoisomers in *cis* or *trans* configuration with respect to carbons 2 and 3 ((−)-epicatechin (*cis*) and (+)-catechin (*trans*)). The most common oligomers (procyanidins) present in edible plants are derived from (−)-epicatechin. The profiles of the oligomers and the relative concentration of each individual procyanidin vary depending on the plant [3]. For example, cocoa (*Theobroma cacao*) synthesizes mostly B-type dimers, whereas mostly A-type

dimers are present in peanuts (*Arachis hypogea* L.). Chemically, the monomers of the B-type dimers are linked through 4→8 carbon–carbon bonds (Fig. 2). Essentially, higher molecular weight oligomers present in cocoa maintain this 4→8-type of bonding generating linear molecules that can twist to the most stable three-dimensional structures. In the case of A-type dimers, the monomers are linked by both a 4→8 carbon–carbon and a 2→O7 ether bond (Fig. 2). In other plants, e.g., in tea (*Camellia sinensis*), flavanols can contain gallate groups (e.g., (−)-epigallocatechin gallate, EGCG) and form complex polymers (tannins), even postharvest, but not simpler linear oligomers.

Even minor changes in isomerization, monomer bonding type, and degree of polymerization can have a major impact on the biological actions of these molecules. Metabolism can lead to secondary compounds that can be responsible for the observed biological actions. Furthermore, procyanidins can bear multiple tridimensional structures that determine the possibility of chemical and physical interactions with proteins or lipids.

Flavanols and procyanidins: bioavailability and metabolism

As with all bioactive substances, the molecular actions of flavanols and procyanidins in animals are largely dependent on their bioavailability at the target tissue. This bioavailability depends on flavanol and procyanidin absorption, metabolism at the gastrointestinal tract, tissue and cellular distribution, and tissue metabolism after absorption. Data from human subjects show that flavanols and procyanidins are stable during gastric transit [4,5], allowing their presence in the gastrointestinal tract at high concentrations. These concentrations of procyanidins seem to persist for several hours after the ingestion of a flavanol-rich food. The possibility that procyanidins could be depolymerized to monomeric flavanols in the stomach and intestine derives from ex vivo models [4,6]. In rats fed a grape-seed extract containing (−)-epicatechin and dimers, trimers, tetramers, and higher molecular weight procyanidins, monomers and dimers are present in the small and large intestine (the presence of larger procyanidins was not
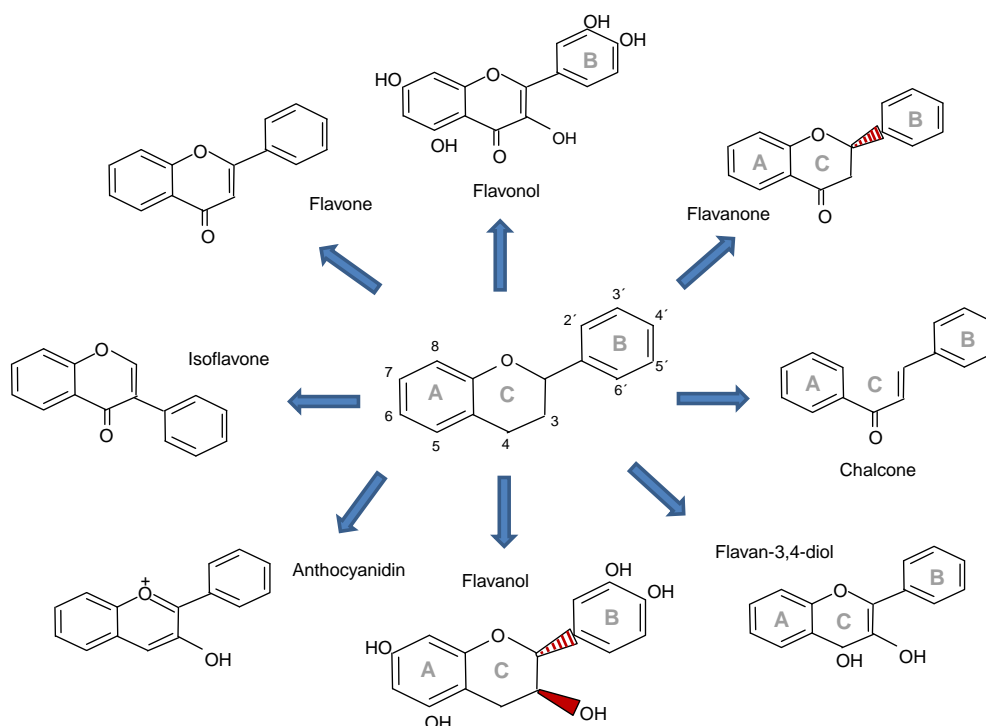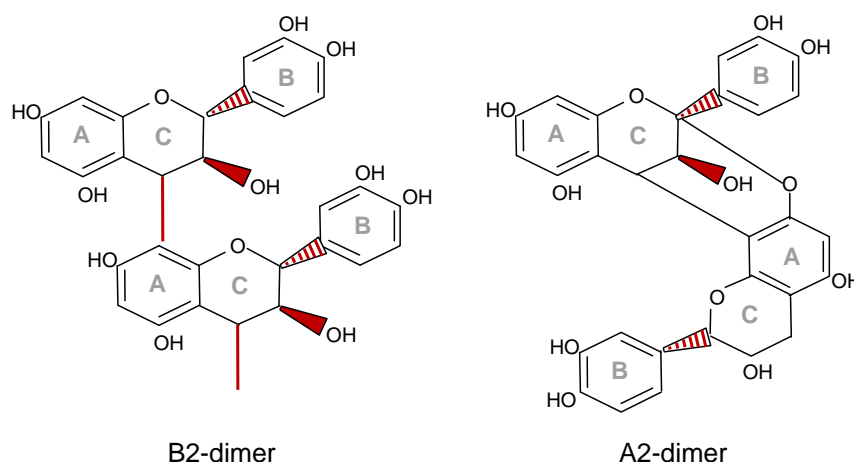

Fig. 1. Chemical structures of the main flavonoid families present in plants that are part of human diets. In the center: the basic generic flavonoid structure showing A, B, and C rings and the numbers for the various positions in the flavan structure. For the flavanol family, the structure of (−)-epicatechin is shown.

B2-dimer   A2-dimer

Fig. 2. Chemical structures of dimeric procyanidins. B2 dimer, (−)-epicatechin-4→8-(−)-epicatechin, showing the possibility of extending the oligomer and forming higher molecular weight procyanidins by binding new (−)-epicatechin molecules through carbons 4 and 8. A2 dimer, (−)-epicatechin-4→8;2→O7-(−)-epicatechin.

investigated) [7]. A recent report suggests that select trimers could also be present in plasma after the intake of a grape-seed extract [8]. However, more convincing evidence is necessary to confirm the plasma presence of trimeric procyanidins. All this evidence suggests that, in vivo, procyanidins are not depolymerized and/or metabolized during upper gastrointestinal tract transit and could reach the large intestine at concentrations that allow their participation in chemical reactions of biological relevance.

In the small intestine, flavanols are extensively glucuronidated and partially methylated [9,10], allowing negligible amounts of native catechin or epicatechin in the mesenteric circulation. In the liver, further glucuronidation, methylation, and sulfation can take place [9,11,12]. Several studies have determined the presence of these conjugates in the plasma and urine of rodents and humans [11,13–15], as well as in rat bile [11] and brain [16]. In plasma, monomers are present as conjugated metabolites (>90%) and nonconjugated molecules (<10%). Total plasma concentrations of (−)-epicatechin plus (−)-epicatechin metabolites were found in the low-micromolar range as soon as 1 h after the consumption of a flavanol-rich food (cocoa) [14,17–20]. The major metabolite of (−)-epicatechin detected in plasma was 4′-O-methyl-epicatechin-7-β-D-glucuronide [20]. It is important to note that regardless of the composition of the procyanidin-containing food ingested, only plasma oligomers of (−)-epicatechin, and not of (+)-catechin, were detected. The simple stereochemical orientation of an OH group in the B ring that distinguishes (−)-epicatechin from (+)-catechin makes a remarkable difference in bioavailability and stresses a high specificity for the interactions of flavanols with cell components. These differences in the bioactivity of (−)-epicatechin and (+)-catechin were observed both in rats [21,22] and in humans [18,23].

Further metabolism can occur in the colon, where microflora can also modify flavanols and procyanidins, including the breakage of the flavan structure to form simple phenolics and ring-fission metabolites [24,25]. In this regard, an increased urinary excretion of four phenolic acids has been found 9–48 h after cocoa consumption in humans [26]. The biological relevance of these metabolites remains to be assessed.

In summary, biological activities (chemical reactions) in need of high flavanol and procyanidin concentrations are possible only in the gastrointestinal tract. In blood and the vasculature, the biological actions that would be feasible are compatible with nanomolar concentrations of nonmetabolized flavanols or low-micromolar concentration of metabolites, e.g., methylated and glucorinated (−)-epicatechin. Given the limited current knowledge, it can be speculated that in most other tissues, flavanols are present at low-nanomolar concentrations and would participate only in highly specific biological processes.

Flavanols and procyanidins in the regulation of cell signaling

A large number of studies have described, in both in vitro and in vivo models, the numerous effects of procyanidin-rich extracts on cell signaling. Although these studies are of significant value as a starting point to define the health benefits of flavanols and procyanidins, they have very limited value when mechanisms of action are discussed, given the difficulty in identifying the molecule(s) responsible for the observed effects. The biological actions and health benefits of food extracts enriched in procyanidins have been reviewed elsewhere [27]. Thus, in this review we summarize current knowledge on the action of pure or highly purified monomers (mostly (−)-epicatechin), their metabolites, and derived procyanidins on cell signaling. The capacity of these compounds to regulate cell signaling is discussed based on their capacity to (a) act as antioxidants and redox regulators or have antioxidant-like properties, (b) interact with signaling proteins, and (c) interact with membranes. These mechanisms are of critical relevance to understand the potential health benefits of flavanols and procyanidins.

Antioxidant and redox actions of flavanols and procyanidins

Flavanols and procyanidins are nevertheless chemically able to prevent oxidation, and their presence/administration has been associated with a decrease in oxidative stress markers in animals and humans; the exact mechanisms behind those decreases are debatable and largely depend on their bioavailability. Ubiquitous oxidant species, generally referred to as reactive oxygen species, or ROS, are essential participants in the physiology of aerobic organisms. ROS can actively participate in the normal regulation of cell signaling, including the triggering of signals involved in adaptive (antioxidant) responses [28,29]. Higher and uncontrolled ROS production leads to extensive oxidation of cell components and irreversible cell/tissue damage underlying the acute pathology of many disease and pathological states. In between, a ROS-mediated imbalance of the cellular redox state should not compromise vital cell functions, but could lead in the long term to the onset of disease, e.g., chronic inflammation [30].

The prevention of oxidative damage generically categorizes a compound as antioxidant. However, the extrapolation of an antioxidant action in vitro to an in vivo condition is not trivial. A possible approach is to segregate "direct antioxidant effects," i.e., free radical scavenging and redox-active metal sequestration, from "indirect antioxidant effects," e.g., regulation of protein synthesis and activities, signaling strategies, etc. Such rationale is meaningful for a discussion about polyphenols and phytochemicals, because it considers the

concentrations (bioavailability) of the molecule to be defined as antioxidant. In this regard, high concentrations are usually required to act as a direct antioxidant but significantly lower concentrations to act as an indirect antioxidant [31–35].

Many plant polyphenols and flavonoids, including flavanols and procyanidins, are often categorized as in vivo antioxidants because: (i) they have chemical structures that support direct antioxidant reactions affecting both the steady-state concentration (production and metabolism) of cell oxidants and the occurrence of oxidant-mediated events [36,37]; (ii) it has been extensively demonstrated that supplementing the diet of experimental animals and humans with flavonoids reduces the oxidation levels in various organs [38–47]; and (iii) significant evidence shows that certain flavanols and procyanidins can provide benefits in pathological situations associated with high oxidant production, for example, hypertension and cardiovascular disease, and affect markers of oxidative stress [44,46]. However, such direct antioxidant actions in vivo are likely to be significant in tissues exposed to high concentrations of polyphenols, e.g., in the digestive tract where flavanol and procyanidin concentrations can reach values in the upper micromolar range. In blood, (−)-epicatechin and its metabolites can reach concentrations in the low-micromolar range. Considering the affinity for radicals (reduction potentials) and the actual concentrations, it can be estimated that (−)-epicatechin will scavenge radicals at a rate about 25 times lower than ascorbate [48]. In most other tissues the concentrations of flavanols and procyanidins are too low to provide any relevant direct antioxidant action compared to other compounds, e.g., glutathione, albumin, ascorbate, and tocopherols [48]. A similar situation is the case for flavanol and procyanidin metal-sequestering actions. Thus, the molecular and physiological mechanisms linking the chemical characteristics of flavonoids with their health effects can be based not only on scavenging free radicals and/or chelating redox-active metals, but also on more complex and specific chemical interactions compatible with the low concentrations found in most tissues [33,44,49,50].

It is important to stress that even under in vitro conditions, the mechanisms involved in the relative radical scavenging actions of flavanol monomers and procyanidins are not trivial. For instance, depending on the model of oxidation used, liposomes are not equivalently protected from chemical oxidation by monomers and procyanidins [51]. Monomers are more efficient than procyanidins, protecting liposomes from oxidation when ferrous iron is the oxidation promoter. On the other hand, procyanidins exert a better antioxidant protection than monomers when a thermo-labile free radical initiator (2,2′-azobis(2,4-dimethylvaleronitrile)) is the oxidant [51]. Thus, the mechanism of oxidation, i.e., pro-oxidant challenge, will define the relative antioxidant capacities of monomer vs hexamer, i.e., if one molecule of hexamer has the antioxidant capacity of one monomer or of six monomers [52]. Moreover, not only the number of subunits but the conformational structure of oligomers will define their relative antioxidant action.

A first example of the mentioned indirect antioxidant effects is the modulation of pro-oxidant enzymes by flavanols and procyanidins. In this regard (−)-epicatechin and related metabolites inhibit the activity of NADPH-oxidase in cultures of human umbilical vein endothelial cells [53,54]. Interestingly, the (−)-epicatechin O-methylated metabolite has structural similarities with apocynin, a typical NADPH-oxidase inhibitor. NADPH-oxidase is also inhibited by various (−)-epicatechin metabolites, i.e., 3′-O-methyl epicatechin, 4′-O-methyl epicatechin, and (−)-epicatechin glucuronide; by procyanidin B2 dimer; and to a lesser extent by (−)-epicatechin. Considering the micromolar concentrations used in cell cultures, this inhibition of NADPH-oxidase could be operative in vivo for the regulation of redox cell signaling and vascular function (see below). The inhibition of NADPH-oxidase by (−)-epicatechin and its metabolites can also occur in other cells, resulting in a diminution in superoxide anion

production. Overall, an increase in superoxide anion can lead not only to a diminution of cell oxidation but also to the regulation of superoxide anion-mediated cell signaling and finally to defining NO availability [55].

Another example of the indirect action of flavanols and procyanidins is the alteration of oxidant production as a result of inhibiting the binding of a ligand to its receptor. This is the case for tumor necrosis factor (TNFα), in which its binding to TNFα receptor 1 leads to the activation of NADPH-oxidase and to the subsequent superoxide anion production [56]. In intestinal cells, (−)-epicatechin, catechin, B2 dimers, and hexameric procyanidins decrease the transient increase in oxidants associated with TNFα-triggered signaling [57]. These effects are further discussed under Cell membranes.

In summary, a direct antioxidant action of flavanols and procyanidins would be limited to tissues/organs in which relatively high concentrations of these compounds can be achieved. Flavanols and procyanidins could support indirect antioxidant effects through the regulation of enzymes that generate oxidants or by preventing protein–receptor coupling that initiates oxidant production.

NF-κB

NF-κB is a ubiquitous transcription factor that regulates many central events in normal cell function and fate. NF-κB is redox sensitive, and in general, oxidants promote and antioxidants inhibit its activation. Flavanols and procyanidins can interfere with NF-κB activation by counterbalancing changes in cell redox state, but also by specific bonding to proteins involved in the NF-κB pathway.

NF-κB regulates the expression of a large family of genes including those encoding proteins involved in innate immunity, inflammation, and the regulation of cell survival. Although of central importance in multiple physiological cell processes [58,59], its chronic activation is associated with inflammatory diseases and cancer [60,61]. The Rel/NF-κB family of proteins in human cells includes c-Rel, RelB, RelA (p65), p50/p105, and p52/p100. In the canonical pathway (Fig. 3), inhibitory IκB proteins bind to dimers of RelA, c-Rel, and p50 forming an inactive complex in the cytosol [60]. Superoxide anion and derived species, e.g., H_2O_2, can oxidize thiol groups in LC8, a protein that prevents IκB processing [62]. LC8 oxidation leads to its dissociation from IκB, which is subsequently phosphorylated on two serines (S32 and S36) by IκB kinases (IKK). IκB phosphorylation targets the protein for ubiquitination and degradation [63]. The released active NF-κB dimer can subsequently translocate to the nucleus and bind to specific κB sites in select gene promoters (reviewed in [60]). In the nucleus, redox regulation also occurs, given the requirement of reduced thiol groups in NF-κB for its optimum binding to DNA.

(−)-Epicatechin and procyanidins can inhibit NF-κB at different levels in the activation pathway as is illustrated in Fig. 3. A decrease in cell oxidants that are involved in NF-κB activation is a potential mechanism of modulation by these compounds. Other specific reactions can also occur in the prevention of NF-κB activation. The interaction of B2 dimer with components of the NF-κB/Rel family of proteins is a paradigm of the capacity of procyanidins to interact specifically with signaling proteins. We have shown that B2 dimer inhibits the binding of NF-κB proteins, i.e., RelA and p50, to its κB DNA consensus sequence in whole cells, nuclear fractions, and purified chemical systems [64–66]. The presence of select OH groups in the B2 dimer (Fig. 4a) is decisive in explaining the chemical characteristics of this inhibition. Molecular modeling of the B2 dimer shows a folded structure in which ring B′ stacks onto ring A, orienting the hydroxyl groups toward the same edge of the molecule (Fig. 4b). This mimics the guanine pairs in the κB DNA sequence that specifically interacts with p50 and RelA. Thus, B2 dimer can establish hydrogen bonds similar to those that the guanine pairs establish with the arginine residues present in the DNA binding regions of both p50 (Arg 54 and Arg 56) and RelA (Arg 33 and Arg 35) (Fig. 4c). These interactions
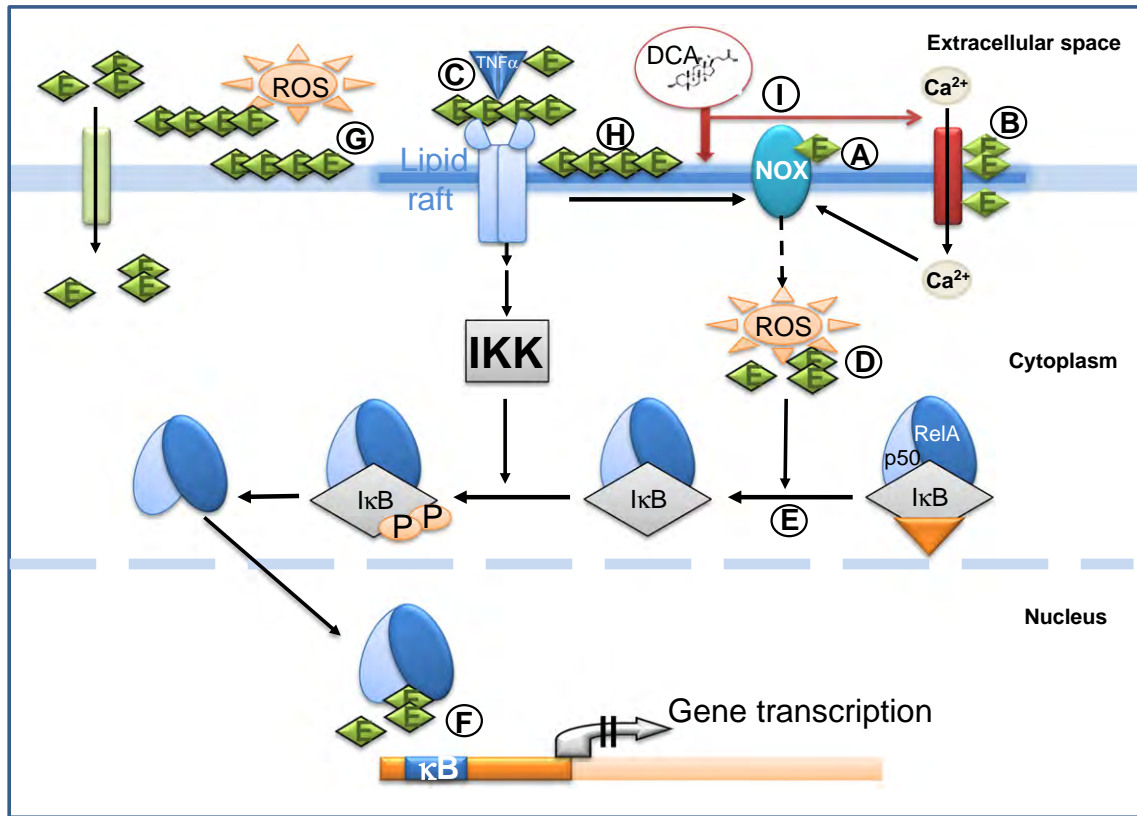
Fig. 3. Redox and nonredox regulation of the NF-κB signaling pathway by (−)-epicatechin and related procyanidins. Epi/catechin monomers (single green diamonds) and dimers (double green diamonds) can be transported inside cells and then act on both intracellular and plasma membrane targets. (−)-Epicatechin and dimers can inhibit NADPH-oxidase and the subsequent superoxide production by: (A) directly binding to the enzyme, (B) regulating calcium influx, or (C) potentially inhibiting the binding of ligands that trigger NADPH-oxidase activation (e.g., TNFα) to their receptors. (D) At high enough concentrations, (−)-epicatechin and dimers could also directly scavenge free radical and related oxidants. (E) A decrease in cell oxidants could prevent the redox-sensitive release of the LC8 inhibitory peptide (orange triangle), allowing the phosphorylation and degradation of IκBα, and the associated release of the active NF-κB complex. (F) Inside the nucleus, both (−)-epicatechin and B dimers can interact with the DNA-binding site in the NF-κB proteins, preventing the interaction of NF-κB with κB sites in gene promoters, thus inhibiting gene transcription. Procyanidins with three or more units (multiple green diamonds) are not transported inside the cells but can interact with membrane components involved directly or indirectly in oxidant production or NF-κB signaling. (G) Procyanidins can act at the extracellular space and at the cell membrane level (e.g., gastrointestinal tract lumen or vascular endothelium), scavenging oxidants. (C) When NF-κB activation occurs secondary to ligand–receptor binding (e.g., TNFα–receptor), procyanidins could prevent the binding of the ligand (stimulus) to its receptor or (H) interact with membrane lipids, promoting changes in membrane biophysical properties that could indirectly affect a receptor's affinity for its ligand. Procyanidins also could prevent receptor-independent NF-κB activation. (I) In the case of secondary bile acid, e.g. deoxycholate, signaling is triggered through changes in cholesterol local concentrations at lipid rafts, which promote calcium influx, and NADPH-oxidase activation. Procyanidins can prevent this series of events through their interaction with membrane lipid rafts.
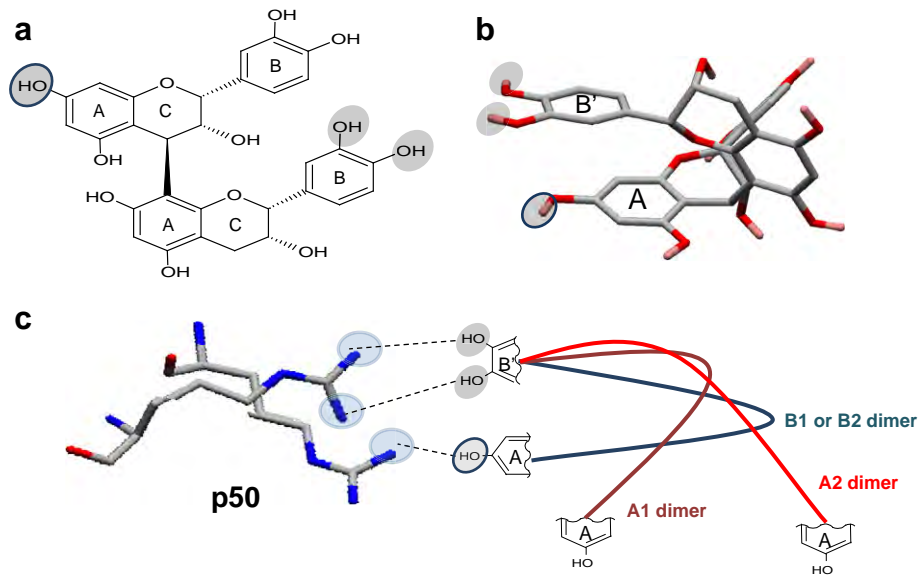
Fig. 4. Interactions of (−)-epicatechin and dimeric procyanidins with NF-κB proteins. (a) Chemical structure of B2 dimer stressing the OH groups that are involved in bonding to NF-κB protein p50. (b) B2 dimer minimum energy conformer. (c) Modeled interactions of dimers A1, A2, B1, and B2 with the amino acids of p50 involved in the interactions with DNA. The shapes of the minimum-energy conformers of the dimers are represented with lines. Adapted from [49,64,66].

between B2 dimer and RelA or p50 explain in part the capacity of B2 dimer to inhibit NF-κB binding both in vitro and in vivo [64].

The binding of B2 dimer to NF-κB protein is stereospecific. Although dimers have similar chemical composition, interunit bonds can determine significant changes in their three-dimensional structure, which can result in differential biological actions. In this regard, we compared the capacity of dimers B1, B2, A1, and A2 to inhibit NF-κB [66]. Dimers B1 and B2, but not A1 and A2, inhibited both NF-κB binding to κB DNA sites and NF-κB-dependent gene transcription. In the B-type dimers, flavanol monomers are linked through 4β→8 carbon–carbon bonds, but A-type dimers have an extra 2→O7 ether bond that does not allow folding of the dimer (Fig. 4c). This does not allow their interaction with RelA and p50 arginines. To a lesser degree, monomers ((−)-epicatechin) can also interact with NF-κB and inhibit TNFα-stimulated and constitutive NF-κB activation in T lymphocytes (Jurkat) and Hodgkin lymphoma cells, respectively [64,67]. Transcription factors that do not present a double guanine in their DNA consensus sequence, e.g., OCT-1, were not inhibited by flavanol monomers and dimers, stressing the specificity of the protein–flavanol interaction [64].

In summary, in vitro data support the capacity of (−)-epicatechin and procyanidins to inhibit NF-κB with high specificity. In addition several studies in which rodents were fed (−)-epicatechin- and procyanidin-rich extracts showed anti-inflammatory and anticancer effects associated with NF-κB inhibition. However, there is still no direct evidence that flavanols or procyanidins inhibit NF-κB in humans under physiological or pathological conditions.
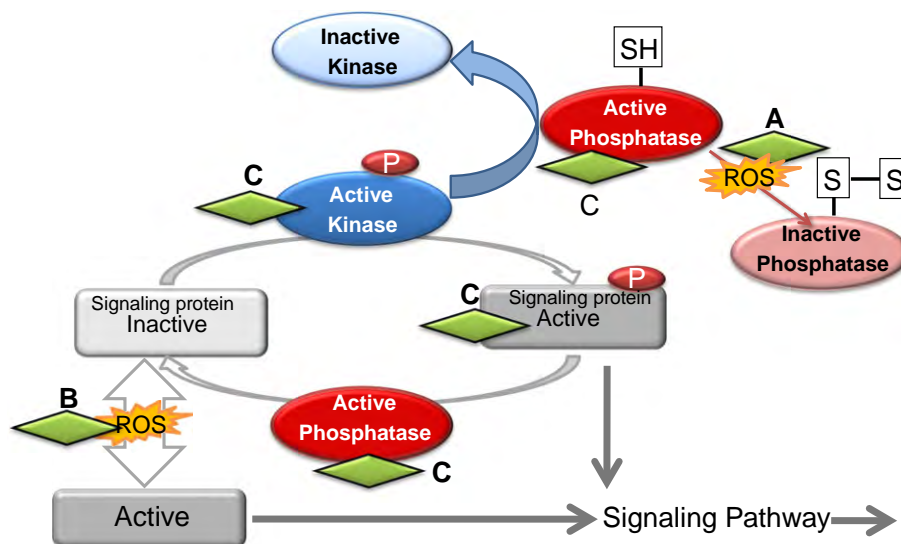
Protein kinases and phosphatases

Protein kinases and phosphatases are key molecules in most signaling pathways. Both types of activities can be modulated by flavanols and procyanidins. Protein kinases are often activated by phosphorylation and inactivated through the action of select phosphatases (Fig. 5). Both kinases and phosphatases are susceptible to redox regulation. For example, the mitogen-activated protein kinase (MAPK) ASK1 is inactive when forming a complex with thioredoxin. Oxidative conditions that change thioredoxin redox status cause the disruption of the complex and the release of ASK1 in its active form [68]. Several protein phosphatases, including those that dephosphorylate MAPKs, are sensitive to redox-regulated inactivation. Members of the family of MAPKs phosphatases and the tyrosine

phosphatase PTP1B are also inhibited through the reversible oxidation of a cysteine group in their active site [69,70]. Inactivation of MAPKs phosphatases can lead to decreased dephosphorylation and persistent activation of JNK and p38, constituting one regulatory point of the well-known sensitivity of JNK and p38 to oxidative stress.

In addition to the regulation of redox-sensitive kinases and phosphatases by (−)-epicatechin and procyanidins through a reduction in oxidant concentrations, these compounds can also interact with signaling proteins, modulating their activity (Fig. 5). The B2 dimer was found to interact with the MAPK kinase MEK [71]. MEK is a protein kinase that phosphorylates its downstream target extracellular signal-regulated protein (ERK) at select serine and tyrosine residues. The MEK/ERK pathway regulates proliferative responses, and its overactivation is considered one critical event in oncogenesis. In fact, B2 dimer inhibited the neoplastic transformation of JB6 P + mouse epidermal cells induced by 12-O-tetradecanoyl-phorbol 13-acetate, probably because of the capacity of B2 dimer to inhibit MEK and the downstream activation of ERK, AP-1, and NF-κB [71]. In the same study, B2 dimer also caused a lower level of MEK phosphorylation, the triggering event in MEK activation. In monocytes B2 dimer decreased the endotoxin-induced expression of cyclooxygenase-2, through the inhibition of MAPK p38, JNK, and ERK and NF-κB [72]. Other flavonoids, i.e., delphinidin and anthocyanidin, with chemical similarities to (−)-epicatechin also inhibited MEK and the upstream kinase Raf [73]. Molecular modeling suggested potential binding sites in both kinases different from that of ATP, which would be an obvious candidate, given the purine-like structure of flavanols. Overall, a keto group in the B ring, and a 3′,4′-catechol group in the C ring seem to be required for optimum flavonoid inhibitory action on in vitro MEK activity. However, a comparison of the conformation and three-dimensional structure of the different compounds would be necessary for more definitive conclusions.

(−)-Epicatechin also modulates the ERK signaling pathway, both in vitro and in vivo. Cisplatin-induced ERK1/2 activation was inhibited by (−)-epicatechin in a cochlear organ of Corti-derived cell line and in a rat model [74]. Relevant to neurodevelopment and synaptic transmission (−)-epicatechin and its metabolite 3′-O-methyl-(−)-epicatechin activated ERK in neuronal cells, as well as the ERK downstream target CREB [75]. Importantly, these effects depended on the (−)-epicatechin concentration, activating ERK at 100–300 nM but inhibiting ERK in the micromolar range [75]. These results stress the caution with which in vitro results should be interpreted, especially

Fig. 5. Potential regulation by (−)-epicatechin and dimeric procyanidins of signaling pathways involving protein kinases and phosphatases. (−)-Epicatechin and dimeric procyanidins could act at different levels in signaling pathways involving protein kinases and phosphatases. They could act: (A) by scavenging oxidant species that inactivate phosphatases, (B) by scavenging oxidant species that inactivate/activate signaling proteins, or (C) by directly interacting with phosphatases, kinases, or other signaling proteins.

taking into consideration the possible flavonoid concentration in the target tissue, the cell type, and the flavonoid metabolism.

Nrf2 and other signaling pathways

Nrf2 is a transcription factor central in the protection of cells against the adverse effects of oxidative and electrophilic stress [76,77]. Nrf2 resides in the cytosol as an inactive complex through its binding to the protein Keap-1. Dissociation of this complex, which occurs as a consequence of Keap-1 cysteine oxidation or cysteine covalent bonding, leads to Nrf2 nuclear translocation and its binding to ARE sequences. In the nucleus Nrf2 binds to the promoters of genes encoding phase II detoxification enzymes and proteins involved in the antioxidant response. Nrf2 is among the signals that link oxidative stress with inflammation and, as a consequence, with cancer and other pathologies of inflammatory origin [30].

In terms of ROS metabolism, Nrf2 seems to play a key role in maintaining active antioxidant pathways in response to constitutive oxidative stress such as that provided by low concentrations of H_2O_2. Several flavonoids activate Nrf2, and, for example, the human cancer prevention associated with a high consumption of green tea has been attributed in part to the capacity of EGCG, a major tea constituent, to activate Nrf2 (reviewed in [78]). Oral (−)-epicatechin administration to mice protects against Aβ25-35-induced hippocampal toxicity [79] and from stroke-associated brain infarcts and neurologic deficits [80]. The latter protective effects were not observed in mice with genetic deficits of Nrf2 and of heme oxygenase-1, a Nrf2-target gene [80]. At nanomolar concentrations, (−)-epicatechin stimulated the Nrf2 signaling pathway in primary cultures of astrocytes and neurons [81]. In the same model, the inhibition of phosphatidylinositol 3-kinase by wortmannin suggested the involvement of this kinase in the activation of Nrf2, but the molecular target of (−)-epicatechin was not identified.

Procyanidins were also shown to inhibit the transformation of the aryl hydrocarbon receptor in vitro and in vivo. Aryl hydrocarbon receptor transformation is involved in the toxic actions of dioxins, a major environmental contaminant. In a cell-free system, B2 and B5 dimers, but not (−)-epicatechin, inhibited dioxin-induced aryl hydrocarbon receptor transformation [82]. A similar inhibitory effect was observed in the liver of mice fed a cacao polyphenol extract and later treated with dioxin. Finally, (−)-epicatechin and procyanidins can also inhibit other signaling pathways: proteomic analysis of the effects of B2 dimer on the formation of lipid-laden macrophages induced by LDL showed that this dimer acts by affecting several signaling pathways [83]. Among them, the B2 dimer inhibited LDL-triggered MAPK p38 activation and up-regulation of both AP-1 mRNA and the antiatherogenic transcription factor PPARγ.

In summary, as indicated for NF-κB, phosphatases, and kinases, other signaling cascades have been shown to be modulated by (−)-epicatechin and procyanidins. In most cases the molecular target(s) of this modulation is not yet identified.

Cell membranes

The interactions with membranes could in part explain the biological actions, including regulation of cell signaling, and the antioxidant effects of large procyanidins that are unlikely to be transported inside cells, i.e., trimers and larger polymers. Flavonoids in general, and procyanidins in particular, interact chemically and physically with artificial and biological membranes [84–86]. In synthetic liposomes, the membrane interactions of various flavonoid families and the relationships of these interactions to their capacity to deactivate hydrophilic and hydrophobic oxidants and to exert membrane protective effects were investigated [87]. Both antioxidant and protective actions were related to the hydrophilicity of the compound, the number of hydroxyl groups, and, for procyanidins, the degree of oligomerization. In intestinal cells in culture, these membrane interactions were associated with the capacity

of larger procyanidins to exert antioxidant, membrane-protective, and anti-inflammatory actions [52,57].

We have proposed that high-molecular-weight procyanidins could have selectivity for interacting with certain zones of the plasma membrane [57]. Procyanidin interactions with lipid rafts, for example, could explain procyanidin capacity to affect cell signaling. Lipid rafts are specialized areas of the cell membrane enriched in sphingolipids and cholesterol that contain a number of proteins that actively participate in cell signaling, including redox cell signaling (e.g., NADPH-oxidase, endothelial nitric oxide synthase (eNOS), heme oxygenase-1, tyrosine kinase receptors, and G-protein-coupled receptors) [88]. By interacting with lipid rafts, flavanols and procyanidins could directly interact with signaling proteins or change the biophysical properties of the lipid bilayer, with consequences for the appropriate signaling functions. Among the limited number of demonstrations of the interactions between flavanols and lipid rafts, EGCG suppressed the expression of the high-affinity IgE receptor FcεRI by interacting with lipid rafts and also by binding to the 67-kDa laminin [89,90]. Consistent with a membrane effect of EGCG, we observed that, in phosphatidylcholine liposomes, EGCG causes lipid ordering at 7 and 18 Å from the membrane surface, whereas (−)-epicatechin has no effect [87]. EGCG, but not (−)-epicatechin, was found to prevent epidermal growth factor (EGF)-mediated activation of the raft-associated EGF receptor in HT29 colon cancer cells [91].

Other evidence that supports a physiologically relevant interaction between procyanidin and lipid rafts is that (−)-epicatechin hexamers prevented a deoxycholate-induced increase in cellular oxidants and the associated alterations in the permeability of Caco-2 cell monolayers [52]. Being that the increase in cell oxidants was at least in part due to the activation of NADPH-oxidase, and considering the location of this enzyme at lipid rafts and the known action of deoxycholate selectively disrupting lipid raft organization, it can be extrapolated that hexameric procyanidins could act at this level. The interactions of hexameric procyanidins with lipid rafts could prevent a deoxycholate-induced mobilization of cholesterol from lipid rafts to other areas of the membrane and the associated alterations in signaling and oxidant balance. In fact, deoxycholate triggers the activation of ERK, p-38, and Akt in various cell types, including intestinal cells, being oxidants in part involved in these events [92,93]. Significantly, preliminary evidence indicates that hexameric procyanidins can prevent deoxycholate-induced ERK, p-38, and Akt phosphorylation (M. da Silva et al., unpublished data). The potential interaction of hexameric procyanidins with lipid rafts is also supported by their capacity to differentially inhibit NF-κB activation depending on the stimuli, which suggests certain selectivity for membrane regions [57].

Overall, current evidence suggests that the interaction of flavonoids with select areas of the membrane could in part explain their capacity to regulate cell signaling and, in particular, oxidant production and redox signaling initiated at lipid rafts. Procyanidins could have selective interactions with lipid raft components, but further research is required to understand the potential interactions of procyanidins with lipid rafts and the biological relevance of these interactions. A relevant conclusion of the above evidence is that membrane effects could be favored by the exposure to higher concentrations of flavanols and procyanidins than intracellular components. This is based on the fact that flavanols and procyanidins in the digestive tract, and monomers in the vascular system, can be present at extracellular concentrations consistent with the proposed membrane-associated mechanisms.

Flavanols and procyanidins: cell signaling and health benefits

Cardiac and vascular

The most significant associations between flavanol- and procyanidin-rich foods and health are those related to cocoa consumption and vascular physiology (reviewed in [94–99]). Pioneered by work by

Waterhouse et al. [100], a number of investigations subsequently demonstrated positive health effects of cocoa and chocolate consumption. Using purified flavanols present in cocoa, i.e., (−)-epicatechin and (+)-catechin, a number of studies in humans and rodents (Table 1) confirm the vascular effects observed using the whole food [20,23,80,101–110]. The bioavailability of NO seems to be a common event underlying the biochemical mechanisms explaining how cocoa and flavanols present in cocoa can affect the vasculature [20,95,111,112]. NO bioavailability to reach target molecules is dependent on its production mediated by several NOS activities and its reaction with superoxide anion [55]. In the following paragraphs we address in what manner in vitro experiments can provide a valid approach to understand how flavanols and procyanidins can affect both NOS activity and superoxide anion production in the vasculature.

The exposure of vascular cells to high-nanomolar/low-micromolar concentrations of flavanols and procyanidins can provide some clues to their effects on vascular function. In vivo levels of flavanols and procyanidins have not been determined in endothelial or smooth muscle cells; however, a membrane effect triggering cell responses is possible at blood (vascular) levels of these compounds (nanomolar/low-micromolar concentrations). In cultured human coronary endothelial cells, the potential existence of a cell-surface acceptor–effector for (−)-epicatechin that could mediate the subsequent activation of eNOS was proposed [113]. The observed eNOS activation was: (i) associated with calcium homeostasis, both independent and dependent on calmodulin-dependent kinase II, and (ii) increased by flavanols and quercetin through the phosphorylation of serine residues. Interestingly, these effects were sensitive to the structure of the flavonoid, i.e., (−)-epicatechin was more effective than (+)-catechin or quercetin. Studies from the same group showed that, at the organ level, (−)-epicatechin limited myocardial infarct size and left ventricular remodeling after a severe myocardial ischemic injury [103]. The mechanism behind this protection was not identified but it was independent of Akt or ERK activation. Recent evidence of the interaction of (−)-epicatechin with membranes is the finding that (−)-epicatechin-mediated cardiac protection was dependent on the stimulation of δ-opioid receptors after an ischemia–reperfusion injury in mice [114]. It is important to mention that cell membranes are central to calcium homeostasis, to the activation of eNOS and NADPH-oxidase, and to the regulation of numerous signaling cascades. In cell cultures, we demonstrated that nanomolar concentrations of (−)-epicatechin, B2 dimer, or C1 trimer regulate calcium homeostasis [115]. The observation that specific metabolites of (−)-epicatechin (3′- and 4′-O-methyl-(−)-epicatechin and (−)-epicatechin glucuronide) can inhibit NADPH-oxidase and consequently diminish superoxide anion production and NO bioavailability [53,54] strongly supports an alternative mechanism for the effects of flavanols on NO-dependent cell signaling and, as a result, on vascular function, inflammation, and hypertension.

Gastrointestinal cancer

Although extensive research has been aimed at unraveling the effects of tea flavanols, i.e., epigallocatechin, epicatechin gallate, and EGCG, on cancer, direct evidence in human populations is scarce. The gastrointestinal tract is a model organ to evaluate the potential anticancer actions of flavanols and procyanidins because: (i) throughout the gastrointestinal tract the ingested parent compounds and their primary metabolites can reach high concentrations, (ii) in the gastrointestinal tract a unique metabolism of these compounds occurs, (iii) high-molecular-weight procyanidins cannot be absorbed, but can exert local effects through their interaction with the plasma membrane of cells in the gastrointestinal tract, and (iv) the risk of cancer in the gastrointestinal tract is related to chronic inflammation and to the diet.

A recent epidemiological study suggests an inverse relationship between the dietary consumption of procyanidins and the risk of colorectal cancer [116]. This potential association is supported by a number of animal studies. In rodent models of ulcerative colitis and colorectal cancer, anti-inflammatory and anticancer properties for food extracts containing procyanidins have been observed [27,117–119]. Epidemiological and in vitro [57] evidence suggests that the oligomers could be more effective than the monomers (epicatechin) as anti-inflammatory/anticarcinogenic molecules in the colon. In fact, the trend for a lower risk of colorectal cancer in humans was not observed for the consumption of monomers, but was found for the procyanidins having 2–10 subunits [120].

Chronic inflammation is a risk for colorectal cancer [121–123], and most inflammation-associated colorectal cancers are characterized by the activation of the transcription factors NF-κB and STAT3, which drives the overactivation of the immune response and the oncogenic process [124]. Procyanidins regulate signals involved in carcinogenesis [27]. Dimeric and hexameric procyanidins inhibit NF-κB in intestinal cells and lymphocytes under proinflammatory conditions [57,64–66]. Both intestinal and immune cells are part of the inflammatory scenario that underlies inflammatory bowel diseases and that constitutes a major risk for colorectal cancer. Dimeric procyanidins B1 and B2, but not A1 and A2, inhibit NF-κB-dependent IL-2 production in Jurkat T cells [64,66]. B2 dimer also inhibits constitutively active NF-κB in Hodgkin lymphoma cells, decreasing cell viability and the production of IL-6, TNFα, and Rantes [65]. Hexameric procyanidins also inhibited TNFα-induced NF-κB activation and associated inducible NOS expression [57]. We recently

Table 1
Cardiac and vascular benefits of (−)-epicatechin and (+)-catechin oral administration in humans and rodents.

Compound	Model/treatment	Effects
(−)-Epicatechin	Healthy men	Increased flow-mediated dilation and peripheral artery tonometry [20]
	Healthy men	Increased plasma S-nitrosothiols and nitrites + reduced plasma endothelin-1 [101]
	Healthy men	Increased brachial artery dilation [23]
	Mice subjected to middle cerebral artery occlusion	Smaller brain infarcts and decreased neurologic deficit scores [80]
	Rats subjected to permanent coronary occlusion	Decreased infarct size [102]
	L-NAME-induced hypertensive rats	Prevented blood pressure increase [103]
	C57BL/6 mice	Increased angiogenesis in hippocampus [104]
(+)-Catechin	Apo E-deficient mice	Decreased progression of atherosclerosis, susceptibility of LDL to oxidation and aggregation [105]
	Apo E-deficient mice	Decreased atherosclerotic lesion, down-regulation of genes related to leukocyte adhesion to endothelium, energy and lipid metabolism, lipid trafficking, etc. [106]
	Apo E-knockout mice	Decreased F2 isoprostanes, superoxide anion, and endothelin-1 in aorta [107]
	OLETF rats	Prevented blood pressure, fasting sugar, and insulinemia increases [108]
	Atherosclerotic mice	Decreased cerebral superoxide staining, restored endothelial function, increased changes in cerebral blood flow during stimulation, and prevented learning decline [109]
	Preatherosclerotic mice	Reduced plaque burden and normalized vascular markers (worsened endothelial dysfunction and increase in leukocyte adhesion when atherosclerosis was established) [110]
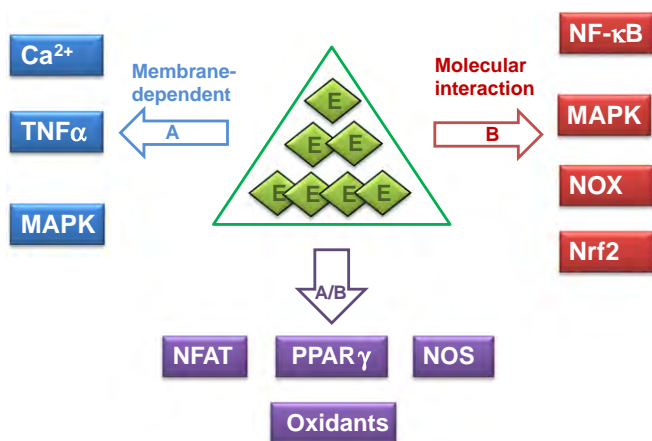
Fig. 6. Schematic summary of the potential actions of (−)-epicatechin and related procyanidins on various signaling pathways.

observed that hexameric procyanidins inhibit bile acid-induced ERK and Akt activation (M. da Silva et al., unpublished). Significantly, ERK regulates proliferation [125], and Akt regulates signals involved in malignant transformation [126,127]. A procyanidin extract (2–13 subunits) isolated from a grape-seed extract induces colorectal cancer cell apoptosis through the down-regulation of antiapoptotic proteins (Bcl-2) and up-regulation of proapoptotic proteins (Bax) [128]. Furthermore, in animal models of chronic inflammation, plant extracts with high procyanidin content, i.e., apples and grapes, have anti-inflammatory and protective actions in models of ulcerative colitis and colorectal cancer [27,117,118]. Nevertheless, the potential benefits of (−)-epicatechin and procyanidins on inflammatory bowel disease and colorectal cancer are backed by both in vitro and experimental animal studies using complex mixtures of these compounds. Further research is necessary to establish the direct relevance of flavanols and procyanidins providing health benefits for the gastrointestinal tract.

Conclusions

Flavanols and procyanidins are molecules ubiquitously present in plants that are consumed by humans and other animals. These compounds were believed to act mostly by providing antioxidant protection by trapping radicals and chelating redox-active metals. However, a significant and increasing body of evidence currently supports the participation of flavanols and procyanidins in the regulation of cell signaling as the means by which these substances affect disease progress (Fig. 6). The mechanisms underlying signaling regulation include the capacity of flavanols and procyanidins to regulate cell oxidant production and antioxidant defenses and hence cell redox state, specific interactions that modulate the activity and biological reactions of cell signaling proteins, and the regulation of membrane-associated cell signaling. The chemical conformation of monomers and the number, bonding, three-dimensional structure, and the type of monomers forming procyanidins have a major impact on the capacity of these molecules to regulate cell signaling. All these actions would be limited by the bioavailability of flavanols at the target tissue, e.g., high-micromolar concentration in the digestive tract, low-micromolar/high-nanomolar concentration in blood, and lower concentrations in other tissues and cells. The protection from cardiac and vascular disease and from cancer that is associated with a high consumption of fruit and vegetables could be in part explained by the capacity of flavanols and related procyanidins to modulate proinflammatory and oncogenic signals.

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