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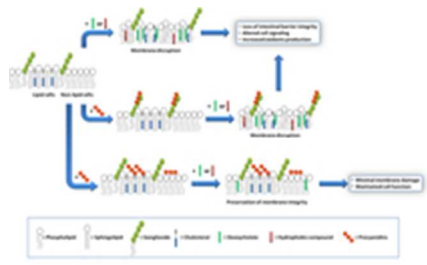


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Procyanidin-lipid interactions decrease the potential of deleterious agents to incorporate in the membrane and disrupt membrane organization and functions.
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Interactions of flavan-3-ols and procyanidins with membranes: mechanisms and physiological relevance.

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ABSTRACT

Flavonoids are a type of phenolic compounds widely present in edible plants. A great number of health benefits has been ascribed to flavonoid consumption in human populations. However, the molecular mechanisms involved in such effects remain to be identified. The flavan-3-ols (-)-epicatechin and (+)-catechin, and their related oligomers (procyanidins) have been thoroughly studied because of their capacity to interact with cell membranes. Starting in these interactions, procyanidins could modulate multiple biochemical processes, such as enzyme activities, receptor-ligand binding, membrane-initiated cell signaling, and molecules transport across membranes. This review focuses on molecular aspects of procyanidin interactions with membrane lipid components, and the resulting protection of the membranes against mechanical and/or oxidative damage, resulting in the maintenance of cell functions.

KEYWORDS. Procyanidin, flavonoid-membrane interactions, lipid rafts, cholesterol, detergents, lipid oxidation

ABBREVIATIONS USED.

AAPH: 2,2'-azobis (2-amidinopropane) hydrochloride; aGM1: asialo ganglioside GM1; AMVN: 2,2'-azobis (2,4-dimethylvaleronitrile); DCA: sodium deoxycholate; DOPC dioleoyl phosphatidylcholine; DPPC: dipalmitoyl phosphatidylcholine; EGCG: epigallocatechin gallate; EGF: epidermal growth factor; MAPK: mitogen-activated protein kinase; PC: phosphatidylcholine; POPC: 1-palmitoyl 2-oleoyl phosphatidylcholine; PS: phosphatidylserine; SM: sphingomyelin

Introduction

Flavonoids are (poly)phenolic compounds synthesized by plants as secondary metabolites. The regular consumption of flavonoid-containing foods and beverages can provide health benefits. In spite of the large amount of research performed up to date, the mechanisms underlying those beneficial effects are not fully understood.

The interaction of flavonoids with membrane components, *i.e.* lipids and proteins may in part account for flavonoids actions on human and animal health. A direct interaction of flavonoids with membrane proteins could affect membrane-associated processes, such as the regulation of enzymes, channels and receptors, and membrane-initiated cell signaling¹. Flavonoids can also bind to lipids and modulate the biophysical properties of membranes, which ultimately could affect the aforementioned membrane-associated processes.

This review will focus on a particular class of flavonoids, the flavan-3-ols and their oligomers, the procyanidins, which are found in large amounts in fruits (*e.g.* berries, apples), cocoa, nuts and beans, and derived foods². Flavan-3-ol and procyanidin daily intake can significantly vary among different populations (*e.g.* 455 and 135 mg/d for men in Spain and Greece, respectively)³. Such variations are explained by, among other factors, nutritional habits, local availability of procyanidin-containing foods, variations within varieties, and seasonal factors. In the United States of America the main dietary sources of procyanidins are apples, cocoa, peanuts, and grapes⁴. Cocoa (*Theobroma cacao*) beans, and peanut (*Arachis hypogea* L.) and grape (*Vitis vinifera*) skins contain almost exclusively (-)-epicatechin-derived procyanidins⁵⁻⁷. The relative content of monomers and procyanidins in cocoa beans was estimated to be 36% of monomers, 17% of dimers and 48% of higher procyanidins (trimers to decamers)⁸. By comparison, peanut skin contains 2.5% monomers, 18% dimers (18%), 34 trimers, and 46% tetramers⁹. As it will be

discussed in the next section, even when cocoa beans and peanut skin procyanidins are built by (-)-epicatechin units, the chemical and three-dimensional structures of these molecules are different in the two plants. These differences may determine distinct interactions with membrane components, and hence different biological effects.

Chemical structure of flavan-3-ols and procyanidins.

Flavonoids are plant secondary metabolites presenting a huge number of chemical structures. Overall, these structures are characterized by the presence of two aromatic rings (A and B) connected by a three-carbon chain forming an oxygenated heterocycle (C ring) (Fig. 1). Based on the substitutions incorporated on this basic skeleton, such as a keto, hydroxyl, methoxyl, or gallate groups, flavonoids are categorized in six families¹⁰. One of them, the flavan-3-ols results from the incorporation of hydroxyl groups in positions 3, 5, 7, 3' and 4' (Fig 1). Regarding the hydroxyl group in position 3, two spatial conformations are possible. The 2,3-*cis* isomer is denominated (-)-epicatechin, whereas the 2,3-*trans* isomer is denominated (+)-catechin (Fig 2). Both monomers can oligomerize through 4 β →8 bonds (e.g. in cocoa beans) or 2 β →O7 bonds (e.g. in peanut skin^{7, 9}) to generate the procyanidins, composed of two or more units¹¹. Procyanidins built up with (-)-epicatechin are more frequently found in nature than those composed of (+)-catechin¹². The biochemical pathway of monomer oligomerization in plants is still not completely elucidated¹³. However, it has been proposed that this process requires the oxidation of flavan-3-ols to their respective quinones, which subsequently link to each other forming the procyanidins^{12, 14}.

Flavan-3-ols and procyanidins are usually represented as planar structures. However, the spatial conformation adopted by these molecules is not planar. In fact, (-)-epicatechin B ring is oriented almost perpendicularly to the rest of the molecule (Fig 2).

Dimer B2 [(-)-epicatechin (4 β →8) (-)-epicatechin] and dimer B1 [(-)-epicatechin (4 β →8) (+)-catechin] adopt similar U-shaped conformations in which the two B rings from the monomeric units are almost stacked (Fig 2). By molecular modeling, we observed that the incorporation of an additional 2 β →O-7 bonding (dimer A2, Fig 2) between the two (-)-epicatechins generates a double-bonded rigid molecule, that does not allow the stacking¹⁵. To notice, the conformation adopted by the dimer A2 [(-)-epicatechin (4 β →8, 2 β →O-7) (-)-epicatechin] is more extended than that of the dimer A1 [(-) epicatechin (4 β →8, 2 β →O-7) (+)-catechin] (Fig 2)¹⁵. This observation implies that not only the additional bonds between the monomers, but also the stereochemistry of the hydroxyl group in position 3 are important in determining the overall shape of the molecule. Larger procyanidins, e.g. cocoa hexamer (Fig 2), adopt extended conformations with (-)-epicatechin monomers arranged in a left-handed helix¹⁴. As mentioned before, differences in procyanidin spatial conformation will lead to differential interactions of these procyanidins with other molecules, such as lipids, proteins, carbohydrates and nucleic acids^{15, 16}.

Depending on the number and characteristics of their substitutions, flavonoids can partition differentially in hydrophilic or lipophilic domains. Among the flavan-3-ols, (+)-catechin and (-)-epicatechin partition almost equally between *n*-octanol and a buffered water solution¹⁷. The incorporation of a hydroxyl group in the 5' of (-)-epicatechin defining the (-)-epigallocatechin, increases the hydrophilicity of the molecule¹⁷. The solubility of procyanidins in water (buffered solutions) increases with the number of monomer units¹⁷. By molecular dynamics modeling of procyanidins hydration, it was evidenced that the monomers, but not the procyanidins, have a tendency to self-associate following hydrophobic interactions¹⁸. In this regard, Pianet *et al.*¹⁸ postulated that “*the higher the degree of polymerization, the greater the number of structural degrees of freedom that*

favor hydration and prevent intermolecular hydrophobic stacking”, thus explaining the greater solubility of higher procyanidins in water.

Flavan-3-ol and procyanidin interactions with model membranes.

A major interest of our research over the last 10 years has been oriented to characterize the interactions of flavan-3-ols and procyanidins with membranes, and to assess the biological/functional relevance of such interactions. Studies were performed using (+)-catechin, (-)-epicatechin and procyanidins purified from cocoa¹⁹ and from peanut skins.⁷

Initial experiments were designed to investigate if the interactions of (-)-epicatechin, (+)-catechin and procyanidins (dimer to hexamer) with membranes could prevent lipid oxidation, given that these compounds have well-recognized antioxidant properties *in vitro*. Given that the antioxidant ability of flavan-3-ols resides mainly in the presence of the catechol moiety in B ring²⁰, we expected that the higher the number of monomer units, the higher would be the antioxidant capacity. Thus, the concentrations of procyanidins were adjusted to achieve equivalent amounts of monomer in the samples. In liposomes composed of phosphatidylcholine (PC) and phosphatidylserine (PS) treated with the generator of lipid-soluble radicals (2,2'-azobis (2,4-dimethylvaleronitrile), AMVN) the antioxidant potential of procyanidins increased linearly with the number of monomer units in the molecule²¹. This size-dependent relationship was not observed when using the generator of water-soluble radicals (2,2'-azobis (2-amidinopropane) hydrochloride, (AAPH)²². Both, AMVN and AAPH are azo-compounds that upon thermal decomposition generate carbon-centered radicals²³ although the site where those radicals are formed is different. Due to its hydrophobicity, AMVN incorporates into lipid bilayers thus generating the radicals within a hydrophobic environment. On the contrary, AAPH is hydrophilic and

generates oxidizing radicals in the water milieu²³. The different procyanidin behavior against these two oxidants suggested that procyanidins may interact with the membrane surface limiting the access of lipid soluble oxidants to the hydrophobic core of the membrane making larger molecules more efficient when preventing membrane oxidation²¹.

Interaction of procyanidins with phospholipids

To substantiate the above-mentioned hypothesis, we next investigated if procyanidins affected membrane physical properties that may limit the accessibility of the oxidants to the core of the lipid bilayer. We observed that procyanidins decreased the surface potential of PC and PS liposomes in a concentration- and chain length-dependent manner²¹. The effects of procyanidins on membrane surface potential depended on the characteristics of the polar headgroup of the phospholipids investigated. Thus, dimer B2 increased liposome surface potential in liposomes composed by only PC¹⁶ and decreased it in liposomes of PC and PS²¹. This difference could be ascribed to both the overall surface charge and the interactions established between the polar headgroup of phospholipids. While at neutral pH PC is a zwitterion, PS is negatively charged. In addition, neighbor PCs establish only weak interactions between their polar headgroups, whereas the coexistence of PC and PS in the membrane not only generates stronger hydrogen bonds between the polar headgroups but also increases lipid packing²⁴. Thus, in membranes containing PC and PS the penetration of dimer B2 into the bilayer may be restricted and confined to the surface. In contrast, the more relaxed surface of PC liposomes may allow the dimer B2 to penetrate deeper into the bilayer, exposing the phosphate groups of PC to the water milieu. Supporting this, dimer A1, which resembles dimer B2 in its spatial conformation, also increased the surface potential of PC liposomes

¹⁶. A different behavior was observed for dimer A2, a molecule that adopts a conformation more extended than those of dimers B2 and A1, displaying a biphasic effect in PC liposomes ¹⁶. Both, trimer C1 [(-)-epicatechin (4 β →8) (-)-epicatechin (4 β →8) (-)-epicatechin] and trimer A [(-)-epicatechin (4 β →8, 2 β →O-7) (-)-epicatechin (4 β →8) (-)-epicatechin], which adopt extended conformations (Fig 2), similarly increased PC liposome surface potential, supporting the hypothesis that their interaction with the membrane occurs at the surface level.

Flavan-3-ols and procyanidins can alternatively modulate membrane fluidity. In liposomes composed of brain PC and containing a fluorescent probe located close to the membrane surface ²⁵, we found that (+)-catechin and (-)-epicatechin have minor effects on membrane fluidity ¹⁷. The effects of (+)-catechin and (-)-epicatechin on membrane fluidity were also dependent on the chemical characteristics of the phospholipids. In liposomes composed of dipalmitoyl phosphatidylcholine (DPPC, a synthetic phospholipid with two saturated acyl chains), (+) and (-)-catechin as well as (+) and (-)-epicatechin promoted membrane rigidification, although the magnitude of that effect was different for each flavan-3-ol ²⁶. (-)-Epicatechin was the most effective in promoting membrane rigidification not only at the membrane lipid-water interface but also at the hydrophobic core ²⁶. On this basis, it was proposed that these geometrical esteroisomers have different lipophilicity, being (-)-epicatechin the most lipophilic of the series, and thus able to reach the deeper sections of the bilayer ²⁶. In line with this, the hydrophilic flavonoid EGCG mainly interacts with the membrane surface and promotes its rigidification, with almost no effect on the hydrophobic core of the bilayer ²⁷. By molecular dynamics simulations, Sirk *et al* ²⁸ showed that (-)-epicatechin could adsorb onto 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) bilayers to a greater extent than EGCG. However, once adsorbed, both flavonoids induced

a lateral expansion of the bilayer below the phosphate group of the phospholipid, compressing their immediately adjacent lipids²⁸. This lateral compression of the membrane surface may account for the rigidifying effect of (-)-epicatechin and EGCG both in dioleoyl phosphatidylcholine (DOPC) and DPPC liposomes (two synthetic phospholipids). The extrapolation of these results to biological membranes should consider that the latter contain a complex mixture of phospholipid species, with different acyl chain lengths and saturation degrees. Consequently, the individual molecules of natural phospholipids will pack differently than in membranes composed of a single PC species. Thus, measurements would represent the average effect of flavonoids on the different phospholipid species, and underscore the need of cautiousness when extrapolating the results obtained in artificial models to physiological conditions.

In contrast to flavan-3-ols monomers, which have minor effects on liposome fluidity, dimer B2 and hexamers markedly decreased the fluidity of the membranes¹⁷. Supporting the notion that procyanidin adsorption would underlie their effects on membrane fluidity, the effect of the procyanidins on membrane surface potential correlated with their ability to rigidify the membrane¹⁷. Dimers A1 and A2, and trimers A and C1 also decreased the fluidity of the bilayer, mainly by establishing contacts with the surface of the membrane¹⁶. To notice, even when the interactions of procyanidins with membranes occurred at the surface, their rigidifying effect propagated into the bilayer and affected the acyl chains of phospholipids¹⁷. Interestingly, the magnitude of the rigidifying effect of the dimers in this region followed the order B2 < A1 << A2 which seems to be associated with the type of folding of their chemical structures. In addition, positive and significant correlations related the effects of the dimers on membrane fluidity at the surface and deepest region of the bilayer. By analyzing the slope of those correlations, it can be suggested that the folded dimers affect mainly the surface of the membrane and to a lesser extent, the hydrophobic

core of the membrane ¹⁶. In the case of dimer A2 that is more extended and thus potentially able to interact with more phospholipids than the other dimers, the rigidifying effect propagated to a similar extent from the surface down to the hydrophobic core of the bilayer ¹⁶. The comparison of the effects of trimers A and C1 on membrane fluidity shows no significant differences between them, neither at the most superficial nor in the deeper region of the bilayer, thus suggesting that the additional bonding in trimer A has no major impact on the overall interaction of this procyanidin with membrane lipids ¹⁶. Supporting this, Yu *et al* demonstrated by solid state ²H- and ³¹P-NMR that trimer C2 [(+)-catechin (4 β →8) (+)-catechin (4 β →8) (+)-catechin] which also adopts an extended conformation in solution ²⁹, caused minimal alterations in membrane fluidity, mostly at the surface level ³⁰. Therefore, it is important to consider the spatial conformation of the procyanidins, and not just their chemical structure and/or the number of monomer units in their structure, to understand the consequences of procyanidins interaction with biological membranes.

We next hypothesized that the interaction of procyanidins with membranes might affect the incorporation of hydrophobic compounds into the bilayer. To assess this possibility, we evaluated if (-)-epicatechin, (+)-catechin, and a series of procyanidins (dimer to hexamer) were capable to prevent the disruption of the bilayer by Triton X-100. This detergent incorporates into the bilayer and causes its disruption in a progressive and controlled manner. The progression of this process can be followed from the changes in the fluorescence of a probe incorporated in the bilayer ³¹. This method provides a quantitative value (C_{50}) which is inversely related to the susceptibility of the membrane to be disrupted ²¹. The pre-incubation of liposomes composed of PC and PS with the procyanidins made liposomes more resistant to disruption ²¹. Experiments were performed using equivalent concentrations of monomers for all the procyanidins assessed. Despite of this, the number of monomers in the procyanidins still correlated with the increase in C_{50}

values ²¹. The comparison of the protective effects of dimers and trimers containing only 4 β →8 bonds versus those having both 4 β →8 and 2 β →O-7 bonds indicates that the more extended the three-dimensional structure, the higher the protection against detergent-mediated membrane disruption ¹⁶.

The above results show that the capacity of procyanidins to maintain membrane integrity when exposed to a detergent is determined not only by the number of monomers in their structure but also their three-dimensional configuration ^{21, 22}. These protective effects of procyanidins against membrane disorganization positively correlates with their capacity to scavenge free radicals ²¹. This suggest that the capacity of large procyanidins to limit the accessibility to the membrane of hydrophobic oxidizing compounds defines an indirect antioxidant effect that would make flavan-3-ols and procyanidins physiological protectors of the gastrointestinal tract ¹⁰.

Interaction of procyanidins with glycolipids

Similar to that discussed before, the capacity of procyanidins to protect membranes from detergent-mediated disorganization relies on the nature of the polar headgroup of membrane lipids. Galactolipids are a family of lipids having a galactose moiety as polar headgroup (Fig. 3). Galactose could establish hydrogen bonding with the procyanidins, thus maintaining the procyanidins close to the surface of the membrane. The protective capacity of hexameric procyanidins against membrane disruption was markedly higher in galactolipids-containing liposomes than in those containing PC and PS ²¹. While the presence of a single galactose in galactolipids favors the interaction of procyanidins with the membrane, lipids containing higher number of sugar moieties impair this interaction. This is the case of complex sphingolipids, e.g. gangliosides, which bear large polar headgroups with four or more sugar moieties. We recently demonstrated such impairment

using liposomes containing the asialoganglioside GM₁ (aGM₁, Fig. 3). This ganglioside bears four carbohydrate moieties in its polar headgroup (two galactose molecules, one glucose and one N-acetyl galactosamine) and is concentrated in certain membrane domains, *i.e.* the lipid rafts³². The presence of aGM₁ turned liposomes more susceptible to disruption by Triton X-100 or sodium deoxycholate (DCA)³³, two amphiphilic but chemically different compounds. Therefore, the protective effects of procyanidins on membranes depend both on the strength and the location of procyanidins interaction with membrane components rather than on the chemical structure of the stressor. The bulky headgroup of aGM₁ protrudes several Å from the membrane surface³⁴, which might keep procyanidins away from the membrane surface. Conversely, the small headgroup of galactolipids may put procyanidins in close contact with the surface of the membrane allowing them to exert their protective effects. Thus, the relative amount of the different kind of lipids in biological membranes will determine not only the strength of procyanidin contacts with the membrane but also their location, and ultimately their potential biological actions (Fig.4).

Interaction of procyanidins with cholesterol

Cholesterol is a key component of animal cell plasma membrane, and among several other functions, it plays a role in the arrangement of membrane lipids^{35, 36}. Cholesterol is particularly abundant in specialized lipid domains of the plasma membrane, the so-called lipid-rafts³⁷, where it interacts preferentially with sphingomyelin (SM, Fig 3) rather than with the phospholipids³⁸. The cholesterol-SM complex adopts a cylindrical, bilayer-forming shape that facilitate lipid raft organization³⁷. In the absence of cholesterol, hexamers bound to liposomes composed of PC and SM exert a mild protective effect against membrane disruption by sodium DCA or Triton X-100³³. The magnitude of this effect was

similar to that previously found in PC liposomes¹⁷. This was not surprising given that, in spite of their different hydrophobic domains, PC and SM share a common polar headgroup, a phosphorylcholine moiety (Fig 3). For that reason, the nature and magnitude of the interactions between the hexamer and the membrane were similar for both types of liposomes. The presence of cholesterol in liposomes was central to procyanidin-membrane interactions. As observed in liposomes containing equimolar amounts of PC, SM, and cholesterol³⁹, the capacity of the hexamer to prevent bilayer disruption by detergents was markedly higher respect to membranes lacking cholesterol³³. Again, the overall protective effect of the hexamer was independent of the detergent used. As we will discuss in the next section, the interaction of the procyanidins with lipid raft components is highly relevant to its biological actions given that lipid rafts concentrate multiple molecules involved in cell signaling¹.

Together, the experimental data obtained in artificial membranes provide mechanistic insights regarding the strength and location of procyanidin binding to membranes, which clearly depends on the characteristics of lipids polar headgroup. Some lipids will favor closer interactions between procyanidins and membranes, shielding them from the aggression by external agents. Others will keep procyanidins far from the surface, decreasing their capacity to protect membranes. Thus, the potential beneficial effects of procyanidins in cells, which have a complex mixture of lipids arranged in dynamic domains, might be higher in those membrane regions containing lipids that favor procyanidins contact with the membrane surface, and minimal in those containing lipids that impede procyanidins contact with the membrane (Fig 4).

Interactions of flavan-3-ols and procyanidins with cell membranes.

A major logic limitation to the effects of procyanidins on biological membranes is their presence at the appropriate place and in the appropriate amounts. The concentration of procyanidins in the post-prandial gastrointestinal tract is relatively high^{6, 40}. Large procyanidins incorporated through the diet are poorly absorbed by the enterocytes, whereas the monomers, rather than the dimers, can reach the bloodstream and other organs^{41, 42}. For this reason, procyanidins with more than three monomeric units remain in the gastrointestinal tract where they can be found as parent compound, with the capacity to interact with the apical membrane of gastrointestinal epithelial cells^{43, 44}.

Interaction of procyanidins with the plasma membrane

Based on our data showing that procyanidins interact with lipids and help to maintain the integrity of synthetic membranes, we extended the investigation to determine whether a similar protective effect may take place in biological membranes. We used a model of intestinal epithelial cells, the human colorectal adenocarcinoma (Caco-2) cells⁴⁵. These cells have the particularity to spontaneously differentiate into polarized, confluent monolayers with functional brush borders and tight cellular junctions⁴⁶. Differentiated Caco-2 cells constitute a valuable and extensively used model for the study of intestinal nutrient and drug transport and metabolism^{45, 47}. In differentiated Caco-2 cells, procyanidins interact with the plasma membrane and these interactions depend on the procyanidin degree of polymerization⁴⁸. Hexameric procyanidin adsorption occurs within 30 min of cell exposure and the interaction is stable. The later is supported by findings that after removal of the hexamer from the incubation medium, the protective actions of the procyanidin are still observed⁴⁸. Considering this, the average orocecal transit times, and the fact that procyanidins are found along the gastrointestinal tract within 1 and 12 h after

consumption⁴⁰, it is possible to speculate that a regular ingestion of procyanidin-containing foods, could provide sustained health effects.

Under physiological conditions, intestinal cells interact with both endogenous and exogenous compounds that potentially might damage their plasma membrane. For example, intestinal cells are exposed to bile salts, a group of endogenous amphiphilic molecules responsible for diet fat emulsification and absorption. One of those compounds is DCA, a secondary bile acid that results from the metabolism of chenodeoxycholic acid by intestinal bacteria. DCA is an amphiphilic molecule that spontaneously intercalates between lipids and potentially damages the integrity of the cell plasma membrane. High chronic concentrations of DCA are associated with an increased risk for colorectal cancer⁴⁹.

In intestinal cell cultures, DCA caused cell death (necrotic and apoptotic) that was prevented by procyanidins^{48, 50, 51}. Importantly, procyanidins adsorb to Caco-2 cell monolayers without affecting paracellular or transcellular transport⁴⁸. The magnitude of procyanidin protective effects against DCA-induced cell damage increases with the number of monomer units forming the procyanidin⁴⁸. These findings are in line with our previous results in liposomes, stressing the relevance of procyanidin-membrane interactions in the protection of the intestinal barrier against mechanical damage.

Bile acids promote an increase in paracellular transport by compromising the integrity of the tight junctions⁵². DCA induced the redistribution of the protein ZO-1⁵³ from the tight junctions towards the cytosol, which was associated with an increase in paracellular transport⁴⁸. Both effects were prevented when cells were treated with the hexamer prior to the exposure to DCA⁴⁸. The maintenance of the integrity of the apical plasma membrane of intestinal cells, as well as the preservation of cell tight junctions, is critical for the correct functioning of the epithelial barrier. It has been reported that both in Caco-2 cells and

normal human intestinal cells, tight junctions determine two kind of hydrophilic pores, the smaller ones with an estimated radii of 5-6 Å and the larger ones with a radii >10 Å⁵⁴. Thus, tight junction pores limit the size of the molecules that can cross the epithelium, and only small water-soluble molecules are allowed to permeate through them^{55, 56}. In the case of tight junction pores being altered and paracellular transport being increased, not only external large, toxic macromolecules but also potentially pathogenic microorganisms may penetrate the intestinal epithelium, initiate a cycle of local inflammation and/or reach the bloodstream and trigger inflammation and damage in other tissues^{57, 58}.

Another undesired effect of bile acids on intestinal cells is the promotion of intracellular oxidant generation. For example, the treatment of isolated crypt epithelium with DCA or of Caco-2 cells with cholic acid stimulated the production of oxidants^{52, 59}. The effect of DCA in promoting oxidant generation in Caco-2 cells was in part due to the activation of the enzyme NADPH oxidase^{48, 52}, which is one of the main sources of cellular superoxide anion. We found that DCA-mediated generation of oxidants could be prevented by the hexamer in a concentration-dependent manner⁴⁸. Accordingly, the hexamer also inhibited the NADPH-dependent increase in oxidants triggered by the pro-inflammatory cytokine tumor necrosis alpha (TNF α)⁶⁰.

The higher oxidant production contributes to DCA-mediated increase of Caco-2 cell barrier permeability⁴⁸. In humans and in experimental animals the permeabilization of the intestinal barrier can lead to disease. In this regard, the loss of intestinal barrier integrity constitutes a major pathological event proposed to underlie the development and/or progression of several human diseases including inflammatory bowel diseases (e.g. ulcerative colitis and Crohn's disease)⁶¹⁻⁶⁴, alcoholic liver disease⁶⁵, obesity-triggered cardiovascular disease⁶⁶, insulin resistance and type 2 diabetes⁶⁷, coeliac disease⁶⁸ and food allergies in general⁶⁹, among others. Therefore, being procyanidins able to protect

intestinal cells from the deleterious effects of select damaging compounds (e.g. DCA, $\text{TNF}\alpha$)⁶⁰, the regular consumption of procyanidin-rich foods may alleviate the symptoms of those intestinal pathologies where the integrity of the intestinal barrier is compromised. Very importantly, given the relevance of the microbiota on intestinal permeability and endotoxin production, the potential effects of procyanidins on intestinal microbiota can also be a major aspect of the beneficial health effects of procyanidins on gastrointestinal health.

Caco-2 cells and the colonic mucosa express high levels of the isoform NADPH oxidase-1, a superoxide anion generating enzyme which activation has been related to colonic inflammation and oncogenesis⁷⁰. This enzyme is located at the plasma membrane and co-localizes with caveolin, which is a characteristic lipid-raft protein^{70, 71}. The mechanism of DCA-mediated NADPH oxidase activation is still under elucidation. Mello-Vieira *et al* recently demonstrated that DCA incorporates into lipid rafts and alters their dynamics⁵¹, an effect that explains the activation of certain lipid raft-associated enzymes observed in DCA-treated cells. In addition, it was demonstrated that the exposure of HCT116 cells to DCA causes the activation of the receptor of the epidermal growth factor (EGF) even in the absence of its ligand⁷². EGF receptor resides in lipid rafts, and its activation leads to the downstream activation of the mitogen-activated protein kinases (MAPKs) ERK1/2, p38 and JNK all participants in bile salts-induced oncogenesis^{73, 74} and apoptosis⁷⁵. In addition, activation of these signaling cascades promote transcription of oncogenes and cell proliferation, and are found to be activated in colorectal cancer⁷⁶⁻⁷⁸. We observed that Caco-2 cell treatment with DCA markedly activated p38, ERK1/2 and Akt (PKB) with an associated increase in intracellular calcium⁵⁰. All these events were prevented by the hexamer in a concentration-dependent manner⁵⁰. Supporting potential anticancer actions of procyanidins the regular consumption of food rich in these compounds has been associated with decreased risks for colorectal cancer⁷⁹.

Interactions of procyanidins with lipid rafts

We next investigated if specific interactions of the hexamer with lipid rafts were involved in the protective effects of this procyanidin. First, we evaluated the impact of the hexamer on Caco-2 cell membrane fluidity as a parameter of procyanidin interaction with the membrane. The hexamer decreased the fluidity of cell apical portion of the plasma membrane, but the effect was restricted to the most superficial level of the membrane, close to the lipid polar headgroups³³. The removal of cholesterol from the membrane with methyl- β -cyclodextrin⁸⁰ prevented the rigidifying effect of the hexamer, and provided additional evidence of cholesterol participation in hexamer binding to membranes³³. Subsequent cell treatment with DCA had a minor effect on hexamer-mediated membrane rigidification. To corroborate the involvement of cholesterol in hexamer-Caco-2 cell membrane interactions, cholesterol distribution in the plasma membrane was evaluated. Cholesterol showed a characteristic punctuated distribution in the plasma membrane that indicates its presence in discrete domains (lipid rafts). These domains were not affected by the hexamer neither in size nor in the pattern of membrane distribution³³. The diffuse localization of cholesterol in DCA-treated cells indicates an alteration in cholesterol distribution, similar to the observed for methyl- β -cyclodextrin³³. Interestingly, when cells were first incubated with the hexamer and subsequently exposed to DCA, the pattern of cholesterol distribution was preserved³³ suggesting that DCA could not reach the same regions of the membrane when the hexamer was present. Similarly, cell pre-incubation with the hexamer prevented cholesterol removal by methyl- β -cyclodextrin. Resembling findings in liposomes, these results suggest that the hexamer interacts with cholesterol and prevents its redistribution and/or removal by external agents. Furthermore, and

provided that the punctuated labeling of cholesterol showed lipid raft distribution, the capacity of the hexamer to prevent its redistribution by DCA indicates that this procyanidin interacts with lipid raft components, effect that explains its ability to prevent the activation of lipid raft-associated cell signals^{50, 60} (Fig 4).

In summary, we obtained evidence indicating that the hexamer, as a model of large procyanidins, can: (a) prevent DCA-mediated deleterious effects in Caco-2 cells, most of them occurring at the lipid raft environment^{48, 50}; (b) interact with membrane lipids and protects them preserving the integrity of the membrane upon DCA treatment^{21, 33}; and (c) interact preferentially with zones of the membranes containing cholesterol, a key component of lipid rafts³³. It is important to stress that the membrane-related effects of procyanidins discussed in this section can be physiologically relevant. The effective concentrations of the hexamer are in the low micromolar range, e.g. 10 μ M, which are concentrations expected in the gastrointestinal tract upon the consumption of procyanidin-rich foods or beverages⁸¹.

Concluding remarks.

Ever since Renaud and Lorgeil suggested red wine consumption as an explanation for the “French paradox”⁸², the incorporation of flavonoid-rich foods and beverages rich into the diet, as well as pharmacological supplementation with these compounds, has been associated with improvements in health. However, there is still limited information on the molecular mechanisms behind the impact of flavonoids on health. This also implies the need to identify the actual compounds acting on the biological system. The complete elucidation of how and where flavonoids may act is an enormous challenge given that they include hundreds of individual molecules that, once ingested and metabolized, render metabolites with still not fully known biological activities.

Over the last decade, our group was interested in elucidating the mechanisms by which flavan-3-ols and procyanidins may act. Monomeric flavan-3-ols and, to a lesser extent, their dimers, can enter cells and exert systemic biological effects. On the other hand, being larger molecules, the procyanidins cannot be absorbed and their presence is mostly restricted to the gastrointestinal tract^{6, 40}. Therefore, effects of procyanidins occur principally in the gastrointestinal lumen in part via their interaction with the exofacial side of enterocyte apical membrane. We have presented extensive evidence demonstrating that procyanidins have a key role in stabilizing membranes, preventing their disruption by chemical and biological agents, and regulating membrane-associated events. In this regard, procyanidins mitigate oxidative stress, the activation of proinflammatory and oncogenic signals, and the permeabilization of the intestinal epithelial barrier. Procyanidins may, therefore, help preserve the function and integrity of the intestinal epithelium, and thereby impact favorably on gastrointestinal health.

In summary the chemical and physical interactions of flavan-3-ols and procyanidins with cell membranes provide explanations consistent with their molecular structure, and tissue presence, based on experimental evidence obtained using synthetic membranes, cell cultures, and animal models. Future studies should be addressed to corroborate the involvement of the proposed mechanisms in the ability of flavan-3-ols and procyanidins to prevent/ameliorate diseases that directly or indirectly involve a loss of intestinal barrier integrity. This prospective research should consider that intact flavan-3-ols and procyanidins could exert other beneficial effects at the gastrointestinal tract such as inhibition of digestive enzymes, changing the microbiota population, and/or direct antioxidant actions. Furthermore, flavan-3-ols and procyanidins can be metabolized by the microbiota to smaller phenolic compounds, which increases the number of molecules potentially responsible for their beneficial effects on health.

ACKNOWLEDGEMENTS.

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LEGEND TO FIGURES.

Figure 1. Structure of flavonoids and flavan-3-ols.

Basic structure of flavonoids showing the A, B and C rings. Numbers define the substitution positions. Hydroxyl groups in positions 3, 5, 7, 3' and 4' define the flavan-3-ol group.

Figure 2. Planar chemical structure and three-dimensional structures of flavan-3-ol and related procyanidins.

Planar chemical and three-dimensional structures of the flavan-3-ol and procyanidins: (-)-epicatechin; (+)-catechin; dimer B2 [(-)-epicatechin-(4 β →8) (-)-epicatechin]; dimer B1 [(-)-epicatechin-(4 β →8) (+)-catechin]; dimer A2 [(-)-epicatechin (4 β →8, 2 β →O-7) (-)-epicatechin]; dimer A1 [(-)-epicatechin (4 β →8, 2 β →O-7) (+)-catechin]; trimer C1 [(-)-epicatechin-(4 β →8) (-)-epicatechin-(4 β →8) (-)-epicatechin]; trimer A [(-)-epicatechin (4 β →8, 2 β →O-7) (-)-epicatechin (4 β →8) (-)-epicatechin]; and hexamer procyanidin [(-)-epicatechin-(4 β →8) [(-)-epicatechin-(4 β →8)]₄ (-)-epicatechin]. Three-dimensional structures were modeled using PRODRG⁸³.

Figure 3. Chemical structure of the lipids discussed in this work.

PC: phosphatidylcholine; PS: phosphatidylserine; SM: sphingomyelin; GL: galactolipid; aGM₁: asialo ganglioside GM₁; Chol: cholesterol.

Figure 4. Schematic representation of procyanidin-cell membrane interactions, and their protective effects against membrane-damaging agents.

The beneficial effects of procyanidins at the intestinal epithelium can be explained by their capacity to modulate the deleterious actions of certain luminal hydrophobic compounds (e.g. DCA). These agents can incorporate into the cell plasma membrane, cause alterations in the bilayer physical properties, and subsequently affect membrane-associated processes. The capacity of procyanidins to protect membrane structure and function from those agents depends, in part, on their interaction with membrane lipids and on the location of these lipids in the bilayer. When bound to lipids with bulky polar headgroups (e.g. gangliosides), procyanidins remain far from the surface, which limits their capacity to exert protective actions. On the contrary, the interaction of procyanidins with cholesterol, abundant at lipid rafts, position procyanidins at the membrane surface. Consequently, procyanidin-cholesterol interactions decrease the potentiality of hydrophobic agents to incorporate in the membrane and trigger the loss of membrane functions.

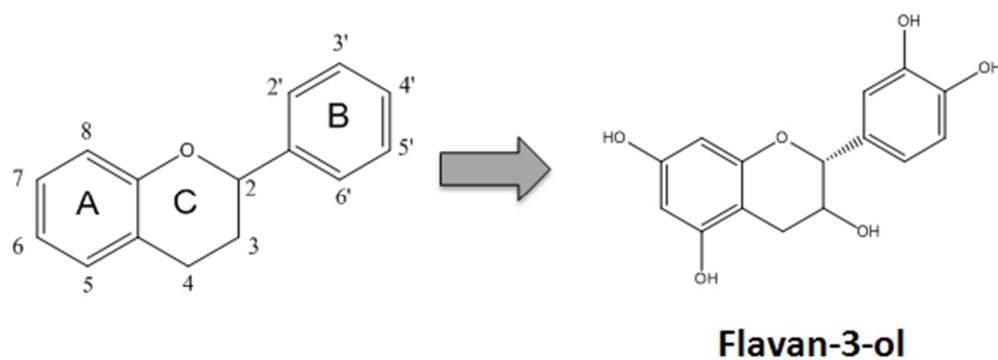


Figure 1. Structure of flavonoids and flavan-3-ols.
Basic structure of flavonoids showing the A, B and C rings. Numbers define the substitution positions.
Hydroxyl groups in positions 3, 5, 7, 3' and 4' define the flavan-3-ol group.
49x18mm (300 x 300 DPI)

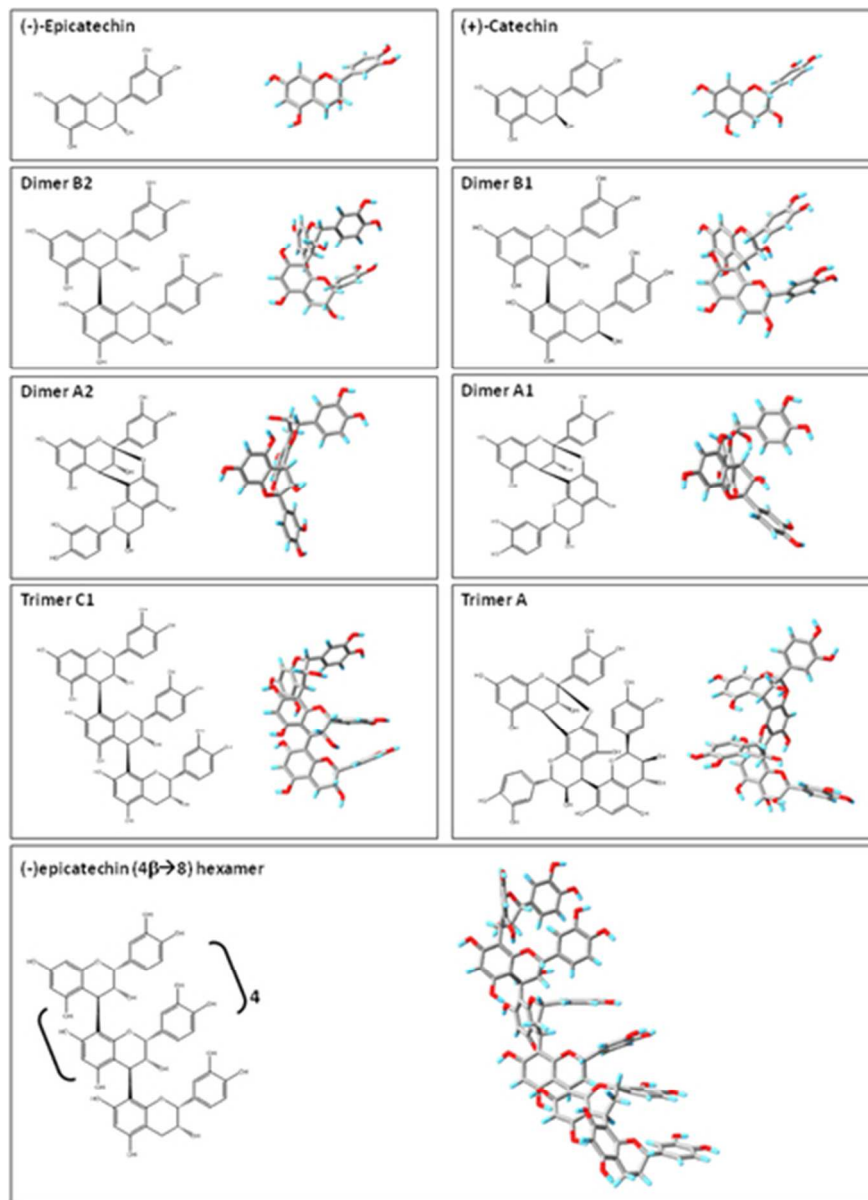


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38x52mm (300 x 300 DPI)

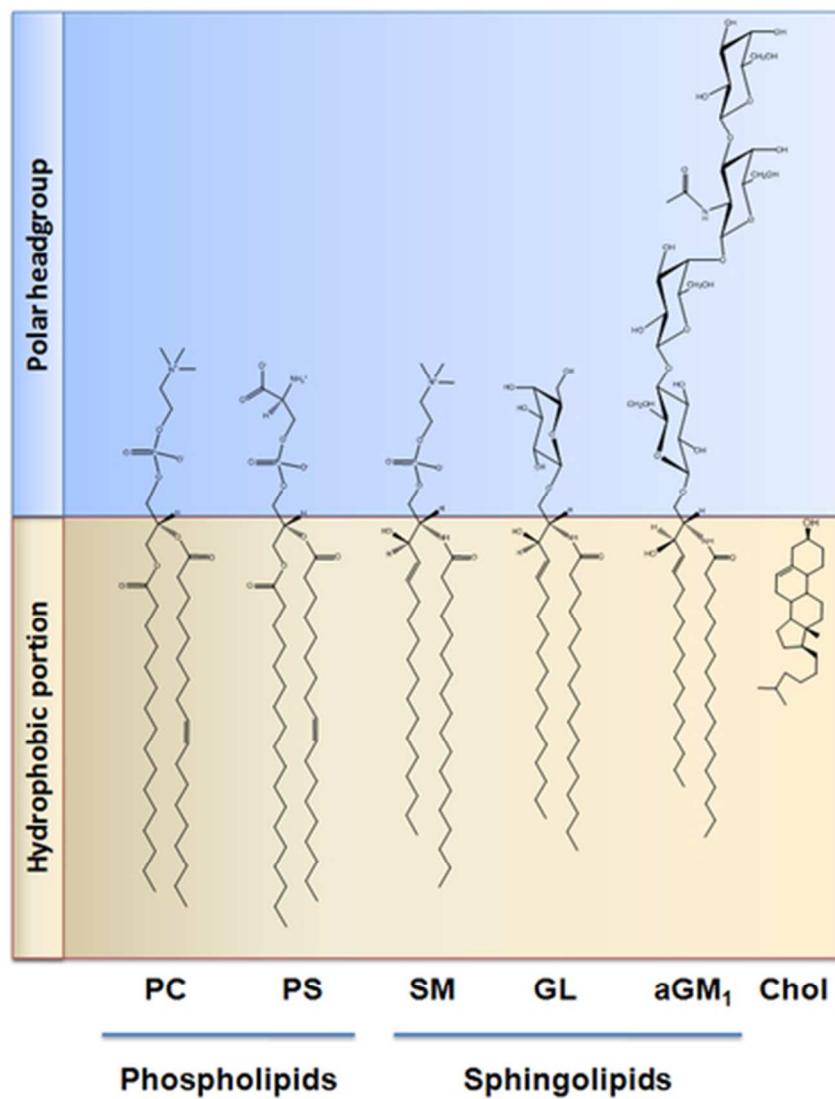


Figure 3. Chemical structure of the lipids discussed in this work. PC: phosphatidylcholine; PS: phosphatidylserine; SM: sphingomyelin; GL: galactolipid; aGM₁: asialo ganglioside GM₁; Chol: cholesterol.

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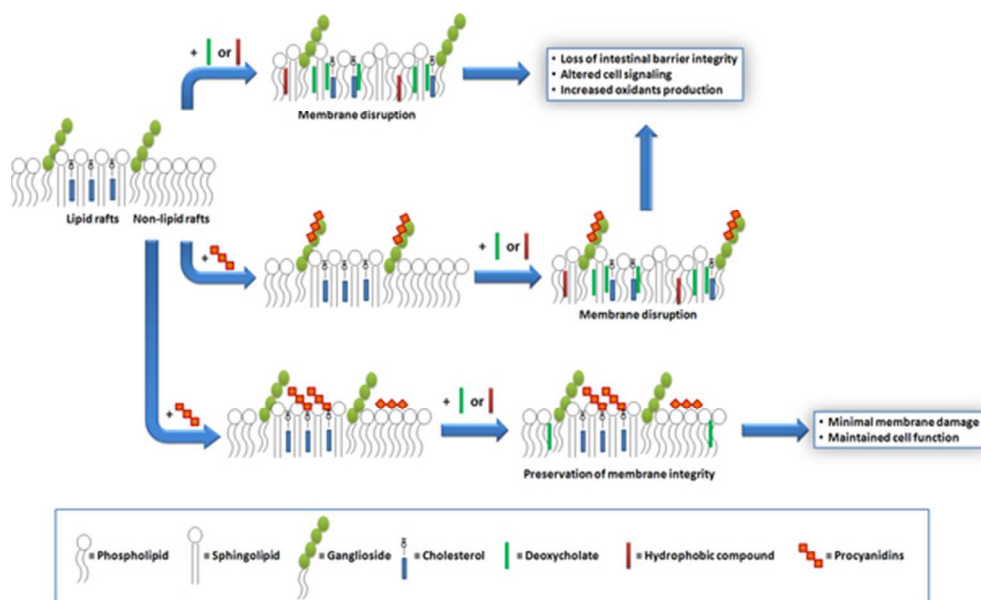


Figure 4. Schematic representation of procyanidin-cell membrane interactions, and their protective effects against membrane-damaging agents.

The beneficial effects of procyanidins at the intestinal epithelium can be explained by their capacity to modulate the deleterious actions of certain luminal hydrophobic compounds (e.g. DCA). These agents can incorporate into the cell plasma membrane, cause alterations in the bilayer physical properties, and subsequently affect membrane-associated processes. The capacity of procyanidins to protect membrane structure and function from those agents depends, in part, on their interaction with membrane lipids and on the location of these lipids in the bilayer. When bound to lipids with bulky polar headgroups (e.g. gangliosides), procyanidins remain far from the surface, which limits their capacity to exert protective actions. On the contrary, the interaction of procyanidins with cholesterol, abundant at lipid rafts, position procyanidins at the membrane surface. Consequently, procyanidin-cholesterol interactions decrease the potentiality of hydrophobic agents to incorporate in the membrane and trigger the loss of membrane functions.

52x30mm (300 x 300 DPI)