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# Procyanidins can interact with Caco-2 cell membrane lipid rafts: Involvement of cholesterol

Sandra V. Verstraeten [a], Grayson K. Jaggers [b,c], Cesar G. Fraga [b,d], Patricia I. Oteiza [b,c,*]

[a] Department of Biological Chemistry and IQUIFIB (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina
[b] Department of Nutrition, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA
[c] Department of Environmental Toxicology, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA
[d] Department of Physical Chemistry-PRALIB (UBA-CONICET), School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

Large procyanidins (more than three subunits) are not absorbed at the gastrointestinal tract but could exert local effects through their interactions with membranes. We previously showed that hexameric procyanidins (Hex), although not entering cells, interact with membranes modulating cell signaling and fate. This paper investigated if Hex, as an example of large procyanidins, can selectively interact with lipid rafts which could in part explain its biological actions. This mechanism was studied in both synthetic membranes (liposomes) and Caco-2 cells. Hex promoted Caco-2 cell membrane rigidification and dehydration, effects that were abolished upon cholesterol depletion with methyl-β-cyclodextrin (MCD). Hex prevented lipid raft structure disruption induced by cholesterol depletion/redistribution by MCD or sodium deoxycholate. Supporting the involvement of cholesterol–Hex bonding in Hex interaction with lipid rafts, the absence of cholesterol markedly decreased the capacity of Hex to prevent deoxycholate- and Triton X-100-mediated disruption of lipid raft-like liposomes. Stressing the functional relevance of this interaction, Hex mitigated lipid raft-associated activation of the extracellular signal-regulated kinases (ERK) 1/2. Results support the capacity of a large procyanidin (Hex) to interact with membrane lipid rafts mainly through Hex–cholesterol bondings. Procyanidin–lipid raft interactions can in part explain the capacity of large procyanidins to modulate cell physiology.



## 1. Introduction

Procyanidins are oligomers of flavan-3-ols which are present in significant amounts in different fruits and vegetables. Regular dietary consumption of procyanidins has been inversely associated with the risk of colorectal cancer [1]. Given their size and polarity, large procyanidins (with three and more catechin/epicatechin subunits) are unlikely to be absorbed at the intestinal epithelium. Being partially metabolized by the gut microbiota to phenolic compounds [2], unmetabolized procyanidins are found throughout the gastrointestinal tract up to the colon [3,4]. Accordingly, the biological actions of intact large procyanidins would be limited to the gastrointestinal tract.

Although not transported inside intestinal cells, large procyanidins can exert certain biological actions through their interactions with the cell plasma membrane [5]. We previously demonstrated that cocoa procyanidins, which are composed by the flavan-3-ols (−)-epicatechin and catechin linked by 4β → 8 bonds, interact with synthetic bilayers altering their physical properties [6]. These interactions depend on the size of the molecule, with larger procyanidins showing stronger interactions. In this regard, the capacity of procyanidins to modify liposome membrane potential correlates with their degree of polymerization [6]. Large procyanidins also regulate important processes in an in vitro model of intestinal epithelium. In this regard, hexameric procyanidins (Hex) inhibited deoxycholate (DCA)-induced permeabilization of Caco-2 cell monolayers, mitigating the underlying deregulation of calcium transport, the activation of NADPH oxidase, and the associated increase in oxidant production [7,8]. Furthermore, Hex attenuates DCA-induced activation of protein kinase B (Akt), mitogen activated kinases, extracellular signal-regulated kinases (ERK) and p38 [7]. Hex also inhibits tumor necrosis alpha (TNFα)-induced increase in oxidant production and the activation of transcription factor NF-κB in Caco-2

*Abbreviations:* 16-AP, 16-(9-anthroyloxy) palmitic acid; 6-AS, 6-(9-anthroyloxy) stearic acid; aGM1, asialoganglioside GM1; BSA, bovine serum albumin; CTX-FITC, FITC-conjugated subunit B of cholera toxin; DCA, sodium deoxycholate; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinases; Spm, sphingomyelin; GP, generalized polarization; Laurdan, 6-dodecanoyl-2-dimethyl aminonaphthalene; MCD, methyl-β-cyclodextrin; PBS, phosphate buffer saline; MEM, minimum essential medium; PC, phosphatidylcholine; PE, phosphatidylethanolamine; R18, octadecyl rhodamine; TEER, transepithelial electrical resistance; TNFα, tumor necrosis alpha

* Corresponding author at: Department of Environmental Toxicology, University of California, Davis. One Shields Avenue, Davis, CA 95616, USA. Tel.: +1 530 754 6074; fax: +1 530 752 8966.
*E-mail address:* poteiza@ucdavis.edu (P.I. Oteiza).

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intestinal cells [9]. Interestingly, several of the molecular targets and events regulated by Hex are associated with lipid rafts (e.g. NADPH oxidase [10], TNFα receptor [11]). This suggests that rather than non-specific interactions, Hex could interact with certain areas of the cell membrane, like lipid rafts, and exert local and specific effects.

Lipid rafts are specialized areas of the plasma membrane characterized by a high content of cholesterol, glycosphingolipids, and sphingomyelin tightly packed in liquid-ordered state. These particular lipid domains are more rigid than the remaining areas of the membrane which are organized in the typical liquid disordered state [12,13]. Lipid rafts recruit specific proteins involved in cell signaling [14], transport, regulation of cell survival [15], preservation of the intestinal barrier integrity [16] and other major cellular events [17].

It has been recently shown that lipid raft disturbance in mouse and human intestinal epithelium precedes the disruption of the epithelial barrier during inflammatory processes [16]. Thus, the interaction of highly polymerized procyanidins with lipid rafts may explain the capacity of Hex to inhibit oxidant production, the activation of proinflammatory signaling cascades, and alterations of intestinal epithelium permeability [7,9]. Furthermore, findings showing that Hex interactions with synthetic membranes vary with changes in the composition of the bilayer (e.g. decreased binding in the presence of negatively charged head groups [6]) suggest a degree of specificity in procyanidin–membrane interactions.

Based on the above, the capacity of hexameric procyanidins to interact with lipid rafts was investigated. These interactions were initially evaluated in Caco-2 cells differentiated into intestinal epithelial cells. Subsequently, the use of liposomes with a lipid raft-like lipid composition, allowed the assessment of the interactions of Hex with particular lipid raft components. Results indicate that Hex interacts with lipid rafts, being the presence of cholesterol essential for this interaction. Binding of Hex to lipid rafts can explain the capacity of large procyanidins to regulate select cell signaling cascades and other events initiated at the cell membrane.

## 2. Materials and methods

### 2.1. Materials

Hexameric procyanidins (Hex) were purified [18,19] and supplied by Mars Incorporated (Hackettstown, NJ). Caco-2 cells were from the American Type Culture Collection (Rockville, MA). Minimum Essential Medium (MEM), non-essential amino acid mixtures, antibiotics, and fetal bovine serum were from Invitrogen Life Technologies (Carlsbad, CA). Primary antibodies for p-ERK1/2 (sc-7383) and ERK1/2 (sc-93) were from Santa Cruz Biotechnology (Santa Cruz, CA). The fluorescent probes 6-(9-anthroyloxy)stearic acid (6-AS), 16-(9-anthroyloxy) palmitic acid (16-AP), 6-dodecanoyl-2-dimethyl aminonaphthalene (Laurdan), and octadecyl rhodamine (R18) were purchased from Invitrogen/Molecular Probes Inc. (Eugene, OR, USA). Porcine brain phosphatidylcholine (PC) was from Avanti Polar Lipids Inc. (Birmingham, AL). Egg yolk phosphatidylethanolamine (PE), bovine brain asialoganglioside GM1 (aGM1), bovine brain sphingomyelin (Spm), bovine serum albumin (BSA), cholesterol, sodium deoxycholate (DCA), Triton X-100, filipin, FITC-conjugated subunit B of cholera toxin (CTX-FITC), methyl-β-cyclodextrin (MCD) and all the other reagents were purchased from Sigma Chem. Co. (St. Louis, MO).

### 2.2. Cell culture and incubations

Caco-2 cells were grown onto 24- or 48-well culture plates or 18 mm glass round coverslips, and cultured at 37 °C in a humidified, 5% $CO_2$ atmosphere in MEM supplemented with non-essential amino acids, 0.1 M sodium pyruvate, 10% (v/v) fetal bovine serum and antibiotics (50 U/ml penicillin, and 50 μg/ml streptomycin). Cells were differentiated by culturing them for 14 days after reaching confluence [8]. The cell culture medium was replaced every 3 d.

### 2.3. Evaluation of plasma membrane fluidity

Cells were incubated at 37 °C for 30 min either in the absence or in the presence of 2.5 mM methyl-β-cyclodextrin (MCD) in serum-free MEM. The medium was replaced by serum-free MEM and cells were further incubated at 37 °C for 30 min in the presence of 10 μM Hex. Samples were subsequently added without or with either 0.2 mM DCA or 0.1 mM Triton X-100 and incubated at 37 °C for 30 min. After media removal, cells were added with 0.2 ml of Hank's balanced salt solution containing 0.3 μM of the fluorescent probes 6-AS or 16-AP. After 15 min of incubation at 37 °C to allow the incorporation of the probe into the plasma membrane, fluorescence anisotropy was measured at 435 nm (λexcitation: 384 nm) in a LS50 spectrofluorometer (PerkinElmer Ltd., Beaconsfield, United Kingdom).

### 2.4. Evaluation of plasma membrane hydration

After cell incubation in the conditions described above, the culture medium was removed and replaced by 0.2 ml of Hank's balanced salt solution containing 0.3 μM of the fluorescent probe Laurdan. Cells were incubated for 15 min at 37 °C to allow probe's incorporation to the membrane. Laurdan generalized polarization (GP) was calculated using the equation:

$$GP = \frac{I_{380} - I_{350}}{I_{380} + I_{350}}$$

where $I_{350}$ and $I_{380}$ are the fluorescence intensities recorded at 430 nm after exciting samples at 350 and 380 nm, respectively.

### 2.5. Cholesterol staining with filipin

Caco-2 cells were grown and differentiated onto 18 mm round glass coverslips, and after replacing the culture media by serum-free MEM, cells were incubated at 37 °C for 30 min in the absence or presence of 10 μM Hex. Samples were next added with 0.2 mM DCA, 0.1 mM Triton X-100, or 2.5 mM MCD and further incubated at 37 °C for 30 min. Cholesterol distribution in membranes was evaluated by filipin staining [20]. Culture media were removed, and cells were fixed for 20 min at room temperature with 1 ml p-formaldehyde 3.7% (w/v). After fixation, samples were washed three times with PBS, and incubated for 20 min at room temperature with 1 ml of glycine (1.5 mg/ml in PBS). Next, cells were permeabilized by incubating samples with 0.5% (v/v) Triton X-100. The excess of detergent was removed by washing cells with PBS, and samples were incubated for 30 min at room temperature in the dark and in the presence of filipin (0.05 mg/ml in PBS). Samples were washed three times with PBS, mounted with 10 μl of glycerol (90% in PBS), sealed with nail polish, excited under UV light and observed through a blue filter in an Olympus IMT-2 (Melville, NY) fluorescence microscope coupled with a Hamamatsu Orca II charge-coupled device camera (Bridge-water, NJ). Captured images were processed using the software Image Pro Plus 5.1 (Media Cybernetics Inc., Bethesda, MD, USA).

### 2.6. Lipid raft staining

Caco-2 cells were grown onto 18 mm round glass coverslips, and incubated in the conditions described above (*Cholesterol staining with filipin*). Lipid rafts were evidenced by the specific reaction of endogenous ganglioside GM1 (a lipid raft marker) with FITC-conjugated cholera toxin (CTX-FITC) [21]. Briefly, after 30 min incubation in the presence of 10 μM Hex followed by 30 min DCA (0.2 mM), Triton X-100 (0.1 mM) or MCD (2.5 mM) treatment, culture medium was

removed, and samples were incubated at 4 °C for 30 min in the presence of 10 μg/ml CTX-FITC in 0.5 ml $NaHCO_3$-free MEM containing 25 mM HEPES and 0.5% (w/v) BSA. Next, samples were fixed for 20 min at room temperature with p-formaldehyde (3.7% w/v), quenched with 1 ml of glycine (1.5 mg/ml in PBS), mounted and observed in a fluorescence microscope.

### 2.7. Western blot analysis

For the preparation of total cell extracts, after the corresponding treatment cells were rinsed with warm PBS, scrapped and centrifuged. The pellet was rinsed with warm PBS, and suspended in 0.2 ml of 50 mM Hepes (pH 7.4), 125 mM KCl containing protease and phosphatase (1 mM $Na_3VO_4$, 10 mM NaF) inhibitors, and 2% (v/v) Igepal. The final concentration of the protease inhibitors was 0.5 mM PMSF, 1 mg/l leupeptin, 1 mg/l pepstatin, 1.5 mg/l aprotinin, and 2 mg/l bestatin. Samples were exposed to one cycle of freezing and thawing, incubated at 4 °C for 30 min, and centrifuged at 15,000 ×g for 30 min. The supernatant was decanted and protein concentration was measured [22]. Aliquots of total cell fractions containing 30 μg protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. Colored (Bio-Rad Laboratories, Hercules, CA) and biotinylated (Cell Signaling Technologies, Danvers, MA) molecular weight standards were ran simultaneously. Membrane non-specific sites were blocked for 2 h in 5% (w/v) non-fat milk, incubated overnight in the presence of the corresponding primary antibodies (1:1000 dilution) in 5% (w/v) BSA in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.6), containing 0.1% (v/v) Tween-20. After incubation for 90 min at room temperature in the presence of the HRP-conjugated secondary antibody (1:10,000 dilution) the conjugates were visualized by chemiluminescence detection in a Phosphoimager 840 (Amersham Pharmacia Biotech. Inc., Piscataway, NJ).

### 2.8. Evaluation of liposome transition to micelles

The protective effect of Hex on detergent-mediated bilayer transition to micelle was evaluated as previously described [6,23]. For this study, two kinds of liposomes were prepared. The first liposome population had a lipid composition similar to enterocyte brush border lipid rafts [24]: cholesterol:aGM1:PE:PC:Spm (32:35:13:11:9% on a molar basis), whereas the second liposome population had PC:Spm:cholesterol (1:1:1 on a molar basis). Both kinds of liposome contained 1.5 mol% of the fluorescent probe R18, and were prepared in 20 mM Tris–HCl, 140 mM NaCl buffer (pH 7.4). Samples containing 0.25 ml of liposomes were placed into 96-well microplates apt for fluorescence measurements and incubated for 10 min at room temperature in the presence of 1.25–20 μM Hex. After incubation, samples were added with 0.5 μl 40 mM DCA or Triton X-100, mixed, and after 2 min the fluorescence emission at 580 nm (λexcitation: 560 nm) was recorded. The addition of the detergent was continued until the achievement of a constant fluorescence emission corresponding to the micellar form of the lipids. Plots were adjusted to sigmoidal curves, and the detergent concentration necessary to reach half of the maximal fluorescence (C50) was calculated for each Hex concentration assessed [6].

### 2.9. Statistics

One-way analysis of variance (ANOVA) followed by Fisher's PLSD (protected least square difference) test was performed using the routines available in StatView 5.0 (SAS Institute, Cary, NC, USA). Two-way ANOVA was performed using the software GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA). A probability (P) value lower than 0.05 was considered as statistically significant.

## 3. Results

### 3.1. Membrane cholesterol is involved in Hex interactions with cell membranes

We previously demonstrated that the evaluation of changes in lipid ordering using the probe 6-AS is a reliable indicator of the interaction of flavonoids with membranes at a superficial level [7,25]. We initially studied if the higher lipid packing induced by Hex in the Caco-2 cell plasma membrane was affected upon cell exposure to DCA or Triton X-100. Both of these compounds are known to promote changes in membrane structure and physical properties [20,26]. When membrane fluidity was evaluated using the probe 6-AS (Fig. 1A), 10 μM Hex caused a significant increase in 6-AS anisotropy ($P < 0.005$ with respect to control cells), indicating a Hex-mediated decrease in membrane fluidity. Cell treatment with DCA or Triton X-100 did not modify the fluidity of the membrane. In cells pre-incubated with Hex, and subsequently treated with DCA or Triton X-100, plasma membrane fluidity was similar to that measured in cells exposed to Hex alone. The integrity of lipid rafts was next altered by partially removing cholesterol with MCD. In cholesterol depleted cells, and in the absence or the presence of DCA or Triton X-100, Hex lost its capacity to change plasma membrane fluidity (Fig. 1A). UV–visible spectra (Supplementary Fig. 1) do not indicate the existence of interactions between Hex and DCA, Triton X-100 or MDC.

To investigate if the reduced fluidity in the Hex-exposed Caco-2 cell plasma membrane was associated with a lower lipid hydration at the water–lipid interface, changes in Laurdan generalized polarization (GP) were evaluated. As depicted in Fig. 1B, Hex increased Laurdan GP, which is indicative of a partial dehydration of the bilayer. Similar to findings with the probe 6-AS, neither DCA nor Triton X-100 modified per se membrane hydration nor affected Hex-mediated decrease in membrane hydration (Fig. 1B). Again, cholesterol removal with MCD prevented Hex-mediated changes in membrane hydration. Together, the above results indicate that membrane cholesterol may be involved in Hex binding to the membrane.

Finally, plasma membrane fluidity was evaluated at a deeper region of the bilayer using the fluorescent probe 16-AP. Supporting the hypothesis that the interaction of Hex with membranes occurs at a superficial level, no alterations in membrane fluidity were observed for all the experimental situations assessed (Fig. 1C).

### 3.2. Hex interacts with lipid rafts

Given the particularly high concentration of cholesterol in lipid rafts, we next investigated whether Hex interacts with plasma membrane lipid rafts. Cholesterol localization in Caco-2 cell plasma membrane was evaluated by staining membranes with the cholesterol-specific probe, filipin. Both in control and Hex-treated cells, cholesterol showed a characteristic punctuated distribution in the plasma membrane (Fig. 2) indicating the presence of cholesterol-enriched domains. In cells exposed only to DCA, filipin labeling showed a more diffuse, homogenous pattern whereas cell preincubation with 10 μM Hex partially prevented DCA-mediated cholesterol redistribution. On the other hand, Triton X-100 did not modify cholesterol distribution, neither in the absence or presence of Hex. In cells where cholesterol was partially removed with MCD, cholesterol labeling pattern was similar to that observed in DCA-treated cells. Again, in cells preincubated with Hex and subsequently treated with MCD, cholesterol removal was partially prevented, with cells showing a pattern of cholesterol labeling similar to that of control cells.

The possibility that Hex may prevent DCA-mediated lipid raft disruption was next investigated. For this purpose, the localization of the endogenous ganglioside GM1, a lipid raft marker, was evaluated from its reaction with CTX-FITC. In control cells, GM1 was observed in discrete regions of the plasma membrane (Fig. 3). In cells pre-incubated

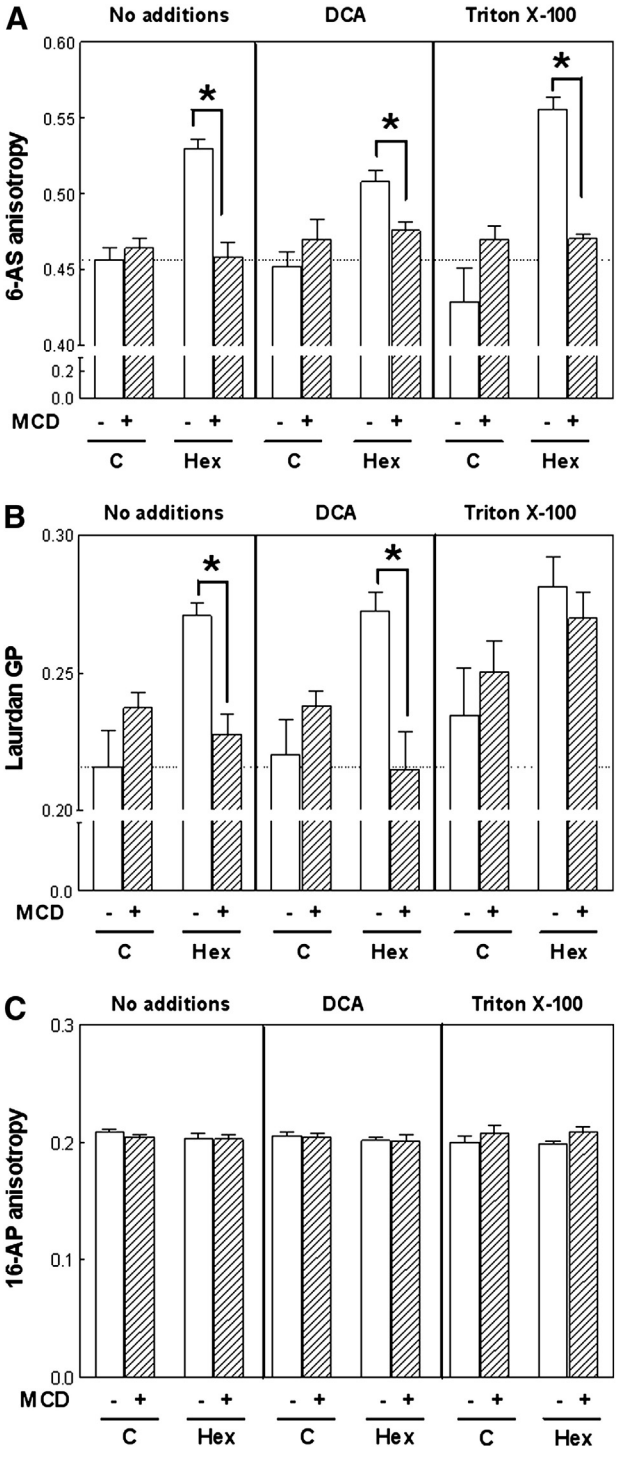


**Fig. 1.** Hex interactions with Caco-2 cell plasma membrane rely on the presence of cholesterol presence in the bilayer. Caco-2 cell plasma membrane cholesterol was partially removed by incubating cells either in the absence (□) or presence (▨) of 2.5 mM MCD for 30 min at 37 °C. Cells were next incubated for 30 min in the absence or presence of 10 μM Hex, and subsequently treated with 0.2 mM DCA or 0.1 mM Triton X-100. The following plasma membrane physical properties were evaluated in intact cells: (A) membrane superficial fluidity (probe 6-AS); (B) membrane hydration (probe Laurdan); and (C) membrane fluidity at the hydrophobic core of bilayer (probe 16-AP). Results are shown as the mean ± SEM of four independent experiments. * indicates a significant difference respect to the value measured in MCD-treated cells ($P < 0.005$, two-way ANOVA test).

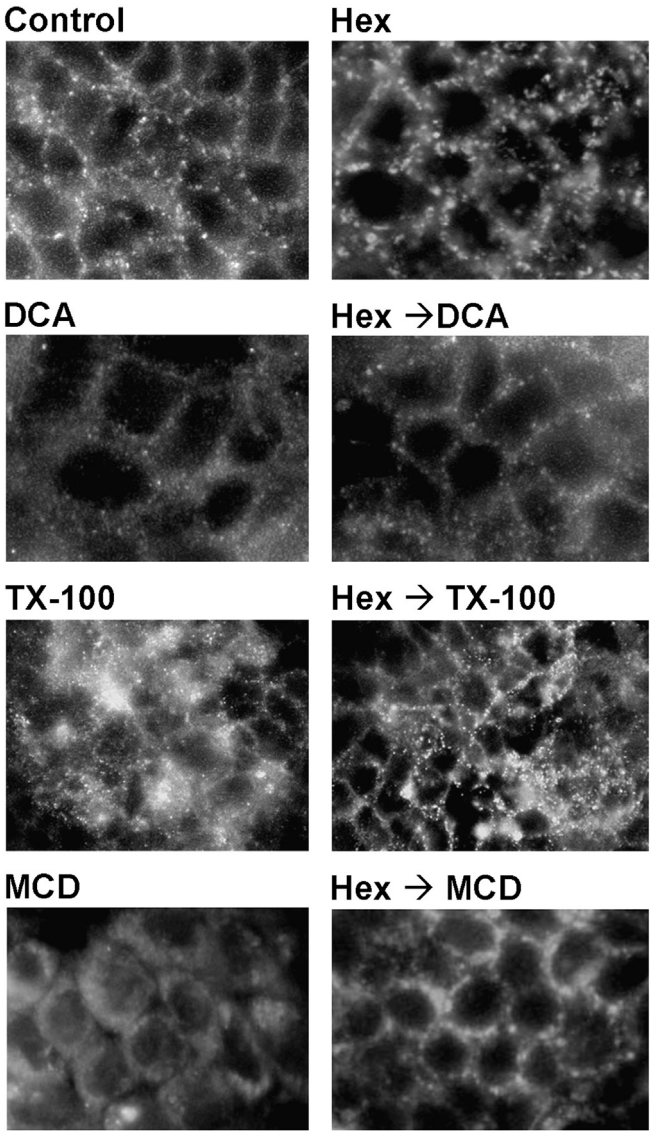


**Fig. 2.** Hex prevents cholesterol redistribution and removal from the plasma membrane. Caco-2 cells were incubated in the absence (C) or presence of 10 μM Hex (Hex). After incubation, cells were treated for 30 min at 37 °C with or without 0.2 mM DCA, 0.1 mM Triton X-100, or 2.5 mM MCD. Cholesterol distribution in the membrane was evidenced by filipin staining, as indicated in Materials and methods, and observed by fluorescence microscopy. Magnification: 400×.

with Hex, GM1 distribution was similar to that in control cells, although the intensity of the fluorescence was consistently lower, suggesting a decreased ability of CTX-FITC to reach GM1. Both in DCA- and MCD-treated cells GM1 showed a different pattern of distribution, being the labeling concentrated in highly fluorescent spots. This redistribution of GM1 due to DCA or MCD was partially prevented in Hex-pretreated cells. Both in the absence and presence of Hex, Triton X-100 did not affect the pattern of GM1 labeling.

### 3.3. Hex inhibits MCD-induced ERK1/2 activation in Caco-2 cells

We previously showed that Hex inhibits DCA-induced activation of the mitogen activated kinase ERK1/2 [7]. DCA was shown to activate ERK by disrupting lipid raft organization [20]. Based on our current findings (Figs. 2 and 3) showing that Hex can prevent MCD-mediated lipid raft disruption, we next investigated if Hex can inhibit MCD-induced ERK activation.

MCD promoted ERK phosphorylation within 15 min of incubation, effect that increased continuously up to 3 h incubation (Fig. 4A). Hex (10 μM) completely (at 30 min) or partially (39% at 3 h) inhibited MCD-induced ERK activation (Fig. 4B). Thus, these findings suggest

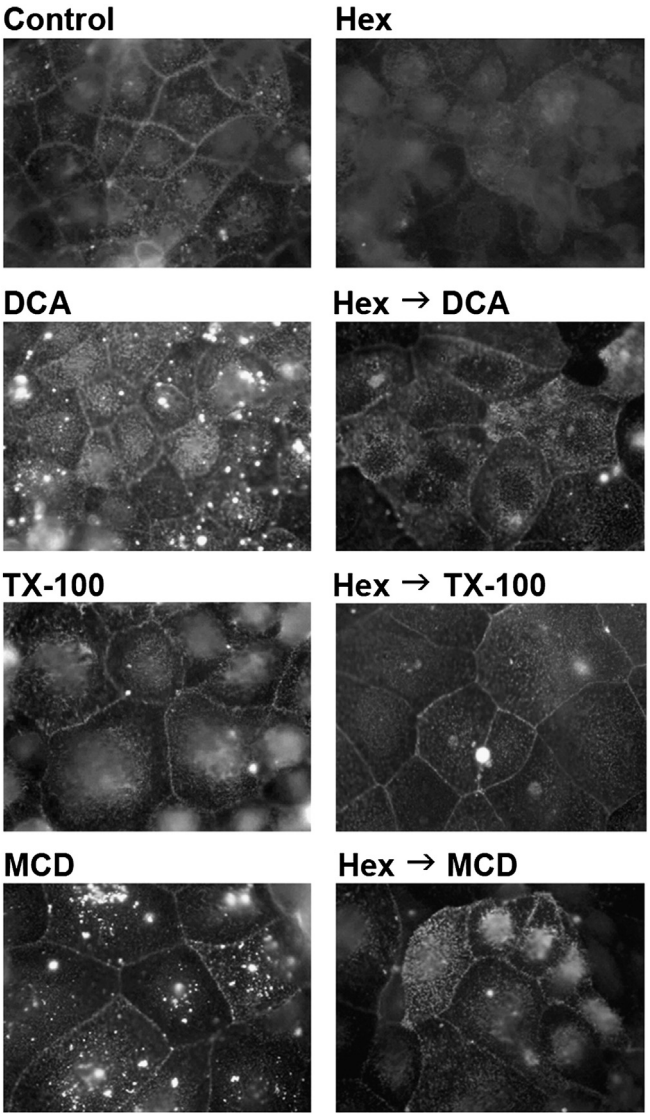


**Fig. 3.** Hex prevents DCA-mediated lipid raft disorganization. Caco-2 cells were incubated in the absence (control) or presence of 10 μM Hex (Hex). After incubation, cells were treated for 30 min at 37 °C with or without 0.2 mM DCA, 0.1 mM Triton X-100, or 2.5 mM MCD. Lipid raft distribution was evaluated by staining with CTX-FITC, as indicated in Materials and methods, and observed by fluorescence microscopy. Magnification: 1000×.

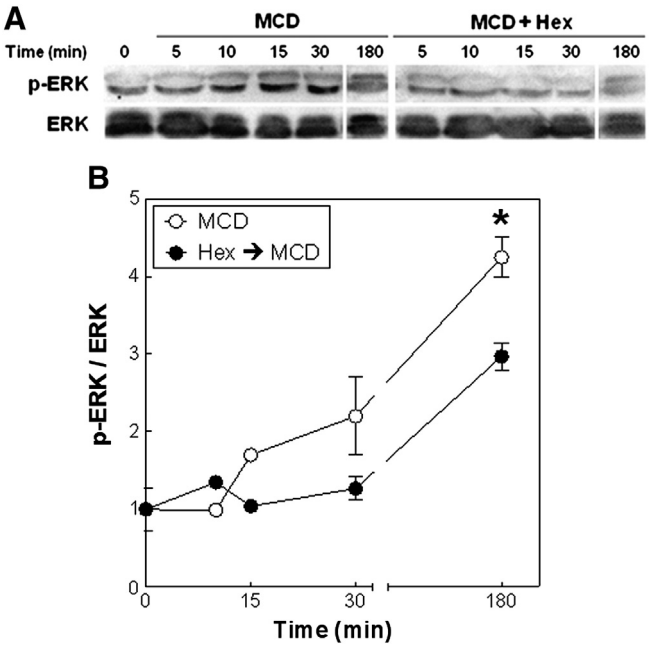


**Fig. 4.** Hex inhibits MCD-induced ERK1/2 phosphorylation. Caco-2 cells were incubated for 30 min at 37 °C in the absence or the presence of 10 μM Hex, and subsequently in the presence of 2.5 mM DCA for 0–3 h. (A) Representative Western blots images showing phosphorylated ERK1/2 (p-ERK) and total ERK (ERK1/2) in total cell extracts. (B) Quantification of p-ERK to total ERK ratio. Results are shown as mean ± SEM of 2–3 independent experiments. * indicates a significant difference ($P < 0.05$) with respect to the value measured in MCD-treated cells (one-way ANOVA test).

that Hex mitigates lipid raft disruption by MCD, and as a consequence prevents ERK activation. This supports the concept that Hex can bind to particular components of lipid rafts and through these interactions modulate cell function.

### *3.4. The differential interaction of Hex with asialoganglioside GM1 and cholesterol affects its capacity to prevent liposome disruption by detergents*

In a previous work [6] we demonstrated that Hex interacts with the polar headgroup of phospholipids and prevents the mechanical disruption of the bilayer caused by membrane treatment with the detergent Triton X-100. This protective effect of Hex was higher when membranes contained galactolipids, a lipid bearing a galactose moiety in their headgroup [6]. Therefore, we next investigated whether the presence of another component of lipid rafts, aGM1, which contains four chained sugar moieties in its polar headgroup, could enhance Hex protective effects on detergent-mediated liposome disruption. Liposomes were prepared using a lipid composition similar to that found in lipid rafts of brush border enterocytes [24].

In these liposomes, Hex prevented in a concentration (1.25–20 μM)-dependent manner DCA (Fig. 5A)- and Triton X-100 (Fig. 5B)-mediated liposome disruption as evidenced from the higher concentration of DCA or Triton X-100 necessary to achieve 50% disruption of liposomes (C50). This protective effect of Hex was significant in both liposome populations regardless of the presence of aGM1, even at the lowest concentration of Hex assessed ($P < 0.005$, one-way ANOVA). Interestingly, in liposomes lacking aGM1, the protective effect of Hex was higher than in liposomes containing aGM1. This difference in the magnitude of Hex-mediated membrane protection was even more pronounced when membranes were disrupted with Triton X-100 (Fig. 5B). These results suggest that the interaction of DCA and Triton-X100 with membranes does not occur at the same domains of the bilayer.

Finally, the role of cholesterol in Hex-mediated prevention of membrane disruption by detergents was investigated in lipid raft-like liposomes. For this purpose liposomes containing PC:Spm (1:1 on a molar basis) with or without cholesterol were used. In the absence of cholesterol, Hex had a modest but significant effect protecting liposomes from DCA ($P < 0.001$ at 20 μM Hex)- or Triton X-100 ($P < 0.01$ at 2.5 μM Hex)-mediated disruption (Fig. 6). When membranes contained cholesterol (PC:Spm:chol, 1:1:1 on a molar basis), the protective effect of Hex was significantly higher compared to cholesterol-free liposomes. This different magnitude of protection was observed for both DCA- and Triton X-100-mediated disruption (Fig. 6). The magnitude of Hex effects was significant ($P < 0.001$) at 2.5 μM concentrations and higher.

Together, the experimental results suggest that Hex differentially interacts with cholesterol and aGM1. While Hex-cholesterol enhances Hex protective effect against detergent-mediated membrane disruption, Hex–aGM1 interaction decreases it.

## 4. Discussion

This work shows that hexameric procyanidins interact with intestinal cell membrane lipid rafts, mainly through their binding to

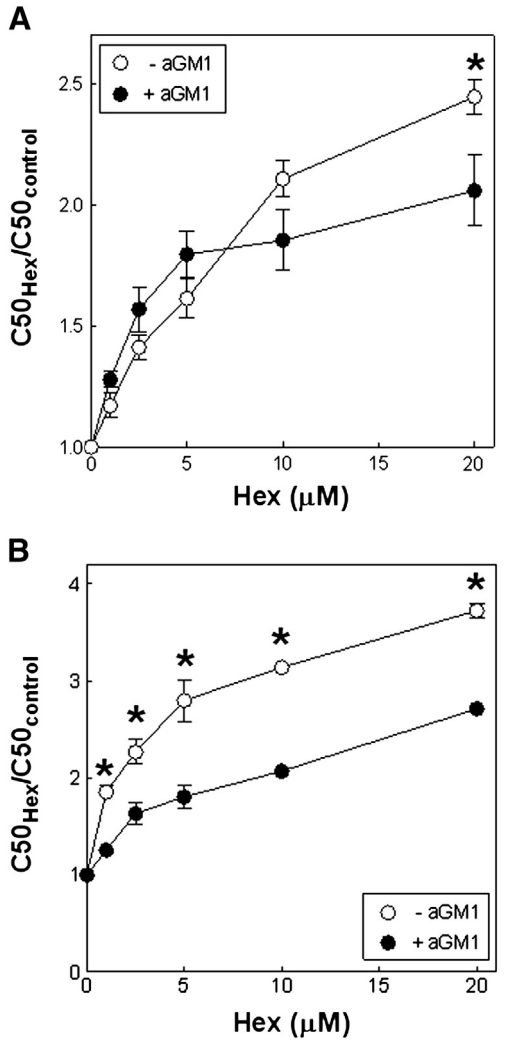


**Fig. 5.** GM1 decreases Hex capacity to prevent detergent-mediated liposome to micelle transition. Brush border lipid raft-like liposomes containing (●) or not (○) the asialoganglioside GM1 (aGM1), and labeled with the fluorescent probe R18 were incubated for 10 min at room temperature in the presence of Hex (1.25–20 μM). After incubation, liposomes were disrupted by the progressive addition of (A) DCA (0–5 mM), or (B) Triton X-100 (0–2 mM). The amount of detergent required to cause a 50% rupture of liposomes (C50) was calculated as indicated in Materials and methods. Results are shown as the ratio of C50 calculated for Hex-treated samples to C50 in control liposomes, and are the mean ± SEM of at least four independent experiments. * indicates a significant difference with respect to the value obtained in liposomes containing aGM1 ($P < 0.01$, two-way ANOVA test).

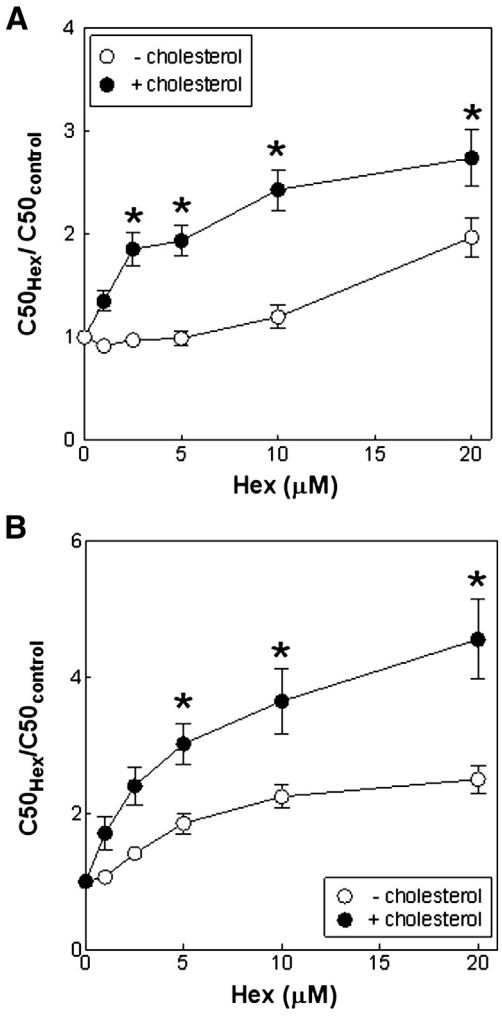


**Fig. 6.** Cholesterol enhances Hex capacity to prevent detergent-mediated liposome to micelle transition. PC:Spm liposomes containing (●) or not (○) cholesterol, and labeled with the fluorescent probe R18 were incubated for 10 min at room temperature in the presence of Hex (1.25–20 μM). After incubation, liposomes were disrupted by the progressive addition of (A) DCA (0–5 mM), or (B) Triton X-100 (0–2 mM). The amount of detergent required to cause a 50% rupture of liposomes (C50) was calculated as indicated in Materials and methods. Results are shown as the ratio of C50 calculated for Hex-treated samples to C50 in control liposomes, and are the mean ± SEM of at least four independent experiments. * indicates a significant difference respect to the value obtained in liposomes lacking cholesterol ($P < 0.001$, two-way ANOVA test).

cholesterol. Given the concentration at lipid rafts of proteins involved in cell signaling, such interactions can explain the capacity of large procyanidins to modulate cell signaling and fate [7–9] even when they cannot enter cells.

The concept that flavonoids could interact with lipid rafts was previously proposed based on the different properties of flavonoids to modulate membrane physical properties [27]. However, the existence in the gastrointestinal tract of a complex mixture of flavonoids/metabolites with different chemical composition, conformation and polymerization degree makes it impossible to predict a unique mechanism for such interactions. Procyanidins can interact with synthetic [6,28] and biological [7,28,29] membranes, having greater interactions with increasing polymerization degree. We currently observed that Hex interacts with Caco-2 cell membranes causing a decrease in membrane fluidity at the surface but not at deeper regions of the bilayer, and also decreasing membrane hydration. Partial removal of membrane cholesterol by cell treatment with MCD prevented Hex-induced changes in Caco-2 cell membrane fluidity. These results suggest that cell membrane cholesterol is required for the binding of Hex to the surface of the bilayer.

We next investigated the potential interactions of Hex with Caco-2 cell lipid rafts using fluorescent labeling of raft components (cholesterol, GM1). MCD-mediated depletion of membrane cholesterol disrupted lipid raft structure (filipin distribution) in Caco-2 cells. A similar effect was induced by DCA, which acts displacing raft cholesterol to other areas of the membrane [20]. As expected, Triton X-100 did not affect both filipin and GM1 pattern of distribution. In fact, Triton X-100 is used to isolate membrane fractions enriched in lipid rafts given its relatively very low capacity to disrupt lipid rafts [30]. Whereas Hex did not affect lipid rafts in Triton X-100-treated cells, it mitigated the actions of MCD and DCA disrupting filipin distribution. Caco-2 cell cholesterol depletion also led to GM1 aggregation, as previously shown in Jurkat T cells using GM1 fluorescence labeling [31]. We observed that MCD and DCA induced GM1 aggregation in Caco-2 cell membranes, effect that was prevented by Hex. The above findings indicate that this procyanidin can interact with lipid rafts preventing the disrupting effects of MCD and DCA.

The interactions of Hex with lipid rafts can explain the capacity of Hex to modulate cell signaling [7,9]. This is in part supported by the finding that Hex inhibited MCD-induced ERK1/2 phosphorylation. Membrane cholesterol depletion modulates ERK activation in different cell types, being the signaling pathway activated [32–34] or inhibited [31] in part depending on the extent of cholesterol depletion [31]. Cholesterol depletion of COS-1 cells by MCD causes a ligand-independent activation (phosphorylation and dimerization) of the epidermal growth factor receptor (EGFR) which leads to the downstream activation of ERK1/2 [34]. DCA also acts promoting EGFR tyrosine phosphorylation and ERK1/2 activation in human colon adenocarcinoma (HCT-116) cells through the promotion of membrane perturbations and membrane cholesterol redistribution [20]. We recently showed that Hex also acts inhibiting DCA-induced ERK phosphorylation in Caco-2 cells [7]. Thus, the capacity of Hex to inhibit DCA- and MCD-mediated ERK activation further supports the relevance of Hex-cholesterol binding, particularly at lipid rafts, in the capacity of Hex to modulate cell signaling.

To further characterize the capacity of Hex to interact with lipid rafts and the nature of these interactions, we used liposomes with a lipid composition resembling that of brush border lipid rafts, and varying the content of GM1 and cholesterol. These interactions were assessed studying the capacity of Hex to inhibit DCA- and Triton X-100-induced liposome disruption with the subsequent lipid rearrangement into micelles. Previous evidence from our laboratory showing that Hex has a preferential interaction with galactolipids [6] suggested that Hex would interact with the carbohydrate moieties of GM1. On the contrary, the presence of GM1 did not afford protection by Hex and even made liposomes more prone to disruption. A plausible explanation for such unexpected result may be related to the fact that the bulky polar headgroup of GM1 protrudes from the membrane surface [35]. Therefore, the interaction of Hex with GM1 may occur far from the water–lipid interface, impeding Hex to protect GM1-containing membrane clusters. Supporting the results obtained in Caco-2 cells, indicating the involvement of cholesterol in Hex binding to membranes, the presence of cholesterol in lipid raft-like liposomes significantly improved the capacity of Hex to prevent detergent-induced liposome disruption.

At the intestinal epithelium non-absorbable large procyanidins can exert important biological actions through their interactions with lipid rafts. Cholesterol plays a role in the regulation of tight junction structure, and is proposed to be essential in the preservation of the permeability of the intestinal epithelial barrier. Removal of cholesterol from Caco-2 cell membranes using MCD decreases transepithelial electrical resistance (TEER), increases monolayer permeability, leading to a redistribution of tight junction proteins [36]. The association of tight junctions to membrane rafts is evidenced by findings that tight junction proteins reside in lipid rafts [37]. Furthermore, lipid raft protein composition is altered in the intestine of humans with ulcerative colitis and in mice with induced colitis [16]. In mice, lipid raft disruption precedes the increased permeability of the intestinal barrier, which stresses the relevance of the preservation of lipid raft structure on intestinal permeability [16]. Accordingly, we previously showed that Hex prevents DCA-induced permeabilization (decreased TEER, increased paracellular transport) of Caco-2 cell monolayers [8]. This can be in part explained by the currently observed interactions of Hex with lipid rafts.

The interaction of Hex with lipid rafts can also explain its capacity to inhibit DCA-induced oxidant increase in Caco-2 cells [7,8], in particular by preventing NADPH oxidase activation [7]. In fact, NADPH oxidase 1 is present in large amounts at the plasma membrane of intestinal epithelial cells [38]. Its co-localization with caveolin in vascular smooth muscle cells [39], and the raft localization of NADPH oxidase subunits gp91(phox) and p47(phox) upon adipokine stimulation in endothelial cells [40] are evidence of its location to lipid rafts within the membrane. Mitigation of oxidant production and oxidative stress by large procyanidins can be an important mechanism on potential procyanidin beneficial effects in processes of chronic colonic inflammation [41] and oncogenesis [42].

In summary, the beneficial actions of large procyanidins on intestinal health [1,7–9] and their capacity to mitigate oxidative stress, intestinal barrier permeabilization, cytokine-induced inflammation, and the activation of oncogenic signals can be in part explained by their capacity to interact with lipid rafts, membrane platforms of cell signaling, trafficking and redox regulation. Even though these molecules cannot be absorbed by intestinal epithelial cells, they can still exert a number of biological actions through specific interactions with lipid raft components (mainly cholesterol) present in the exofacial leaflet of the plasma membrane.

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