Free Radical Biology & Medicine 52 (2012) 151–159

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Large procyanidins prevent bile-acid-induced oxidant production and membrane-initiated ERK1/2, p38, and Akt activation in Caco-2 cells

Mathieu Da Silva [a,b,1], Grayson K. Jaggers [a,b,1], Sandra V. Verstraeten [c], Alejandra G. Erlejman [d], Cesar G. Fraga [a,e], Patricia I. Oteiza [a,b,*]

[a] Department of Nutrition, University of California, Davis, CA 95616, USA
[b] Department of Environmental Toxicology, University of California, Davis, CA 95616, USA
[c] IIHMNO–IQUIFIB–Department of Biological Chemistry (UBA–CONICET)
[d] Department of Biological Chemistry, School of Exact and Natural Sciences, University of Buenos Aires–CONICET, Buenos Aires, Argentina
[e] Physical Chemistry–IBIMOL, School of Pharmacy and Biochemistry, University of Buenos Aires–CONICET, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 12 September 2011
Revised 30 September 2011
Accepted 7 October 2011
Available online 19 October 2011

Keywords:
Procyanidins
Flavonoids
Intestinal epithelial cells
Bile acids
Free radicals
NADPH oxidase
Membrane
MAPK
Colorectal cancer

ABSTRACT

Procyanidins are oligomers of flavanol subunits present in large amounts in fruits and vegetables. Their consumption is associated with health benefits against colonic inflammation and colorectal cancer (CRC). Large procyanidins (with more than three subunits) are not absorbed by intestinal epithelial cells but could exert biological actions through their interactions with the cell membrane. This study investigated the capacity of hexameric procyanidins (Hex) to prevent oncogenic events initiated by deoxycholic acid (DCA), a secondary bile acid linked to the promotion of CRC. Hex interacted with Caco-2 cell membranes preferentially at the water–lipid interface. Hex (2.5–20 μM) inhibited DCA-triggered increase in cellular calcium, NADPH oxidase activation, and oxidant production. DCA promoted the activation of protein kinase B (Akt), of the mitogen-activated protein kinases ERK1/2 and p38, and of the downstream transcription factor AP-1. This activation was not triggered by calcium or oxidant increases. Hex caused a dose-dependent inhibition of DCA-mediated activation of all these signals. DCA also triggered alterations in the cell monolayer morphology and apoptotic cell death, events that were delayed by Hex. The capacity of large procyanidins to interact with the cell membrane and prevent those cell membrane-associated events can in part explain the beneficial effects of procyanidins on CRC.

A high consumption of fruit and vegetables in general, and of polyphenols in particular, is inversely related to colorectal cancer (CRC)[2] risk [1–5]. Among polyphenols, this relationship has been found for the procyanidins, which are oligomers composed of flavanol units [3].

Procyanidins are among the most abundant polyphenols in human diets given that they are present in high concentrations in various edible plants (e.g., grapes, cocoa, tea, and apples). Procyanidin chemical characteristics and composition are highly dependent on the type of plant; for example, in cocoa, procyanidins are oligomers mostly composed of units of the flavanol (−)-epicatechin, linked by 4β→8 bonds, which make linearly bound molecules (Fig. 1). The biological activities of these molecules will be dependent not only on their chemical composition, but also on their tridimensional structures [6,7].

Significant in vivo and in vitro evidence supports the concept that high concentrations of deoxycholic acid (DCA) in the colon are associated with the promotion of CRC [8]. Diets enriched in procyanidins could prevent DCA-induced colonic inflammation and CRC [6,7,9–11]. In an attempt to characterize the mechanisms that could explain these protective effects, we previously showed that a hexameric procyanidin fraction (Hex) isolated from cocoa protects Caco-2 cell monolayers from DCA-mediated oxidant production, intestinal epithelial cell monolayer disruption, and cytotoxicity [9]. Hex also acts by inhibiting transcription factor NF-κB activation triggered by tumor necrosis factor α [10]. Based on this evidence, we proposed that procyanidins could exert significant biological effects in part through their capacity to interact with the outer surface of the cell membrane [12,13]. These mechanisms would be physiologically relevant in the gastrointestinal tract, where procyanidins can be present at micromolar concentrations [14]. Furthermore, procyanidins larger than three flavanol units (herein named large procyanidins) are not absorbed by cells [15], limiting

Abbreviations: Akt, protein kinase B; 16-AP, 16-(9-anthroyloxy)palmitic acid; 6-AS, 6-(9-anthroyloxy)stearic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl ester); CRC, colorectal cancer; DCA, deoxycholate; DHDCF, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; DCF, oxidized DHDCF; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; Fura-2-AM, Fura-2-pentakis(acetoxymethyl) ester; L-NAME, N^G-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; NOS, nitric oxide synthase; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline.

* Corresponding author. Fax: +530 752 8966.
E-mail address: poteiza@ucdavis.edu (P.I. Oteiza).

[1] These authors contributed equally to this article.

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.10.436

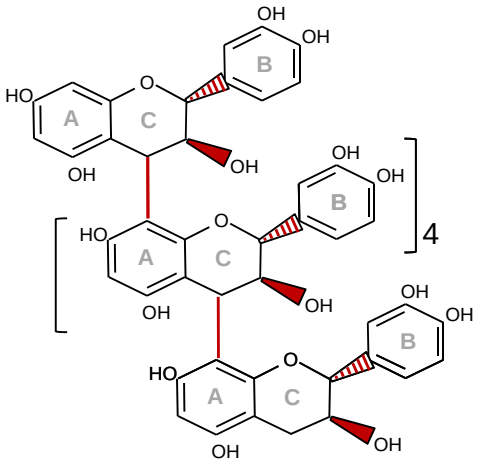

Fig. 1. Chemical structure of a hexameric procyanidin comprising subunits of (−)-epicatechin, linked by 4β→8 bonds.

their effects to membranes. Through these membrane interactions, procyanidins could regulate oxidant-sensitive signals and/or other signals (e.g., protein kinase B (Akt) and the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases (ERK) 1/2 and p38) initiated at the membrane level [16]. In line with these membrane-related actions, it has been proposed that the toxic effects of DCA are associated with its capacity to alter membrane lipid rafts, affecting the local concentration of cholesterol, subsequently altering lipid raft-associated signals [17]. Thus, the interactions of procyanidins with cell membranes could prevent or reverse DCA-induced membrane lipid raft alterations and the associated activation of cell signals.

In terms of cancer development, it has been reported that DCA causes the activation of several signaling pathways in CRC cells that could be a target of regulation by procyanidins. In HTC116 cells, DCA increases the ERK1/2 phosphorylation via activation of the epidermal growth factor receptor (EGFR) [17,18]. This activation occurs independent of the ligand binding to the receptor and was ascribed to the capacity of DCA to affect the lipid environment of the receptor. In HM3 colon cancer cells, DCA activates the EGFR/protein kinase C (PKC)/Ras/Raf-1/MEK1/ERK1/2 cascade and pathways involving the activation of MAPK p38 and Akt [17]. Significantly, the activation of these signaling pathways can induce cell proliferation and promote the expression of genes involved in oncogenesis, particularly related to CRC [19,20]. In this regard, in esophageal cancer cells, DCA induces ERK1/2-, p38-, and transcription factor AP-1-mediated cyclooxygenase-2 expression [21] and the expression of MUC2 in CRC cells [22]. From a mechanistic prospective, the activation of ERK1/2, p38, and Akt by DCA could be mediated by both DCA-mediated membrane effects [17] and its capacity to increase cellular oxidant levels [9,23].

In this study we investigated, in Caco-2 cells differentiated to intestinal epithelial cells, the capacity of Hex, as an example of large procyanidins, to (i) prevent the DCA-mediated activation of the oncogenic signals ERK/p38/AP-1 and Akt and (ii) regulate the mechanisms mediating such activation, i.e., membrane interactions, calcium mobilization, and increased production of oxidants. The obtained results are in agreement with the hypothesis that large procyanidins could in part mediate the beneficial effects on CRC provided by diets rich in fruits and vegetables.

Materials and methods

Materials

Hexameric procyanidins (Hex) were purified [24,25] and supplied by Mars, Inc. (Hackettstown, NJ, USA). Caco-2 cells were from the American Type Culture Collection (Rockville, MD, USA). Cell culture media and reagents and Pluronic-127 were from Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies for p-ERK1/2 (sc-7383), ERK1/2 (sc-93), p38 (sc-7149), PARP (sc-7150), and β-tubulin (sc-9104) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for p-Akt (9271S), Akt (9262), and p-p38 (9211) were from Cell Signaling Technology (Danvers, MA, USA). Fura-2-AM, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DHDCF), propidium iodide, 6-(9-anthroyloxy)stearic acid (6-AS), and 16-(9-anthroyloxy)palmitic acid (16-AP) were from Invitrogen/Molecular Probes (Eugene, OR, USA). DCA, apocynin, 1,2-bis(2-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA-AM), Ro-32-0432, and N^G-nitro-L-arginine methyl ester (L-NAME) were from Sigma Chemical Co. (St. Louis, MO, USA). The oligonucleotide containing the consensus sequences for AP-1 (5′-CGCTTGATGAGTCAGCCG-GAA-3′), and the reagents for the electrophoretic mobility shift assay (EMSA) were obtained from Promega (Madison, WI, USA). The Cell Death Detection ELISA Plus kit was from Roche Applied Science (Indianapolis, IN, USA).

Cell culture and incubations

Caco-2 cells were cultured at 37 °C under a 5% (v/v) CO_2 atmosphere in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). For the experiments, the cells were used 10 days after confluence to allow their differentiation in intestinal epithelial cells. The medium was replaced every 3 days. DCA and Hex concentrations and general incubation conditions were as previously established [9]. Briefly, cells were washed, and the medium was replaced by fetal bovine serum-free MEM. Cells were then preincubated for 30 min in the presence of Hex at the concentrations described for each experiment, after which 0.2 mM DCA was added to the cell culture. After the corresponding incubation, cells were harvested at various time points and processed according to each determination. Under the experimental conditions used, Hex (up to 100 μM) did not affect cell viability (data not shown).

Plasma membrane fluidity

Cell culture medium was replaced by 0.2 ml serum-free MEM, and cells were preincubated at 37 °C either in the absence or in the presence of 10 μM Hex for 30 min. Next, cells were treated with 0.2 mM DCA and further incubated at 37 °C for 30 min. The culture medium was removed, and cells were treated with 0.2 ml of Hanks' balanced salt solution containing 0.3 μM fluorescent probe 6-AS or 16-AP. After 15 min incubation at 37 °C, fluorescence polarization was registered at 435 nm ($\lambda_{excitation}$ 384 nm) in a LS50 spectrofluorimeter (PerkinElmer, Beaconsfield, UK).

Cell oxidant levels

Cell oxidant levels were evaluated using the probe DHDCF. Caco-2 cells (1×10^5) were grown in 12-well plates. For experiments, cells were preincubated for 30 min in the absence or the presence of 2.5–10 μM Hex, or the corresponding inhibitor (0.1 mM apocynin, 0.5 mM L-NAME, 1 μM Ro-32-0432, or 50 μM BAPTA-AM), and further incubated for 60 min with DCA. The medium was discarded, and the cells were rinsed with phosphate-buffered saline (PBS) and incubated in 500 μl MEM containing 10 μM DHDCF. After 30 min of incubation at 37 °C, the medium was removed; cells were rinsed with PBS and then incubated in 300 μl PBS containing 0.1% (v/v) Igepal. Then, the cells were sonicated and the fluorescence of the mixture was determined ($\lambda_{excitation}$ 475 nm; $\lambda_{emission}$ 525 nm). To measure DNA content, samples were subsequently incubated with 50 μM propidium iodide for 20 min at room temperature, and the fluorescence ($\lambda_{excitation}$ 538 nm, $\lambda_{emission}$

590 nm) was measured. Results are expressed as the ratio of DCF fluorescence/propidium iodide fluorescence.

Determination of intracellular calcium levels

Cells were loaded with 5 μM Fura-2-AM containing the nonionic detergent Pluronic F-127 for 60 min. After loading, the medium was removed; cells were washed twice with PBS and incubated for 30 min with or without 10 μM Hex and a further 30 min without or with 0.2 mM DCA. The medium was subsequently removed; cells were rinsed with PBS and cellular calcium was measured as previously described [26]. Results are expressed as the ratio of the fluorescence measured at $\lambda_{excitation}$ 340 nm and $\lambda_{excitation}$ 380 nm ($\lambda_{emission}$ 510 nm).

Western blot analysis

For the preparation of total cell extracts, after the corresponding treatments, cells were rinsed with PBS, scraped, and centrifuged. The pellet was rinsed with PBS and suspended in 200 μl of 50 mM Hepes (pH 7.4), 125 mM KCl, which contained protease and phosphatase inhibitors, and 2% (v/v) Igepal. The final concentration of the protease inhibitors was 0.5 mM phenylmethanesulfonyl fluoride, 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 [27]. Aliquots of total cell fractions containing 25–50 μg protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to polyvinylidene difluoride membranes. Colored molecular weight standards (GE Healthcare, Piscataway, NJ, USA) were run simultaneously. Membranes were blotted for 2 h in 5% (w/v) nonfat milk and incubated overnight in the presence of the corresponding antibodies (1:1000 dilution) in 5% (w/v) bovine serum albumin 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 secondary antibody (horseradish peroxidase-conjugated, 1:10,000 dilution), the conjugates were visualized by chemiluminescence detection in a Phosphoimager 840 (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

EMSA

Nuclear fractions were isolated as previously described [28,29] with minor modifications [10]. For the EMSA, the oligonucleotide containing the consensus sequence for AP-1 was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified using Chroma Spin-10 columns. Samples were incubated with the labeled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 1× binding buffer (5× binding buffer: 50 mM Tris–HCl buffer, pH 7.5, containing 20% (v/v) glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, and 0.25 mg/ml poly(dI–dC)). The products were separated by electrophoresis in a 6% (w/v) nondenaturing polyacrylamide gel using 0.5× TBE (Tris–borate 45 mM, EDTA 1 mM) as the running buffer. The gels were dried and the radioactivity was quantified in a Phosphoimager 840 (Amersham Pharmacia Biotech).

Evaluation of apoptosis

Apoptosis was evaluated using the Cell Death Detection ELISA Plus (Roche Applied Science), which quantifies cytoplasmic histone DNA fragments (mono- and oligonucleosomes). The assay was done following the manufacturer's protocol. Values ($A_{405\ nm} - A_{490\ nm}$) were referred to those obtained for control untreated cells.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using StatView 5.0 (SAS Institute, Cary, NC, USA). Fisher's least significance difference test was used to examine differences between group means. A p value of <0.05 was considered statistically significant. Data are shown as means ± SEM.

Results

Hex interacts with Caco-2 cell plasma membranes

The interactions of Hex with membranes were initially investigated. We previously described that using liposomes as a model of synthetic membranes, changes in lipid ordering induced by flavonoids can be measured with the probes 6-AS and 16-AP [12]. The anthroyloxy group in 6-AS is localized approximately at 7 Å from the surface of the bilayer, whereas 16-AP senses changes in lipid ordering at 18 Å from the membrane surface. DCA (0.2 mM) did not alter 6-AS and 16-AP fluorescence polarization, which indicates that under the present experimental conditions, DCA does not affect Caco-2 membrane fluidity (Fig. 2). Hex caused an increase in 6-AS fluorescence polarization that was not modified by the subsequent treatment of cells with DCA (Fig. 2). By contrast, 16-AP fluorescence polarization was not affected by Hex or DCA. These results indicate that Hex interacts with Caco-2 cell plasma membranes preferentially at the water–lipid interface; the strength of these interactions is such that DCA cannot displace Hex from its binding sites.

Hex prevents DCA-induced increase in oxidants in Caco-2 cells

Given the potential antioxidant action of Hex, cell oxidants were evaluated with the probe DHDCF, which crosses the cell membrane and fluoresces when it is oxidized inside the cells to DCF. DCA caused a 42% ($p<0.01$) increase in DCF fluorescence that was prevented by preincubation with 10 μM Hex. The increase in cell oxidants was dependent on NADPH oxidase, protein kinase C, and intracellular calcium, as indicated by the inhibition of the fluorescence increase by apocynin, Ro32-0432, and BAPTA-AM (Fig. 3A). The nitric oxide synthase (NOS) inhibitor L-NAME caused a partial (40%) inhibition of DCA-mediated increase in DCF fluorescence.

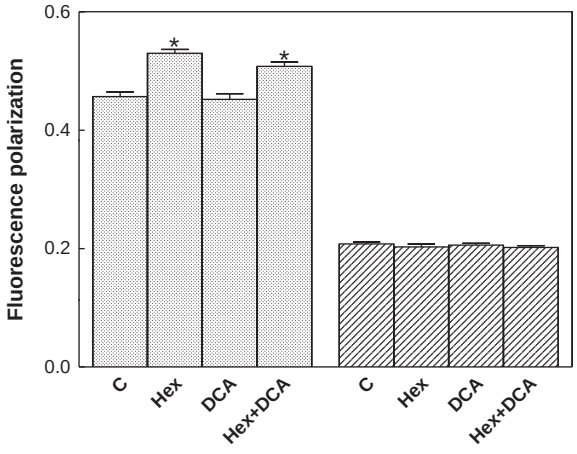

Fig. 2. Hex interacts with Caco-2 cell membranes. Caco-2 cells were incubated for 30 min at 37 °C in the absence (C) or the presence of 10 μM Hex (Hex). Subsequently, the cells were incubated in the absence or presence of 0.2 mM DCA for another 30 min. Plasma membrane fluidity was evaluated from the changes in 6-AS (left) and 16-AP (right) fluorescence polarization as indicated under Materials and methods. Results are shown as means ± SEM of four independent experiments. *$p<0.05$, significantly different from the value measured in control cells (one-way ANOVA).

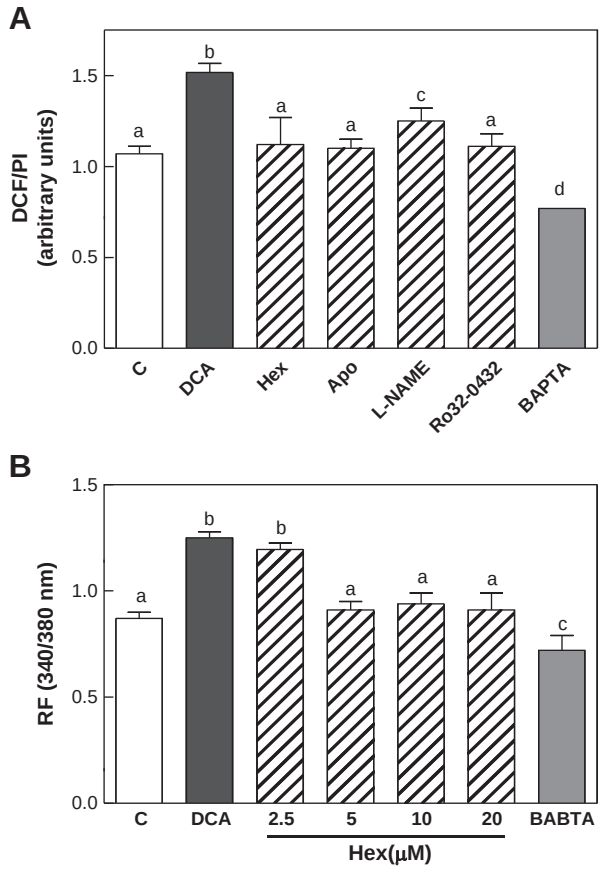

Fig. 3. Hex inhibits DCA-induced increase in cell oxidants and calcium levels. (A) Caco-2 cells were incubated for 30 min at 37 °C in the absence or the presence of 10 μM Hex, 0.1 mM apocynin (Apo), 0.5 mM L-NAME, 1 μM Ro-32-0432, or 50 μM BAPTA-AM. Subsequently, the cells were incubated in the absence (C) or presence of 0.2 mM DCA and incubated for a further 30 min. Oxidant levels were determined as described under Materials and methods. DCF fluorescence was normalized to the propidium iodide (PI) fluorescence. (B) Caco-2 cells were incubated for 30 min at 37 °C in the absence or the presence of Hex (2.5–20 μM) and further treated for 30 min with 0.2 mM DCA. Intracellular calcium was evaluated from the increase in Fura-2-AM fluorescence ratio at 340 and 380 nm ($\lambda_{emission}$ 510 nm) as described under Materials and methods. Results are shown as means ± SEM of four independent experiments. Values having different letters are significantly different ($p<0.05$, one-way ANOVA).

Hex prevents DCA-mediated cell calcium mobilization in Caco-2 cells

Given that calcium can be the upstream regulator of NADPH oxidase, NOS, and PKC, the effects of Hex on intracellular calcium levels were next investigated using the probe Fura-2-AM. After Caco-2 cells were incubated for 30 min in the presence of 0.2 mM DCA, a significant increase (44%, $p<0.02$) in Fura-2-AM fluorescence was observed (Fig. 3B). Whereas preincubation with 2.5 μM Hex had no effect, 5–20 μM Hex completely prevented the increase in Fura-2-AM fluorescence. As expected, pretreating the cells with the calcium chelator BAPTA-AM abolished DCA-induced cellular calcium increase.

Hex prevents DCA-mediated activation of the kinases ERK1/2, p38, and Akt and of transcription factor AP-1 in Caco-2 cells

Given the capacity of DCA to activate ERK1/2, p38, and Akt [17,21], we next investigated the modulation of the phosphorylation of these signals by Hex. DCA caused a time (0–6 h)-dependent increase in the phosphorylation levels of ERK1/2, p38, and Akt in Caco-2 cells (Figs. 4A and C). Although showing different kinetics of activation, the three kinases showed significant increases in their phosphorylation after 3 h incubation with DCA (81, 61, and 30% higher than controls for ERK1/2, p38, and Akt, respectively). Thus, this time point was subsequently used to evaluate the effects of Hex and various inhibitors on DCA-induced kinase phosphorylation. The activation of transcription factor AP-1, a downstream target of ERK1/2 and p38, was investigated. AP-1–DNA binding in nuclear fractions, evaluated by EMSA, was threefold higher after 3 and 6 h of incubation with 0.2 mM DCA (Figs. 4B and C).

We next investigated the potential association of DCA-induced kinase phosphorylation with the previously observed increase in cell oxidation. The NADPH oxidase inhibitor apocynin, the NOS inhibitor L-NAME, the PKC inhibitor Ro-32-0432, and the calcium chelator BAPTA-AM had no significant effects on DCA-induced ERK, p38, or Akt phosphorylation. Only a partial inhibition of ERK1/2 phosphorylation by L-NAME was observed (Fig. 4D).

The inhibition of DCA-induced ERK, p38, and Akt phosphorylation by preincubation with Hex was dose (2.5–20 μM)-dependent (Fig. 5A). Hex completely prevented the DCA-induced increase in phosphorylation levels of ERK1/2 and Akt with DI_{50} of 3.8 and 3.2 μM, respectively. The DI_{50} for p38 phosphorylation was under 2.5 μM, but no complete inhibition of p38 phosphorylation was observed with up to 20 μM Hex. Hex prevented DCA-induced AP-1 activation at all concentrations tested, with a DI_{50} <2.5 μM (Fig. 5B).

Hex partially prevents DCA-mediated activation of apoptotic events in Caco-2 cells

Morphologically, Caco-2 cell monolayers start showing evidence of alterations after 3 h of incubation with DCA (Fig. 6A). Preincubation in the presence of Hex (10 μM) prevented DCA-induced morphological alterations. After 6 h, cells treated with DCA showed morphological evidence of epithelial disruption and apoptotic cell death that was not significantly prevented by the Hex. Nevertheless, in cells preincubated with Hex the epithelium was more preserved (Fig. 6A).

Apoptotic cell death was next evaluated by measuring the presence of mono- and oligonucleosomes, which are formed during the apoptotic DNA cleavage. After 6 h incubation with DCA, a significant increase (30%) in mono- and oligonucleosomes was observed; this increase was prevented by 10 μM Hex (Fig. 6B). The cleavage of the caspase-3 protein substrate PARP was next evaluated as a parameter of caspase-3 activation, which is an early event in the apoptotic cascade. PARP cleavage was observed after 2–6 h incubation with 0.2 mM DCA. Treatment with Hex (10 μM) delayed caspase-3 activation, given that PARP cleavage was observed only after 6 h incubation (Fig. 6C).

Discussion

This study shows that procyanidins can protect Caco-2 cells differentiated into epithelial intestinal cells from DCA-induced activation of the oncogenic signals ERK, p38, Akt, and AP-1. This protection occurs in parallel with other membrane-related effects, as antioxidant actions and prevention of calcium mobilization. The fact that kinase phosphorylation, NADPH oxidase, and calcium mobilization are events initiated at membrane lipid rafts implicates potential molecular interactions of hexameric procyanidins with lipid raft components.

The use of Caco-2 cells differentiated into epithelial intestinal cells challenged by DCA, a proposed initiator of CRC and other intestinal diseases, is a viable model in which the potential beneficial effects of a food constituent can be evaluated under close to physiological conditions. The fact that large procyanidins are not absorbed and only partially metabolized in the stomach and small intestine [30] would allow them to reach the colon at relatively high concentrations [31]. This provides a rationale for exposing cells to Hex concentrations in the micromolar range. It should be considered that a molecule the size of a (−)-epicatechin hexamer is likely to remain outside the

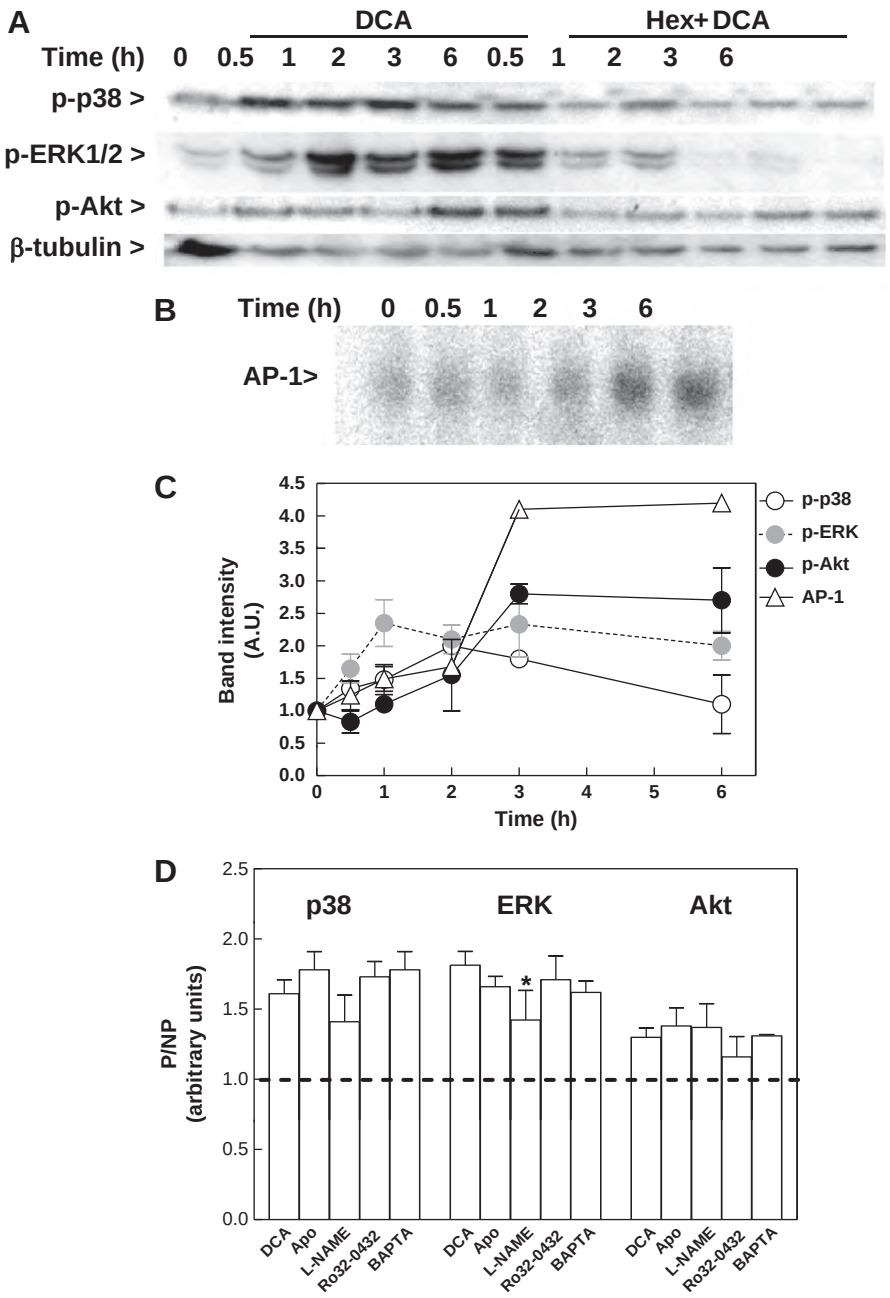

Fig. 4. Hex inhibits DCA-induced p38, ERK1/2, and Akt phosphorylation and AP-1 activation: kinetics and underlying mechanisms. (A) Caco-2 cells were incubated for 30 min at 37 °C in the absence or the presence of 10 μM Hex and subsequently in the presence of 0.2 mM DCA for 0–6 h. Western blot images show phosphorylated p38 (p-p38), phosphorylated ERK1/2 (p-ERK), phosphorylated Akt (p-Akt), and β-tubulin in total cell extracts. (B) Kinetics of DCA-induced AP-1–DNA binding evaluated by EMSA. (C) After quantification of Western blot and EMSA bands; results are shown as means ± SEM of three independent experiments. (D) Caco-2 cells were incubated for 3 h at 37 °C in the absence or the presence 0.1 mM apocynin, 0.5 mM L-NAME, 1 μM Ro-32-0432, or 50 μM BAPTA-AM. Results are shown as means ± SEM of three independent experiments ($p<0.05$ one-way ANOVA).

cells and consequently promote effects by interacting with the outer face of the cell membrane.

Hexameric procyanidins interact with Caco-2 cell membranes in what seems to be the foundation of Hex biochemical actions. This was already demonstrated in experiments in which Hex increases the transepithelial electrical resistance of polarized Caco-2 monolayers [9]. The Hex–membrane interaction seems to be rather superficial as suggested by the fact that Hex caused membrane rigidification as sensed with a probe located 7 Å from the surface (6-AS) but not with the probe located at 18 Å (16-AP). This suggests that the interaction should occur in zones of the membrane with decreased lipid mobility, e.g., the lipid rafts that are membrane zones with high lipid and protein complexity. This is backed by previous results showing that in simpler membranes, i.e., phosphatidylcholine liposomes, Hex increases both 6-AS and 16-AP fluorescence polarization [12]. In accordance, the phenolic compound resveratrol was demonstrated to be absorbed into the cells by mechanisms mediated by membrane lipid rafts [32].

When the antioxidant capacity of Hex was investigated, it was observed that Hex prevented DCA-mediated oxidant increase. It was previously reported that DCA increases cell oxidant production in both differentiated Caco-2 [9] and HCT-116 (a CRC cell line) cells [33]. In the latter the origin of oxidants was ascribed to a mitochondrial superoxide anion production that occurs after 4 h of incubation in the presence of DCA at concentrations of 0.4 mM and higher [33]. The fact that under our experimental conditions the origin of oxidants

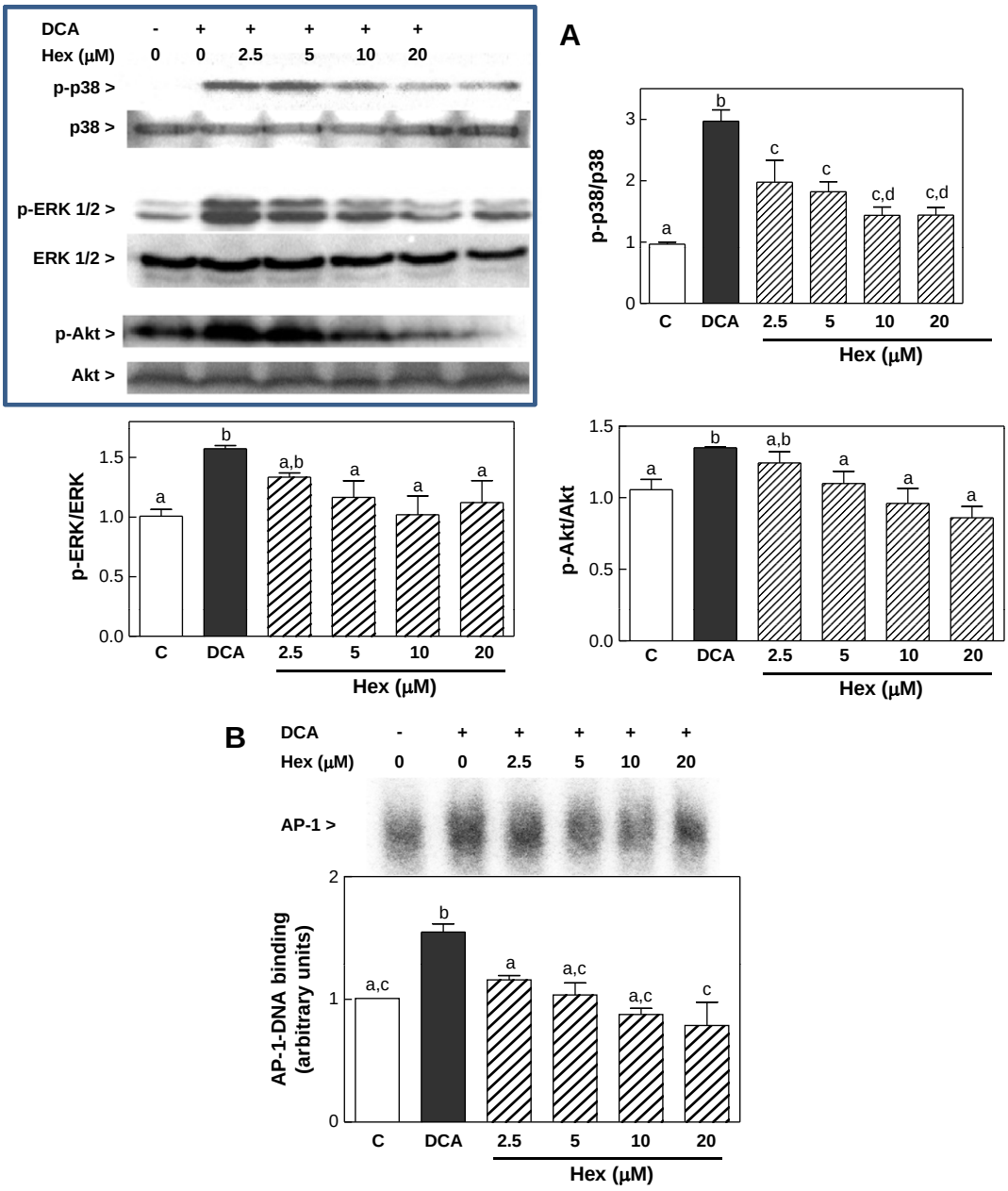

Fig. 5. Hex inhibits DCA-induced p38, ERK1/2, and Akt phosphorylation and AP-1 activation: dose-dependent manner. Caco-2 cells were incubated for 30 min at 37 °C in the absence or the presence of 10 μM Hex. Subsequently, cells were treated with 0.2 mM DCA and incubated for a further 3 h. (A) Western blots, representative images and quantifications, for phosphorylated p38 (p-p38), phosphorylated ERK1/2 (p-ERK), phosphorylated Akt (p-Akt), and nonphosphorylated p38, ERK1/2, and Akt in total fractions. (B) EMSA for AP-1, representative image and quantitation. For (A) and (B) bands were quantified and results were referred to control (C) untreated cell values (set at 1). For Western blots (A) results were expressed as the ratio of phosphorylated/nonphosphorylated protein levels. Results are shown as means ± SEM of three or four independent experiments. Values having different superscripts are significantly different ($p<0.05$, one-way ANOVA).

is the membrane-associated enzyme NADPH oxidase provides an explanation for the rapid (30 min) increase in cell oxidant levels at lower DCA concentration (0.2 mM).

Concerning the association between DCA-mediated oxidation production and intestinal health, it is important to point out that of the five NADPH oxidase activities that have been described in various cell types and tissues [34,35], NADPH oxidase 1 is highly expressed in Caco-2 cells and in the colonic mucosa, and it has been proposed that its overactivation could have a major role in colonic inflammation and oncogenesis [36]. In addition, NADPH oxidase 1 was described as being localized at the plasma membrane [37] and as colocalizing with caveolin [38]. Thus, DCA-induced alterations in lipid rafts could mediate the activation of NADPH oxidase 1, whereas Hex–membrane interactions could prevent it. Significantly, another of the observed DCA-mediated effects was calcium mobilization. DCA was previously shown to promote extracellular calcium influx in human gastric cells [39], and trimeric procyanidins regulated calcium mobilization and calcium-dependent oxidant production in Jurkat T cells [40]. Changes in cellular calcium levels can lead to the activation of PKC [41], NADPH oxidase, and NOS [42], and all these events support a membrane-dependent cell oxidant generation that is preventable by Hex. It should be stressed that, given the relevance of calcium as a second messenger, the inhibition by Hex of DCA-induced cellular calcium increase could also be important in preventing the activation of calcium-triggered signaling cascades. In summary, the capacity of high-molecular-weight procyanidins to interact with synthetic and cell membranes [12,13,43,44] and prevent DCA-induced lipid raft alterations can be the mechanism underlying their capacity

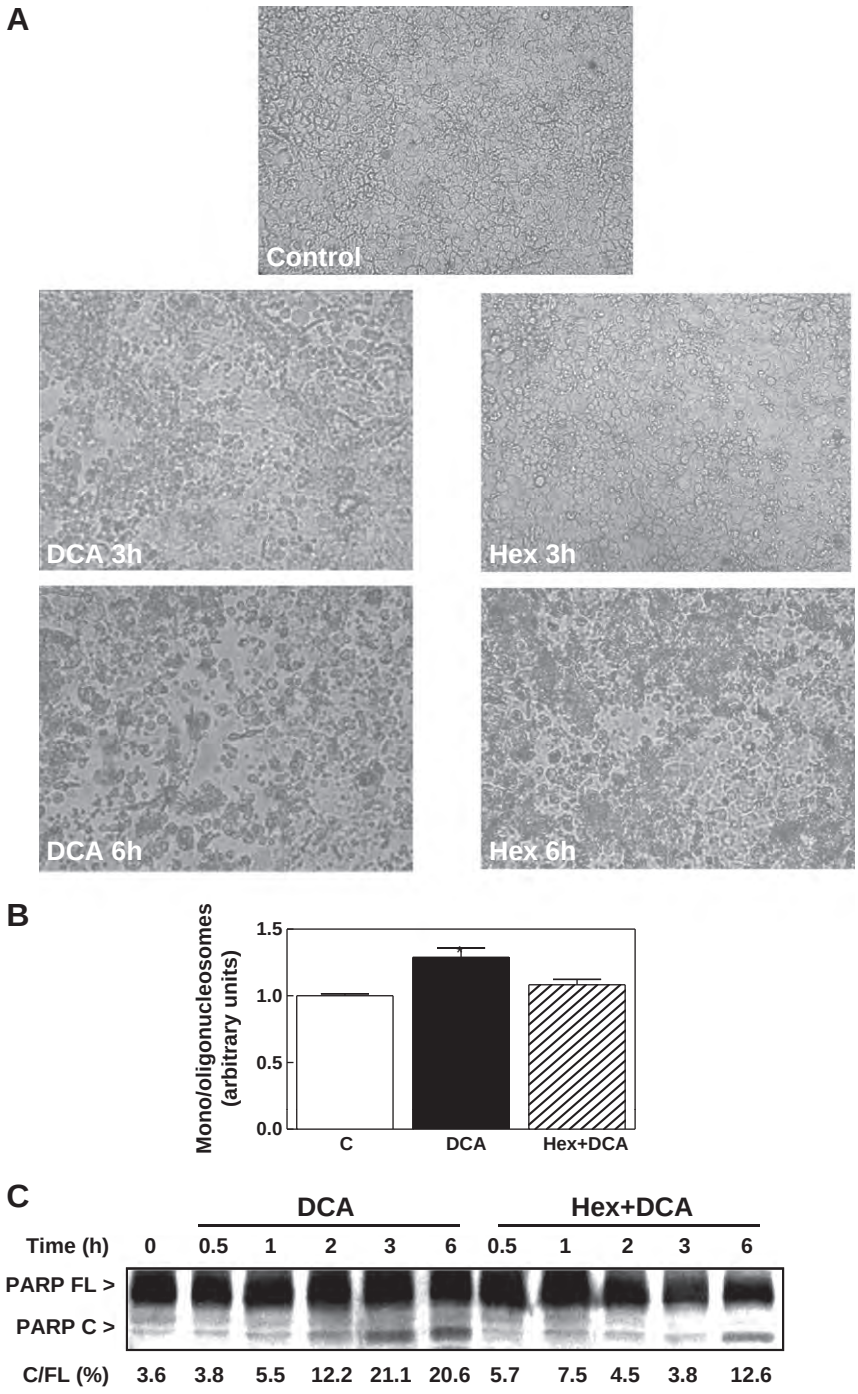

Fig. 6. Hex delays DCA-induced Caco-2 cell apoptosis. Caco-2 cells were incubated for 30 min at 37 °C in the absence or the presence of 10 μM Hex. Subsequently, the cells were treated with 0.2 mM DCA and incubated for a further 0–12 h. (A) Phase-contrast microscopy for cells incubated in the absence of additions (control), in the presence of DCA for 3 (DCA 3 h) and 6 h (DCA 6 h), or in the presence of DCA and Hex for 3 (Hex 3 h) and 6 h (Hex 6 h). (B) Apoptotic cell death was evaluated by measuring mono- and oligonucleosomes in cells incubated for 6 h in control medium (C) or in the presence of 0.2 mM DCA without (DCA) or with (Hex + DCA) 30 min preincubation with 10 μM Hex. $^{*}p < 0.05$. (C) Western blots of full-length (FL) and cleaved (C) PARP, in cells incubated for 0–6 h in the presence of DCA or in the presence of 0.2 mM DCA after 30 min preincubation with 10 μM Hex (Hex + DCA).

to prevent a DCA-mediated increase in cellular calcium and oxidants. It is important to indicate that as previously suggested the prevention of oxidation by Hex is not related to a direct antioxidant action, i.e., free radical scavenging or metal chelation, but to an indirect antioxidant effect as could be the inhibition of oxidant production by changes in lipid structure and/or specific actions on proteins, i.e., NADPH oxidase or NOS regulation [14,16,45–47]. However, at the intestinal lumen, large procyanidins could exert a direct antioxidant effect toward oxidants present in food or generated as a consequence of the digestive process.

DCA was reported to induce the activation of oncogenic signaling pathways involving ERK, p38, and Akt in various cell types [22,48–50]. In the intestine, the activation of ERK, p38, and Akt is involved in DCA-induced increased expression of MUC2, a mucin normally produced by intestinal goblet cells but abnormally expressed in CRC [22]. In Barret esophagus and esophageal adenocarcinoma cells, DCA caused an increased expression of cyclooxygenase-2, secondary to the activation of ERK, Akt, and AP-1, which was reverted by antioxidants [48]. Under the current experimental conditions, the inhibition of NADPH oxidase and NOS, and of calcium mobilization,

did not have a significant effect on DCA-induced ERK, p38, and Akt activation. Nevertheless, Hex prevented the increased phosphorylation of ERK, p38, and Akt induced by DCA and the associated activation of AP-1. Supporting a lipid raft-dependent effect of Hex, it was demonstrated that the DCA-induced phosphorylation of ERK is mediated by the upstream activation of the EGFR, which has been shown to occur independent of the ligand binding [17]. EGFR is located in lipid rafts, and the local effect of DCA displacing cholesterol from lipid rafts to other areas of the membrane is proposed to generate changes in the receptor's membrane environment leading to ligand-independent activation [17].

DCA induces apoptotic cell death in intestinal cells. Extensive damage to the mucosa as a consequence of epithelial cell death could cause an increased cell proliferation as a mechanism of repair. If uncontrolled, this could in the long term lead to the development of CRC. We observed that DCA rapidly caused the activation of caspase-3 in Caco-2 cells, whereas Hex treatment significantly delayed its activation. Caspase-3 is a cysteine protease that plays a key role in the apoptotic cascade by cleaving specific substrates, which triggers the organized dismantling of various cellular structures and compartments. One of these events is DNA fragmentation, a final stage in the apoptotic cascade, which was also delayed by Hex. Thus, Hex was protective against DCA-induced oxidative stress and activation of oncogenic signals, and delayed apoptotic cell death, and could protect from uncontrolled proliferation if cells were chronically exposed to high DCA concentrations.

The obtained results support the hypothesis that large procyanidins can prevent DCA-induced events that are closely involved in the development of CRC. At the intestinal lumen, Hex can provide a direct (free radical-scavenging) antioxidant effect, which would develop into a decrease in oncogenic mechanisms secondary to oxidative stress. By interacting with the membrane of intestinal cells, large procyanidins can (i) prevent DCA-induced activation of oncogenic signals (ERK, p38, Akt, and AP-1) and (ii) prevent DCA-induced calcium mobilization, NADPH oxidase activation, and oxidant production. All these events being initiated or developed at lipid rafts, the specific interaction of Hex and possibly other large procyanidins with lipid rafts is strongly suggested.

Extrapolating the effects of Hex preventing DCA-mediated triggering of oncogenic signals to a more general condition, it is possible to suggest that procyanidin-rich diets or pharmacological interventions with procyanidins could benefit gastrointestinal health. The mechanisms supporting these benefits involve the capacity of procyanidins to interact with cell membranes and modulate oxidant production and calcium mobilization and signals that define cell fate and function.

Acknowledgments

This work was supported by grants from the CHNR–State of California Vitamin Price Fixing Consumer Settlement Fund, USA, and from the University of Buenos Aires (01/1111), Argentina. We thank Mars, Inc., USA, for kindly providing the hexameric procyanidin fraction. C.G.F., S.V.V., and A.G.E. are members of CIC and P.I.O. is a correspondent researcher of CIC, CONICET, Argentina.

References

[1] Franceschi, S.; Favero, A.; La Vecchia, C.; Negri, E.; Conti, E.; Montella, M.; Giacosa, A.; Nanni, O.; Decarli, A. Food groups and risk of colorectal cancer in Italy. *Int. J. Cancer* **72:**56–61; 1997.

[2] Michels, K. B.; Giovannucci, E.; Chan, A. T.; Singhania, R.; Fuchs, C. S.; Willett, W. C. Fruit and vegetable consumption and colorectal adenomas in the Nurses' Health Study. *Cancer Res.* **66:**3942–3953; 2006.

[3] Rossi, M.; Negri, E.; Parpinel, M.; Lagiou, P.; Bosetti, C.; Talamini, R.; Montella, M.; Giacosa, A.; Franceschi, S.; La Vecchia, C. Proanthocyanidins and the risk of colorectal cancer in Italy. *Cancer Causes Control* **21:**243–250; 2010.

[4] Terry, P.; Giovannucci, E.; Michels, K. B.; Bergkvist, L.; Hansen, H.; Holmberg, L.; Wolk, A. Fruit, vegetables, dietary fiber, and risk of colorectal cancer. *J. Natl. Cancer Inst.* **93:**525–533; 2001.

[5] Rossi, M.; Negri, E.; Talamini, R.; Bosetti, C.; Parpinel, M.; Gnagnarella, P.; Franceschi, S.; Dal Maso, L.; Montella, M.; Giacosa, A.; La Vecchia, C. Flavonoids and colorectal cancer in Italy. *Cancer Epidemiol. Biomarkers Prev.* **15:**1555–1558; 2006.

[6] Mackenzie, G. G.; Adamo, A. M.; Decker, N. P.; Oteiza, P. I. Dimeric procyanidin B2 inhibits constitutively active NF-kappaB in Hodgkin's lymphoma cells independently of the presence of IkappaB mutations. *Biochem. Pharmacol.* **75:**1461–1471; 2008.

[7] Mackenzie, G. G.; Carrasquedo, F.; Delfino, J. M.; Keen, C. L.; Fraga, C. G.; Oteiza, P. I. Epicatechin, catechin, and dimeric procyanidins inhibit PMA-induced NF-kappaB activation at multiple steps in Jurkat T cells. *FASEB J.* **18:**167–169; 2004.

[8] Debruyne, P. R.; Bruyneel, E. A.; Li, X.; Zimber, A.; Gespach, C.; Mareel, M. M. The role of bile acids in carcinogenesis. *Mutat. Res.* **480–481:**359–369; 2001.

[9] Erlejman, A. G.; Fraga, C. G.; Oteiza, P. I. Procyanidins protect Caco-2 cells from bile acid- and oxidant-induced damage. *Free Radic. Biol. Med.* **41:**1247–1256; 2006.

[10] Erlejman, A. G.; Jaggers, G.; Fraga, C. G.; Oteiza, P. I. TNFα-induced NF-κB activation and cell oxidant production are modulated by hexameric procyanidins in Caco-2 cells. *Arch. Biochem. Biophys.* **476:**186–195; 2008.

[11] Mackenzie, G. G.; Delfino, J. M.; Keen, C. L.; Fraga, C. G.; Oteiza, P. I. Dimeric procyanidins are inhibitors of NF-kappaB-DNA binding. *Biochem. Pharmacol.* **78:**1252–1262; 2009.

[12] Erlejman, A. G.; Verstraeten, S. V.; Fraga, C. G.; Oteiza, P. I. The interaction of flavonoids with membranes: potential determinant of flavonoid antioxidant effects. *Free. Radic. Res.* **38:**1311–1320; 2004.

[13] Verstraeten, S. V.; Keen, C. L.; Schmitz, H. H.; Fraga, C. G.; Oteiza, P. I. Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and disruption of the bilayer structure. *Free Radic. Biol. Med.* **34:**84–92; 2003.

[14] Fraga, C. G.; Galleano, M.; Verstraeten, S. V.; Oteiza, P. I. Basic biochemical mechanisms behind the health benefits of polyphenols. *Mol. Aspects Med.* **31:**435–445; 2010.

[15] Serra, A.; Macia, A.; Romero, M. P.; Angles, N.; Morello, J. R.; Motilva, M. J. Distribution of procyanidins and their metabolites in rat plasma and tissues after an acute intake of hazelnut extract. *Food Funct.* **2:**562–568; 2011.

[16] Fraga, C. G.; Oteiza, P. I. Dietary flavonoids: role of (−)-epicatechin and related procyanidins in cell signaling. *Free Radic. Biol. Med.* **51:**813–823; 2011.

[17] Jean-Louis, S.; Akare, S.; Ali, M. A.; Mash Jr., E. A.; Meuillet, E.; Martinez, J. D. Deoxycholic acid induces intracellular signaling through membrane perturbations. *J. Biol. Chem.* **281:**14948–14960; 2006.

[18] Im, E.; Martinez, J. D. Ursodeoxycholic acid (UDCA) can inhibit deoxycholic acid (DCA)-induced apoptosis via modulation of EGFR/Raf-1/ERK signaling in human colon cancer cells. *J. Nutr.* **134:**483–486; 2004.

[19] Fang, J. Y.; Richardson, B. C. The MAPK signalling pathways and colorectal cancer. *Lancet Oncol.* **6:**322–327; 2005.

[20] Saif, M. W.; Chu, E. Biology of colorectal cancer. *Cancer J.* **16:**196–201; 2010.

[21] Looby, E.; Abdel-Latif, M. M.; Athie-Morales, V.; Duggan, S.; Long, A.; Kelleher, D. Deoxycholate induces COX-2 expression via Erk1/2-, p38-MAPK- and AP-1-dependent mechanisms in esophageal cancer cells. *BMC Cancer* **9:**190; 2009.

[22] Lee, H. Y.; Crawley, S.; Hokari, R.; Kwon, S.; Kim, Y. S. Bile acid regulates MUC2 transcription in colon cancer cells via positive EGFR/PKC/Ras/ERK/CREB, PI3K/Akt/IkappaB/NF-kappaB and p38/MSK1/CREB pathways and negative JNK/c-Jun/AP-1 pathway. *Int. J. Oncol.* **36:**941–953; 2010.

[23] Araki, Y.; Katoh, T.; Ogawa, A.; Bamba, S.; Andoh, A.; Koyama, S.; Fujiyama, Y.; Bamba, T. Bile acid modulates transepithelial permeability via the generation of reactive oxygen species in the Caco-2 cell line. *Free Radic. Biol. Med.* **39:**769–780; 2005.

[24] Adamson, G. E.; Lazarus, S. A.; Mitchell, A. E.; Prior, R. L.; Cao, G.; Jacobs, P. H.; Kremers, B. G.; Hammerstone, J. F.; Rucker, R. B.; Ritter, K. A.; Schmitz, H. H. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agric. Food Chem.* **47:**4184–4188; 1999.

[25] Hammerstone, J. F.; Lazarus, S. A.; Schmitz, H. H. Procyanidin content and variation in some commonly consumed foods. *J. Nutr.* **130:**2086S–2092S; 2000.

[26] Mackenzie, G. G.; Oteiza, P. I. Zinc and the cytoskeleton in the neuronal modulation of transcription factor NFAT. *J. Cell. Physiol.* **210:**246–256; 2007.

[27] Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72:**248–254; 1976.

[28] Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11:**1475–1489; 1983.

[29] Osborn, L.; Kunkel, S.; Nabel, G. J. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc. Natl. Acad. Sci. U. S. A.* **86:**2336–2340; 1989.

[30] Tsang, C.; Auger, C.; Mullen, W.; Bornet, A.; Rouanet, J. M.; Crozier, A.; Teissedre, P. L. The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *Br. J. Nutr.* **94:**170–181; 2005.

[31] Rios, L. Y.; Bennett, R. N.; Lazarus, S. A.; Remesy, C.; Scalbert, A.; Williamson, G. Cocoa procyanidins are stable during gastric transit in humans. *Am. J. Clin. Nutr.* **76:**1106–1110; 2002.

[32] Colin, D.; Limagne, E.; Jeanningros, S.; Jacquel, A.; Lizard, G.; Athias, A.; Gambert, P.; Hichami, A.; Latruffe, N.; Solary, E.; Delmas, D. Endocytosis of resveratrol via lipid rafts and activation of downstream signaling pathways in cancer cells. *Cancer Prev. Res.* **4:**1095–1106 (Philadelphia); 2011.

[33] Payne, C. M.; Weber, C.; Crowley-Skillicorn, C.; Dvorak, K.; Bernstein, H.; Bernstein, C.; Holubec, H.; Dvorakova, B.; Garewal, H. Deoxycholate induces mitochondrial oxidative stress and activates NF-kappaB through multiple mechanisms in HCT-116 colon epithelial cells. *Carcinogenesis* **28:**215–222; 2007.
[34] Lambeth, J. D. NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.* **4:** 181–189; 2004.
[35] Brown, D. I.; Griendling, K. K. Nox proteins in signal transduction. *Free Radic. Biol. Med.* **47:**1239–1253; 2009.
[36] Rokutan, K.; Kawahara, T.; Kuwano, Y.; Tominaga, K.; Nishida, K.; Teshima-Kondo, S. Nox enzymes and oxidative stress in the immunopathology of the gastrointestinal tract. *Semin. Immunopathol.* **30:**315–327; 2008.
[37] Helmcke, I.; Heumuller, S.; Tikkanen, R.; Schroder, K.; Brandes, R. P. Identification of structural elements in Nox1 and Nox4 controlling localization and activity. *Antioxid. Redox Signal.* **11:**1279–1287; 2009.
[38] Hilenski, L. L.; Clempus, R. E.; Quinn, M. T.; Lambeth, J. D.; Griendling, K. K. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **24:**677–683; 2004.
[39] Kokoska, E. R.; Smith, G. S.; Wolff, A. B.; Deshpande, Y.; Rieckenberg, C. L.; Banan, A.; Miller, T. A. Role of calcium in adaptive cytoprotection and cell injury induced by deoxycholate in human gastric cells. *Am. J. Physiol.* **275:**G322–G330; 1998.
[40] Verstraeten, S. V.; Mackenzie, G. G.; Oteiza, P. I.; Fraga, C. G. (−)-Epicatechin and related procyanidins modulate intracellular calcium and prevent oxidation in Jurkat T cells. *Free. Radic. Res.* **42:**864–872; 2008.
[41] Looby, E.; Long, A.; Kelleher, D.; Volkov, Y. Bile acid deoxycholate induces differential subcellular localisation of the PKC isoenzymes beta 1, epsilon and delta in colonic epithelial cells in a sodium butyrate insensitive manner. *Int. J. Cancer* **114:**887–895; 2005.
[42] Trebak, M.; Ginnan, R.; Singer, H. A.; Jourd'heuil, D. Interplay between calcium and reactive oxygen/nitrogen species: an essential paradigm for vascular smooth muscle signaling. *Antioxid. Redox Signal.* **12:**657–674; 2010.
[43] Oteiza, P. I.; Erlejman, A. G.; Verstraeten, S. V.; Keen, C. L.; Fraga, C. G. Flavonoid–membrane interactions: a protective role of flavonoids at the membrane surface? *Clin. Dev. Immunol.* **12:**19–25; 2005.
[44] Verstraeten, S. V.; Hammerstone, J. F.; Keen, C. L.; Fraga, C. G.; Oteiza, P. I. Antioxidant and membrane effects of procyanidin dimers and trimers isolated from peanut and cocoa. *J. Agric. Food Chem.* **53:**5041–5048; 2005.
[45] Fraga, C. G. Plant polyphenols: how to translate their in vitro antioxidant actions to in vivo conditions. *IUBMB Life* **59:**308–315; 2007.
[46] Fraga, C. G.; Oteiza, P. I. Dietary flavonoids: role of (−)-epicatechin and related procyanidins in cell signaling. *Free Radic. Biol. Med.* **51:**813–823; 2011.
[47] Galleano, M.; Verstraeten, S. V.; Oteiza, P. I.; Fraga, C. G. Antioxidant actions of flavonoids: thermodynamic and kinetic analysis. *Arch. Biochem. Biophys.* **501:**23–30; 2010.
[48] Song, S.; Guha, S.; Liu, K.; Buttar, N. S.; Bresalier, R. S. COX-2 induction by unconjugated bile acids involves reactive oxygen species-mediated signalling pathways in Barrett's oesophagus and oesophageal adenocarcinoma. *Gut* **56:**1512–1521; 2007.
[49] Allen, K.; Kim, N. D.; Moon, J. O.; Copple, B. L. Upregulation of early growth response factor-1 by bile acids requires mitogen-activated protein kinase signaling. *Toxicol. Appl. Pharmacol.* **243:**63–67; 2010.
[50] Qiao, D.; Stratagouleas, E. D.; Martinez, J. D. Activation and role of mitogen-activated protein kinases in deoxycholic acid-induced apoptosis. *Carcinogenesis* **22:**35–41; 2001.