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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

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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.

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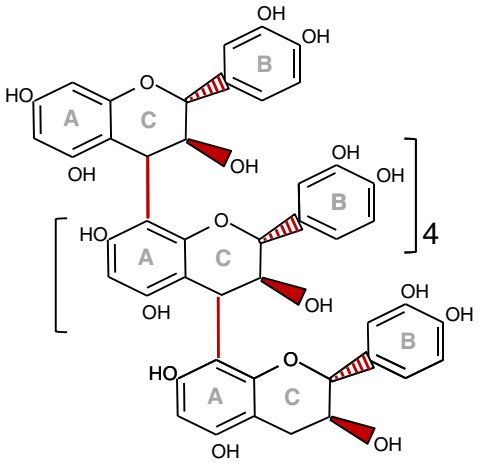

Fig. 1. Chemical structure of a hexameric procyanidin comprising subunits of (−)-epicatechin, linked by 4β→8 bonds.

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, $\lambda_{emission}$