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Cocoa procyanidins with different degrees of polymerization possess distinct activities in models of colonic inflammation

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Abstract

Procyanidins are available in the diet from sources such as cocoa and grapes. Procyanidins are unique in that they are comprised of repeating monomeric units and can exist in various degrees of polymerization. The degree of polymerization plays a role in determining the biological activities of procyanidins. However, generalizations cannot be made regarding the correlation between procyanidin structure and bioactivity because the size–activity relationship appears to be system dependent. Our aim was to screen fractions of procyanidins with differing degrees of polymerization *in vitro* for anti-inflammatory activities in models of colonic inflammation. Monomeric, oligomeric and polymeric cocoa procyanidin fractions were screened using cell models of disrupted membrane integrity and inflammation in human colon cells. High-molecular-weight polymeric procyanidins were the most effective at preserving membrane integrity and reducing secretion of interleukin-8 in response to inflammatory stimuli. Conversely, oligomeric procyanidins appeared to be the least effective. These results suggest that polymeric cocoa procyanidins may be the most effective for preventing loss of gut barrier function and epithelial inflammation, which are critical steps in the pathogenesis of metabolic endotoxemia, inflammatory bowel disease and colon cancer. Therefore, further investigations of the potential health-protective benefits of cocoa procyanidins with distinct degrees of polymerization, particularly high-molecular-weight procyanidins, are warranted.

Keywords: Cocoa; Procyanidins; Degree of polymerization; Inflammation; Colon; Permeability

1. Introduction

Flavan-3-ols, also referred to as flavanols or procyanidins (PCs), are a subclass of flavonoids comprised of monomers [(±)-catechin (C), (−)-epicatechin (EC), etc.], oligomers and polymers. Flavanols are characterized by their degree of polymerization (DP, the number of monomeric residues in an oligomer or polymer) [1] and the mean DP (mDP), the average DP of all flavanols in a matrix. PCs are widely available in the diet from sources such as cocoa, grapes, apples and berries [2]. Examples of flavan-3-ols found in cocoa are shown in Fig. 1. PCs have been widely studied for their biological activities related to prevention or amelioration of acute and chronic diseases. DP appears to play a role in determining PC efficacy in models of disease including inflammation [3–7] and cancer [8–14]. Unfortunately, no broad generalizations can be made regarding the correlation between PC structure and bioactivity because the DP–activity relationship appears to be system dependent. In some cases, activity is directly proportional to DP; in other cases, the reverse is true [15]. Moreover, in some systems, there appears to be an "optimum DP," above and below which activity is reduced.

Bioavailability, which is roughly inversely proportional to DP, represents one of the factors further complicating the interpretation of PC biological activity. Reported oral bioavailability is generally <10% for monomers (although up to 55% has been reported for cocoa catechins) [16,17], much lower for small PCs and essentially 0% for large PCs [17–20]. *In vitro* assays that predict a positive DP–activity relationship may not translate to *in vivo* feeding studies, where poor bioavailability may severely limit tissue exposure and subsequent bioactivity of large PCs. Interestingly, however, the relative bioactivity of PCs *in vivo* does not necessarily correspond to their relative bioavailability [21,22].

In vitro screening assays may be most useful for predicting PC activities in the lumen or epithelium of the gastrointestinal tract,

Abbreviations: C, (±)-catechin; EC, (−)-epicatechin; AOM, azoxymethane; CE, cocoa extract; DP, degree of polymerization; DSS, dextran sodium sulfate; DMSO, dimethylsulfoxide; FITC, fluorescein isothiocyanate; IL, interleukin; mDP, mean degree of polymerization; M, monomer-rich fraction; O, oligomer- rich fraction; P, polymer-rich fraction; PCs, procyanidins; TNF, tumor necrosis factor.

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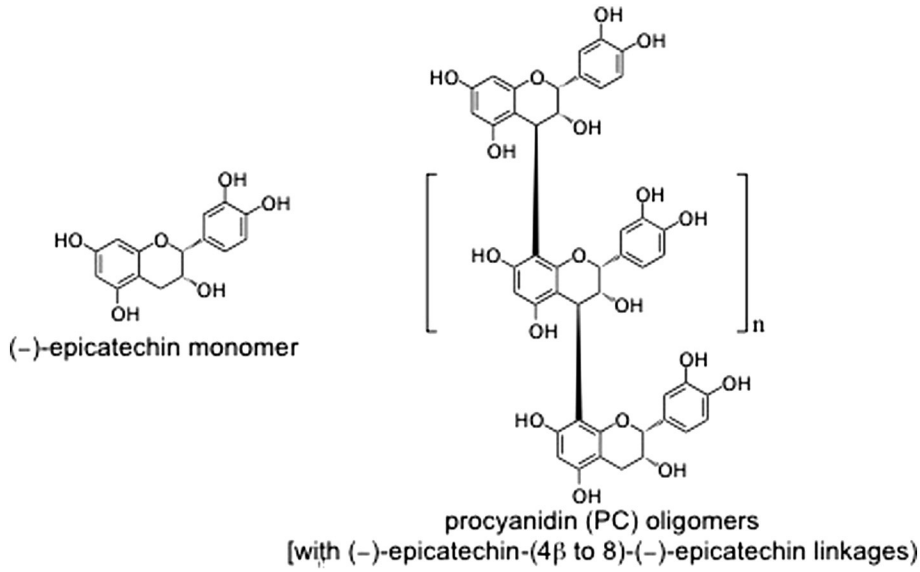

Fig. 1. Structures of selected representative flavan-3-ols found in cocoa: EC monomer and PC oligomers comprised of EC monomers linked (4β → 8).

where direct contact with higher concentrations of PCs is possible. Such activities include, but are not limited to, inhibition of luminal and brush border digestive enzymes; modulation of intestinal barrier function and endotoxin uptake; and modulation of intestinal inflammation, proliferation and apoptosis. These activities are critical mediators of obesity, diabetes, cancer and inflammatory diseases that can be modulated by PCs [23,24].

Cocoa is one of the most flavanol-rich food products and contains PCs of a wide range of DP [1,25–29]. Recently, we successfully fractionated a cocoa PC extract into monomer-, oligomer- and polymer-rich fractions and reported that the oligomeric fraction appears to provide enhanced protection against diet-induced obesity and type-2 diabetes compared to monomeric or polymeric PCs [22]. These data prompted us to screen these fractions of similarly sized cocoa PCs *in vitro* for anti-inflammatory activities in models of colonic inflammation. We hypothesized that distinct cocoa PC fractions (i.e., groups of similar PCs) containing distinct mDPs would exhibit distinct anti-inflammatory activities.

2. Materials and methods

2.1. Cocoa PC fractions

A PC-rich cocoa extract (CE) and fractions with distinct flavanol compositions (monomer-, oligomer- and polymer-rich fractions) were produced from commercially available cocoa powder as described previously [22]. The concentrations and enrichment of specific cocoa PCs in each fraction are presented in Supplementary Table 1. The normal-phase high-performance liquid chromatography (HPLC) flavanol profiles of CE and cocoa PC fractions are shown in Supplementary Fig. 1. Additionally, a cocoa polymer fraction with greater enrichment of high-molecular-weight (MW) PCs (92% by weight of DP7+) was a generous gift from The Hershey Co. (Hershey, PA, USA; the fraction was originally prepared for Hershey by Planta Analytica, Danbury, CT, USA). The composition of this fraction is shown in Table 1.

Table 1
Composition of a CE fraction enriched for high-MW polymeric PCs (DP 7+).

DP	% (w/w)
7	21
8	20
9	20
10	15
11	9
12	7
Other	8

2.2. Normal-phase HPLC analysis

The PC composition of the DP7+ fraction was evaluated by normal-phase HPLC profiling [22,30]. Analyses were performed on an Agilent Technologies (Santa Clara, CA, USA) 1260 Infinity HPLC equipped with a solvent degasser, quaternary pump, an autosampler with temperature control, a thermostat column compartment and a fluorescence detector (FLD). Separations were carried out using a Develosil Diol column (100 Å, 250 × 4.6 mm, 5-μm particle size) equipped with a Luna HILIC guard column (4 × 3.0-mm ID SecurityGuard cartridge and cartridge holder) (both from Phenomenex, Torrance, CA, USA). The column temperature was 35°C. Binary gradient elution employing 2% acetic acid (v/v) in acetonitrile (ACN, phase A) and 2% acetic acid (v/v) and 3% ddH_2O (v/v) in MeOH (phase B) was performed at a flow rate of 1 mL/min. The gradient was as follows: 93% A at 0 min, 93% A at 3 min, 62.4% A at 60 min, 0.0% A at 63 min, 0.0% A at 70 min, 93.0% A at 76 min, 7.0% B at 0 min, 7.0% B at 3 min, 37.6% B at 60 min, 100.0% B at 63 min, 100.0% B at 70 min and 7.0% B at 76 min. FLD excitation and emission wavelengths were 230 nm and 321 nm, respectively. The DP7+ fraction was prepared at 10 mg/ml in acetone:water:acetic acid (70:28:2, v/v/v) immediately prior to analysis. Samples were held at 5°C in the autosampler before injection. Injection volume was 5 μl. Mixtures of authentic standards consisting of monomers (DP 1: C, EC, ECG), PC oligomers (dimers–hexamers) and PC polymers (heptamers–decamers) were prepared and used as a reference for comparison of elution profiles as described previously [22].

2.3. Cell culture conditions

Caco-2 and HT-29 human colon cancer cells (American Type Culture Collection, Manassas, VA, USA) were maintained in subconfluence in Dulbecco's modification of Eagle's medium or McCoy's 5A medium, respectively. All media were supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO_2 atmosphere. Cells were subcultured by trypsinization.

2.4. Colon permeability assay

The ability of cocoa fractions to mitigate colon permeability *in vitro* was examined by measuring the apical to basolateral flux of fluorescein isothiocyanate–dextran (FITC-D) across differentiated Caco-2 cell monolayers as described previously [31]. In brief, Caco-2 cells were seeded in polycarbonate transwell inserts (0.33-cm^2 area and 0.4-μm pore size; Corning Life Sciences, Tewksbury, MA, USA) and allowed to reach confluence and differentiate for 21 days. Only monolayers with a transepithelial electrical resistance of greater than 500 Ω cm^2 were used for experiments [32]. Differentiated monolayers were treated with final concentrations of 100 μg/ml CE, 100 μg/ml cocoa PC fractions, 10–25 μg/ml DP7+ cocoa fraction, or vehicle only (dimethylsulfoxide, DMSO) 2 h prior to addition of 2% dextran sodium sulfate (DSS, average MW=40,000 Da; MP Biomedicals, Solon, OH, USA) to the media to induce loss of epithelial membrane integrity [33]. Cells were then co-incubated for an additional 48 h. FITC-labeled dextran (MW=4000 Da; Sigma-Aldrich, St. Louis, MO, USA) was added to the apical compartment at a final concentration of 1 mg/ml, and cells were incubated for 6 h. Basolateral media (50 μl) were removed every 30 min, and fluorescence was