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Modulation of MPP+ uptake by procyanidins in Caco-2 cells: Involvement of oxidation/reduction reactions

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Abstract **It is becoming increasingly evident that the absorption of certain nutrients and drugs and their effects are largely influenced by the concomitant ingestion of other substances. As various xeno- and endobiotics belong to the class of organic cations, the aim of this work was to study the modulation of the intestinal apical uptake of organic cations by diet procyanidins.**

Five procyanidin fractions with different structural complexity were obtained after fractionation of a grape seed extract. The effect of these compounds on 1-methyl-4-phenylpyridinium (MPP^+) uptake was evaluated in Caco-2 cells.

Apical uptake of ^3H-MPP^+ by Caco-2 cells was increased by a 60 min exposure to 600 μg ml^{-1} of procyanidin fractions, that increase being positively related with procyanidins structural complexity. It was verified that ^3H-MPP^+ uptake increased with preincubation time. It was speculated that procyanidins were oxidized during preincubation, this change could interfered with transport activity. Tested oxidizing agents showed that the redox state of the transporter could affect its activity. Additionally, *trans*-stimulation experiments showed that catechin and fraction I (the simpler fraction) can use the same transporter as MPP^+. The results are compatible with the hypothesis of these compounds being competitive inhibitors of MPP^+ transport.

In conclusion, procyanidins are capable to modulate MPP^+ apical uptake in Caco-2 cells, this transport being most probably modulated through oxidation–reduction phenomena. Interactions between these compounds and drugs present in the diet may affect their absorption and bioavailability. Both the concentration and complexity of the procyanidin compounds should be taken into account in medical practice.
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1. Introduction

Procyanidins are a class of polyphenolic polymers composed of flavan-3-ol units (catechin and epicatechin). Red wine, apples, tea and cocoa or chocolate are among the richest food sources of procyanidins [1]. Although the dietary intake of flavanols is still unknown because of their structural complexity, consumption of flavanols is estimated to vary from several tens to several hundreds of milligrams per day, depending on the diet [2]. Little is known about absorption and uptake of the oligomeric procyanidin and the interaction of procyanidins with the intestinal absorption of organic cations, despite their common occurrence in diet. It is becoming increasingly evident that the absorption of certain nutrients and drugs and their effects may be influenced by the concomitant ingestion of other substances.

Because biological membranes prevent transmembrane diffusion of the majority of organic molecules that bear net charges, and many of the organic cations are polar and positively charged at physiological pH, membrane-bound transport systems are generally involved in the absorption, distribution, and elimination of these compounds. Thus, intestinal transporters may play a crucial role in limiting and/or promoting the absorption or secretion of organic cations. Indeed, many organic cations are incompletely absorbed after oral administration and may also be actively secreted in the intestine [3].

Various xeno- and endobiotics belong to the class of organic cations. Drugs from a wide array of therapeutic groups, including antihistamines, skeletal muscle relaxants, calcium channel blockers, and β-adrenoceptor blocking agents, are organic cations. In addition, several endogenous bioactive amines (such as catecholamines, 5-hydroxytryptamine and histamine), and some vitamins (such as thiamine and riboflavin) are also organic cations.

It has been recently advanced [4,5] that the intestinal uptake of organic cations is modulated by beverages such as red wine and tea, which are rich in procyanidins, and that these beverages increase the uptake of organic cations, the form in which most vitamins, nutrients and xenobiotics pass the intestinal barrier.

The aim of this work was to characterize the modulation of the intestinal apical uptake of organic cations by procyanidins of different structural complexity. Caco-2 cells, an enterocyte-like cell line derived from a human colonic adenocarcinoma, were used as an intestinal model. This human intestinal epithelial cell line forms confluent monolayers of well-differentiated enterocyte-like cells with the functional properties of transporting epithelia [6–10].

The low molecular weight organic cation 1-methyl-4-phenylpyridinium iodide (MPP^+) was chosen as substrate for this work. This compound has been extensively employed to study the handling of organic cations by other organs and is not subjected to metabolism [11]. It has been found to be a very good substrate of several distinct transporters for organic cations:

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Abbreviations: MPP^+, 1-methyl-4-phenylpyridinium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; DTT, dithiothreitol; PAO, phenylarsine oxide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid)

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the neuronal serotonin transporter, SERT [12], the extraneuronal monoamine transporter, EMT (also known as OCT3) [13,14], the organic cation transporter type 1, OCT1 [14] and the organic cation transporter type 2, OCT2 [14].

2. Materials and methods

2.1. Materials

^{3}H-MPP$^+$ (*N*-[methyl-^{3}H]-4-phenylpyridinium acetate; specific activity 82 Ci mmol^{-1}) (New England Nuclear Chemicals, Dreieich, Germany); MPP$^+$ iodide (Research Biochemicals International, Natick, MA, USA); Triton X-100 (Merck, Darmstadt, Germany); (+)-catechin hydrate, tris (tris-(hydroxymethyl)-aminomethane hydrochloride), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide), DTT (dithiothreitol), PAO (phenylarsine oxide) and DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)) (all from Sigma, St. Louis, MO, USA).

2.2. Grape seed extract

Condensed tannins were extracted from *Vitis vinifera* grape seed tissues with an ethanol/water/chloroform solution (1:1:2, v/v/v) using a blender (Ultra-Turrax) as described elsewhere [15]. The 50% aqueous ethanol upper layer, containing polyphenols, was separated from the chloroform layer containing chlorophylls, lipids and other undesirable compounds. Ethanol was removed using a rotary evaporator and the resulting aqueous solution, containing the polyphenolic compounds, was extracted with ethyl acetate, followed by precipitation with hexane, in order to obtain the procyanidin oligomers, according to the procedure described in the literature [16].

2.3. Fractionation of grape seed procyanidins

The grape seed extract was fractionated through a TSK Toyopearl HW-40(s) gel column (250 mm × 16 mm i.d., with 0.8 ml min^{-1} methanol as eluent) according to the procedure described in the literature with some modifications [17]. Fractions were all obtained after elution with 99.8% (v/v) methanol; the first 120 ml, corresponding to the elution of catechin monomers, were eliminated, and elution was followed over 5 h in order to elute the procyanidin oligomers; all the fractions were mixed with deionized water; the solvent was eliminated using a rotary evaporator under reduced pressure at 30 °C and then freeze dried. Procyanidins were dissolved in ethanol and maintained at −80 °C until use. The resulting solids were analysed by laser secondary ionization mass spectrometry (LSIMS).

2.4. LSIMS analysis

A few mg of each sample was dissolved in the minimum volume of anhydrous MeOH and then dissolved in a matrix of glycerol. The LSIMS spectra were recorded using a VG Autospec EQ mass spectrometer, equipped with a Cs$^+$ gun in negative mode (beam energy 35 keV). Calibration was performed with caesium iodide (200–4000 Da) (Table 1).

2.5. Cells and culture conditions

The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC37-HTB, Rockville, MD, USA) and was used between passage number 15 and 84. Caco-2 cells were maintained in a humidified atmosphere of 5% CO_2–95% air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO, USA) supplemented with 15% fetal bovine serum, 25 mmol l^{-1} HEPES, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin B (all from Sigma). Culture medium was changed every 2–3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin–EDTA, 5 min, 37 °C), split 1:3, and subcultured in plastic culture dishes (21 cm^2; ∅60 mm; Corning Costar, Corning, NY). For the experiments, the Caco-2 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ∅ 16 mm; Corning Costar). For 24 h before the experiment, the cell medium was free of fetal bovine serum. Uptake studies were generally performed 9–11 days after the cells formed a monolayer. Each square centimetre contained about 300–900 μg cell protein.

2.6. Transport studies

The transport experiments were performed in Hanks' medium with the following composition (in mmol l^{-1}): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 1.0 $MgCl_2$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 0.25 $CaCl_2$, 0.15 Tris–HCl, and 1.0 sodium butyrate, pH 7.4.

Initially, the growth medium was aspirated and the cells were washed with Hanks' medium at 37 °C; then the cell monolayers were preincubated in Hanks' medium at 37 °C. Transport studies were performed in cells cultured on plastic supports, ^{3}H-MPP$^+$ being applied to the medium facing the apical cell membrane. Uptake was initiated by the addition of 0.3 ml medium at 37 °C containing 200 nM ^{3}H-MPP$^+$. Incubation was stopped after 5 min by placing the cells on ice and rinsing them with 0.5 ml ice-cold Hanks' medium. The cells were then solubilized with 0.3 ml 0.1% (v/v) Triton X-100 (in 5 mmol l^{-1} Tris–HCl, pH 7.4), at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting.

2.6.1. Effect of compounds. Compounds to be tested were present during both the preincubation and incubation periods. Controls for all treatments were run in the presence of the solvent (1% EtOH for procyanidins fractions, catechin). Procyanidin oxidation was achieved by exposure of a small amount of procyanidin solution to air, at room temperature, for 7 days. The resulting power was ressuspended in the same volume.

2.7. Determination of cell viability

Cell viability was assessed by the MTT assay [18]. This is a colorimetric assay dependent on the cellular reduction of MTT to a blue formazan product by the mitochondrial dehydrogenase of viable cells. The intensity of the blue color is a measure of cell viability.

Briefly, culture medium was removed and cells were preincubated with the tested compounds in Hanks' medium at 37°. This treatment was removed and the cells were incubated for 3 h with 500 μl of Hanks' medium and 50 μl of MTT (5 mg/mL in PBS). This solution was carefully aspirated and the formazan produced by mitochondrial dehydrogenase activity dissolved in DMSO. The tested compounds were present only during preincubation periods.

Absorbance at 660 nm corresponds to unspecific reduction of MTT. The difference in absorbance between 540 and 660 nm was calculated and registered. Results were expressed in % of control.

2.8. Protein determination

The protein content of cell monolayers was determined as described by [19] with human serum albumin as standard.

2.9. Calculations and statistics

Values are expressed as the arithmetic means ± S.E.M. Statistical significance of the difference between various groups was evaluated by one-way analysis variance (ANOVA) followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. Differences were considered to be significant when $P < 0.05$.

Table 1
Average molecular weights (M_W) and general composition of procyanidins in grape seeds fractions, determined by LSIMS

	Recovery time	Average M_W (Da)	Procyanidin composition
Fraction I	2:30–4	600	Catechins (traces), dimers, epicatechin *O*-gallate B2-3″-*O*-gallate (traces)
Fraction II	4–4:35	800	Dimers, B2-3″-*O*-gallate
Fraction III	4:35–6	900	B2-3″-*O*-gallate (traces) trimers
Fraction IV	6–7	1000	Trimers, tetramers
Fraction V	7–8	1200	Tetramers

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