Free Radical Biology & Medicine 52 (2012) 1750–1759

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

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

Original Contribution

Transport and bioactivity of cyanidin 3-glucoside into the vascular endothelium

Lovro Ziberna [a,b], Federica Tramer [a], Spela Moze [c], Urska Vrhovsek [d], Fulvio Mattivi [d], Sabina Passamonti [a,*]

[a] Department of Life Sciences, University of Trieste, 34127 Trieste, Italy
[b] Institute of Pharmacology and Experimental Toxicology, Faculty of Medicine, University of Ljubljana, 1000 Ljubljana, Slovenia
[c] Biotechnical Faculty, University of Ljubljana, 1000 Ljubljana, Slovenia
[d] Food Quality and Nutrition Area, IASMA Research and Innovation Center, Edmund Mach Foundation, 38010 San Michele all'Adige, Italy

ARTICLE INFO

Article history:
Received 28 September 2011
Revised 13 February 2012
Accepted 19 February 2012
Available online 3 March 2012

Keywords:
Oxidative stress
Antioxidants
Cyanidin 3-glucoside
Vascular endothelium
Bilitranslocase

ABSTRACT

Flavonoids are dietary components involved in decreasing oxidative stress in the vascular endothelium and thus the risk of endothelial dysfunction. However, their very low concentrations in plasma place this role in doubt. Thus, a relationship between the effective intracellular concentration of flavonoids and their bioactivity needs to be assessed. This study examined the uptake of physiological concentrations of cyanidin 3-glucoside, a widespread dietary flavonoid, into human vascular endothelial cells. Furthermore, the involvement of the membrane transporter bilitranslocase (TC No. 2.A.65.1.1) as the key underlying molecular mechanism for membrane transport was investigated by using purified anti-sequence antibodies binding at the extracellular domain of the protein. The experimental observations were carried out in isolated plasma membrane vesicles and intact endothelial cells from human endothelial cells (EA.hy926) and on an ischemia–reperfusion model in isolated rat hearts. Cyanidin 3-glucoside was transported via bilitranslocase into endothelial cells, where it acted as a powerful intracellular antioxidant and a cardioprotective agent in the reperfusion phase after ischemia. These findings suggest that dietary flavonoids, despite their limited oral bioavailability and very low post-absorption plasma concentrations, may provide protection against oxidative stress-based cardiovascular diseases. Bilitranslocase, by mediating the cellular uptake of some flavonoids, is thus a key factor in their protective activity on endothelial function.

Flavonoids are polyaromatic compounds synthesized in plants, including edible fruits, vegetables, and grains [1]. Epidemiological evidence supports the view that dietary intake of flavonoid-rich food is associated with a reduced incidence of cardiovascular diseases [2,3].

Cellular redox imbalance is thought to underlie endothelial dysfunction [4,5], which initiates a plethora of mechanisms leading to cardiovascular disease [5–7]. Therefore, the protective properties of flavonoids on the cardiovascular system have been mainly linked to their antioxidant activity [8,9], exerted either directly as free radical scavengers or indirectly as modulators of pro- and antioxidant enzymes [10]. However, the limited bioavailability of dietary flavonoids results in very low plasma concentrations (0.1–1 μM) [11], placing in doubt the possibility that their intracellular concentrations are of any relevance to endothelial function [12].

Membrane transport of flavonoids is an important issue in understanding the vascular bioactivity of flavonoids. The open questions are, do flavonoids enter into endothelial cells and, if so, to what extent? Starting from the fact that water-soluble compounds, such as dietary flavonoids, require specific transport proteins for their translocation through the plasma membrane [13], the further, more specific point is to know which plasma membrane transporters have molecular features suitable for high-affinity transport of flavonoids, a requirement to deal with their very low physiological plasma concentration. In a previous study, the bilirubin-specific transporter named bilitranslocase (TCDB 2.A.65.1.1) [14] was found to act as a flavonoid transporter in the vascular endothelium [15] and mediating endothelium-dependent vasodilation activity induced by anthocyanins [16]. In this study, the aim was to further characterize bilitranslocase-mediated transport of flavonoids into endothelial cells and to assess whether this step is critical in terms of the cardiovascular effects of these dietary compounds.

We chose cyanidin 3-glucoside (C3G)[1] (Fig. 1A), because it is one of the most widespread dietary flavonoids, present in many vegetables and fruits, including grapes. Fruit extracts rich in C3G-containing grape extracts have been found to induce favorable effects on endothelial function in healthy subjects [17,18] and to provide cardioprotection in the ischemia–reperfusion model [19]. Previous study showed that C3G is a competitive inhibitor of rat liver bilitranslocase [20], therefore likely to be transported by bilitranslocase also into the vascular endothelial cells.

Abbreviations: ABAP, 2,2′-azobis (2-amidinopropane) dihydrochloride; BSP, bromosulfalein; C3G, cyanidin 3-glucoside; ECG, electrocardiogram; HBSS, Hanks' balanced salt solution; K-H, Krebs–Henseleit solution; LDH, lactate dehydrogenase; P3G, peonidin 3-glucoside; PBS, phosphate-buffered saline; QRS, complex of waves Q, R and S in the electrocardiogram; ROS, reactive oxygen species.

* Corresponding author. Fax: +39 0405583691.
E-mail address: spassamonti@units.it (S. Passamonti).

0891-5849/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2012.02.027

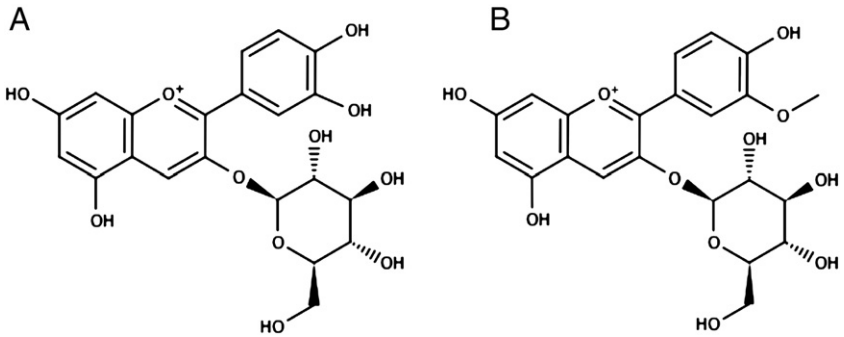

Fig. 1. Chemical structures of (A) cyanidin 3-glucoside (C3G) and (B) peonidin 3-glucoside (P3G).

Here we show for the first time that C3G enters into endothelial cells in the range of its reported postabsorption plasma concentration [21,22] and only 1 min after its application to the cells. These observations were translated into two experimental models of bioactivity and were essentially abolished by an anti-sequence bilitranslocase antibody. As a whole, these data confirm the principle that membrane transporters are essential for targeting hydrophilic, dietary antioxidants to the vascular endothelium, where they activate complex mechanisms relevant for cardiovascular health.

Materials and methods

Chemicals

C3G and all other anthocyanins used as analytical standards were purchased from Polyphenols Laboratories AS (Sandnes, Norway). Formic acid (LC–MS; Fluka), methanol (LC–MS; Chromasolv, Fluka), and deionized water were used for chromatography. Other materials used included Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), Hanks' balanced salt solution (HBSS), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP), bromosulfophthalein, valinomycin, and rabbit immunoglobulins (Sigma–Aldrich, Steinheim, Germany); fetal bovine serum, L-glutamine, penicillin–streptomycin solution, trypsin, and EDTA (EuroClone, Milan, Italy); and Krebs–Henseleit solution (K-H; composition in mM: 118.6 NaCl, 4.7 KCl, 11.1 glucose, 25 $NaHCO_3$, 1.66 $MgSO_4$, 1.2 NaH_2PO_4, and 2.52 $CaCl_2$, all from Merck, Darmstadt, Germany).

Animals and cell lines

Rats (*Rattus norvegicus,* adult males, 10–12 weeks of age, 260–280 g, $n=25$) bred at the Animal House of the Faculty of Medicine at the University of Ljubljana (Slovenia) were kept in climate-controlled areas (22–24 °C, 50–60% humidity) under specific-pathogen-free conditions, with 12-h light/dark cycles and given ad libitum access to a standard laboratory diet (Altromin 1320; Altromin, Lage, Germany) and water. All animal procedures and study protocols were conducted in accordance with permission issued by the Veterinary Administration of the Republic of Slovenia (Permit SI-No. 34401-23/2009/3), which conforms with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). The human endothelial cell line (EA.hy926) was purchased from the American Type Culture Collection (Rockville, MD, USA).

Bilitranslocase antibodies

Polyclonal bilitranslocase antibodies were obtained from rabbits immunized with a multiantigen peptide (EDSQGQHLSSF) corresponding to segment 65–75 of the primary structure of bilitranslocase. The antibodies, purified from rabbit immune sera using affinity chromatography, as described earlier [23], were the same as those used in a previous study with EA.hy926 cells (antibody A) [15].

Cell cultures

EA.hy926 cells were cultured in complete DMEM (i.e., supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and 1 mM penicillin–streptomycin). Cells were grown in an incubator at 37 °C in a humidified atmosphere (95% air and 5% carbon dioxide).

Preparation of plasma membrane vesicles

Cells grown to confluence in six flasks (150 cm^2) were harvested by incubation with 0.05% trypsin and 0.02% EDTA for 5 min, collected in 50 ml DMEM, and centrifuged at 600*g* at 20 °C for 5 min. Cells were resuspended in 3 ml ice-cold homogenization buffer (0.25 M sucrose, 0.2 mM $CaCl_2$, 10 mM Hepes, pH 7.4) and sonicated 3×30 s, separated by 40-s intervals, using a Branson sonifier (Branson Ultrasonic Corp., Danbury, CT, USA) equipped with a microtip, at 2.8 A. Lysed cells were brought to 30 ml with washing buffer (homogenization buffer with 1 mM EDTA) and centrifuged at 1000*g* for 10 min. The supernatant was centrifuged at 20,000*g* for 30 min. The pellet was suspended in 3.5 ml washing buffer and loaded on a discontinuous gradient (20 and 40% (mass:vol) sucrose in 10 mM Hepes, pH 7.4) and centrifuged at 50,000*g* for 150 min. Plasma membrane vesicles were collected at the 20-to-40% interface, diluted in washing buffer, and centrifuged again at 70,000*g* for 40 min. Final pellets were resuspended in washing buffer at [protein] = 1.7 mg/ml and stored at −80 °C.

Assay of bilitranslocase transport activity

Bilitranslocase transport activity was assayed spectrophotometrically as described previously [20,24]. In detail, 4.5 µl vesicles (7.65 µg protein) was added to a stirred cuvette containing 2 ml assay medium (0.1 M potassium phosphate, pH 8.0, at 20 °C) with various bromosulfalein (BSP) concentrations (in the range 4–40 µM), without or with 6.8 µM cyanidin 3-glucoside as a reversible inhibitor. This addition caused an instantaneous fall in absorbance (recorded at λ 580–514 nm). After the attainment of a steady state (4 s), a second fall in absorbance was brought about by adding the K^+-specific ionophore valinomycin (10 µg in 2 µl methanol). The ensuing K^+ diffusion potential drove the substrate into the vesicles. The slope of the linear phase of this decrease in absorbance, lasting about 1 s, is referred to as electrogenic BSP uptake and is related to bilitranslocase transport activity. Under these conditions, the pH in the assay medium is constant throughout the test [24].