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ORIGINAL CONTRIBUTION

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Absorption and metabolism of anthocyanin cyanidin-3-glucoside in the isolated rat small intestine is not influenced by ethanol

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Summary Anthocyanins are receiving renewed attention for their positive health attributes. High intakes and an adequate absorption rate of anthocyanins are necessary for efficient protection, though other dietary agents might influence absorption efficacy. The aim of this study was to investigate intestinal handling of luminally administered cyanidin-3-glucoside in the absence and presence of ethanol in an isolated preparation of luminally and vascularly perfused rat small intestine. A synthetic perfusate free from blood components was used as vascular medium, with a perfluorocarbon as the oxygen carrier. Luminal media consisted of a bicarbonate buffered sodium chloride solution in water or in water/ethanol (95/5), spiked with cyanidin-3-glucoside. Absorption rate of cyanidin-3-glucoside was 4.3 % ± 3.2 (n = 5). Ethanol (5 %) had no significant influence on absorption rate (2.9 % ± 1.8, n = 5). Irrespective of the presence of ethanol, the majority of the absorbed cyanidin-3-glucoside appeared unchanged, besides some cyanidin-3-glucoside-conjugate.

Key words cyanidin-3-glucoside – anthocyanin – intestinal metabolism – intestinal absorption – rat

Introduction

Anthocyanins are major phenolic compounds from fruits, beans, cereals, vegetables and beverages like juices and especially red wine [1, 2]. These flavonoids are receiving renewed attention for their positive health attributes. Consumption of anthocyanins is associated with a reversal of age-dependent neuronal degeneration [3], reduced platelet aggregation [4] as well as significant vaso-protective and anti-inflammatory effects [5]. Although our diet contains relatively high amounts of these protective anthocyanins, available research on absorption and metabolic handling is scarce. Few studies have been performed with mixtures of anthocyanins from *Vaccinium myrtillus* extracts [6], elderberry juice [7] and red wine [8]. In all these studies, low anthocyanin absorption rates were estimated. Recently, human and animal studies focused on absorption of purified cyanidin glycosides [9–11]. These authors observed intact cyanidin glycosides in plasma and tissue compartments and also estimated very low absorption rates.

However, high intakes and an adequate absorption rate of anthocyanins would be necessary for efficient protection. Many factors affect the fate of ingested compounds. Besides the dose, the matrix in which the compounds are ingested is of great importance. It might be conceivable that other dietary agents influence the efficacy of anthocyanin absorption. The presence of compounds liable to bind or solubilize anthocyanins could modify the extent of absorption and metabolism. Ethanol, in particular, might affect absorption or influence metabolism. Consequently, it has been speculated that the presence of alcohol in red wine improves flavonoid availability by increasing intestinal absorp-

tion [12]. Yet, the impact of ethanol on intestinal absorption of anthocyanins has not been investigated.

The aim of the present study was to evaluate absorption and metabolism of cyanidin-3-glucoside in the presence and absence of ethanol by using an isolated rat small intestine (duodenum, jejunum and ileum) [13, 14]. This organ perfusion model facilitates direct investigation of luminal disappearance and venous appearance of administered compounds, thereby allowing the estimation of intestinal absorption.

Materials and methods

Animals

Male Sprague-Dawley rats (CD-rats), 40 d old and weighing about 170 g, were obtained from Charles River (Sulzfeld, Germany). Rats were fed a cornstarch-based cyanidin-3-glucoside-free synthetic diet (Altromin C-1000, Altromin International GmbH, Lage, Germany) for 5 days. Animals were provided with free access to tap water and food. For perfusions with cyanidin-3-glucoside animals weighed 213.1 ± 7.1 g (n = 5) and for perfusions with cyanidin-3-glucoside and ethanol 217.2 ± 15.2 g (n = 5).

Vascularly and luminally perfused rat small intestine

The small intestine was prepared in rats as described elsewhere [14, 15]. Luminal media consisted of 114.6 ± 23.7 µmol/L cyanidin-3-glucoside (amount applied 3780.2 ± 782.3 nmol, n = 5), 135 mmol/L NaCl, 20 mmol/L $NaHCO_3$ and 10 mmol/L ascorbic acid at pH 7.2. In case of perfusions with cyanidin-3-glucoside and ethanol (5 mL per 95 mL luminal media), luminal media were spiked with 130.1 ± 39.6 µmol/L cyanidin-3-glucoside (amount applied 4294.0 ± 1308.2 nmol, n = 5).

Vascular perfusion medium consisted of a perfluorotributylamine (ABCR, Karlsruhe, Germany) emulsion in Krebs-buffer containing 10 mmol/L glucose and additional 0.6 mmol/L glutamine, gassed with 5 % carbon dioxide in oxygen (pH 7.4). The perfluorotributylamine (200 g/L) was emulsified with polyoxyproylene-polyoxyethylene copolymer (25.6 g/L Pluronic, F-68®, BASF, Ludwigshafen, Germany) in sterile, pyrogene-free water, using a high-pressure homogenizer (Mouton-Gaulin LAB 60/60–10TBS, APV Gaulin GmbH, Lübeck, Germany) to an average diameter of 0.2 µm.

Oxygen uptake and acid-base homeostasis were carefully controlled (Clark pO_2-electrode and pH-electrode integrated in an ABL 30 Acid-Base Analyzer; Radiometer, Copenhagen, Denmark). Glucose, lactate and pyruvate were determined photometrically by using enzymatic test kits (Monotest; Boehringer Mannheim, Germany). For glucose the MPR3 Glucose/GOD-Perid® test kit (glucose oxidase, peroxidase; ABTS®; Boehringer Mannheim, Germany), for lactate the MPR3 lactate test kit (lactate dehydrogenase; NAD^+) and for pyruvate the MPR1 pyruvate test kit (lactate dehydrogenase; NADH) were used.

The study was approved by the Regierungspräsidium Stuttgart, Germany.

Sampling and sample preparation

Vascular (50 mL) and luminal (5 mL) aliquots were obtained and the entire isolated small intestine as well as the mesenteric blood vessels were harvested for analyses of cyanidin-3-glucoside and its conjugates with RP-HPLC with electrochemical (coulometric) detection after sample preparation as described below.

Vascular samples

Of each vascular sample, 1 mL was centrifuged at 10 000 x *g* for 10 min (Sigma 112, Sigma Laborzentrifugen GmbH, Osterode, Germany). The supernatant was separated, the pellet resuspended with 0.1 mL ethanol and centrifuged again at 10 000 x *g* for 10 min. The combined supernatants (0.9 mL) were analyzed by HPLC. Cyanidin-3-glucoside showed a recovery from vascular media of 101.4 ± 3.7 % (means ± SD, n = 3). After the perfusion experiment, cyanidin-3-glucoside was stabilized in the vascular samples by addition of 17.6 mg ascorbic acid (resulting in a concentration of 2 mmol/L) and 5 mL hydrochloric acid (2 mol/L).

Luminal samples

After centrifugation at 2800 x *g* for 20 min (Hermle ZK 364; Kontron, Zürich, Switzerland), the supernatant was separated. The pellet was extracted twice with acidified methanol/water (1:1, 200 mmol/L hydrochloric acid) by sonication for 10 min and then centrifuged again at 2800 x *g* for 10 min. The volume of the combined supernatants was defined and the solution was analyzed by HPLC. Cyanidin-3-glucoside recovery from luminal media was 90.2 ± 0.4 % (means ± SD, n = 4). After sampling the pH was immediately adjusted to 1 with 2 mol/L hydrochloric acid.

Small intestinal tissue and blood vessels

After lyophilization of the entire small intestine, the tissue was cut into small pieces and subsequently extracted by sonication with 4.5 mL and 6.0 mL of an acidified methanol-water mixture (1:1, 200 mmol/L hydrochloric acid). After centrifugation (10 000 x *g* for 10 min), the extracts were pooled and the volume adjusted to 8–9 mL

(the exact volume was defined). The blood vessels were lyophilized and extracted like the small intestinal tissue except that the extraction volume was 3.0 mL (twice) and the extracts were adjusted to about 5 mL (the exact volume was defined). Cyanidin-3-glucoside exhibited a recovery from the small intestinal tissue and blood vessels of 83.6 ± 3.2 % (means ± SD, n = 3).

Analytical procedures

Gradient HPLC system with coulometric detection

The HPLC system (Sykam, Gilching, Germany) consisted of a solvent delivery system S 1100, an HPLC controller S 2000, a low pressure gradient mixer S 8110, an autoinjector Marathon Basic$^+$ (Spark, Emmen, The Netherlands) with a 100 µL loop and an ESA Coulochem electrochemical detector (model 5100A with guard cell 5020 and analytical cell 5011; Max Stevenson scientific & medical equipment, Berlin, Germany) at + 900 mV. Continuous on-line monitoring and data quantification was performed with a Chromatopac C-R5A data processor (Shimadzu Corp., Kyoto, Japan). Separation was carried out on a Gromsil ODS-4 HE column (125 mm x 4.0 mm I. D., 5 µm, Grom Analytik & HPLC GmbH, Herrenberg, Germany), with a flow rate of 0.9 mL/min. Elution of cyanidin-3-glucoside (about 11 min) and of aglycone cyanidin (about 19 min) was achieved using the following gradient conditions: 0–3 min, 0 % B; 3–8 min, 0–12 % B; 12–17 min, 12–17 % B; 17–24 min, 17–38 % B. Eluent A consisted of acetonitrile/water/formic acid (3/87/10, v/v/v); eluent B was composed of acetonitrile/water/formic acid (50/40/10, v/v/v). An injection volume of 50 µL (10 µL in case of luminal samples) resulted in a detection limit of 30 nmol/L and a quantification limit of 100 nmol/L for cyanidin-3-glucoside. In case of the aglycone cyanidin, detection limit was 50 nmol/L, quantification limit 150 nmol/L.

Naringin (naringenin-7-glucoside) was used as an internal standard in concentrations of 2.46 mmol/L for luminal and 24.6 µmol/L for vascular samples as well as for small intestinal tissue and for blood vessels.

Gradient HPLC system with MS detection (LC-MS)

For the identification of cyanidin-3-glucoside and conjugates we used a clean-up procedure for vascular samples and gradient HPLC system combined with a MS detector in the electro-spray-ionization mode (ESI$^+$, ESI$^-$) as described earlier [16]. Luminal samples were enriched tenfold by evaporation with nitrogen at 37 °C prior to the LC-ESI-MS analysis.

Cleavage of cyanidin-3-glucoside conjugates

Conjugates such as glucuronides and sulfates were analyzed as cyanidin-3-glucoside after enzymatic cleavage according to Sfakianos et al. [17], with modifications as described below. For cleavage of glucuronides, 0.15 mL of K_2HPO_4 (1 mol/L), 0.05 mL NaOH (2 mol/L) and 0.1 mL glucuronidase solution (248 Fishman units; *E. coli*, Sigma-Aldrich, Deisenhofen, Germany) were added to 0.6 mL sample solution. Subsequent cleavage of sulfate conjugates was performed with 0.0625 mL glucuronidase-sulfatase solution (265 units; *Helix pomatia*, Sigma-Aldrich, Deisenhofen, Germany) and 0.5 mL sample solution (glucuronidase incubation) in an acetate buffer (0.0625 mL, 0.2 mol/L, pH 4.5). Mixtures were incubated for 15 min (glucuronides) and 45 min (sulfates) at 37 °C. (Glucuronidase: β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31; Sulfatase: aryl-sulfate sulfohydrolase, EC 3.1.6.1).

Since sulfate and glucuronide conjugates of anthocyanins are not commercially available, the applicability of the enzymatic cleavage in luminal perfusate was confirmed by the conversion of the sulfate and glucuronide conjugates of 4-nitrophenol. The cleavage of 4-nitrophenol glucuronide resulted in 4-nitrophenol recovery of 96.6 ± 1.8 % (mean ± SD, n = 3), the cleavage of 4-nitrophenol sulfate resulted in a recovery of 102.0 ± 0.5 % (mean ± SD, n = 3).

Chemicals and solvents

All chemicals used were of analytical grade. Solvents for HPLC analysis were of HPLC grade. Cyanidin-3-glucoside and cyanidin were purchased from Extrasynthèse (Genay, France), naringin, 4-nitrophenol, 4-nitrophenol-glucuronide and -sulfate from Fluka (Buchs, Switzerland).

Calculations

Oxygen and glucose consumptions were calculated from arterio-venous concentration differences (ΔC), the corresponding flow rates and the dry weight (DW) of the entire small intestine used in the experiment and given as fluxes (µmol x min^{-1} x (g dry intestine)$^{-1}$, means ± SD) according to the following equation:

$$\text{Flux} = \frac{\Delta C\ [\text{nmol mL}^{-1}] \times \text{flow}\ [\text{mL min}^{-1}]}{\text{DW}\ [\text{g}]}$$

Statistical differences of absorption rates and viability parameters were determined using student's t-test for unpaired observations. P values less than 0.05 were considered to indicate significant differences.

Results

In control perfusion experiments with anthocyanin-free basic perfusion media, neither cyanidin-3-glucoside nor anthocyanin metabolites were detected. Stability of cyanidin-3-glucoside in the luminal (95.5 ± 6.8 %, n = 3) and vascular perfusate (91.6 ± 13.6 %, n = 3) was confirmed for 1.5 h at 37 °C. The recovery of cyanidin-3-glucoside from the perfusates and the tissues was nearly complete.

All organ preparations were viable during the perfusion experiments, confirmed by repeatedly measuring oxygen uptake, glucose-lactate handling and acid-base homeostasis. No significant differences were observed in comparing viability data from experiments with and without ethanol[1].

In both experimental groups, most of the luminally administered cyanidin-3-glucoside left the organ preparation via luminal efflux (Table 1). The main compound in the luminal and vascular effluent was unchanged cyanidin-3-glucoside. Small amounts of cyanidin-3-glucoside were absorbed in the conjugated form. Minor amounts of the applied substance and its conjugates were found in the gut tissue extracts. Enzymatic cleavage of perfusates and tissue extracts with glucuronidase yielded an increase in cyanidin-3-glucoside concentration. Sulfatase cleavage following glucuronidase incubation only yielded slightly higher cyanidin-3-glucoside concentrations in the intestinal tissue (Table 1). The enzymatic treatments indicate that the conjugates were predominantly glucuronides. However, LC-ESI-MS analyses enabled neither characterization of a sulfate nor a glucuronide-conjugate.

Absorption rates of cyanidin-3-glucoside with (4.3 ± 3.2 %) and without ethanol (2.9 ± 1.8 %) were not significantly different. The recovery over five experiments with ethanol was 73.2 % and 68.8 % without ethanol, respectively.

1 Viability parameters (means ± SD, n = 5): perfusion experiments without and with ethanol: oxygen consumption: 4.7 ± 1.0 (5.3 ± 1.2) µmol x min^{-1} x g^{-1}, lactate-pyruvate ratio: 37.9 ± 16.4 (32.5 ± 14.4); glucose consumption: 6.1 ± 2.4 (6.6 ± 2.8) µmol x min^{-1} x g^{-1}; arterial pressure 67 ± 6 (68 ± 11) mm Hg; arterial pH: 7.51 ± 0.03 (7.48 ± 0.02); venous pH: 7.39 ± 0.04 (7.34 ± 0.04).

Table 1 Distribution of cyanidin-3-glucoside and its conjugates in the luminal, vascular and tissue compartments after perfusion experiments of 1 h with the isolated rat small intestine[1]. N. D. is under detection limit

	Without ethanol		With ethanol	
	nmol ± SD	% ± SD[2]	nmol ± SD	% ± SD[3]
Luminal loss cyanidin-3-glucoside	2197.7±402.7	59.1±11.7	2580.0±507.1	66.8±6.1
glucuronides	251.9±363.9	6.5±9.9	27.0±60.3	1.6±2.8
sulfates	N. D.		N. D.	
total	2449.6±515.6	65.6±13.2	2607.0±473.7	68.4±9.0
Vascular uptake cyanidin-3-glucoside	100.9±64.4	2.7±1.5	118.4±66.3	3.6±2.4
glucuronides	9.0±15.5	0.2±0.4	14.3±22.9	0.7±0.9
sulfates	N. D.		N. D.	
total	109.9±79.1	2.9±1.8	132.7±84.0	4.3±3.2
Intestinal tissue cyanidin-3-glucoside	11.1±6.1	0.3±0.2	9.8±8.7	0.3±0.2
glucuronides	N. D.		3.1±2.9	0.1±0.1
sulfates	N. D.		1.6±2.2	0.1±0.1
total	11.1±6.1	0.3±0.2	14.5±11.0	0.5±0.2
Blood vessels cyanidin-3-glucoside	1.2±0.8	0.04±0.03	1.3±1.2	0.03±0.03
glucuronides	0.2±0.4	0.004±0.01	0.1±0.1	0.002±0.004
sulfates	N. D.		N. D.	
total	1.4±1.0	0.04±0.03	1.4±1.1	0.03±0.03
recovery	2572.0±527.4	68.8±13.0	2755.6±445.1	73.2±8.2

[1] 4294.0 ± 1308.2 nmol of cyanidin-3-glucoside with 5 % ethanol and 3780.2 ± 782.3 nmol of cyanidin-3-glucoside without ethanol in luminal media were applied, each in five perfusion experiments
[2] Based on the mean dosage of 3780.2 ± 782.3 nmol
[3] Based on the mean dosage of 4294.0 ± 1308.2 nmol

Discussion

To assess the influence of ethanol on intestinal absorption and metabolism of cyanidin-3-glucoside, we used an isolated preparation of a vascularly and luminally perfused rat small intestine, characterized by fully maintained tissue viability. Intact mucosal morphology without loss of villous tip cells after 120 min perfusion has previously been demonstrated in histologic specimens of the isolated intestinal preparations perfused in the same way as in the present study [18]. As in earlier studies, viability and functional integrity of the intestinal preparation were carefully and continuously controlled [14, 15]. The functional integrity of the intestinal preparation was monitored and the viability confirmed in agreement with data from earlier studies using the same model [16, 19, 20].

Control perfusions with basic media without cyanidin-3-glucoside confirmed that the small intestine of the experimental animals did not contain any anthocyanins sequestered from food. It is also to be emphasized that in the present study the administered amount of cyanidin-3-glucoside (3780 and 4294 nmol equivalent to 1.70 and 1.93 mg) was in the physiological range and might be easily taken up by a normal meal (30 mg of elderberry [21], 2 mL of blood orange juice [22] or 5 g of sweet cherries [23].

The incomplete total recoveries of cyanidin-3-glucoside after perfusion experiments (about 70 %) was also observed in experimental studies with anthocyanins [24]. Cyanidin-3-glucoside might be metabolized to substances which escape electrochemical detection under the present conditions. In most anthocyanin studies no recoveries were indicated [6–11]. The incomplete recovery and moreover the low absorption rates might explain lack of quantitative data in the literature concerning absorption of anthocyanins.

The low absorption rate of cyanidin-3-glucoside and conjugates in the present study (2.9 % and 4.3 %, without and with ethanol, respectively) is in good agreement with earlier observations gained from human feeding experiments performed with mixtures of anthocyanins from red wine ranging from 1 % to 6.7 % [8]. In feeding experiments with rats, bioavailability of an anthocyanine mixture from *Vaccinium myrtillus* extracts was calculated somewhat lower (1.2 %) [6]. In contrast to these and to our results, absorption rate of pure cyanidin-3-glucoside [9–11] and anthocyanins from elderberries [24] in human and animal studies was estimated to be extremely low.

From human studies, it was concluded that absorption of aglycones is enhanced by conjugation with glucose, possibly by absorption via the sodium-dependent glucose transport system (SGLT1) [25]. Absorption of anthocyanidin glycosides from black currant was suggested to occur through the hexose transport pathway [10]. In contrast, the 7-glucoside of genistein, an isoflavone, was absorbed far less than the aglycone and a passive diffusion for the transepithelial transport was proposed [19, 20]. For cyanidin-3-glucoside absorption, both transport processes are conceivable.

It is generally proposed that flavonoid glycosides are hydrolyzed to the corresponding less polar aglycones prior to gastrointestinal absorption [26–28] and an ability of mammals to hydrolyze flavonoid glycosides to the corresponding aglycones has been repeatedly reported [20, 29–31]. However, cyanidin-3-glucoside was not hydrolyzed by small intestinal β-glucosidase and only traces (at the detection limit) of the aglycone cyanidin in a vascular perfusate were found. These results are in line with recent observations from human and animal studies [9, 11].

Conjugates of cyanidin-3-glucoside were analyzed after enzymatic cleavage. It must be emphasized that none of the enzymes, neither the glucuronidase from *E. coli* nor the glucuronidase-sulfatase from *Helix pomatia*, were able to cleave the β-glucoside cyanidin-3-glucoside, despite an apparent β-glucosidase activity of these enzymes [20]. Our observations were confirmed by incubation experiments with cyanidin-3-glucoside and β-glucosidase [11]. Irrespective of the presence of ethanol, enzymatic cleavage of perfusates and tissue extracts with glucuronidase yielded an increase in cyanidin-3-glucoside concentration, which was not significantly different between the experimental groups. Subsequent incubation with glucuronidase-sulfatase did not further increase cyanidin-3-glucoside concentration. Therefore, we suppose formation of glucuronide or diglucuronide conjugates during perfusion experiments. With LC-ESI-mass spectrometry, however, we could not identify any such metabolites. Metabolites of cyanidin-3-glucosides have been also observed in an animal study [9], but so far no glucuronide conjugates have been characterized.

It has been supposed that the presence of ethanol in red wine might improve flavonoid availability [12]. It is conceivable that ethanol affects absorption by solubilizing anthocyanins or acting as a mucosal barrier breaker [32, 33]. In the ethanol experiments, cyanidin-3-glucoside was solubilized in the luminal medium spiked with 5 % ethanol. Wine contains about 12 % ethanol, which is diluted during the digestion process. To investigate effects of ethanol on intestinal handling, we decided therefore, to work with a 5 % ethanolic solution, as a convenient basis.

Our experiments clearly indicate that ethanol has no significant influence on absorption of the anthocyanin cyanidin-3-glucoside. For other phenolic compounds similar observations were reported. Human studies with dealcoholized and reconstituted red wine indicated that coingestion of ethanol did not affect extent of catechin [34, 35], caffeic, protocatechuic and 4–0-methyl gallic acid absorption [36].

Cyanidin-3-glucoside, this potent pharmacologically active plant component, is absorbed in the small intestine, mainly as intact β-glucoside. Yet, the extent of absorption is low. The present study under well-controlled experimental conditions reveals for the first time that ethanol has no influence on the amount of cyanidin-3-glucoside absorbed. However, low absorption rates of anthocyanins obviously seem to be high enough to exert beneficial physiological activity, reported from feeding studies [3–5].

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