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ORIGINAL ARTICLE

Phloridzin improves absorption of genistin in isolated rat small intestine

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Summary *Background & aims*: Cancer-protective effects of isoflavones like genistin or genistein are well known. High intakes and an adequate absorption rate of isoflavones are necessary for efficient chemoprevention, though other dietary agents might increase absorption efficacy. The aim of this study was to investigate the effect of phloridzin, an inhibitor of the sodium-dependent glucose transporter (SGLT1), on genistin absorption and metabolism.

Methods: Phloridzin and genistin were luminally administered 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 oxygen carrier. Luminal media consisted of a bicarbonate buffered sodium chloride solution spiked with genistin ($24.5\,\mu$mol/l) and phloridzin (1 mmol/l).

Results: In previous experiments, genistin absorption rate of 17.2% has been observed. In the present study, phloridzin administered simultaneously with genistin, increased genistin uptake 2.5 fold (44.5%).

Conclusion: The naturally occurring substance phloridzin, present in apples, thus considerably amplify genistin absorption. These effects might offer a promising novel method in designing functional foods for cancer prevention by combining genistin- and phloridzin-containing foods.

Introduction

Ovarian hormones have important stimulating influences on breast and prostate cancer development.[1] High concentrations of blood estrogen and increased urinary excretion rates are associated with increased breast cancer risk.[2,3] Epidemiological and experimental studies suggest that consumption of isoflavone-containing food is associated with reduced rates of breast and prostate cancer.[4–7] Isoflavones are weakly estrogenic[6,8] and compete with endogenous estrogens for β-receptor binding.[4,9] Accordingly, isoflavones are currently advocated in the prevention of

hormone-dependent cancers.[10,11] However, high intakes and an adequate absorption rate of isoflavones would be necessary for efficient chemoprevention. It might be conceivable that other dietary agents can reduce the amounts of isoflavones needing to be eaten if they increase the efficacy of isoflavone absorption and disposition. Before searching for such compounds we decided to characterize the small intestinal handling of genistin (genistein glucoside) in an isolated perfused rat organ model, in order to determine its baseline absorption rate.[12] In the present study, we were particularly interested in discovering, by use of the sugar transport inhibitor phloridzin, whether genistin is absorbed via the sodium-dependent glucose carrier system SGLT1. An absorption of quercetin glucosides via this glucose transporter has recently been suggested.[13] To test this hypothesis for the glucoside genistin, we investigated small intestinal handling of genistin in the presence of phloridzin. If genistin is actually absorbed via this transporter, absorption rate in the presence of phloridzin should dramatically decrease compared to baseline absorption rates which were investigated in a previous study using the same intestinal model.[12]

Materials and methods

Animals

Three male Sprague-Dawley rats (CD-rats, Charles River, Sulzfeld, Germany), 30-day old and weighing about 130 g were fed a cornstarch-based isoflavone-free synthetic diet (Altromin C-1000, Altromin International GmbH, Lage, Germany) for 14 days to eliminate isoflavones from their bodies. This was confirmed in perfusion experiments with basic luminal media. Animals were provided with free access to tap water and food. At perfusion experiments, animals weighed 217 ± 14.2 g.

Vascularly and luminally perfused rat small intestine

The small intestine was prepared as described elsewhere.[14–16] The luminal media was spiked with 24.5 µmol/l genistin and 1 mmol/l phloridzin. Luminal flow rates were 0.5 ml/min.

Oxygen uptake and acid-base homeostasis (Clark pO_2-electrode and pH-electrode integrated in an ABL 30 Acid–Base Analyzer; Radiometer, Copenhagen, Denmark) were carefully controlled. 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 associated blood vessels were harvested for analyses of genistin, genistein and their conjugates with RP-HPLC with UV and MS detection after sample preparation as described below.

Vascular samples: Of each vascular sample, 2 ml were spiked with internal standard 4-nitrophenol (50 µl of a 170 µM solution) and prepared as described earlier.[17] Genistin and genistein exhibited a recovery of $100.1 \pm 3.9\%$ and $97.2 \pm 3.0\%$, respectively (means $\pm$ SD).

Luminal samples: As internal standard 4-nitrophenol (20 µl of a 23.5 mmol/l solution) was added to the luminal effluent of a 10 min period and prepared as described earlier.[17] Genistin and genistein recovery was $100.3 \pm 1.8\%$ and $99.9 \pm 1.2\%$, respectively (means $\pm$ SD).

Small intestinal tissue and blood vessels: After lyophilization, the tissue was powdered using a pestle and a mortar, and defatted by duplicate extraction with 10 ml hexane. The pellet was extracted three times with 3 ml (1 ml in case of blood vessels) methanol/acetic acid (3%) (1:1) and centrifuged at 2800g for 20 min. The extracts were pooled and the volume adjusted to 10 ml (3 ml in case of blood vessels). Genistin and genistein recovery was $100.4 \pm 3.9\%$ and $100.0 \pm 4.8\%$, respectively (means $\pm$ SD) for small intestinal tissue and $99.4 \pm 4.4\%$ and $96.4 \pm 9.9\%$, respectively (means $\pm$ SD) for blood vessels.

Clean up for LC-MS identification of genistin and genistein conjugates: 12 ml of the vascular perfusate were acidified (hydrochloric acid) to pH 6.5 and centrifuged at 11 600g for 30 min. The clear supernatant was evaporated to 3 ml at 22°C. After addition of 60 µl phosphoric acid (16 mol/l), the concentrate was drawn through a non-conditioned Nexus® column (Varian GmbH, Darmstadt, Germany) under low vacuum. After column rinsing with 1 ml water, the conjugates were eluted with 2 ml methanol. The eluate was diluted with 0.25 ml of water and then concentrated under a gentle flow of

nitrogen to 0.5 ml. This concentrate was used for LC-MS analysis.

Analytical procedures

Gradient HPLC-system with UV detection: The HPLC-system (Sykam, Gilching, Germany) consisted of a micro-solvent delivery system S 1100, a low-pressure gradient mixer S 8110, equipped with an autosampler (Spark Triathlon, Emmen, The Netherlands; 50 µl filling loop). Gradient control and continuous on-line monitoring and data quantitation were performed with Pyramid-Software (Axxiom Chromatography, Moorpark, CA). UV-absorbance was monitored with a UVIS 200 (Linear, Freemont, CA) at 262 nm with a flow cell of 10 µl. A 125 mm long, 2.0 mm i.d. Grom-Sil ODS-3 (particle size 3 µm) column was used (Grom, Herrenberg, Germany). The column was at 40°C (column oven S 4110; Sykam, Gilching, Germany), with a flow rate of 0.3 ml/min. The eluents were composed of 0.2% acetic acid in H_2O (A) and 0.2% acetic acid in acetonitrile (B). The elution conditions were as follows: 0–2 min 5% B, 2–9 min 5–15% B, 9–20 min 15–60% B, 20–22 min 60–5% B. An injection volume of 25 µl genistin and genistein solution resulted in a detection limit of 11 and 5 nmol/l and a quantitation limit of 20 and 9 nmol/l, respectively.

Gradient HPLC-system with MS-detection (LC-MS): For the identification of genistin and genistein conjugates we used a gradient HPLC-system series 1100 (Hewlett Packard, Böblingen, Germany) combined with a MS-detector in the ESI-mode (Micro Mass Platform II, Mass Lynx 4.0, Manchester, UK) was used. Source temperature was maintained at 120°C, cone voltage at 45 V and acceleration lens potential at 0.5 kV, respectively. Negative ion characterization was performed in the m/e range of 120–800 at a scan rate of 0.5 scans/s, and a multiplier voltage of 650 V. Separation was carried out in a Inertsil ODS-2 column (250 mm × 4.6 mm, 5 µm, VDS-Optilab, Berlin, Germany) at 40°C, with a flow rate of 0.9 ml/min using ammonium formiate buffer (5 mM, solvent A) and acetonitrile:100 mM ammonium formiate (95:5, solvent B). The elution conditions were as follows: 0–2 min 5% B, 2–9 min 5–25% B, 9–15 min 25–52% B, 15–28 min 52–70% B, 28–30 min 70–5% B. Injection volume was 70 µl.

Cleavage of isoflavone conjugates: Genistin and genistein conjugates were analyzed as genistein after enzymatic cleavage according to Sfakianos et al.[18] with modifications as described below. 0.25 ml of a potassium phosphate buffer (0.2 mol/l, pH 6.8 for glucuronidase and pH 7.1 for sulfatase) and 0.1 ml glucuronidase solution (220 Fishman

units) or 0.02 ml arylsulfatase solution (0.3 units), respectively, were added to 0.5 ml sample solution.

The applicability of the enzymatic cleavage in cleaned-up fluorocarbon emulsion was confirmed by the conversion of 4-nitrophenol glucuronide and 4-nitrophenol sulfate with β-glucuronidase and arylsulfatase, respectively. The cleavage of 4-nitrophenol glucuronide resulted in 4-nitrophenol recovery of 100.1%, the cleavage of 4-nitrophenol sulfate resulted in a recovery of 99.2%.

Chemicals and solvents

All chemicals used were of analytical grade. Solvents for HPLC-analysis were of HPLC-grade. Genistin was purchased from Extrasynthese (Genay, France), Phloridzin from Fluka (Buchs, Switzerland), Genistein and β-glucuronidase from Sigma-Aldrich (Steinheim, Germany).

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 ($\mu mol \times min^{-1} \times$ (g dry intestine)$^{-1}$, means $\pm$ SD) according to the following equation:

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

Viability parameters and absorption rates of genistin and its metabolites were compared using multivariated ANOVA and subsequent Student's *t*-test for unpaired observations. *P* values less than 0.05 were considered as significant differences.

Results

Genistin and genistein were stable in the luminal and vascular perfusate for 10 h at 37°C. 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 genistin/phloridzin and genistin perfusion experiments*.[15]

Table 1 Distribution of genistin, genistein and genistein glucuronide in the luminal, vascular and tissue compartments after perfusion experiments with isolated rat small intestine with phloridzin*.

	nmol	%[†]
Luminal loss		
Genistin	220.5 ± 46.4	30.0 ± 6.3
Genistein	156.9 ± 27.4	21.3 ± 3.7
Glucuronide	4.1 ± 4.1	0.5 ± 0.6
Total	381.5 ± 22.1	51.8 ± 3.0
Vascular uptake		
Genistin	64.9 ± 15.7	8.8 ± 2.1
Genistein	181.2 ± 30.9	24.6 ± 4.2
Glucuronide	81.4 ± 18.1	11.1 ± 2.5
Total	327.5 ± 39.1	44.5 ± 5.3
Intestinal tissue		
Genistin	9.3 ± 3.0	1.2 ± 0.4
Genistein	26.8 ± 10.6	3.7 ± 1.4
Glucuronide	11.4 ± 4.0	1.6 ± 0.5
Total	47.5 ± 16.5	6.5 ± 2.2
Blood vessels		
Genistin	1.6 ± 0.2	0.2 ± 0.0
Genistein	3.7 ± 1.7	0.2 ± 0.0
Glucuronide	2.4 ± 1.4	0.3 ± 0.2
Total	7.7 ± 0.1	1.0 ± 0.0
Recovery	764.2 ± 33.6	103.8 ± 4.6

*735.9 ± 0.0 nmol of genistin simultaneously with 30 μmol phloridzin were applied in three perfusion experiments of 60 min. Values are given as means ± SD.
[†]Based on the mean dosage of 735.9 ± 0.0 nmol.

In experiments with genistin/phloridzin, only half of the luminally administered genistin was lost via the luminal perfusate (Table 1), the major components being genistin, followed by genistein and genistein glucuronide. At the vascular side, 44.5% of applied genistin was found. Genistein was the major component, followed by genistein glucuronide and unmetabolized genistin. Glucuronidation rate during perfusion experiments was 13.4%. Genistein glucuronide was preferentially absorbed to the vascular side. Minute amounts of the genistin administered were located in the intestinal tissue and the blood vessels. Figure 1 gives a schematic presentation of the small intestinal handling of genistin applied simultaneously with phloridzin.

Genistein glucuronide and genistin in the vascular effluent were verified by LC-MS. Characteristic fragment ions of genistin were the molecular ion [M–H]− at m/e 431 and the aglycone ion [genistein–H]− at m/e 269. The mass spectra of genistein

glucuronide showed the molecular ion [M–H]− at m/e 445 and the signal for the aglycone fragment [M–glucuronic acid–H]− as base peak at m/e 269.

The results of the present study were compared with the results gained from experiments with pure genistin given in Table 2.[12] The amounts of glucuronides formed in the presence of phloridzin (13.5%) were significantly lower than observed with genistin only (36.6%). In this study with phloridzin, nearly all of the genistein glucuronide formed was transported to the vascular side, whereas in experiments with genistin only, the glucuronide conjugate was preferentially secreted into the luminal perfusate. Total genistein absorption significantly increased about 250% compared to results of experiments without phloridzin.

Discussion

Viability of the isolated rat small intestine has been proven for over 120 min.[14] Each of the six perfusion experiments was done with a freshly isolated rat small intestine, to ensure viability of the organ preparation.

During the experiments tissue viability was maintained and mucosal morphology remained intact without loss of villous tip cells, indicating that the results are physiologically robust.

Importantly, the administered amounts of genistin (735.9 nmol) was in the physiological range corresponding well to its usual nutritional delivery in food (about 4 g of tofu or 6 g of tempeh[19]) at normal dietary intakes in the Western world. For a reliable inhibition of the SGLT1 we applied phloridzin, an apple ingredient, in concentrations of 1 mmol/l.

In experiments with everted intestinal sac preparations, transepithelial transport of numerous phenolic monoglucoside conjugates by the SGLT1 was confirmed.[20–22] It is known that phloridzin completely inhibits active transport of glucose and glycoside conjugates by SGLT1,[20,23] even at lower concentrations like 0.5 mmol/l.[24] And indeed, by implicating phloridzin we intended to inhibit the SGLT1, in order to avoid a SGLT1-mediated transport of the β-glucoside genistin. According to the original hypothesis, we should have expected a lower absorption of genistin in the presence of phloridzin. Yet, surprisingly total genistein absorption significantly increased about 250% compared to results of experiments without phloridzin (Fig. 2). This means that promotion of genistin transport by the SGLT1 carrier system cannot explain values for the higher absorption rates of genistin and its
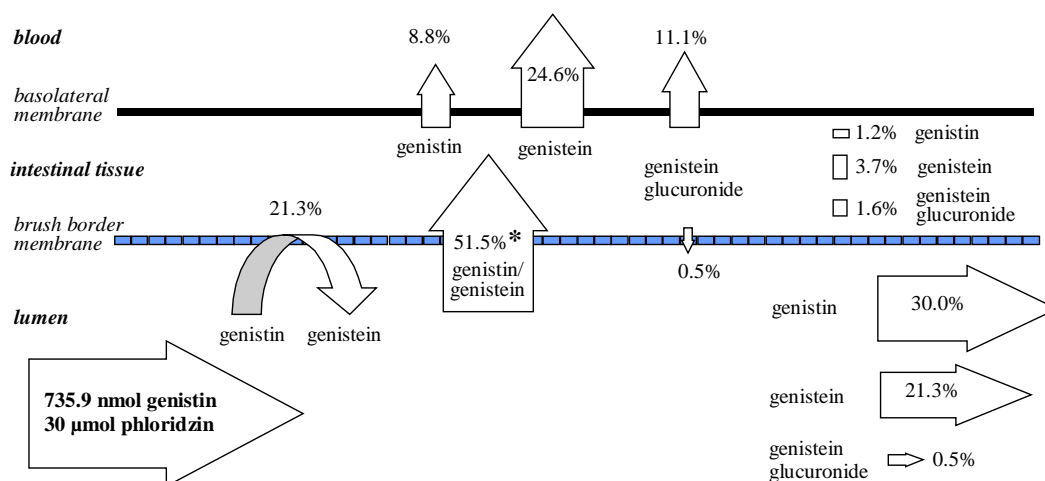
Figure 1 Schematic presentation of the small intestinal handling of genistin applied simultaneously with phloridzin. Values are means of three experiments. Recovery of genistein compounds from three experiments was $103.8 \pm 4.6\%$. Genistin was presumably hydrolyzed (21.3%) in the lumen by enzymes bound to the brush border membrane (e.g. lactase phloridzin hydrolase). *Value calculated from absorbed, secreted and tissue located genistein compounds.

Table 2 Distribution of genistin, genistein and genistein glucuronide in the luminal, vascular and tissue compartments after perfusion experiments with isolated rat small intestine without phloridzin 0* (modified from Andlauer et al.[12]).

	nmol	%†
Luminal loss		
Genistin	306.6 ± 58.5	43.0 ± 8.0
Genistein	112.9 ± 33.7	15.8 ± 4.7
Glucuronide	151.2 ± 37.6	21.2 ± 5.4
Total	570.7 ± 52.0	80.0 ± 6.7
Vascular uptake		
Genistin	6.1 ± 3.2	0.9 ± 0.4
Genistein	10.6 ± 2.4	1.5 ± 0.3
Glucuronide	105.9 ± 15.2	14.8 ± 2.2
Total	122.6 ± 17.6	17.2 ± 2.6
Intestinal tissue		
Genistin	0.6 ± 0.1	0.1 ± 0.0
Genistein	5.5 ± 2.0	0.8 ± 0.3
Glucuronide	3.1 ± 0.9	0.5 ± 0.2
Total	9.2 ± 2.9	1.4 ± 0.4
Blood vessels		
Genistin	0.06 ± 0.00	0.01 ± 0.01
Genistein	0.08 ± 0.05	0.01 ± 0.01
Glucuronide	0.73 ± 0.24	0.10 ± 0.03
Total	0.87 ± 0.26	0.12 ± 0.04
Recovery	703.4 ± 31.9	98.7 ± 3.9

*713.1 ± 3.6 nmol of genistin were applied in three perfusion experiments of 60 min. Values are given as means $\pm$ SD.
†Based on the mean dosage of 713.1 ± 3.6 nmol.
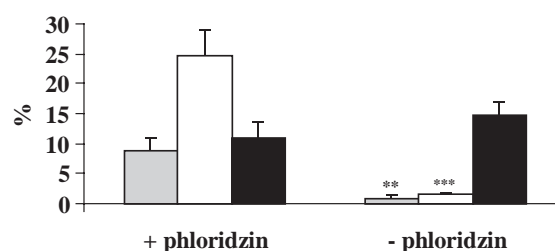
Figure 2 Genistin, genistein and genistein glucuronide uptake (gray, white and black bars, respectively) to the vascular side during 60 min of perfusion. Luminally, 713.1 ± 3.6 nmol genistin were applied without phloridzin (−phloridzin[12]) and 735.9 ± 0.0 nmol simultaneously with 30 μmol phloridzin (+ phloridzin), each in three perfusion experiments. Significant differences were observed for absorption of genistin ($P < 0.01$) and genistein ($P < 0.001$).

metabolites in the presence of phloridzin. It has been shown from work in isolated cells that genistin is not transported from the apical to the basolateral side, whereas it was actively transported in the opposite direction,[25] suggesting secretion of genistin, possibly by the P-glycoprotein and the multidrug resistance protein (MRP). Phloridzin or its aglycone phloretin, which we have detected as an intestinal metabolite of phloridzin (data not shown), might inhibit these transport proteins in the brush border membrane, an interaction which has been repeatedly reported for other flavonoids.[26,27] The evidence that phloridzin specifically influence these transport proteins has to be established by further experimental studies.

The amounts of glucuronides formed in the presence of phloridzin (13.5%) were significantly lower than observed with genistin only (36.6%) (Fig. 2). In rat everted small intestine, similar observations were made for 4-nitrophenol, acetaminophen and 1-naphthol.[28] Consequently, a competitive inhibition of glucuronidation by phloridzin and its intestinal metabolite phloretin was suggested. In this respect, it is to note that isoflavone glucuronides were reported to be physiologically less active than the non-conjugated aglycones.[29]

In the present study with phloridzin, nearly all of the genistein glucuronide formed was transported to the vascular side, whereas in experiments with genistin only, the glucuronide conjugate was preferentially secreted into the luminal perfusate.[12] For the transport of anionic compounds like phenol glucuronides, the involvement of the MRP transporter has been repeatedly confirmed.[30–32] Consequently, the high efflux of glucuronide[12] but also that of genistein glucoside[25] might seriously limit total intestinal absorption of genistin. Therefore, natural food ingredients like phloridzin or phloretin which decrease glucuronidation rate and inhibit secretion of genistin and its metabolites may dramatically promote absorption rate of isoflavones. These effects might offer a promising novel method in designing functional foods for cancer prevention by combining isoflavone- and phloridzin-containing foods like soy and apple. A functional food based upon soy and apple might have useful nutritional advantages. Folk wisdom suggests that an apple a day keeps the doctor away— but how much better might be apple and soy together?

Acknowledgements

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