Absorption and Metabolism of Genistein in Isolated Rat Small Intestine

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ABSTRACT Studies suggest a variety of biological effects for the isoflavonoid genistein, but there is little information regarding small intestinal absorption and metabolism. The aim of this study was to investigate the absorption and metabolism of luminally administered genistein in an isolated preparation of luminally and vascularily 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 genistein (12 μmol/L). Viability was maintained during the entire perfusion as indicated by no significant differences between genistein and control perfusions for perfusion pressure, lactate-pyruvate ratio, oxygen uptake and acid-base homeostasis. Luminal disappearance rate of genistein did not change throughout the entire perfusion time. After a significant increase until about 30 min, vascular appearance rate of total genistein reached an equilibrium. The intestinal absorption of luminally administered genistein was 46.6%, corresponding to an average uptake of 2.9 nmol · min⁻¹ · g dry intestine⁻¹. The majority (31.3%) of the absorbed genistein appeared as genistein glucuronide, also recovered as the main metabolite on the luminal side (13.3%). Only small amounts of genistein (26.6%) and genistein glucuronide (2.9%) were found in the intestinal tissue. The results demonstrate a favorable uptake of genistein, a pertinent addition to the ongoing discussion about health benefits of isoflavones. J. Nutr. 130: 843–846, 2000.

KEY WORDS: • intestinal absorption • genistein • isoflavone • intestinal metabolism • rats

Isoflavones, major dietary components of soybeans (Barnes et al. 1994a, Wang and Murphy 1994), have recently received great attention because of their proposed health-related and clinical benefits like estrogen receptor binding (Adlercreutz 1990, Barnes et al. 1994b) and natural killer cell activation (Zhang et al. 1999). Soy and its bioactive component, genistein (4’,5,7-trihydroxyisoflavone), have been the subject of numerous investigations (Coward et al. 1993, Herman et al. 1995, Record et al. 1995, Wei et al. 1995).

Intestinal absorption is a prerequisite for a possible causal relationship between genistein intake and its proposed chemopreventive action. Available research has chiefly been directed to examinations of urinary, biliary and fecal excretion (Kelly et al. 1995, King et al. 1996, Sfakianos et al. 1997). Information on in vivo absorption and metabolism of isoflavones is scarce.

The aim of this study was to evaluate intestinal absorption and metabolism of genistein, employing an isolated rat small intestine perfusion model (Hummel et al. 1997, Plauth et al. 1991). This 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) were obtained from Charles River (Sulzfeld, Germany). Rats were fed a cornstarch-based isoflavonoid-free synthetic diet (Altromin C-1000; Altromin International GmbH, Lage, Germany) for 14 d to allow elimination of circulating genistein from the previously offered soy-based diet. Rats were provided with free access to tap water and food.

Vascularly and luminally perfused rat small intestine. The small intestine (duodenum, jejunum, ileum) was prepared in rats as described elsewhere (Hartmann et al. 1984, Plauth et al. 1991). Briefly, small intestine was prepared in six rats for perfusion with genistein (n = 3, weight 223 g ± 8.9) and for control perfusions with basic luminal media (n = 3, 229 g ± 7.8) in narketan® (Chassot AG, Bern, Germany)-xylazin® (Vetimex, Bladel, Netherlands) narcosis after overnight food-deprivation. After cannulation of the superior mesenteric artery and the portal vein, the small intestine was vascularly perfused with an artificial oxygen carrier (vide infra). Subsequently, the intestine was excised, intestinal lumen was cannulated and rinsed free with warm saline (155 mmol/L NaCl). The isolated small intestine was transferred to a warmed tissue bath (37°C) and allowed to equilibrate for 30 min. The experiment was started after filling the intestinal lumen with a 7 mL bolus of luminal media (126 μmol/L genistein, none in the case of controls, respectively, and 135 mmol/L NaCl, 20 mmol/L NaHCO₃, pH 7.2) as described elsewhere (Hartmann et al. 1984, Plauth et al. 1991). The isolated small intestine was transferred to a warmed tissue bath (37°C) and allowed to equilibrate for 30 min. The experiment was started after filling the intestinal lumen with a 7 mL bolus of luminal media (126 μmol/L genistein, none in the case of controls, respectively, and 135 mmol/L NaCl, 20 mmol/L NaHCO₃, pH 7.2) with sampling over 60 min. Perfusion was carried out according to single-pass technique. In this mode the flow rates were 5 mL/min vascularly (venous) and 0.5 mL/min luminally.

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 poloxypolypropylene-poloxoxyethylenecopolymer (Phuronic, F-68®, 25.6 g/L; ABTS, Boehringer Mannheim, Germany) in sterile, pyrogen-free water, using a high-pressure homogenizer (Mounot-Gaulin LAB 60/60–10TBS, APV Gaulin GmbH, Lübeck, Germany) to an average diameter of 0.2 μm.
The viability of the model was carefully controlled by repeatedly measuring oxygen uptake and acid-base homeostasis using a Clark pO2-electrode and a 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®), 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 ethical committee of the Regierungspräsidium Stuttgart, Germany.

Sampling and sample preparation. Arterial, venous and luminal aliquots were taken every 10 min and analyzed for genistein and genistein conjugates with RP-HPLC after sample preparation as described below. At the end of the study, the entire portion of the isolated small intestine as well as the blood vessels were obtained for analyses of genistein and its conjugates.

Vascular samples. Of each vascular (venous) sample, 2 mL was centrifuged at 2800 x g for 40 min. The supernatant was separated, and the pellet was extracted with 0.4 mL ethanol by sonication for 20 min and centrifuged at 2800 x g for 20 min. The unified supernatants were analyzed by HPLC.

Small intestinal tissue. After lyophilization, the tissue was powdered using a pestle and a mortar, and defatted by extraction with 10 mL hexane twice. The supernatants were joined and extracted with methanol to rule out any loss of genistein. The pellet was extracted three times with 4 mL methanol/water (1:1) and centrifuged at 2800 x g for 20 min. The extracts were pooled and adjusted to 13 mL. Genistein recovery was 100.0 ± 4.8% (means ± SD, n = 3).

Blood vessels. They were lyophilized and defatted like the small intestinal tissue. Genistein was extracted three times according to the intestinal tissue, but only with 2 mL of methanol/water. The extracts were pooled and adjusted to 7 mL. Genistein recovery was 94.6 ± 9.9% (means ± SD, n = 3).

Analytical procedures. HPLC: 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 Triohanol, 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. Isocratic elution of genistein with an elution time of about 65 min was achieved with water/acetonitrile/tetrahydrofuran/formic acid (69.5:20:8.5:2) as eluent. An injection volume of 25 μL genistein solution resulted in a detection limit of 10 nmol/L and a quantitation limit of 30 nmol/L, respectively.

Cleavage of genistein conjugates. Genistein conjugates such as glucuronides and sulfates were analyzed as genistein after enzymatic cleavage according to Sfakianos et al. (1997), 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 glucuronide solution (220 Fishman units) or 0.02 mL arylsulfatase solution (0.3 U), 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. Genistein and the enzymes β-glucuronidase and arylsulfatase were obtained from Sigma-Aldrich (Deisenhofen, Germany).

Calculations. Fluxes (nmol·min⁻¹·g dry intestine⁻¹, means ± SD), were calculated from arterio-venous and proximo-distal concentration differences (ΔC), respectively, the corresponding flow rates and the dry weight (DW) of the entire small intestine used in the experiment according to the following equation:

\[
\text{Flux} = \frac{\Delta C \times \text{flow} \times \text{DW}}{\text{g}}
\]

Positive fluxes or rates denote appearance and negative disappearance.

Statistical evaluations were performed by ANOVA and subsequent Student's t test of the paired observations. P-values < 0.05 were considered to indicate significant differences.

RESULTS

With genistein-free basic perfusion media (control), no genistein was detected. Genistein was added to the luminal and vascular perfusate to verify stability. No genistein degradation was observed after 5 h at 37°C.

Viability of the organ preparation was confirmed in all perfusion studies by repeatedly measuring oxygen uptake, glucose-lactate handling and acid-base homeostasis. No significant differences in viability data could be observed between genistein and control perfusion experiments.

The luminal disappearance rate of genistein was higher than the vascular appearance rate, resulting in a net disappearance of 0.3 nmol·min⁻¹·g⁻¹. The luminal disappearance rate showed no significant differences over the entire perfusion time (Fig. 1A). However, the transfer of the free and glucuronidated genistein from the gut tissue to the vascular side revealed a steady, significant increase until about 30 min when an apparent steady state was established (Fig. 1B). Consequently genistein was enriched in the intestinal tissue preparation during the first 30 min of the perfusion time. After steady state was approached (30 min), luminal disappearance and vascular appearance rates were well-balanced. The flux (secretion) of genistein glucuronide back to the luminal side (Fig. 1C) peaked after a significant increase between 20 and 30 min to be decreased significantly during the remaining period. In all perfusion periods, the vascular appearance rate of genistein glucuronide was about two times the value of its luminal appearance rate.

In a single pass through the isolated small intestine, 46.4% of the luminally administered genistein was extracted, calculated as the difference from the administered genistein and the luminal loss (Table 1). Of the administered genistein, 40.6% appeared at the vascular side; 5.5% was located in the intestinal tissue; and only small amounts were found in the blood vessels (0.1%).

Most of the absorbed genistein appeared vascularity as glucuronide (31.3% of administered genistein), less as free genistein (9.3%). In the luminal effluent, free genistein (40.3%) prevailed glucuronidated genistein (13.3%). In the intestinal tissue, free and glucuronidated genistein were...
present in similar amounts (both about 3%). No genistein-sulfate conjugates could be identified by conversion of luminal and vascular perfusates as well as gut tissue extracts with arylsulfatase. Total genistein recovery was 99.8%.

DISCUSSION

To assess the intestinal absorption of genistein, we used an isolated preparation of 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 could be demonstrated in histologic specimens of the isolated intestinal preparations perfused in the same way as in the present study (Plauth et al. 1992). In earlier studies, viability and functional integrity of the intestinal preparation were continuously controlled in terms of maintenance of regular perfusion pressure, stable lactate-pyruvate ratio, stable arterial pH and preservation of distinct luminal and vascular compartments (Hartmann et al. 1984, Plauth et al. 1991). The functional integrity of our intestinal preparation was similarly monitored and the viability confirmed. The viability data showed no significant differences between genistein and control perfusion experiments and are in agreement with data from earlier studies using the same model (Hummel 1998).

By using a blood cell- and plasma-free synthetic medium, the model used facilitates the selective study of genistein absorption and metabolism in the intestinal tissue without interference with the changing metabolism of erythrocytes and plasma enzymes in the perfusion medium.

Control perfusions with basic media without genistein confirmed that the small intestine of the experimental animals contained no sequestered genistein from the food. Importantly, a complete recovery of genistein from luminal and vascular perfusion media as well from the intestinal tissue was obtained (Table 1).

An initial, not significant increase (P = 0.18) of the luminal disappearance rate after the first 10 min is notable (Fig. 1A). Yet it is simply a consequence of the experimental design. In fact, the small intestine is filled with the luminal medium with a higher rate as the perfusion flow rate subsequently used. The observed negative balance of genistein and genistein glucuronide fluxes over the total perfusion time indicates that some free genistein and genistein glucuronide remain in the intestinal tissue.

Isoflavones are known to be extensively transformed by phase II enzymes, especially by glucuronosyltransferases (Lundh 1990). From earlier studies, the glucuronidation of isoflavones was thought to be liver-specific as in the case with

| TABLE 1 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | nmol            | %              |
| Luminal effluent |                 |                |
| genistein        | 148.7 ± 18.9    | 40.3 ± 5.4     |
| genistein glucuronide | 49.3 ± 9.9    | 13.3 ± 2.3     |
| total             | 198.0 ± 15.2    | 53.6 ± 3.4     |
| Vascular side    |                 |                |
| genistein        | 34.4 ± 7.9      | 9.3 ± 1.7      |
| genistein glucuronide | 115.5 ± 10.9   | 31.3 ± 3.8     |
| total             | 149.9 ± 6.8     | 40.6 ± 2.5     |
| Intestinal tissue|                 |                |
| genistein        | 9.5 ± 6.6       | 2.6 ± 1.8      |
| genistein glucuronide | 10.7 ± 4.0    | 2.9 ± 1.1      |
| total             | 20.2 ± 10.6     | 5.5 ± 3.0      |
| Blood vessels    |                 |                |
| genistein        | 0.1 ± 0.1       | 0.03 ± 0.0     |
| genistein glucuronide | 0.3 ± 0.1     | 0.08 ± 0.0     |
| total             | 0.4 ± 0.1       | 0.11 ± 0.0     |
| recovery          | 368.5 ± 8.2     | 99.8 ± 2.8     |

1 369.3 ± 16.1 nmol of genistein were applied in three perfusion experiments of 60 min. Recoveries are given as means ± sd.

2 Based on the mean dosage of 369.3 ± 16.1 nmol.
most steroidal estrogens and does not occur in the small intestine (Axelson et al. 1984). In contrast, the results of the present study show that glucuronidation occurs also in the small intestinal wall. These results are in agreement with those of Sfakianos et al. (1997), who used everted intestinal sac preparations. In line with this earlier study, no genistein sulfate conjugates were found in our study. However, Yasuda et al. (1996), who studied metabolism of genistein in the whole rat, observed sulfate conjugates. Our results strongly indicate that no sulfate conjugation occurs in the small intestine, but it might occur in the liver.

In the luminal perfusate, genistein was stable for 5 h. This indicates that no extracellular glucuronosyltransferase is present luminally. Thus genistein glucuronide formed inside the mucosal cell is secreted into the lumen (Fig. 1C).

Genistein glucuronide is preferentially released into the vascular perfusate, while only about a third was secreted into the luminal perfusate. Similar observations were made with 1-naphthol in studies with isolated perfused small intestinal segments of the rat (De Vries et al. 1989). It seems unlikely that highly charged glucuronides (pK$_a$ = 2–3; Dutton 1980) are released from the metabolizing compartment by passive diffusion. The findings might be explained by specialized transport carriers for phenol glucuronides in the brush border and the basolateral membrane (De Vries et al. 1989, Koster and Noordhoek 1983). The observed decrease after 30 min of the luminal genistein glucuronide appearance rate is puzzling indeed. A limitation of the glucuronosyltransferase cosubstrate UDP-glucuronic acid is unlikely since vascular appearance rate of genistein glucuronide remains constant. Nevertheless the luminal disappearance rates of genistein after 30 min parallel those of luminal appearance rates of genistein glucuronide, both showing a decreasing tendency with a total of about 1 nmol 1 min$^{-1}$ g dry intestine$^{-1}$. It is conceivable that the basolateral glucuronide transporter possesses a higher affinity for genistein glucuronide compared with the transporter at the brush border membrane.

In the present study, the measured rate of total genistein absorption was 46% in a single-pass perfusion, which is in good agreement with earlier observations gained from a bile duct-cannulated rat model (Sfakianos et al. 1997); the appearance in the bile was 40–50%. The intestinal absorption experienced in the present study is somewhat higher than found in human feeding experiments with soymilk having 13–35%, depending upon the gut microflora (Xu et al. 1995).

Genistein should be susceptible to C-ring cleavage by the intestinal bacterial flora (Griffiths and Smith 1972). However, complete recovery (99.8%) of genistein was obtained, which indicates that no microbial degradation occurred in the small intestine.

In conclusion, our findings support the notion that genistein is fairly bioavailable. The presented novel implication of the model might facilitate direct ex vivo investigations of intestinal handling of phytochemicals, thereby allowing future research on their absorption, transport, distribution and metabolism. Indeed such knowledge is a prerequisite to critically elaborate upon the particular role of phytochemicals in human health and disease.

ACKNOWLEDGMENTS

We are indebted to Dr. Ing. R.-P. Franke and W. Röhlke, Central Institut for Biomedical Technique, Department Biomaterials, University of Ulm, Germany, for the production of the perfluorocarbon emulsion. We are grateful for the support of this work by Institut Danone für Ernährung, Germany.

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