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ABSORPTION AND METABOLISM OF PHENOLIC PHYTOCHEMICALS IN THE ISOLATED RAT SMALL INTESTINE WITH SPECIAL REFERENCE TO ISOFLAVONES AND ANTHOCYANINS – A REVIEW

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Studies suggest a variety of biological effects for isoflavones and anthocyanins, but there is little information regarding small intestinal absorption and metabolism. In this article, absorption and metabolism studies of selected phytochemicals, especially isoflavones and anthocyanins are reviewed.

To assess small intestinal absorption an isolated preparation of luminally and vascularly perfused rat small intestine was used, with a synthetic perfusate free from blood components as vascular medium. Luminal media consisted of a buffered sodium chloride solution spiked with the phytochemicals. Viability and functional integrity of the organ preparation was maintained during the entire perfusion. Venous and luminal aliquots were analyzed for the phytochemicals with HPLC.

Complex foods (tofu) were predigested with an *in vitro* digestion system using pepsin and pancreatin before perfusion experiments.

Vascular uptake of the lipophilic isoflavone genistein was higher than for the more hydrophilic glycosides genistin, daidzin and cyanidin-3-glucoside. The isoflavone glycosides were hydrolyzed to their aglycons, which appeared at the vascular and luminal side. In contrast, anthocyanidin glucosides were not cleaved during perfusion experiments. All of the investigated phytochemicals were partly glucuronidated. These conjugates were vascularly absorbed and luminally secreted.

Tofu ingredients influenced isoflavone uptake, extent of glucuronic conjugation and distribution of glucuronides in the small intestine. Ethanol, in contrast, had no significant influence on cyanidin-3-glucoside absorption.

The investigated phytochemicals are absorbed at various extent in the small intestine, partly as conjugates. The implication of a simulated digestion and the use of the isolated perfused rat small intestine are suitable tools to investigate and evaluate the influence of food matrix on the intestinal handling of phytochemicals.

INTRODUCTION

During the last 30 years, research in the field of nutrition and chronic disease causation has led to exciting, significant progress in providing an understanding of specific risk factors and chemopreventive agents. The major health problems considered are cardiovascular diseases and the nutritionally linked cancers. One aspect involved in initiation and development of both cardiovascular diseases and cancers noted are abnormal oxidative processes, leading to the generation of radicals. In part, the protective role of vegetables and fruits is thus to provide antioxidant vitamins and specific phytochemicals that display a powerful inhibition in oxidative reactions. Epidemiological studies as well as laboratory experimentation have yielded sound data and evidence in support of the fact that vegetables and fruits and the specific antioxidants therein account mechanistically for inhibition [Weisburger, 1998].

It is to remember that with a normal diet the intake of phytochemicals is about 1-2 g a day [Ames *et al.*, 1990]. This represents a chemical cocktail of 5 000-10 000 substances, not taken into account that they can also influence each other in their effects [Ames *et al.*, 1990].

Reliable knowledge of the pharmacokinetics and biovailability of phytochemicals is mandatory to evaluate their role as chemopreventive agents. It should be remembered that for some years ago absorption of phytochemicals from gut lumen was considered to be inconsequential. This opinion is now revized because of growing body of experimental evidence indicating significant absorption of various phytochemicals from the gut lumen [Hollman, 1997; Ueno *et al.*, 1983; Das & Griffiths, 1969; Piskula *et al.*, 1999; Izumi *et al.*, 2000; Hollman *et al.*, 1996].

The assessment of bioavailability is a critical issue. Recent research has focused on urinary and biliary excretion, whereas little attention has been paid to blood, plasma, and tissue values after oral ingestion of phytochemicals, probably because sufficiently sensitive and selective analytical methods are lacking. Following a controlled tea and fried onion diet, it was concluded that flavonols in plasma and urine reflect short-term flavonol intake and that they might be valuable as biomarkers, to distinguish between high and low flavonol consumption in epidemiologic studies [deVries *et al.*, 1998].

Previously only free flavonoids without a sugar molecule, the so-called aglycones, were thought to be able

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to pass through the gut wall [Kühnau, 1976]. However, in recent studies it could be demonstrated that the quercetin glycosides from onions were absorbed far better than the pure aglycone [Hollman & Katan, 1997]. One proposed explanation for the rapid and better absorption of the quercetin glucoside is that the glucose transport pathway provides a route for absorption of glycosylated species in the small intestine, as shown in model studies for phenolic glucosides [Mizuma & Awazu, 1998]. It has been also suggested that flavonoid glycosides are capable of interacting with the sodium dependent galactose-glucose transport pathway [Gee *et al.*, 1997].

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 phytochemical absorption. The presence of compounds liable to bind or solubilize phytochemical could modify the extent of absorption and metabolism. Ingestion of green or black tea significantly increased the total plasma antioxidant capacity, whereas tea consumed with milk totally abolished this effect (For reference cf. [Hollman *et al.*, 1997]). The inhibitory effect was thought to be due to the complexation of tea polyphenols by milk proteins [Serafini *et al.*, 1996]. In contrast, others found no effect of milk on blood catechins or quercetin, yet it can not be excluded that an inhibitory effect of milk on plasma antioxidant activity is due to a reduction of the absorption of antioxidants other than catechins or quercetin [Hollman *et al.*, 1997; van het Hof *et al.*, 1998]. 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 absorption [Ruf *et al.*, 1995]. Yet, the impact of ethanol on intestinal absorption of anthocyanins has not been investigated.

THE ISOLATED RAT SMALL INTESTINE - A MODEL FOR THE ASSESSMENT OF PHYTOCHEMICAL ABSORPTION

In order to assess intestinal handling of phytochemicals we employed an *ex vivo* isolated vascularly and luminally perfused rat small intestine (Figure 1) [Plauth *et al.*, 1992]. Briefly, the small intestine (duodenum, jejunum, ileum) was prepared in rats in narketan® (Chassot AG, Bern, CH) - xylazin® (Vetimex, Bladel, NL) 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. The isolated small intestine was transferred to a warmed tissue bath (37°C) and allowed equilibrating for 30 min. The experiment was started after filling the intestinal lumen with a bolus of luminal media (digested tofu (*vide infra*) or buffered saline spiked with phytochemicals or not, in the case of controls, respectively), 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.

The vascular perfusion medium consisted of a perfluorotributylamine (ABCR, Karlsruhe, Germany) emulsion in Krebs-buffer containing glucose and glutamine, gassed with 5% carbon dioxide in oxygen. The perfluorotributylamine was emulsified with polyoxyproylene-polyoxyethylene copolymer (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). The linear oxygen dissociation and chemical inertness renders the perfluorotributylamine emulsion as an ideal oxygen carrier for the intestinal mucosa with high oxygen turnover.

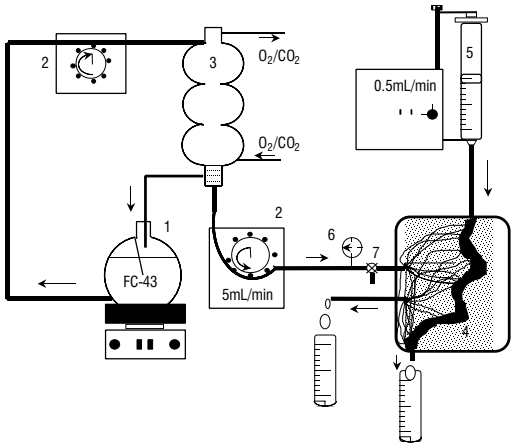

FIGURE 1. Schematic illustration of the perfusion model: 1: perfusate reservoir with magnetic stir; 2: roller pumps; 3: oxygenator; 4: perfusion chamber with organ preparation, humidified and temperature controlled; 5: precision pump for luminal perfusate; 6: pressure gauge (modified from [Kolb, 2001]).

In all studies, viability and functional integrity of the organ preparation were continuously monitored in terms of maintenance of regular perfusion pressure, stable lactate--pyruvate-ratio, regular oxygen uptake and glucose consumption and acid-base homeostasis. No significant differences in viability data could be observed between perfusions with phytochemicals and control perfusion experiments. Viability and functional integrity were controlled by repeatedly measuring oxygen uptake and acid--base homeostasis using a Clark pO_2-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®; 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 *ex vivo* organ preparation facilitates direct investigation of luminal disappearance and venous appearance of administered compounds, thereby allowing the assessment of intestinal absorption under strictly controlled conditions. Chemically defined perfusion media facilitate a constant supply of substrates and phytochemicals to the intestine.

DIGESTION MODEL

Food structure and food components which are able to bind phytochemicals like dietary fiber or proteins might

influence the extent of absorption and metabolism. To assess the influence of the food matrix, the food was predigested before perfusion experiments.

Tofu digestion. Chewing was simulated by pressing the tofu through a sieve (0.7 mm). Tofu was suspended with 0.1 mol/L HCl flat bottom glass flask and stirred magnetically for 5 min at 37°C in a water bath. The pH was adjusted to 1.9 and pepsin solution was added. After 1 h, the digestion was stopped by increasing pH to 7.4 with NaOH. To the reaction mixture solutions of $NaHCO_3$ and pancreatin were added. Pancreatin from porcine pancreas is a mixture of many enzymes, including amylase, trypsin, chymotrypsin, lipase, ribonuclease and carboxypeptidase. The pancreatin digestion was carried out for 1 h at 37°C. After digestion, bile salts (cholic acid, deoxycholic acid) were added. This enzymatic hydrolysate was used for luminal perfusion.

During the digestion procedure, protein digestion was controlled and confirmed by amino acid OPA-HPLC analytic (LKB, Bromma, Sweden) [Graser *et al.*, 1985] and analysis of soluble nitrogen by elemental analyzer (Elemental Analyzer Antek 7000 V; Antek Instruments Inc., Houston, Texas) [Grimble *et al.*, 1988].

During incubation of the mashed tofu with pepsin, soluble nitrogen containing compounds but not free amino acids (Figure 2) increased significantly, indicating an increasing degree of protein digestion. After addition of the exopeptidase pancreatin, both, nitrogen containing, soluble compounds and free amino acids increased significantly and reached a plateau after about 2 h. During the enzymic incubation, the stability of isoflavones was confirmed by HPLC-analysis. Neither the acidic nor the enzymatic conditions of the simulated predigestion hydrolyzed the glucosides or malonyl-glucosides.

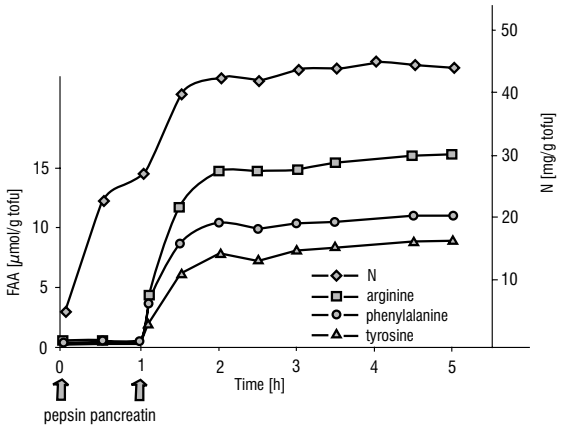

FIGURE 2. Soluble nitrogen containing compounds (N, calculated as nitrogen, n=3) and free amino acids (FAA: tyrosine, phenylalanine, arginine, n=2) during incubation of the mashed tofu with pepsin and pancreatin. Arrows indicate addition of pepsin (pH 1.9) and pancreatin (pH 7.4).

ANALYTICAL PROCEDURES

Cleavage of isoflavone glucuronide and sulfate conjugates. Isoflavone and anthocyanin glucuronide and sulfate conjugates were analyzed after enzymatic cleavage according to Sfakianos *et al.* [1997], with modifications as described previously [Andlauer *et al.*, 2000 a; c; d].

HPLC. Phytochemicals were analyzed with gradient HPLC combined with UV/Vis or MS-detection (LC-MS) as described previously [Andlauer *et al.*, 2000a; c; d].

CALCULATIONS

Fluxes (μmol x min^{-1} x (g dry intestine)$^{-1}$, means ± SD) were calculated from arterio-venous concentration differences (ΔC in nmol mL^{-1}), the corresponding flow rates (in mL min^{-1}) of luminal and vascular media and the dry weight (DW in g) of the entire small intestine used in the experiment according to the following equation:

$$\text{Flux} = (\Delta C \times \text{flow}) / \text{DW}$$

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.

PERFUSION EXPERIMENTS WITH ISOFLAVONES

Isoflavones, major dietary components from soybeans [Barnes *et al.*, 1994a; Wang & Murphy, 1994] have recently raised great attention because of their proposed health--related and clinical benefits such as estrogen receptor binding [Barnes *et al.*, 1994b; Adlercreutz, 1990], radical scavenging [Vedavanam *et al.*, 1999], anti-proliferative and growth inhibiting effects on cancer cells [Booth *et al.*, 1999]. There have been numerous investigations into the antioxidative and anticancer activity of soy and its bioactive isoflavones [Wei *et al.*, 1995; Record *et al.*, 1995; Herman *et al.*, 1995; Coward *et al.*, 1993].

Genistein. Perfusions with genistein were carried out with luminal media containing 6.8, 12.3 and 24.5 μmol/L, respectively. In a single pass through the isolated small intestine, 43.6% of the luminally administered genistein was extracted, calculated as the difference from the administered genistein and the luminal loss (Figure 3). Of the administered genistein, 36.8% appeared at the vascular side; 6.5% was located in the intestinal tissue; and only small amounts were found in the blood vessels (0.1%).

Most of the absorbed genistein appeared vascularly as glucuronide (27.2% of administered genistein), less as free genistein (9.6%). In the luminal effluent, free genistein (47.7%) prevailed glucuronidated genistein (8.7%). In the intestinal tissue, free and glucuronidated genistein were

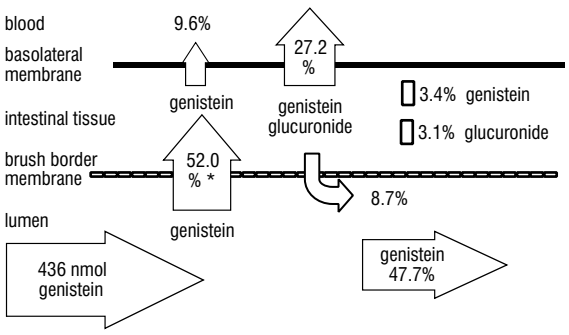

FIGURE 3. Schematic presentation of the intestinal handling of genistein in rat perfused small intestine. Values are means of nine experiments. Recovery of genistein was 99.9 ± 2.1%. * Value calculated from absorbed, secreted and tissue located genistein compounds.

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 99.9%.

Genistin. Luminal media contained genistein concentrations of 5.9, 12.0 and 23.8 μmol/L, respectively. Most of the luminally administerd genistin left the organ preparation *via* luminal efflux (Figure 4). Main compounds in the luminal effluent were genistin, followed by genistein and genistein glucuronide. Genistein glucuronide was verified *via* LC-MS by 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. Nearly 15% of genistin administered (mean of all 9 experiments) appeared at the vascular side, chiefly as genistein glucuronide but also as genistein and as unmetabolised genistin. Genistein glucuronide and genistin in the vascular effluent were also 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. Genistein glucuronide was preferentially secreted to the luminal side. Minute amounts of the genistin administered were located in the intestinal tissue and the blood vessels as unchanged genistin, genistein and genistein glucuronide.

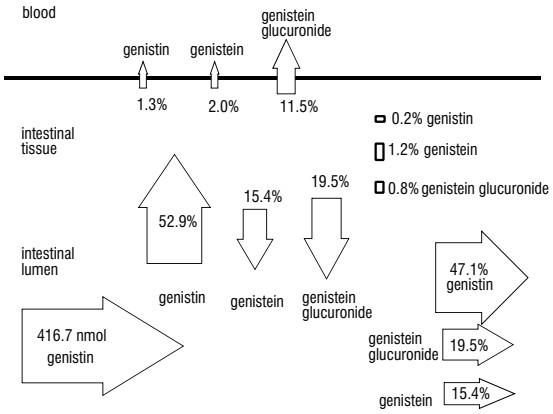

FIGURE 4. Schematic presentation of the intestinal handling of genistin in rat perfused small intestine. Values are means of nine experiments. Recovery of genistin was 99.9 ± 2.1%. * Value calculated from absorbed, secreted and tissue located genistein compounds.

Luminal disappearance and vascular appearance rates of genistin as well as vascular and luminal appearance rates of metabolites increased proportionally to increasing luminal genistin concentrations applied (Table 1).

In none of the luminal and vascular perfusates or gut tissue extracts mixed glucurono-sulfo-conjugates, sulfate conjugates of genistein and glucuronide or sulfates conjugates of genistin were detectable with LC-MS.

TABLE 1. The average fluxes (n=3) of genistin and its metabolites over the perfusion time of 60 min (nmol x min^{-1} x g^{-1}, mean ± SD) (modified from [Andlauer *et al.*, 2000a]).

Applied genistin [nmol ± SD]	176.8 ± 1.8	360.1 ± 0.0	713.1 ± 3.6
Luminal disappearance	2.0 ± 0.1	3.2 ± 0.3	8.4 ± 1.0
Luminal appearance (secretion)	0.9 ± 0.3	2.1 ± 0.3	5.3 ± 0.3
Vascular appearance	0.5 ± 0.0	0.9 ± 0.1	2.5 ± 0.3

Tofu. Soybeans and therefore soy-based foods like tofu have an extremely variable isoflavone content (ranging from about 200 μg/g to over 3 500 μg/g [Tsukamoto *et al.*, 1995]), depending on variety and environmental conditions [Wang & Murphy, 1994; Tsukamoto *et al.*, 1995]. Therefore, isoflavone content of the digested tofu was verified before perfusion.

Tofu was incubated with pepsin and pancreatin. During the enzymic incubation, the stability of isoflavones was confirmed by HPLC-analysis. Neither the acidic nor the enzymatic conditions of the simulated predigestion hydrolyzed the naturally occurring glucosides and malonyl--glucosides. After the addition of bile acids, isoflavone content of digested tofu was analyzed. Mean (n=3) isoflavone content of 1.0 g digested tofu was 760.7 nmol genistin, 84.8 nmol genistein, 339.1 nmol malonyl-genistin, 366.7 nmol daidzin, 47.5 nmol daidzein and 158.6 nmol malonyl-daidzin (a total of 1 184.6 nmol genistein compounds and 572.8 nmol daidzein compounds), respectively. For the assessment of intestinal absorption and metabolism of isoflavones, this predigested tofu was perfused through the isolated rat organ preparation.

Following luminal perfusion of predigested tofu, 91.2% of genistein compounds (Figure 5) and 92.0% of daidzein compounds were eliminated *via* luminal efflux (Figure 6). 65.0% of administered malonyl-genistin and 68.3% of malonyl-daidzin were found unchanged in the luminal effluent. Genistein (337.7%) and daidzein (189.7%) content of the luminal effluent strongly increased, due to cleavage of the corresponding glucosides and malonyl--glucosides which concurrently decreased. In the luminal effluent, 12.2% of total genistein and 11.6% of total daidzein compounds were conjugated with glucuronic acid, calculated from genistein (daidzein, respectively) after acid hydrolysis minus genistein, genistin and malonyl-genistin (daidzein, daidzin and malonyl-daidzin, respectively) from direct HPLC-analysis prior to hydrolysis. For cleavage of conjugates in the luminal effluent we used acid hydolysis, since enzymatic cleavage was incomplete.

Eight percent of the applied genistein compounds appeared at the vascular side, chiefly as genistein (4.4%), but also as genistin (2.1%) and genistein glucuronide (1.5%). The majority of the absorbed daidzein compounds was found as free daidzein (4.7%), less as daidzin (2.6%)

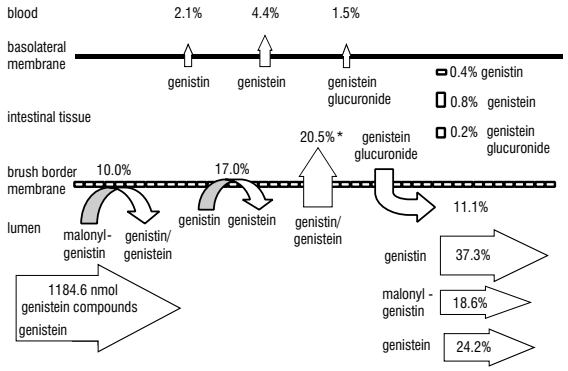

FIGURE 5. Schematic presentation of the intestinal handling of genistein compounds from tofu in rat perfused small intestine. Recovery of genistein compounds from three experiments was 100.6 ± 0.6%. * Value calculated from absorbed, secreted and tissue located genistein compounds (modified from [Andlauer *et al.*, 2000a]).

and daidzein glucuronide (1.6%). Irrespective whether derived from daidzein or genistein, about 20% of the absorbed isoflavones appeared vascularly as glucuronide conjugates. Only small amounts of genistein (1.4%) and daidzein (1.6%) compounds were located in the small intestinal tissue. Total recoveries of genistein and daidzein compounds were 100.6% and 102.6%, respectively.

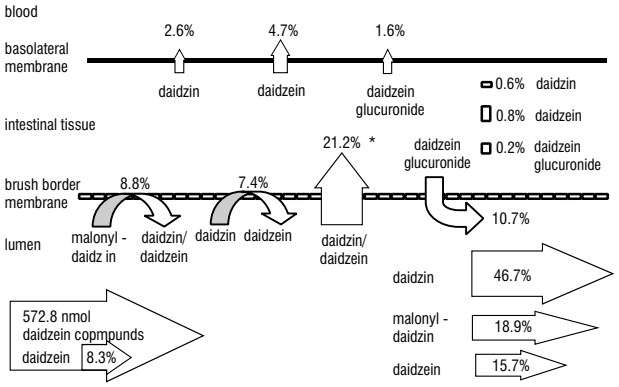

FIGURE 6. Schematic presentation of the intestinal handling of daidzein compounds from tofu in rat perfused small intestine. Recovery of daidzein compounds from three experiments was 102.6 ± 0.2%. * Value calculated from absorbed, secreted and tissue located daidzein compounds (modified from [Andlauer *et al*., 2000a]).

In the luminal and vascular perfusates as well as gut tissue extracts no mixed glucurono-sulfo-conjugates, no sulfate conjugates of isoflavones and no glucuronide or sulfates conjugates of the glycosides, no malony--lated isoflavones, equol, o-desmethylangolensin and p-ethylphenol were detectable by LC-MS.

EVALUATION OF ISOFLAVONE PERFUSIONS

The measured rate of total genistein absorption was 44% 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%.

In genistin perfusion experiments, the absorption rate of total genistein compounds was about 15%. Human feeding experiments with soymilk showed absorption rates of 14.6% [Lu *et al.*, 1995], 10% and 37% [Xu *et al.*, 1995], respectively. In feeding experiments with rats, bioavailability of genistin calculated from the urinary excretion of genistein and genistein derivatives was about 22% [King & Bursill, 1998], indicating that other parts of the digestive tract might be involved in the absorption processes.

It was proposed that flavonoid glycosides are hydrolyzed to the corresponding less polar aglycones prior to gastrointestinal absorption [Manach *et al.*, 1996; Yasuda *et al.*, 1996; Setchell *et al.*, 1998]. In the presented studies, however, genistin and daidzin were partly absorbed without previous cleavage which contradicts the above assumption. Supporting our observation, phloretin and quercetin have recently been shown to occur as glycosides in human plasma [Paganga & Rice-Evans, 1997].

Transepithelial transport of naphthol- and 4-nitrophenol--monoglucoside conjugates by the sodium dependant glucose transporter (SGLT1) was confirmed in experiments with everted intestinal sac preparations [Mizuma *et al.*, 1994; Mizuma & Awazu, 1998]. From human studies it was concluded that quercetin absorption is enhanced by conjugation with glucose, possibly by absorption *via* the glucose carrier [Hollman *et al.*, 1999]. Genistin, as genistein-7-monoglucoside might also be absorbed by this transport system. Yet, the absorption rate of genistein [Andlauer *et al.*, 2000d] was not enhanced due to conjugation with glucose. An increase in luminal genistin disappearance rates was seen in proportion to the concentrations luminally applied, thus rendering a transepithelial transport of genistin by diffusion more likely than an active transport by a carrier system.

Microorganisms of the rat and human intestine are able to cleave β-glucosides [Hackett, 1986]. However, since genistin remains stable in the luminal effluent, a microbial cleavage to genistein during genistin perfusion is unlikely and genistein luminally appearing was more likely hydrolyzed in the small intestinal tissue and subsequently secreted.

Additionally, genistein and genistin should be susceptible to C-ring cleavage by the intestinal bacterial flora [Griffiths & Smith, 1972]. However, complete recovery of genistein and genistin was obtained, which indicates that no microbial degradation occurred in the small intestine.

In tofu perfusions, mean recoveries from three experiments for daidzein and for genistein compounds were somewhat over 100%, which might be explained by small amounts of acetyl-glucosides of genistein and daidzein, observable with LC-MS, but not quantifiable with HPLC--UV. Acetyl-glucosides might be cleaved to quantifiable glucosides during small intestinal passage.

The measured absorption rate of genistein compounds derived from tofu (8.0%) is in fair agreement with earlier observations gained from human feeding experiments with soy milk corresponding to 14.6% [Lu *et al.*, 1995], 9% [Xu *et al.*, 1994], 10% [Xu *et al.*, 1995] and 16% with tofu, calculated from the urinary recovery [Xu *et al.*, 2000]. Absorption rate of pure genistin was higher (17.2%). It appears thus, that the tofu matrix decreases the genistin absorption rate. On the other hand, the relative absorption rate of genistin is difficult to calculate since malonyl--genistin is partly cleaved to yield both, genistin and genistein. In previous studies, daidzein revealed a better bioavailability than genistein in human studies (16% [Xu *et al.*, 1995], 21% [Xu *et al.*, 1994], 51% [Xu *et al.*, 2000]). In the presented study, however, daidzein showed about the same absorption rate ($p<0.05$) than genistein compounds (8.9%, 8.0%, respectively); the absolute absorption of daidzein compounds being much lower, because of lower content of daidzein compounds in the tofu. The effect that absorption rate of genistein equals that of daidzein in the small intestinal preparation might be explained by the greater gut microbial degradation of genistein compared with daidzein in *in vivo* models [Xu *et al.*, 1995]. Food derived differences in isoflavone absorption rates might be explained by the varying conjugation patterns of isoflavones (not conjugated, glucosylated, malonyl-glucosylated) in different soy products [Coward *et al.*, 1993; Wang & Murphy, 1994]. In this respect, it is notable that flavonoid and isoflavonoid glycosides are poorly absorbed in the small intestine compared with their aglycones, due to higher hydrophilicity and greater molecular weight [Xu *et al.*, 1995; Hutchins *et al.*, 1995].

The intestinal handlings of genistein and daidzein compounds are summarized in Figure 5 and Figure 6, respectively. It is obvious that genistin, the main genistein compound, is either absorbed from the lumen partly unhydrolyzed and directly transported (by an unknown transporter or diffusion) or partly hydrolyzed in the intestinal tissue and subsequently transported to the vascular side as genistein.

Malonyl-genistin as well as part of tofu derived genistin are presumably hydrolyzed in the lumen by enzymes bound to the brush border membrane like lactase phloridzin hydrolase [Day *et al.*, 2000]. Genistein secretion from intestinal tissue can be excluded because of the high luminal concentration of genistein provided, assuming that no active transport exists [Walle *et al.*, 1999a]. Daidzin and daidzein, both cleavage products of malonyl-daidzin are partly absorbed during small intestinal passage. In the small intestine, daidzin might be partly cleaved to daidzein. Daidzin and daidzein are transported to the vascular side. Dominating compound in the luminal effluent is daidzin coming from digested tofu and conceivably from malonyl--daidzin, which might be converted during intestinal passage (also to daidzein). A secretion of daidzein into the higher concentrated luminal perfusate is not likely when considering that no active transport is existing.

Obviously, genistin was hydrolyzed to a higher extent than daidzin, resulting in a considerable increase in genistein (338%) in the luminal perfusate. Keeping with the fact that genistin is stable in the luminal effluent and thus any microbial degradation can be excluded, we propose therefore a glycosidic cleavage as repeatedly reported [Griffiths, 1982; Booth *et al.*, 1957; Day *et al.*, 1998; Ioku *et al.*, 1998]. β-Glucoside-cleaving enzymes include the intracellularly occurring β-glucuronidase [Andlauer *et al.*, 2000b] and a broad-specificity cytosolic β-glucosidase [McMahon *et al.*, 1997; Day *et al.*, 1998] as well as the lactase phloridzin hydrolase, which is present on the luminal side of the brush border membrane [Day *et al.*, 2000]. From the low aglycone concentrations on the blood side and the high luminal aglycone concentrations we exclude a back transport of genistein and daidzein from the intestinal tissue to the luminal side. Therefore, the high luminal genistein and daidzein concentrations are more likely from the luminal cleavage of glucosides by the lactase phloridzin hydrolase than from secreted aglycones coming from cytosolic cleavage.

Isoflavones are known to be extensively transformed by phase II enzymes, especially by UDP glucuronosyltransferase (2.4.1.17) [Lundh, 1990]. From earlier studies, the glucuronidation of isoflavones was thought to be liver-specific as in the case with most steroidal estrogens [Axelson *et al.*, 1984]. However, recently several investigators have shown that this phase II biotransfor--mation also occurs in the gut tissue [Koster & Noordhoek, 1983; Mizuma & Awazu, 1998; Chowdhury *et al.*, 1985]. The presented results [Andlauer *et al.*, 2000d] and that gained from experiments with everted intestinal sac preparations [Sfakianos *et al.*, 1997], provide evidence that isoflavone aglycones are glucuronidated in the small intestinal tissue. Neither other conjugates nor conjugates of the 7-glucosides were found in any perfusion experiment, in contrast to results from a study obtained after oral administration of daidzin to whole rats, reporting sulfate, disulfate and sulfo-glucuronide conjugates of daidzein [Yasuda *et al.*, 1994].

In the luminal perfusate, isoflavones were stable for 5 h. This indicates that no extracellular glucuronosyltransferase was present luminally. Thus isoflavone glucuronides formed inside the mucosal cell are secreted into the lumen.

In genistin and tofu perfusions, formed glucuronides are preferentially secreted into the luminal perfusate. In contrast to genistein perfusions, where 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 [DeVries *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 [DeVries *et al.*, 1989; Koster & Noordhoek, 1983]. Actually, for glucuronides of the flavonoid chrysin, an active transport *via* the multi drug resitance protein MRP2 pump could be described in human intestinal cell line Caco-2 [Walle *et al.*, 1999b].

In experiments with everted rat intestine, it was recently shown that secreted glucuronides are reabsorbed in the colon, and thus, not lost for the organism [Inoue *et al.*, 1999].

Tofu ingredients obviously influenced genistin uptake, extent of glucuronic conjugation and distribution of glucuronides in the intestine.

PERFUSION EXPERIMENTS WITH ANTHOCYANINS

Anthocyanins are major phenolic compounds from fruits, beans, cereals, vegetables and beverages like juices and especially red wine [Kühnau, 1976; Ghiselli *et al.*, 1998]. These flavonoids are receiving renewed attention for their positive health attributes. Consumption of anthocyanins is associated with a reversal of age-dependent neuronal degeneration [Joseph *et al.*, 1999], reduced platelet aggregation [Keevil *et al.*, 2000] as well as significant vaso-protective and anti--inflammatory effects [Lietti & Forni, 1976]. 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 [Morazzoni *et al.*, 1991], elderberry juice [Cao & Prior, 1999] and red wine [Lapidot *et al.*, 1998]. In all these studies, low anthocyanin absorption rates were estimated. Recently, human and animal studies focused on absorption of purified cyanidin glycosides [Tsuda *et al.*, 1999; Matsumoto *et al.*, 2001; Miyazawa *et al.*, 1999]. These authors observed intact cyanidin glycosides in plasma and tissue compartments and also estimated very low absorption rates.

In 1992, a fascinating hypothesis was postulated: the s.c. "French Paradox" [Renaud & DeLorgeril, 1992]. It claims that French subjects who have similar intakes of saturated fatty acids, similar risk factors and comparable plasma cholesterol levels exhibit a much lower incidence of death from CHD than US or West European subjects with comparable intakes of fat. It could be demonstrated that consumption of red wine was the only dietary factor that

showed a negative correlation with CHD. In subsequent reports the remarkable effects of red wine have been confirmed and possible underlying mechanisms discussed [Frankel *et al.*, 1993; Maxwell *et al.*, 1994; Groenbaek *et al.*, 1995; Serafini *et al.*, 1998]. It is to emphasize that in recent communications similar effects were observed with grape extracts only [Day *et al.*, 1997]. The essential question remains still unanswered: whether alcohol *per se* Groenbaek *et al.*, 1995; Kiechl *et al.*, 1998] the nonalcoholic fraction of wine (grapes), represented mainly by phenolic compounds, or a mixture of both, are responsible for the protective effect.

Our experiments were designed to investigate the effect of ethanol on absorption rate of cyanidin-3-glucoside in the small intestine [Andlauer *et al.*, 2003].

Cyanidin-3-glucoside. To assess the influence of ethanol on cyanidin-3-glucoside absorption, in one experimental group 114.6 ± 23.7 μmol/L cyanidin-3-glucoside (= 3 780.2 ± 782.3 nmol per perfusion experiment, n=5) were applied. In the second group, luminal media was spiked with 130.1 ± 39.6 μmol/L cyanidin-3-glucoside (amount applied 4 294.0 ± 1 308.2 nmol, n=5) and additionally with 5% ethanol (v/v) in the luminal media.

In both experimental groups, most of the luminally administered cyanidin-3-glucoside left the organ preparation *via* luminal efflux (65.6% and 68.4% in perfusions with ethanol, respectively) (Figure 7). Main compound in the luminal and vascular effluent was unchanged cyanidin--3-glucoside 2.9% and 4.3% in perfusions with ethanol, respectively). Small amounts of cyanidin-3-glucoside were absorbed in 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 a weak increase in cyanidin--3-glucoside concentration. Sulfatase cleavage following glucuronidase incubation did only yield slightly higher cyanidin-3-glucoside concentrations in intestinal tissue. 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.

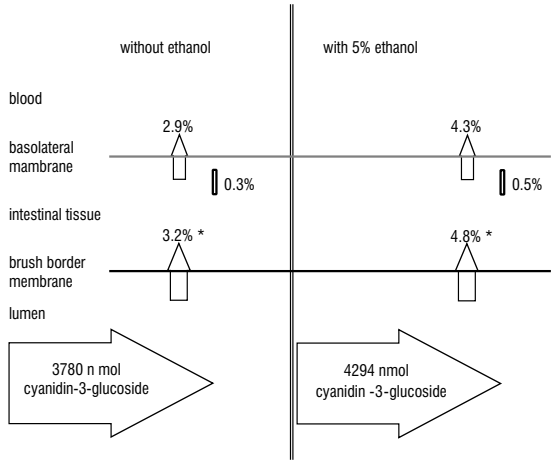

FIGURE 7. Left side: Schematic presentation of the intestinal handling of cyanidin-3-glucoside without ethanol in rat perfused small intestine. Recovery of cyanidin-3-glucoside from five experiments was 68.8 ± 13.0%. Right side: Schematic presentation of the intestinal handling of cyanidin-3-glucoside with ethanol (5% in luminal solution) in rat perfused small intestine. Recovery of cyanidin-3-glucoside from five experiments was 73.2 ± 8.2%. * Values calculated from absorbed and tissue located cyanidin-3-glucoside compounds.

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.

Elderberry extract. For these perfusion experiments a freeze dried elderberry extract was used. This extract contained cyanidin-3-glucoside-5-sambubiosid, cyanidin--3-glucoside, cyanidin-3-sambubiosid besides some rutin. Cyanidin-3-glucoside and cyanidin-3-sambubiosid could not be separated with the selected HPLC-conditions and were consequently quantified together.

Luminal perfusion media contained cyanidin-3--glucoside/cyanidin-3-sambubiosid (127.6 ± 19.6 μmol/L (= 4 211.6 ± 646.9 nmol per perfusion experiment, n=3) and 9.1 ± 1.8 μmol/L cyanidin-3-glucoside-5-sambubiosid (= 301.1 ± 59.8 nmol per perfusion experiment). Vascular absorbance of cyanidin-3-glucoside/cyanidin-3-sambubiosid was 2.4 ± 0.3% and the recovery of these compounds was about 70%. Cyanidin-3-glucoside-5-sambubiosid was somewhat better absorbed (3.8 ± 1.8%) and showed a recovery of nearly 94%. Most of the administered anthocyanins left the organ preparation *via* luminal efflux. Anthocyanins of elderberry extract were absorbed as glycosides, conjugation of anthocyanins was not observed.

EVALUATION OF ANTHOCYANIN PERFUSIONS

The incomplete total recoveries after perfusion experiments (about 70%) of cyanidin-3-glucoside and cyanidin-3-glucoside/cyanidin-3-sambubiosid from elderberry was observed in other experimental studies with anthocyanins [Murkovic *et al.*, 2000]. These anthocyanidin--glycosides might be metabolized to substances which escape electrochemical detection under the present conditions. In most anthocyanin studies no recoveries were indicated [Morazzoni *et al.*, 1991; Cao & Prior, 1999; Lapidot *et al.*, 1998; Tsuda *et al.*, 1999; Matsumoto *et al.*, 2001; Miyazawa *et al.*, 1999]. 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 rates of cyanidin-3-glucoside (2.9% and 4.3%, without and with ethanol, respectively) and cyanidin-3-glucoside/cyanidin-3-sambubiosid (2.4%) and cyanidin-3-glucoside-5-sambubiosid from elderberry extract (3.8%) are in good agreement with earlier observations gained from human feeding experiments performed with mixtures of anthocyanins from red wine ranging from 1.0% to 6.7% [Lapidot *et al.*, 1998]. In feeding experiments with rats, bioavailability of an anthocyanin mixture from *Vaccinium myrtillus* extracts was calculated somewhat lower (1.2%) [Morazzoni *et al.*, 1991]. In contrast to these and to our results, absorption rate of pure cyanidin-3-glucoside [Tsuda *et al.*, 1999; Matsumoto *et al.*, 2001; Miyazawa *et al.*, 1999] and anthocyanins from elderberries [Murkovic *et al.*, 2000] 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) [Hollman *et al.*, 1999]. Absorption of anthocyanidin glycosides from black currant was suggested to occur through the hexose transport pathway [Matsumoto *et al.*, 2001]. 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 [Andlauer *et al.*, 2000c; d]. For cyanidin-3-glucoside absorption, both transport processes are conceivable.

Anthocyanin glycosides were 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 [Tsuda *et al.*, 1999; Miyazawa *et al.*, 1999].

Conjugates of anthocyanidin glycosides were analyzed after enzymatic cleavage. It is to emphasize, that none of the enzymes, neither the glucuronidase from *E. coli* nor the glucuronidase-sulfatase from *Helix pomatia* were able to cleave the β-glycoside from anthocyanins, despite an apparent β-glucosidase activity of these enzymes [Andlauer *et al.*, 2000c]. Our observations were confirmed by incubation experiments with cyanidin-3-glucoside and β-glucosidase [Miyazawa *et al.*, 1999]. 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 got no mass spectra of such metabolites. Metabolites of cyanidin-3--glucosides have been also observed in an animal study [Tsuda *et al.*, 1999], but so far no glucuronide conjugates have been characterized. In perfusion experiments with elderberry extract, no conjugates were detected.

It has been supposed that the presence of ethanol in red wine might improve flavonoid availability [Ruf *et al.*, 1995]. It is conceivable that ethanol affects absorption by solubilizing anthocyanins or acting as a mucosal barrier braker [Beck *et al.*, 1986; Morris *et al.*, 1989]. 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 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 observation were reported. Human studies with dealcoholized and reconstituted red wine indicated that coingestion of ethanol did not affect extent of catechin [Bell *et al.*, 2000; Donovan *et al.*, 1999], caffeic, protocatechuic and 4-0-methyl gallic acid absorption [Caccetta *et al.*, 2000].

Anthocyanin glycosides, potent pharmacologically active plant components are absorbed in the small intestine, mainly as intact β-glucoside. Yet, extent of absorption is low. The presented studies 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 [Joseph *et al.*, 1999; Keevil *et al.*, 2000; Lietti & Forni, 1976].

CONCLUSIONS

The presented results from *ex vivo* studies support the notion that isoflavones and anthocyanins are absorbed and metabolized in the small intestine.

Studies with the isolated rat small intestine are conducted under well controlled and defined conditions. Chemically defined perfusion media facilitate a constant supply of substrates and phytochemicals to the intestine. The linear oxygen dissociation and chemical inertness renders the perfluorotributylamine emulsion as an ideal oxygen carrier for the intestinal mucosa with high oxygen turnover. With this *ex vivo*-model, small number of experiments are sufficient for significant results.

The implication of a simulated digestion and the use of the isolated perfused rat small intestine are suitable tools to investigate and evaluate the influence of food matrix on intestinal handling of phytochemicals. The isolated perfused small intestinal preparation facilitates investigations of intestinal handling of phytochemicals, thereby allowing future research on their absorption, transport, distribution and metabolism. This knowledge is a prerequisite to evaluate the particular role of phytochemicals in human health and disease.

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