Metabolism of Flavonoids via Enteric Recycling: Role of Intestinal Disposition

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ABSTRACT

The purpose of this study was to determine the importance of intestinal disposition in the first-pass metabolism of flavonoids. A four-site perfused rat intestinal model, rat liver and intestinal microsomes, Caco-2 cell microsomes, and the Caco-2 cell culture model were used. In the four-site model, ~28% of perfused aglycones were absorbed (~450 nmol/30 min). Both absorption and subsequent excretion of metabolites were rapid and site-dependent (p < 0.05). Maximal amounts of intestinal conjugates excreted per 30 min were 61 and 150 nmol for genistein and apigenin, respectively. Maximal amounts of biliary conjugates excreted per 30 min were 50 and 30 nmol for genistein and apigenin, respectively. Microsomes, prepared from Caco-2 cells, rat intestine, and rat liver, always glucuronidated genistein faster than genistein (p < 0.05). In addition, rat jejunal microsomes glucuronidated both flavonoids faster (p < 0.05) than rat intestinal microsomes prepared from other regions. When comparing glucuronidation in different organs, jejunal microsomes often but not always glucuronidated both flavonoids faster than liver microsomes. In the Caco-2 model, both flavonoids were rapidly absorbed and rapidly conjugated, and the conjugates were excreted apically and basolaterally. Similar to the four-site perfusion model, apigenin conjugates were excreted much faster than genistein conjugates (>2.5 times for glucuronic acid, >4.5 times for sulfate; p < 0.05). In conclusion, intestinal disposition may be more important than hepatic disposition in the first-pass metabolism of flavonoids such as apigenin. In conjunction with enterohepatic recycling, enteric recycling may be used to explain why flavonoids have poor systemic bioavailabilities.

Genistein, a soy isoflavone, is being tested for its ability to prevent prostate cancer (Steele et al., 1995; Kelloff et al., 2001). In vitro studies have shown that it can inhibit cancer cell growth via a variety of mechanisms (Kurzer and Xu, 1997; Birt et al., 2001; Yang et al., 2001). However, the bioavailability of genistein is poor (Xu et al., 1994, 1995; King et al., 1996, King and Bursill, 1998; Setchell et al., 2001). Poor bioavailability of genistein is a serious concern because most plasma isoflavones are present as conjugated forms, and in vivo plasma concentrations of aglycones (unconjugated isoflavone) are in the range of 0.01 to 0.4 μM (Setchell et al., 2001; Busby et al., 2002), significantly less than the IC50 or EC50 values of 5 to 50 μM commonly reported for its anticancer effects in vitro (Kurzer and Xu, 1997; Birt et al., 2001; Yang et al., 2001). Prostate concentrations of isoflavones (mostly conjugated) have been determined and they are within the same range of the plasma concentrations (Morton et al., 1997; Hong et al., 2002). Genistein is contained in a large number of dietary phytoestrogen products, and its consumption may accelerate as more women seek alternatives to the traditional hormone replacement therapy.

Apigenin, a flavone analog of genistein, also has anticancer activities (Wang et al., 2000; Gupta et al., 2001). Apigenin is also poorly bioavailable (Birt et al., 2001), but less is known about its absorption and metabolism than genistein. Limited studies in rats suggest that it is more extensively metabolized than genistein (Liu and Hu, 2002). In HepG2 cells, apigenin was also metabolized rapidly via UDP-glucuronosyltransferase UGT1A1 (Galiatovic et al., 1999; Walle and Walle, 2002). Similarly, the in vitro anticancer effects of apigenin are represented by IC50 or EC50 values in the micromolar range, significantly higher than its in vivo concentration achievable after diet. Transdermal delivery of apigenin to prevent skin cancer has been used to avoid extensive metabolism and the results indicated that it was not metabolized significantly in skin (Li et al., 1996).

Extensive first-pass metabolism is the main reason for their poor bioavailabilities, because genistein and apigenin were rapidly absorbed (Liu and Hu, 2002). In rats and humans, the main metabolites of genistein are 7-OH-glucuronic acid and 4′-OH-sulfate (King et al., 1996; King and Bursill, 1998; Setchell et al., 2001).
Liver conjugates of genistein and apigenin are partially excreted into the urine or eliminated through the bile (King et al., 1996), whereas intestinal conjugates were excreted by the rat enterocytes (Andlauer et al., 2000; Liu and Hu, 2002). In rat and human cells, the major metabolites of apigenin are also glucuronidated and sulfated conjugates (Galijatovic et al., 1999; Liu and Hu, 2002), although the exact structures of these conjugates have not been determined.

In our previous study, extensive phase II metabolism of genistein and apigenin in the intestine was demonstrated (Liu and Hu, 2002). We also proposed that enteric recycling, which involves reconversion of conjugates excreted by the intestine, is an important component of flavonoids disposition process in vivo. However, relative contribution of liver versus intestine to the low bioavailability of flavonoids was not determined. Therefore, the main purpose of the present study was to determine relative contribution of intestinal disposition versus hepatic disposition to the overall disposition of flavonoids.

Materials and Methods

Materials. Cloned Caco-2 cells, TC7, were a kind gift from Dr. Monique Rouset (Institut National de la Santé et de la Recherche Médicale U178, Villejuif, France). Apigenin and genistein were purchased from Indofine Chemicals (Somerville, NJ). β-Glucuronidase with (catalog no. G1512) or without sulfatase (catalog no. G7396), sulfatase without glucuronidase (catalog no. S1629), uridine diphosphoglucuronic acid, alamethicin, β-saccharin-1,4-lactone monobromide, magnesium chloride, Triton, and Hanks' balanced salt solution (HBSS, powder form) were purchased from Sigma-Aldrich (St Louis, MO). [14C]PEG-4000 was obtained from PerkinElmer Life Sciences (Boston, MA). All other materials (typically analytical grade or better) were used as received.

Cell Culture. The culture conditions for growing Caco-2 cells have been described previously (Hu et al., 1994a,b; Liu and Hu, 2002). The seeding density (100,000 cells/cm²), growth media (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum), and quality control criteria were all implemented in the present study as they were described previously (Hu et al., 1994a,b). Caco-2 TC7 cells were fed every other day, and the monolayers were ready for experiments from 19 to 22 days postseeding.

Transport Experiments in the Caco-2 Cell Culture Model. Experiments in triplicate were performed in pH 7.4 HBSS (Hu et al., 1994a,b). The protocol for performing cell culture experiments was the same as described previously (Liu and Hu, 2002). Briefly, the cell monolayers were washed three times with 37°C, pH 7.4 HBSS. The transepithelial electrical resistance values were measured, and those with transepithelial electrical resistance values less than 500 ohms × cm² were discarded. The monolayers were incubated with the buffer for 1 h and the incubation medium was then aspirated. Afterward, the solution containing the compound of interest was loaded onto the apical side and amounts of transepithelial transport were followed as a function of time. Two donor (or apical) samples were taken at the beginning and at the end of an experiment, and four receiver (or basolateral) samples (650 μl) were taken every 30 min, followed by the addition of 650 μl of fresh buffer to keep the constant volume at the receiver side. Methanol (200 μl) containing 50 μM testosterone was added to each sample as internal standard. Afterward, the mixture was centrifuged at 13,000 rpm for 15 min, and the supernatant was analyzed by HPLC (Table 1).

Animals. Male Sprague-Dawley rats (70–110 days old) weighing between 260 and 350 g were from Simonsen Laboratory (Gilroy, CA). The rats were fed with Teklad F6 rodent diet (W) from Harlan Laboratories (Madison, WI). The rats were fasted overnight before the day of the experiment. No flavonoids were found in pH 7.4 HBSS buffer that had been perfused through a segment of jejunum, indicating minimal presence of dietary flavonoids in the intestine.

Animal Surgery. The procedures were approved by Washington State University’s Institutional Animal Care and Uses Committee. The intestinal surgical procedures were modified from our previous publications (Hu et al., 1988, 1998), in that we perfused four segments of the intestine simultaneously and therefore called a “four-site (perfusion) model”, and added bile and jugular vein cannulation. The circulation to the liver and intestine was not disturbed in this model. Here is a brief description of the surgical procedures. Anesthesia was induced by an i.m. injection of ketamine (75 mg/kg), xylazine (5 mg/ml), and acepromazine (2 mg/kg), and maintenance dose of ketamine was infused into the jugular vein during the perfusion experiment to sustain the anesthetic condition. After the rat was anesthetized, it was put over a heating blanket and under a heating lamp to keep its normal body temperature. A 1-cm cut was made to expose the jugular vein. After the vein was separated from the surrounding tissue, a fine-pointed surgical scissors was used to cut the vein and a cannula made of polyethylene-20 tubing was inserted and secured with sterilized black silk suture. After the rat abdominal cavity was opened by a midline incision of ~4 cm, the bile duct was located near the duodenum. A fine-pointed scissors was used to cut the duct and polyethylene-10 tubing was inserted into the bile duct. When bile flow out of the cannulation was seen without restriction, the cannula was secured with sutures. After the bile and jugular vein were cannulated, we then cannulated four segments of the intestine, each with two cannulae. After each cannula was inserted, it was secured with a sterilized black suture before the next cannula was inserted and secured. First, the abdomen was located as the intestinal segment immediately adjacent to the stomach, and two cannulae at ~10 cm apart were inserted into two ends of the duodenum and secured with suture. Next, the jejunal wall was located below the duodenum, and the first cannula was inserted at ~4 cm below the duodenal outlet cannula, whereas the second cannula was inserted at ~10 cm below the first jejunal cannula. Then, the terminal ileum was located by identifying the ileocecal junction of the rat intestine. The outlet cannula was inserted into the ileum at ~2 cm above the junction, and the inlet cannula was inserted ~10 cm above the outlet cannula. Last, the colon inlet cannula was inserted into the colon at ~2 cm below the junction, and the outlet cannula was inserted through the anus. After cannulation, the small intestinal segments were placed carefully into the abdominal cavity, avoiding crimping or kinking of the segments to the best of our ability. The incision was then covered by a normal saline-wetted paper towel. A piece of plastic wrap was put on the towel to keep the intestinal segments moist. In addition to the careful surgery, caution was also exercised to keep the inlet and outlet cannulate at the same height to avoid gravitational flow. To keep the temperature of the perfusate constant, the inlet cannulate was insulated and kept warm by a 37°C circulating water bath.

Transport and Metabolism Experiments in Perfused Rat Intestinal Model. This is a single-pass perfusion method. Four

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent</th>
<th>Glucuronic Acid</th>
<th>Sulfate</th>
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<tr>
<td>Apigenin</td>
<td>14.3</td>
<td>4.7</td>
<td>11.0</td>
</tr>
<tr>
<td>Genistein</td>
<td>14.7</td>
<td>4.5</td>
<td>10.5</td>
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segments of the intestine (duodenum, upper jejunum, terminal ileum, and colon) were perfused simultaneously with a perfusate containing the compound of interest and PEG-4000 (as water flux marker) using an infusion pump (Harvard Apparatus, Cambridge, MA) at a flow rate of 0.382 ml/min. After a 30-min washout period, which is usually sufficient to achieve the steady-state absorption, four samples were collected from the outlet cannulae every 30 min afterward. Bile samples (about 1 ml) were collected before perfusion started and every 30 min afterward. Blood samples (400 µl) were taken before the perfusion started, and at 30, 60, 90, 120, and 150 min, which happened to be the time we collected perfusate and bile samples. After perfusion, the length of the intestine was measured as described previously (Hu et al., 1988, 1998). The outlet concentrations of test compounds in the luminal perfusate (or perfusate) were determined by HPLC (Table 1), and radioactivity of labeled PEG in the perfusate was determined by liquid scintillation spectrophotometer. Bile and plasma samples were diluted (1:3) with buffer, added glucuronidase + sulfatase, and reacted for 4 h to release the aglycones for HPLC measurement.

Rat Intestinal Microsomes Preparation. The rats, which were fasted overnight with access only to water, were euthanized with sodium phenobarbital (200 mg/kg). Segments of eight rat intestines were cut and separated using the following protocol: for small intestine, first 10 cm, duodenum; last 20 cm, ileum; the rest of the small intestine, jejunum; and for large intestine, cecum, dissected; colon, used for colon microsome preparations. After the same segments were pipped from eight rats, each segment was washed with the ice-cold washing solution, which consists of ice-cold saline plus reducing agent dithiothreitol (1 mM). The segments were then cut open lengthwise to flush any remaining fecal material away with washing solution. Opened intestine was placed into the ice-cold solution A, which consists of 8 mM KH2PO4, 5.6 mM Na2HPO4, 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, and 0.04 mg/ml phenylmethylsulfonyl fluoride (PMSF), and washed twice with it. The intestinal strips were then blotted dried and scraped, and the scraped mucosal cells were put into the ice-cold solution B, which consists of 8 mM KH2PO4, 5.6 mM Na2HPO4, 1.5 mM EDTA, and 0.5 mM dithiothreitol and 0.04 mg/ml PMSF. Cells were collected by centrifugation at 18,500 g at 4 °C for 15 min at 4 °C, the supernatant was removed with a Pasteur pipette and the fat layer and pellet were discarded. The microsomes were resuspended in 250 mM sucrose solution, and separated into microcentrifuge tubes, and stored at −80°C until use.

Caco-2 Cell Microsomes. We used a procedure similar to that used for preparing rat intestinal microsomes. We harvested about 80 million Caco-2 cells (9 days postseeding) and washed the cells twice using the washing buffer. The cells are then processed the same way as described previously (Hu et al., 1998, 1998). The outlet concentrations of test compounds in the luminal perfusate (or perfusate) were determined by HPLC (Table 1), and radioactivity of labeled PEG in the perfusate was determined by liquid scintillation spectrophotometer. Bile and plasma samples were diluted (1:3) with buffer, added glucuronidase + sulfatase, and reacted for 4 h to release the aglycones for HPLC measurement.

Western Blotting. The expression of proteins was confirmed by Western Blotting using 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% fat-free milk in tris-buffered saline with 0.1% Tween 20 (PBST) and probed with primary antibodies against the relevant target proteins. Antibody specificity was confirmed by competition experiments using blocking peptides.

Sample Preparation. To confirm the formation of a particular conjugate, we selectively extracted samples with methylene chloride to remove >90% of aglycones. The resulting sample was then divided into two parts, one of which was analyzed directly, whereas the other part was analyzed after glucuronidase or sulfatase hydrolysis. The difference in amount of aglycones found in these two samples was the amount of metabolites formed. The relationship between the peak areas of the metabolites before hydrolysis and the peak areas of aglycones after the hydrolysis is used to establish the conversion factor used to quantify the amounts of apigenin and genistein conjugates as described previously (Liu and Hu, 2002).

HPLC Analysis of Isoflavones and Their Conjugates. The conditions for HPLC analysis of isoflavones were modified based on a previously published method (Liu and Hu, 2002). The conditions for analyzing apigenin, genistein, and their conjugates were as follows: system, Hewlett Packard 1090 with diode array detector and Hewlett Packard Chemstation; column, Aqua (Phenomenex, Gilroy, CA), 5 μm, 150 × 0.45 cm; mobile phase A, 0.1 mM pH 2.5 phosphate buffer; mobile phase B, 100% acetonitrile; gradient, 0 to 3 min, 20% B, 2 to 22, 20 to 49% B, 22 to 26, 49% B; wavelength, 254 nm (for genistein and the internal standard) and 340 nm (for apigenin); and injection volume, 200 μl. There was a 4-min interval between the end of the run and the next injection to allow the column to be reequilibrated with 20% mobile phase B. The retention time of flavonoids and their conjugates are listed in Table 1.

Data Analysis. In the perfused rat intestinal model, amounts of genistein or apigenin absorbed (Mₘₐₜ) were expressed as follows:

\[
M_{ab} = Q \cdot (CA_{in} - CA_{out})
\]

where \(Q\) is the flow rate (in milliliters per minute), \(\tau\) is the sampling interval (30 min), and \(CA_{in}\) and \(CA_{out}\) are the inlet and outlet concentrations of aglycones corrected for water flux using \(^{15}\)C)PEG-4000, respectively. We have assigned amounts disappeared as the amounts absorbed because our previous studies have indicated that these compounds are stable in the intestinal perfusate (Liu and Hu, 2002) and that the conjugating enzymes are located inside the enterocytes.

Amounts of conjugated excreted into the intestinal lumen (Mₖᵢₜ) were expressed as follows:

\[
M_{k} = Q \cdot CM_{out}
\]

where \(CM_{out}\) is the outlet concentrations (in nanomoles per milliliter) of metabolites corrected for water flux, and \(Q\) and \(\tau\) are the same as defined for eq. 1.

Amounts of conjugated excreted via the bile (Mₜₜₜ) were expressed as follows:

\[
M_{b} = V \cdot CM_{b}
\]

where \(CM_{b}\) is the bile concentrations (in nanomoles per milliliter) of metabolites, and \(V\) is the volume of bile collected over a 30-min time period.
The percent absorbed and percent metabolized values were calculated as follows:

\[ \text{% absorbed in the intestine} = \frac{M_{ab}}{M_{\text{total}}} \]  
\[ \text{% metabolites excreted in the intestine} = \frac{M_{\text{met}}}{M_{\text{total}}} \]

where \( M_{\text{total}} \) is the total amount of compound perfused over a 30-min time period.

Rates of metabolism were expressed as amount of metabolites formed per minute per milligram of protein or nanomoles per minute per milligram.

One-way ANOVA or an unpaired Student’s t-test (Microsoft Excel) was used to analyze the data. The prior level of significance was set at 5%, or \( p < 0.05 \).

**Results**

**Regional Absorption and Metabolism of Genistein and Apigenin (Fig. 1).** Absorption of genistein and apigenin (35 \( \mu \)M each) was rapid with minimal effects on water flux (i.e., water flux less than 0.5%/cm of perfused intestinal segment), but there were differences between amounts absorbed of these two compounds in different regions of the intestine. For genistein, amounts absorbed in duodenum (44% of perfused amounts) and colon (35%) were higher \( (p < 0.05) \) than that in jejunum (16%) and terminal ileum (18%). For apigenin, amounts absorbed was the highest in the colon (40%) and the lowest in the terminal ileum (21%) \( (p < 0.05); \) Fig. 1A). There were also significant differences in conjugates excretion. For example, larger amounts of apigenin conjugates were excreted \( (p < 0.05) \) than genistein conjugates in all regions of the intestine (Fig. 1B). In addition, for both compounds, conjugates excretion was much higher in duodenum and jejunum than that in terminal ileum and colon \( (p < 0.05); \) Fig. 1B). The maximum differences in excretion were about 25-fold for apigenin (53% of \( M_{ab} \) excreted as conjugates in jejunum, and 2% in colon) and 100-fold for genistein (7.9% of \( M_{ab} \) excreted as conjugates in duodenum, and 0.04% in colon). We also determined the amounts of genistein and apigenin absorbed during perfusion (35 \( \mu \)M each, \( M_{\text{total}} = 1604 \, \text{nmol} \) for all four segments every 30 min or 401 nmol for each segment every 30 min), and found that \( M_{ab} \) was approximately equal \( (448 \pm 31 \, \text{nmol}) \) for genistein, and \( 476 \pm 31 \, \text{nmol} \) for apigenin; \( p > 0.1 \). Finally, genistein conjugates were found in the plasma at amounts at least 8 times higher than apigenin conjugates (Fig. 1C).

**Intestinal versus Biliary Excretion of Phase II Conjugates.** Intact aglycones were not found in the bile or plasma collected during the perfusion experiments, even though large percentages of aglycones were absorbed (Fig. 1). Therefore, we analyzed amounts of conjugates excreted in bile after their hydrolysis with \( \beta \)-glucuronidase + sulfatase (Fig. 2). The results indicated that for genistein amounts of conjugates excreted into the intestinal lumen or \( M_{\text{met}} \) (52 nmol or 13% of \( M_{ab} \), on average and 61 nmol maximum per 30 min) were similar to that excreted into the bile or \( M_{\text{bile}} \) (45 nmol or 11% of \( M_{ab} \), on average and 50 nmol maximum) (Fig. 2A). In contrast, for apigenin, \( M_{\text{met}} \) (118 nmol or 33% of \( M_{ab} \) on average and 127 nmol maximum) were much larger than \( M_{\text{bile}} \) (27 nmol or 7% of \( M_{ab} \) on average and 29 nmol maximum) (Fig. 2B). The highest observed conjugated genistein concentration in bile was about 110 \( \mu \)M with an average steady-state concentration at approximately 80 \( \mu \)M, whereas the highest observed conjugated apigenin concentration in bile was slightly more than 75 \( \mu \)M with an average at ap-
Flavonoids are broadly distributed in the fruit and vegetables, and their consumption is associated with a variety of

proximoately 50 μM. The steady-state concentrations of genistein and apigenin conjugates in bile were significantly higher than the starting perfusate concentration of 35 μM.

**Metabolism of Genistein and Apigenin in Intestinal Microsomes of Different Regions.** Metabolism of genistein and apigenin (35 μM each) was determined using intestinal microsomes prepared from different regions of the rat intestine (i.e., duodenum, jejunum, ileum, and colon) (Fig. 3). The results indicated that there were significant differences in the metabolism of these two compounds using microsomes prepared from different regions of the rat intestine. Similar to what was observed in the perfusion studies, apigenin was metabolized faster than genistein. However, metabolism of these two compounds was always the highest in jejunal microsomes, and occurred readily in the colon, even though very little or no metabolites were found in the perfusate. The difference in metabolism rate between jejunum and colon was 5.5-fold for apigenin and 4.9-fold for genistein. In contrast, the differences in amounts of metabolites excreted in jejunal versus colon microsomes were 10 times more than that of genistein.

**Metabolism of Genistein and Apigenin in Caco-2 Microsomes.** Metabolism of genistein and apigenin (35 μM each at the donor side) were metabolized into glucuronidated and sulfated metabolites during their transport across the Caco-2 cell monolayers. More apigenin was conjugated than genistein (Fig. 5). Approximately equal amounts of glucuronidated metabolites and sulfated metabolites were found, but glucuronidated metabolites were slightly higher (Fig. 5). Conjugated metabolites were excreted into both apical and basolateral sides (Table 2). Amounts of apigenin glucuronic acid found in the basolateral and apical media were 3.5 times of genistein glucuronic acid, whereas amounts of apigenin sulfate found in the apical media were 10 times more than that of genistein sulfate.

**Discussion**

Flavonoids are broadly distributed in the fruit and vegetables, and their consumption is associated with a variety of

Fig. 2. Relative contribution of intestinal (slashed columns) versus biliary (solid columns) excretion of genistein (A) and apigenin (B) conjugates (n = 4). Amounts of metabolites excreted in the perfusate (Mper) and bile (Mbil) over a 30-min sample interval were calculated as described under Data Analysis using eqs. 2 and 3. In the figures, the time (e.g., 60 min) indicates the time when the sampling ends. Each column represents the average of four determinations and the error bar is the S.E.M. According to one-way ANOVA, there was no statistically significant effect of time in A or B. In A, amounts of genistein conjugates excreted into the lumen were similar to amounts of genistein conjugates excreted into the bile. In B, the amounts of apigenin conjugates excreted into the lumen were significantly higher than amounts of apigenin conjugates excreted into the bile (indicated by arrows).

Fig. 3. Metabolism of genistein (solid columns) and apigenin (slashed columns) in microsomes prepared from different regions of the rat intestine (n = 3). The concentration of both aglycones was 35 μM and the reaction time was 30 min. Each column represents the average of four determinations and the error bar is the S.D. There were statistically significant differences (p < 0.05) between rate of metabolism using microsomes prepared from different regions of the intestine for apigenin (labeled as "a" columns) and genistein (labeled as "b" columns), according to a one-way ANOVA analysis. In microsomes prepared from the same region of the intestine, rates of apigenin metabolism was significant higher (p < 0.05) than genistein (indicated by arrows).
health benefits, including anticancer, protection of the cardiovascular system, and many others (for reviews, see Kurzer and Xu, 1997; Birt et al., 2001; Yang et al., 2001; and Harborne and Williams, 2000). Although these claims are backed by a variety of in vitro studies, it is commonly known that flavonoids are extensively metabolized after oral administration to humans and rodents (Birt et al., 2001; Setchell et al., 2001; Yang et al., 2001). It is generally believed that the main site of flavonoid metabolism is liver (Kurzer and Xu, 1997). The main evidence in support of this claim is that bile and/or feces contained large amounts of conjugates (Xu et al., 1995; King et al., 1996). Several groups of investigators also showed recently that intestine metabolized flavonoids and excreted their conjugates (Crespy et al., 1999; Andlauer et al., 2000; Liu and Hu, 2002). In addition to extensive metabolism in gut and liver, one of the reasons why flavonoids have poor bioavailabilities is because they also participate in metabolic recycling processes. The best known recycling process (i.e., enterohepatic recycling), which has been shown to be important for the disposition of many drugs (Roberts et al., 2002), is proposed as one of the main reasons why these compounds have poor bioavailabilities. For flavonoids, which also undergo extensive intestinal conjugation and excretion (Crespy et al., 1999; Andlauer et al., 2000; Liu and Hu, 2002), we have proposed that enteric recycling involving microbial hydrolysis of flavonoid conjugates excreted by the enterocytes is also very important in their disposition (Liu and Hu, 2002). Which one of these two recycling processes is more important in the disposition of flavonoids has not been determined. However, the important role played by the intestinal microflora in these recycling processes has been confirmed by Schneider et al. (2000) who showed significant differences in the pattern of flavonoid metabolites recovered in urine, depending on whether or not bacteria are present in the gut. It is generally believed that these recycling processes may provide a prolonged exposure period for dietary flavonoids. Therefore, the purpose of this study is to determine the importance of intestinal disposition in the first-pass metabolism of flavonoids. The results of our studies may also allow us to determine whether enteric recycling process may be more important than enterohepatic recycling process in the first-pass metabolism of flavonoids.

In the present studies, we determined the excretion of phase II conjugates of flavonoids (i.e., genistein and apigenin) from the liver and from a four-site perfusion model (50% of total intestinal length). For apigenin (35 μM), much more conjugates were excreted from the intestine (33% of M_ab) than from the bile (7% of M_ab), which together accounted for 40% of M_ab. Assuming approximately equal amounts of phase II conjugates are excreted into the intestinal lumen and the basolateral domain (and into the portal vein), which was supported by our Caco-2 results (Table 2), intestinal metabolism could contribute to at least 66% of apigenin metabolism. Hence, intestinal conjugation seems to be the most important component for the first-pass metabolism of apigenin. This conclusion is supported by microsomal
studies using rat liver and intestinal microsomes, where apigenin was glucuronidated in the intestinal microsomes at a rate equal to (concentration 2.5 μM) or faster than (concentrations from 5 to 100 μM) its glucuronidation in the liver microsomes. It is further supported by the fact that absorption of apigenin should be completed in the upper small intestine, where apigenin was conjugated more extensively than terminal ileum and colon. If absorption only occurred in the upper small intestine, more intestinal metabolism than shown (33% of metabolite excretion) is likely, because rat duodenum excreted 41% of $M_{ab}$ and jejunum excreted 53% of $M_{ab}$ (Fig. 1B).

For genistein, approximately equal amounts of conjugates were excreted from perfused intestinal segment (13% of $M_{ab}$) and bile (11% of $M_{ab}$), which together accounted for more than 24% of absorbed amounts ($M_{ab}$). Even though the contribution of intestinal disposition to overall first-pass metabolism of genistein is apparent and significant, it is more difficult to precisely measure the contribution of intestinal disposition. For example, a first examination of the data suggests that intestinal conjugation may account for 26% of $M_{ab}$ (assuming equal amounts were excreted into the lumen and basolateral domain), and therefore is not the most important. However, a closer examination of the data reveals that the actual contribution could be much higher. This is due to the fact that genistein would have been absorbed in the upper small intestine after oral administration (top 20–30 cm), where the metabolism is much more extensive than what occurred in terminal ileum and colon. Indeed, up to 40% (average of 28%) of $M_{ab}$ was excreted into the jejunum as genistein conjugates. If that was the case, 56% of absorbed amounts could be conjugated in the upper small intestine, surpassing metabolism in liver. Furthermore, the concentration of genistein in the enterocytes is likely to be much higher than the concentration of genistein in the portal vein, which could be used to argue that apparent rates of metabolism of genistein in the intestinal microsomes could be higher than that in the liver microsomes.

Taken together, these data strongly support the hypothesis that intestinal disposition contributed more to the poor bioavailability of certain flavonoids than hepatic disposition. The contribution of hepatic disposition to the overall disposition of a flavonoid is likely to depend on its structure. Hepatic metabolism may be more important for flavonoids that are not as extensively metabolized in the gut or that are mainly absorbed in the larger intestine. Because intestinal disposition primarily involves conjugation and subsequent excretion of the conjugates, enteric recycling of flavonoids is at least as important as, if not more important than, the enterohepatic recycling in the first-pass metabolism process. At present, the biological implication of this discovery is not clear, but our findings may lend support to the hypothesis that flavonoids function to maintain proper levels of expression of phase II enzymes in the gut, liver, and probably other target sites (Birt et al., 2001; Yang et al., 2001). Another function of the enteric recycling process is that it prolongs the systemic exposure to dietary flavonoids, which is similar to the enterohepatic recycling process. However, the enteric recycling process may provide more constant exposure because gallbladder only empties with a meal.

We were somewhat surprised to observe that the magnitudes of differences between conjugate excretion in intact models (rat intestine or Caco-2 cells) were much higher than that in the corresponding microsomes. For example, apigenin was glucuronidated 10 times faster (11-fold difference) than genistein in the Caco-2 cell microsomes, but excretion rates of apigenin glucuronic acid in the Caco-2 cell monolayers was only 2.5 times faster. On the other hand, the differences in glucuronidation rates between jejunum and colon microsomes were 5.5-fold for apigenin and 4.9-fold for genistein. In contrast, the differences in amounts of conjugates excreted in jejunum versus colon were 25-fold for apigenin and 100-fold for genistein. These results suggest that glucuronidation is not the only important factor that determines how much metabolites are excreted into the intestinal lumen. Rather, transporters of flavonoid conjugates play a significant role in determining how much of the conjugates are excreted into the intestinal lumen and the basolateral intracellular space. Intestinal transport of glucuronidated and sulfated conjugates are poorly defined, but the involvement of multidrug resistance-related protein and related anion transporters in their efflux are likely, because conjugates are transported
this way in the liver. Studies are currently ongoing to determine the mechanisms of conjugate excretion. In conclusion, intestinal disposition of flavonoids is affected by both conjugating enzyme activities and specificities and capacities of efflux transporters for conjugated metabolites. Intestinal disposition may be more important than hepatic disposition in the first-pass metabolism of flavonoids.

In conjunction with enterohepatic recycling, enteric recycling may be used to explain why flavonoids have poor systemic bioavailabilities.

References


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