Metabolism of Flavonoids via Enteric Recycling: Mechanistic Studies of Disposition of Apigenin in the Caco-2 Cell Culture Model

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ABSTRACT
The purpose of this study was to determine the mechanisms responsible for intestinal disposition of apigenin in the human Caco-2 cell culture model. The results indicated that most of the absorbed apigenin (10 μM) were conjugated and only a small fraction was transported intact. The amounts of conjugates excreted, especially that of the sulfate, were dependent on days-post-seeding. Apical efflux of apigenin sulfate did not change with concentration of apigenin (4 to 40 μM), whereas its basolateral efflux increased (p < 0.01) with concentration and plateaued at about 25 μM. In contrast, sulfate formation rates in cell lysate increased with concentration and plateaued at 25 μM and were 4 to 6 times faster than the corresponding excretion rates. Formation and polarized excretion rates of glucuronidated apigenin increased with apigenin concentration but formation rates were usually 2.5 to 6 times faster than the corresponding excretion rates. Inhibitors of multidrug resistance-related proteins (MRPs) such as leukotriene C4 and MK-571, which inhibited glucuronidation of apigenin at a high concentration (>25 μM), significantly decreased excretion of both apigenin conjugates, and higher concentrations of MK-571 increased the extent of inhibition. In contrast, an organic anion transporter (OAT) inhibitor estrone sulfate only inhibited excretion of apigenin sulfate. In conclusion, we have shown for the first time that intestinal efflux is the rate-limiting step in the intestinal excretion of phase II conjugates of flavones. Furthermore, MRP and OAT are involved in the intestinal efflux of these hydrophilic phase II conjugates.

Apigenin is an important and bioactive common flavonoid that is present in a variety of plants, vegetables, fruits, and herbs as well as their extracts, which are commonly marketed as diet and herbal supplements. Consumption of a diet rich in flavonoids is associated with a variety of health benefits (Harborne and Williams, 2000; Birt et al., 2001; Yang et al., 2001). Apigenin has demonstrated anticancer activities (Wang et al., 2000; Gupta et al., 2001; Ross and Kasum, 2002). Recently, apigenin has been shown to have the potential to prevent prostate cancer via cell cycle regulation, apoptosis, inhibition of androgen synthesis, or inhibition of oncogenic protein kinases (Lee et al., 1998; Makela et al., 1998; Knowles et al., 2000; Wang et al., 2000; Gupta et al., 2001, 2002; Kobayashi et al., 2002). However, apigenin, like many other flavonoids that are part of popular herbal supplements such as ginkgo biloba, is poorly bioavailable (Birt et al., 2001; Ross and Kasum, 2002), and little is known about its absorption and metabolism. Like many other flavonoids, 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. Studies in rats and Caco-2 cells suggest that it is rapidly absorbed but more extensively metabolized than genistein, an isoflavone analog of apigenin (Liu and Hu, 2002; Chen et al., 2003). In HepG2 cells, apigenin was also metabolized rapidly via UGT1A1 (Galigatovic et al., 1999; Walle and Walle, 2002). 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).

Previously, we have proposed that repeated shuffling of flavonoids through a duo recycling scheme involving both enteric and enterohepatic recycling is the reason for extensive metabolism of apigenin (Liu and Hu, 2002). This metabolic scheme is supported by studies from independent groups of investigators, who also showed that intestine conjugated flavonoids and excreted their conjugates (Crespy et al., 1999; Andlauer et al., 2000). More recently, we have shown that intestinal disposition of apigenin is more important than hepatic disposition of apigenin, in that more apigenin conjugates were excreted into the rat lumen directly than those excreted via the bile (Chen et al., 2003). However,

ABBREVIATIONS: HBSS, Hanks’ balanced salt solution; HPLC, high pressure liquid chromatography; MRP, multidrug resistance-related protein; OAT, organic anion transporter; UGT, UDP-glucuronosyltransferase.
intestinal metabolism of flavonoids is poorly understood, and mechanistic studies that systematically study the intestinal metabolism of a flavone and enteric excretion of its phase II conjugates are lacking. For example, the mechanisms responsible for efflux of phase II conjugates of flavonoids from the enterocytes have not been determined. Studies of effects of cellular differentiation and of changes in apigenin concentration on intestinal phase II conjugates of flavones are also lacking.

In this report, we systematically determined the pathways and kinetics of apigenin disposition and mechanisms responsible for metabolism of apigenin and excretion of apigenin conjugates in the Caco-2 cell monolayers. We have chosen the Caco-2 cell model system because it is an established model of human intestinal epithelium. It was also chosen because apigenin is rapidly metabolized in this model, which allowed us to accurately measure amounts of apigenin metabolites excreted as a function of time.

Materials and Methods

Materials. Cloned Caco-2 cells, TC7, were a kind gift from Dr. Monique Rousset of INSERM U178 (Villejuif, France). MK-571 and leukotriene C4 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Apigenin was purchased from Indofine Chemicals (Somerville, NJ). β-Glucuronidase with (catalog no. G1512) or without (catalog no. G7396) sulfatase, sulfatase without glucuronidase (catalog no. S1629), uridine diphosphoglucuronic acid, alamethicin, β-saccharic-1,4-lactone monohydrate, magnesium chloride, estrone sulfate, and Hanks’ balanced salt solution (HBSS; powder form) were purchased from Sigma-Aldrich (St Louis, MO). 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; Liu and Hu, 2002). Caco-2 TC7 cells were fed every other day, and the monolayers were ready for experiments from 19 to 22 days post-seeding except in the days-post-seeding studies.

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 from those 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 on to the apical side, and amounts of transepithelial transport were followed as a function of time. Four donor samples (650 μl) and four receiver samples (650 μl) were taken every 30 min, followed by the addition of 650 μl of donor solution to the donor side or 650 μl of fresh buffer at the receiver side. As an internal standard and preservative, 200 μl of methanol/acetoniitrole containing 50 μM of testosterone was added to each sample. Afterward, the mixture was centrifuged at 13,000 rpm for 15 min, and the supernatant was analyzed by HPLC (see below).

Caco-2 Cell Lysate. Caco-2 cell monolayers (6) were put in 6 ml of 50 mM potassium phosphate (pH 7.4 buffer), and sonicated in Aquasonic 150D sonicator (VWR Scientific, Bristol, CT) for 20 min at the maximum power (135 average watts) in an ice-cold water bath. The resulting cell lysate is then harvest and used fresh in the experiments.

Lysate Protein Concentration. Protein concentration of lysate was determined using the Bio-Rad protein assay (Hercules, CA), using bovine serum albumin as standards.

Measurement of UGT Activities Using Cell Lysate. The incubation procedures for measuring UDP-glucuronosyltransferase (UGT) activities using cell lysate were as follows: 1) mix 135 μl of cell lysate (final concentration = 0.7 mg of protein/ml), 3’-phosphoadenylyl sulfate (100 μM), and substrate of an appropriate concentration (total volume 100 μl) for 30 min; 2) stop the reaction by the addition of 25 μl of 94% acetonitrile/6% glacial acetic acid containing 100 μM testosterone as an internal standard; 3) centrifuge the sample at 13,000 rpm for 15 min; and 4) assay the sample via HPLC as described below.

HPLC Analysis of Apigenin and Its Conjugates. The conditions for HPLC analysis of isoflavones were modified based on a previously published method (Chen et al., 2003). The conditions for analyzing apigenin and its conjugates were: system, HP 1090 with diode array detector and HP Chemstation; column, Aquapak Phenomenex, Torrence, CA), 5 μm, 150 × 0.45 cm; mobile phase A, 0.04% (v/v) phosphoric acid plus 0.06% (v/v) triethylamine (pH 2.8); mobile phase B, 100% acetonitrile; gradient, 0 to 3 min, 20% B, 3 to 22, 20 to 49% B, 22 to 23, 49% B, wavelength 340 nm; injection volume, 200 μl. There was a 5 min interval between the end of the run and the next injection to allow the column to be re-equilibrated with 20% mobile phase B. The retention time of apigenin was 14.3 min, apigenin sulfate 11.0 min, and apigenin glucuronic acid 4.7 min.

Data Analysis. Rate of apigenin transport or rate of apigenin conjugate excretion was determined using linear regression analysis of amounts transported or excreted versus time plots.

One-way analysis of variance 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

Apical-to-Basolateral Transport and Metabolite Excretion As a Function of Days-Post-Seeding in Intact Caco-2 Cell Monolayers. We followed the transport and metabolism of apigenin (10 μM) as a function of days-post-seeding (Fig. 1). The results indicated that amounts of intact apigenin transported from the apical to-basolateral side, rate of excretion of conjugates from both the apical and basolateral sides, increased approximately linearly with time (Fig. 1). The total amounts of sulfate and glucuronidated metabolites excreted were significantly (p < 0.05) dependent on days-post-seeding (Fig. 2A). Maximal amounts of metabolites were excreted at 15 days post-seeding, and the maximal amounts of intact apigenin were transported at 9 days post-seeding (Fig. 2A). The rates of metabolite excretion were also significantly dependent on days-post-seeding and the maximal excretion rate or maximal amounts excreted occurred at 15 days post-seeding (Fig. 2B). In contrast to the total rate, only basolateral excretion rates reached maximum at 15 days post-seeding whereas apical excretion rates appear to have...
two maxima at 15 and 24 days post-seeding (Fig. 2C). In addition to the dependence on days-post-seeding, more glucuronidated metabolites were excreted into the basolateral media, whereas more sulfated metabolites were excreted into the apical medium (Fig. 1). Last, higher total amounts of sulfated metabolites were excreted than glucuronidated metabolites at an apigenin concentration of 10 μM (Fig. 2B, which was primarily due to much faster apical excretion of sulfates (Fig. 2C).

**Effects of Concentrations on the Disposition of Apigenin in Intact Cell Monolayers.** An increase in apical concentrations of apigenin from 5 to 40 μM significantly increased the apical-to-basolateral transport rates of apigenin, basolateral efflux rates of glucuronidated and sulfated conjugates, and apical efflux rates of glucuronidated conjugates, but not apical efflux rates of sulfated conjugates. At concentrations of 5 μM or less, the rates of apigenin transport (in intact form) were quite low. The transport rates increased linearly with the concentration from 5 to 15 μM, after which the increase in transport rate accelerated (as signified by the rates above the straight line in Fig. 3A). In contrast, the basolateral excretion rates of both conjugates increased with concentration but gradually plateaued at higher concentrations (Fig. 3B). On the apical membrane, excretion rates of glucuronic acid increased but excretion rates of sulfate did not change significantly with concentration (Fig. 3C). In general, higher percentages of conjugates were excreted as sulfates at lower concentrations of apigenin but as glucuronic acid at higher concentrations (Fig. 3, B and C).
Effect of Concentrations on the Metabolism of Apigenin in Caco-2 Cell Lysate. Conjugated metabolism of apigenin was measured as a function of concentrations (2.5–50 μM) for 30 min (for sulfation) or 60 min (for glucuronidation) after preliminary studies demonstrated that amounts of metabolites were linear with time for at least 90 min (not shown). The results indicated that glucuronidation rates increased with concentration and started to approach but did not reach saturation (Fig. 4A). In contrast, sulfation appeared to have reached saturation and started to show some level of substrate inhibition at high concentrations (≥50 μM) (Fig. 4B). When the conjugation reaction rates were converted to nanomoles per hour per Caco-2 cell monolayer, these conjugation formation rates in cell lysate were substantially higher (2- to 6-fold) than the total excretion rates of conjugates from the Caco-2 cells (Fig. 4).

Effects of MRP Inhibitors on the Disposition of Apigenin in Caco-2 Cell Lysate. We were interested in the determination of effects of MRP inhibitors leukotriene C4 and MK-571 on the efflux of conjugated apigenin since MRPs are

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known to transport glucuronic and sulfated conjugates in the liver by the hepatocytes (Konig et al., 1999). Therefore, we first determined whether these inhibitors would affect the conjugation of apigenin via the UGT and sulfotransferase pathways. To our surprise, MK-571 inhibited glucuronidation of apigenin at a concentration (25–50 μM) previously used to study the efflux mechanisms of these conjugates (Vaidyanathan and Walle, 2001; O’Leary et al., 2003) (Fig. 5). On the other hand, leukotriene C4 did not inhibit the glucuronidation of apigenin. Last, neither MK-571 (up to 100 μM) nor leukotriene C4 (up to 1.6 μM) inhibited the sulfation of apigenin.

**Effects of MRP Inhibitors on the Disposition of Apigenin in Intact Caco-2 Cells.** MRP inhibitors leukotriene C4 and MK-571 (the latter also inhibits glucuronidation at high concentrations) were used to determine the mechanisms by which conjugated apigenin is transported out of the Caco-2 cells. The results indicated that 0.1 μM leukotriene C4 significantly ($p < 0.05$) decreased the apical efflux rate of apigenin sulfate but significantly increased ($p < 0.05$) its basolateral efflux rates when it is present on the basolateral side. Presence of this inhibitor at the apical side only or at both sides only slightly increased its basolateral efflux ($p > 0.05$). Effects of leukotriene C4 on excretion of glucuronidated metabolites were more complex, but the effects were generally moderate. For apical efflux of glucuronidated apigenin, presence of leukotriene C4 at both sides of the monolayers decreased its efflux ($p < 0.05$) but the effect was moderate (32%). The presence of leukotriene C4 at the basolateral side, however, significantly increased (up to 51%, $p < 0.05$) the basolateral efflux rate of apigenin glucuronic acid (Fig. 6).

Unlike leukotriene C4, 10 μM MK-571, which is not expected to inhibit the formation of glucuronidated metabolites because of the low concentration, significantly ($p < 0.05$) inhibited efflux of glucuronidated conjugates (31% for basolateral and 44% for apical). MK-571 at 10 μM also inhibited apical efflux of sulfated metabolites by 61% ($p < 0.05$) but only slightly decreased basolateral efflux of sulfated metabolites (20%, $p > 0.05$). When the concentration of MK-571 was increased to 50 μM, polarized excretion of both conjugates further decreased. Use of MK-571 and leukotriene C4 greatly further reduced ($p < 0.05$) apical efflux of these conjugates when compared with using these inhibitors alone, but the combination did not enhance the extent of basolateral excretion.

**Effects of OAT Inhibitors on the Transport and Metabolism of Apigenin.** Estrone sulfate has been shown to be the substrate of many prototypical anion transporters in the kidney and to a lesser extent in the liver cannicular mem-

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**Fig. 4.** Effects of concentration on the formation and excretion rates of apigenin glucuronic acid (panel A) and apigenin sulfate (panel B). Formation rates (hollow symbols with solid curves) of apigenin sulfate (diamonds) and apigenin glucuronic acid (squares) were measured in Caco-2 cell lysate, obtained from cell monolayers (21–22 days post-seeding). Excretion rates (solid symbols with dashed curves) of apigenin conjugates were measured separately from the formation rates using cell monolayers of the same ages. To facilitate comparison, both rates were normalized to nanomoles per hour per cell monolayer or nanomoles per hour in the figure. We assumed that the activities of various enzymes in the cell lysate were the same as those present in intact cells, which created a slight negative bias since some enzyme activities are expected to be lost during lysate preparations. Each point represents the average of three determinations, and the error bar is the standard deviation of the mean. The regression lines represent the trend of the data and are not based on a kinetic model of metabolism or transport.

**Fig. 5.** Effects of different concentrations of MK-571 and leukotriene C4 (LTC4) on the formation rates of apigenin (10 μM) conjugates in Caco-2 cell lysate. Effects of MK-571 concentrations on the formation rates of sulfate (diamonds) and glucuronic acid (squares) were determined as a function of MK-571 (A) or leukotriene C4 (B) in Caco-2 cell lysate. The maximal MRP inhibitor concentrations used for cell lysate experiments were at least 2 times the maximal concentrations used in the intact cell studies. Each point (bar) represents the average of three determinations, and the error bar is the standard deviation of the mean. The star symbol indicates that the rate of metabolism is significantly less than the control.
Fig. 6. Effects of MRP inhibitors on transport and metabolism of apigenin (10 μM). Control experiments in this figure are the experiments performed in the absence of any MRP inhibitors. MRP inhibitor leukotriene C₄ (LTC₄) or MK-571 was used alone or in combination with one another. We also used different concentrations of MK-571. We measured the apical-to-basolateral transport rate of apigenin (panel A), basolateral excretion rate (panel B), and apical excretion (panel C) rates of apigenin glucuronic acid (shaded bars) and apigenin sulfate (solid bars). Each bar represents the average of three determinations, and the error bar is the standard deviation of the mean. The star symbol indicates that the rate of metabolism is significantly different from the control.
brane (Sekine et al., 2000). We found that the presence of the estrone sulfate (10 μM) in the apical and basolateral medium did not significant affect excretion of apigenin glucuronic acid but significantly decreased apical and basolateral excretion of apigenin sulfate by 76 and 46% (p < 0.01), respectively (Fig. 6).

Discussion

Apigenin is extensively metabolized in vivo and in cell culture models, and it is metabolized via the dual recycling scheme involving both enteric recycling and enterohepatic recycling (Galijatovic et al., 1999; Liu and Hu, 2002; Walle and Walle, 2002; Chen et al., 2003). Here we determined intestinal disposition of apigenin and the mechanisms by which its metabolites are excreted in the Caco-2 cells to gain better understanding of processes responsible for the intestinal flavonoid disposition.

We first defined a time window for the proposed studies by determining how transport and metabolism change as a function of days-post-seeding. Changes in days-post-seeding were shown to affect peptide transporter function and proline activities (Hu et al., 1995). The results indicated that most of absorbed apigenin were conjugated, regardless of days-post-seeding (Fig. 1). The structures of the conjugates appeared to be the same also at different days-post-seeding because of similarities in: 1) metabolites’ retention times; 2) the retention times of metabolites’ hydrolysis products; and 3) the UV spectra of the metabolites (not shown). The types of flavonoid metabolites found here are similar to the types of flavonoid conjugates found by Walle and coworkers (Galijatovic et al., 1999). Since the excretion rates were similar from 21 to 24 days post-seeding (Fig. 2), the later experiments were performed using cell monolayers of 19 to 22 days post-seeding.

We also determined the effects of concentration on the disposition of apigenin and found that cellular conjugation was saturable (a plot of concentration versus passive absorption rates deviated positively) (Fig. 3A). In intact cell monolayers, types of metabolites formed, and how they were effluxed depended on the concentration (Fig. 3, B and C). At low concentrations, the primary metabolite is apigenin sulfate, which is mainly excreted apically (Fig. 3, B and C). At high concentrations, the primary metabolites became apigenin glucuronic acid, which was preferentially excreted basolaterally (Fig. 3, B and C). This suggests that the apical efflux transporter has high affinity and low capacity for apigenin sulfate, because apical efflux of apigenin sulfate was constant even though sulfate formation rates increased with concentrations (Fig. 3, B and C, and Fig. 4B).

To explain the apparent lack of correlations between cellular sulfate formation rates and its excretion rates, we hypothesized that conjugate excretion could be rate-limited by their efflux rates. We found that the excretion rates of apigenin sulfate are much slower than its formation rates, especially at higher concentrations (Fig. 4B). This important finding supports our hypothesis, which is novel since the current theory of metabolite production asserts that enzyme level is the most important factor in determining how much metabolite is produced. Therefore, when changes in phase II metabolite levels are observed in vivo or in vitro, the most likely experiments to do at the present are the determination of phase II enzyme expression levels. Our finding suggests that it will be necessary to determine the expression levels of efflux transporters as well.

Our hypothesis that cellular excretion of conjugates is rate-limited by their efflux will have significant impacts on drug induction and drug-drug interaction studies, if it is proven in vivo. Traditionally, phase II metabolism-related interaction studies always focus on the induction or the inhibition of enzymes. The results presented here suggest that it will be valuable to determine the induction or inhibition of the efflux transporters in drug-drug interaction studies involving hydrophilic phase II conjugates.

We believe that MRPs and OATs are involved in the efflux of hydrophilic conjugates, based on what is known about their transport in kidney and liver (Konig et al., 1999; Sekine et al., 2000). We found that leukotriene C₄ inhibited apical efflux of both apigenin conjugates, but mildly increased basolateral efflux of apigenin conjugates. We also found that MK-571 (10 μM) was a highly effective inhibitor of apical efflux of apigenin conjugates but was only mildly effective against basolateral efflux of apigenin conjugates. When used at a higher concentration, MK-571 (50 μM) was more effective against the excretion of conjugated metabolites, but its inhibition of cellular excretion of glucuronic acid is somewhat complicated by the fact that MK-571 also inhibited glucuronidation at this concentration. Taken together, these results support the hypothesis that the apical efflux is mediated by MRP and possibly other OATs, whereas basolateral efflux of these conjugates are probably mediated by an OAT since leukotriene C₄ was capable of stimulating efflux of both conjugates. The nature of this basolateral efflux transporter is currently unknown, but it may be similar to an organic anion transporter called rOAT1 because rOAT1 could interact with both estrone sulfate (inhibition) and leukotriene C₄ (stimulation) (Sekine et al., 2000).

The finding that intestinal Caco-2 cells secreted glucuronidated and sulfated metabolites via MRPs and estrone sulfate-sensitive organic transporter is novel. In general, mechanisms responsible for intestinal efflux of phase II conjugates of xenobiotics are poorly understood. We have hypothesized that these conjugates will be effluxed from the intestinal cells via MRPs based on the fact that cannicular MRPs transported many conjugates into the bile (Konig et al., 1999). Previously, only efflux of glutathione conjugates from the intestinal Caco-2 cells via MRP was shown (Gotoh et al., 2000; Terlouw et al., 2001). Now we have shown that MRPs, probably MRP2 based on the fact that MRP2 is located apically (Konig et al., 1999), are capable of excreting sulfated and glucuronidated conjugates of flavonoids. We have also shown that efflux of sulfates but not glucuronides may be mediated by the organic anion transporter similar to rOAT1. Based on previous observations that conjugates of many flavonoids are excreted into the lumen (Crespy et al., 1999; Andlauer et al., 2000; Liu and Hu, 2002; Chen et al., 2003), MRP2 and an estrogen sulfate-sensitive transporter may be responsible for apical excretion of many flavonoid conjugates. Further studies are underway to define the structural characteristics of suitable flavonoid conjugates.

In summary, we have shown for the first time that intestinal excretion of hydrophilic phase II conjugates is rate-limited by the efflux transporters. We are the first to demonstrate that intestinal MRP and possibly estrone sulfate-sensitive organic transporter.
sensitive OATs are involved in the apical and basolateral efflux of hydrophilic phase II conjugates of apigenin.

References


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