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Transport of the Flavonoid Chrysin and Its Conjugated Metabolites by the Human Intestinal Cell Line Caco-2

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ABSTRACT. Chrysin (5,7-dihydroxyflavone), a natural product present in our daily diet, is a potent inhibitor of drug-metabolizing enzymes. However, its oral bioavailability is not known. This study examined the intestinal epithelial transport of chrysin (20 μM), using the human colonic cell line Caco-2 as a model of human intestinal absorption. The apical to basolateral flux of chrysin, with an apparent permeability coefficient (P_{app}) during the first hour of $6.9 \pm 1.6 \times 10^{-6}$ cm · sec^{-1} (mean ± SEM), was more than 10-fold higher than for the paracellular transport marker mannitol, $0.42 \pm 0.12 \times 10^{-6}$ cm · sec^{-1}. Interestingly, the reverse, basolateral to apical flux of chrysin, $P_{app} = 14.1 \pm 1.6 \times 10^{-6}$ cm · sec^{-1}, was about 2-fold higher than the apical to basolateral flux ($P < 0.01$). In transport studies beyond 1 hr, there was a rapid decline in P_{app}. This correlated with the appearance of two metabolites, M1 (chrysin glucuronide) and M2 (chrysin sulfate), identified by enzymatic hydrolysis procedures and HPLC. Following apical loading of chrysin, as much as 90% of M1 + M2 appeared on the apical side, thus indicating clear efflux of the chrysin metabolites. The addition of the anion transport inhibitor MK-571 (50 μM) on the apical side produced a 71% ($P < 0.0001$) and 20% ($P < 0.05$) inhibition of the efflux of M1 and M2, respectively, suggesting the involvement of the multidrug resistance protein MRP2 pump. Indeed, using specific antibodies, MRP2 was in fact detected by western blotting in Caco-2 plasma membranes, whereas MRP1 was not. These observations suggest that chrysin has favorable membrane transport properties but that its intestinal absorption may be seriously limited by surprisingly efficient glucuronidation and sulfation by the enterocytes and almost quantitative efflux by MRP2 of the metabolites formed. BIOCHEM PHARMACOL **58**;3: 431–438, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. chrysin; bioflavonoids; flavonoids; Caco-2 cells; intestinal absorption; glucuronidation; sulfation; efflux; MRP2; cMOAT

Flavonoids are polyphenolic compounds present in many fruits, vegetables, and beverages [1, 2]. They have been strongly implicated as protective in coronary heart disease and stroke [3, 4] as well as cancer [5, 6]. One flavonoid, chrysin (Fig. 1), present at high levels in honey and propolis [7], has also been shown recently to be a potent inhibitor of human immunodeficiency virus activation in models of latent infection [8]. The target of this effect appears to be casein kinase II [9]. Chrysin has also been shown to be a potent inhibitor of drug-metabolizing enzymes, including CYP1A1 [10] and the P-form phenolsulfotransferase [11], both involved in carcinogen bioactivation.

Whether any of the *in vitro* effects can be extrapolated to the *in vivo* situation is unknown, as the systemic availability of chrysin after oral ingestion has not been investigated. Chrysin, however, can undergo metabolic transformation and is metabolized extensively by rat liver microsomes [12]. The resulting products were shown to be mono- and dihydroxylated chrysin, with the oxidation site being the B-ring. It should be pointed out, however, that this metabolism occurred in microsomes from Aroclor 1254-induced rats; microsomes from uninduced rats showed very little metabolism of chrysin.

In the present study, we used the human adenocarcinoma colonic cell line Caco-2, a well accepted model of human intestinal absorption [13, 14], recently used for studies of quercetin [15], to study the transport of chrysin. Although chrysin, from these observations, appeared to have favorable membrane transport properties, its absorption may still be seriously limited by surprisingly efficient conjugative metabolism by the intestinal epithelial cells.

MATERIALS AND METHODS

Materials

Chrysin, β-glucuronidase from bovine liver (essentially sulfatase-free), D-saccharic acid 1,4-lactone, sulfatase from *Aerobacter aerogenes* (no detectable β-glucuronidase activity at pH 7), and estrone 3-sulfate were obtained from the

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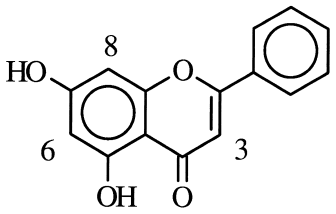

FIG. 1. Chemical structure of chrysin (5,7-dihydroxyflavone).

Sigma Chemical Co., and D-[^{14}C]mannitol (57 mCi/mmol) was purchased from Amersham Life Science. MK-571 was a gift from Dr. A. W. Ford-Hutchinson, Merck-Frosst Centre for Therapeutic Research.

The polyclonal antibody EAG5, raised against the MRP2-specific carboxy terminus of human MRP2 [16, 17], was a gift from Dr. D. Keppler (Krebsforschungszentrum, Heidelberg). The monoclonal antibodies MRPm6 and M_2I-4, raised against bacterial fusion proteins containing the carboxy terminal and carboxy proximal end of MRP* (MRP1) [18] and an internal epitope of cMOAT (MRP2) [19], respectively, were obtained from the Kamiya Biomedical Co. QCRL-1, monoclonal MRP1 antibodies raised against doxorubicin-selected H69 cell membranes [20, 21], were a gift from Dr. S. P. C. Cole and are commercially available from Centocor Diagnostics. Secondary antibodies, HRP-conjugated goat anti-rabbit and anti-mouse antibodies, and LumiGLO chemiluminescence substrate were from Kirkegaard & Perry Laboratories. Electrophoresis and blotting supplies were from Bio-Rad Laboratories. Pre-stained molecular weight marker mixture and α_2-macroglobulin (M_r 180 kDa) were purchased from the Sigma Chemical Co.

Cell Culture

Caco-2 cells from the American Type Culture Collection (ATCC) were cultured in Eagle's Minimum Essential Medium with 10% fetal bovine serum, nonessential amino acids, and antibiotics as previously described [15, 22]. For transport studies, the cells were seeded in 12 mm i.d. Transwell polycarbonate inserts (Corning Costar Corp.) in 12-well plates at a density of 10^5 cells/insert. Cells were used for transport experiments at passage 30–44 at 20–31 days after seeding. Inserts were used for transport experiments when the transepithelial electrical resistance exceeded 300 $\Omega \cdot cm^2$. Hep G2 cells from ATCC were grown as previously described [23]. PANC-1 cells from ATCC were grown in RPMI-1640 medium with 10% fetal bovine serum [24], penicillin, and streptomycin.

* *Abbreviations*: MRP, multidrug resistance protein; cMOAT, canalicular multispecific organic anion transporter; TBST, Tris-buffered saline with 0.05% Tween 20; HRP, horseradish peroxidase; and HBSS, Hanks' balanced salt solution with 25 mM HEPES, pH 7.4.

Transport Experiments

The cell layers were washed twice for 30 min with warm HBSS. Chrysin was dissolved in ethanol and diluted to 20 μM with HBSS (final ethanol concentration 0.4%). This loading solution was added to either the apical or the basolateral chamber (0.5 or 1.5 mL, respectively). HBSS was added on the other side. In the experiments involving MK-571, a selective inhibitor of the anionic MRP pumps [25–27], the drug was dissolved in HBSS and added to the apical side. All solutions were made fresh immediately prior to each experiment. A small amount of [^{14}C]mannitol was added to the apical chamber. Samples were withdrawn from both chambers for analysis of chrysin and metabolites by HPLC. At the end of each experiment, basolateral samples were also analyzed for mannitol transport by liquid scintillation counting.

Although the intestinal chrysin concentration after dietary intake of this flavonoid is not known, we selected 20 μM as being reasonable and below that employed in two animal studies [7, 28]. In a previous study, we selected 50 μM quercetin as a reasonable oral concentration of this flavonoid [15].

Sample Analysis

Chrysin and its metabolites in the transport buffer were separated and quantified without further purification by reversed-phase HPLC. A Symmetry C_{18} column (3.9 × 150 mm, Waters) with a μBondapak C_{18} Guard-Pak precolumn insert (Waters) and a mobile phase of 55% methanol in 1 M acetic acid at a flow rate of 0.9 mL/min was used. Detection was by UV at 280 nm.

Enzymatic Hydrolysis of Chrysin Conjugates M1 and M2

Transport medium from the apical side of Caco-2 inserts 6 hr after basolateral loading of 20 μM chrysin was used for these experiments. When testing for the presence of a chrysin glucuronide, the pH was adjusted to 4.5 with 1 M sodium acetate buffer. Beef liver β-glucuronidase (2 mg; 1300 U) and, in some samples, the β-glucuronidase inhibitor d-saccharic acid 1,4-lactone (1 mg) [29] were added. Control samples were incubated in the absence of enzyme. After incubation in a shaking water bath at 37° for 24 hr, the samples were analyzed by HPLC as described above after solid phase extraction (Oasis HLB extraction cartridges, Waters). When testing for the presence of a chrysin sulfate conjugate, the transport medium was kept at pH 7.4. Sulfatase (0.35 μL; 0.006 U) and, in some samples, the sulfatase inhibitor estrone 3-sulfate (200 μM) [30] were added. Control samples were incubated in the absence of enzyme. The samples were incubated at 37° for 30 min and subjected to solid phase extraction and HPLC analysis.

Calculations and Statistics

Chrysin and the chrysin metabolites were quantified by peak area measurement in comparison to known amounts of chrysin. As no synthetic chrysin glucuronide or sulfate was available, the quantitation of these compounds assumes equal molar absorption as for chrysin itself. Although a very slight shift in the UV absorption profile was observed for these metabolites as compared with chrysin, this assumption appears reasonable.

The apparent permeability coefficients (P_{app}), expressed in cm/sec [13], were calculated as $\Delta Q/\Delta t \times 1/60 \times 1/A \times 1/C_0$, where $\Delta Q/\Delta t$ is the permeability rate (μg/min), A is the surface area of the membrane (cm^2), and C_0 is the initial concentration in the donor chamber (μg/mL). One-hour samples were used for P_{app} calculations. The statistical significance of differences between different transport directions or treatments was evaluated using Student's two-tailed paired *t*-test with a significance level of $P < 0.05$.

Preparation of Membrane-Enriched Cell Fractions for Immunoblotting

Cell membrane fractions were prepared essentially as described by Almquist *et al.* [31]. Caco-2 cells (grown in 100-mm dishes) were rinsed with PBS, scraped off the dishes, and collected by centrifugation. The cell pellet was resuspended in 10 mM Tris buffer, pH 7.4, containing KCl, $MgCl_2$, protease inhibitors, RNase A and DNase I (50×10^6 cells/mL). After 10 min on ice, the cells were sonicated twice for 5 sec each time, and the homogenate was centrifuged at 800 *g* for 15 min at 4°. The supernatant then was centrifuged at 100,000 *g* for 20 min at 4° to give a pellet enriched in cell membranes. This pellet was resuspended in Tris with sucrose and protease inhibitors and stored at −80°. Hep G2 and PANC-1 cell membranes were prepared identically.

Immunoblotting

Samples of membrane-enriched cell fractions were diluted 1:2 with sample treatment buffer, agitated for 30 min at room temperature, and loaded on 7.5% SDS-polyacrylamide gels. After electrophoresis [32], the proteins were transferred to nitrocellulose [33] and probed for MRP2 [34]. After 1 hr blocking with 10% nonfat milk powder in TBST, the blots were incubated with EAG5 MRP2 primary antibodies at a 1 to 20,000 dilution in milk-blot overnight at room temperature. After three TBST washes of 10 min each, the blots were incubated with secondary antibody (HRP-conjugated goat anti-rabbit, 1 to 1000 dilution in milk-blot) for 1 hr. After thorough washing the antibody binding was determined by ECL detection.

With the monoclonal MRP1 and MRP2 antibodies MRPm6 and M_2I-4, the sample loading, electrophoresis, and transfer were identical to the procedures above, except that polyvinylidene difluoride membranes were used. After overnight blocking at 4° with 2% BSA in TBST, primary antibody incubations with MRPm6 and M_2I-4 (MRP1 and MRP2, respectively), at a dilution of 1 to 200, were done for 1 hr at room temperature. The secondary antibody, HRP-conjugated goat anti-mouse IgG, was incubated with the blot for 1 hr at a 1 to 1000 dilution.

With the monoclonal QCRL-1 MRP1 antibody, blocking of the nitrocellulose membranes was done with 1% BSA and 5% fetal bovine serum in TBST [21]. Incubation with the primary antibody at a dilution of 1 to 1000 was for 2 hr at room temperature. The HRP-conjugated goat anti-mouse antibodies were used at a 1 to 10,000 dilution for 1 hr.

RESULTS

After apical loading of chrysin (20 μM), chrysin could be detected on the basolateral (receiving) side as early as 30 min after loading and throughout the 6-hr incubation period (retention time 9.9 min) (Fig. 2A). Similar amounts of two chrysin metabolites, M1 and M2, also were detected. Surprisingly, both M1 and M2 were detected at much higher concentrations on the apical (loading) side (Fig. 2B). The chromatograms also suggested that M1 and M2 each may consist of one major and one minor isomer.

M1 and M2, appearing in the apical solution, were identified as glucuronic acid and sulfate conjugates of chrysin, respectively, by enzymatic hydrolysis, as detailed in Materials and Methods. Thus, incubation with beef liver β-glucuronidase led to the disappearance of the M1 peak with a concomitant increase in the chrysin peak but with no change in the M2 peak. This hydrolytic reaction was inhibited by the β-glucuronidase-specific inhibitor D-glucaro-1,4-lactone [29]. When incubations were done with bacterial aryl sulfatase, the M2 peak disappeared with a concomitant increase in chrysin but with no change in the M1 peak. This hydrolytic reaction was inhibited by the selective sulfatase inhibitor estrone sulfate [30].

A more complete and quantitative account of the fluxes of chrysin and its metabolites M1 and M2 after both apical and basolateral loading of chrysin is shown in panels A–D of Fig. 3. After apical loading, the chrysin concentrations on the apical side decreased from 20 μM at time zero to 12 μM at 1 hr and only 4 μM at 3 hr and 2 μM at 6 hr (Fig. 3A). Only a small fraction of this loss was due to transport to the basolateral side (Fig. 3B). Most of this loss was due to the formation of M1 and M2 by the Caco-2 cells and subsequent efflux to the apical side (Fig. 3A). Although chrysin was accumulated extensively by the Caco-2 cells with a cell/medium concentration ratio of about 10, this accumulation had only a modest effect on the mass balance. After basolateral loading of chrysin, the rate of loss of chrysin from the loading side appeared less (Fig. 3C), presumably due to the 3-fold larger volume on the basolateral compared with the apical side. However, the main profile was very similar to apical loading, with high concentrations of both M1 and M2 appearing on the apical side

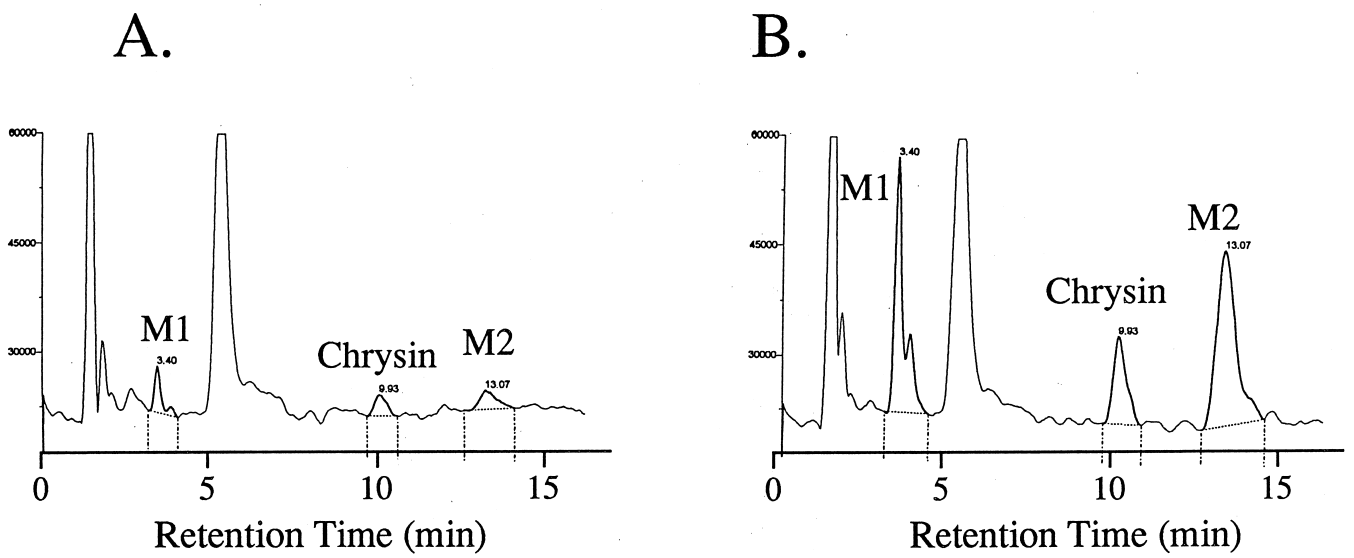

FIG. 2. HPLC of chrysin and chrysin metabolites M1 and M2 in the transport medium of Caco-2 cells after apical loading of 20 μM chrysin. (A) Basolateral sample; and (B) apical sample, after a 6-hr incubation. The y-axis indicates absorption (280 nm) expressed in arbitrary units (same scale in A and B).

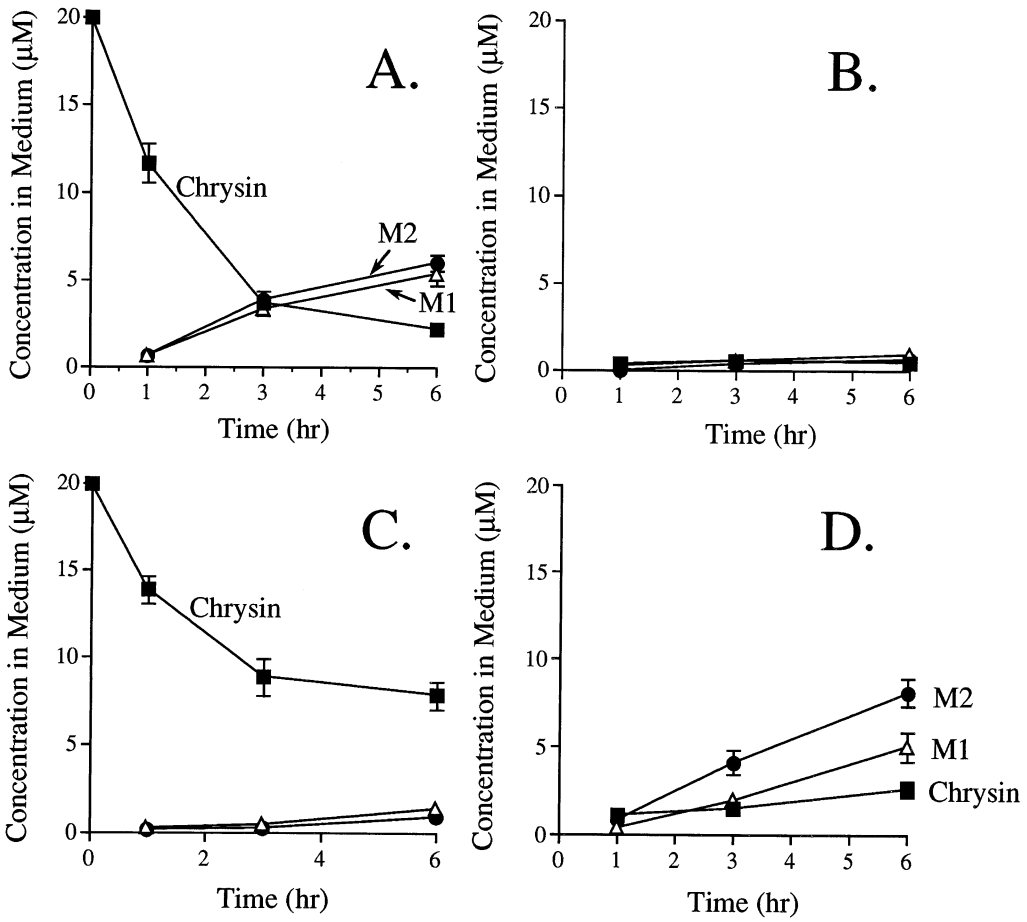

FIG. 3. Concentrations of chrysin and chrysin metabolites M1 and M2 in the transport medium of Caco-2 cells during 6-hr incubations. (A) Apical loading, apical sample; (B) apical loading, basolateral sample; (C) basolateral loading, basolateral sample; and (D) basolateral loading, apical sample. The values shown are means ± SEM (N = 11).

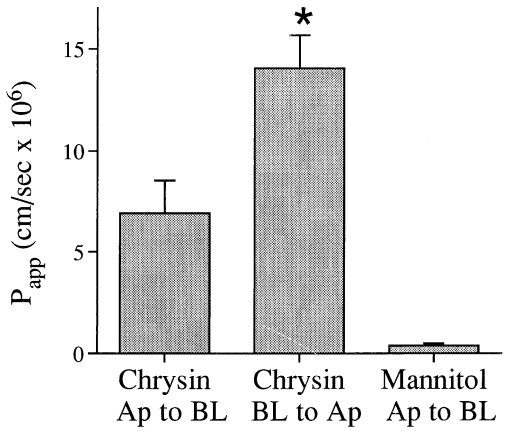

FIG. 4. Apparent permeability (P_{app}) for chrysin and mannitol in Caco-2 cells. Values are means ± SEM (N = 8) of 1-hr transport experiments. Ap to BL: apical to basolateral transport; BL to Ap: basolateral to apical transport. Key: (*) significantly higher than Ap to BL, $P < 0.01$.

(Fig. 3D) and low concentrations of these metabolites appearing on the basolateral side (Fig. 3C).

The early time course indicated that chrysin transport may be linear with time up to 1 hr, with barely detectable levels of metabolites M1 and M2. Thus, calculation of an apparent permeability coefficient (P_{app}) over this time should yield a value representative of the transport of chrysin itself with minimal influence of metabolism. This is summarized in Fig. 4 for both apical and basolateral loading of chrysin and apical loading of the paracellular transport marker mannitol. The apical flux of chrysin ($6.9 \pm 1.6 \times 10^{-6}$ cm · sec^{-1}) was more than 10-fold higher than that of mannitol ($0.42 \pm 0.12 \times 10^{-6}$ cm · sec^{-1}). Interestingly, the basolateral flux of chrysin ($14.1 \pm 1.6 \times 10^{-6}$ cm · sec^{-1}) was 2-fold higher than the apical to basolateral flux ($P < 0.01$).

As the metabolites being effluxed, i.e. M1 and M2, are anionic glucuronic acid and sulfate conjugates of chrysin, the effect of a selective inhibitor of the anionic multidrug resistance protein MRP pumps, MK-571, was investigated [25–27]. As demonstrated in Fig. 5, there was a concentration-dependent inhibition of the flux of both M1 and M2, although M1 was considerably more prone to inhibition. Thus, 5 μM MK-571 inhibited M1 efflux by about 31% ($P < 0.01$) and 50 μM by about 71% ($P < 0.0001$). M2 inhibition was observed only at 50 μM MK-571, and this effect was very modest (about 25%), although statistically significant ($P < 0.05$).

To be able to demonstrate whether an MRP pump is present in the Caco-2 cells, plasma membranes were prepared by brief sonication of the cells in the presence of a mixture of protease inhibitors, RNase and DNase, followed by differential centrifugation [31]. Hep G2 and PANC-1 cells were treated similarly. Figure 6 shows an immunoblot using the MRP2-specific polyclonal antibody EAG5. Caco-2 membranes (lanes 1 and 2) showed a band at about 190 kDa, i.e. consistent with MRP2. As a positive control, Hep G2 cell membranes (lane 4) also contained the 190-kDa band. An additional lower M_r band was also visible in these preparations and is most likely a contaminating protein from the 100,000 *g* supernatant (lane 3). The M_2I-4 monoclonal MRP2 antibodies gave very similar results, i.e. 190-kDa bands for both Caco-2 and Hep G2 membranes (data not shown).

When Caco-2 cell membranes were probed with the MRP1-selective antibodies MRPm6 (Kamiya) and QCRL-1 [20, 21], the Caco-2 cell membranes did not show any distinct band, whereas the MRP1-positive control PANC-1 cell membranes [24] gave a strong signal at about 190 kDa (data not shown).

DISCUSSION

For flavonoids in general there is little information on biological membrane transport. In this study, we demon-

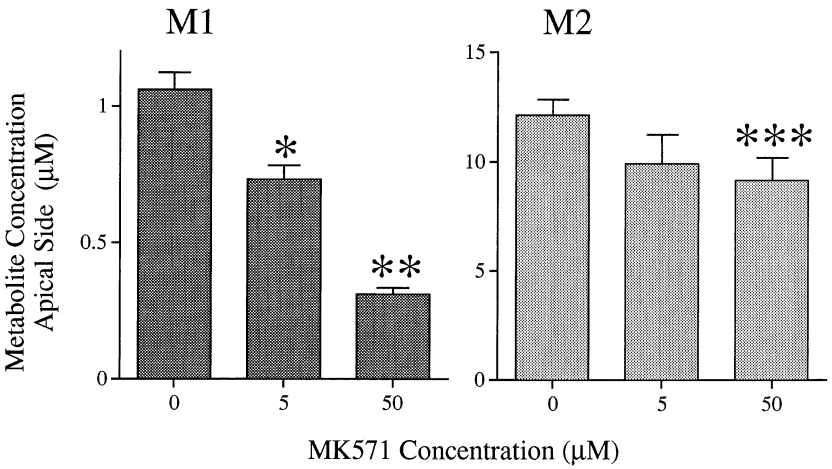

FIG. 5. Inhibition of Caco-2 efflux of chrysin metabolites M1 and M2 by the MRP inhibitor MK-571. Chrysin (20 μM) and MK-571 (0, 5, or 50 μM) were added to the apical side. After a 3-hr incubation, the transport medium from the apical side was analyzed for chrysin, M1, and M2 by HPLC as in Fig. 2. Mean values ± SEM (N = 5) are shown. Key: (*) significantly lower than 0 μM MK-571 ($P < 0.01$); (**) significantly lower than 0 μM MK-571 ($P < 0.0001$); and (***) significantly lower than 0 μM MK-571 ($P < 0.05$).

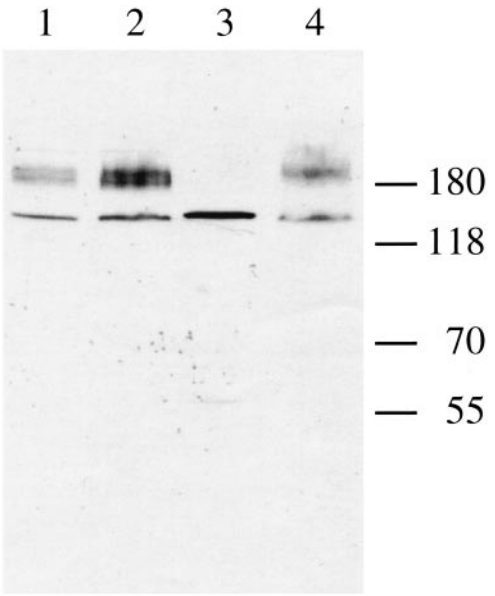

FIG. 6. Immunoblot analysis of Caco-2 cell membranes (lane 1, 100 μg protein, and lane 2, 200 μg), Caco-2 cytosol (lane 3, 50 μg), and Hep G2 cell membranes (lane 4, 200 μg), using EAG5 MRP2-specific polyclonal antiserum (diluted 1:20,000). M_r standards (kDa) are shown to the right.

strated a rather favorable P_{app} for chrysin. Thus, a value of 6.9×10^{-6} cm · sec^{-1} for the net apical to basolateral flux, according to data by Artursson and Karlsson [35], suggests virtually complete absorption of chrysin in humans. This P_{app} value is about 20% higher than previously found for quercetin [15], using identical experimental conditions. This difference may be expected, based on the fact that chrysin is a more hydrophobic molecule than quercetin.

The initial transport rate for chrysin during the first hour diminished greatly at longer incubation times, coinciding with the appearance of two conjugated metabolites of chrysin, M1 and M2. M1 and M2 were identified as a glucuronic acid and a sulfate conjugate, respectively, using specific enzymatic hydrolyses, i.e. with β-glucuronidase and sulfatase, and selective inhibitors of these enzymes. Although these chrysin metabolites were not unexpected, the rate of their formation in the Caco-2 cells was surprisingly high, considering that this cell line, in general, is not thought to express high levels of drug-metabolizing enzymes [36]. As the flavonoids are common components of our diet, it is conceivable that conjugating enzymes specialized for metabolizing these compounds are expressed in the intestinal epithelial cells. In the study of transport and metabolism by Caco-2 cells of L-α-methyldopa, for which, as for chrysin, glucuronidation and sulfation are major metabolic pathways, only the basolateral samples could be analyzed for metabolites [37]. It would be quite interesting to repeat this study to determine if the glucuronide and sulfate metabolites of this drug, like those of chrysin, showed major efflux to the apical side.

Previous studies in Caco-2 cells have shown well defined efflux mechanisms due to P-glycoprotein, expressed on the apical membrane [38]. For vinblastine [38], cyclosporin [39], and digoxin [40], as well as taxol [22], this mechanism reduces the intestinal absorption. The observation in this study of a rather dramatic efflux of a glucuronic acid and a sulfate conjugate in the Caco-2 cells has received less attention. The mechanism for this efflux is not likely to involve P-glycoprotein, but rather may involve the relatively recently cloned MRP1 and MRP2 pumps, which both have high propensity for anionic substrates [25–27]. The inhibition of this efflux by MK-571, a selective inhibitor of both of these transporters [25–27], strongly supports this contention. In polarized epithelial cells, such as hepatocytes and proximal tubule epithelial cells of the kidney, MRP2 is localized strictly to the apical domains, whereas MRP1 appears to be localized to the lateral domains [25–27]. Our observations with the efflux of chrysin metabolites in the Caco-2 cells are therefore suggesting MRP2 as the transporter. The much more potent inhibition of the glucuronide than the sulfate efflux by MK-571 should be emphasized. Based on studies in the vascularly perfused mouse small intestine by Wollenberg and Rummel in 1984 [41], a separate transporter of certain sulfate conjugates may exist in the intestine.

Chrysin itself also showed preferential flux from the basolateral to the apical side, although only about 2-fold. Similar efflux-directed transport was observed previously for quercetin and its mono- and diglucosides [15]. The mechanism(s) for this effect is not known, but does not appear to involve P-glycoprotein, as verapamil was without inhibitory effect (data not shown). Potential inhibition of such an outwardly directed transporter by components of our diet may lead to increased absorption of flavonoids.

Through the use of two MRP2-specific antibodies, the polyclonal EAG5 [16, 17] and the monoclonal M_2I-4 [19], we were able to detect the MRP2 transporter in a Caco-2 cell plasma membrane preparation. Hep G2 cell membranes, serving as a positive control, also showed the presence of a 190-kDa band with this antibody. These cells have been demonstrated previously to contain MRP2, with very low expression of MRP1 [17]. Whereas MRP2 and its rat isoform mrp2 are primarily expressed in the liver [27], low levels of mrp2 mRNA have been detected by northern blot analysis in the ileum and duodenum of Wistar rats [42] and in the duodenum and jejunum of Sprague–Dawley rats [43]. Using two MRP1-specific antibodies, we did not find any evidence for the MRP1 transporter in the Caco-2 cell membranes, whereas membranes from PANC-1 cells, shown to contain MRP1 [24], gave a band at 190 kDa. In contrast, a recent paper showed the presence of an MRP isoform in Caco-2 cells [44]. Although the monoclonal antibody used was raised against an amino-terminal portion of MRP1, its specificity for MRP1 versus MRP2 has not been shown.

Although the observations made in this study suggest that chrysin has favorable properties for biological membrane penetration, surprisingly efficient metabolism by the Caco-2 cells still may limit the oral bioavailability of

chrysin in humans. Based on our observations in this study, a more in-depth study of the glucuronidation and sulfation of chrysin in Caco-2 and Hep G2 cells is in progress and a clinical study to evaluate the oral bioavailability of chrysin has been initiated.

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