Transport and Metabolism of the Tea Flavonoid (–)-Epicatechin by the Human Intestinal Cell Line Caco-2

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Purpose: Tea flavonoids, including (–)-epicatechin (EC), have been suggested to have chemopreventive properties in cancer. However, there is limited knowledge of the oral bioavailability of these dietary compounds. The purpose of this study was to gain a better understanding of the absorption of EC.
Methods: The intestinal epithelial membrane transport of EC was examined using the monolayer of the human Caco-2 cell line grown in Transwells, a common model of intestinal absorption. EC and its metabolites were measured by high performance liquid chromatography with diode array detection.
Results: EC showed no apical to basolateral absorption at concentrations ranging from 5 to 50 μM. In contrast, EC demonstrated basolateral to apical efflux with a P_{app} value of $0.67 \pm 0.05 \times 10^{-6}$ cm/sec, i.e., slightly higher than for mannitol, $0.50 \pm 0.30 \times 10^{-6}$ cm/sec, a paracellular transport marker. There was a 50% reduction in the efflux of EC in the presence of 50 μM MK-571, a competitive inhibitor of the MRP2 transporter expressed in the apical membrane of Caco-2 cells. Most important, the presence of 50 μM MK-571 resulted in clearly measurable apical to basolateral absorption of EC with a P_{app} of $0.31 \pm 0.06 \times 10^{-6}$ cm/sec. Two polar metabolites, M1 and M2, were formed from EC, both of which appeared exclusively on the apical side. MK-571 (50 μM) dramatically inhibited the transport for both of these metabolites. Incubations with inorganic $^{35}SO_4^{2-}$ and hydrolysis by aryl sulfatase strongly suggested that these metabolites were sulfate conjugates.
Conclusions: These results suggest an important role for the multispecific organic anion transporter MRP2 in the bioavailability of EC and possibly other tea flavonoids.

KEY WORDS: tea flavonoids; epicatechin; transport; metabolism; Caco-2 cells.

INTRODUCTION

Flavonoids are naturally occurring polyphenols present in many fruits, vegetables, and in common beverages like tea and wine (1,2). (–)-Epicatechin (EC) (Fig. 1) belongs to the catechin (flavan-3-ols) group of flavonoids and is present together with other flavonoids in substantial amounts in green tea, red wine, chocolates, and many fruits (3). Experimental and epidemiological data have provided evidence that the polyphenolic components present in green tea afford protection against cancer initiation and its subsequent progression and development (4). Proposed mechanisms include inhibition of certain drug metabolizing enzymes (5), radical scavenging properties (6), and the ability to inhibit growth-related signal transduction pathways (7,8).

To understand the beneficial effects of these flavonoids to human health, its absorption must be established. There is only limited knowledge on the bioavailability of these naturally occurring compounds. Although EC and its metabolites have been found in human plasma and urine (9–12), the absolute oral bioavailability is not known.

In the present study, we used the human colonic cell line Caco-2, a well accepted model of intestinal absorption (13–16) to study the transport of EC. The Caco-2 cells have previously been used to study the transport of two other flavonoids, i.e., quercetin and chrysin (17,18). In contrast to quercetin and chrysin, EC showed no absorption, but rather efflux when loaded on the basolateral side. It also formed sulfate conjugates, which appeared to be effluxed by the apical transporter MRP2.

MATERIALS AND METHODS

Materials

D-[1-^{14}C]-Mannitol (57.0 mCi/mmol) and [^{35}S]-sodium sulfate (10-100 mCi/mmol) were purchased from Amersham Life Science, Inc. (Arlington Heights, IL). (–)-Epicatechin (EC), quercetin, chrysin, verapamil, and sulfatase from *Aerobacter aerogenes* were obtained from Sigma Chemical Co. (St. Louis, MO). MK-571 was a gift from Dr. A. W. Ford-Hutchinson, Merck-Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada. Trifluoroacetic acid was of spectrophotometric grade from Aldrich Chemical Co. (Milwaukee, WI); fetal bovine serum from Atlanta Biologicals (Norcross, GA), and Hanks' Balanced Salts Solution (HBSS) and other cell culture medium components were obtained from Cellgro, Mediatech, Fischer Scientific (Pittsburgh, PA).

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, 1% nonessential amino acids, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin and were grown in a humidified atmosphere of 5% CO_2 at 37°C.

For most transport studies, Caco-2 cells were seeded in 12 mm i.d. Transwell® inserts (polycarbonate membrane, 0.4 μM pore size, Corning Costar Corp., Corning, NY) in 12-well plates at a density of 10^5 cells/cm^2. Some transport experiments were done in 24 mm i.d. Transwell inserts in 6-well plates.

Transport Experiments

Caco-2 cells in Transwells at passage 50–80 were used for experiments 20–30 days post seeding. Transepithelial electrical resistance (TEER) values across the cell monolayers were measured using a Millicell-ERS Voltohmmeter (Millipore Corp, Bedford, MA). Inserts with TEER values > 350 Ω cm^2 in culture medium were selected for transport experiments. The inserts were washed twice for 30 min with warm transport buffer, HBSS containing 25 mM of HEPES, pH 7.4. TEER values were also obtained after completion of transport experiments. The paracellular transport marker [^{14}C]mannitol

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ABBREVIATIONS: HBSS, Hanks' balance salt solution; EC, epicatechin; TEER, transepithelial electrical resistance; CAT, (+)-catechin.

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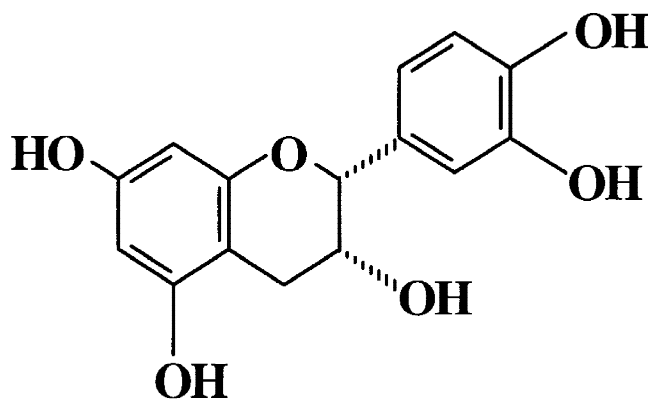

Fig. 1. Structure of (–)-epicatechin (EC).

was added to the apical side of all inserts for the assessment of monolayer integrity. Aliquots of the basolateral medium were assayed for transport of [^{14}C]mannitol by measuring the radioactivity.

Transepithelial permeability of EC was measured at a 50 μM concentration unless stated otherwise. A 5 mM stock solution of EC in DMSO was diluted to 50 μM in transport buffer. Transport buffer containing EC was added to either the apical (0.5 ml) or basolateral (1.5 ml) side of the inserts, whereas the receiving chamber contained the corresponding volume of transport buffer. Upon the end of the 3-h incubation at 37°C, samples were collected from both sides of the cell monolayer and analyzed for flavonoid content by high performance liquid chromatography (HPLC) after solid-phase extraction.

Solid-Phase Extraction

Solid-phase extraction was done using Oasis HLB 1cc C18 Extraction Cartridges (Waters Corp, Milford, MA). The cartridges were washed with 1 ml methanol and equilibrated with 1 ml water. After loading 0.5 ml of the sample, the cartridges were washed with 5% methanol and eluted with 2 ml 100% methanol. The methanol eluate was dried under N_2 gas at 40°C, and the sample was redissolved in 250 μl mobile phase for HPLC analysis.

HPLC Analysis

EC and its metabolites, chrysin and quercetin were analyzed by reversed phase HPLC of 150 μl samples on a Millennium HPLC system (Waters Corp, Milford, MA) with a Symmetry C18 column, 3.9 × 150 mm, and a model 996 photodiode array detector. The mobile phase for EC and its metabolites consisted of 20% methanol in 0.3% trifluoroacetic acid at a flow rate of 0.9 ml/min with detection at 278 nm. Quantitation was done by peak area measurements in comparison with a standard curve for EC. In some experiments (+)-Catechin (CAT) was included as an analytical standard.

The mobile phase for chrysin consisted of 55% methanol in 0.3% trifluoroacetic acid at a flow rate of 0.9 ml/min with detection at 268 nm. The mobile phase for quercetin consisted of 35% methanol and 5% acetic acid at a flow rate of 0.9 ml/min with detection at 370 nm. Quantitation was done by peak area measurements in comparison with a standard curve for chrysin and quercetin, respectively.

Inhibition of Transport

These experiments were carried out as described above in transport studies with some modifications. MK-571 and verapamil were used at 50 μM concentration as selective inhibitors of MRP1/MRP2 (19,20) and P-glycoprotein (21,22), respectively. The cells were preincubated with the inhibitors for 30 min. After the preincubation, the inhibitors were added to both sides of the membrane and EC (50 μM) was added to one side.

Calculation and Statistics

The apparent permeability coefficient (P_{app}) expressed in cm/sec was determined as:

$$P_{app} = dC/dt \times V/(A \times C_0),$$

where dC/dt is the change in concentration on the receiving side over time (μM/s), V is the volume of the solution in the receiving compartment (cm^3), A is the surface area of the membrane (1.13 cm^2) and C_0 is the initial concentration in the donor chamber (μM) (16). The effect of transport inhibitors on P_{app} was expressed relative to control. The statistical significance of differences between treatments was evaluated by using two-tailed paired student t tests with a significance level of $P < 0.05$.

Formation of [^{35}S]-Labeled Sulfate Conjugates

In some experiments, Caco-2 cells grown in Transwells were incubated with 1 mM sodium [^{35}S]sulfate with or without 50 μM EC in buffer on the basolateral side. After 3 and 6 h, the apical side buffer was harvested and subjected to solid phase extraction. The reconstituted samples were subject to HPLC analysis as above. HPLC eluate 1 min fractions were collected for 30 min, and the content of radioactivity measured by liquid scintillation spectrometry after the addition of Biodegradable Counting Scintillant (Amersham, U.K.).

Hydrolysis of Sulfate Conjugates

EC basolateral to apical transport was done as before and the samples collected, dried under N_2 gas, and redissolved in 0.5 ml pH 7.4 Tris buffer. The samples were then incubated at 37°C for 6 h in the presence or absence (control) of sulfatase (0.5 μl; 0.006 U) and analyzed after solid-phase extraction by HPLC.

Cellular Uptake Studies

For all cellular uptake studies, confluent monolayers of Caco-2 cells were grown in plastic dishes. After washing the monolayers twice with HBSS, the cells were incubated with 50 μM EC, 25 μM chrysin, or 25 μM quercetin in HBSS. The uptake was halted after 1 h by rapidly aspirating the buffer, and cells were rinsed three times with ice-cold buffer. The cells were then scraped off the dishes and extracted thrice with 1 ml methanol by vortex mixing for 1 min and centrifugation at 14,000 g for 2 min. The combined supernatants were evaporated to dryness under N_2 and reconstituted in the mobile phase and subjected to HPLC analysis as above.

RESULTS

EC (Fig. 1) was used for this study based on its stability under the experimental conditions used. Consistent with previous observations (23), we found that EC was the most stable among all the tea flavonoids at 37°C and pH 7.4. This was followed closely by (–)-epicatechin-3-gallate. The other two tea flavonoids, (–)-epigallocatechin and (–)-epigallocatechin-3-gallate, were unstable under these conditions (data not shown). The concentrations of EC employed in this study of 10 to 50 μM were judged to be a realistic value for concentrations of dietary flavonoids in the intestinal lumen (17). EC was soluble up to a concentration of at least 200 μM in the transport buffer.

When EC (10 to 50 μM) was loaded on the apical side of the Caco-2 cells grown on Transwells, EC could not be detected on the basolateral (receiving) side even after a 3 h incubation (Fig. 2A). CAT was added to the samples as an analytical standard after the transport but before HPLC. Interestingly, after basolateral loading of EC, it was clearly detected on the apical side. Along with EC, metabolites, M1 and M2 were also detected, only on the apical side (Fig. 2B). There were also trace quantities of two more metabolites, m_1 and m_2.

A summary of the flux for both apical and basolateral loading of EC and apical loading of the paracellular transport marker mannitol is shown in Fig. 3. The efflux of EC was linear with time for up to three hours. Its apparent permeability coefficient (P_{app}) of $0.67 \pm 0.05 \times 10^{-6}$ cm sec (mean ± SE; $n = 6$) was similar to that of mannitol, $0.58 \pm 0.06 \times 10^{-6}$ cm/sec There was no absorption of EC. The basolateral to apical P_{app} values were identical for EC concentrations ranging from 10 to 100 μM. The P_{app} for mannitol was identical whether loaded on the apical or basolateral side (data not shown).

The lack of apical to basolateral transport of EC was further investigated by determining cellular accumulation of EC into the Caco-2 cells grown in culture dishes. Chrysin was found to be extensively metabolized by the Caco-2 cells (18,24) and quercetin, which had been shown to be transported by the Caco-2 cells (17), were used as positive controls. Consistent with the transport experiments, there was no accumulation of EC in Caco-2 cells after a 1-h incubation, and no EC metabolites detected by reversed phase HPLC. In sharp contrast, large amounts of chrysin and quercetin were found in the Caco-2 cell extracts after 1 h by HPLC (data not shown). In fact, as much as 40% of chrysin and 41% of quercetin could be found in the cell extracts, corresponding to 2500 pmoles/mg protein of chrysin and 2158 pmoles/mg protein for quercetin, respectively. Even when the incubation time was extended to 24 h, there was no cellular accumulation of EC or metabolites formed.

Because there was a strong direction dependence in the EC transport, the involvement of transporters was investigated. The apical to basolateral transport (absorptive) and the basolateral to apical transport (efflux) were performed in the presence of selective inhibitors of transporters. Verapam-

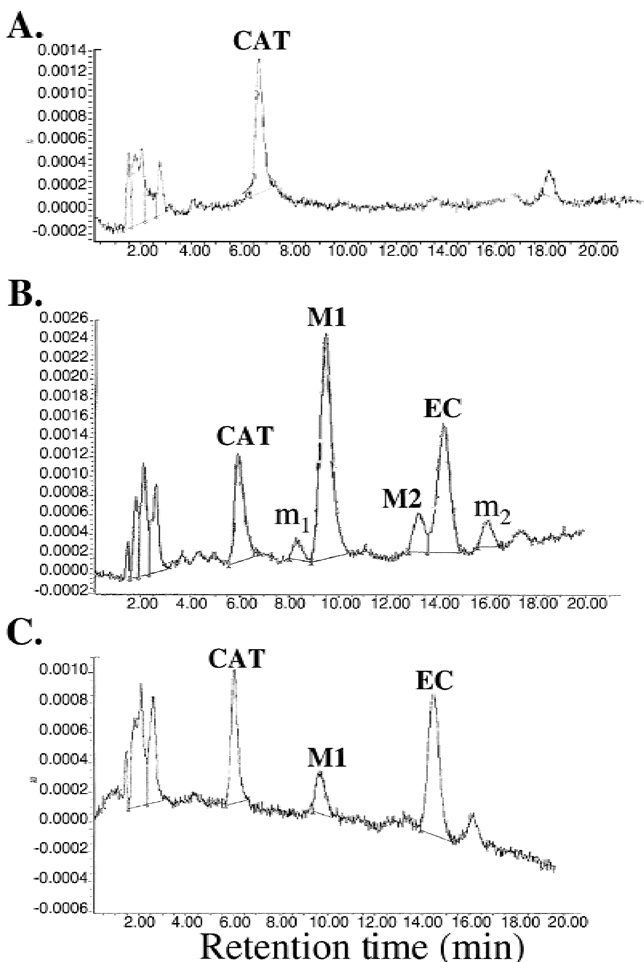

Fig. 2. Reversed phase HPLC of EC in 3-h Caco-2 cell transport experiments. **(A)** Apical loading, basolateral sample. **(B)** Basolateral loading, apical sample. **(C)** Basolateral sample, apical loading in the presence of the transport inhibitor MK-571. [EC]: 50 μM, [MK-571]: 50 μM; M1 and M2 are EC metabolites; m_1 and m_2 may be two additional metabolites; CAT is (+)-catechin, used as an analytical standard.

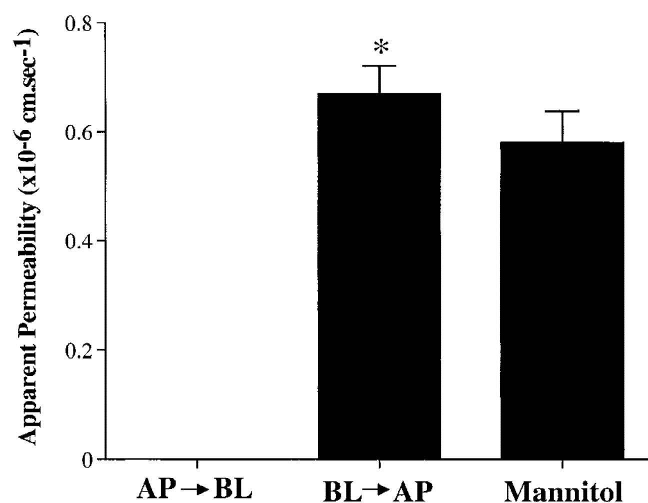

Fig. 3. Transepithelial flux of EC (50 μM) and the paracellular transport marker [^{14}C]mannitol in Caco-2 cells. Values are mean ± SEM ($n = 6$) of 3-h transport experiments. AP→BL: apical to basolateral transport, BL→AP: basolateral to apical transport. (*) Indicates significantly higher than AP→BL, $P < 0.01$. The mannitol flux was measured in AP→BL direction.

il, a P-glycoprotein inhibitor (21,22) did not significantly affect the efflux of EC, P_{app} of 0.64 ± 0.29 × 10^{-6} cm/sec, versus control 0.67 ± 0.05 ×10^{-6} cm/sec (Fig. 4A). In contrast, MK-571, an MRP selective inhibitor (19,20) inhibited about 50 % of the efflux of EC (P <0.001). When the apical to basolateral absorption of EC was studied in the presence of these inhibitors, verapamil had no effect, but the presence of MK-571 caused the appearance of EC on the basolateral side with a P_{app} of 0.30 ± 0.20 × 10^{-6} cm/sec, Fig. 4B.

On the basis of the observation in Fig. 2B, several metabolites of EC are formed by the Caco-2 cells when loading EC on the basolateral side, which appeared to be effectively effluxed into the apical side. For structure identification, larger amounts of M1 and M2 were generated by doing the experiments in 24 mm Transwell inserts. To determine if the metabolites were sulfate conjugates, basolateral to apical transport experiments were done with 50 μM EC in the presence of [^{35}S]-labeled inorganic sulfate in the cell culture medium, similar to a previous study (25). The results demonstrated the formation of radiolabeled M1 (the major metabolite) and M2 (the minor metabolite), consistent with sulfate conjugates (Fig. 5). Further evidence that these metabolites were sulfate conjugates was based on enzymatic hydrolysis. Thus, incubations of the apical sample after basolateral loading of EC with aryl sulfatase resulted in a 71% and 35% reduction in the amount of M1 and M2 formation respectively, whereas the EC amount increased by 4-fold.

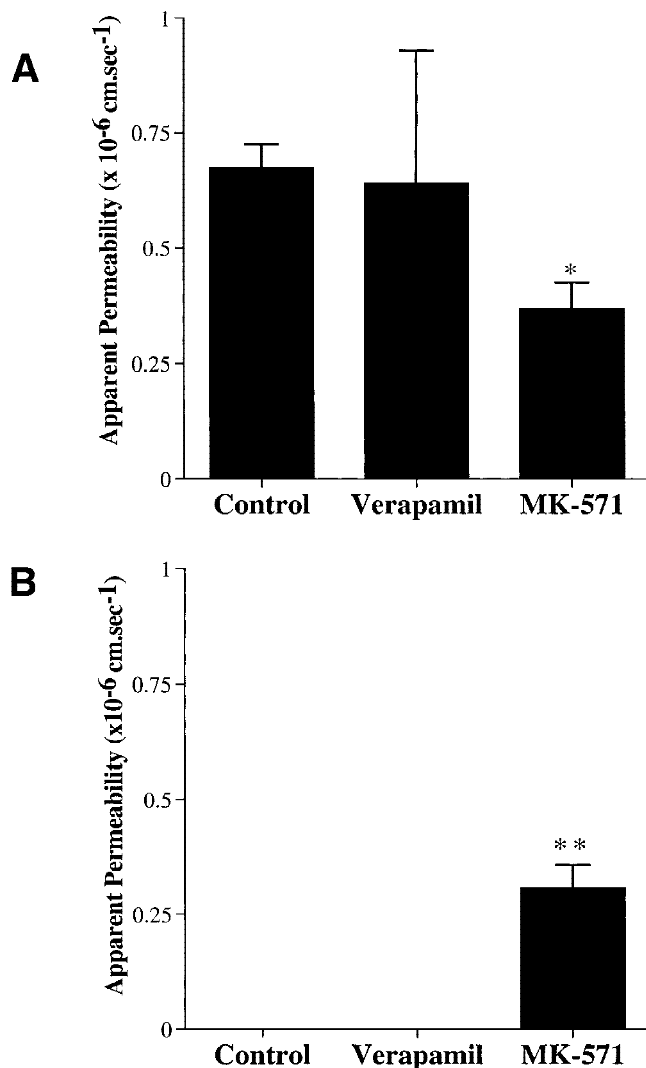

Fig. 4. EC transport in Caco-2 cells in the presence of transport inhibitors. 50 μM EC ± inhibitors were loaded on **(A)** the basolateral side or **(B)** the apical side. Samples were collected for analysis after 3 h. Values are mean ± SEM (n = 5–7). (*), significantly lower than control (P < 0.01); (**) significantly higher than control (P < 0.01). [Verapamil]: 50 μM, [MK-571]: 50 μM.

The effect of the selective MRP transport inhibitor MK-571 on the efflux of the two anionic sulfate conjugates M1 and M2 was then determined. As observed in Fig. 2C, the efflux of M1 and M2 was almost completely abolished (Fig. 6).

DISCUSSION

It is essential to know the bioavailability of flavonoids to understand their potential actions *in vivo.* Although EC has been detected in plasma and urine in humans after ingestion of decaffeinated green tea (9–12), the oral bioavailability is not known. To determine the mechanism(s) of the absorption of EC, we have used the human Caco-2 cell monolayer, a well-accepted model of human intestinal absorption (13–16).

While no apical to basolateral transport of EC was detected in the Caco-2 cell monolayer over the concentration range of 5–50 μM, we observed a basolateral to apical efflux of a magnitude similar to that of the paracellular transport marker mannitol. The lack of ability of EC to penetrate the apical membrane was demonstrated in cell uptake experiments. Thus there was no accumulation of EC in the Caco-2 cells, when the cells, grown on regular plastic dishes, were exposed to EC over 1 to 24 h. The uptake of quercetin, a structurally similar flavonoid with five hydroxyl groups like EC, and which is known to be transported by Caco-2 cells (17), and that of chrysin, which is extensively metabolized by the Caco-2 cells (18,24), was extensive under identical conditions. Thus an efflux pump localized on the apical membrane, with a high specificity for EC compared to other flavonoids, must be responsible for these observations.

Previous studies in Caco-2 cells have shown the presence of well-defined efflux pumps in the apical membrane. One is P-glycoprotein, which is responsible for the efflux of many hydrophobic compounds like vinblastine (26) and taxol (27). Previous studies in our laboratory have also shown the presence of MRP2 (18,28) in the apical membrane of the Caco-2 cell monolayer. The lack of effect of verapamil on EC trans-

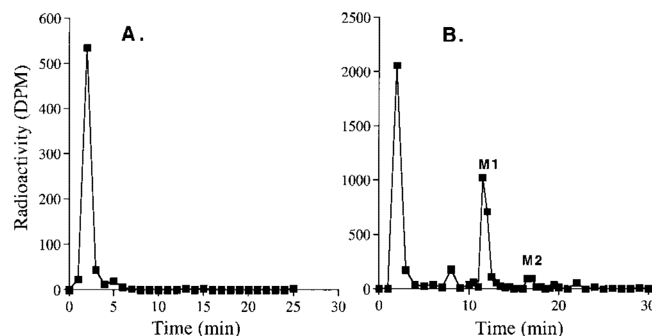

Fig. 5. Reversed phase HPLC of EC metabolites after a 6 h incubation of Caco-2 cells in the presence of $Na_2^{35}SO_4$ with 1-min fractions collected and analyzed for radioactivity. **(A)** Control without EC and **(B)** with 50 μM EC.

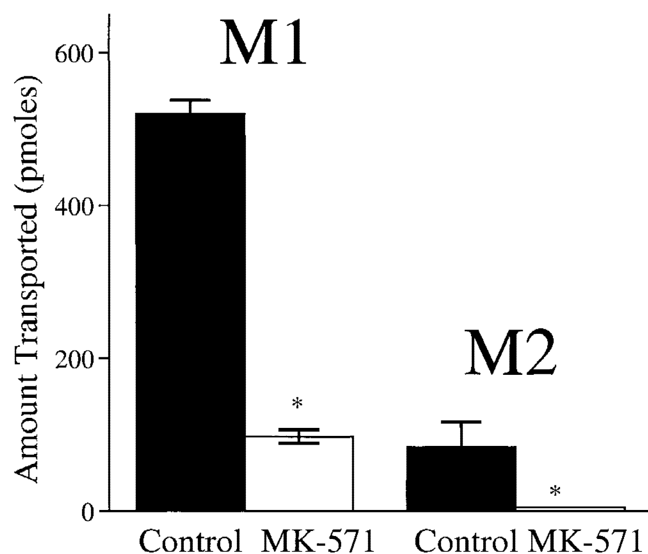

Fig. 6. Effect of MK-571 on the formation and efflux of metabolites M1 and M2. After basolateral loading of 50 μM EC either without (closed bars) or in the presence of 50 μM MK-571 (open bars), the apical 3-h samples were analyzed for EC metabolites. Values are mean ± SEM, $n = 3$. * Significantly lower than control; $P < 0.001$.

port rules out P-glycoprotein as a mechanism. On the other hand, the inhibitory effect of MK-571 seems to implicate MRP2 as responsible for EC efflux. This was greatly strengthened by the observation that apical to basolateral absorption of EC could be observed, although rather low, in the presence of MK-571. The modest 50% inhibition of EC efflux by MK-571 is much less than would be expected for MRP2 transport (19,20). This might be due to the fact that EC being a neutral molecule, is not a typical substrate for MRP2. This should require further studies; preferentially in MRP2 transfected cells and potential cotransport with glutathione (29) should be considered. Although no other efflux transporters on the apical membrane of enterocytes are clearly known at this time, this cannot be ruled out. In addition, the Caco-2 cells efficiently metabolized EC. Two polar metabolites, M1 and M2, were subsequently effluxed into the apical side like EC. Our findings that M1 and M2 became radioactively labeled when incubations were done in the presence of inorganic $^{35}SO_4^{2-}$ strongly indicate that both of these metabolites are anionic sulfate conjugates. This was further confirmed when these metabolites were hydrolyzed by aryl sulfatase. Previous studies have suggested that sulfate conjugation of EC may predominate in humans (11) but glucuronidation in rats (30). The present study appears to be first to actually separate sulfate conjugates of EC, by HPLC. The inhibitory potency of MK-571 on the efflux of M1 and M2 was much greater than for EC, consistent with their anionic nature, an important characteristic for MRP2 substrates (31).

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