JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Tea Polyphenols Inhibit the Transport of Dietary Phenolic Acids Mediated by the Monocarboxylic Acid Transporter (MCT) in Intestinal Caco-2 Cell Monolayers

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It was previously reported that a fluorescent marker dye, fluorescein, is transported via the monocarboxylic acid transporter (MCT). Fluorescein transport was competitively inhibited by MCT substrates such as ferulic and salicylic acids. Tea polyphenols, in particular, epigallocatechin gallate (EGCg) and epicatechin gallate (ECg), inhibited the transport of fluorescein. Tea polyphenols also inhibited the transport of salicylic and ferulic acids, suggesting tea polyphenols might be substrates of MCT. However, the transepithelial flux of tea polyphenols was much lower than that of the MCT substrates and was inversely correlated with the paracellular permeability of Caco-2 cell monolayers. These findings suggest that tea polyphenols are not substrates but inhibitors of MCT. Furthermore, the transepithelial transport of these polyphenols is mainly via paracellular diffusion. However, directional transport of ECg and EGCg from the basolateral to the apical side was observed, indicating that the behavior of tea polyphenols in the intestinal epithelium is complex.

KEYWORDS: Tea polyphenols; catechins; monocarboxylic acid transporter; fluorescein; Caco-2

INTRODUCTION

In our previous study (*1*) using Caco-2 cells as an in vitro model of intestinal absorption and metabolism (*2*−*4*), we established that the fluorescent marker dye fluorescein is transported across a monolayer of cells by the intestinal monocarboxylic acid transporter (MCT). Transport in the apical to basolateral direction was both pH- and concentration-dependent in a saturable manner. Measuring the competitive effect of dietary substances on the transport of fluorescein is a useful means of identifying compounds that can be transported by MCT. MCT-mediated transport of dietary phenolic acids, such as ferulic acid, was demonstrated by this method (*5*).

Catechins are abundant acylated flavonoids found in tea. The major component is (−)-epigallocatechin gallate (EGCg), which makes up ∼50% of the catechin content, together with (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), and (−)-epicatechin gallate (ECg). These catechins have been reported to act as antioxidative (*6*, *7*), antitumor, antimutagenic (*8*), and antihypertensive agents (*9*). Because the physiological importance of tea polyphenols depends on their availability, estimating the efficiency of intestinal absorption is important in assessing their potential dietary benefit.

A number of studies have been published on the availability of dietary polyphenols. Although various metabolites of polyphenols were detected in the plasma and urine (*10*−*13*), the first limiting step for ingested polyphenols must be their intestinal absorption. Until recently the partition coefficient (log octanol/water) was thought to govern their transport across intestinal epithelial cells, because passive diffusion seemed to be the major mechanism in the permeation process (*14*). However, although the precise mechanism of transport remains unclear, the possible involvement of the sodium-dependent glucose transporter (SGLT-1) in the absorption of polyphenolic compounds has been reported (*15*). We have found that tea polyphenols inhibit the transport of fluorescein across a monolayer of epithelial cells. In this paper, we have used fluorescein as a marker to study whether the MCT-mediated transport mechanism is involved in the intestinal absorption of tea polyphenols.

MATERIALS AND METHODS

Materials. The human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Fetal calf serum, glutamine, nonessential amino acids, penicillin, and streptomycin (10000 units/mL and 10 mg/mL in 0.9% sodium chloride, respectively), phosphate-buffered saline, and Hank's balanced salt solution (HBSS) were all purchased from Invitorogen Corp. (Carlsbad, CA). Type I collagen was purchased from Nitta Gelatin Inc. (Osaka, Japan). Plastic dishes, plates,

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10.1021/jf034894t CCC: $25.00
Published on Web 11/05/2003

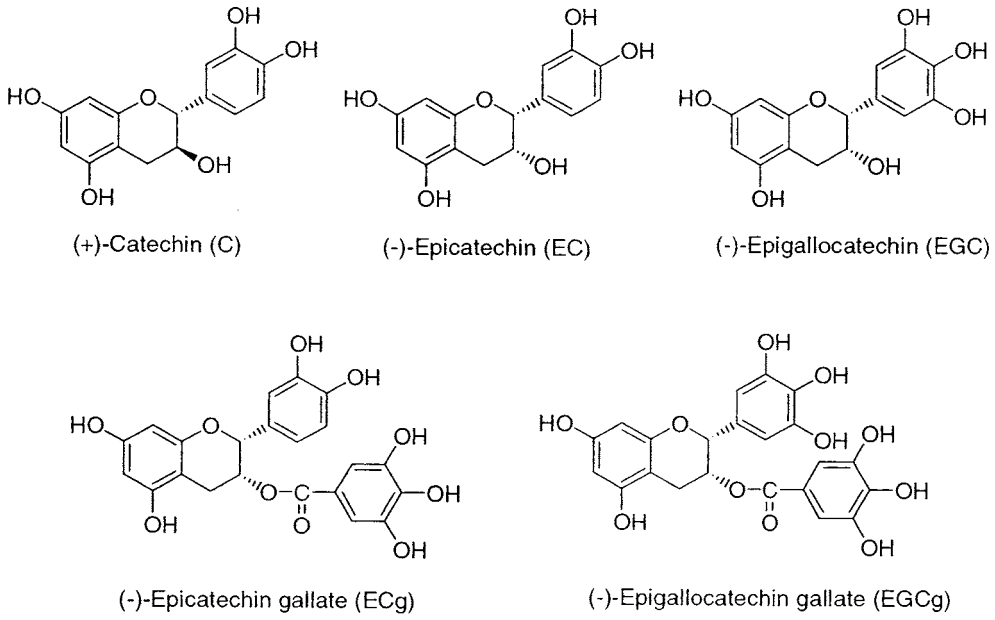

Figure 1. Chemical structures of tea polyphenols.

and Transwell inserts with 0.4-μm polycarbonate membranes 12 mm in diameter were obtained from Corning (Corning, NY). C, EC, EGC, ECg, and EGCg were obtained from Mitsui Norin Co. (Shizuoka, Japan). All other chemicals used in this study were of analytical grade.

Cell Culture. Caco-2 cells were cultured in DMEM containing 10% fetal calf serum, 1% nonessential amino acids, 4 mM L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin, together with sodium bicarbonate to adjust the pH to 7.4. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The monolayers became confluent 6−7 days after the seeding with 1×10^5 cells per 100-mm dish, and the cells were passaged at a split ratio of 4 to 8 by treatment with 0.1% trypsin and 0.02% EDTA acid in HBSS. All cells used were between passages 53 and 78.

Measurement of Transepithelial Electrical Resistance (TER). Cells were grown for TER measurement in Transwell inserts with the semipermeable membrane first coated with type I collagen (12-mm diameter and 0.4-μm pore size, Corning Costar, Corning, NY). The cells were seeded at a density of $1 \times 10^5/cm^2$, and the medium was changed every 1 or 2 days. Monolayers were formed after 2 weeks of culturing. The integrity of the cell layer was evaluated by measurement of TER with Millicell-ERS equipment (Millipore, MA). Monolayers with TER of $>250\ \Omega \cdot cm^2$ were used for the experiments. The TER of the monolayer was measured before and after an assay sample was added to the insert.

Inhibition of the Transport of Fluorescein, Salicylic Acid, and Ferulic Acid. The basal chamber of a Transwell insert was filled with 1.5 mL of HBSS (pH 7.4, 37 °C), and 0.5 mL of the test solution (pH 6.0, 37 °C) containing 10 mmol/L of each catechin was added to the apical side of the insert. After equilibration at 37 °C, 0.1 mmol/L of fluorescein (in HBSS, pH 6.0) was added to the apical side and incubated for 30 min at 37 °C. Then the basal solution containing transported fluorescein was taken and replaced by the same amount of fresh HBSS (pH 7.4) every 10 min. The fluorescein transported by the cells was determined using a fluorescence spectrophotometer (Spectral Max Gemini, Molecular Devices Corp., Sunnyvale, CA) at an excitation wavelength of 490 nm and an emission wavelength of 514 nm.

To determine the inhibitory effect of catechins on the transport of salicylic acid or ferulic acid, 0.1 mmol/L salicylic acid or ferulic acid (HBSS, pH 6.0) was added to the apical side in the presence of each catechin at 10 mmol/L. The Caco-2 cells were incubated for 30 min at 37 °C, and then the amount of transported salicylic acid or ferulic acid was measured by HPLC with an electrochemical detector (ECD) fitted with an ESA coulometric detection system (ESA Inc., Boston, MA).

Transepithelial Transport of Catechins. To measure the apical-to-basolateral flux of catechins, 1.5 mL of HBSS (pH 7.4, 37 °C) was added to the basal chamber of the Transwell insert, and 0.5 mL of the test solution (pH 6.0, 37 °C) containing each catechin (10 mmol/L) was added to the apical side. After incubation for an appropriate time at 37 °C, the basal solution was collected. The amount of catechin transported by Caco-2 cells was estimated by HPLC-ECD with an ESA coulometric detection system.

To examine the basolateral-to-apical transport, HBSS (pH 6.0, 37 °C) was put on the apical side, and 1.5 mL of the test solution (pH 7.4, 37 °C) containing each catechin was added to the basolateral side.

Chromatographic Conditions. HPLC-ECD fitted with an coulometric detection system was used to analyze the samples in two dimensions (chromatographic and voltammetric). Resolution was achieved by arranging several coulometric detectors in series, set to different potentials (*16*). HPLC gradient pumps (Shimadzu, Kyoto, Japan) were coupled with two cell packs in series, each containing four porous graphite working electrodes. Chromatographic separation was performed on a C18 column (ODS150, MC Medical, Inc., Tokyo, Japan). The mobile phase A (solvent A) was 50 mM sodium acetate containing 5% methanol (pH 3.0 adjusted with phosphoric acid), whereas mobile phase B (solvent B) was 50 mM sodium acetate containing 40% acetonitrile and 20% methanol (pH 3.5 adjusted with phosphoric acid). The elution (0.6 mL/min) profile was as follows: 0−28.5 min, 85% solvent A/15% solvent B to 20% solvent A/80% solvent B; 28.5−31 min, 20% solvent A/80% solvent B to 100% solvent B. For analysis of salicylic acid the eight electrode detector potentials were set from 500 to 920 mV in increments of 60 mV. Likewise, for ferulic acid the potentials were set from 0 to 700 mV in increments of 100 mV, and for each catechin five electrode detector potentials were used from 0 to 400 mV in increments of 100 mV.

Data Analysis. The permeation rate [nmol min^{-1} (mg of protein)$^{-1}$], J, was evaluated from the slope of the initial linear part of plots of the amount transported [nmol min^{-1} (mg of protein)$^{-1}$] against time (in min), calculated by linear regression analysis. Results are expressed as the means ± standard deviation (SD). Statistical analysis was done with Student's two-tailed t test, and differences with $P < 0.01$ were considered to be significant.

RESULTS

Effect of Tea Polyphenols on Fluorescein Transport across Caco-2 Cell Monolayers. When tea polyphenols C, EC, ECg, EGC, and EGCg (**Figure 1**) mixed with fluorescein were added to the apical solution, the transport of fluorescein across the monolayer was markedly inhibited. The relative permeation

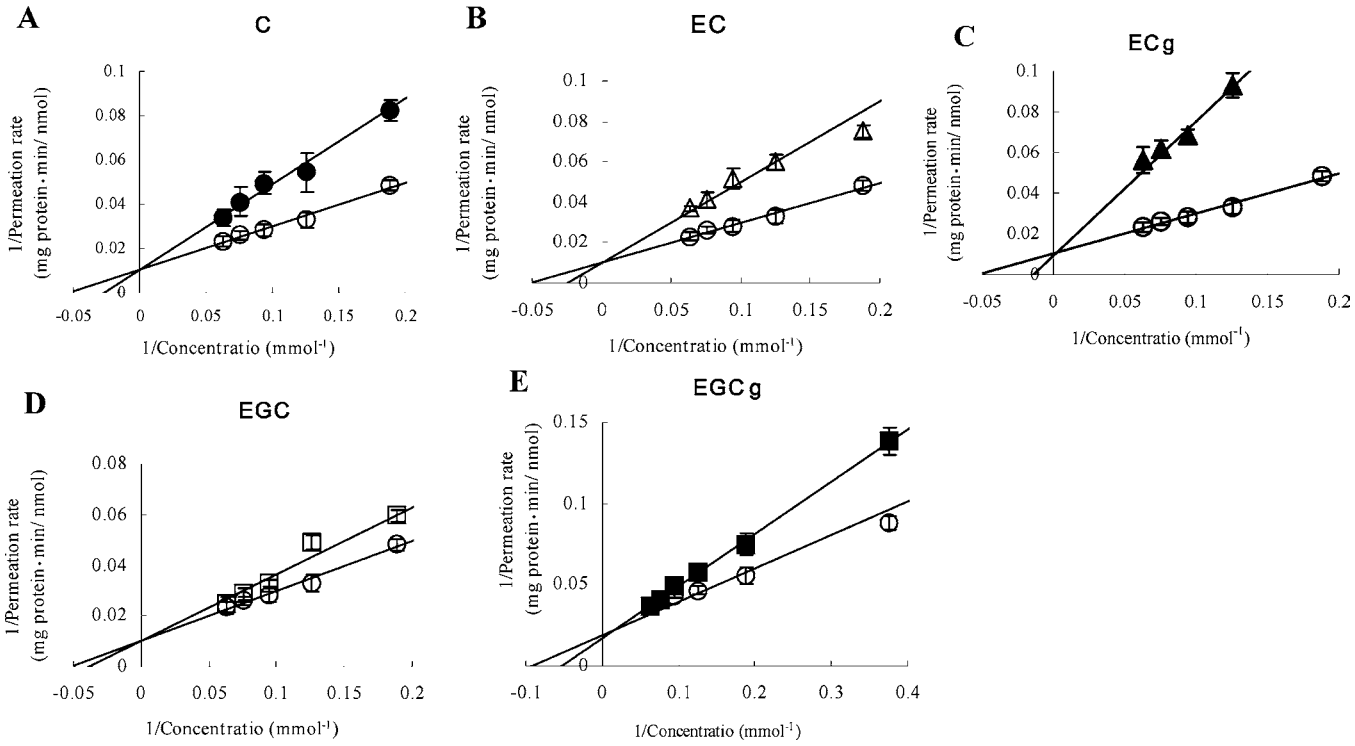

Figure 2. Lineweaver–Burk plots of tea polyphenols for the transport of fluorescein across Caco-2 cell monolayers. The permeation coefficients of C (**A**), EC (**B**), ECg (**C**), EGC (**D**), and EGCg (**E**) were measured in the absence of added compounds (○) and in the presence of each tea polyphenol (●, C; △, EC; ▲, ECg; □, EGC; ■, EGCg). Each point is the mean ± SEM of three or more experiments.

Table 1. Effects of Tea Polyphenols on Fluorescein Transport across Caco-2 Cell Monolayers

compd	concn (mM)	relative permeation[a] (% of control)
C	10	64.8 ± 5.3*
EC	10	59.9 ± 4.9*
ECg	10	50.2 ± 6.8*
EGC	10	86.7 ± 7.1
EGCg	10	32.0 ± 3.4*

[a] Fluorescein transport was measured at 37 °C for 30 min by incubating Caco-2 cells in the absence or presence of a compound at the concentration indicated (apical pH, 6.0; basolateral pH, 7.4). Values are the means ± SD of three or more experiments. *, $P < 0.01$.

Table 2. Inhibition Constant of Tea Polyphenols on Fluorescein Transport across Caco-2 Cell Monolayers

compd	K_i[a] (mM)	compd	K_i[a] (mM)
C	17.2 ± 2.7	EGC	27.9 ± 5.0
EC	14.8 ± 3.2	EGCg	2.5 ± 0.6
ECg	4.9 ± 0.6		

[a] Values are the mean ± SD of three or more experiments.

(percentage of control) is shown in **Table 1**. The order of the inhibitory activity was EGCg > ECg > EC > C > EGC. **Figure 2** shows the effect of each catechin (EGCg, 5 mM; other catechin, 10 mM) on the permeation of fluorescein in terms of a Lineweaver−Burk plot. All of the tea polyphenols tested competitively inhibited the transport of fluorescein. The inhibition constant (K_i) values were calculated and are shown in **Table 2**. Addition of the galloyl ester group to the basic triphenol structure of tea polyphenols is likely to enhance the inhibitory activity. The K_i values of EGCg and ECg were close to that of ferulic acid (2.99 ± 0.08 mM). We have found that ferulic acid was a good substrate of MCT, sharing the transporter with fluorescein (*5*). These results suggest that EGCg and ECg are transported across Caco-2 cells via MCT as substrates such as ferulic acid or fluorescein.

Effects of Tea Polyphenols on Phenolic Acid Transport across Caco-2 Cell Monolayers. The amounts of permeated salicylic acid and ferulic acid were measured by HPLC-ECD with a coulometric detection system, and the standard chromatograms showing resolution of the transported salicylic acid or ferulic acid in the basolateral solution are presented in **Figure 3**. The dominant oxidation potential was 740 mV for salicylic acid and 400 mV for ferulic acid, and the detection limit was <0.5 pmol on the column for both salicylic acid and ferulic acid. When tea polyphenols were added to the apical solution containing an MCT substrate, such as salicylic acid or ferulic acid (*5*, *17*), the transport of these phenolic acids was also inhibited. The relative permeation (percentage of control) is shown in **Table 3**. These results suggest that tea polyphenols share MCT with phenolic acids as well as fluorescein. The inhibitory effects of ECg and EGCg were most potent among the tea polyphenols, again indicating that a galloyl ester group was important for the MCT-mediated transport.

Characteristics of Transepithelial Transport of Tea Polyphenols. Transepithelial transport of tea polyphenols was then analyzed. A standard chromatogram showing the resolution of the transported tea polyphenols in the basolateral solution is presented in **Figure 3**. Each catechin was determined at a detection limit of <0.5 pmol on the column. The dominant oxidation potential values for tea polyphenols were 100 mV for C, 100 mV for EC, 200 mV for ECg, 0 mV for EGC, and 100 mV for EGCg. The purity of the peaks was assessed using

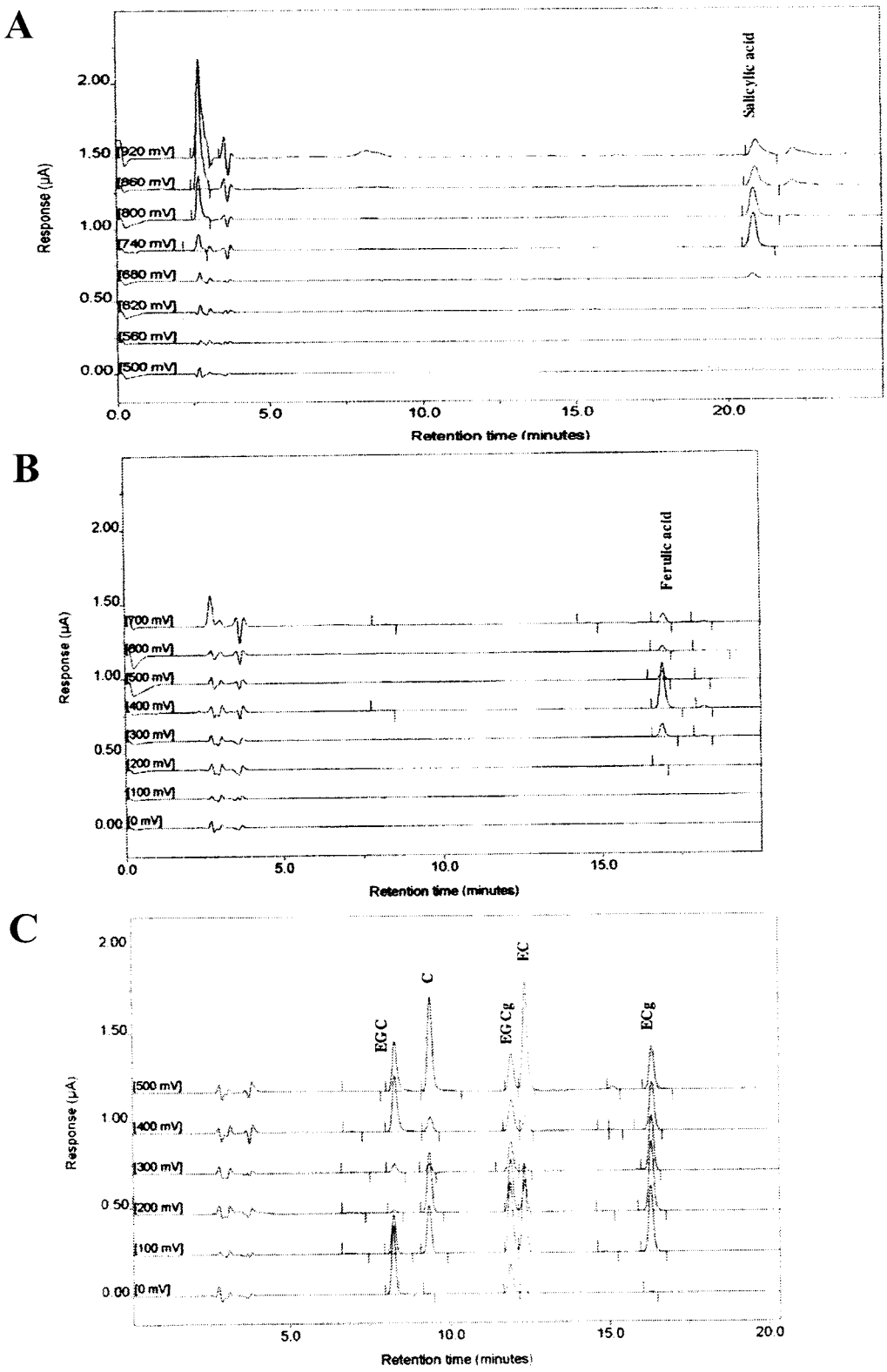

Figure 3. Chromatograms of salicylic acid (**A**), ferulic acid (**B**), and tea polyphenol (**C**) transported across Caco-2 cell monolayers.

peak area ratios for the adjacent oxidation channels (lower or upper) to the dominant oxidation channel, because the voltammetric response of the analyte across these channels was unique for each compound (*16*).

Surprisingly, the apical-to-basolateral flux of tea polyphenols was much lower than that of ferulic acid (*5*) in the presence of an inward proton gradient (**Table 4**), suggesting that the mechanism of permeation is different. To determine whether the MCT-mediated transport mechanism is involved in the transport of tea polyphenols, the bidirectional permeation of tea polyphenols in both apical-to-basolateral and basolateral-to-apical directions across Caco-2 cell monolayers was examined (apical pH, 6.0; basolateral pH, 7.4). As shown in **Table 4**, the initial permeation rates in the apical-to-basolateral direction, $J_{ap \rightarrow bl}$, were similar among the tea polyphenols except for EGCg, suggesting that catechol and gallyl and galloyl ester groups of

Table 3. Effects of Tea Polyphenols on MCT-Mediated Transport across Caco-2 Cell Monolayers

	relative permeation[a] (% of control)	
compd	salicylic acid	ferulic acid
C	84.5 ± 3.8*	69.9 ± 3.5*
EC	87.5 ± 4.5	65.3 ± 4.3*
ECg	78.1 ± 4.8*	56.4 ± 4.3*
EGC	87.2 ± 5.0	72.1 ± 4.1*
EGCg	67.9 ± 3.3*	47.6 ± 5.3*

[a] The amount of salicylic acid or ferulic acid transported was measured at 37 °C for 30 min by incubating Caco-2 cells in the absence or presence of each catechin (10 mM) (apical pH, 6.0; basolateral pH, 7.4). Values are the mean ± SD of three or more experiments. *, $P < 0.01$.

Table 4. Bidirectional Transepithelial Transport of Tea Polyphenols across Caco-2 Cell Monolayers

	permeation rate[a] (nmol/min/mg of protein)	
compd	$J_{ap\rightarrow bl}$	$J_{bl\rightarrow ap}$
C	0.59 ± 0.11	0.73 ± 0.12
EC	0.49 ± 0.09	0.50 ± 0.05
ECg	0.39 ± 0.12	1.54 ± 0.17
EGC	0.49 ± 0.10	0.44 ± 0.13
EGCg	0.22 ± 0.03	0.42 ± 0.05

[a] Each polyphenol (10 mM) was loaded on the apical side of Caco-2 cells. The amount of polyphenol transported was then measured at 37 °C for 60 min by incubation with a Caco-2 cell monolayer (apical pH, 6.0; basolateral pH, 7.4). Each value represents the mean ± SD of three or more experiments.

tea polyphenols do not cause critical changes in the transepithelial transport of tea polyphenols in Caco-2 cells. The initial permeation rates in the basolateral-to-apical direction, $J_{bl\rightarrow ap}$, were also similar among the tea polyphenols except for ECg and EGCg.

Interestingly, the $J_{bl\rightarrow ap}$ of ECg was ~4-fold the $J_{ap\rightarrow bl}$ (**Table 4**). A similar trend was observed in the case of EGCg, indicating that polarized efflux of ECg and EGCg from the basal to apical side of the Caco-2 cell monolayers might occur.

Paracellular Transport of Tea Polyphenols across the Caco-2 Cell Monolayers. Caco-2 cell monolayers exhibiting different TER values were prepared by treating the cells with cytochalasin D (*1*). The apical-to-basolateral transport of tea polyphenols was then characterized by using these monolayers. As illustrated in **Figure 4**, the transepithelial flux of all of the tea polyphenols tested was inversely correlated with the TER, suggesting that tea polyphenols permeate across Caco-2 cell monolayers via the paracellular pathways. Distribution studies using Caco-2 cell monolayers without cytochalasin treatment showed that >99% of apically loaded tea polyphenols remained on that side even after 60 min of incubation (data not shown). This suggests that the intestinal absorption of tea polyphenols is restricted when the epithelial tight junction is tight enough. This result is in good agreement with the previous findings that tea polyphenols were mainly located in the intestinal mucosa after oral administration (*18*).

DISCUSSION

Tea polyphenols inhibited fluorescein transport across the Caco-2 cell monolayers by a competitive mechanism (**Table 1**; **Figure 2**). The transport of such phenolic acids as salicylic and ferulic acids was also inhibited by tea polyphenols (**Table 3**). In previous papers (*1*, *5*), we have found that fluorescein and ferulic acid are transported via the MCT. We therefore speculated that tea polyphenols were also MCT substrates, thereby inhibiting the transport of fluorescein and ferulic acid in a competitive manner.

However, the structures of the tea polyphenols are quite different from those of substrates known to be recognized by MCT. A monoanionic carboxyl group and an unpolar side chain or aromatic hydrophobic portion are thought to be necessary components of a substrate for MCTs (*19*). The structure of EGCg and ECg at an apical pH of 6.0 is illustrated in **Figure 1**. The pK_a values for the hydroxy or ketone groups are estimated to be >8.38 ± 0.15 by a calculating procedure for a breaking ring system. Clearly this would not fulfill the structural criteria for an MCT substrate. It is therefore unlikely that tea polyphenols are transported by MCT as substrates. However, they may well act as inhibitors of the transporter.

Several proton-coupled MCTs, named after their substrate specificity for short-chain or aromatic monocarboxylic acids, have been fully characterized at the molecular level. Currently, nine isoforms of MCT have been identified (*20*), but only MCT1−MCT4 are characterized in terms of their substrate and inhibitor kinetics. Of the inhibitors already known, three types were found to be most effective: (i) hydrophobic substrate analogues, as exemplified by α-cyano-4-hydroxycinnamic acid (CHC); (ii) general inhibitors of anion transport, such as 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS); and (iii) flavonoids and their derivatives, such as quercetin, phloretin, and benzbromarone (*21*). The transporter responsible for fluorescein transport is not likely to be MCT1−MCT4, because transport was not inhibited by lactic acid, a good substrate for MCT1−MCT4 (*1*, *22*). It is possible that Caco-2 intestinal cells possess a novel MCT that transports fluorescein and phenolic acids. Because tea polyphenols are acylated flavonoids that are structurally related to quercetin, it may not be surprising that these substances are effective inhibitors of MCT.

If tea polyphenols are not substrates of MCT, we might expect the transepithelial flux of tea polyphenols to be lower than that of the MCT substrates. The mechanism for the intestinal transport of tea polyphenols was therefore investigated. As expected, the transepithelial flux of tea polyphenols was much lower than that of ferulic acid: $J_{ap\rightarrow bl}$ of tea polyphenols was 0.22−0.59 nmol/min/mg of protein (**Table 4**), whereas that of ferulic acid was 9.79 nmol/min/mg of protein (*5*). Considering these results, the transport of tea polyphenols was likely to occur via the paracellular pathway, whereas that of phenolic acids would be via the MCT-mediated transcellular pathway.

Transport studies of tea polyphenols in the apical-to-basolateral and basolateral-to-apical direction, however, demonstrated two types of transport pattern: a nonpolarized transport of C, EC, and EGC and the basolateral-to-apical transport of ECg and EGCg (**Table 4**). The reason for this polarized transport is unknown. The TER of the Caco-2 monolayers was rapidly lowered by adding ECg and EGCg to the basal chamber (data not shown). Thus, the greater $J_{bl\rightarrow ap}$ over $J_{ap\rightarrow bl}$ of ECg and EGCg could be explained in terms of increased paracellular permeability of the Caco-2 cell monolayer caused by the tea polyphenols. Hashimoto and co-workers, using a liposome system, found that the amount of tea polyphenols incorporated into lipid bilayers in the order ECg > EGCg > EC > EGC (*23*). ECg and EGCg, in particular, were found to interact with liposomes and locate at the surface of the lipid bilayers. A high affinity of ECg and EGCg for the lipid bilayer may cause some critical changes in the membrane structure, resulting in a rapid increase in tight junction permeability of Caco-2 cell monolayer. Indeed, the interaction of tea polyphenol with the membrane

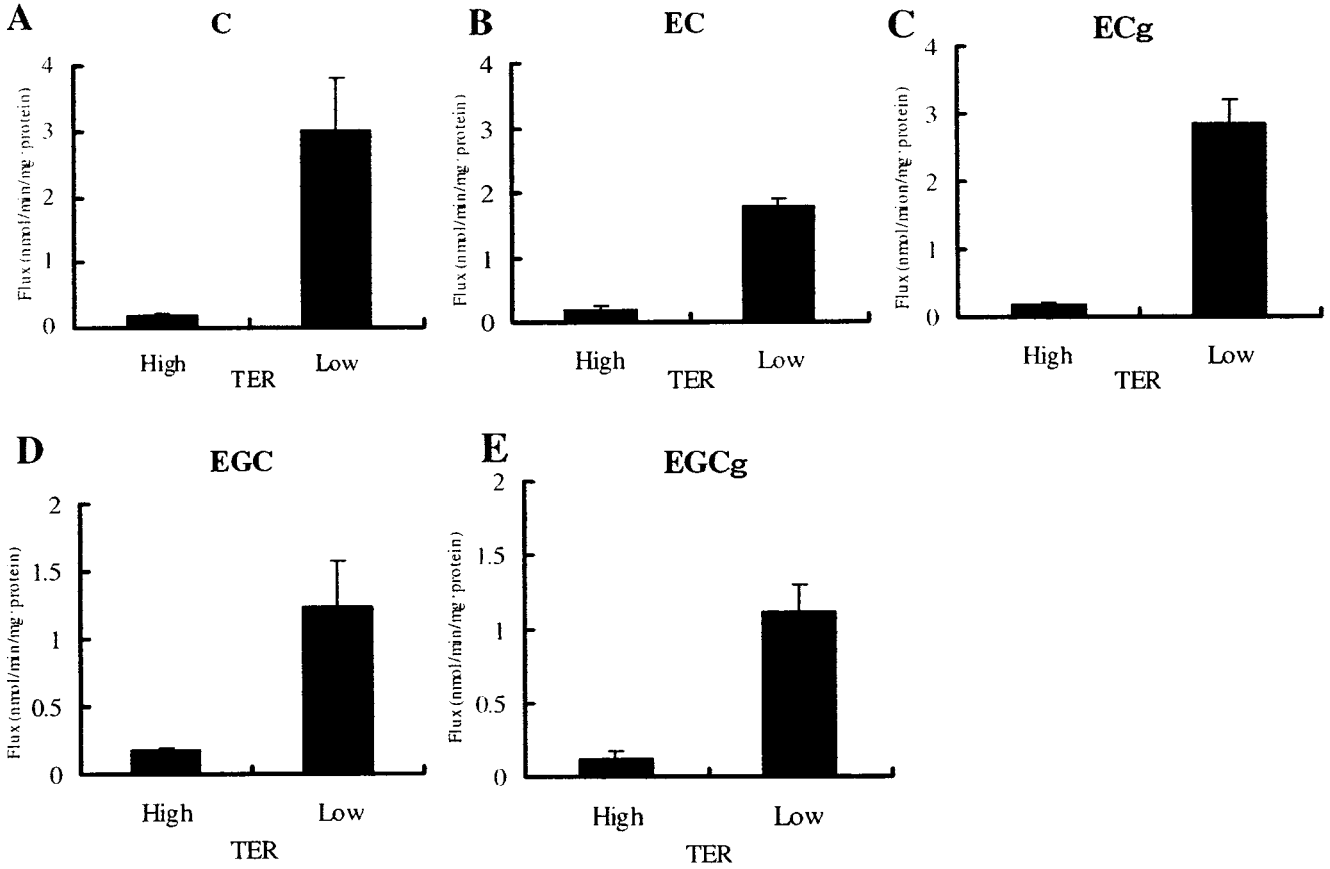

Figure 4. Correlation between TER and the transepithelial flux of each tea polyphenol. Each tea polyphenol (10 mM) was loaded on the apical side, and the apical side to the basolateral flux was measured at 37 °C (apical pH, 6.0; basolateral pH, 7.4). TER values are indicated as follows: (**A**) C (high, 1516 ± 61; low, 242 ± 17); (**B**) EC (high, 1418 ± 130; low, 287 ± 23); (**C**) ECg (high, 1308 ± 172; low, 278 ± 9); (**D**) EGC (high, 1223 ± 23; low, 287 ± 46); (**E**) EGCg (high, 1243 ± 144; low, 270 ± 21). Each point is the mean ± SD of three experiments.

bilayer might differ on the apical or basolateral side of the epithelial sheet. Further analysis is required to establish the intracellular signal transduction and cytoskeletal changes caused by tea polyphenols. Other tea polyphenols (C, EC, and EGC), which did not alter the tight junction permeability, exhibited nonpolarized flux as observed in the case of Lucifer Yellow, a paracellularly transported fluorescent marker (*1*).

Addition of a galloyl ester moiety to the basic triphenol structure of a tea polyphenol (e.g., ECg or EGCg) may allow excretion to the apical side by efflux transporters such as P-glycoprotein (P-gp) and the multidrug resistance related proteins (MRPs) (*24*). Recently, Hong et al. (*25*) have reported that EGCg is metabolized in the intestinal HT29 cells and then pumped out by MRPs.

Tea polyphenols were reported to exert some of their physiological functions in the intestinal tract. For example, tea polyphenols inhibited intestinal α-glucosidase or sucrase, which may be the main mechanism for their suppressive effects on the increase in the plasma glucose level after a meal (*26*, *27*). The inhibition of intestinal glucose transporter SGLT1 by tea polyphenols may also participate cooperatively in reducing the blood glucose level (*28*, *29*). Inhibition of intestinal glucose transporter (*29*) and multidrug-resistant P-gp (*30*) by direct interaction with tea polyphenols has been suggested. However, the mechanism for the inhibition of MCT by tea polyphenols is not yet known. Further analysis using photoaffinity labeling, for example, would be necessary to reveal the mechanism. In the present study, tea polyphenols were found to inhibit monocarboxylic acid transporters, emphasizing the diverse physiological effect of tea polyphenols on the intestinal uptake of nutrients.

In conclusion, we have demonstrated that tea polyphenols are slowly transported via the paracellular pathway, although some other polarized transport mechanisms for tea polyphenols might also be present. Further studies on the transport characteristics of dietary polyphenols and the effect of interactions between different polyphenolic compounds on their bioavailability are necessary to clarify the benefits of dietary polyphenols on human health.

ABBREVIATIONS USED

DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; TER, transepithelial electrical resistance; MCT, monocarboxylic acid transporter; ECD, electrochemical detector; C, catechin; EC, epicatechin; EGC, epigallocatechin; ECg, epicatechin gallate; EGCg, epigallocatechin gallate

ACKNOWLEDGMENT

We thank K. Hagiwara for support during this work.

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Received for review August 5, 2003. Revised manuscript received September 24, 2003. Accepted September 29, 2003.

JF034894T