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Transepithelial Transport of Chlorogenic Acid, Caffeic Acid, and Their Colonic Metabolites in Intestinal Caco-2 Cell Monolayers

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Both chlorogenic and caffeic acids exhibited nonsaturable transport in Caco-2 cells, whereas caffeic acid also showed proton-coupled polarized absorption. Thus, the absorption efficiency of caffeic acid was greater than that of chlorogenic acid. Polarized transport of caffeic acid was inhibited by substrates of MCT such as benzoic and acetic acids. Almost all of the apically loaded chlorogenic and caffeic acid was retained on the apical side, and the transepithelial flux was inversely correlated with the paracellular permeability of Caco-2 cells. These results indicate that transport was mainly via paracellular diffusion, although caffeic acid was absorbed to a lesser extent by the monocarboxylic acid transporter (MCT). Furthermore, *m*-coumaric acid and 3-(*m*-hydroxyphenyl)propionic acid, the main metabolites of chlorogenic and caffeic acid by colonic microflora, competitively inhibited the transport of fluorescein, a known substrate of MCT. This suggests that their absorption could also be mediated by MCT. These findings have exemplified the physiological importance of MCT-mediated absorption in both phenolic acids per se and their colonic metabolites.

KEYWORDS: Chlorogenic acid; caffeic acid; monocarboxylic acid transporter; colonic metabolite; Caco-2

INTRODUCTION

Dietary polyphenols have been widely assumed to be beneficial to human health by exerting various biological effects such as free radical scavenging, metal chelation, modulation of enzymatic activity, and altering signal transduction pathways (*1*−*6*). Epidemiological studies have also highlighted the association between the consumption of polyphenol-rich food and beverages and the prevention of various human diseases (*7*, *8*).

A major class of phenolic compounds are the hydroxycinnnamic acids, which are ubiquitously found in fruits, vegetables, and cereals (*9*−*12*). They mainly exist in an esterified form with organic acids, sugars, and lipids (*1*, *12*). Caffeic acid is the major representative of the hydroxycinnamic acids and occurs in foods mainly as chlorogenic acid (5-caffeoylquinic acid), an ester of quinic acid. Coffee is a major source of chlorogenic acid in the diet, daily intake of chlorogenic acid in coffee drinkers being ∼1 g. Another dietary source of chlorogenic acid is fruits such as apples, pears, and berries (*13*).

Chlorogenic acid and caffeic acid have vicinal hydroxyl groups on an aromatic residue and exhibit antioxidant activities and antimutagenic and carcinogenic effects in vitro (*14*, *15*). Indeed, this is consistent with the reported inverse correlation between coffee intake and colon cancer in some epidemiologic studies (*16*−*18*). The physiological importance of chlorogenic acid and caffeic acid depends on their availability for intestinal absorption and subsequent interaction with target tissues. A number of recent studies have focused on the absorption and metabolism of chlorogenic acid and caffeic acid in rats and humans (*15*, *19*−*24*). It has been reported that caffeic acid is more efficiently absorbed than chlorogenic acid. Caffeic acid that undergoes absorption appears to be glucuronidated or sulfated as well as *O*-methylated to ferulic acid or isoferulic acid. The gut microflora may metabolize these compounds further to give phenolics such as *m*-coumaric acid and derivatives of phenylpropionic and hippuric acid (*15*, *21*, *24*). In contrast, most ingested chlorogenic acid reaches the colon intact and is most likely cleaved into caffeic and quinic acids by esterase activity of the colonic microflora. The free quinic acid is then absorbed and metabolized in a manner similar to caffeic acid (*21*, *23*, *24*). Several studies have focused on the significance of the microbial metabolites, as well as ingested parent compounds, as physiologically relevant bioactive components in vivo (*15*, *21*, *24*). However, the absorption characteristics of chlorogenic and caffeic acids and their colonic metabolites are still obscure, because only passive diffusion seems to be involved in the permeation process (*25*, *26*).

Recently we have reported that phenolic acids such as ferulic and *p*-coumaric acids are absorbed by the monocarboxylic acid transporter (MCT) in Caco-2 cells (*27*, *28*). Furthermore, by investigating structural effects of phenolic acid on fluorescein transport, which is also the substrate of MCT, we concluded that caffeic acid is likely to have a lower affinity for MCT (*29*,

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30). This study was designed to investigate and clarify the absorption mechanisms of chlorogenic and caffeic acids using Caco-2 cells as an in vitro model of intestinal absorption and metabolism (*31*−*33*). The study provides further insight into the nature of the health benefits of these compounds in vivo.

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 Invitrogen Corp. (Carlsbad, CA). Type I collagen was purchased from Nitta Gelatin Inc. (Osaka, Japan). Plastic dishes, plates, and Transwell inserts with 0.4-μm polycarbonate membranes (12 mm in diameter) were obtained from Corning (Corning, NY). Chlorogenic and caffeic acids were from Wako Pure Chemicals Inc., Ltd. (Osaka, 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 seeding with 1×10^5 cells per 100-mm dish. Cells were passaged at a split ratio of 4−8 by treatment with 0.1% trypsin and 0.02% EDTA acid in HBSS. All cells used were between passages 55 and 73.

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 culturing for 2 weeks. The integrity of the cell layer was evaluated by measurement of TER with Millicell-ERS equipment (Millipore, MA). Monolayers with TER of $>250\ \Omega \bullet cm^2$ were used for the experiments. The TER of the monolayer was measured before and after an assay sample was added to the insert.

Transepithelial Transport Experiments. To measure the apical-to-basolateral permeability, 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 or 7.4, 37 °C) containing chlorogenic or caffeic acid (5 mM) was added to the apical side. At the designated time after incubation at 37 °C, the basal solution was collected, and subsequently an equal volume of HBSS was replaced. The amount of chlorogenic or caffeic acid transported by Caco-2 cells was estimated by HPLC with electrochemical detection (ECD) with an ESA coulometric detection system (ESA Inc., Boston, MA). The results were expressed in terms of specific permeability ($\mu L/cm^2$), which was calculated as the amount transported divided by the initial concentration in the donor compartment.

To examine the basolateral-to-apical transport, HBSS (pH 6.0 or 7.4, 37 °C) was added to the apical side, and 1.5 mL of the test solution (pH 7.4, 37 °C) containing chlorogenic or caffeic acid (5mM) was added to the basolateral side.

Chromatographic Conditions. HPLC-ECD fitted with a 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 (*34*). 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). 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: 0−5 min, 100% solvent A/0% solvent B; 5−28.5 min, 100% solvent A/0% solvent B → 20% solvent A/80% solvent B; 28.5−31 min, 0% solvent A/100% solvent B. For analysis of chlorogenic and caffeic acids the eight electrode detector potentials were set from 0 to 700 mV in increments of 100 mV.

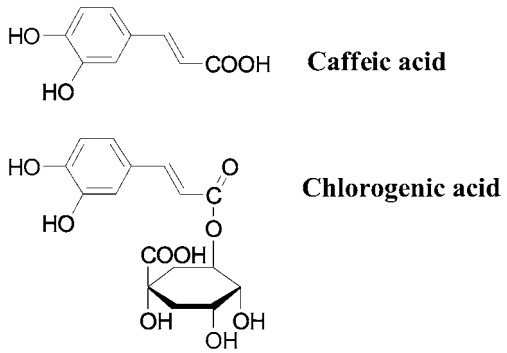

Figure 1. Chemical structures of chlorogenic and caffeic acids.

Distribution of Chlorogenic Acid and Caffeic Acid after Transport Experiments. At the end of the transport experiments, the level of chlorogenic acid and caffeic acid in the apical and basolateral solutions was measured. The monolayer cells were rinsed with HBSS (pH 6.0 or 7.4) and extracted with methanol/solvent A (10:1, v/v) for 30 min. Chlorogenic acid and caffeic acid in this extract were measured and used as an index of the intracellular fractions taken up by the Caco-2 cells.

Inhibition of the Transport of Fluorescein. 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 compound 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 40 min at 37 °C. The basal solution, containing transported fluorescein, was replaced by the same amount of fresh HBSS (pH 7.4) every 10 min. The fluorescein transported by the cells was estimated with a fluorescence spectrophotometer (Spectral Max Gemini, Molecular Devices Corp., Sunnyvale, CA) at an excitation wavelength of 490 nm and emission wavelength of 514 nm.

Data Analysis. The slope of the initial linear portion of the amount transported (nmol/min/mg of protein) versus time (min) curves, calculated by linear regression analysis, was defiend as the permeation rate (nmol/min/mg of protein), *J*. Results are expressed as the means ± SD. Statistical analysis was done with Student's two-tailed *t* test, and differences with $P < 0.01$ were considered to be significant.

RESULTS

HPLC Analysis of Chlorogenic and Caffeic Acids Transported across Caco-2 Cell Monolayers. Representative chromatograms of chlorogenic acid and caffeic acid transported (**Figure 1**) into the basolateral solution are presented in **Figure 2**. Chlorogenic acid and caffeic acid were determined at a detection limit of <0.5 pmol, and the reproducibility was good without requiring any sample pretreatment. Purity of the peaks was assessed using peak area ratio accuracies for the oxidation channels (lower or upper) adjacent to the dominant oxidation channel. The voltammetric response of the analyte across these channels was unique for each compound, and >70% ratio accuracy was considered to support the peak purity (*34*). The retention time (RT) and dominant oxidation potential for chlorogenic and caffeic acids are 17.5 min and 200 mV and 18.9 min and 100mV, respectively.

Characteristics of Transepithelial Transport of Chlorogenic and Caffeic Acids. Bidirectional permeation of chlorogenic acid and caffeic acid across Caco-2 cell monolayers was examined in the presence and absence of an inwardly directed proton gradient (**Figure 3**). The permeation of chlorogenic acid

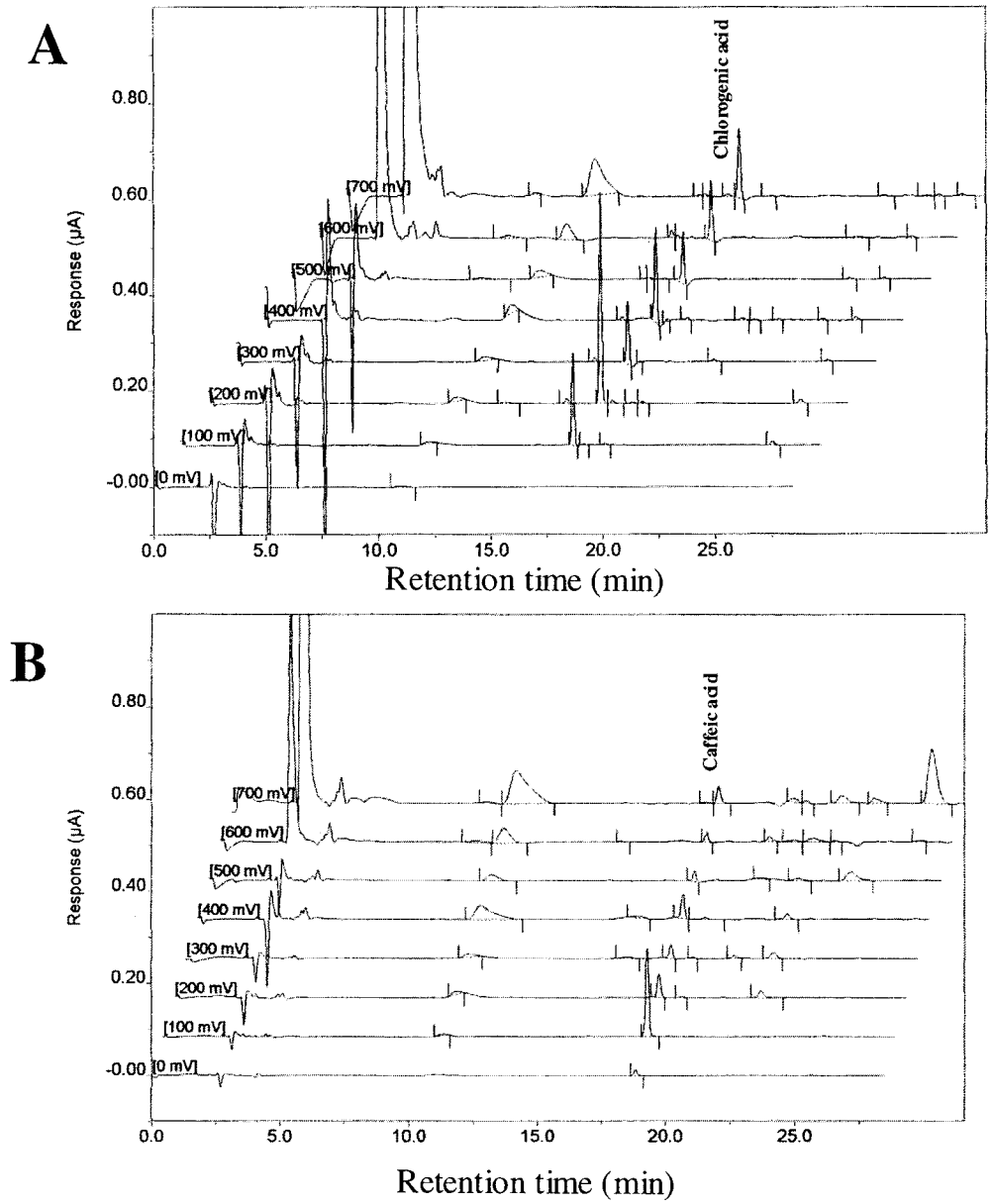

Figure 2. Chromatograms of chlorogenic acid (**A**) and caffeic acid (**B**) transported across Caco-2 cell monolayers.

in the apical-to-basolateral direction, $J_{ap \rightarrow bl}$ (0.16 nmol/min/mg of protein) was similar to that in the basolateral-to-apical direction, $J_{bl \rightarrow ap}$ (0.13 nmol/min/mg of protein) in the presence of a proton gradient (apical pH, 6.0; basolateral pH, 7.4). However, in the absence of a proton gradient (apical pH, 7.4; basolateral pH, 7.4), $J_{ap \rightarrow bl}$ (0.59 nmol/min/mg of protein) was greater than $J_{bl \rightarrow ap}$ (0.16 nmol/min/mg of protein), which is characteristic of polarized transport. In contrast, caffeic acid exhibited a pH-dependent directional transport from the apical to the basolateral side ($J_{ap \rightarrow bl}$ and $J_{bl \rightarrow ap}$ being 2.69 and 0.56 nmol/min/mg of protein, respectively, with an apical pH of 6.0 and a basolateral pH of 7.4). However, $J_{ap \rightarrow bl}$ was the same as $J_{bl \rightarrow ap}$ in the absence of a proton gradient (0.51 nmol/min/mg of protein; apical pH, 7.4; basolateral pH, 7.4). These results are consistent with those of the MCT substrates such as ferulic acid, *p*-coumaric acid, and fluorescein (*27*, *28*, *30*).

Distribution of Chlorogenic and Caffeic Acids. After the transport experiments in the absence of a proton gradient (apical pH, 7.4; basolateral pH, 7.4), >99% of apically loaded chlorogenic and caffeic acids was retained on the apical side, suggesting they were restricted by the tight junctions (**Table 1**). In the presence of a proton gradient (apical pH, 6.0; basolateral pH, 7.4), a considerable amount of caffeic acid was

Table 1. Distribution of Chlorogenic and Caffeic Acids after Transepithelial Transport Experiments in the Presence and Absence of a Proton Gradient[a]

	% compound recovered from		
sample pH gradient	apical side	basolateral side	cells
chlorogenic acid			
6.0/7.4	99.69 ± 0.10	0.06 ± 0.01	0.25 ± 0.10
7.4/7.4	99.76 ± 0.19	0.12 ± 0.02	0.12 ± 0.17
caffeic acid			
6.0/7.4	97.55 ± 0.11*	1.57 ± 0.03*	0.88 ± 0.08*
7.4/7.4	99.55 ± 0.01	0.20 ± 0.01	0.25 ± 0.02

[a] Transepithelial transport experiments were done as described under Materials and Methods both in the presence and in the absence of a proton gradient (apical pH, 6.0 or 7.4; basolateral pH, 7.4). Values are the mean ± SD of three or more experiments. An asterisk (*) indicates significant difference from the result in the absence of the proton gradient ($P < 0.001$).

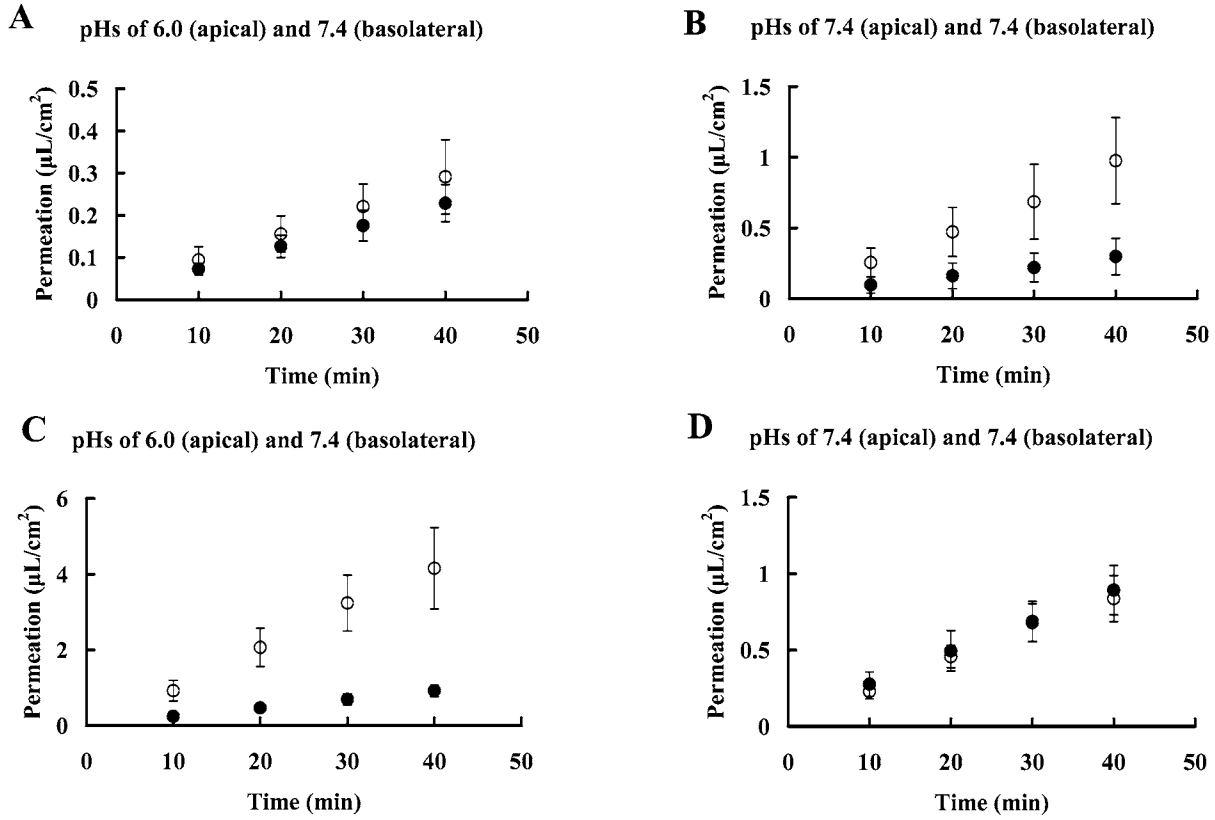

Figure 3. Characteristics of transepithelial transport of chlorogenic acid (**A**, **B**) and caffeic acid (**C**, **D**) across Caco-2 cell monolayers. Permeation of chlorogenic and caffeic acids (5 mM) from the apical side to the basolateral side (○) and from the basolateral side to the apical side (●) was measured at 37 °C both in the presence (**A**, **C**) and in the absence (**B**, **D**) of a proton gradient (apical pH, 6.0 or 7.4; basolateral pH, 7.4). Each point is the mean ± SD of three experiments.

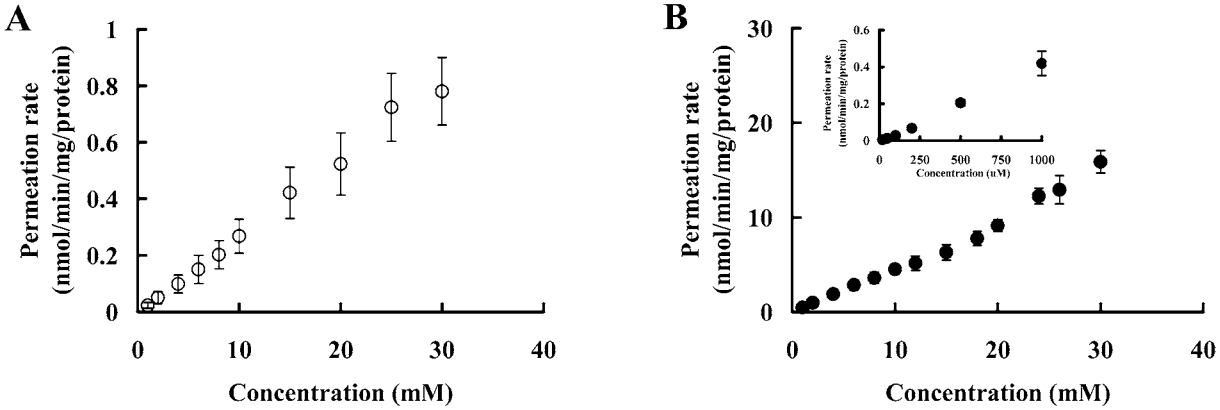

Figure 4. Concentration dependence of chlorogenic acid (**A**, ○) and caffeic acid (**B**, ●) transport across Caco-2 cell monolayers in the presence of a proton gradient. The initial permeation rate is shown. Values are the mean ± SD of three or more experiments.

transported into the basolateral side, although >99% of apically loaded chlorogenic acid was retained on the apical side. Caffeic acid is likely to be taken up and transported into the basolateral side according to a pH gradient, whereas chlorogenic acid seems to be restricted by the tight junction irrespective of a pH gradient.

Concentration Dependence of Chlorogenic and Caffeic Acids Transport. Figure 4 shows the relationship between the initial permeation rate of chlorogenic and caffeic acids and their concentrations (apical pH, 6.0; basolateral pH, 7.4). The permeation rates of both chlorogenic and caffeic acids were concentration dependent and were not saturable, even at 30 mM, suggesting that passive diffusion might be involved. These results are also in good agreement with the distribution study that showed both chlorogenic and caffeic acids are mainly permeated via paracellular diffusion. Lucifer Yellow, a marker compound for paracellular transport, also showed this trend (*30*).

Inhibition of Directional Transport of Caffeic Acid. To investigate the transport characteristics responsible for proton-coupled polarized transport of caffeic acid (apical pH, 6.0; basolateral pH, 7.4), we added 1 mM caffeic acid to the apical chamber and the effects of various compounds on the permeation of caffeic acid were examined (**Table 2**). A metabolic inhibitor, NaN_3 (10 mM), strongly reduced the permeation of caffeic acid. The substrates for MCTs such as benzoic acid and acetic acid significantly inhibited the permeation of caffeic acid, although lactic acid, a good substrate for MCT1−MCT4 (*35*), had no effect on caffeic acid transport. The order of the inhibitory activity of the MCT substrates was benzoic acid > acetic acid

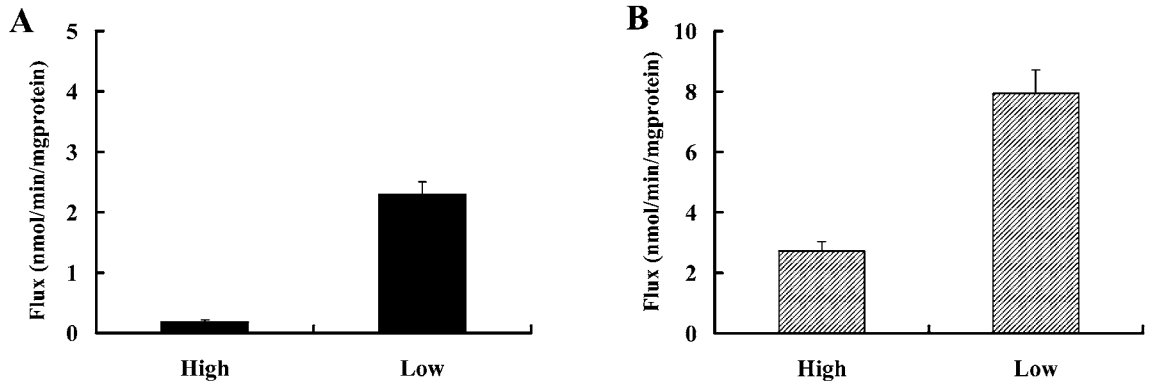

Figure 5. Correlation between TER and the transepithelial flux of chlorogenic (**A**) acid and caffeic acid (**B**). Chlorogenic and caffeic acids (5 mM) were loaded in the apical side, and the flux from the apical side to the basolateral side was measured at 37 °C (apical pH, 6.0; basolateral pH, 7.4). TER values are indicated as follows: (**A**) high, 737 ± 18, and low, 294 ± 24; (**B**) high, 757 ± 19, and low, 273 ± 6. Each point is the mean ± SD of three experiments.

Table 2. Effects of Various Compounds on Caffeic Acid Transport across Caco-2 Cell Monolayers in the Presence of a Proton Gradient[a]

	concn (mM)	relative permeation (% of control)
NaN_3	10	27.6 ± 2.2*
benzoic acid	20	30.0 ± 2.9*
lactic acid	20	114.2 ± 8.9
acetic acid	20	53.5 ± 4.3*

[a] The amount of caffeic acid transported was measured at 37 °C for 40 min by incubating Caco-2 cells in the absence or presence of each compound at the concentration indicated (apical pH, 6.0; basolateral pH, 7.4). Each value represents the mean ± SD of three or more experiments. *, $P < 0.01$.

> lactic acid. This order was the same as that for ferulic and *p*-coumaric acid transport, clearly demonstrating that caffeic acid is transported at least in part by MCT (*27*, *28*).

Paracellualr Transport of Chlorogenic and Caffeic Acids across the Caco-2 Cell Monolayers. Caco-2 cell monolayers exhibiting different TER values were prepared by treating the cells with cytochalasin D (*30*). The apical-to-basolateral transport of chlorogenic and caffeic acids was then characterized by using these monolayers (apical pH, 6.0; basolateral pH, 7.4). As illustrated in **Figure 5**, the transepithelial flux of both chlorogenic and caffeic acids was inversely correlated with the TER, suggesting that they permeate across Caco-2 cell monolayers via the paracellular pathways. This finding also suggests that the intestinal absorption of chlorogenic and caffeic acids is restricted when the epithelial tight junction is tight enough.

Effect of the Colonic Metabolites of Chlorogenic and Caffeic Acids on Fluorescein Transport across Caco-2 Cell Monolayers. When the metabolites of colonic microbial origin such as *p*-benzoic, *m*-coumaric, 3-(*p*-hydroxyphenyl)-, 3-(*o*-hydroxyphenyl)-, and 3-(*m*-hydroxyphenyl)propionic acids were mixed with fluorescein (*21*, *24*) and added to the apical solution, they inhibited fluorescein transport across the monolayer. However, *m*-benzoic acid, 3-(3,4-dihydroxyphenyl)propionic acid, and derivatives of hippuric acid did not display any such inhibitory effect (**Table 3**). The order of the inhibitory activity was *p*-benzoic acid > *m*-coumaric acid > 3-*o*- or 3-(*p*-hydroxyphenyl)propionic acid > 3-(*m*-hydroxyphenyl)propionic acid. **Figure 6** shows the effects of *m*-coumaric acid and 3-(*m*-hydroxyphenyl)propionic acid on the permeation of fluorescein in terms of a Lineweaver−Burk plot. Both compounds competitively inhibited the transport of fluorescein. The inhibition constant (K_i) values were 3.35 ± 0.39 mM for *m*-coumaric acid and 18.3 ± 3.3 mM for 3-(*m*-hydroxyphenyl)propionic acid.

Table 3. Effects of Various Colonic Metabolites and Their Derivatives on Fluorescein Transport across Caco-2 Cell Monolayers[a]

compound	relative permeation (% of control)
3-(*p*-hydroxyphenyl)propionic acid	68.5 ± 7.7*
3-(*o*-hydroxyphenyl)propionic acid	64.0 ± 7.0*
3-(*m*-hydroxyphenyl)propionic acid	83.0 ± 3.6*
3-(3,4-dihydroxyphenyl)propionic acid	107.9 ± 8.8
3-(4-hydroxy-3-methoxyphenyl)propionic acid	43.0 ± 5.5*
p-hydroxybenzoic acid	34.5 ± 7.1*
m-hydroxybenzoic acid	97.1 ± 7.6
m-coumaric acid	43.7 ± 2.1*
caffeic acid	115.8 ± 12.1
hippuric acid	104.0 ± 3.7
4-hydroxyhippuric acid	108.6 ± 8.7

[a] The amount of fluorescein transported was measured at 37 °C for 40 min by incubating Caco-2 cells in the absence or presence of each compound (10 mM, apical pH, 6.0; basolateral pH, 7.4). Values are the mean ± SD of three or more experiments. *, $P < 0.01$. *p*-Hydroxybenzoic acid, *m*-hydroxybenzoic acid, *m*-coumaric acid, and caffeic acid were investigated in our previous studies (*29*, *30*).

The K_i value of *m*-coumaric acid was close to that of ferulic acid (2.99 ± 0.08 mM), whereas the K_i value of 3-(*m*-hydroxyphenyl)propionic acid was close to that of *p*-coumaric acid (27.2 ± 4.3 mM). We have found that both ferulic and *p*-coumaric acid are good substrates of MCT, sharing the transporter with fluorescein (*27*, *28*). These results suggest that *m*-coumaric and 3-(*m*-hydroxyphenyl)propionic acids, the main metabolites of chlorogenic and caffeic acid by the gut microflora (*21*, *24*), are also transported across Caco-2 cells via MCT.

DISCUSSION

A monoanionic carboxyl group and a nonpolar side chain or aromatic hydrophobic moiety are thought to be necessary components of a substrate for MCTs (*36*). Chlorogenic acid, having a monocarboxylic group in the portion of quinic acid and the aromatic portion inside, might seem to fulfill the structural criteria for an MCT substrate (**Figure 1**), although the ester group might cause some critical effect on interaction with the MCT molecule. However, it was not known whether MCT could recognize and transport chlorogenic acid in Caco-2 cells. Hence, the mechanism for the intestinal transport of chlorogenic acid was investigated. The transepithelial flux ($J_{ap \rightarrow bl}$) of chlorogenic acid (0.13−0.59 nmol/min/mg of protein) was much lower than that of ferulic acid (9.79 nmol/min/mg of protein) (*27*). However, the transepithelial flux of chlorogenic acid (0.16−0.22 nmol/min/m of protein) was nearly the same as that of gallic acid and tea catechin (0.22−0.59 nmol/min/

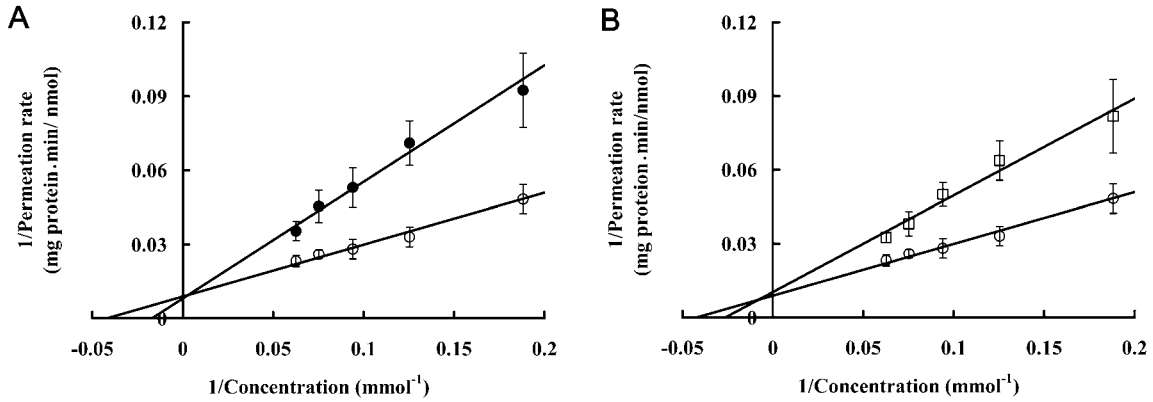

Figure 6. Lineweaver–Burk plots for the transport of fluorescein across Caco-2 cell monolayers. The permeation coefficient was measured in the absence of added compounds (○) and in the presence of 5 mM *m*-coumaric acid (●, **A**) or 10 mM 3-(*m*-hydroxyphenyl)propionic acid (□, **B**). Each point is the mean ± SEM of three or more experiments.

mg of protein) (*28*, *37*). Because gallic acid and tea catechin are thought to be transported via paracellular diffusion (*28*, *37*), it was expected that chlorogenic acid would also be permeated via paracellular diffusion across Caco-2 cell monolayers. Evidence from the distribution study (**Table 1**) and correlation between TER and the permeability of chlorogenic acid (**Figure 5**) also support this hypothesis.

Directional transport of chlorogenic acid in the apical-to-basolateral direction was demonstrated, only in the absence of the inwardly directed proton gradient (**Figure 3**). The reason for this polarized transport is not clear because the mechanism responsible for proton-uncoupled transport is not fully understood. Neither NaN_3 (10 mM), HCO_3^- (25 mM), nor benzoic acid (20 mM) inhibited the directional transport, suggesting that neither the MCT nor the anion exchange transporter is involved (data not shown). Apically loaded chlorogenic acid caused the TER of the Caco-2 monolayer to fall only in the absence of a proton gradient (data not shown). Thus, the greater $J_{ap\rightarrow bl}$ over $J_{bl\rightarrow ap}$ would be ascribed to the increased paracellular permeability of a Caco-2 cell monolayer (apical pH, 7.4; basolateral pH, 7.4).

The transepithelial flux of caffeic acid ($J_{ap\rightarrow bl}$; 2.69 nmol/min/mg of protein, apical pH, 6.0; basolateral pH, 7.4) was also lower than that of ferulic acid ($J_{ap\rightarrow bl}$; 9.79 nmol/min/mg of protein), although the permeation rate was higher than that of chlorogenic acid ($J_{ap\rightarrow bl}$; 0.13−0.59 nmol/min/mg of protein). Ingested caffeic acid is also likely to be primarily absorbed via the paracellular pathway, because transport increased linearly and did not plateau even at 30 mM (**Figure 4**). Moreover, the results of the distribution study (**Table 1**) and correlation between TER and the permeability (**Figure 5**) also support this conclusion. However, caffeic acid exhibited proton-coupled polarized transport in the apical-to-basolateral direction. This transport is presumably mediated by MCT, as is the case for ferulic and *p*-coumaric acids (*27*, *28*). Hydroxylation of a phenolic acid would be predicted to decrease affinity for MCT (*29*). Caffeic acid, a dihydroxy derivative of cinnamic acid, may still have some affinity for MCT. In contrast, gallic acid, a trihydroxy derivative of benzoic acid, would have no affinity for MCT and permeate only via paracelluar diffusion. Caffeic acid has been reported to be more efficiently absorbed than chlorogenic acid, but the mechanism is unknown (*15*). We speculate that the absorption efficiency of caffeic acid would be attributed to its MCT-mediated absorption.

Recent studies have attempted to address the problem of how microbial metabolites of ingested polyphenols, as well as the parent compounds, can act as physiologically relevant bioactive components in vivo (*21*, *24*, *38*, *39*). It has been reported that the majority of ingested chlorogenic acid is not absorbed in the proximal part of gut, but reaches the large intestine, where it is hydrolyzed to caffeic acid and quinic acid by microbial esterases (*21*, *23*). Furthermore, caffeic acid and quinic acid are metabolized by the gut microflora to give *m*-coumaric acid, derivatives of phenylpropionic acid such as 3-(3,4-dihydroxyphenyl)-propionic and 3-(*m*-hydroxyphenyl)propionic acid, *m*-hydroxybenzoic acid, and derivatives of hippuric acid (*21*, *24*). Because these metabolites still possess a free phenolic group, they could have significant reducing and antioxidant properties and therefore protect against oxidative stress, although the monophenolic structure is generally a poorer antioxidant than ortho-diphenolic structures. There is still, however, a need for comprehensive data concerning their absorption and distribution around the whole body (*21*, *39*).

We have speculated that these metabolites might be MCT substrates, because they have a monoanionic carboxyl group and an aromatic hydrophobic portion (*36*). We have established that 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 (*30*). MCT-mediated transport of dietary phenolic acids such as ferulic and *p*-coumaric acids was demonstrated by this method (*27*, *28*). Therefore, the effect of these metabolites on fluorescein transport was examined. As expected, *m*-coumaric acid and derivatives of phenylpropionic acid markedly inhibited fluorescein transport across Caco-2 cells, although *m*-hydroxybenzoic acid and a derivative of hippuric acid did not (**Table 3**). The order of the inhibitory activity of derivatives of phenylpropionic acid was 3-(*o*-hydroxyphenyl)propionic acid (relative permeation value of 64.0%) > 3-(*p*-hydroxyphenyl)propionic acid (68.5%) > 3-(*m*-hydroxyphenyl)propionic acid (83.0%) >3-(3,4-dihydroxyphenyl)propionic acid (107.9%). Because the inhibitory activity of 3-(4-hydroxy-3-methoxyphenyl)propionic acid (43.0%) was 2-fold greater than that of 3-(3,4-dihydroxyphenyl)propionic acid, meta-hydroxylation of the substrate appears to decrease the affinity for MCT, which is consistent with previous results (*29*). Furthermore, *m*-coumaric acid and 3-(*m*-hydroxyphenyl)-propionic acid, major colonic metabolites formed after chrologenic acid intake (*21*), competitively inhibited fluorescein transport (**Figure 6**), indicating that they are transported across Caco-2 cells via MCT. Additional experiments are necessary to reveal the MCT-mediated absorption of these colonic metabolites.

Regular consumption of cereal bran and bran-enriched products would result in the ingestion of significant amounts

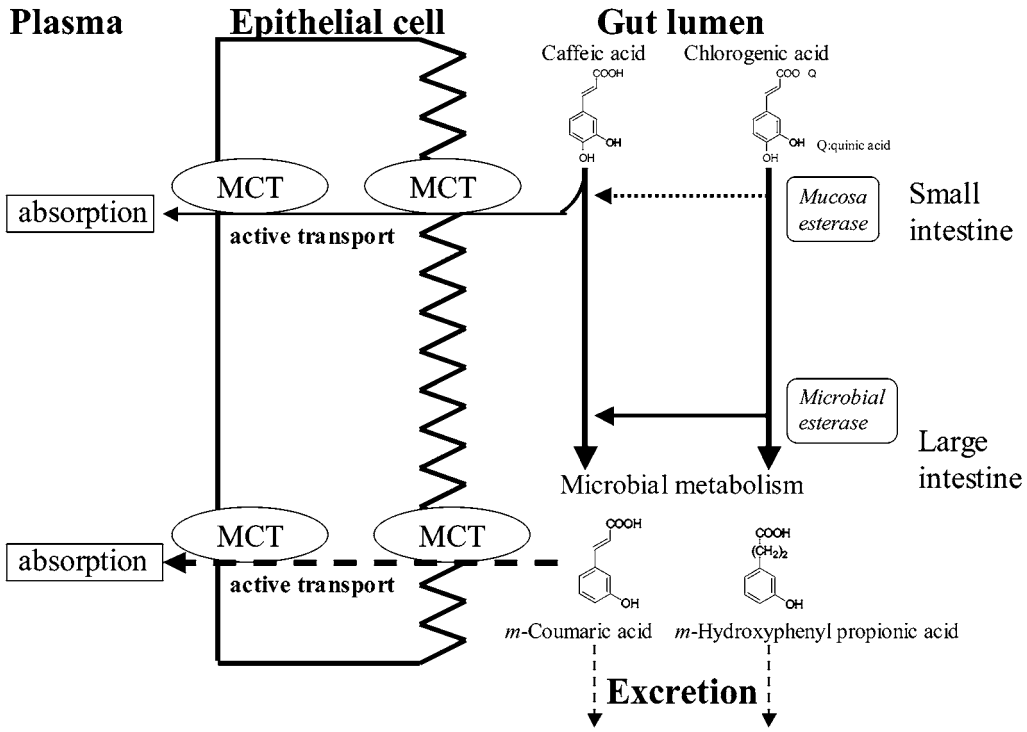

Figure 7. Possible routes for absorption of ingested chlorogenic and caffeic acid.

of hydroxycinnamic acids, in particular, ester-linked ferulic and diferulic acids (*40*, *41*). Diferulic acid in the gut lumen, liberated by intestinal cinnamoyl esterase, could be absorbed directly. In contrast, undigested bran reaching the large intestine would form diferulic acid by microflora xylanases and cinnamoyl esterase, followed by either absorption or further transformation by colonic microflora (*41*). A recent study reported that chlorogenic acid is mainly hydrolyzed to caffeic acid by esterase in human colonic microflora and then absorbed (*19*). Indeed, an esterase activity that catalyzes this reaction has been demonstrated in humans (*23*, *42*). However, in another human study, some chlorogenic acid was reported to be absorbed intact (*20*). The results obtained in this study, together with data from the literature, suggest that the major portion of chlorogenic acid reaches the large intestine, and *m*-coumaric and 3-(*m*-hydroxyphenyl)propionic acid, the principal metabolites formed by the colonic microflora, are actively absorbed by MCT. Absorption of intact chlorogenic acid is restricted by the tight junction. Caffeic acid, liberated from chlorogenic acid by intestinal mucosa esterase (*43*), is not only absorbed via paracellular diffusion (e.g., chlorogenic acid) but also actively absorbed by MCT. The putative mechanism for absorption of both chlorogenic and caffeic acids is shown in **Figure 7**. The proposed scheme is consistent with the highly efficient absorption of aromatic colonic metabolites after oral administration (*44*), the low recovery of intact chlorogenic acid (*15*), and the greater absorption of caffeic acid over chlorogenic acid reported previously (*15*).

Currently, nine isoforms of MCT have been identified (*35*), but only MCT1−MCT4 are characterized in terms of their substrate and inhibitor kinetics. Each MCT isoform is likely to have a unique biological role, which is related to the different tissue distribution. MCT1 is the most well studied isoform and is found in almost all tissues, that is, heart, skeletal muscle, small intestine, colon, liver, brain, spinal cord, testis, ovary, placenta, and adrenal gland (*45*). Both MCT1 and MCT2 were also reported to be located in the colon (*46*). Caco-2 intestinal cells might possess a novel MCT, which transports phenolic acids and fluorescein (*30*). Further identification and characterization of MCT responsible for phenolic acid absorption are necessary to clarify the health benefits of dietary phenolic acids.

Additionally, paracellular absorption has been recognized to be modulated by conutrients and food-derived substances ingested. Solvent drag caused by rapid increase of the luminal glucose immediately after the meal (*47*), and TER decreasing dietary substances such as capsianoside or other food-derived substances (*48*, *49*), increased the paracellular permeability dramatically. Actually in the meal, intact chlorogenic and caffeic acids ingested with the food substances mentioned above might be absorbed more efficiently via the paracellular pathways, exerting effective antioxidant activity in human body.

In conclusion, we have demonstrated that both chlorogenic and caffeic acids are mainly transported via the paracelluar pathways, although caffeic acid is partially transported by MCT. Furthermore, we have indicated that the metabolites of chlorogenic and caffeic acids formed by gut microflora are absorbed by MCT, suggesting the physiological significance of MCT-mediated absorption. Further studies on MCT-mediated transport of dietary substance and their colonic metabolites are required to fully understand the health effects of dietary polyphenolic compounds.

ABBREVIATIONS USED

HBSS, Hanks' balanced salt solution; TER, transepithelial electrical resistance; MCT, monocarboxylic acid transporter; ECD, electrochemical detector.

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