Transepithelial Transport of Rosmarinic Acid in Intestinal Caco-2 Cell Monolayers

Yutaka Konishī1,2 and Shoko Kobayashi2

1Applied BioResearch Center, Research and Development Department, Kirin Brewery Co., Ltd.,
3 Miyahara-cho, Takasaki-shi, Gunma 370-1295, Japan
2Department of Food and Life Science, Takasaki University of Health and Welfare, Takasaki 370-0033, Japan

Received October 29, 2004; Accepted November 25, 2004

The absorption characteristics of rosmarinic acid (RA) were examined by measuring permeation across Caco-2 cell monolayers using an HPLC-electrochemical detector (ECD) fitted with a coulometric detection system. RA exhibited nonsaturable transport even at 30 mM, and the permeation at 5 mM in the apical-to-basolateral direction, \( J_{\text{ap-b}} \), was 0.13 nmol/min/mg of protein. This permeation rate is nearly the same as that of 5 mM chlorogenic acid (CLA) and gallic acid, which are paracellularly transported compounds. Almost all of the apically loaded RA was retained on the apical side, and \( J_{\text{ap-b}} \) was inversely correlated with paracellular permeability. These results indicate that RA transport was mainly via paracellular diffusion, and the intestinal absorption efficiency of RA was low. Furthermore, RA appeared to be unsusceptible to hydrolysis by mucosa esterase in Caco-2 cells. These results, together with our previous work \( J. \) Agric. Food Chem., 52, 2518–2526 (2004), suggest that the majority of RA is further metabolized and degraded into \( m \)-coumaric and hydroxylated phenylpropionic acids by gut microflora, which are then efficiently absorbed and distributed by the monocarboxylic acid transporter (MCT) within the body. The potential of orally administered RA in vivo will be further investigated.

Key words: rosmarinic acid; paracellular diffusion; monocarboxylic acid transporter; HPLC-electrochemical detector (ECD); Caco-2

Rosmarinic acid (RA) is an ester of caffeic acid (CA) and 3,4-dihydroxyphenyllactic acid, and is commonly distributed in species of the Boraginaceae and the subfamily Nepetoideae of Lamiaeae, as well as other higher plant families.1) In plants, RA is biosynthesized from phenylalanine and tyrosine, and is thought to act as a constitutively accumulated defense compound against stress.1) Herbal medicine made from Perilla frutescens extract, which contains RA as the major constituent, has been used primarily in the treatment of inflammatory diseases, clinical depression, and anxiety-related disorders such as anxiety neurosis and anxiety hysteria.2) RA has been reported to have a number of biological activities in vitro, including antiviral, antibacterial, antioxidant, anti-inflammatory, and antiallergenic activities.3) Recent in vivo studies have also reported that orally administered RA exhibited anti-allergenic and anti-carcinogenic effects within the body.4–6) But, the biological activity in vivo after oral RA administration is dependent on intestinal absorption and subsequent interaction with target tissues. There are some data concerning the absorption, metabolism, distribution, and excretion of RA,7–10) but the absorption characteristics and bioactive compounds in vivo are still unknown.

Recently we found that measuring the competitive effect of dietary substances on the transport of fluorescein in Caco-2 cell monolayers, a substrate of the monocarboxylic acid transporter (MCT), is a useful means of identifying compounds that can be transported by the MCT.11) We found that dietary phenolic acids, such as ferulic and \( p \)-coumaric acids, are transported by MCT-mediated active absorption using this method.12,13) Hitherto, transepithelially passive diffusion, dependent on the partition coefficient (log octanol/water) was thought to be the major mechanism in the permeation process for these compounds.14) Furthermore, we have reported that caffeic acid (CA) and chlorogenic acid (CLA) are mainly absorbed via the paracellular pathway,15) whereas \( m \)-coumaric acid and \( m \)-hydroxyphenylpropionic acid (mHPP), the major colonic metabolites of CA and CLA, are absorbed by the MCT in Caco-2 cells.15,16) This study was designed to identify the absorption mechanism of RA, by directly measuring transepithelial transport across Caco-2 cells as an in vitro model of intestinal absorption and metabolism.17–19)

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1) To whom correspondence should be addressed. Tel: +81-45-788-7588; Fax: +81-45-788-4047; E-mail: konishiy@kirin.co.jp

Abbreviations: HBSS, Hanks' balanced salt solution; TER, transepithelial electrical resistance; MCT, monocarboxylic acid transporter; ECD, electrochemical detector; RA, rosmarinic acid; CA, caffeic acid; CLA, chlorogenic acid; mHPP, \( m \)-hydroxyphenylpropionic acid; Pgp, P-glycoprotein; MRP, multidrug resistance-associated protein; EGCg, epigallocatechin gallate
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 (10,000 units/ml in 0.9% NaCl), streptomycin (10 μg/ml in 0.9% NaCl), phosphate-buffered saline, and Hank’s balanced salt solution (HBSS) were all purchased from Invitrogen (Carlsbad, CA). Type I collagen was purchased from Nitta Gelatin (Osaka, Japan). Plastic dishes, plates, and Transwell inserts with 0.4-μm polycarbonate membranes (12 mm in diameter) were obtained from Corning (Corning, NY). RA was from Sigma-Aldrich (St. Louis, MO). 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 I.U./ml penicillin, and 50 μg/ml streptomycin (pH 7.4) in a humidified atmosphere of 5% CO2 at 37°C. All cells used were between passages 57 and 62.

Transepithelial transport experiments. Cells were grown in Transwells with the semipermeable membrane first coated with type I collagen (12-mm diameter and 0.4-μm pore size, Corning Costar, NY). The cells were seeded at a density of 1 x 105/cm2, and the monolayers were formed after culturing for 2 weeks. The integrity of the cell layer was evaluated by measurement of transepithelial electrical resistance (TER) with Millicell-ERS equipment (Millipore, MA). Monolayers with a TER of more than 250 Ω•cm2 were used in transepithelial transport experiments.

To measure 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 RA (5 mM) was added to the apical side. After incubation for a designated period of time, the basal solution was collected and then replaced with an equal volume of HBSS. The amount of RA transported by Caco-2 cells was estimated using a HPLC-electrochemical detector (ECD) with an ESA coulometric detection system (ESA, Boston, MA). The results were expressed in terms of specific permeability (μl/cm2), which was calculated as the amount of compound transported divided by the initial concentration in the donor compartment.

To examine 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) was added to the basolateral side. Inhibitors of efflux pumps, P-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP), such as MK571 or verapamil, respectively, were added to both sides of the Caco-2 cell monolayers, and the effects on the basolateral-to-apical transport of RA were examined.

Chromatographic conditions. HPLC-ECD fitted with a coulometric detection system was used for analysis, as previously reported. Chromatographic separation was performed on a C18 column (ODS150, MC Medical, Tokyo, Japan). Mobile phase A (Solvent A) was 50 mM sodium acetate containing 5% methanol (pH 3.0), while mobile phase B (Solvent B) was 50 mM sodium acetate containing 40% acetonitrile and 20% methanol (pH 3.5). The elution profile (0.6 ml/min) was as follows: 0–28.5 min, linear gradient from 85% solvent A/15% solvent B to 20% solvent A/80% solvent B; 28.5–31 min, isocratic elution 0% solvent A/100% solvent B; 31–35 min, isocratic elution 85% solvent A/15% solvent B. To measure the amount of RA, eight electrode detector potentials were used from 0 to 700 mV in increments of 100 mV.

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

Correlation between the TER and the permeation of RA. The TER is often used as an approximate indicator of the “tightness” of intercellular junctions, which mainly reflects the development of tight junction and is correlated with paracellular permeation in Caco-2 cells. We prepared Caco-2 cell monolayers with high or low TERs by cytchalasin D treatment (100 μg/ml) and examined the relationship between the TER and the transepithelial flux of RA.

Detection of phenolic acid liberated from RA or methylferulate incubated with Caco-2 cell monolayers. Methyl ferulate (1 mM) or RA (5 mM) was loaded on the apical side, and the formation of ferulate or CA in the apical side was measured after 40 min or 3 h incubation at 37°C (apical pH, 6.0; basolateral pH, 7.4). To measure the amount of methyl ferulate and ferulate with HPLC-ECD, mobile phase A (Solvent A) was 50 mM sodium acetate containing 5% methanol (pH 3.0), while mobile phase B (Solvent B) was 50 mM sodium acetate containing 40% acetonitrile and 40% methanol (pH 3.5). The elution profile and eight electrode detector potentials were the same as those of RA.

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

**Results**

**HPLC analysis of RA transported across Caco-2 cell monolayers**

Representative chromatograms of RA transported into the basolateral solution are presented in Fig. 1. RA was determined at a detection limit < 0.5 pmol on the column, and reproducibility was good without requiring any sample pre-treatment. Purity of the peaks was assessed using the peak area ratio accuracy for the adjacent oxidation channels (lower or upper) to the dominant oxidation channel. The voltammetric response of the analyte across these channels was unique for each compound. Greater than 70% ratio accuracy was considered to support peak purity.21) The retention time (RT) and dominant oxidation potential for RA were 20.3 min and 200 mV respectively.

**Characteristics of transepithelial transport of RA**

Bidirectional permeation of RA (5 mM) across Caco-2 cell monolayers was examined in the presence and absence of an inwardly directed proton gradient (apical pH, 6.0 or 7.4; basolateral pH, 7.4) (Fig. 2). Permeation from the apical to the basolateral side, $J_{\text{ap→bl}}$, in the presence of a proton gradient (0.13 nmol/min/mg protein) was nearly the same as that of $J_{\text{ap→bl}}$, in the absence of a proton gradient (0.15 nmol/min/mg protein). Permeation from the basolateral to the apical side, $J_{\text{bl→ap}}$, in the presence of a proton gradient (0.23 nmol/min/mg protein) was also the same as that of $J_{\text{bl→ap}}$ in the absence of a proton gradient (0.22 nmol/min/mg protein).

**Distribution of RA**

After the transport experiments both in the presence and the absence of a proton gradient (apical pH, 6.0 or 7.4; basolateral pH, 7.4), > 99% of apically-loaded RA was retained on the apical side, suggesting that RA is restricted by the tight junctions (Table 1). Levels of apical, basolateral, and intracellular fractions as between the presence and the absence of a proton gradient were not significantly different.

**Concentration and paracellular permeability dependence of RA transport**

Figure 3A shows the relationship between the initial permeation rate of RA and concentration (apical pH, 6.0 or 7.4; basolateral pH, 7.4), > 99% of apically-loaded RA was retained on the apical side, suggesting that RA is restricted by the tight junctions (Table 1). Levels of apical, basolateral, and intracellular fractions as between the presence and the absence of a proton gradient were not significantly different.

![Chromatograms of RA Transported Across Caco-2 Cell Monolayers](image-url)
As illustrated in Fig. 3B, the transepithelial flux of RA in Caco-2 cell monolayers with a low TER was about 15 times greater than that with a high TER (1.90 nmol/min/mg protein for low TER and 0.13 nmol/min/mg protein for high TER). The flux of RA was inversely correlated with the TER, suggesting that RA permeates across Caco-2 cells via the paracellular pathways.

The effect of inhibitors on the basolateral-to-apical transport of RA

The effects of verapamil, an inhibitor of Pgp-mediated transport, and MK-571, an inhibitor of MRP-mediated transport, on the basolateral-to-apical transport of RA, were examined. Treatment with NaN₃ (10 mM), verapamil (50 μM), or MK-571 (50 μM) failed to inhibit RA transport in the basolateral-to-apical direction. Indeed, a slight increase in the permeation of RA was observed (Table 2).

Release of phenolic acid from RA or methylferulate by differentiated Caco-2 cell monolayers

Formation of ferulate (RT: 14.2 min, dominant oxidation potential: 400 mV) from methyl ferulate (RT: 22.3 min, dominant oxidation potential: 400 mV)
was detected. The level of conversion increased with prolonged incubation, indicating that Caco-2 cells exhibit esterase activity (Fig. 4A). In contrast, CA was not formed from apically loaded RA during a 3 h incubation period with Caco-2 cells (Fig. 4B).

**Discussion**

There are several reports in the literature concerning the absorption and metabolism of RA. Various conjugated forms or metabolic products of RA have been detected in the plasma and urine. Orally administered RA in rats and humans resulted in the formation of conjugated and/or methylated forms, as well as degradation products such as CA, ferulic acid, mHPP, m-coumaric acid, and their sulfated or glucuronide derivatives. A scheme for the metabolism of RA has been proposed in which cleavage of the ester bond and p-dehydroxylation is mediated by gut microflora in the large intestine, and CA and m-coumaric acid formed from RA is then absorbed and metabolized further in the liver or kidney. By topical administration of RA to the skin in rats, RA was absorbed percutaneously and distributed in the blood, skin, bone, and muscle. However, for ingested RA, the first limiting step must be intestinal absorption, although the precise mechanism remains unclear. Recently we found MCT-mediated active absorption of dietary phenolic acids, such as ferulic and p-coumaric acids. The necessary molecular components for an MCT substrate are thought to be a monoanionic carboxyl group and a nonpolar side chain or aromatic hydrophobic moiety. RA, having a monocarboxylic group in the 3,4-dihydroxyphenyllactic acid moiety and an aromatic group, might seem to fulfill the structural criteria for an MCT substrate (Fig. 1), but

**Table 2.** Effects of Various Compounds on RA Efflux Across Caco-2 Cell Monolayers in the Presence of a Proton Gradient

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative permeation (% of control)</th>
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<tbody>
<tr>
<td>NaN1 10 mM</td>
<td>121.5 ± 5.3*</td>
</tr>
<tr>
<td>Verapamil 50 μM</td>
<td>133.0 ± 7.9*</td>
</tr>
<tr>
<td>MK-571 50 μM</td>
<td>124.3 ± 6.2*</td>
</tr>
</tbody>
</table>

The amount of RA transported in the basolateral-to-apical direction was measured at 37°C for 40 min by incubating Caco-2 cells in the presence of each compound at the concentrations indicated (apical pH, 6.0; basolateral pH, 7.4). Each value represents the mean ± SD of three or more experiments. Significantly different from the control value (*P < 0.01).
there is a paucity of experimental data to verify whether RA is recognized and transported by MCT. Hence the mechanism for its intestinal transport was investigated (Fig. 2).

The apical-to-basolateral flux, $J_{ap\rightarrow bl}$, of 5 mM RA (0.13 nmol/min/mg-protein) was much lower than that of 1 mM ferulic acid (9.79 nmol/min/mg-protein), a known substrate of MCT. But, the $J_{ap\rightarrow bl}$ of 5 mM RA was nearly the same as those of 5 mM CLA and gallic acid (0.16 nmol/min/mg-protein). Because gallic acid and CLA are transported via paracellular pathways, it was expected that RA would also be absorbed via paracellular diffusion across Caco-2 cell monolayers. This idea is supported by the fact that RA transport increased linearly with concentration and did not plateau even at 30 mM (Fig. 3A). Moreover, the results of the distribution study (Table 1) and the correlation between TER and permeability (Fig. 3) indicate that RA is restricted by a tight junction, irrespective of the proton gradient. Although CLA fulfills the structural criteria for an MCT substrate, like RA, it has no affinity for MCT and is permeated irrespective of the proton gradient. Therefore, CLA and RA might prevent interaction with the MCT molecule.

Efflux of RA in the basolateral-to-apical direction might have taken place (Fig. 2). Hence we examined the effects of verapamil and MK-571 on RA transport (Table 2). The addition of NaN$_3$ (10 mM), verapamil (50 μM), or MK-571 (50 μM) did not inhibit RA transport in the basolateral-to-apical direction. In fact, a slight increase in the permeation of RA was detected. The reason for this is not clear, but we did observe that these inhibitors caused the TER of Caco-2 cell monolayers to fall. After the experiment, the TER value relative to the initial was 0.48 for NaN$_3$, 0.27 for verapamil, and 0.42 for MK-571. Furthermore, basolaterally loaded RA decreased the TER (apical pH, 6.0; basolateral pH 7.4, relative TER value of 0.63, apical pH, 7.4; basolateral pH 7.4, relative TER value of 0.68), while apically loaded RA somewhat increased the TER (apical pH, 6.0; basolateral pH 7.4, relative TER value of 1.46, apical pH, 7.4; basolateral pH 7.4, relative TER value of 1.15). Thus the greater $J_{bl\rightarrow ap}$ over $J_{ap\rightarrow bl}$ can be ascribed to the increased paracellular permeability of Caco-2 cell monolayers, which also matches the paracellular permeation of RA mentioned above. Taken together, these results suggest that specific efflux systems such as Pgp and MRP are not involved in the permeation of RA.

The contribution to biological effects of the microbial metabolites of ingested polyphenols has been studied. Particular attention has focused on those metabolites that are poorly absorbed in the small intestine, such as catechin, quercetin, and CA. The intestinal absorption of CLA and CA is low, because they are mainly absorbed via paracellular pathways and are restricted by the epithelial tight junction. But CA is absorbed by MCT in part, which can be ascribed to the higher absorption efficiency of CA than of CLA. It has been reported that the majority of CLA is not absorbed in the proximal part of the gut, but reaches the large intestine where it is hydrolyzed to CA and quinic acid by colonic microbial esterases. Previously we reported that CA and quinic acid are metabolized by gut microflora to give m-ferulic acid, 3,4-dihydroxyphenylpropionic acid, and mHPP, which are absorbed by MCT. Since these metabolites still possess a free phenolic group, they might have significant reducing and antioxidant properties and thus protect against oxidative stress. We have found possible routes for the absorption of ingested CLA or CA and have addressed the physiological significance of their colonic metabolites, which are efficiently absorbed and distributed within the body by MCT as bioactive compounds in vivo. It has been reported that esterase activity capable of hydrolyzing dietary hydroxycinnamates and related compounds such as RA and CLA is distributed along the gastrointestinal tract of mammals. Human intestinal Caco-2 cells also have the capacity to metabolize dietary hydroxycinnamates, including phase I (de-esterification) and phase II (glucuronidation, sulfation, and O-methylation). The susceptibility of RA to hydrolysis by a mucosa esterase in Caco-2 cells was examined. RA was not formed from RA during an incubation period of up to 3 h with Caco-2 cells, but ferulate was liberated from methyl ferulate in a time-dependent manner, which indicates that the Caco-2 cells exhibit esterase activity (Fig. 4). This suggests that RA is susceptible to hydrolysis by an esterase of gut microflora, rather than an esterase of the mucosa.

The results obtained in this study, together with our previous work, could give new insight into the absorption characteristics of RA with their colonic microbial metabolites (Fig. 5). Absorption of intact RA is restricted by the tight junction. CA, liberated from RA by colonic microbial esterase, is not only absorbed via paracellular diffusion, but is also actively absorbed by MCT, although to a lesser extent. Furthermore, CA is metabolized to form mHPP and m-ferulic acid, which are also actively absorbed by MCT. Until now, the biological activity of mHPP or m-ferulic acid has scarcely been reported, because research concerning the physiological function of food constituents has focused on the biologically active constituents of the food itself. mHPP and m-ferulic acid are generated by colonic microflora from ingested food materials, especially poorly absorbed. Hence we proposed to call them “metabo-nutrients”, since this nomenclature distinguishes them from classical nutrients, such as sugars, lipids, amino acids, minerals, and vitamins. In the future the metabo-nutrients should also be investigated for health benefits. The putative mechanism of absorption and metabolism of RA in the gut exemplifies the physiological significance of MCT-mediated absorption and distribution in relation to the metabo-nutrients in vivo.
Fig. 5. Proposed Pathways for the Absorption of Ingested RA.

In rats administered RA (50 mg or 138.7 μmol/kg), recovery of RA and methyl-RA from the urine was 0.44% and 1.60% respectively, which indicates low efficiency of absorption. Similar results were obtained using human subjects. These results are consistent with the proposed scheme involving paracellularly passive absorption. We found previously that the absorption efficiency of epigallocatechin gallate (EGCG) is low in Caco-2 cells as well as in animals and humans, because it is absorbed via the paracellular pathways. The proposed scheme for the absorption of RA resembles that of EGCG. But, RA was present mainly as conjugated RA and methyl-RA in the femoral artery. Nevertheless, it is not yet known whether conjugated RA is also present in the portal vein. It is possible that intact RA, which is paracellularly absorbed, becomes conjugated in the liver. Furthermore, the discrepancy between the in vitro Caco-2 cell system and in vivo studies might originate from differences in the evaluation methods employed. Indeed, conjugated gallic acid was detected in the portal vein of rats after oral administration, although gallic acid was paracellularly absorbed. No conjugated form of gallic acid was detected in Caco-2 cells. Further studies to investigate in vivo intestinal absorption characteristics and bioavailability are required.

Over the past decade, there has been enormous research interest in the potential health benefits of dietary flavonoids in humans. Many studies have focused on the absorption and metabolism of flavonoids to examine their bioactive compounds in vivo and the mechanism by which they might exert their physiological effects. In comparison with flavonoids, however, phenolic acids have not been extensively studied, and are not currently considered to be of great nutritional interest in spite of their wide distribution in grains, vegetables, and fruits. The relative oral bioavailability of p-coumaric acid to gallic acid is about 70 in rats after oral administration. This finding illustrates the high absorption efficiency of MCT-mediated transport in vivo. Thus phenolic acids, which are rapidly absorbed and distributed intact within the body, are now receiving considerable attention. The absorption characteristics of dietary functional phenolic compounds that are available on an industrial scale, such as CLA and RA, have been investigated. The absorption efficiencies of these compounds was low due to a lack of affinity for MCT. Both CLA and RA are absorbed via paracellular diffusion. To evaluate the impact of dietary RA on human health, further identification and characterization of bioactive compounds in vivo of ingested RA are required.

Acknowledgment

We thank Mrs. K. Hagiwara for her support during this work.

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