Stereoselective and Carrier-Mediated Transport of Monocarboxylic Acids Across Caco-2 Cells

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Purpose. To characterize the transport mechanism of monocarboxylic acids across intestinal epithelial cells by examining the stereoselectivity of the transcellular transport of several chiral monocarboxylic acids.
Methods. The transport of monocarboxylic acids was examined using monolayers of human adenocarcinoma cell line, Caco-2 cells.
Results. The permeability of L-[^{14}C]lactic acid at a tracer concentration (1 μM) exhibited pH- and concentration-dependencies and was significantly greater than that of the D-isomer. The permeabilities of both L-/D-[^{14}C]lactic acids involve saturable and nonsaturable processes; the saturable process showed a higher affinity and a lower capacity for L-lactic acid compared with the D-isomer, while no difference between the isomers was seen for the nonsaturable process. The transport of L-lactic acid was inhibited by chiral monocarboxylic acids such as (*R*)/(*S*)-mandelic acids and (*R*)/(*S*)-ibuprofen in a stereoselective manner. Mutually competitive inhibition was observed between L-lactic acid and (*S*)-mandelic acid.
Conclusions. Some chiral monocarboxylic acids are transported across the intestinal epithelial cells in a stereoselective manner by the specific carrier-mediated transport mechanism.

KEY WORDS: carrier-mediated transport; lactic acid; stereoselectivity; Caco-2 cells; monocarboxylic acid.

INTRODUCTION

Stereoselectivities have been frequently reported in pharmacokinetic, pharmacodynamic and toxicologic studies with chiral drugs, for example in drug-receptor interaction, drug metabolism and serum protein binding (1,2). However, little is known about the stereoselectivity in intestinal absorption.

Recently we demonstrated that intestinal membrane transport of monocarboxylic acids occurs via two carrier-mediated processes that are energized by a proton gradient and/or a bicarbonate gradient, using isolated intestinal brush-border membrane vesicles (BBMVs) and human adenocarcinoma cell line, Caco-2 (3–7). Similar transport mechanisms of propionate (8) and lactate (9) have been also reported. We have emphasized that such pH-dependent and carrier-mediated transport may predominate over passive diffusion according to pH-partition theory (10) in the intestinal absorption of monocarboxylic acids. Since it is possible that the transporter(s) may discriminate the chirality of drugs, observation of differences in the permeability of enantiomers would provide further evidence of the physiological importance of a carrier-mediated transport.

The purpose of the present study is to characterize the transport of monocarboxylic acids across intestinal epithelial cells by examining stereoselectivity in the transport of chiral monocarboxylic acids using monolayers of Caco-2 cells. We chose L-/D-lactic acids for the present study, since firstly, they have stereoselective inhibitory effects on the intestinal brush-border membrane transport of monocarboxylic acids (3,4), secondly, they are hardly metabolized or racemized during the transport process, and thirdly, the radio-labeled compounds are commercially available.

MATERIALS AND METHODS

Materials

The Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). Polycarbonate membrane Transwell clusters, 11.2 mm in diameter and 3.0 μm pore size, were purchased from Costar (Bedford, MA). L-[^{14}C]Lactic acid (5.55 GBq/mmol) and D-[^{14}C]lactic acid (2.04 GBq/mmol), and [^{3}H]mannitol (1110 GBq/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO), and New England Nuclear (Boston, MA), respectively. Unlabeled L-/D-lactic acids were obtained from Sigma Chemicals (St. Louis, MO), and (*S*)- and (*R*)-ibuprofen from Aldrich Chemical Co. (Milwaukee, WI) and Research Biochemicals Inc. (Natick, MA), respectively. All other chemicals were of reagent grade and commercially available.

Cell Culture and Transport Experiments

The cultivation of Caco-2 cells was performed as described previously (5). All cells used in this study were between passages 33 and 59. A typical transport experiment was as follows. The cells grown on the polycarbonate membrane were washed twice with Hanks' balanced salt solution without sodium ions (sodium-free HBSS; 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM choline Cl, 0.385 mM K_2HPO_4, 30 mM D-glucose and 10 mM HEPES for pH 7.3 or 10 mM MES for pH 6.0 and the osmolarity was 315 mOsm/kg). To initiate transport, sodium-free HBSS (pH 7.3, 37°C) was put on the basolateral side (receiver side) and test solution (pH 6.0 or 7.3, 37°C) containing a radio-labeled or non-labeled substrate was loaded on the apical side (donor side) of a cell insert. At designated times, solution was removed from the receiver side. For the quantification of the drugs in the cells, cells were solubilized with 0.5 ml of Solvable (NEN, Boston, MA). The amount of radio-labeled or non-labeled compound transported or taken up by cells was estimated by radioactivity measurement or HPLC assay and expressed as permeability (μl/mg protein) obtained by dividing the transported amount by the initial concentration in the donor compartment and correcting for the cellular protein amount. Each result represents the mean ± S.E.M. of three experiments using the same cultivation

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ABBREVIATIONS: BBMVs, brush-border membrane vesicles; HBSS, Hank's balanced salt solution; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.

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of Caco-2 cells. Radioactivity was determined by a liquid scintillation counter (LSC-1,000, Aloka Co. Ltd., Tokyo, Japan). The HPLC systems for (*S*)/(*R*)-mandelic acid and (*S*)/(*R*)-ibuprofen were as follows: the analytical column was Inertsil ODS-2, 4.6 id. × 250 mm (GL Science Co. Ltd., Tokyo), and the mobile phase was a mixture of 10 mM acetate buffer (pH 6.0) and methanol (80/20) for mandelic acids, and a mixture of 10 mM acetate buffer (pH 5.0) and acetonitrile (50/50) for ibuprofen, at a flow rate of 1.0 ml/min and at 40°C. Mandelic acids and ibuprofen were detected by at 220 nm and 229 nm, respectively. Cellular protein was measured by the method of Lowry *et al.* (11) with bovine serum albumin as a standard.

Assessment of Chiral Inversion and Metabolism of Lactic Acids

The chiral inversion and metabolism of L-/D-[^{14}C]lactic acids by Caco-2 cells were assessed by analyzing the radioactivity in the receiver side after each transport experiment with enantioselective HPLC techniques. Intact and metabolized L-/D-[^{14}C]lactic acids were analyzed by HPLC with a chiral column (Chiral pak MA(+) 4.6 id. × 50 mm, Daicel Chemical Industries, Tokyo) and quantified by measuring radioactivity in each fraction of eluted solution. The mobile phase used was 2 mM $CuSO_4$ aq. at a flow rate of 0.5 ml/min at room temperature.

Data Analysis

The permeability coefficient (μl/min/mg protein) was evaluated from the slope of the initial linear portion of permeability versus time curves by linear regression analysis. To estimate the kinetic parameters for the saturable transport, the transport rate (J) was fitted to the following equation, consisting of both saturable and the nonsaturable-linear terms, by using the nonlinear least-square regression analysis program, MULTI (12).

$$J = J_{max} \times S/(K_t + S) + k_d \times S \quad (1)$$

where J_{max} is the maximum transport rate for the carrier-mediated process, S is the concentration of substrate, K_t is the half-saturation concentration, and k_d is the first-order rate constant. Statistical analysis was performed by using Student's two-tailed t test.

RESULTS

pH and Sodium Ion Dependence of L-/D-Lactic Acid Transport

As shown in Fig. 1, the permeability of L-[^{14}C]lactic acid increased linearly with time after an initial lag period of a few minutes. Under the sodium-free condition, the transcellular transport of L-[^{14}C]lactic acid was examined at the apical pH of 6.0 or 7.3, while the pH on the basolateral side was kept at 7.3, which is close to the intracellular pH of Caco-2 cells (5). The permeability coefficient of L-[^{14}C]lactic acid at the apical pH of 6.0, 1.25 ± 0.004 μl/min/mg protein, was significantly higher than that observed at the apical pH of 7.3 (0.699 ± 0.029 μl/min/mg protein). Sodium ions did not affect the permeability coefficient (1.26 ± 0.019 μl/min/mg protein). Accordingly, in the following experiments, transport studies were performed at apical and basolateral pH values of 6.0 and 7.3, respectively, and in the absence of sodium ions to exclude the possible minor effects of sodium ions as observed in the membrane vesicle study (9). Figure 1 also shows time-courses of the transcellular transport of D-[^{14}C]lactic acids (1 μM) from the apical to the basolateral side. The permeability coefficient of D-[^{14}C]lactic acid, 0.626 ± 0.078 μl/min/mg protein, was 50% of that of the L-isomer. Since the permeability coefficient of L-/D-[^{14}C]lactic acids were significantly higher than that of [^{3}H]mannitol (0.087 ± 0.003 μl/min/mg protein), which represents the paracellular permeability, L-/D-lactic acids transport occurred mainly by transcellular permeation rather than through the paracellular pathway.

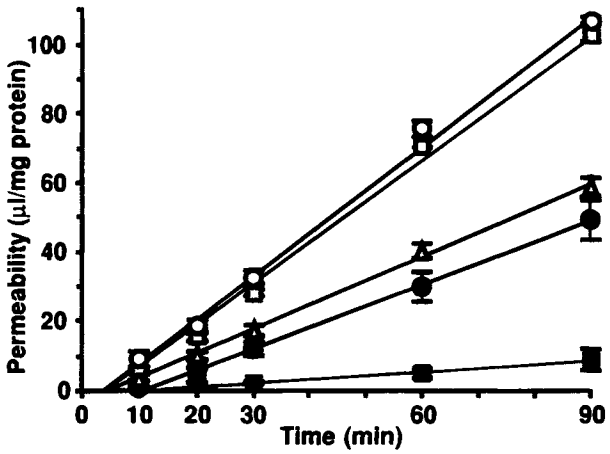

Fig. 1. Time courses for of L-/D-[^{14}C]lactic acids and [^{3}H]mannitol transport across Caco-2 cell monolayers. Permeability of L-[^{14}C]lactic acid (1 μM) was measured at 37°C by incubating Caco-2 monolayers in sodium-free HBSS buffer at an apical pH of 6.0 (○) or 7.3 (△) with a constant basolateral pH 7.3, respectively, containing 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM choline Cl, 0.385 mM K_2HPO_4, 25 mM D-glucose and 10 mM Hepes for pH 7.3 or 10 mM Mes for pH 6.0. When sodium-containing HBSS buffer at an apical pH of 6.0 (□) and a basolateral pH of 7.3 was used, NaCl and Na_2HPO_4 were contained instead of choline Cl and K_2HPO_4. Permeability of D-[^{14}C]lactic acid (1 μM; ●) and [^{3}H]mannitol (33 nM; ■) was measured at 37°C by incubating Caco-2 monolayers in sodium-free HBSS buffer at an apical side pH of 6.0 and a basolateral side pH of 7.3. An aliquot was withdrawn from the basolateral side at the times indicated. Each point represents the mean ± S.E.M. of three experiments.

Concentration Dependence

Figure 2 shows the relationship between the initial transport rate of L-/D-[^{14}C]lactic acids and their concentrations in the medium from 1 μM to 40 mM. The results indicate that the permeation rate of both isomers consists of a saturable process at lower concentrations and a nonsaturable process that is observed at higher concentrations. At low concentration (tracer level, 1 μM), the permeability of the L-isomer across the Caco-2 cell monolayer was greater than that of the D-isomer, while the isomers showed similar behavior at an intermediate concentration (1 mM), and at a higher concentration, the D-isomer exhibited greater permeability than the L-isomer. Kinetic analysis of the concentration-dependent permeation of L-/D-

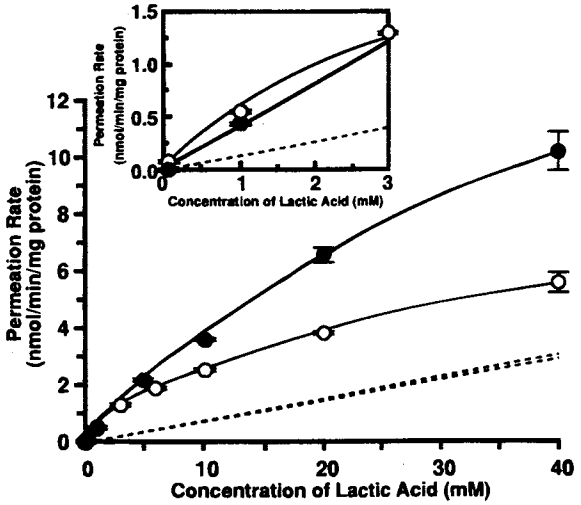

Fig. 2. Concentration dependence of L-(○)/D-(●) lactic acids transport across Caco-2 cell monolayers. The incubation conditions were identical to those described in the legend to Fig. 1. The broken line represents the transport rate for the nonsaturable component calculated from the kinetic parameters obtained as mentioned in the results. Each point represents the mean ± S.E.M. of three experiments.

lactic acids gave J_{max}, K_t and k_d values of 2.21 ± 0.22 nmol/min/mg protein, 3.75 ± 0.40 mM and 0.09 ± 0.01 μl/min/mg protein (mean ± S.D.) for L-lactic acid, and 8.44 ± 2.67 nmol/min/mg protein, 23.9 ± 6.41 mM and 0.12 ± 0.03 μl/min/mg protein for the D-isomer, respectively. The saturable process for L-lactic acid showed higher affinity and lower capacity compared with that for the D-isomer, although the nonsaturable process was comparable between isomers.

Chiral Inversion and Metabolism Test

Figure 3 shows the radio-HPLC pattern of the basolateral side solution of Caco-2 cells at the end of a experiment with L-/D-[^{14}C]lactic acids at the concentration of 1 μM. The radioactivity was recovered completely from the cells, and the apical and basolateral solutions at the end of each experiment, 90 min after initiation. Retention times of L-/D-[^{14}C]lactic acids were about 9.0 and 12.5 minutes, respectively, according to the manufacturer of the column, and authentic L-/D-isomers were clearly separated. No peak other than those of the intact materials was observed, suggesting that chiral inversion and metabolism did not occur during L-/D-lactic acids transport.

Structural Specificity of the Transporter

As shown in Table 1, the permeabilities of L-/D-[^{14}C]lactic acids were reduced by L-/D-lactic acids and (*S*)/(*R*)-mandelic acids in a stereoselective manner. Permeability of L-[^{14}C]lactic acid (1 μM) in the presence of unlabeled L-/D-lactic acids amounted to 37.3% and 76.7% of the control value, respectively. A stereoselective inhibitory effect of (*S*)/(*R*)-ibuprofen at 5 mM on L-[^{14}C]lactic acid transport was also observed. In addition, L-[^{14}C]lactic acid transport was significantly reduced in the presence of benzoic acid which is transported by a carrier-mediated transport mechanism in Caco-2 cells (5).

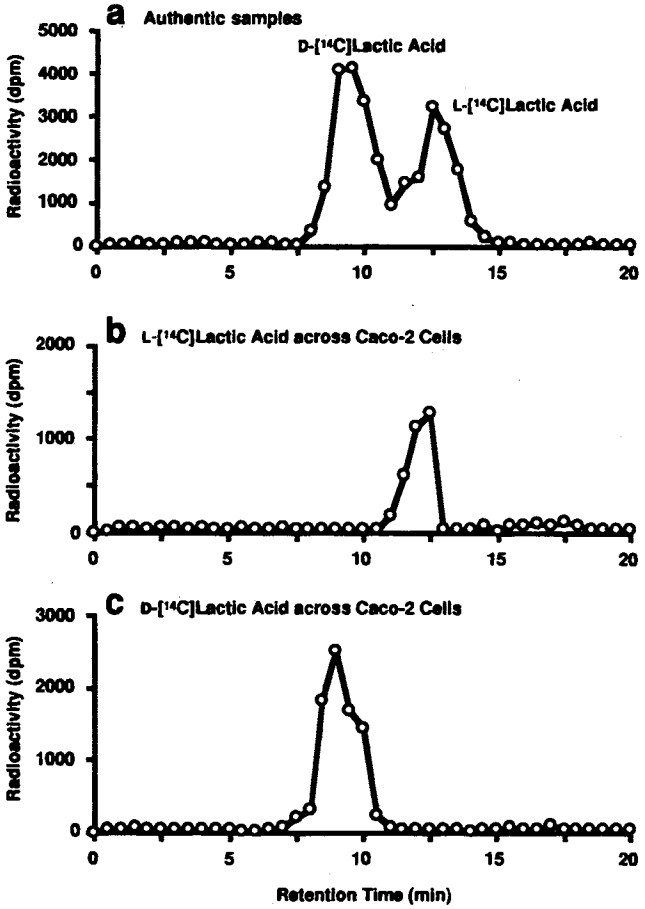

Fig. 3. Radio-HPLC patterns of L-/D-[^{14}C]lactic acids transported across Caco-2 cell monolayers. (a), (b), and (c) represent the results obtained with authentic L-/D-[^{14}C]lactic acids, L-[^{14}C]lactic acid, and D-[^{14}C]lactic acid transported across Caco-2 cell monoplayers from the apical to the basolateral side, respectively.

Transport of (*S*)/(*R*)-Mandelic Acids and (*S*)/(*R*)-Ibuprofen

Figure 4 shows a permeability for the apical to basolateral transport of (*S*)/(*R*)-mandelic acids (1 mM) and (*S*)/(*R*)-ibuprofen (1 mM). The permeability coefficient of (*S*)-mandelic acid (0.144 ± 0.006 μl/min/mg protein) was significantly higher than that of the (*R*)-isomer (0.106 ± 0.003 μl/min/mg protein). Permeability of (*S*)-mandelic acid from 1 mM to 20 mM was saturable (data not shown) and the evaluated kinetic parameters were: J_{max} of 3.56 ± 1.19 nmol/min/mg protein, K_t of 9.51 ± 4.28 mM, and k_d of 0.10 ± 0.02 μl/min/mg protein (mean ± S.D.). The permeability coefficients of (*S*) and (*R*)-ibuprofen were 10.07 ± 0.59 μl/min/mg protein and 9.57 ± 0.81 μl/min/mg protein, respectively, with no significant difference between the isomers.

Kinetic Analysis of Inhibitory Effect

To study the mechanism of lactic acid transport inhibition by monocarboxylic acids, the mutual inhibitory effects between L-lactic acid and (*S*)-mandelic acid were kinetically analyzed. Figure 5a shows Lineweaver-Burk plots for the effect of (*S*)-mandelic acid on L-lactic acid permeation rate after subtraction

Table I. Inhibitory Effect of Various Compounds on L/D-[^{14}C]Lactic Acid Transport

Inhibitor	Concentration (mM)	Relative Transport Rate (% of Control)[a] L-[^{14}C]Lactic Acid	D-[^{14}C]Lactic Acid
L-Lactic acid	10	37.3 ± 3.11[b]	42.6 ± 0.86[b]
D-Lactic acid	10	76.7 ± 1.27[b]	73.8 ± 2.93[b]
(*S*)-Mandelic acid	10	35.7 ± 1.07[b]	45.1 ± 0.55[b]
(*R*)-Mandelic acid	10	78.7 ± 0.62[b]	80.5 ± 1.23[b]
(*S*)-Ibuprofen	1	70.6 ± 1.94[b]	
	5	24.0 ± 1.95[b]	
(*R*)-Ibuprofen	1	73.3 ± 4.14[b]	
	5	35.2 ± 2.86[b]	
Benzoic acid	10	21.7 ± 1.76[b]	25.7 ± 1.66[b]

Note: Transport of L-/D-[^{14}C]lactic acid was measured at 37°C for 90 min by incubating Caco-2 cells in sodium-free HBSS buffer (apical pH 6.0, basolateral pH 7.3) in the presence of each inhibitor. The other incubation conditions were the same as described in the legend to Fig. 1.

[a] Each value represents the mean ± S.E.M. of three experiments and is expressed as percentage of the control.

[b] Significantly different from the control value by Student's *t* test ($p < 0.05$).

of the non-saturable component. Figure 5b shows the effect of L-lactic acid on the permeation rate of (*S*)-mandelic acid. Mutually competitive inhibitions were observed and the evaluated inhibition constants, K_i, of L-lactic acid and (*S*)-mandelic acid were 4.10 and 8.58 mM, respectively.

DISCUSSION

In the present study, stereoselective transcellular transport of several chiral monocarboxylic acids were examined using monolayers of Caco-2 cells in order to characterize the transport mechanism of monocarboxylic acids across intestinal epithelial cells.

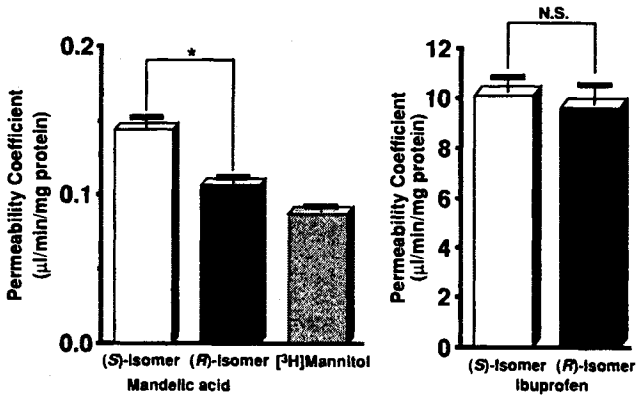

Fig. 4. Permeability coefficient for the transcellular transport of (*S*)/(*R*)-mandelic acids(1 mM), [^{3}H]mannitol(33 nM) and (*S*)/(*R*)-ibuprofen(1 mM) from apical to basolateral sides across Caco-2 monolayer cells. The incubation conditions were identical to those described in the legend to Fig. 1. Each point represents the mean ± S.E.M. of three experiments. *Significantly different between isomers by Student's *t* test ($p < 0.05$).

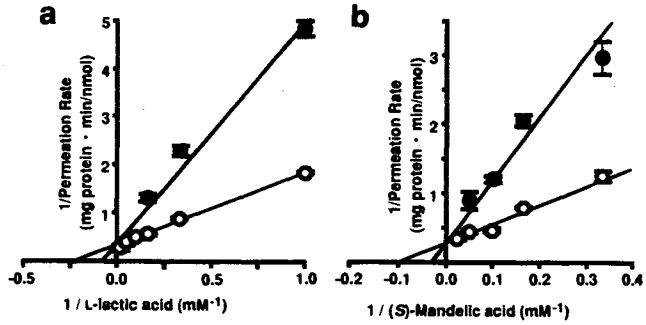

Fig. 5. Lineweaver-Burk plots for the transport of L-lactic acid and (*S*)-mandelic acid across Caco-2 cell monolayers. (a): The transport was measured in the absence (○) or presence of 5.7 mM (*S*)-mandelic acid (●). (b): The transport was measured in the absence (○) or presence of 10 mM L-lactic acid (●). The incubation conditions were identical to those described in the legend to Fig. 1. Each point represents the mean ± S.E.M. of three experiments.

The permeation rate of L-[^{14}C]lactic acid at the apical pH of 6.0 was 1.8 times faster than that observed in the absence of a pH gradient (pH 7.3), whereas sodium ions caused no change. Accordingly, the transport of L-[^{14}C]lactic acid across Caco-2 cell monolayers is Na^+-independent, but pH-dependent. Tiruppathi *et al.* (9) reported that lactate transport in rabbit intestinal brush-border membrane was mainly energized by a proton gradient, and less significant by a sodium gradient. Therefore, the transporter responsible for lactic acid in Caco-2 cells may be the same transporter characterized by them. Kinetic analysis of the concentration-dependence revealed a higher affinity and a lower capacity for L-lactic acid compared with the D-isomer, although the nonsaturable process was similar for the L-/D-isomers. The results suggest that, at a low concentration, L-lactic acid is more efficiently transported, while the D-isomer exhibits a higher transport rate than the L-isomer at a high concentration. Accordingly, stereoselective transport may not be observed at an intermediate concentration, around 1 mM. Although when two compounds shares the same transporter, maximum transport rates should be comparable in a usual enzyme kinetics, the difference of J_{max} between L- and D-isomers may be accounted for by the difference of the permeability across the cell membrane between the isomers after the binding to the common transporter. Consistent with their observations, the L-[^{14}C]lactic acid transport was inhibited by 10 mM L-/D-lactic acids in a stereoselective manner. Stereoselectivity of lactic acid transport was also consistent with our previous finding of differential inhibitory effects between L-/D-lactic acids on intestinal membrane transport of monocarboxylic acids such as nicotinic acid and acetic acid by rabbit and rat jejunal BBMVs and Caco-2 cells (3,4). All of these results suggest that the L-isomer has a higher affinity for the transporter than the D-isomer. Since the remaining radioactivity in Caco-2 cells at the end of experiment (90 min) was very low (less than 0.6% of loaded radioactivity on the apical side), it is suggested that the rate limited process is in the apical membrane transport, although the effect of the basolateral membrane permeation on the net transcellular transport process cannot be neglected. L-[^{14}C]lactic acid transport was reduced more markedly by (*S*)-mandelic acid than by the (*R*)-isomer. Since the inhibitory effects of (*S*)/(*R*)-mandelic acids were comparable

with those of L-/D-lactic acids at the same concentration, mandelic acid and lactic acid seem to be similar in affinity for the transporter. Moreover, permeability of (*S*)-mandelic acid was significantly greater than that of the (*R*)-isomer, as expected. Lactic acid has a methyl group instead of the phenyl group of mandelic acid, but these compounds have a common structure with hydroxyl and carboxyl groups at the α-carbon chiral center. The chirality of L-lactic acid and (*S*)-mandelic acid might be a more important factor than the substituent group in determining the affinity for the monocarboxylic acid transporter. L-[^{14}C]lactic acid transport was also reduced by (*S*)-ibuprofen more than by the (*R*)-isomer. These stereoselective inhibitory potencies suggest that mandelic acid and ibuprofen are also transported by a specific carrier-mediated transport mechanism across Caco-2 cells. Although the permeability of (*S*) and (*R*)-ibuprofen had no significant difference between the isomers at 1 mM, the differential permeability between isomers could be observed at an appropriate concentration of them.

Mutual competitive inhibition between L-lactic acid and (*S*)-mandelic acid indicates that L-lactic acid and (*S*)-mandelic acid share a common transport system in intestinal epithelial cells. Previously, we examined the inhibitory effect of various monocarboxylic acids such as acetic acid, lactic acid, nicotinic acid, propionic acid and benzolic acid on the transport of monocarboxylic acid (3–7), and demonstrated mutual competitive inhibitions among these monocarboxylic acids. Accordingly, this transporter is supposed to be specific for certain organic anions having one carboxylic acid moiety, and all the compounds tested might be transported by a common transporter at least partially to the lactic acid transport system. Recently, we demonstrated a functional presence of a monocarboxylate transporter, MCT1, for lactic acid and pyruvic acid in rat intestine (13,14), suggesting that MCT1 may play a role in the stereoselective transport observed in the present study. However, since it was demonstrated that MCT1 localizes at the basolateral membrane of cecum in Chinese hamster (15), further studies such as cellular distribution of MCT1 in Caco-2 cells would be required to ascribe the observed transport phenomena to the function of MCT1.

In conclusion, we have clarified that stereoselective transcellular transport of L-/D-lactic acid across a Caco-2 monolayer is performed by the pH-dependent transporter specific for monocarboxylic acids. Furthermore, several monocarboxylic acids were demonstrated to share a common transporter with lactic acid. Therefore, intestinal absorption of many acidic drugs may be accounted for by the specific pH-dependent transporter, rather than by passive diffusion according to pH-partition theory.

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