Transepithelial Transport of the Bioactive Tripeptide, Val-Pro-Pro, in Human Intestinal Caco-2 Cell Monolayers

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Some of the food-derived tripeptides with angiotensin converting enzyme (ACE)-inhibitory activity have been reported to be hypotensive after being orally administered. The mechanism for the intestinal transport of these tripeptides was studied by using monolayer-cultured human intestinal Caco-2 cells which express many enterocyte-like functions including the peptide transporter(PepT1)-mediated transport system. Val-Pro-Pro, an ACE-inhibitory peptide from fermented milk, was used as a model tripeptide. A significant amount of intact Val-Pro-Pro was transported across the Caco-2 cell monolayer. This transport was hardly inhibited by a competitive substrate for PepT1. Since no intact Val-Pro-Pro was detected in the cells, Val-Pro-Pro apically taken by Caco-2 cells via PepT1 was likely to have been quickly hydrolyzed by intracellular peptidases, producing free Val and Pro. These findings suggest that PepT1-mediated transport was not involved in the transepithelial transport of intact Val-Pro-Pro. Paracellular diffusion is suggested to have been the main mechanism for the transport of intact Val-Pro-Pro across the Caco-2 cell monolayer.

Key words: tripeptide; bioactive peptide; peptide transporter; paracellular pathway; Caco-2 cell

A number of physiologically active peptides have been discovered in the hydrolyzates of various food proteins. Those peptides include opiate, hypotensive, mineral absorption stimulatory, and immunomodulatory types. Among them, hypotensive peptides, particularly angiotensin-converting enzyme (ACE)-inhibitory peptides, have been paid considerable attention, because they are thought to be promising ingredients of functional foods that would be helpful for people with mild hypertension. Although a variety of food-derived peptides with ACE-inhibitory activity have been found by many investigators with an in vitro assay, ACE-inhibitory peptides which show a real antihypertensive effect in vivo are limited. Fujita et al. have demonstrated that many of the reported peptides were mere substrates of ACE and showed apparent inhibitory activity by competing with a synthetic substrate that was used for the enzyme assay. However, some of the reported peptides definitely had real inhibitory activity against ACE, showing antihypertensive effects when intravenously injected or even orally administered. Several di- and tripeptides, for example the ones having Pro or Trp as the C-terminal amino acid, have been reported to be real inhibitors and showed antihypertensive activity in vivo. Ile-Lys-Pro from bonito muscle, and Ile-Lys-Trp from chicken muscle, and Ile-Pro and Val-Pro-Pro from fermented milk are examples of these real inhibitors. These tripeptides have been reported to lower the blood pressure in spontaneously hypertensive rats (SHR) by oral administration.

In order to express their activity in vivo, these peptides must be absorbed across the intestinal epithelium. However, the mechanism for transepithelial oligopeptide transport in the intestinal tract is not yet fully understood. Pappenheimer et al. have demonstrated that octapeptides could be paracellularly absorbed across the intestinal epithelium. Adsor et al., using D-Phe-Gly, D-Phe2-Gly and D-Phe3-Gly, concluded that the flux of these peptides was predominantly paracellular. On the other hand, we have previously observed that a transcytotic process may be involved in the transepithelial transport of hydrophobic nonapeptides, while the paracellular route could be the major pathway for tetrapeptides. A similar transcytotic mechanism for basic oligopeptides has been reported by Sai et al. In addition to these absorption routes, the intestinal epithelium has another route specific for di- and tripeptides; this is a peptide transport system via the

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proton-dependent transporter, PepT1.\textsuperscript{16,17} PepT1 transports di- and tripeptides very efficiently, and is also known to be responsible for the transepithelial transport of such antibiotics as β-lactam compounds.\textsuperscript{18} It may be possible for the antihypertensive tripeptides to be absorbed transcellularly, at least partly, via this peptide transporter. However, the contribution of the transporter-mediated transport system to the transepithelial transport of intact tripeptides is still obscure.

We have investigated in this study the mechanism for the transepithelial transport of an antihypertensive tripeptide by using the human intestinal Caco-2 cell monolayer\textsuperscript{19} as a model of the intestinal epithelium. Val-Pro-Pro, which has already been used as an ingredient of a commercial fermented milk product with prospective hypotensive activity, was used as a model tripeptide.

Materials and Methods

Materials for cell culture. The human colon adenocarcinoma cell line, Caco-2, was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmacies (Tokyo, Japan). Fetal calf serum (FCS), phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were purchased from Gibco (Gaithersburg, MD, U.S.A.), and non-essential amino acids (NEAA) were from Cosmo Bio (Tokyo, Japan). Penicillin-streptomycin (10,000 units/ml and 10 mg/ml in 0.9% sodium chloride, respectively), araphamene, Gly-Pro, Wortmannin and phenylarsine oxide were all purchased from Sigma (St. Louis, MO, USA). Collagen type I was purchased from Nitta Gelatin (Osaka, Japan), N-(2-hydroxyethyl) piperazine-N'2-ethanesulfonic acid (HEPES) was from Nacalai Tesque (Kyoto, Japan), and 2-morpholinoethanesulfonic acid (MES) was from Merck (Darmstadt, Germany). Val-Pro-Pro chemically synthesized and chromatographically purified was obtained from Sawady Technology Co. (Tokyo, Japan). All other chemicals were of guaranteed grade. Plastic dishes and plates were from Corning (NY, USA), and a Transwell insert with a 0.40-μm polycarbonate membrane of 12 mm in diameter was purchased from Costar (Bedford, MA, U.S.A.).

Cell culture. Caco-2 cells were cultured in DMEM supplemented with 10% FCS, 1% NEAA, 2% glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin, together with an appropriate amount of sodium bicarbonate. They were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. The monolayer became confluent 3 to 4 days after seeding at \(7 \times 10^6\) cells per 60-mm dish, and the cells were passaged at a split ratio of 4 to 8 by trypsinizing with 0.1% trypsin and 0.02% EDTA in PBS. All the cells used in this study were between passages 40 and 70.

Cells for the transport study were grown in a Transwell insert with a membrane coated with type I collagen. The collagen coating was performed as described by Hashimoto and Shimizu.\textsuperscript{20} The cells were seeded at a density of \(4 \times 10^5\) cells/cm², the medium being changed every other day. The cell layers were used for the experiments after culturing for 14 days. The integrity of the cell layer was evaluated beforehand by measuring the transepithelial electrical resistance (TER).

Transport studies. The cell monolayers grown in the Transwell insert (12 mm in diameter) were gently rinsed with HBSS, before 500 μl of HBSS (pH adjusted to 6.0 with MES) was added to the apical side of the Transwell insert, and the insert placed in a well containing 1 ml of HBSS (pH adjusted to 7.4 with HEPES). After incubating for 30 min at a constant temperature of 37°C, HBSS on the apical side of the cell monolayer was replaced by the same volume (500 μl) of a peptide solution. After further incubating for an appropriate time, the apical and basolateral solutions were taken, and the peptide concentration in each was determined by high-performance liquid chromatography (HPLC).

High-performance liquid chromatography (HPLC). An LC-6A HPLC system (Shimadzu, Kyoto, Japan) equipped with a YMC-Pack ODS-AQ312 column (6 × 150 mm; YMC, Kyoto, Japan) and a UV detector at 210 nm were used. A sample solution was applied to the column that had been equilibrated with 0.09% (v/v) trifluoroacetic acid and was eluted with a linear gradient of acetonitrile at a flow rate of 1.0 ml/min. Val-Pro-Pro was determined by measuring the peak area.

Free amino acid analysis. Caco-2 cell monolayers after the transport experiments were washed with ice-cold PBS(-) and then trypsinized with 0.1% trypsin to suspend the cells. The cells were washed three times with ice-cold PBS(-) and then homogenized by ultrasonication. The homogenate was centrifuged at 15,000 rpm for 30 min. The amino acid content of the supernatant was measured with an L-8500 amino acid analyzer (Hitachi, Japan). Free amino acids in the apical and basolateral chambers were measured by directly applying the apical and basolateral solutions to the amino acid analysis.

Statistical analyses. Data were subjected to an analysis of variance and means separation by Scheffé's tests. Statistical studies were performed with Statgraphic software (Statistical Graphic Co. and Graphic Software Systems Inc., Rockville, MD, U.S.A.).
Results

Transepithelial flux of Val-Pro-Pro across the Caco-2 cell monolayer

Culturing on a semipermeable filter for longer than 5 days resulted in our Caco-2 cells forming monolayers with a transepithelial electrical resistance (TER) value of 100-150 ohm × cm², indicating that an integrated epithelial cell monolayer had been successfully formed. Since the PepT1 activity in Caco-2 cells was highest after culturing the cell layer for 2 weeks (data not shown), the subsequent experiments were carried out by using cell layers cultured for 14-15 days.

The transepithelial transport rate (apical-to-basolateral flux) for intact Val-Pro-Pro was measured. Val-Pro-Pro was added to the apical chamber at a final concentration of 1.0 mM. After incubating at 37°C for an appropriate time, the solution in the basal chamber was taken, and the amount of intact peptide appearing in the basal solution was determined by HPLC. Figure 1 shows the amount of Val-Pro-Pro transepithially transported to the basolateral chamber. The amount linearly increased up to 90 min. The transepithelial flux of Val-Pro-Pro was therefore evaluated in the following experiments by measuring the basolateral concentration of Val-Pro-Pro after 60 min of incubation. Less than 2% of Val-Pro-Pro added to the apical chamber was observed to be transported to the basolateral side in 60 min.

Contribution of transporter-mediated transport to the transepithelial flux of Val-Pro-Pro

The effect of the Val-Pro-Pro concentration on its transepithelial flux was examined. As shown in Fig. 2, The flux was not saturable when the apical concentration of the peptide was 4 mM or lower. Since the Km value for PepT1 has been reported to be 0.1-4 mM, the non-saturable pattern in Fig. 2 suggests that this transport was not a transporter-mediated process.

The apical-to-basolateral flux of Val-Pro-Pro was measured in the presence of 10 times the concentration (10 mM) of Gly-Pro. Gly-Pro is a good substrate for the peptide transporter with a low Km value, and is fairly resistant to brush border peptidases. Gly-Pro has therefore often been used to analyze the peptide transporter functions. The flux of Val-Pro-Pro was not inhibited by Gly-Pro (Fig. 3). The presence of arphamenine A, which is also a substrate for the peptide transporter with a low Km value, slightly reduced the basolateral concentration of Val-Pro-Pro, although the decrease in the flux was not significant. These results suggest that the peptide transporter did not play a significant role in the transepithelial transport of Val-Pro-Pro in the Caco-2 cell monolayer.
Fig. 4. Effect of Inhibitors of Transcytosis on the Transepithelial Transport of Val-Pro-Pro (VPP) Across the Caco-2 Cell Monolayer.

Caco-2 cell monolayers were pretreated with each inhibitor for 60 min before the transport experiment. VPP (1 mM) was then added to the apical solution in the presence or absence of an inhibitor. The concentration of VPP in the basolateral solution was measured after a 60-min incubation. Each value is the mean ± S.E. (n = 3).

Fig. 5. Apical-to-basolateral (AP-to-BL) and Basolateral-to-apical (BL-to-AP) Flux for Val-Pro-Pro (VPP) Across the Caco-2 Cell Monolayer.

VPP (1 mM) was added either to the apical or basolateral solution, and the concentration of VPP in the basolateral or apical solution was measured after a 60-min incubation. Each value is the mean ± S.E. (n = 3).

Effect of endocytosis inhibitors on the transepithelial flux of Val-Pro-Pro

The transcytotic process is initiated by endocytosis at the apical cell membrane. The effects of wortmannin\(^2\)\(^4\) and phenylarsine oxide,\(^2\)\(^5\) both inhibitors of the endocytic process, on the apical-to-basolateral transport of Val-Pro-Pro were examined. As shown in Fig. 4, these inhibitors did not significantly inhibit the transport of Val-Pro-Pro, indicating that transcytotic process was not involved in the transepithelial transport of Val-Pro-Pro in the Caco-2 cell monolayer.

Apical-to-basolateral and basolateral-to-apical transport of Val-Pro-Pro

The degree of apical-to-basolateral (AP-BL) and of basolateral-to-apical (BL-AP) flux of Val-Pro-Pro across the Caco-2 cell monolayer was compared. As shown in Fig. 5, the AP-BL flux was slightly higher than the BL-AP flux, although the difference was not significant, indicating that the transport was not unidirectional.

Table 1. Distribution of Val-Pro-Pro and Free Val in the Caco-2 Cell Monolayer System after a 60-min Incubation with 1 mM Val-Pro-Pro Added to the Apical Chamber

<table>
<thead>
<tr>
<th></th>
<th>Amount (nmol)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val-Pro-Pro</td>
<td>428.5 ± 35.0</td>
<td>84.23</td>
</tr>
<tr>
<td>Val</td>
<td>25.5 ± 1.5</td>
<td>5.01</td>
</tr>
<tr>
<td>Cell fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val-Pro-Pro</td>
<td>n.d.</td>
<td>0.00</td>
</tr>
<tr>
<td>Val</td>
<td>42.3 ± 4.0</td>
<td>8.32</td>
</tr>
<tr>
<td>Basolateral solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val-Pro-Pro</td>
<td>9.8 ± 0.3</td>
<td>1.93</td>
</tr>
<tr>
<td>Val</td>
<td>2.6 ± 0.3</td>
<td>0.51</td>
</tr>
<tr>
<td>Total Val</td>
<td>508.7</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Five hundred \(\mu\)L of a Val-Pro-Pro solution (1 mM) was added to the apical solution. After incubating for 60 min, the Val-Pro-Pro and free Val contents in the apical/basal solutions and in the cell monolayer were measured.

\* Converted into the equivalent amount (nmol) of Val.

Hydrolysis of Val-Pro-Pro during the process of transepithelial transport across the Caco-2 cell monolayer

Val-Pro-Pro at 1 mM was added to the apical chamber of the Caco-2 cell monolayer, and the concentrations of intact Val-Pro-Pro and of free amino acids (Val and Pro) in the Caco-2 cells as well as in the apical/basolateral solutions were determined. The distribution of Val-Pro-Pro and free Val after
the transport experiment for 60 min is summarized in Table 1. The intracellular concentrations of free Val and Pro are also shown in Fig. 6. About 15% of Val-Pro-Pro disappeared from the apical chamber by either hydrolysis or transport (Table 1). However, the amount of intact Val-Pro-Pro appearing in the basolateral solution after a 60-min incubation was less than 2%. Intact Val-Pro-Pro was not detectable in the cell fraction (Table 1), whereas the intracellular free Val and Pro concentrations were markedly increased (Fig. 6). The amount of intracellular Val corresponded to about 8% of Val-Pro-Pro added to the apical solution. Since this increase was significantly inhibited by adding the competitor peptide, Gly-Gly (Fig. 6), the increased levels of intracellular Val and Pro are thought to have been due to the transporter-mediated uptake of Val-Pro-Pro and the subsequent digestion by intracellular peptidases. A small amount of free Val was also detected in the apical solution, suggesting that a small portion (about 5%) of Val-Pro-Pro had been hydrolyzed by surface peptidases in the apical cell membrane, although that intracellular free Val had been released from the cells to the apical solution cannot be ruled out.

**Discussion**

Three major mechanisms have been recognized for the intestinal transport of oligopeptides. The first one is the transporter (PepT1)-mediated transport for di- and tripeptides. Irrespective of the amino acid sequence, di- and tripeptides can be actively transported by PepT1. This is basically a degradative pathway for those peptides. Secondly, transcytosis has been reported to transport certain peptides. The third mechanism is paracellular transport through the intercellular junctions. This is an energy-independent passive transport mechanism and would be applicable to the absorption of water-soluble low-molecular-weight substances including minerals and oligopeptides. It would be possible for Val-Pro-Pro to be transported via any of these three pathways.

The human intestinal epithelial cell, Caco-2, is known to express a variety of small intestinal cell functions, and has been used as a model of the small intestinal epithelium. Caco-2 cells express many intestinal enzymes and transporters that include the peptide transporter, PepT1. Transcytotic activity has also been observed in Caco-2 cells. The tight junction which regulates the paracellular transport of the cell monolayer has also been expressed in Caco-2 cell monolayers when the cells were cultured on a semipermeable filter. Although the activities of PepT1 and transcytosis in Caco-2 cells may be relatively low compared with those of epithelial cells isolated from animal intestine, Caco-2 is one of the most useful cell lines at present as a model of the small intestinal epithelial cell. We therefore used this cell monolayer to analyze the absorption behavior of Val-Pro-Pro in the intestinal epithelium.

The present results demonstrate that transepithelial transport of intact Val-Pro-Pro across Caco-2 cell monolayers was hardly inhibited by the addition of a competitive substrate for PepT1 such as Gly-Pro and arphamenine A. This suggests that PepT1-mediated transport was not the major pathway for transepithelial transport of the intact Val-Pro-Pro. Although Val-Pro-Pro was apically taken into Caco-2 cells via PepT1, the internalized peptide is likely to have been quickly hydrolyzed by intracellular peptidases, producing free Val and Pro. The intracellular ratio of Val and Pro of 1:1 (Fig. 6) suggests that Val-Pro-Pro was first cleaved to Val and Pro-Pro by intracellular aminopeptidases. Transcytosis is also not likely to have been involved in the transepithelial transport of intact Val-Pro-Pro. Although Val-Pro-Pro is composed of hydrophobic amino acids, a peptide with only three amino acid residues may not be hydrophobic enough to be transported via adsorptive transcytosis.

These results suggest the paracellular route to be the intestinal absorption pathway for intact Val-Pro-Pro. Paracellular diffusion is regulated by the tight junction, which is a dynamic cell device composed of such proteins as occludin, claudin, ZO-1 and ZO-2. Since these tight junction-related proteins are associated with a cytoskeletal structure such as actin filaments, the tight junction and paracellular transport are regulatable by various factors which affect the cellular structure and functions. Madara and Stafford have reported that the tight junctional permeability of human intestinal T84 cell monolayers was increased by interferon-γ. We have observed that an interferon-γ treatment of Caco-2 cell monolayers decreased their TER value by 65%. This TER decrease was accompanied by a 2.5 times increase in the permeability of the monolayer for Val-Pro-Pro (unpublished results). On the other hand, PepT1 activity in the interferon-γ treated Caco-2 cells did not change. These results indicate that Val-Pro-Pro transport was mainly via the paracellular pathway.

Madara and Pappenheimer have demonstrated that the sodium-coupled transport of such nutrients as glucose and amino acids triggered an alteration in the cytoskeletal structure, thereby increasing the paracellular permeability of the nutrients. We have observed that such food ingredients as sweet pepper contained certain substances which could increase the tight-junction permeability. These findings suggest that the amount of bioactive tripeptides paracellularly absorbed during food intake would be higher than that expected from the results of simple *in vitro* transport experiments. Conradi et al. have compared the paracellular permeability of the Caco-2 cell monolayer with that of rat intestinal mucosa. They
concluded that the paracellular transport of a small solute was greater in vivo than that predicted from the Caco-2 cell permeability. This also suggests that the absorption rate for an intact tripeptide in vivo would be much higher than the value obtained in the present study with Caco-2 cells.

It is not likely that any tripeptide can be transepithelially transported in an intact form. The transepithelial flux of intact tripeptides would be strongly dependent on their structure and properties. The susceptibility to cellular peptidases would be the most important factor determining the bioavailability of oligopeptides. If a peptide has a sequence highly susceptible to surface peptidases, transepithelial transport of the intact peptide would be negligible. For example, when Leu-Ala-Pro, a tripeptide susceptible to Caco-2 peptidases, was added to the apical chamber, 70% of the peptide disappeared from the apical solution after a 60-min incubation, and no detectable amount of intact Leu-Ala-Pro was obtained in the basolateral solution (unpublished results). In addition to Val-Pro-Pro, Ile-Lys-Pro and Ile-Lys-Trp have been reported to be antihypertensive by orally administering at a relatively low dose.6,10 We have preliminarily observed that these peptides were also fairly resistant to Caco-2 peptidases and that significant amounts could be detected in the basolateral chamber after transport experiments. The sequences, X-Pro-Pro, X-Lys-Pro and X-Lys-Trp, are likely to be particularly resistant to intestinal peptidases. Masuda et al.39 have reported that Ile-Pro-Pro and Val-Pro-Pro were detectable in the abdominal aorta of SHR 6 hours after administering sour milk containing these peptides, indicating the stable nature of these X-Pro-Pro tripeptides. The high in vivo activity of these peptides may be, at least partly, due to their low susceptibility to the peptidases.

In conclusion, the present study indicates that an ACE-inhibitory tripeptide, Val-Pro-Pro, could be transported across the Caco-2 cell monolayer in an intact form. The mechanism for this transport was mainly via paracellular diffusion, and transcytosis and transporter-mediated transport are not likely to have played a major role in the transepithelial transport of the “intact” tripeptide, although a small portion of the intact tripeptide could be transcellularly transported via PepT1 (and via an unidentified transporter system in the basolateral membrane) like certain peptide drugs and antibiotics.39 This possibility cannot be ruled out since the tripeptide has remarkably high resistance to hydrolysis by intestinal peptidases.

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References

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