Effects of Capsaicin on Cellular Damage and Monolayer Permeability in Human Intestinal Caco-2 Cells

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Recent studies suggest that capsaicin (Cap), a major constituent of hot pepper, may affect the function and permeability of the intestinal mucosa in vitro. However, the relationships between the dose of Cap and the barrier and/or transporter functions on intestinal epithelial cells are unknown. The aim of this study was to investigate whether Cap initiates cellular injury and alter epithelial permeability in Caco-2 cells. Cellular toxicity, as measured using a lactate dehydrogenase release assay, was not observed at high concentrations of Cap (up to 300 μM). When cell viability was measured by a WST-1 assay (tetrazolium salt-based assay), damage to Caco-2 monolayers was observed at doses of 200 and 300 μM of Cap. The barrier function of tight junctions was assessed by measuring transepithelial electrical resistance (TEER) in Caco-2 cells. Treatment of Caco-2 cells with Cap at doses above 100 μM significantly decreased the TEER compared to treatment with buffer alone for 2 h (p<0.05). We next examined the effects of Cap on the activity of P-glycoprotein (P-gp) found on transcellular transporters. At doses of 100 and 200 μM, Cap inhibited the transport of rhodamine 123 by P-gp-mediated efflux in Caco-2 cells. Cap thus exhibited inhibitory effects on P-gp. The results of this study indicate that Cap, a dietary phytochemical, causes functional and structural changes in Caco-2 cell monolayers at nontoxic doses (less than 100 μM of Cap). The concomitant administration of Cap with drugs that are substrates of P-gp might increase the plasma concentrations of such drugs.

Key words capsaicin; Caco-2; P-glycoprotein; tight junction; food–drug interaction

Some dietary constituents and phytochemicals have recently been identified as important factors affecting drug disposition. The effects of these factors on drug absorption are thought to vary depending on the epithelial barrier functions and transporters. Ingestion of many types of herbal supplements results in interactions with drugs administered concomitantly.

The intestinal mucosal epithelium constitutes the major barrier (including tight junctions (TJ) and efflux transporters) to the absorption of drugs that are administrated orally. A TJ forms a paracellular barrier on the lateral membranes of adjacent cells and acts as a structural barrier to the paracellular passage of water-soluble molecules and bacteria.1,2) Disruption of the TJ barrier allows paracellular penetration of toxic luminal substances such as anticancer drugs and nonsteroidal anti-inflammatory drugs (NSAIDs),3,4) which promote gastrointestinal mucosal injury.

During absorption, dietary supplements can interact with the efflux transporters present in enterocytes, and marked interaction may occur with drugs administrated concomitantly. A role has been suggested for P-glycoprotein (P-gp) in the intestinal absorption and prehepatic elimination of numerous xenobiotics, including drugs.5) Both its location on the apical membrane of enterocytes and its function as an efflux pump suggest an important role for the intestinal P-gp in modulating permeability to drugs.

Capsaicin (Cap) is a major component of hot pepper, a widely consumed spice in Southeast Asian countries. Cap has previously been shown to be a substrate of P-gp6) and to inhibit P-gp.7) Cap also has chemopreventive and chemoprotective properties8,9) and has been found to affect the permeability of human colon carcinoma cell line (HCT-8) in intestinal epithelial monolayers.10) Although high doses of Cap cause mucosal damage through direct cytotoxic effects, the effects of various doses of Cap on the gastrointestinal TJ barrier and P-gp are unknown.

In the present study, we examined the effects of Cap on the intestinal epithelial TJ barrier and P-gp using a filter-grown Caco-2 intestinal epithelial monolayer. Caco-2 cells grown on permeable inserts form TJ and attain many of the morphological and functional characteristics of small intestinal enteroctyes5) and are widely used as an in vitro model in drug-absorption studies. This cellular model system can be used to determine the effects of Cap on paracellular and transcellular permeability.

MATERIALS AND METHODS

Materials Dulbecco’s modified Eagle’s medium (DMEM), trypsin, penicillin, streptomycin, and Hank’s balanced salt solution (HBSS) were purchased from Invitrogen Corp. (CA, U.S.A.). Fetal bovine serum (FBS) was purchased from BioSource International Inc. (CA, U.S.A.). Millipore-PCF 3.0 μm permeable filters (12 mm) were purchased from Millipore Corp. (MA, U.S.A.). Rhodamine 123 (Rh123) was purchased from Sigma-Aldrich Inc. (MO, U.S.A.). Cap was kindly provided by Maruishi Pharmaceutical Co., Ltd. (Osaka, Japan). MTX “LDH” kit was purchased from Kyokuto Pharmaceutical Industrial Co. (Tokyo, Japan). Cell Counting Kit was purchased from DOJINDO Laboratories (Kumamoto, Japan). All other chemicals were of reagent grade.

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Cell Cultures Caco-2 cells (passage 52—60), from a human colonic carcinoma, were grown in a culture medium composed of DMEM with 4.5 mg/ml glucose, 50 U/ml streptomycin, 50 U/ml penicillin, 4 mmol/l glutamine, and 10% FBS in 25-cm² tissue culture flasks (Becton-Dickinson Ind., NJ, U.S.A.). The cells were maintained at 37 °C in an atmosphere of 5% CO₂/95% air in a CO₂ incubator (NAPCO Inc., NC, U.S.A.) until they reached 80—90% confluence, usually in 5—7 d. The cells were subcultured by partial digestion with 0.25% trypsin and 1 mmol/l EDTA in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) solution. The Caco-2 cells were then detached from the stock cultures by trypsin digestion, washed once by centrifugation, resuspended, and subcultured in 10 ml of medium in culture flasks at a concentration of 1×10⁵ cells/ml. The cultures were observed on a regular basis under an inverted light microscope to monitor growth and contamination. For growth on filters, high-density Caco-2 cells (2.0×10⁵ cells/ml) were plated on nitrocellulose-based Millicell-HA filters. The Caco-2 cells were fed with culture medium every alternate day and then daily for 18 or 25 d by replacing 0.4 ml of the medium in the apical chamber and 0.6 ml of the medium in the basolateral chamber. They were monitored regularly for confluence by measuring transepithelial electrical resistance (TEER).

Cellular Toxicity Test Lactate Dehydrogenase (LDH) Release Assay: LDH activity was measured in Ca²⁺- and Mg²⁺-free PBS solution. The assay is based on the oxidation of NADH in the presence of pyruvate. Briefly, Caco-2 cells (5×10⁴ cells/well) were plated on a 96-well multiplate (SUMITOMO BAKELITE Co., Ltd., Tokyo, Japan) and treated with Cap for 72 h. The LDH reagent was added to each well. Following 2-h incubation at 37 °C, the stop solution (5 mmol/l, 1 : 9) was added to each well. Following 3-h incubation at 37 °C, absorbance at 450 nm (reference at 630 nm) was determined by a cell proliferation assay using WST-1 reagent (Thermo LabSystems, MA, U.S.A.). Cell monolayers with TEER values below 300 Ω·cm² were not used. The apical or basolateral solution was changed to 5% ethanol/HBSS containing Rh123 (loading dose of 5 μM) with or without Cap. Samples were obtained after 120 min by moving the cell monolayer to a new receiver well containing fresh HBSS. The samples were diluted with 500 μl HBSS, and fluorescence was determined at 485/546 nm (excitation/emission) on a fluorometer (HITACHI, F-3000, Tokyo, Japan). All the transport experiments were performed in triplicate. The TEER values were measured after each experiment.

Cap dissolved in 5% ethanol was diluted with 5% ethanol in HBSS to prepare samples with 0—300 μM of Cap. Control samples included 5% ethanol in HBSS. Apparent permeability coefficient (P_app) was determined according to the following equation: 

\[ P_{\text{app}} = \frac{dQ/dt}{A_{\text{ch}}C_0} \] (cm/s). 

where \( dQ/dt \) (nmol/s) is the flux rate, \( A \) (cm²) is the effective surface area of the cell monolayer, and \( C_0 \) (nmol/ml) is the initial drug concentration (5000 nmol/ml) in the donor chamber.

Data Analysis Results are expressed as means±S.D. Statistical analyses were performed using Student’s unpaired t-test and Tukey multiple comparison test, and differences were regarded as significant at the p<0.05 level.

RESULTS

Cellular Damage by Cap LDH Method The cytotoxic effects of ethanol, which was used to enhance the solubility of Cap, were determined by measuring LDH release in Caco-2 cells. Ethanol slightly increased the LDH release by Caco-2 cells at various concentrations (2.5%, 5.0%, and 10% in HBSS) compared with the control (HBSS) (Fig. 1). Cap at various concentrations (0—300 μM), including that dissolved in 5% ethanol/HBSS, did not increase LDH release (Fig. 1).

WST-1 Method There was a marked decrease in cell viability at 200 and 300 μM Cap compared with the decreases observed when the medium and Cap concentrations below 100 μM were used. Percentage cell viability was 32% and
Caco-2 monolayers were treated with Cap for 2 h. Values are mean±S.D. (n=3–4). Experiments were repeated in triplicate to ensure reproducibility. ○, Cap 0 μM; □, 50 μM; ■, 100 μM; ■, 200 μM; ▲, 300 μM. ∗, p<0.05; ∗∗, p<0.01, compared with the control (5% ethanol/HBSS). Cap, capsaicin; TEER, transepithelial electrical resistance.

DISCUSSION

One important function of the intestinal epithelia is to protect against mucosal penetration of toxic compounds, bacteria, and bacterial byproducts as well as dietetic additives present in the intestinal lumen. The intestinal epithelium has barrier functions against and performs efflux transport of these substances. The TJ acts as a structural barrier against paracellular permeation of luminal compounds. 1,2 Disruption of the TJ barrier causes increased epithelial penetration by toxic luminal substances that may promote mucosal injury. P-gp is an active efflux transporter and is abundantly expressed in the intestinal epithelium as one of many important epithelial barriers. Intestinal P-gp also plays a significant role in absorption and presystemic elimination of many peroral xenobiotics, including drugs. The study of the factors regulating these barrier functions is thus important from the perspective of both pharmacology and toxicology.

The efficiencies of the absorption of orally administered drugs can be affected by foods, including supplements. Healthy foods contain multiple nutrients, nonnutrients, and phytochemicals. The pharmacological effects of Cap, which include protection of the gastric mucosa 3 and vasodilation in rats 4 have been previously investigated, and recent research on Cap receptors 5,6 has suggested its potential function as an analgesic. Besides these neuronal and vascular effects of Cap, direct interactions of Cap with gastrointestinal epithelia are likely to occur. However, such direct interactions have not yet been clearly determined.

We first examined the effects of ethanol, used as a solvent, on Caco-2 cell monolayers. No toxic effects were observed with ethanol at concentrations less than 10% with LDH release method (Fig. 1). At the doses used (300 μM), Cap did not significantly increase the LDH release by Caco-2 cells (Fig. 1). However, Cap at concentrations of 200 and 300 μM markedly decreased cell viability in the WST-1 method, sug-

Table 1. Apparent Permeability Coefficients (P_app ×10⁻⁵ cm/s) of Rh123 Transport across Caco-2 Cell Monolayers

<table>
<thead>
<tr>
<th>Sample</th>
<th>A→B</th>
<th>B→A</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Ethanol</td>
<td>1 h</td>
<td>0.128±0.030 1.33±0.21</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>0.116±0.019 1.42±0.16</td>
</tr>
<tr>
<td>10 μM Cap</td>
<td>1 h</td>
<td>0.120±0.024 1.43±0.18</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>0.157±0.097 1.35±0.05</td>
</tr>
<tr>
<td>50 μM Cap</td>
<td>1 h</td>
<td>0.117±0.003 1.27±0.19</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>0.176±0.046 1.30±0.13</td>
</tr>
<tr>
<td>100 μM Cap</td>
<td>1 h</td>
<td>0.139±0.057 1.00±0.23*</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>0.155±0.011* 1.08±0.20*</td>
</tr>
<tr>
<td>200 μM Cap</td>
<td>1 h</td>
<td>0.160±0.012 1.03±0.08**</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>0.175±0.014** 1.44±0.12</td>
</tr>
<tr>
<td>300 μM Cap</td>
<td>1 h</td>
<td>0.333±0.009** 1.11±0.19</td>
</tr>
<tr>
<td></td>
<td>2 h</td>
<td>0.691±0.073** 1.36±0.12</td>
</tr>
</tbody>
</table>

Values are means±S.D (n=4–6). Experiments were repeated in triplicate to ensure reproducibility. ∗, p<0.05; ∗∗, p<0.01, compared with 5% ethanol. A→B (left), apical to basolateral chamber; B→A (right), basolateral to apical chamber; Rh123, rhodamine123; Cap, capsaicin.
gesting that cell damage might be observed with exposure to higher concentrations of Cap (Fig. 2). At concentrations of up to 100 μM, Cap did not exhibit cell damage after 2-h incubation. The WST-1 assay is used for measuring the proliferation and viability of cells, and LDH release assay for monitoring cell death due to cell membrane destruction. For these reasons, we consider the WST-1 assay more sensitive in determining cell damage than the LDH release assay.

We next examined the effects of Cap on the barrier function of monolayers of Caco-2 cells in vitro. Paracellular permeability has been correlated with decrease in intestinal epithelial resistance.17) The use of TEER as a measure of the integrity of membrane barriers has also been reported in studies evaluating the damage caused by drugs such as fenadine · HCl or indomethacin.18,19) In addition, TEER measurements have been reported to indicate the permeability of cell monolayers and their barrier properties.20,21) Therefore, we used TEER values to monitor the damage induced by Cap in Caco-2 cells. The confluent Caco-2 cell monolayers cultured for approximately 20 d and used in the experiments were subjected to 0—300 μM Cap in 5% EtOH/HBSS for 2 h. Significant damage with decrease in TEER was induced in these Caco-2 cell monolayers by treatment with 100, 200, and 300 μM Cap for 2 h (Fig. 3). These experiments thus demonstrated the possibility of direct damage to Caco-2 cells in vitro at high concentrations of Cap. In the Caco-2 monolayer experiments, when 300 μM Cap was added, Caco-2 cells were disrupted (Table 1). In the present study, we demonstrated that high doses of Cap had direct cytotoxic effects on epithelial cells at the gastrointestinal surface, with resultant rupture of epithelial layers and formation of large open wounds on the epithelial surface. Thus, the Cap-induced increase in TJ permeability was due to an irreversible change in the TJ barrier, and may have been caused by cell death or the formation of large open wounds on the epithelial surface.

Recent studies have suggested that altered intestinal epithelial TJ permeability may be a significant risk factor for many diseases such as Crohn’s disease,23) NSAID-associated enteritis,22) and diarrheal syndromes.23) It has been reported that in these disorders, increase in intestinal epithelial TJ permeability allows paracellular penetration of toxic luminal substances, aggravating intestinal inflammation and mucosal injury.22,23) Consumption of Cap in these clinical conditions may enhance increase in TJ permeability, resulting in greater mucosal penetration of luminal substances and further worsening of intestinal inflammation.

In the present study, we also evaluated the effects of Cap exposure on P-gp function in Caco-2 cell monolayers. We measured the permeability of Rh123 as a substrate of P-gp. The fluorescent dye Rh123 is frequently used as a P-gp index substrate. A good correlation between Rh123 transport and P-gp function has been reported.24) We observed that Cap was capable of inhibiting P-gp efflux activity at a concentration of 100 μM, a nontoxic concentration, with incubation for 2 h (Table 1). We confirmed that verapamil (100 μM), a P-gp inhibitor, also inhibited the B→A transport of Rh123 across Caco-2 cells (data not shown). In addition, we found that Cap may inhibit P-gp function without decreasing TEER at 100 μM in 1 h (Table 1). It is possible that dietary Cap affects the bioavailability of co-administered drugs by inhibiting intestinal P-gp function. We reported that the bioavail-

ability of digoxin which is a substrate of P-gp increased by oral co-administration of Cap.25) Cap may also simultaneously cause opening of TJ. In this study, Cap induced TJ opening and inhibition of P-gp in a dose-dependent and exposure-time-dependent manner. However, Cap might not inhibit P-gp function at concentrations that induced opening of the Caco-2 TJ barrier, with the exception of exposure to 100 μM Cap for 2 h.

The apicolaterally located TJ forms a paracellular seal or barrier between the lateral membranes of the adjacent cells and acts as structural barrier against the paracellular penetration.26) In this study, the disruption of the TJ barrier by cell damage or cytotoxicity at high doses of Cap allows an increase in epithelial penetration (A→B). And also, Cap at high levels may have a direct effect on the protein structure including TJ-related proteins. Caco-2 monolayers may lose the efflux function of P-gp protein located basolateral side of Caco-2 monolayers connected with the results of a marked decrease in cell viability by treatment with high doses of Cap (Fig. 2). Although the mechanisms leading to the barrier disruption remain unknown, current thinking suggests that both the apical and basolateral membranes of Caco-2 monolayers can respond to the cytotoxic action of Cap.

In conclusion, the results of this study indicate that Cap inhibits P-gp function in the intestinal epithelium at low nontoxic doses and causes opening of the intestinal epithelial TJ barrier at high doses. Since ingestion of Cap can be expected to markedly alter the permeabilities of drugs, the use of Cap during drug administration should be evaluated.

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REFERENCES