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Allyl Isothiocyanate, a Pungent Ingredient of Wasabi and Mustard Oil, Impairs Gastric Paracellular Barrier in Primary Cultures from the Rat Stomach via TRPA1-Independent Pathway

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Additional information is available at the end of the chapter

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1. Introduction

Patients with peptic ulcer and functional dyspepsia avoid food intake of chilies, wasabi, and mustard oil by advised medical staffs, because the prevalent notion is that those condiments would lead to the aggravation of gastric ulcers or stomach pain. However, chilies, wasabi, and mustard oil are known to have pharmacological effects such as the ability to improve appetite and digestion traditionally. It was reported that capsaicin, a pungent ingredient of chilies, induced gastric mucosal protection, accelerated gastric healing, and regulated gastric acid secretion via capsaicin-sensitive sensory neurons from animal and human studies [1-5]. Recently, it has been shown that allyl isothiocyanate, a pungent ingredient of wasabi and mustard oil, has the protective and the aggravating effects of gastric mucosal damages in rats [6-8]. Although the underlying mechanism was investigated, it remained to be inconsistent effects of allyl isothiocyanate on gastric mucosal defense mechanisms.

Gastric mucosal defense mechanisms are essential for preventing potentially harmful elements such as acid, pepsin, and *Helicobacter pylori* (*H. pylori*), present in the gastric lumen from gaining access to the gastric mucosa. Tight junctions, which is classified as epithelial barrier in gastric mucosal defense [9], are dynamic structures located at the most apical region of cell-cell contact points. Interconnected by tight junctions, gastric epithelial cells form tight junction barrier, preventing back diffusion of acid and pepsin. Tight junction proteins are comprised of ZO-1,

occludin, claudins, and junctional adhesion molecules (JAMs) [10, 11]. Occludin was the first identified transmembrane protein of tight junctions. Recently, the claudin family is supposedly composed of at least 24 members in mice and human. In the stomach, it has been reported that ZO-1, occludin, claudin-3, 4, 7, and 11 are expressed [12-14]. In addition, electrical resistance, which is an indicator for tight junction barrier, in gastric mucosa was shown the highest in the gastrointestinal tract, suggesting that tight junction barrier play critical roles in gastric mucosal defenses [11]. Indeed, it was reported that the disruption of tight junction complexes were attributed to gastric mucosal damages induced by aspirin in animal model and cell culture studies [14, 15].

1.1. Aim

The aim of the present study is (1) to develop primary cultures of gastric epithelial cells from rats that enable to investigate tight junction barrier, (2) to examine the influence of allyl isothiocyanate (AITC) on tight junction barrier using primary cultures from the rat stomachs, as compared with action of capsaicin. In this paper, it is suggested that allyl isothiocyanate breaks gastric tight junction barrier. In addition, we have established confluent primary cultures from rat stomachs for investigating tight junction barrier of gastric mucosa.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley strain rats (SLC, Hamamatsu, Japan) weighing 180-220 g were used. Animals were housed under controlled environmental conditions (temperature at $24\pm 2^{\circ}\text{C}$ and light on 7:00 am to 7:00 pm) and fed commercial mouse chow MF (Oriental Yeast, Tokyo, Japan). Animal experiments were performed in compliance with the "Guiding Principles for the Care and Use of Laboratory Animals" approved by the Japanese Pharmacological Society and the guidelines approved by the Ethical Committee on Animal Care and Animal Experimentation of Josai International University (#12). Animals were anesthetized using sodium pentobarbital before the isolation of tissues, and euthanized by over dose of sodium pentobarbital.

2.2. Buffers for cell isolation

Medium A contained (in mM) 0.5 NaH_2PO_4 , 1.0 Na_2HPO_4 , 20 NaHCO_3 , 70 NaCl , 5 KCl , 11 glucose, 50 HEPES, 2 Na_2EDTA , and 20 mg/ml BSA (fraction V). Medium B contained (in mM) 0.5 NaH_2PO_4 , 1.0 Na_2HPO_4 , 20 NaHCO_3 , 70 NaCl , 5 KCl , 11 glucose, 50 HEPES, 20 mg/ml BSA (fraction V), 1.0 CaCl_2 , and 1.5 MgCl_2 . Medium C contained (in mM) 0.5 NaH_2PO_4 , 1.0 Na_2HPO_4 , 20 NaHCO_3 , 70 NaCl , 5 KCl , 11 glucose, 50 HEPES, 1 mg/ml BSA (fraction V), 1.0 CaCl_2 , 1.5 MgCl_2 , and 0.5 dithiothreitol [16, 17].

2.3. Cell isolation

Cell isolation from the rat stomach was according to the methods described by Tani. et al. [16, 18] and modified by us to collect parietal cells and chief cells [19], where were located at the middle and bottom of gastric glands, from the rat stomach. In brief, three non-fasted rats were anesthetized using sodium pentobarbital. The stomach was excised, everted, and tied at both esophagus and pylorus. The everted sac was filled with 2 ml of Medium A, containing 2.5 mg/ml of protease E and placed in Medium A for 30 min (fraction 1), followed by Medium B for 120 min in a shaking water bath at 37°C (fractions 2-4). Isolated cells from digestion fractions 4 were pelleted at 240x g in a TOMY EIX-136 centrifuge (Tokyo, Japan), re-suspended in Medium C. The cells were centrifuged at 500x g at room temperature for 10 min and then re-suspended in 1:1 mixture of Ham's F-12 and Dulbecco's minimum essential medium (DMEM/F-12), supplemented with heat-inactivated 10 % fetal bovine serum (FBS), 8 µg/ml insulin, 1 µg/ml hydrocortisone, 100 U/mL penicillin, 100 U/ mL streptomycin, and 0.25 µg/ml amphotericin B.

2.4. Primary cultured gastric epithelial cells

Isolated cells from rat stomachs were plated at density of 3.6×10^5 cells/cm² in collagen-coated Transwell filters, 35 mm-cultures dishes, and 60 mm-culture dishes. Those cells were incubated in DMEM/F12 supplemented with 10% FBS, 8 µg/ml insulin, 1 µg/ml hydrocortisone, 100 U/ mL penicillin, 100 U/ mL streptomycin, and 0.25 µg/ml amphotericin B under 5 % CO₂ in air at 37 °C by 4 days.

2.5. RGM-1 cells culture

RGM-1 cells, established by Dr. Matsui et al. [Institute of Physical and Chemical Science (RIKEN) Cell Bank and Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan], are non-transformed gastric surface epithelial cells [20]. RGM-1 cells were cultured in DMEM/ F-12 supplemented with 10 % FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.25 µg/ ml amphotericin B. RGM-1 cells were plated at density of 2.8×10^4 cells/cm² in non-coated Transwell filters and incubated in 5 % CO₂ in air at 37 °C by 14-15 days.

2.6. Immunofluorescence microscopy

For histochemical identification of the isolated and 4 day-cultured cells, periodic acid-Sciff reaction (for mucus surface cells), succinic dehydrogenase activity (for parietal cells), and immunofluorescence test for pepsinogen II (for chief cells) were used [21]. Dispersed cells immediately after isolation were pelleted, re-suspended in OCT compound, and then frozen in isopentane cooled with liquid nitrogen. Frozen dispersed cells were sectioned on a cryostat (Leica, Bannockburn, IL, USA) at a thickness of 4 µm. The sections were thaw-mounted on slides glasses. Surface cells were identified by red color (neutral mucins) of the large granules when stained with periodic acid-Sciff stain [22]. Succinic dehydrogenase activity was determined by the methods of Nachlas et al [23]. The sections were

incubated in the medium containing 0.2 M phosphate buffer, 0.25 M succinic acid (disodium salt), and nitro-blue tetrazolium (1 mg/ml) for 50 min at 37°C. After incubation, the sections were counterstained cell nuclei by 2 % methyl green for 15 min at 60 min. Parietal cells containing an unusual number of mitochondria among gastric epithelial cells were identified by bluish purple [24]. For immunofluorescence study of pepsinogen II for identification of chief cells, the section was incubated for 1 hr with blocking buffer containing (in mM) 150 NaCl, 10 NaH₂PO₄, 2 mg/ml of gelatin, 0.5 % fish gelatin, and 2 % BSA (globulin-free). Antibody staining was done at room temperature for 2 hr with anti-pepsinogen II antibody (BioDesign, Saco, ME). Evaluation of staining was done using a Nikon TE300 microscope (MicroVideo Instruments, Avon, MA) outfitted with an Orca charge-coupled device camera (Hamamatus Photonics) and IP Lab (Scanalytics, Fairfax, VA) image processing software. In contrast, cells grown on the 35 mm-dish were fixed for 10 min at room temperature with 4 % formaldehyde in 0.2 M phosphate buffer (pH 7.4). Fixed cells were washed with PBS, permeabilized with 0.25 % Triton X-100 containing 0.02 % saponin for 4 min at 4 °C, and then the above procedure were conducted from incubation with blocking buffer to do immunostaining and identification of each cell type. Antibody staining was done at room temperature for 2 hr with anti-cytokeratine 8/18 antibody (Novocastra, Newcastle, UK) for identification of epithelial cells and anti-vimentin antibody for fibroblasts (Novocastra, Newcastle, UK) [25, 26]. Evaluation of cell purity was done by counting the total cell number, as identified by methyl green and propidium iodide staining of nuclei, against the number of cells stained with periodic acid-Schiff, nitro blue tetrazolium, and above specific antibodies. Approximately 1,000 cells/slide were evaluated.

2.7. Electrophysiological analysis of primary cultures and RGM-1 cells

Transepithelial electrical resistance (TER) was measured in Transwell filter chambers using a “Milli-cell” ERS system (Millipore, Billerica, MA, USA). The background resistance of chambers containing medium alone was subtracted from the value of all experimental conditions. TER was evaluated 1) at 1-4 days after seeding in primary cultured epithelial cells from the rat stomach and at 4, 7, 10, 14 days after seeding in RGM-1 cells, and 2) when capsaicin, allyl isothiocyanate, cinnamaldehyde, and icilin were applied into apical compartment at every 30 min for 3 hr after apical application in 4 day-primary cultures.

2.8. Measurement of permeability in confluent primary cultures and RGM-1 cells

Mucosal (Apical or top well) to serosal (nutrient or bottom well) fluxes of mannitol were done using Transwell filters containing confluent monolayers at day 4. For these studies, 3 mM mannitol was added to the luminal solution and 3 mM D-glucose to the serosal solution. After equilibration for 30 min, 2 mCi of [³H]-mannitol (15-30 Ci.mmol, NEM Life Science Products, Boston, MA) was added to the mucosal solution and the cells were returned to 37°C in the incubator. Triplicate wells were sampled for each treatment at 1 to 3 hr after the addition of

labeled mannitol. The concentration of mannitol in the serosal solution was determined by liquid scintillation as described previously in detail [26].

2.9. Fluorescence microscopy and confocal microscope for occludin, ZO-1, and claudin 4

Gastric epithelial cells grown on Transwell filters were fixed for 10 min at room temperature with 4 % formaldehyde in 0.2 M phosphate buffer (pH 7.4). Fixed cells were washed with PBS, permeabilized with 0.25 % Triton X-100 containing 0.02 % saponin for 4 min at 4 °C. Samples were then labeled with either rabbit anti-occludin antibody, rabbit anti-ZO-1 antibody, or mouse anti-claudin 4 antibody, these were followed by incubation with Gel-PBS containing with 1:200 diluted secondary FITC-conjugated goat anti-rabbit IgG or FITC-conjugated donkey anti-mouse IgG. These samples were mounted in Vectashield (Vector Labs, CA, USA). Fluorescence images were collected using an Axioskop 2 plus microscope with a plan-NEOFLUAR 40x objective. The data was analyzed using AxioVision LE Rel 4.6.3 software (Carl Zeiss Vision, Germany). Images were converted to TIFF format and composites of images were prepared using Adobe Photoshop Elements 2.0 (Adobe Co., CA, USA).

2.10. Measurement of cell viability

Cell viability was evaluated by a colorimetric assay using crystal violet [28]. In brief, primary cultured epithelial cells after apical application of pungent ingredients such as capsaicin and allyl isothiocyanate were washed with PBS to remove dead cells, fixed with methanol, air-dried, and stained with crystal violet. Stained cells were solubilized and the absorbance was measured at 590 nm using 1420 Multilabel Counter (Perkin Elmer, Shelton, CT, USA).

2.11. Reverse transcription-polymerase chain reaction

Total RNA was isolated by using an RNeasy kit (Qiagen, CA, USA) according to the manufacturer's protocol. In brief, either the confluent monolayer of primary cultured rat gastric epithelial cells, which were grown for 4 days after plating, the confluent monolayer of RGM-1 cells, or freshly isolated rat dorsal root ganglia was immediately submerged in Buffer RLT (Qiagen), which inhibited RNase activation, and was homogenized by using a Multi-beads shocker (Yasui Kikai, Osaka, Japan). Reverse transcription-polymerase chain reaction (RT-PCR) was performed by using a one-step RT-PCR Kit (Qiagen) and a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) for 35 cycles (TRPA1, TRPV1, and GAPDH) under the following conditions: reverse transcription at 50°C for 30 min, initial denaturation; 15 min at 95°C and then 30 sec at 94°C, followed by a 30 sec annealing step at 56°C for TRPA1, TRPV1, and GAPDH and 1 min elongation at 72°C. The primers sequences were 5'-CCC CAC TAC ATT GGG CTG CA-3' and 5'-CCG CTG TCC AGG CAC ATC TT-3' for rat TRPA1, 5'-TCG TCT ACC TCG TGT TCT TGT TTG-3' and 5'-CCA GAT GTT CTT GCT CTC TTG TGC-3' for rat TRPV1, and 5'-TCC CTC AAG ATT GTC AGC AA-3' and 5'-AGA TCC ACA ACG GAT ACA TT-3' for rat GAPDH. The PCR products were separated on 3% (wt/vol) agarose gel in Tris-acetate EDTA buffer, stained with ethidium bromide, and analyzed by LAS 3000 (FUJIFILM, Tokyo, Japan). The sequence of the PCR product was analyzed using the BLAST program (NCBI).

2.12. Materials

BSA (globulin-free), crystal violet, deoxyribonuclease 1, ethidium bromide, fish gelatin, hydrocortisone, insulin, methyl green, nitro blue tetrazolium, periodic acid, protease, and icilin were from Sigma-Aldrich (MO, USA). Absolute ethanol, allyl isothiocyanate, BSA (fraction V), capsaicin, cell culture media, cinnamaldehyde, and dimethyl sulfoxide (DMSO) were from Wako Pure Chemical Industries, Inc. (Osaka, Japan). Rabbit polyclonal anti-occludin antibody, rabbit polyclonal anti-ZO-1 antibody, and monoclonal anti-claudin 4 antibody were from Zymed Laboratories (CA, USA). FITC-conjugated goat anti-rabbit IgG, FITC-conjugated donkey anti-mouse IgG, normal goat serum, and normal donkey serum were from Jackson Immune Research Laboratories (PA, USA). Serum was from GibcoBRL (CA, USA). Propidium iodide was from Molecular Probes (OR, USA). Sodium pentobarbital was from Dainippon Sumitomo Pharma Co. (Osaka, Japan). The 35 mm-and 60 mm-dish, and a collagen type I from rat tail were from Beckton Dickinson Biosciences (MA, USA). Transwell with filter (0.4 μ m) was from Corning (MA, USA). Allyl isothiocyanate, capsaicin, and cinnamaldehyde were dissolved in absolute ethanol prior to dilution in cell culture medium, respectively. Icilin was dissolved in DMSO, followed by absolute ethanol prior to dilution in cell culture medium. The final concentrations of either ethanol or DMSO in the apical compartment of Transwell were less than 0.1%. The vehicles used had no pharmacological effects on the epithelial barrier in cell cultures.

2.13. Statistics

Values are presented as means \pm S.E.M. for three or more independent experiments.. Statistical analysis of data were done with SigmaStat software (Jandel Scientific Software, CA, USA) using a two-tailed Student's *t*-test between two groups, and multiple comparisons against a single control group were made by one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of isolated cells from the rat gastric mucosa

Isolated cells from the rat stomach were prepared as described in methods and suspended in medium C. The identification of isolated fraction consisted of chief cells (35.6 ± 5.8 %), parietal cells (31.8 ± 2.6 %), and surface cells (10.3 ± 0.9 %) determined by fluorescent and light microscopy (Table 1). The remaining ~23 % of the cells are likely to be the non-identified immature cell, including proliferative cells and occasional endocrine cells. In addition, those isolated cells became a confluent cultures on the collagen-coating dishes by 4 days after seeding, whose cells were stained with anti-cytokeratine 8/18 antibody, an epithelial marker (data not shown), and with no staining anti-vimentin antibody, a fibroblast marker (0.11 ± 0.11 %). These results suggested that primary cultures from the rat stom-

ach are mainly constituted of epithelial cells, including mucus surface cells, chief cells, and parietal cells, respectively.

Cell type	Values (%)	n
Chief cells	35.6±5.8	4
Parietal cells	31.8±2.6	4
Surface cells	10.3±0.9	4

Table 1. Quantification of each cell type (%) in isolated cells from the rat stomach

3.2. Tight junction barrier is formed in primary cultures from the rat stomach

When isolated gastric epithelial cells were plated, cells attached to Transwell filters by 2 days after seeding. Primary cultured epithelial cells completely covered the Transwell filters by 4 days after seeding, which were similar morphological features when cells were grown on plastic dishes (data not shown). In contrast, RGM-1 cells, which are widely used for the investigation of gastric epithelial physiology as a non-transformed gastric surface epithelial cell lines, were also attached and proliferated on Transwell filters, and formed a confluent monolayer by 4 to 5 days after seeding. Therefore, to determine whether those cultures were an appropriate model to investigate tight junction barrier, we first confirmed to form functional tight junctions. Transepithelial electrical resistance (TER) was quantified. We found that as primary cultures reached confluence at 3 to 4 days after seeding, there was a progressive increase in TER (Fig. 1A). TER at 4 days after seeding was 3069.6±582.0 Ohm.cm². However, RGM-1 cells did not show any increased TER, even though confluence was observed after 4 days after seeding. TER was less than about 60.0 Ohm.cm² throughout the experiment (Fig. 1B).

To investigate why primary cultured epithelial cells, but not RGM-1 cells, have a great high TER, we examined the localization of occludin and ZO-1 for tight junctions in the both cultures. Interestingly, it was found that the localization of occludin and ZO-1 were continuously observed at the cell-cell contact region in primary cultures from the rat stomach (Fig. 2A and B). In RGM-1 cells, occludin was no expressed at the tight junction region, although ZO-1 was only continuously expressed at the cell-cell contact point (Fig. 2C and D). It is suggested that primary cultures from the rat stomach, but not RGM-1 cells, form functional tight junction barrier.

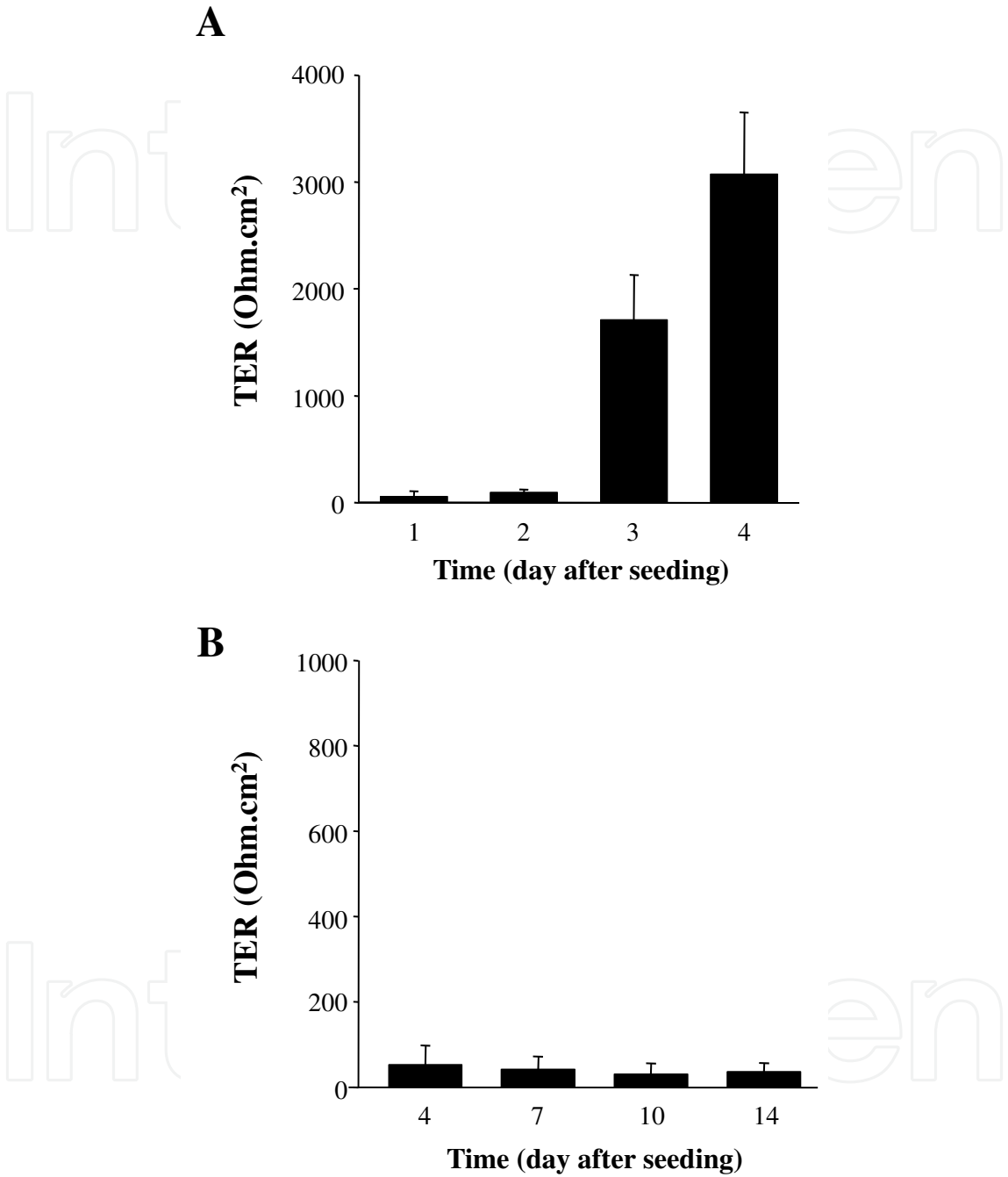


Figure 1. Progressive increased TER in primary cultures from the rat stomach, but not RGM-1 cells. Isolated cells from the rat stomach and RGM-1 cells were grown on Transwell filters. TER was measured from 1 to 4 days after seeding of primary cultures (A) and at 4, 7, 10, 14 days after seeding of RGM-1 cells (B). Data represent means±S.E.M. from 4 independent experiments. Note that primary cultures from the rat stomach produced a great high TER after 3 to 4 days after seeding, when they formed a confluent monolayer.

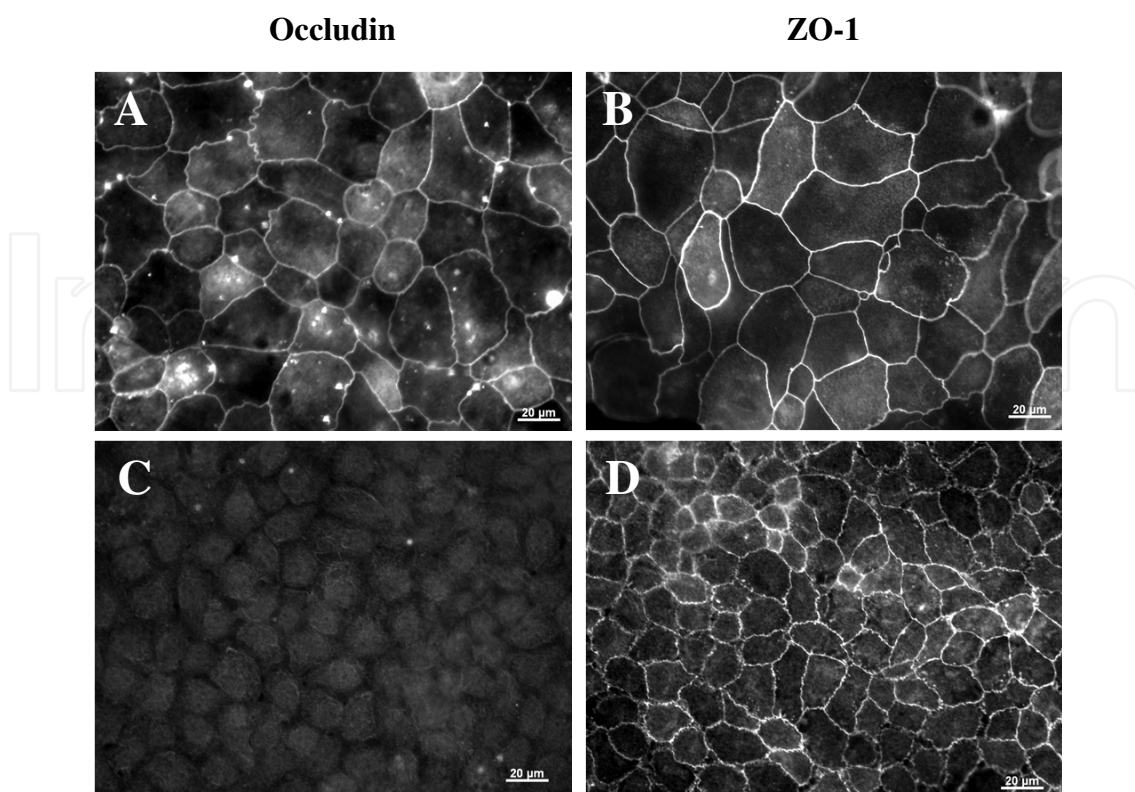


Figure 2. Immunolocalization of occludin and ZO-1 in primary cultures from the rat stomach and RGM-1 cells. The expression is anti-occludin staining (A, C) and anti-ZO-1 staining (B, D) for tight junction. Primary cultures were grown to confluent by 4 days after seeding (A, B), and RGM-1 cells were grown confluent enough by 14 days after seeding on Transwell filters (C, D). Those cells were fixed directly on the filter, incubated with each specific antibody, and evaluated by a fluorescence microscope. Note that the localization of the both occludin and ZO-1 was observed at the entire region of cell-cell contact in primary cultures from the rat stomach, yet occludin, but not ZO-1, did not localize at the cell-cell contact region in RGM-1 cells. Bar (A, B, C, D)=20 μ m.

3.3. Allyl isothiocyanate alters tight junction barrier in primary cultures from rat stomachs

To determine the effects of pungent ingredients such as allyl isothiocyanate and capsaicin on tight junction barrier, we next examined TER and the mannitol flux using confluent cultures at 4 days after seeding. Allyl isothiocyanate and capsaicin are applied into the apical side (luminal or top compartment), because the apical side of gastric epithelial cells would be exposed to those pungent ingredients when people intake condiments such as wasabi, mustard oil, chili etc. It was found that 100 μ M of allyl isothiocyanate induced a progressive decreased in TER in a time-dependent manner as compared with control (Fig. 3A). TER loss was apparent within 1 hr and fell by about 60 % following 3 hr of application. However, the lower concentration of allyl isothiocyanate (1 and 10 μ M) did not affect TER. In contrast, capsaicin failed to affect TER in primary cultures from the rat stomach, even at high concentration (300 μ M) of capsaicin (Fig.3C). Permeability was inversely correlated to TER, where cells applied with 100 μ M of allyl isothiocyanate produced most permeable cultures among all groups (Fig. 4A and B). Permeability is no change when the lower concentration of allyl isothiocyanate (10 μ M) and 300 μ M of capsaicin were applied.

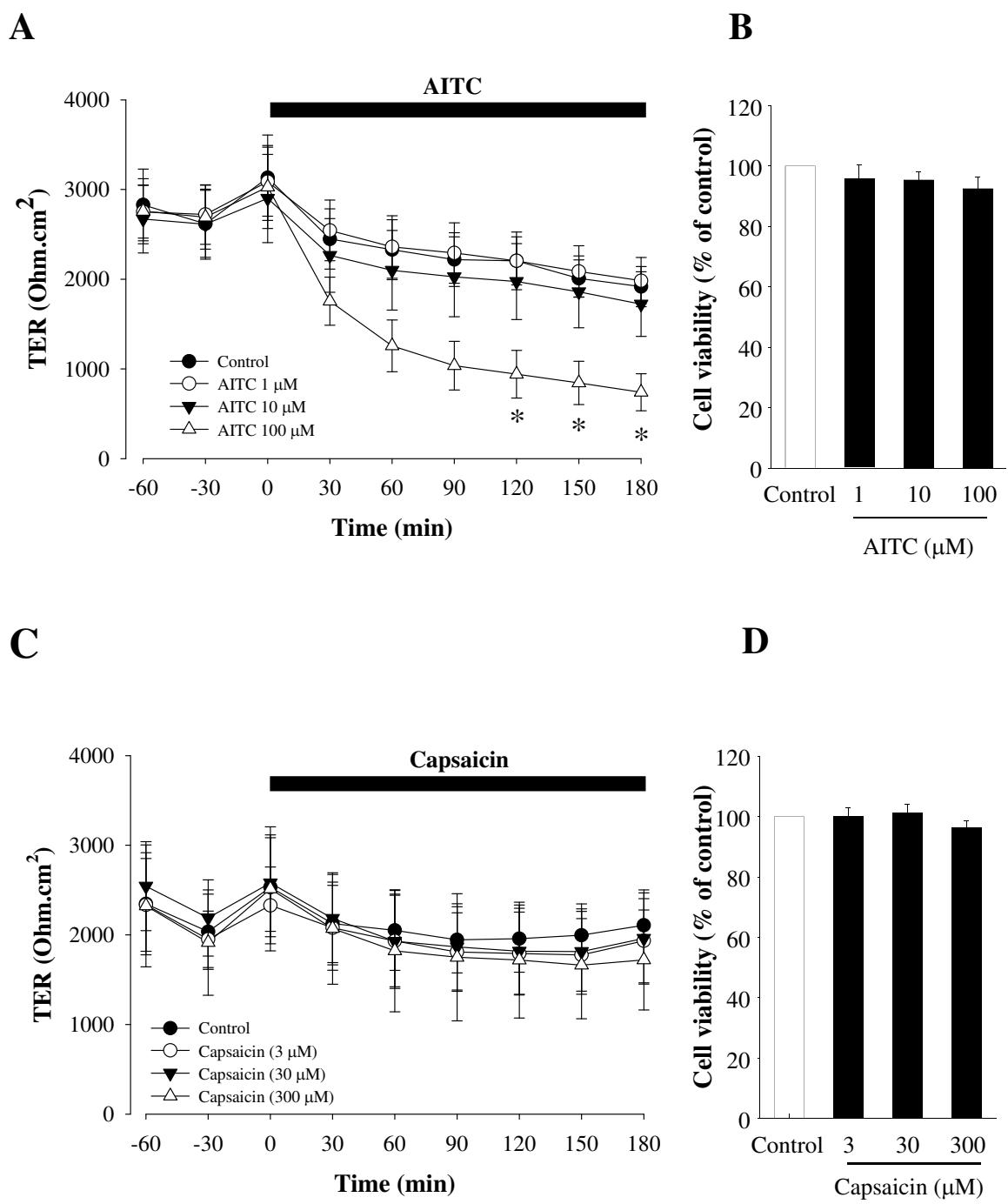
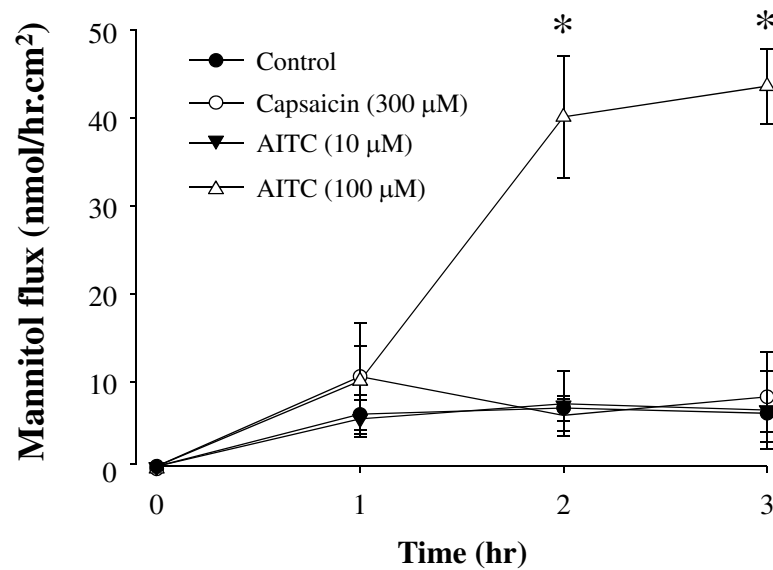


Figure 3. Effect of allyl isothiocyanate and capsaicin on TER and cell viability in primary cultures from the rat stomach. Transwell-grown primary cultures from the rat stomach were incubated for 3 hr with allyl-isothiocyanate (A, B) and capsaicin (C, D) each at the indicated concentrations in the apical side. Data for the time-course analysis of TER (A, C) and cell viability (B, D) represent means \pm S.E.M. from 3~6 independent experiments. * $P < 0.05$ by ANOVA with Dunnett's multiple comparisons test compared with control. Note that the high dose (100 μ M) of allyl-isothiocyanate in the apical side produced a significant decrease in TER in primary cultures from the rat stomach with no affecting cell viability, although the lower dose of allyl-isothiocyanate (1 and 10 μ M) did not any alter in TER and cell viability. In addition, notice that capsaicin did not affect TER and cell viability in primary cultures.

A



B

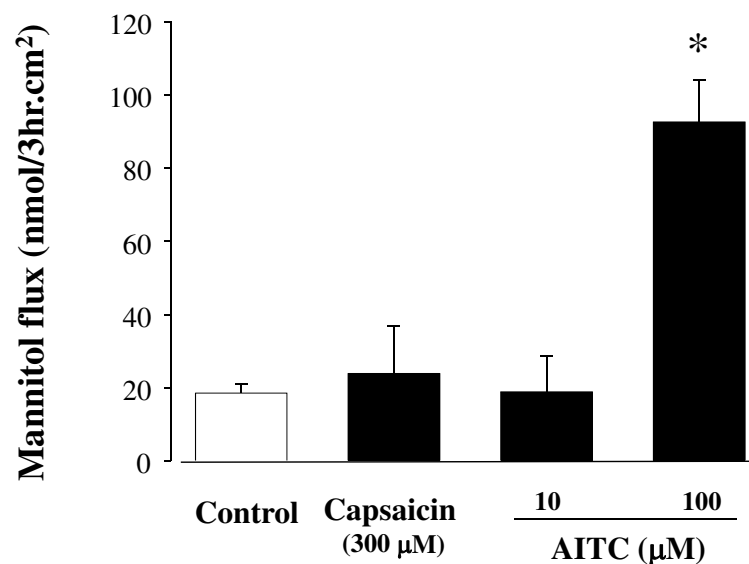


Figure 4. Effect of capsaicin and allyl isothiocyanate on ^3H -mannitol flux in primary cultures from the rat stomach. Isolated cells from the rat stomach were grown to confluence at 4 days after seeding on Transwell filters. The permeability of ^3H -mannitol (MW=182.17) was measured as the flux from the apical to basolateral chambers each hour for 3 hours. **A:** data indicate the ^3H -mannitol flux and presented as means \pm S.E.M. of values determines every 1 hr after apical application of capsaicin and allyl isothiocyanate from 3 independent experiments. *P<0.05 by ANOVA with Dunnett's multiple comparisons test compared with control. **B:** data show total ^3H -mannitol flux for 3 hr after apical application of capsaicin and allyl isothiocyanate from 3 independent experiments. *P<0.05 by ANOVA with Dunnett's multiple comparisons test compared with control. Note that primary cultures from the rat stomach show a low permeability in nature, however the apical application of high concentration of AITC (100 µM), but not low concentration of AITC (10 µM) and capsaicin, produced a high permeable monolayer in primary cultures from the rat stomach.

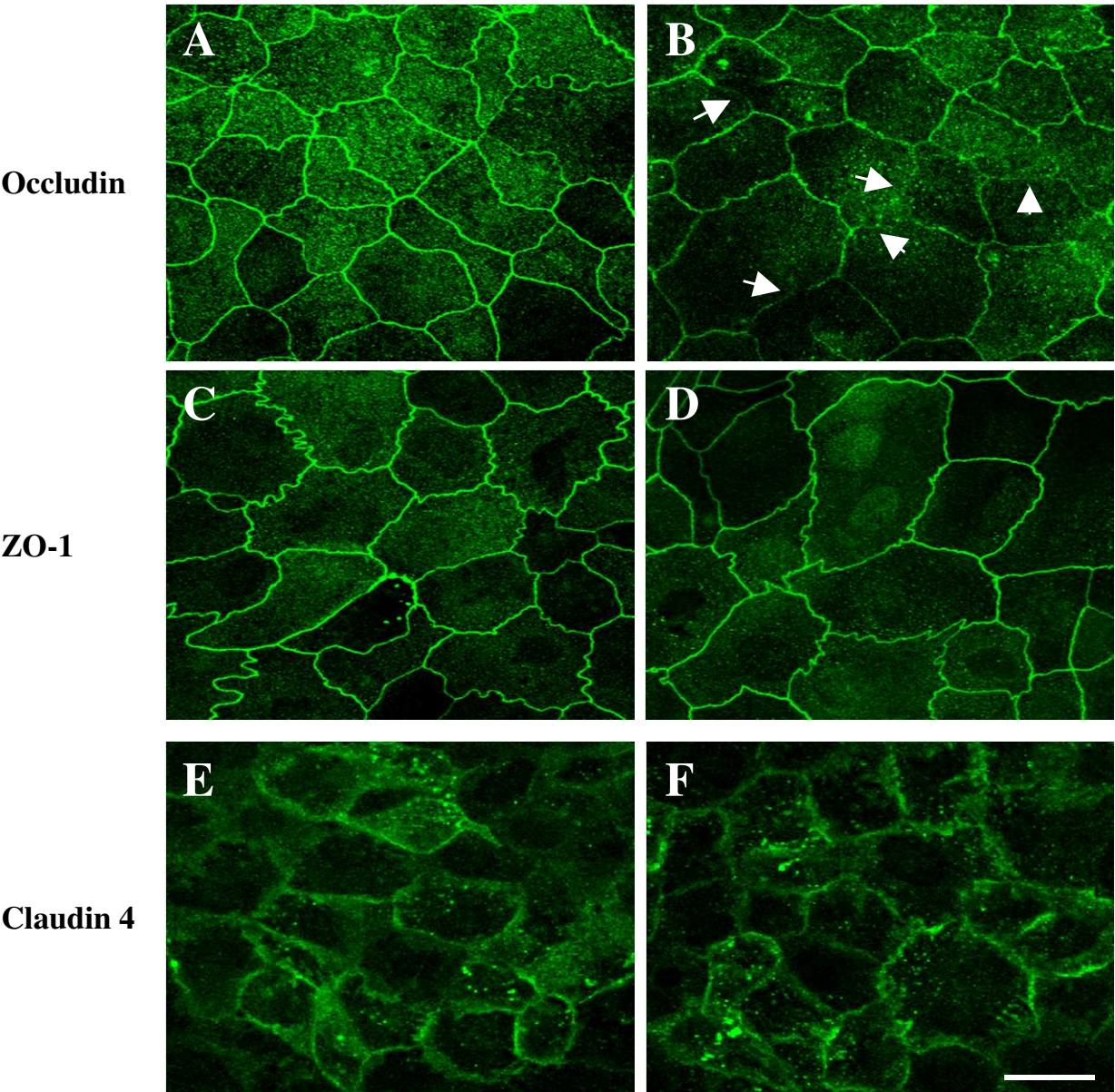


Figure 5. Discontinuous expression of occludin in response to high concentration of allyl isothiocyanate (AITC) in primary cultured epithelial cells from the rat stomach. The green label is anti-occludin antibody (A, B), anti-ZO-1 antibody (C, D), or, anti-claudin 4 antibody (E,F) staining for tight junction. AITC (100 μ M) or control (containing 0.1% ethanol) was added for 3 hr in the apical side of transwell-grown primary cultures. Cells were fixed directly on the transwell filter, followed by incubation with anti-occludin antibody, anti-ZO-1 antibody, or, anti-claudin 4 antibody, and evaluated by fluorescence microscope. Note that the continuous expression of occludin, ZO-1, and, claudin 4 at the cell-cell contact region in primary cultures were observed in control, yet the application of AITC (100 mM) induced the discontinuous expression of occludin, but not ZO-1 and claudin 4, at the cell-cell contact region (arrows). Bar=20 μ m.

To confirm the loss of tight junction barrier in response to allyl isothiocyanate, we assessed localization of ZO-1, occludin, and claudin 4 for tight junctions in primary cultures by immunocytochemistry. Those proteins are well-characterized tight junction components in the stomach and has been reported that dislocation of tight junction proteins lead to the pathogenesis of gastrointestinal tract such as *H. pylori*-induced gastritis [29]. When vehicle

was applied for 3hr, ZO-1, occludin, and claudin 4 were located to the cellular margins (Fig. 5A, C, and E). In contrast, the distribution of occludin was aberrant with discontinuous expression of the cell-cell contact point, when 100 μ M of allyl isothiocyanate was applied for 3 hr (Fig. 5B). However, the distribution of ZO-1 and claudin 4 applied by allyl isothiocyanate was found no deference as compared with vehicle-treated groups (Fig. 5D and F).

To determine that the decreased TER and the mannitol flux were not due to allyl isothiocyanate-induced alterations in cell viability, we measured the cell viability of primary cultures at the end of each experiment. Fig. 3B and D demonstrate that primary cultures with allyl isothiocyanate or capsaicin did not significantly alter cell viability, suggesting that in this system, TER loss following application of allyl isothiocyanate is likely due to modulation of tight junction barrier.

3.4. Allyl isothiocyanate-induced disruption of tight junction barrier is produced via TRPA1-independent pathway

Recently, allyl isothiocyanate is known as an activator of transient receptor potential A1 (TRPA1), which is considered to be a chemosensor in several sensory tissues including gastrointestinal tract [30]. Additionally, it has been well known that TRPA1 is co-expressed with TRPV1, which is ion channel targeted by capsaicin, on neuronal cells such as dorsal root ganglia and trigeminal ganglia. Therefore, to investigate the possibility that loss of tight junction barrier in response to allyl isothiocyanate was attributed to TRPA1 in primary cultures from rat stomachs, we examined (1) the mRNA expression of TRPA1 and TRPV1, and (2) the effect of cinnamaldehyde and icilin, other TRPA1 channel activators, on TER in primary cultures from rat stomachs. We found that not only TRPV1 mRNA (predicted size: 347 bp) but also TRPA1 mRNA (predicted size: 487 bp) were expressed in the primary cultures from rat stomachs (Fig. 6A). By contrast, TRPV1 mRNA was only expressed in RGM-1 cells (Fig. 6B), which is agreement with previous reports [31, 32]. In positive controls samples from the rat dorsal root ganglia, TRPA1 and TRPV1 mRNA were also observed (data not shown). The mRNA expression levels of glyceraldehyde-3-phosphate-dehydrogenase were unchanged among all tested samples so that the mRNA expression analysis by using RT-PCR was done appropriately, suggesting that TRPA1 and TRPV1 are periphery expressed at mRNA levels in native gastric epithelial cells in rats. Therefore, we investigate the effect of cinnamaldehyde and icilin, the other TRPA1 activators, on TER. When apical application of neither cinnamaldehyde (3-300 μ M) nor icilin (1-100 μ M) for 3 hr induced decrease in TER (Fig. 7A, C) and cell viability (Fig. 7B, D) in primary cultures from rat stomachs. In addition, TER reduction in response to 100 μ M of allyl isothiocyanate was not inhibited in the presence of 10 μ M of ruthenium red, which is a nonselective TRP channel inhibitor (data not shown) [33]. These results suggested that allyl isothiocyanate breaks tight junction barrier in primary cultures not via the pathway of TRPA1 channels activation.

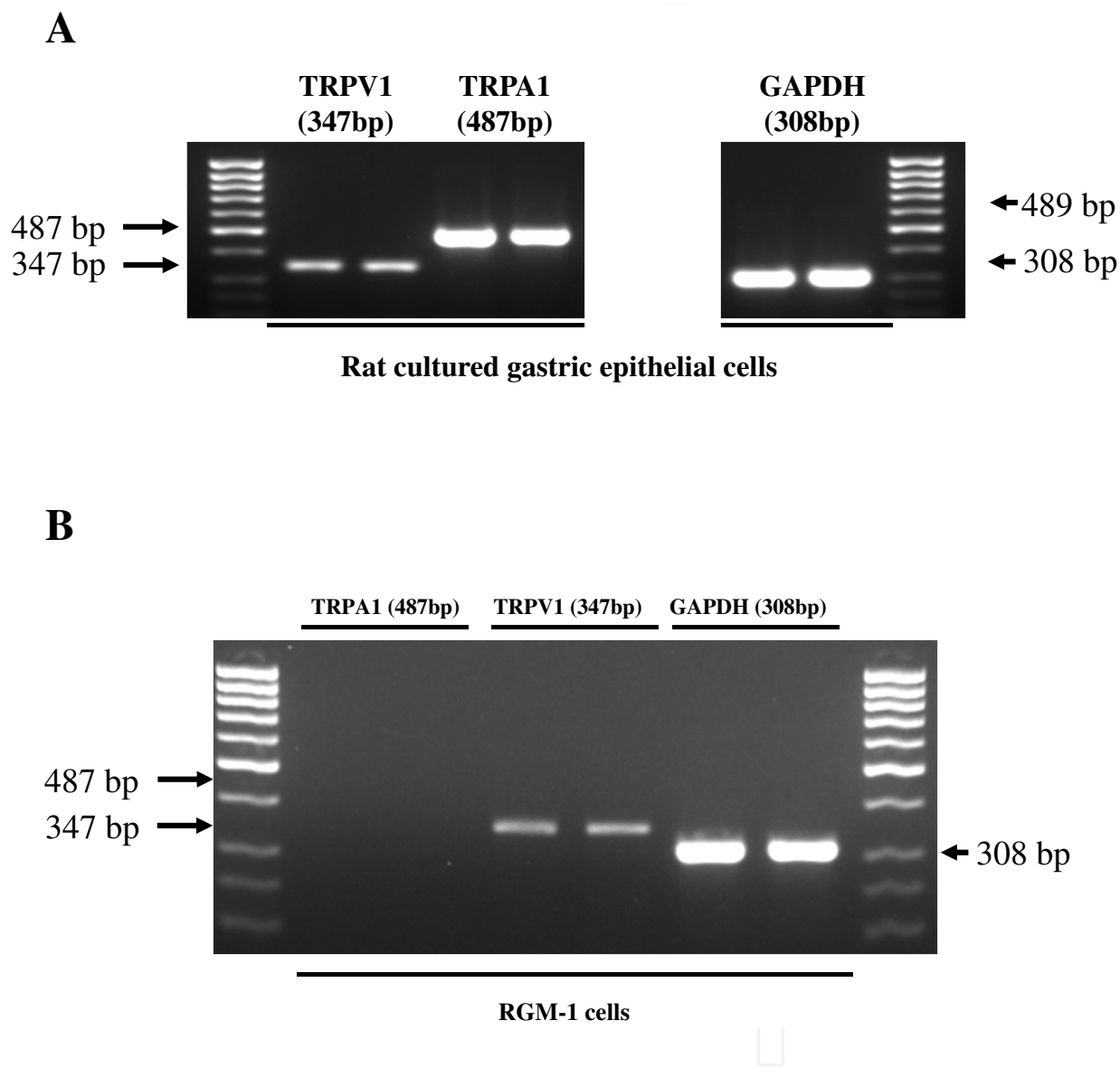


Figure 6. Expression of TRPV1, TRPA1, and, TRPM8 mRNA in primary cultures from the rat stomach, and the expression of TRPV1 and TRPA1 mRNA in RGM-1 cells by RT-PCR. Total RNA was isolated from rat dorsal root ganglia, primary cultures from the rat stomach, and RGM-1 cells. Data showed the mRNA expression of TRPV1 and TRPA1 in primary cultures from the rat stomach (A), and in RGM-1 cells (B). The mRNA levels were analyzed by PCR (35 cycles) using primers for TRPA1, TRPV1, and GAPDH. Rat DRG was used as positive control samples for TRPA1, TRPV1, and TRPM8 mRNA. Predicted sizes of PCR products are TRPA1 (487 bp), TRPV1 (347 bp), and GAPDH (308 bp), respectively. M: marker. GAPDH was the control for assay efficiency.

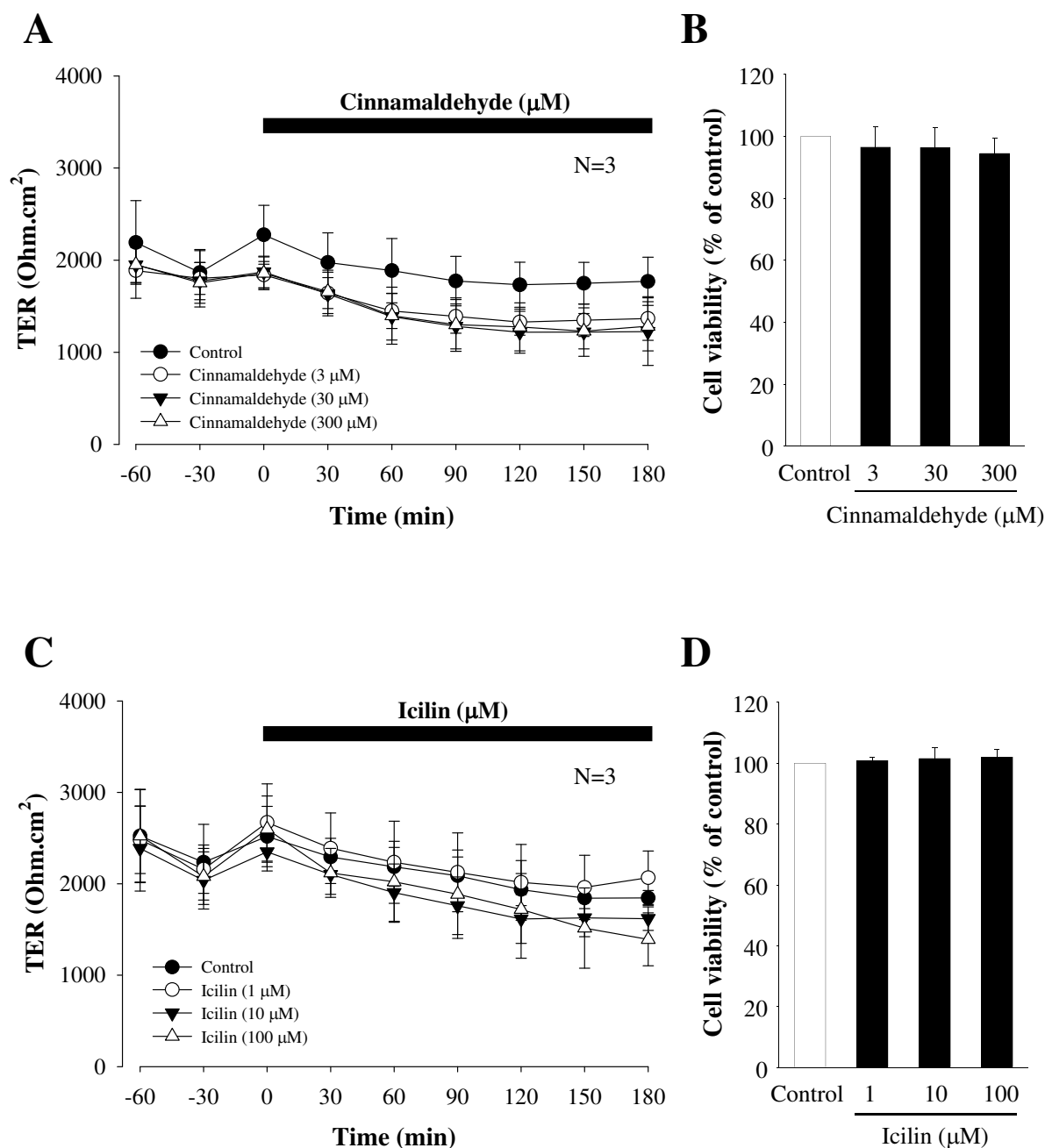


Figure 7. Effect of cinnamaldehyde and icilin, the other TRPA1 antagonist, on TER and cell viability in primary cultures from the rat stomach on AITC-induced TER reduction and cell viability in primary cultures from the rat stomach. Trans-well-grown primary cultures from the rat stomach were incubated for 3 hr with cinnamaldehyde (A, B) and icilin (C, D) each at the indicated concentrations in the apical side. Data for the time-course analysis (A, C) and cell viability (B, D) represent means \pm S.E.M. from 3 independent experiments. Note that the application of cinnamaldehyde and icilin, which are TRPA1 agonists, did not affect TER and cell viability.

4. Discussion

Previous investigations into mechanism by which noxious substances, including acid, *H. pylori*, non-steroidal anti-inflammatory drugs (NSAIDs), and dietary nitrate, disrupts the tight junctions barrier have been limited by the lack of testable gastric epithelial cells models that form functional tight junction barrier. The present study has utilized a biologically relevant in vitro model of the influence of condiments such as wasabi, mustard oil, and chilies on gastric epithelial cell to demonstrate that exposure of allyl isothiocyanate, a pungent ingredient of wasabi and mustard oil, induced a progressive loss of TER which is followed by disruption of occludin at the levels of the tight junctions, but not capsaicin which is a pungent ingredient of chilies. Our results have also identified TRPA1, which is activated by allyl isothiocyanate and are expressed in native rat gastric epithelia cells, is not involved in tight junction barrier dysfunction.

4.1. Confluent primary cultures with a great high TER

Isolated cells from the rat stomach, which are consisted of not only surface cells but also parietal cells, chief cells, and non-identified immature cells, were grown a confluent monolayer with a great high TER ($>2000 \text{ Ohm.cm}^2$), whose cultures are also identified as epithelial cells. In previous reports, TER in gastric mucosa was shown the highest in the gastrointestinal tract ($>2000 \text{ Ohm.cm}^2$) to prevent mucosal damages from exposure of luminal acid and pepsin [11], suggesting that tight junction barrier plays critical roles in gastric mucosal defenses as epithelial barriers. Although MDCK cells, T-84 cells, AGS cells, and MNK 28 cells [34] are frequently used to study aspect tight junction barrier, those cells are not of gastric origin or gastric adenocarcinoma cells so that those experimental data might not faithfully replicate the physiology of gastric epithelial cells. In addition, RGM-1 cells as a non-transformed cell line established from the rat stomach is a very useful cell culture models for investigation of gastric epithelial physiology [20, 28]. However, we found that RGM-1 cells is not able to form tight junction barrier with a high TER, although our results revealed that occludin expression was not located at cell-cell contact region in RGM-1 cells, whereas ZO-1 expression was continuously located at tight junctions regions. It has been reported that occludin-/-mice produced histological abnormalities in the gastric epithelium, which were complete loss of parietal and mucus cell hyperplasia, suggesting that occludin is involved in not only epithelial barrier formation but also epithelial differentiation in the stomach [35, 36]. It has been shown that the expression of tight junction proteins are at the cell-cell contact points which is identified as mature epithelial or endothelial cells in physiological condition, whereas down regulation of tight junction proteins is associated with survival and metastatic potential in human gastric cancer [37]. Shimokawa et al. [38] reported that RGM-1 cells had no secretory granules and the abundance of polyribosomes by using electron microscopic analysis, suggesting that RGM-1 cells are undifferentiated and proliferating mucous progenitor cells, not mature and differentiated mucous neck cells. In agreement with those previous reports, the lack of occludin expression at tight junction regions in RGM-1 cells is due to characterize as immature gastric epithelial cell line such as proliferating mucous progenitor cells. So that primary cultures from

the rat stomach was mimicked to the gastric epithelium, which is stand against noxious substances such as gastric acid and pepsin.

4.2. Allyl isothiocyanate, but not capsaicin, breaks tight junction barrier, which is independent on TRPA1

We next explored the alteration of tight junction barrier in response to allyl isothiocyanate and capsaicin in primary cultures from the rat stomach. That is because the digestive properties of capsaicin and allyl isothiocyanate were attributed to enhancement of digestive functions such as acid secretion [5], motility [39, 40], and mucosal blood flow [41, 42]. However, little is known those pungent ingredients of condiments affect gastric epithelial barrier. Although some reports showed that very high concentration of capsaicin (5 mM) in luminal side decreased in TER in the human intestinal epithelial cell line HCT-8 [43], there is no report about allyl isothiocyanate. In the present studies, it was observed the luminal application of capsaicin (300 μ M) did not have any changes in tight junction barrier, whereas the luminal application of allyl isothiocyanate at high concentration (100 μ M) induced the decreased tight junction barrier in primacy cultures from the rat stomach. Yet, the low concentration of allyl isothiocyanate (<10 μ M) did not provide any alteration of tight junction barrier in primary cultures. Indeed, the localization of tight junction proteins including occludin, ZO-1, and claudin 4 were continuously expressed at the cell-cell region in control group. However, the discontinuous expression of occludin, but not ZO-1 and claudin 4, was observed in the group treated with high concentration of allyl isothiocyanate in primary cultures, suggesting that dislocation of occludin provide the loss of tight junction barrier in response to allyl isothiocyanate.

It has been reported that TRPA1 is expressed primarily in small diameter, nociceptive neurons, where its activation likely contributes to a variety of sensory processes, including thermal nociception and inflammatory hyperalgesia [44]. TRPA1 is an excitatory ion channels targeted by irritant compounds derived from plants including wasabi, mustard, and cinnamon [45]. TRPA1 expression has been demonstrated in gastrointestinal tract [40, 46], especially it was showed that TRPA1 protein expression in gastric sensory neurons in rat using immunohistochemistry [47]. Although little was known about the expression of TRPA1 in non-neuronal cells, Nozawa et al. [48] and Kono et al. [49] recently reported that TRPA1 was expressed in serotonin-containing enterochromaffin cells in the rat small intestine and rat intestinal epithelial cells. These findings let us speculate that gastric epithelial cells express TRPA1 functionally. Interestingly, in the present study, TRPA1 mRNA was also found to be clearly expressed in primary cultures from the rat stomach which are epithelial cells identified with the anti-cytokeratine 8/18 antibody, although there was no observation of the expression of TRPA1 mRNA in RGM-1 cells. These data indicated that TRPA1 was located on not only gastric sensory neuron but also native gastric epithelial cells. Therefore, we asked if the loss of tight junction barrier in response to allyl isothiocyanate was mediated by TRPA1 in primary cultures. It was analyzed by using the other TRPA1 activator cinnamaldehyde and icilin [50], so that it was observed those TRPA1 activator did not provide the loss of tight junction barrier at all. Additionally, it was also observed allyl isothiocyanate-induced tight junction's alteration

was not dose-dependently, and was not inhibited by the pretreatment of a non-selective TRP blocker ruthenium red (data not shown), suggesting that the loss of tight junctions in response to allyl isothiocyanate was not mediated by TRPA1 in primary cultures of the rat stomach.

5. Conclusion

In conclusion, it is suggested that allyl-isothiocyanate breaks gastric tight junction barrier via TRPA1-independent pathways. In addition, we have established a unique technique to form the confluent primary cultures from rat stomachs with a great high TER.

Terminology

TJ: tight junction

RGM-1 cells: rat gastric epithelial cell line

TER: Transepithelial electrical resistance

TRPV1: transient receptor potential vanilloid type 1

TRPA1: transient receptor potential ankyrin 1

ZO-1: zonula occludens-1

S.E.M: standard error of mean

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