Subepithelial Serotonin Reduces Small Intestinal Epithelial Cell Tightness via Reduction of Occluding Expression

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ABSTRACT

Background: *The precise pathogenesis of irritable bowel syndrome (IBS) remains unresolved; however, recent studies have reported that patients with diarrhea-predominant IBS exhibit an increased small intestinal permeability and increased number of enterochromaffin cells containing high 5-hydroxytryptamine (5HT; serotonin) levels. In this study, we investigated whether 5HT has the potential to modulate small intestinal epithelial cell permeability, focusing on tight junction-associated proteins.*

Methods: *The differentiated Caco-2 cell monolayer on porous filters (Millicell) was used. Then, 5HT was added to the lower Millicell compartment for 7 days. Intestinal epithelial cell permeability was assessed by measuring the flux of paracellular permeability markers. We further assessed the expression of occludin in the 5HT-stimulated Caco-2 monolayer.*

Results: *We found that 5HT did not affect the viability of Caco-2 cells at concentrations up to 100 μM during the experimental period. Administration of 5HT to the basal side of Caco-2 cells increased the flux of ^{3}H-labeled mannitol (182 Da) but did not increase that of FITC-dextran (4000 Da). Among the tight junction proteins, the expression of occludin was specifically decreased by stimulation with 5HT at a concentration of 100 μM.*

Conclusion: *In conclusion, excessive 5HT in the basal side increased the permeability of intestinal epithelial cells via reduction of occludin expression.*

Keywords: *Irritable bowel syndrome, serotonin, tight junction, epithelial cell, permeability*

INTRODUCTION

About 5-20% of people in the world have been reported to be affected by irritable bowel syndrome (IBS).[1,2] Particularly in Asia, the incidence of IBS has recently been increasing in young individuals.[3] IBS often interferes with daily life because of symptoms such as constipation, diarrhea, abdominal pain, and discomfort associated with frequent defecation. According to the Rome IV diagnostic criteria, IBS is classified into four types based on the frequency of defecation and stool properties; constipation-predominant IBS (IBS-C), diarrhea-predominant IBS (IBS-D), mixed type of IBS (IBS-M), and unclassified IBS (IBS-U).[4] Multiple factors are involved in the development of IBS, including not only diet and stress but also organ hypersensitivity, abnormal immune responses, and abnormal brain–gut interactions.[5] However, the precise pathogenesis of IBS is not clear.

Recently, the involvement of 5-hydroxytryptamine (5HT; serotonin) in the pathogenesis of IBS has been reported.[6-8] It is synthesized from tryptophan and stored mainly in the gastrointestinal tract. In the human body, 95% of 5HT is stored in enterochromaffin (EC) cells in the gastrointestinal tract, and the remainder is stored in central nerves and platelets in the blood.[9,10] The 5HT stored in the gastrointestinal tract is secreted from the basal membrane of EC cells following various stimulations[2,11] and reabsorbed by the intestinal epithelial cells and peripheral nerves via serotonin reuptake transporter, which maintains homeostasis of the 5HT concentration in the submucosal environment.[12,13] In this way, 5HT acts as a neurotransmitter,[12,13] however, its local effects in the gastrointestinal tract are unknown.

Aside from an increased concentration of 5HT in the small intestine, excessive peristalsis and increased permeability

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of the gastrointestinal system are thought to be involved in the pathogenesis of IBS-D. Previous in vivo studies have shown increased mucosal permeability of the small intestine in patients with IBS-D[5,14-16] as well as in patients with inflammatory bowel disease and those who exhibit diarrhea symptoms.[17] However, the relationship between increased 5HT concentration in the small intestine and mucosal permeability is not clear.

Therefore, we investigated the direct influences of 5HT, a neurotransmitter, on epithelial cell permeability using an in vitro model of the small intestine. Since mucosal permeability is closely related to the tight junction (TJ), TJ proteins' expression was also evaluated.

MATERIALS AND METHODS

Cell Line and Culture Medium

Caco-2 cells (purchased from the American Type Culture Collection, Manassas, VA), a well-differentiated human colorectal adenocarcinoma cell line, were grown in 75 cm^2 plastic flasks (Thermo Fischer Scientific, Waltham, MA, USA) at 37° in a humidified air 5% CO_2 atmosphere. The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA), 4.5 g/L glucose, 1% non-essential amino acids, 2% (v/v) L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco). The medium was replaced every 72 h. Caco-2 cells were cultured and differentiated into enterocyte-like cells for 3 weeks after confluence using a pore size of 0.4 μm in clusters of 24 or 12 wells of Millicell Hanging Cell Culture inserts (Millipore, Billerica, MA, USA). Cells were seeded at 2×10^5 cells or 5×10^5 per well and cultured until post-confluence and full differentiation.

Main Points

- *Recent studies have reported that patients with diarrhea-predominant irritable bowel syndrome exhibit an increased small intestinal permeability and increased number of enterochromaffin cells containing high serotonin levels. However, the relationship between the 2 phenomena is not clear yet.*
- *We investigated whether serotonin has a potential to modulate small intestinal epithelial cell permeability, focusing on tight junction- associated proteins using Caco-2 monolayer.*
- *We found that the excessive serotonin in the basal side directly increased the permeability of intestinal epithelial cells via reduction of occludin expression.*
- *The excessive serotonin might be a therapeutic target of irritable bowel syndrome.*

Reagents

DMEM/high-glucose, phosphate-buffered saline (PBS), and FBS were purchased from Invitrogen (Carlsbad, CA, USA). Fluorescein isothiocyanate (FITC)-labeled dextran (molecular mass of 4000 Da, FD-4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). ^{3}H-labeled mannitol was purchased from PerkinElmer (Waltham, MA, USA). The primary antibodies specific for TJ proteins (claudin-2, occludin, and zonula occludens-1; ZO-1), anti-actin antibody, and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). E-cadherin was obtained from R&D systems (McKinley Place, MN, USA).

Cell Viability

Various concentrations of 5HT (0, 10, and 100 μM) were added to differentiated Caco-2 cells in a 96-well plate. According to the manufacturer's instructions, cell viability was assessed using the Cell-Counting Kit-8 (WST-8 Assay Kit; Dojindo Laboratories, Kumamoto, Japan). Briefly, Caco-2 cells were washed twice with PBS, and WST-8 solution was added. Cells were then incubated for 1 h at 37°C, and the optical density of each well was read at 450 nm using a microplate reader (Spectra Max M2; Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed as a percentage of untreated control cultures.

Transepithelial Electrical Resistance

Transepithelial electrical resistance (TEER) was measured routinely before and after experiments using a Millicell-ERS meter (Millipore Corporation, Bedford, MA, USA) connected to a pair of chopstick electrodes to ensure the integrity of the intestinal epithelial monolayers formed on Millicells. When the TEER exceeded 700 Ω m^2, Caco-2 monolayers were used as fully differentiated and tightly integrated Caco-2 cells.

Paracellular Permeability

Paracellular permeability was assessed by the flux of FITC-conjugated dextran (FD-4) or ^{3}H-labeled mannitol across the Caco-2 monolayer from the apical to the basolateral chamber. The fluorescent intensity of FD-4 or the rate of radioactive counts of ^{3}H-labeled mannitol (count per minute) was measured by a fluorescent microplate reader (Spectra Max M2; Molecular Devices) or a liquid scintillation counter (Tri-Carb2810, PerkinElmer), respectively.

As a positive control, cytokine mix (a mixture of interferon-γ: IFN-γ 10nM and tumor necrosis factor-α: TNF-α 10nM; R&D systems) was used as a stimulant.[18,19]

Western Blotting

Caco-2 cells (5.0×10^5 cells/well) were cultured on permeable filter membranes with a pore size of 0.4 μm and a surface area of 1.1 cm^2 in clusters of 12 wells (Millicell Hanging Cell Culture). After 3 weeks, 5HT (0, 10, or 100 μM) was added to basal chambers and incubated for 7 days. Caco-2 cells were rinsed with ice-cold PBS twice, and the cell pellet was lysed with Lysis Buffer (Cell Lytic M; Sigma-Aldrich) and collected with a cell scraper. Cellular debris was removed by centrifugation at 14 000 *g* at 4°C for 15 min. Protein from the sample was electrophoresed on 10% SDS-PAGE gels for 30 min at 250 V and transferred to the nitrocellulose membrane (Invitrogen Japan, Tokyo, Japan) in 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20 at room temperature. Primary antibodies specific for the TJ proteins occludin, claudin-2, ZO-1, and E-cadherin were used. The immune complexes were detected by a Western Blot Luminol Reagent Kit (ECL Plus; GE Health Bio-Sciences, Tokyo, Japan), and quantification was performed using Image J software (National Institutes of Health, Bethesda, MD, USA)

Statistical Analysis

All statistical analyses were performed using two-tailed unpaired Student's *t*-tests with the Graph Pad PRISM 6 (GraphPad Software, SanDiego, CA, USA) statistical program. A *P*-value of less than .05 was considered statistically significant.

RESULTS

High-dose 5HT for 7 days did not decrease cell viability

As shown in Figure 1, 5HT at a concentration of up to 1 mM did not affect cell viability within 7 days, whereas 10 mM of 5HT for 7 days significantly decreased the cell viability. We used 10 and 100 μM 5HT in this study based on previous reports that the serum concentration of 5HT in humans is 0.2-2.6 μM.[20,21]

High-dose 5HT increased paracellular permeability

TEER was measured as an indicator of TJ formation. Caco-2 cells exhibiting TEER > 700 Ω m^2 were used in this experiment. As shown in Figure 2A, 5HT at a concentration of 10 or 100 μM or cytokine mixture (CM) did not affect TEER for 7 days.

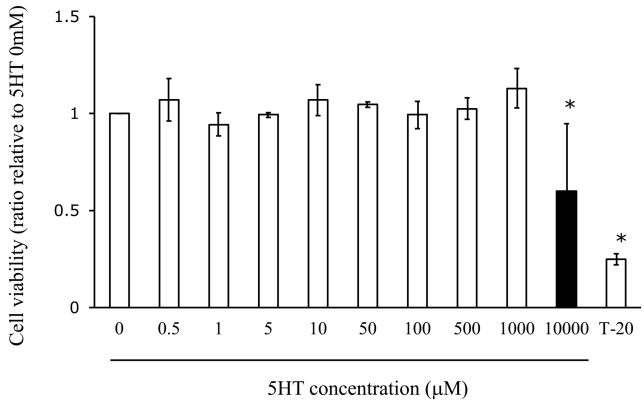

Figure 1. High-dose 5HT for 7 days did not decrease cell viability. Differentiated Caco-2 cells were treated with various concentrations of serotonin for 7 days, and the cell viability was determined using WST-8 assay. Data are presented as ratio relative to 5HT 0 μM (means + SEM of three independent experiments performed in triplicate). *P < .05 versus 5HT 0 μM. 5HT, serotonin; T20, Tween20 (positive control).

To assess the effect of 5HT on paracellular permeability, we used FITC-conjugated dextran (molecular weight of 4000 Da, FD4) and ^{3}H-labeled mannitol (183 Da) as flux indicators. We found that administration of 5HT to the basal side at a concentration of 10 μM did not result in higher FD4 or mannitol flux than that of the control group (Figure 2B and 2C). Although 100 μM of 5HT or CM did not increase FD4 flux, it significantly increased mannitol flux.

The exposure of 5HT on the apical side of Caco-2 cells did not affect these fluxes (data not shown).

5HT Specifically Reduced Occludin Expression

To clarify the underlying mechanism by which 5HT increases epithelial cell permeability, we assessed the expression of typical TJ structural proteins, occludin, claudin, ZO-1, and E-cadherin by the Western blotting. We found that 100 μM of 5HT results in significantly lower occludin expression than that of the control group ($P < .05$), however, no differences were observed for the other TJ proteins (Figure 3A and 3B).

DISCUSSION

In this nerve-free in vitro experiment, we found for the first time that 5HT directly induces the increase of small intestinal epithelial cell permeability via a reduction in occludin protein expression without affecting cell viability. Previous reports have indicated that 5HT exhibits various biological effects via receptors (5HT2B, 3, 4, and

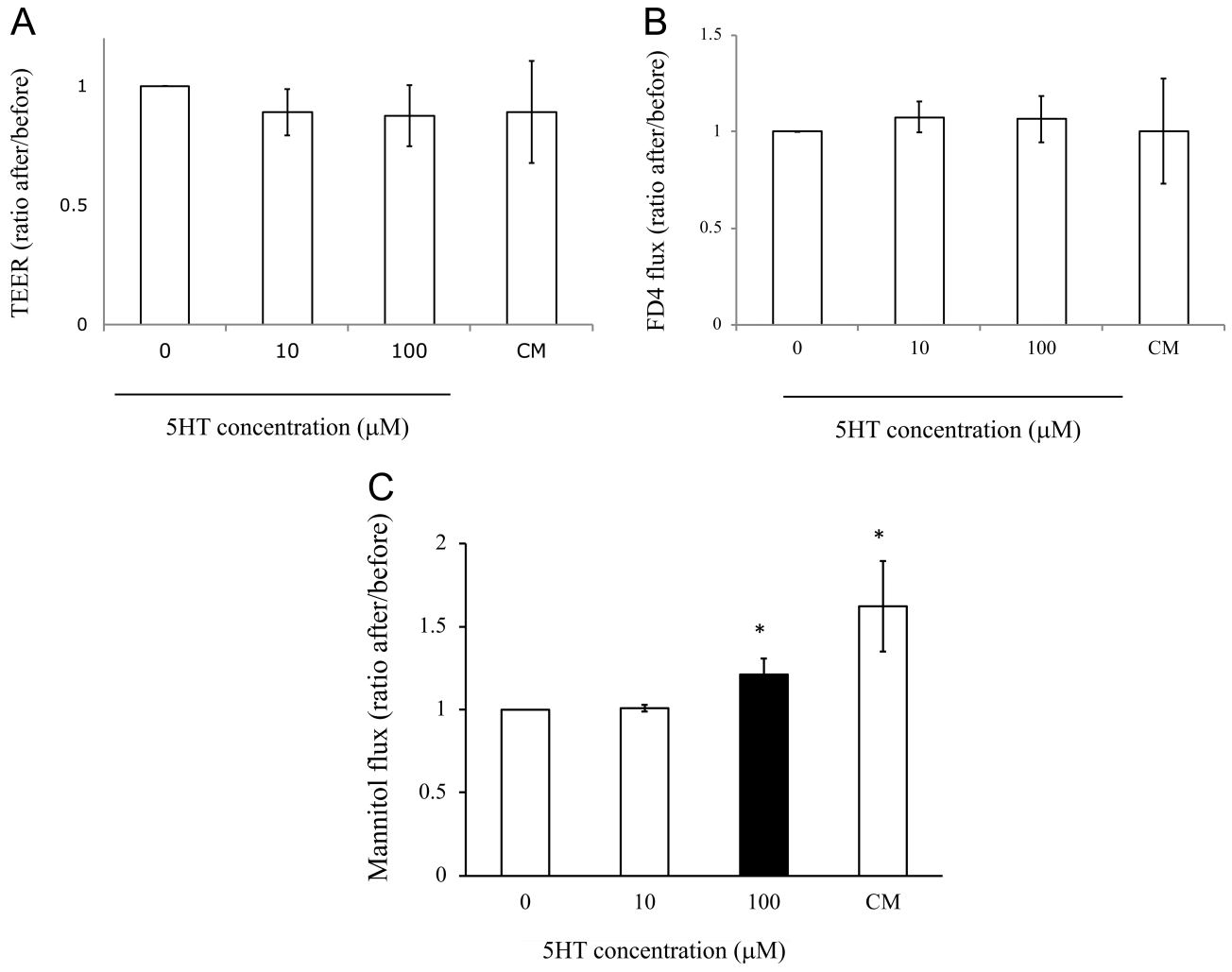

Figure 2. High-dose 5HT increased paracellular permeability. Transepithelial electrical resistance (TEER) was measured before and after the addition of 5HT for 7 days using a Millicell-ERS meter. The ratio of TEER after and before the addition of 5HT was compared (A). After stimulation with 5HT for 7 days, FD4 was added to the apical side of Caco-2 cells for 6 h, and the fluorescent intensity of FD4 flux to the basolateral chamber was measured by a fluorescent microplate reader (B). ^{3}H-mannitol was added to the apical side of Caco-2 cells for 3 h, and a liquid scintillation counter measured the mannitol flux to the basolateral chamber. Cytokine mixture was used as a positive control (C). In each graph, data are presented as ratio after 5HT addition relative to before 5HT addition (means + SEM of three independent experiments performed in triplicate.) *P < .05 versus before 5HT addition. 5HT, serotonin; CM, cytokine mixture.

7) on nerves, including the central nervous system,[22] and excessive 5HT are involved in diarrheal diseases' pathogenesis, such as IBS and ulcerative colitis.[23] Therefore, the relationship between 5HT and neurons has been extensively investigated. In this study, we showed that excessive 5HT directly affects the function of intestinal epithelial cells without affecting cell viability.

To prevent bacterial or bile acid intrusion into the intestinal submucosa, tightness of intestinal epithelial cells is necessary. In this study, we assessed epithelial cell permeability using TEER, FD4 flux, and mannitol flux. Although 5HT did not alter TEER or FD4 (4000 Da) flux, it significantly increased the flux of mannitol (182 Da), suggesting that 5HT selectively increased epithelial cell permeability based on molecular weight without affecting cell viability. Regarding the mechanism of this size-selective disruption of epithelial cell integrity, only a few reports, including ours[24,25,26] have been reported, and the precise mechanism of this phenomenon is not clear.

Since the tightness of epithelial cells is mainly controlled by TJ-associated proteins, we investigated the effect of 5HT on the expression of TJ-associated proteins

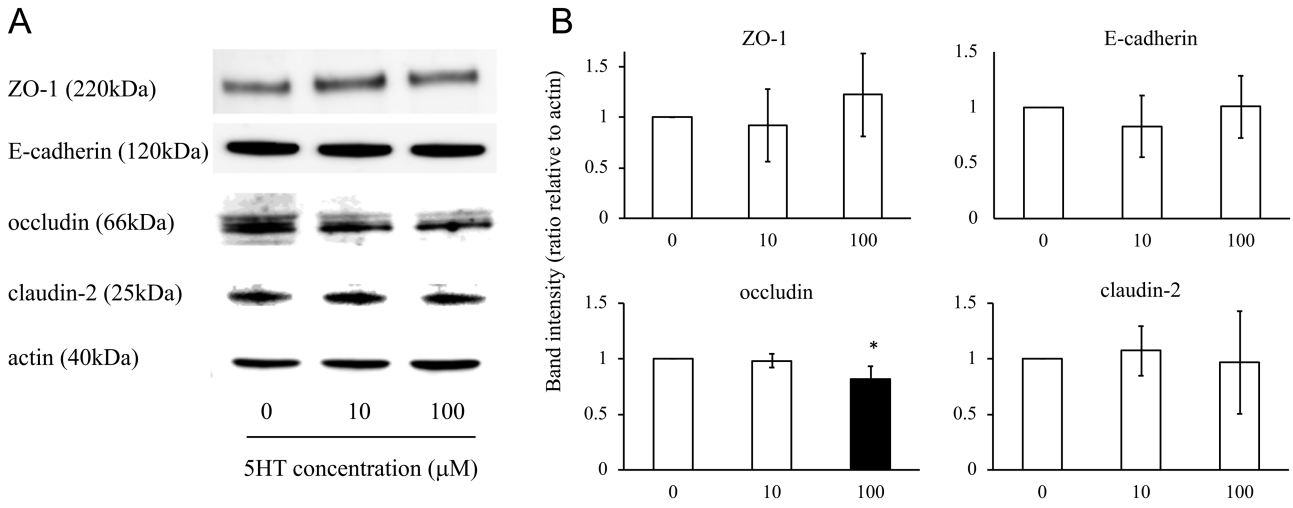

Figure 3. 5HT specifically reduced occludin expression. Differentiated Caco-2 cells were incubated with 5HT (0, 10, or 100 μM) on Millicell Hanging Cell Culture inserts for 3 weeks, and the expression levels of occludin, claudin-2, ZO-1, and E-cadherin were measured by Western blotting. Representative images of three western blots are presented (A). The expression of each protein was also quantified by densitometry with actin as a control (B). The densitometry values are shown as a ratio to the band intensity of actin (means + SEM of three independent experiments performed in triplicate). *P < .05 versus 5HT 0 μM.

and found that 5HT specifically reduces the expression of occludin without affecting the expression of other TJ-associated proteins. The mechanism behind this phenomenon is not clear. In our preliminary study, 5HT induces oxidative stress in Caco-2 cells (data not shown). Among amino acid residues of TJ-associated proteins, the sulfur-containing amino acids, cysteine, and methionine are particularly susceptible to oxidative stress[27] and the cysteine residue of occludin has been reported to be modified by oxidative stress.[28] Therefore, we speculated that 5HT-induced oxidative stress might modify the amino acid residue of occludin, resulting in the reduction of occludin expression. Interestingly, our previous study showed that aspirin, an analgesic, specifically reduces ZO-1 expression by oxidative modification of cysteine but not that in occluding,[29] suggests that the type of generated reactive oxygen species differs depending on stimulants the different species might modify different amino acid. However, this issue should be studied further.

One limitation of this study is that we used differentiated Caco-2 cells, which reportedly serve as a structural and functional model of the normal small intestine and suitable to assess the barrier function. Although Caco-2 is originally a colon cancer cell line, TJ-associated protein has been shown to involve in cancer cell invasion or migration.[30] Therefore, the results of this study may not reflect the biological conditions even if TJs is sufficiently formed. Second, we used a relatively higher concentration of 5HT than the reported concentration of 5HT in the blood. Since the 5HT concentration at the basolateral side of the epithelium in the human body cannot be measured, it is not clear whether the concentration employed in this study is adequate. Third, as previously mentioned, the mechanism by which 5HT reduces occludin expression is not clear. We speculated that 5HT-induced oxidative stress might be involved in this phenomenon, but future experiments are necessary to elucidate the precise mechanism. Fourth, in this study, we investigated the expression of TJ-associated proteins at 7 days after 5HT addition. Since the expression of TJ-associated proteins is dynamically changed overtime,[29] the expression and the role of another TJ-associated protein other than occludin might be involved at shorter time after 5HT stimulation.

Taken together, excessive 5HT in patients with IBS-D might directly reduce intestinal epithelial cell integrity via the reduction of occludin.

Ethics Committee Approval: The study was conducted at Kyoto Prefectural University of Medicine using commercially available cell line. Therefore, no ethical statement is available.

Informed Consent: The study was conducted at Kyoto Prefectural University of Medicine using commercially available cell line. Therefore, no informed consent is available.

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Author Contributions: Concept – OH, YN; Design – OH, YN, AS; Supervision – OH, YN, YI; Data Collection and/or Processing – HH,

MA, YY, YU; Analysis and/or Interpretation – OH, HH, TI, TT; Literature Search – HH, KK, KK, KU; Writing Manuscript – HH, OH; Critical Review - YN, AS, YI.

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Conflict of Interest: The authors have declared that no conflicts of interest exist.

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