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Water intake releases serotonin from enterochromaffin cells in rat jejunal villi

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Running title: Serotonin controls mesenteric lymph formation

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Abbreviations: ethylenediaminetetraacetic acid, EDTA; innate lymphoid cells, ILC; interleukin, IL; 5-hydroxytryptamine, 5-HT; tryptophan hydroxylase 1, Tph 1; IL-7 receptor, IL-7R; regulatory T cells, Treg; physiological saline solution, PSS; fluorescence-activated cell sorting, FACS; RAR-related orphan receptor-γt, RORγt; antibody, Ab; phycoerythrin, PE; phosphate buffered saline, PBS; quantitative reverse transcription–polymerase chain reaction, qRT-PCR; nitric oxide, NO; standard errors of the mean, SEM; not significant, NS

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Abstract

The present study aims to investigate the roles of water intake in serotonin production and release in rat jejunum. We evaluated the changes in concentrations of serotonin in the portal vein and mesenteric lymph vessel induced by the intragastric administration of distilled water. The density of granules in enterochromaffin cells and the immunoreactivity of serotonin in the jejunal villi were investigated before and after water intake. The effects of intravenous administration of serotonin and/or ketanserin on mesenteric lymph flow and concentrations of albumin and IL-22 in the lymph were also addressed. Water intake increased serotonin concentration in portal vein but not in the mesenteric lymph vessel. The flux of serotonin through portal vein was significantly larger than that through mesenteric lymph vessel. Water intake decreased the density of granules in the enterochromaffin cells and increased the immunoreactivity of serotonin in the jejunal villi. The intravenous administration of serotonin increased significantly mesenteric lymph flow and the concentrations of albumin and IL-22; both were significantly reduced by the intravenous pretreatment with ketanserin. We showed that serotonin released from enterochromaffin cells by water intake was mainly transported through portal vein. Additionally, serotonin in blood was found to increase mesenteric lymph formation with permeant albumin in the jejunal villi via the activation of 5-HT_2 receptor.

New & Noteworthy

Water intake released serotonin from enterochromaffin cells in rat jejunal villi. The serotonin was transported mainly through the portal vein. Intravenous administration of serotonin increased mesenteric lymph volume and concentration of albumin in the lymph, suggesting that serotonin in blood regulates physiologically mesenteric lymph formation. The responses of serotonin were produced via an activation of 5-HT_2 receptor. Serotonin controls the innate immunity by the upregulation of IL-22 mRNA in ILC-3.

Introduction

Mesenteric lymph vessels in many kinds of animals show heart-like spontaneous contractions, resulting in active lymph transport [2, 21, 24, 25]. The mesenteric lymph vessels have thick smooth muscle layers in their walls which are innervated by autonomic nervous fibers [19, 22, 23, 25]. To keep the spontaneous contractions of smooth muscles, vasa vasorum are penetrated into just under endothelial layers of the lymphatic wall, supplying oxygen to the cells in the wall [19]. Albumin, permeating through the venular walls of the jejunal microcirculation into the interstitial fluid, enhances mesenteric lymph formation by increasing the colloid osmotic pressure [18, 25, 28, 32]. However, the detailed mechanisms for the physiological regulation of lymph formation in mesenteric lymphatic system remain unsolved. From pharmacological points of view, serotonin is one of most potent vasoconstrictors in mesenteric lymph vessels [20, 25], resulting in acceleration of the lymph transport with increasing the rhythmic contractions of lymphatic smooth muscles. However, there is no study on the serotonin-mediated regulation of lymph formation in mesenteric lymphatic system.

The traditional Japanese health care system recommends that a suitable volume of water should be consumed every day by drinking green tea or eating miso-soup [9]. However, physiological and immunological mechanisms in support to this practice are unknown. We previously conducted experiments in rats and rabbits to investigate the effects of water intake on the jejunal-originated lymph flow, the concentrations of albumin, and the activation of type 3 innate lymphoid cells (ILC3)-mediated secretion of interleukin 22 (IL-22) in the lymph [18]. In that study, water intake increased significantly mesenteric lymph flow and the total flux of albumin and IL-22 in the lymph. In addition, we demonstrated that the water intake-dependent shear stress stimulation released ATP from myofibroblast cells in rat jejunum, and ATP increased IL-22 mRNA and the immunoreactivity of IL-22 in the jejunal villi [7].

Serotonin, a vasoactive substance, is a neurotransmitter and hormone that contributes to the regulation of various physiological functions in the central nervous system and their respective organ systems. Peripheral serotonin is predominantly produced by enterochromaffin cells in the intestinal tract [3, 5, 8, 14, 30, 37]. Furthermore, serotonin in blood is taken up and stored in platelets [29].

In this study, we aimed to evaluate roles of water intake on the release of serotonin from the enterochromaffin cells and the effects of water intake-dependent release of serotonin on IL-22 mRNA and the immunoreactivity of IL-22 in rat jejunal villi. Additionally, we evaluated the physiological role of blood serotonin on lymph formation in the jejunal microcirculation, along with the effects of water intake and a 5-HT_2 receptor antagonist, ketanserin, on changes in the concentration of platelets in the portal and femoral venous blood.

Materials and Methods

In Vivo Rat Experiments

All experiments in the present study were performed with the approval of the Shinshu University Animal Care and Use Committee.

Male Sprague–Dawley rats (10-12 weeks-old; Japan SLC, Tokyo, Japan) were fed a standard pellet diet and water ad libitum. Anesthesia was maintained with 2.0-3.0 % isoflurane (Dainippon Sumitomo Pharma, Tokyo, Japan), which was titrated to effect after tracheostomy. A catheter was inserted into the femoral vein to perfuse physiological saline solution (PSS; Otsuka Pharma, Tokyo, Japan). To minimize hemodynamic changes in the jejunal microcirculation, the intravenous infusion of PSS was stopped during experimentation [18].

To collect the blood of portal vein or the lymph from mesenteric lymph vessels, the abdomen was opened by cutting along the midline, and mesenteric adipose and connective tissues were removed to expose portal vein and jejunal-originated mesenteric lymph vessels. A very small venular catheter (0.22-0.26 mm, inner diameter) was inserted into the portal vein at the junction with spleen-originated branch of portal vein. The blood volume through portal vein was measured by both the Evans blue dye dilution method and the domestically made drop counter with larger venular catheter (0.95 mm, inner diameter) method in another *in vivo* experiment. In other rats, a small polyethylene catheter (0.5-0.6 mm, inner diameter) was inserted centripetally into the jejunal-originated mesenteric lymph vessel to collect the lymph. To evaluate the effects of water intake on the concentrations of serotonin in the poral vein and mesenteric lymph, intragastric administration of distilled water (3 mL, 15 or 45 min) was performed using a gastric tube inserted through the mouth to stomach. Distilled water was selected because a classical study demonstrated that 5 ml of distilled water injected into rat stomach produced a significant increase in lymph flow through the thoracic duct [31]. We used the distilled water in the previous studies of water intake [7, 18] according to the classical study protocol. Moreover, in the preliminary experiments, we confirmed that intragastric administration of PSS (3 mL) significantly increased the mesenteric lymph flow only 3-4 h after the administration. The delayed response of mesenteric lymph flow may be associated with factors such as the hemodynamics of jejunal microcirculation, movement in the small intestine, and discontinuation of intravenous infusion of PSS. Therefore, we selected the distilled water which produced a rapid (in ~ 15 min) increase in rat mesenteric lymph flow after intragastric administration. The volume of distilled water was determined by the maximum rat intragastric volume. The blood of portal vein and mesenteric lymph were collected at 1, 15, and 60 min after the administration of distilled water, and the blood and lymph concentrations of serotonin, albumin and IL-22 were measured.

Other *in vivo* experiments were conducted to evaluate the effects of intravenous administration of serotonin (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M, 0.3 mL, catalogue no 153-95-0, Sigma-Aldrich,

St. Louis, MO, USA), and/or a selective antagonist of the 5-HT_2 receptor, ketanserin (10^{-6}, 10^{-7} M, 0.3 mL, catalogue no K4138, Sigma-Aldrich, MO, USA) on mesenteric lymph volume and the concentrations of albumin and IL-22 in the lymph. These drugs were administered into the femoral veins.

Measurement of the concentrations of serotonin in portal venous blood and mesenteric lymph

The portal venous blood and mesenteric lymph were obtained by adding sodium citrate as an antithrombogenic agent followed by centrifugation. The supernatants were collected and measured the concentrations of serotonin using the serotonin assay kit with serotonin polyclonal antibody (ADI-900-175, Enzo Life Sciences, Lause, Switzerland).

Measurements of albumin and IL-22 in mesenteric lymph

The mesenteric lymph collected with heparin sulfate was centrifuged, and the supernatants were used to measure the concentrations of albumin and IL-22 using the rat albumin enzyme-linked immunosorbent assay (ELISA) kits (catalogue no AKAL-120, FUJIFILM Wako, Gunma, Japan) and a mouse/rat IL-22 Quantikine ELISA kit (catalogue no M2200, R&D Systems, Minneapolis, MN, USA), respectively.

Measurement of the concentration of platelets in the portal and femoral venous blood

The portal and femoral venous blood were collected with ethylenediaminetetraacetic acid (EDTA, 2 mg/mL) as an antithrombogenic agent. The concentration of platelets in the venous blood was measured using a blood cell counter (XT-2000iv, Sysmex, Kobe, Japan).

Cell isolation

Male Sprague–Dawley rats (10-12 weeks-old; Japan SLC, Tokyo) were fasted overnight and then were given water ad libitum for 2 h, which is suitable for evaluating whether or not the gene activation of ILC-3 was induced [18]. The rats were euthanized under anesthesia with isoflurane, and their small intestines were isolated. The jejunum was cut in half along the length of the small intestine. The preparation was washed with excess PSS to remove stools and the mucus, and then followed by incubation for 30 min at 37°C with vigorous shaking in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution supplemented with 5 mM EDTA and 1 mM dithiothreitol to remove the epithelial layer. To isolate lamina propria cells, the remaining the jejunum was chopped into small pieces with a scalpel and digested for 30 min at 37 °C with gentle shaking in RPMI-1640 medium supplemented with 5% fetal bovine serum, 0.5 mg/mL collagenase IV (Sigma, St. Louis, MO, USA), and 50 U/mL DNase (Wako, Tokyo, Japan). Cells were subjected to 40%-70% Percoll gradient centrifugation, and cells from the middle layer were harvested and used for the *in vitro* experiment.

Flow cytometry

Flow cytometry was used in order to isolate the non-T and non-B cells from the lamina propria

in jejunal villi, and mainly including ILC-3, which were evaluated for the IL-22 mRNA expression. The following fluorochrome-conjugated antibodies (Abs) were used for flow cytometry: phycoerythrin (PE) mouse anti-rat CD-3, PE anti-human/mouse RAR-related orphan receptor gamma t (RORγt), and PE rat Ig2A, K isotype control, were purchased from eBioscience (San Diego, CA, USA); fluorescein isothiocyanate mouse anti-rat CD-45R and PerCP/Cy5.5 anti-rat CD-45, were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). The Ghost Rad 780 viability dye was purchased from Tonbo Biosciences (San Diego, CA, USA). Cells were stained on ice in the presence of an anti-CD132 Ab to block Fc-mediated nonspecific staining. To detect RORγt and CD127 (IL-7Ra), the cells were loaded with anti-human/mouse RORγt Ab and CD127 Ab (catalogue no FAB8484R100, R&D Ltd., Pittsburgh, USA), respectively, and then developed with the PE-labelled anti-mouse Ig-G Ab according to the manufacturer's instructions. Rat T and B lymphocytes were identified using the mouse anti-rat CD-3 Ab and mouse anti-rat CD-45R Ab, respectively. Fluorescence-activated cell sorting (FACS) analysis was performed using a BD FACS Canto II flow cytometer (Ver1.1, Diva6.1, BD Bioscience, San Diego, CA, USA). The gating strategy for isolating ILC-3 from the cells in the lamina propria was as follows: 1) isolation of the live cells, 2) gating and isolation of CD-45 positive cells, and then 3) gating and isolation of CD-45R- and CD-3-negative and RORγt-positive cells.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The non-T and non-B cells, and mainly ILC-3 were collected as CD-45-positive, CD-45R- and CD-3-negative cells isolated the lamina propria of jejunal villi. The cells were confirmed to include ILC-3 by the presence of RORγt and CD127 markers using flow cytometry. Total RNA was extracted from the cells isolated from the jejunal lamina propria, using the Isogen reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. The Superscript first-strand synthesis kit (catalogue no AM1710, Invitrogen, Carlsbad, CA, USA) and 1.0 μg of total RNA were used to synthesize cDNA. The following primer pairs (SIGMA Genosis, Tokyo, Japan) were used for each specific reaction: IL-22 (NM 031144, 150 bp); β-actin (NM 00119188, 294 bp). cDNA was diluted five-fold before PCR amplification. Quantitative qRT-PCR was performed using a Light Cycler (Roche Diagnostics, Burgess Hill, UK) according to the manufacturer's instructions. Negative controls were included in each reaction, and the PCR products obtained with each primer pair was subjected to melting curve analysis. Data were analyzed with the Light Cycler analysis software or the ΔΔCt method analysis.

Immunohistochemistry

To evaluate the effects of intragastric administration of distilled water (3 mL) on serotonin, tryptophan hydroxylase 1 (Tph 1), or IL-22 immunoreactivities in the jejunal epithelial layers and lamina propria, the jejunum loaded with water intake during 15 for serotonin and Tph immunoreactivities or 45 min for IL-22 immunoreactivity were rapidly isolated and fixed with

4% paraformaldehyde in PBS overnight. The fixed samples were sectioned, then the slices were washed three times with PBS and incubated for 2 h at room temperature with primary polyclonal Abs to 1:5000 diluted FITC-labelled anti-serotonin (catalogue no S5545, Sigma-Aldrich, St Louis, USA), anti-Tph 1 (catalogue no bs-1215R, Bioss, Boston, USA), or 1:1000 diluted IL-22 (ab203211, abcam, Cambridge, UK). The specificity of antibodies was confirmed with the instrumental explanation in each catalogue. After being washed three times in PBS, the tissue slices were mounted with ProLong Gold antifade reagent and 4'-6-diamidino-2-2-phenylindole (DAPI, catalogue no P36935, Invitrogen) to counterstain the cell nuclei. The slices were examined with a fluorescent microscope (KEYENSE, BZ9000, Osaka, Japan), and photographed.

Enterochromaffin cells staining

To observe the enterochromaffin cells in the jejunum, Grimelius' method with $AgNO_3$ solution was used to identify the Ag-sensitive granules in the enterochromaffin cells. The jejunum was rapidly isolated and fixed with 4% paraformaldehyde in PBS overnight. The fixed samples were sectioned. The slices were washed three times with PBS and incubated at 50 ^{o}C for 1 min with 0.03% $AgNO_3$ solution. The slices were examined with the microscope (KEYENSE, BZ9000, Osaka, Japan), and photographed.

Density measurement

To quantitate the immunoreactivity data taken with the same brightness, high-resolution digital photomicrographs were processed using Scion Image analysis program [10]. The constant area of each photomicrograph was outlined on a gray scale image at the same density and processed for density measurement. The results are expressed in arbitrary units (mean density per pixel).

Drugs

All salts were obtained from Wako (Tokyo, Japan). Heparin sulfate was purchased from Mochida Pharmaceutical Co. (Tokyo, Japan). Serotonin hydrochloride, ketanserine tartrate salt, and methysergide hydrochloride were purchased from Sigma-Aldrich (St Louis, USA). Drug concentrations were described as the final concentration in PBS.

Statistical analysis

All results were expressed as the mean ± standard error (SEM). Statistical analyses were performed using Student's *t*-test for paired or unpaired results or one-way analysis of variance, followed by Duncan's post-hoc test, as appropriate. A value of $p < 0.05$ was considered statistically significant.

Results

Water intake accelerated serotonin release from enterochromaffin cells in rat jejunal villi.

To examine whether the intragastric administration of distilled water (3 mL, 15 min) releases serotonin in rat jejunal villi, we investigated the immunoreactivity of serotonin in the jejunal villi (Fig. 1A). As shown in the Figure 1A-1, the serotonin immunoreactivity was observed slightly in the interepithelial layer in the control without water intake. The intragastric administration of distilled water increased markedly the serotonin immunoreactivity in the jejunal lamina propria (Fig. 1A-1). Density analysis showed that water intake accelerated significantly the serotonin immunoreactivity (control, 99.3 $\pm$ 2.1 vs. water intake, 135.4 $\pm$ 3.9, n = 10, $p < 0.01$, Figure 1A-2).

In contrast, the tryptophan hydroxylase 1 (Tph 1) immunoreactivity, which is the regulatory enzyme for the production of serotonin in the enterochromaffin cells, was not markedly changed with water intake (3 mL, 15 min) (Fig. 1B-1). The right panel in the Figure 1B-1 demonstrates the specificity of the Tph 1 antibody in the photomicrograph stained without no antibody. Water intake produced no significant change in the Tph 1 immunoreactivity in the jejunal villi (control, 100.9 $\pm$ 5.4 vs. water intake, 112.1 $\pm$ 4.8, n = 10, NS, Fig. 1B-2).

Additionally, the Ag-sensitive black-colored granules in the enterochromaffin cells in jejunal villi clearly disappeared after water intake (Fig 1C-1). Density analysis showed that water intake significantly decreased the granules in enterochromaffin cells (control, 163.6 $\pm$ 4.5 vs. water intake, 131.9 $\pm$ 4.4, n = 10, $p < 0.01$, Figure 1C-2).

Water intake increased the concentration of serotonin and total flux of serotonin in rat portal venous blood.

Figure 2A-1 shows the effects of intragastric administration of distilled water (3 mL) on the concentration of serotonin in the portal venous blood. Serotonin concentration increased significantly in first 15 min after the administration (339.1 $\pm$ 46.6 ng/mL vs. control without water intake, 170.0 $\pm$ 22.5 ng/mL, n = 4, $p < 0.05$). The water intake-mediated increase in the serotonin concentration returned to normal 30 min after the administration (15-30 min, 220.1 $\pm$ 25.4 ng/mL, n = 4 vs. the control, NS; 30-45 min, 175.0 $\pm$ 27.8 ng/mL, n = 4 vs. the control, NS).

Figure 2A-2 demonstrates the effects of water intake (3 mL) on the blood volume through portal vein collected over the periods of 15 min. Similar to the changes in serotonin concentration, water intake significantly increased the venous blood volume collected during the first 15 min after water intake (control without water intake, 7.0 $\pm$ 0.2 mL/15 min, n = 4; 0-15 min, 10.0 $\pm$ 0.5 mL/15 min, n = 4 vs. control, $p < 0.01$; 15-30 min, 7.4 $\pm$ 0.4 mL/15 min, n =4 vs. control, NS; 30-45 min, 7.0 $\pm$ 0.3 mL/15 min, n = 4 vs. control, NS).

The total flux of serotonin through the portal vein, which is the multiplication of the concentration of serotonin and the venous blood volume together, was significantly augmented

with water intake (Fig. 2A-3; control without water intake, 1163.1 ± 146.8 ng/15 min, n = 4; 0-15 min, 3626.7 ± 165.4 ng/15 min, n = 4 vs. control, $p < 0.01$; 15-30 min, 1171.4 ± 104.3 ng/15 min, n = 4 vs. control, NS; 30-45 min, 1190.4 ± 144.3 ng/15 min, n = 4 vs. the control, NS).

In contrast, water intake (3 mL) caused no significant change in the concentration of platelets in the portal venous blood (control, 84.5 ± 3.2 x 10^4 /μL, n = 6; 0-15 min, 82.9 ± 1.6 x 10^4 /μL, n = 6; 15-30 min, 81.5 ± 1.9 x 10^4 /μL, n = 6; 30-45 min, 80.2 ± 1.4 x 10^4 /μL, n = 6; each value vs. control, NS).

Additionally, in the control experiments of rats inserted a gastric tube without water intake, we observed no change in the portal venous blood volume and the concentration of serotonin in the portal vein.

Water intake produced no significant change in the concentration of serotonin in rat mesenteric lymph.

Figure 2B-1 shows the concentration of serotonin in the mesenteric lymph collected over the periods of 15 min. The intragastric administration of distilled water (3 mL) produced no significant change in the concentration of serotonin for the mesenteric lymph during 0-45 min after water intake (control without water intake, 19.3 ± 1.7 ng/mL, n = 4; 0-15 min, 19.4 ± 1.6 ng/mL, n = 4; 15-30 min, 17.9 ± 1.2 ng/mL, n = 4; 30-45 min, 19.8 ± 0.6 ng/mL, n = 4; NS between each value).

To evaluate the mechanisms for no significant change in the concentration of serotonin, we investigated the effect of water intake (3 mL) on the mesenteric lymph volume collected over periods of 15 min (Fig. 2B-2). Similar to our previously demonstrated data [18], water intake significantly increased the mesenteric lymph volume during 0-15 min after the administration of distilled water (control without water intake, 43.0 ± 3.6 μL/15 min, n = 4; 0-15 min, 207.3 ± 9.3 μL/15 min, n = 4 vs. control, $p < 0.01$; 15-30 min, 45.1 ± 3.6 μL/15 min, n = 4 vs. control, NS; 30-45 min, 41.5 ± 3.1 μL/15 min, n = 4 vs. control, NS). Although water intake did not significantly affect the concentration of serotonin in the lymph, it accelerated the lymph volume through mesenteric lymph vessel.

Similar to the finding of the portal vein, the total flux of serotonin through the mesenteric lymph vessel increased significantly during 0-15 min after water intake (Fig. 2B-3; control without water intake, 807.3 ± 56.1 pg/15 min, n = 4; 0-15 min, 4100.7 ± 187.1 pg/15 min, n = 4 vs. control, $p < 0.01$; 15-30 min, 816.1 ± 41.7 pg/15 min, n = 4 vs. control, NS; 30-45 min, 806.8 ± 33.6 pg/15 min, n = 4 vs. control, NS). Compared with the total flux obtained with the portal vein, the total flux of serotonin through mesenteric lymph vessel was markedly lower.

In addition, in the control experiments of rats inserted a gastric tube without water intake, no significant changes in mesenteric lymph flow and the concentration of serotonin in the lymph vessel were observed.

Intravenous administration of serotonin increased the mesenteric lymph volume in a dose-dependent manner.

Figure 3A-1 demonstrates a representative recording for the effect of intravenous one-time administration of serotonin (10^{-5} M, 0.3mL) on the mesenteric lymph volume collected over the periods of 60 min.

Figure 3A-2 shows the summarized data (control, 100 % = 155.0 $\pm$ 42.5 μL/60 min; 0-60 min, 422.5 $\pm$ 113.4 %, n = 4 vs. the control, $p < 0.05$; 60-120 min, 112.5 $\pm$ 22.5 %, n = 4 vs the control, NS). The mesenteric lymph volume increased significantly in the first 60 min after the intravenous administration of serotonin. The increased lymph volume returned to around the control volume within 120 min.

Figure 3B is the dose-response relationship between intravenous one-time administration of serotonin and mesenteric lymph volume collected during the first 60 min after the administration (control, 100 % = 155.0 $\pm$ 42.5 μL/60 min; 10^{-5} M, 422.5 $\pm$ 113.4 % vs. the control, n = 4, $p < 0.05$; 10^{-6} M, 242.5 $\pm$ 40.3 % vs. the control, n = 4, $p < 0.05$; 10^{-7} M, 170.0 $\pm$ 44.2 % vs. 10^{-8} M, 120.0 $\pm$ 10.8 %, n = 4, $p < 0.05$, 10^{-8} M vs 10^{-6} M, n = 4, $p < 0.05$).

Intravenous administration of serotonin increased the concentrations of albumin and IL-22 in the mesenteric lymph.

The concentration of albumin in the mesenteric lymph was significantly increased in the first 60 min after intravenous one-time administration of 10^{-5} M serotonin (0.3 mL) and returned to approximately the control concentration within 120 min (Fig. 3C; control, 100 % = 15.9 $\pm$ 2.0 mg/mL; 0-60 min 178.5 $\pm$ 11.2 %, n = 4 vs. the control, $p < 0.01$; 60-120 min 79.0 $\pm$ 5.1 %, n = 4 vs. the control, NS).

Figure 3D is the summarized data for the effect of intravenous one-time administration of 10^{-5} M serotonin (0.3 mL) on the concentration of IL-22 in the lymph collected over 60 min. Similar to the finding of albumin, the serotonin produced a significant increase in the concentration of IL-22 in the lymph collected during the first 60 min after the administration (100 % = 447.5 $\pm$ 60.5 pg/mL; 0-60 min, 125.3 $\pm$ 9.9 % vs. the control, n = 4, $p < 0.05$; 60-120 min, 106.3 $\pm$ 10.9 % vs. the control, n = 4, NS). However, the intravenous administration of serotonin did not significantly change in the concentration of IL-22 in the lymph collected during 60-120 min after the administration.

Intravenous one-time administration of ketanserin significantly decreased the mesenteric lymph volume.

Figure 4A-1 shows a representative recording for the effect of intravenous one-time administration of 10^{-6} M ketanserin (0.3 mL) on the mesenteric lymph volume collected over 60 min. Ketanserin markedly decreased the mesenteric lymph volume collected from 0 to 120 min after the administration, as summarized in Fig. 4A-2 (control = 100 %, 178.7 $\pm$ 33.3 μL/ 60 min;

0-60 min, 27.3 ± 2.2 % vs. the control, n = 4, $p < 0.01$; 60-120 min, 31.0 ± 5.6 % vs. the control, n = 4, $p < 0.01$). Thus, the pharmacological effect of ketanserin concentration on the mesenteric lymph volume continues to be longer than that of serotonin.

Intravenous one-time administration of ketanserin significantly decreased the concentration of albumin, but not IL-22 in the mesenteric lymph.

The intravenous one-time administration of 10^{-6} M serotonin (0.3 mL) decreased the concentration of albumin in the mesenteric lymph collected during 0-120 min (Fig. 4B, control 100 % = 15.7 ± 0.5 mg/mL, n = 4; 0-60 min, 71.8 ± 6.8 % vs. the control, n = 4, $p < 0.01$; 60-120 min, 75.1 ± 5.2 % vs. the control, n = 4, $p < 0.01$).

In contrast, the concentration of IL-22 in the mesenteric lymph collected over 60 min was not significantly changed with the intravenous one-time administration of 10^{-6} M ketanserin (0.3 mL) (Fig. 4C, control 100 % = 407.5 ± 60.5 pg/mL; 0-60 min, 121.0 ± 18.0 % vs. the control, n = 4, NS; 60-120 min, 111.5 ± 11.6 % vs. the control, n = 4, NS).

Pretreatment with ketanserin suppressed the intravenous one-time administration of serotonin-mediated increases in mesenteric lymph volume and concentration of albumin in the lymph.

We conducted rat *in vivo* experiment to investigate whether intravenous pretreatment with ketanserin (10^{-6} M) inhibits the responses to intravenous one-time administration of serotonin (10^{-5} M, 0.3 mL). As shown in Figs. 1 and 2, the water intake released serotonin in the jejunal villi and increased mesenteric lymph volume from 0 to 15 min after the water intake; therefore, in the present experiment, the mesenteric lymph was collected over the periods of 15 min. Intravenous one-time administration of serotonin (10^{-5} M, 0.3 mL) with pretreatment of 10^{-6} M ketanserin did not change the mesenteric lymph volume in 0-15 min (Fig. 5A-1). Figure 5A-2 is the summarized data. The continuous intravenous treatment with ketanserin (10^{-6} M) completely suppressed the serotonin-mediated increase in mesenteric lymph volume (control, 100 % = 45.8 ± 4.6 μL/15 min, n = 4; 0-15 min, 93.9 ± 27.0 %, n = 4 vs. control, NS; 15-30 min, 93.9 ± 29.0 %, n = 4 vs. control, NS; 30-45 min, 80.9 ± 31.2 %, n = 4 vs. control, NS).

Figure 5B demonstrates the effect of continuous intravenous treatment with ketanserin (10^{-6} M) on the serotonin-mediated increase in the concentration of albumin in mesenteric lymph (Fig. 3C). Pretreatment with ketanserin slightly, but not significantly, decreased the serotonin-mediated increase in the concentration of albumin in the lymph collected over the periods of 0-30 min after the water intake. In contrast, during 30-45 min after the administration, the concentration of albumin in the lymph decreased significantly with the pretreatment of ketanserin (control, 100 % = 7.4 ± 1.6 mg/mL, n = 4; 0-15 min, 86.3 ± 9.8 %, n = 4 vs. control, NS; 15-30 min, 65.6 ± 16.6 %, n = 4 vs control, NS; 30-45 min, 50.3 ± 9.9 %, n = 4 vs control, $p < 0.01$).

Figure 5C shows the effect of continuous intravenous treatment with ketanserin (10^{-6} M) on

the serotonin-mediated changes in the concentration of IL-22 in mesenteric lymph. Unlike the data of Fig. 3D, pretreatment with ketanserin did not change the serotonin-mediated changes in the concentration of IL-22 in the lymph collected over the periods of 0-45 min (control, 100 % = 232.5 ± 18.0 pg/mL, n = 4; 0-15 min, 95.7 ± 9.0 %, n = 4 vs. control, NS; 15-30 min, 96.1 ± 10.7 %, n = 4 vs. control, NS; 30-45 min, 88.9 ± 11.4 %, n = 4 vs. the control, NS).

The intravenous administration of ketanserin (10^{-6} M) produced no significant change in the concentration of platelets in the femoral venous blood (control, 81.8 ± 1.0 x 10^4 /μL, n = 6; 0-15 min, 81.6 ± 0.4 x 10^4 /μL, n = 6; 15-30 min, 80.2 ± 0.5 x 10^4 /μL, n = 6; 30-45 min, 80.7 ± 1.0 x 10^4 /μL, n = 6; each value vs. control, NS).

Serotonin upregulated the IL-22 mRNA in ILC-3 in rat jejunal villi.

To evaluate critical roles of serotonin in the ILC-3-dependent regulation for mucosal immunity in the jejunal villi, we investigated the effects of serotonin on the IL-22 mRNA in ILC-3 in rat jejunal villi.

ILC-3 were isolated as non-T, non-B, and RORγt-positive cells (blue dots in Fig 6A-1) using flow cytometry. The percentage of number for RORγt-positive cells was 9.5 ± 2.1% (2.4 ± 1.1 x 10^6 cells, n = 8) of the total numbers of non-T and non-B cells. To confirm the RORγt-positive cells as ILC-3, we investigated the presence of CD127 marker on CD45- and RORγt-positive cells with flow cytometry. Figure 6A-2 shows the representative data for the CD 127 distribution on the CD45- and RORγ-positive cells. Most of the cells expressed CD 127. Thus, we confirmed that the non-T, non-B, and RORγt-positive cells included with ILC-3.

Using the non-T, non-B, and RORγt-positive cells, we evaluated the effect of serotonin on the IL-22 mRNA expression in the ILC-3. Serotonin at a concentration of 10^{-6} M significantly increased the expression of IL-22 mRNA in the ILC-3 (Fig. 6B, control, 1.00 ± 0.01 vs. serotonin, 1.27 ± 0.06, n = 30, $p < 0.01$).

Serotonin increased the immunoreactivity of IL-22 in jejunal villi.

To confirm the serotonin-mediated upregulation of IL-22 mRNA in ILC-3, we additionally investigated the effects of water intake (3 mL, 45 min) on the immunoreactivity of IL-22 in the jejunal villi with or without continuous intravenous treatment with ketanserin (10^{-6} M, 45 min) or a non-selective 5-HT receptor antagonist, methysergide (10^{-6} M, 45 min). In this study we used the stimulation of 3 mL water intake for 45 min because the 15 min water intake produced no significant increase in the immunoreactivity of IL-22.

As shown in Fig. 6C-1 & 6D-1, the water intake for 45 min markedly increased the immunoreactivity of IL-22 in the jejunal villi. Intravenous pretreatment with methysergide (Fig. 6C-1) markedly reduced the water intake-mediated increase in the immunoreactivity of IL-22. Figure 6C-2 shows the summarized data with density analysis (control without water intake, 113.6 ± 3.9, n = 10; water intake, 162.8 ± 3.7, n = 10 vs. control, $p < 0.01$; water intake in the

pretreatment with methysergide, 125.3 $\pm$ 2.2, n = 10 vs. water intake only, $p < 0.05$).

On the other hand, the intravenous pretreatment with ketanserin produced only a slight change in the water intake-mediated increase in the immunoreactivity of IL-22 (Fig. 6D-1). Figure 6D-2 is the summarized data with density analysis (control without water intake,106.9 $\pm$ 2.3, n = 10; water intake, 166.1 $\pm$ 4.5, n = 10, $p < 0.01$; water intake in the pretreatment with ketanserin, 153.9 $\pm$ 6.2, n = 10, vs. control, NS).

Discussion

Water intake accelerated serotonin release from enterochromaffin cells in rat jejunal villi.

In this study, we demonstrated that water intake (3 mL, 15 min) increased the immunoreactivity of serotonin in the lamina propria of rat jejunal villi (Fig. 1A). Additionally, the Ag-sensitive black-colored granules in the enterochromaffin cells in jejunal villi clearly disappeared after water intake (Fig. 1C). The findings suggest that serotonin may be released by the stimulation of water intake from the enterochromaffin cells in the jejunal villi. However, water intake produced no significant change in the levels of Tph 1 (the enzyme involved in serotonin synthesis) immunoreactivity in the enterochromaffin cells.

There exists an important question whether the 5-HT immunoreactivity in the lamina propria of jejunal villi may be related to the signal colocalized with platelets within blood vessels or the 5-HT signal bind with larger molecules in the lamina propria. Because 5-HT is a small molecule, it might be easily washed out in the washing steps during immunohistochemical staining. We confirmed that no platelets were observed in the lamina propria of jejunal villi and the mesenteric lymph vessel after the water intake. Therefore, we suggest that the 5-HT immunoreactivity in the lamina propria 15 min after the water intake (Fig. 1A-1) might be related 5-HT, which is bound to larger molecules such as fatty acids stored in the lamina propria [35, 39]. However, the possibility that the platelets within blood vessels in the lamina propria absorbed the released 5-HT and then contributed to be the 5-HT immunoreactivity in the jejunal villi, might be not able to rule out. We should be needed in the future to conduct additional experiments in order to clarify the possibility.

In the previous study, we reported that the endothelium was highly permeable for plasma albumin in rat jejunal microcirculation, which accelerated the transport of drained water into the lacteal vessels in the jejunal villi [18]. Hence, the combination of the drained water and the permeant albumin may produce higher bulk flow, resulting in the stimulation of the myofibroblast cells under the epithelial layer in the jejunal villi by mechanical force [7]. It is known that gut epithelial enterochromaffin cells are mechanosensitive and require the Piezo2 cation channel to convert mechanical force into serotonin release [1, 34]. Thus, water intake may produce great bulk flow within the jejunal lamina propria, which stimulated mechanical stress on the interepithelial enterochromaffin cells that released serotonin into the jejunal villi. However, the mechanisms for water intake-mediated serotonin release in enterochromaffin cells will be needed to investigate in the future.

In agreement with the findings, water intake produced a significant increase in the concentration of serotonin through the portal venous blood in the first 15 min after water intake (Fig. 2A). In the first 15 min after water intake, the blood volume through the portal vein also increased significantly. Similar to the portal blood volume, water intake significantly increased

mesenteric lymph volume in the first 15 min after water intake. The absolute value of increased mesenteric lymph volume was very small, being approximately 1/50 of the increased portal blood flow. However, the concentration of serotonin in the mesenteric lymph did not change during 0-45 min after water intake. Therefore, the total flux of serotonin through portal vein was approximately 1,000 times larger than that through mesenteric lymph vessel. The lower concentration of serotonin and the smaller amount of lymph volume through the mesenteric lymph vessel may be related to the present experimental protocol in which the lymph volume was measured with one mesenteric lymph vessel cannulated. Another possible mechanism by which the concentration of serotonin in mesenteric lymph is very low and unchanged with water intake may be related to the evidence that serotonin is easily conjugated with long-chain fatty acids [35, 39]. Hence, the high concentration of long-chain fatty acids in the mesenteric lymph [18] may contribute to maintaining a lower concentration of serotonin in the lymph. However, we should confirm in the future the hypothesis by measuring the concentration of free serotonin in the mesenteric lymph after chemical liberation of serotonin from the serotonin-fatty acid complex.

This finding may be related to the fact that the smooth muscles in the mesenteric lymph vessel walls are very sensitive to serotonin, compared with the other lymph vessels. Approximately 10 nM level of serotonin accelerated spontaneous contractions and ~ 1 μM level of serotonin-induced spastic contraction of the lymphatic smooth muscles, resulting the stop of active lymph transport [20, 25]. Thus, the lower level of serotonin concentration in the mesenteric lymph produced by long-chain fatty acids may become physiologically useful to maintain lymph propulsion through the lymph vessels.

Moreover, there is the evidence that the circulatory time of blood flow through the portal vein is faster than the lymph flow through mesenteric lymph vessels [24, 25]. This may produce a quick diffusion of interstitial fluid containing the released serotonin in the jejunal villi, which may contribute to producing the water intake-mediated increase in the concentration of serotonin through portal venous blood. Thus, water intake-released serotonin in rat jejunal villi is transported mainly through the portal vein into the systemic circulation. The conclusion may be supported by our finding that water intake produced no significant change in the concentration of platelets in the portal venous blood. The dramatic change in volume in the portal vein may not contribute to an increase in the concentration of serotonin in the portal vein through platelet activation and platelet serotonin release.

Serotonin is also known to be one of the vasodilation agents in the portal venous system, and thus regulates blood flow [4, 11]. Moreover, the mesenteric vein isolated from portal hypertensive rats are hyper-responsive to serotonin. Serotonin may play a crucial role in the pathophysiology of the hyperkinetic syndrome in patients with portal hypertension [11]. Thus, water intake may play a crucial role in the regulation of portal blood dynamics under physiological and

pathophysiological conditions through the activation of the serotonin release from the jejunal villi. **Serotonin circulating through systemic circulation may be a crucial regulator in the mesenteric lymph formation via the activation of 5-HT_2 receptors.**

The present study suggests that the water intake-released serotonin from enterochromaffin cells in rat jejunal villi transported mainly through the portal vein into systemic circulation. Based on these findings, we aimed to evaluate the physiological roles of serotonin circulating through systemic circulation in the jejunal blood and mesenteric lymph circulation in rats. Smooth muscles of mesenteric lymph vessels are markedly sensitive to serotonin [16, 17, 20, 25]. Serotonin generates contractions of the isolated bovine mesenteric lymphatics via the activation of the 5-HT_2 receptors [15]. 5-HT_4 receptor is also involved the endothelium-independent smooth muscle relaxation of the mesenteric lymph vessel [16]. Stimulation of 5-HT_1-like receptor also produces an endogenous nitric oxide (NO)-dependent relaxation in the smooth muscle of lymph node [17]. Overall, serotonin mainly controls lymph flow in the mesenteric lymphatic system [16, 17, 20, 25] in physiological conditions. However, the physiological roles of serotonin in lymph formation of mesenteric lymph vessels remain unclear.

In the present study, intravenous administration of serotonin significantly increased the mesenteric lymph volume in a dose-dependent manner (10^{-8}–10^{-5} M, Fig. 3B). Serotonin (10^{-5} M) also increased the concentration of albumin in the lymph. Similarly, the concentration of IL-22 in the lymph also increased with the intravenous administration of 10^{-5} M serotonin. Michel *et al*. estimated with theoretical value of hydraulic permeability and oncotic pressure in rat mesenteric venules and then demonstrated that 0.26 mM of serotonin increased the permeability by inducing openings in the venular endothelium [15]. In the present study, we confirmed their hypothesis with the findings that 10^{-5} M serotonin increased mesenteric lymph volume and albumin content in the mesenteric lymph. Hence, serotonin may physiologically regulate the permeability of the venules in the jejunal microcirculation for fluid and albumin.

The permeability of the venular walls for macromolecules such as albumin under physiological condition is well known [15, 28, 32, 36, 38]. Based on the studies, microvascular pressure including hydrostatic and oncotic pressure through venular walls, microvascular blood flow, segmental permeability variations, neutrophil function, pericyte activity, barrier function of basement membrane, subcellar matrix, myosin light chain kinase-dependent contractility of venular cells, and endothelial gap formation play as regulators of the permeability of the venules for albumin *in vivo*. The mode of action for the 5-HT-mediated increase in the permeability of the venules for albumin in the present experiments remained unsolved. We would need to conduct additional experiments in order to clarify the mechanisms for 5-HT-mediated hyperpermeability of albumin in rat jejunal villi.

The intravenous administration of 10^{-6} M ketanserin, a 5-HT_2 receptor antagonist, caused a

significant decrease in the mesenteric lymph volume and the concentration of albumin in the lymph in physiological condition (Fig. 4). Additionally, intravenous pretreatment with 10^{-6} M ketanserin significantly reduced the intravenous one-time administration of serotonin (10^{-5} M, 0.3 mL)-mediated increases in the mesenteric lymph volume, and the concentrations of albumin in the lymph (Fig. 5). The 10^{-6} M ketanserin produced no significant change in the concentration of platelets in the femoral venous blood. Thus, in our experiment, the ketanserin-mediated inhibition for serotonin release from platelets may not contribute to reducing the one-time administration of serotonin responses. Moreover, intravenous treatment with ketanserin decreased the concentration of albumin in the lymph collected during 30-45 min after water intake. The finding may be related to the difference in action time of each drug between one-time administration of serotonin and continuous intravenous administration of ketanserin.

In summary, serotonin in circulatory blood secreted from enterochromaffin cells in the jejunal villi, regulates the permeability of fluid and permeant albumin through the venules via the activation of 5-HT_2 receptors. Thus, serotonin plays a crucial role in physiological regulation of lymph formation in the mesenteric lymph vessels.

However, we will be, in the future, needed to evaluate whether the platelet-, neurotransmitters- or signaling molecules-derived serotonin contribute to the findings that the intravenous administration of ketanserin itself decreased the mesenteric lymph volume and the concentration of albumin in the lymph (Fig. 4). In addition, the possibility that water intake releases other vasoactive substances in addition to serotonin in the jejunal villi, which might contribute to the increase in mesenteric lymph flow is not ruled out in the present experiments. We would need in the future to evaluate the possibility.

Serotonin upregulated IL-22 mRNA in the ILC-3 and increased the immunoreactivity of IL-22 in the lamina propria of rat jejunal villi.

Another important aspect of the present study is that IL-22 mRNA in ILC-3 and the immunoreactivity of IL-22 in the lamina propria of jejunal villi were increased by serotonin and water intake, respectively (Fig. 6). We believe that it may be the first in the field of mucosal immunology to report the serotonin-mediated upregulation of IL-22 mRNA expression in ILC-3. Hence, serotonin regulates the immunological function of ILC-3 in the lamina propria of jejunal villi. The evidence is agreed with the finding that water intake-released serotonin increased the immunoreactivity of IL-22 in the jejunal villi. In addition, the serotonin-mediated increase in immunoreactivity of IL-22 in the jejunal villi may be mainly produced via the activation of the others 5-HT receptors, other than the 5-HT_2 receptor, because methysergide but not keanserin blocked the water intake-mediated increase in the immunoreactivity of IL-22. The evidence may be also related to the finding that intravenous administration of serotonin increased the total flux of IL-22 through the mesenteric lymph

vessel (Fig. 3). The serotonin-mediated increase in the transport of albumin through the venular wall in the jejunal microcirculation may contribute, in part, to the increase in the concentration of IL-22 in the mesenteric lymph because most of IL-22 released from ILC-3 was combined with permeant albumin. We previously demonstrated that the intragastric administration of distilled water in anaesthetized rats also induced a significant increase in the total flux of IL-22 through the jejunal-originated mesenteric lymph vessels, resulting in transport of IL-22 into the venous blood through the thoracic duct [18].

The water intake-released serotonin-dependent IL-22 transport into systemic circulation through the mesenteric lymph vessels may contribute to regulate the barrier mechanisms of the epithelial layer of small intestine [12, 33] and promoting intestinal-stem-cell-mediated epithelial regeneration [13]. Based on the findings that IL-22 from the mesenteric lymph vessels passes into the bloodstream, IL-22 may also contribute to the maintaining and promotion of innate immunity in the body. Recent studies on IL-22 [6, 26] strongly support the hypothesis that IL-22 plays a role in innate immunity. However, the effects of serotonin on mucosal and innate immunity are less well understood except for the findings that mast cells are known to produce serotonin, while T cells, dendric cells, and macrophages express 5-HT_7 receptor [27]. The physiological roles of serotonin in mucosal and innate immunity should be addressed in the future.

Author contributions

T.O. wrote the manuscript, designed the experiments, and analyzed the data. Y.K., M.H., and T-W-A. designed the experiments, analyzed the data, and revised manuscript. R.K., K.A., N.A., S.N., D.M., Y.Y., and M.K., performed the experiments, and analyzed data.

Data availability

All relevant data are available from the corresponding author on request.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Figure Legends

Figure 1

A1: Representative photomicrographs for the effect of intragastric administration of distilled water (3 mL, 15 min) on serotonin immunohistochemical expression in the jejunal villi. Each scale bar is 20 μm.

A2: Summarised data (n = 10) with density analysis for serotonin immunohistochemical expression in the jejunal villi. ** $p < 0.01$.

B1: Representative photomicrographs for the water intake (3 mL, 15 min) on tryptophan hydroxylase (Tph)-1 immunohistochemical expression in the jejunal villi. Each scale bar is 20 μm. NS not significant.

B2: Summarised data (n = 10) with density analysis for Tph-1 immunohistochemical expression in the jejunal villi

C1: Representative photomicrographs with higher magnification for Ag-sensitive black-coloured granules in enterochromaffin cells in the jejunal villi stained with Grimelius staining method. The left two panels show the control photomicrographs without water intake. The right two panels are the photomicrographs with water intake (3 mL, 15 min). Each scale bar is 10 μm.

C2: Summarised data (n = 10) with density analysis for the granule expression in enterochromaffin cells in the jejunal villi. ** $p < 0.01$.

Figure 2

A1: The intragastric administration of distilled water (3 mL) increased rapidly the concentration of serotonin in rat portal venous blood collected during 0–15 min after water intake. The ordinate is the concentration of serotonin (ng/mL). The abscissa shows the collected time course (min).

* $p < 0.05$, NS not significant.

A2: The intragastric administration of distilled water (3 mL) increased rapidly the blood flow rate in the portal vein during 0–15 min after water intake. The ordinate shows the blood flow rate in portal vein (mL/15 min). The abscissa is the collected time course (min).

** $p < 0.01$, NS not significant.

A3: The intragastric administration of distilled water (3 mL) increased rapidly the total flux of serotonin through the portal vein during 0–15 min after water intake. The ordinate shows the total flux of serotonin through the portal vein (ng/15 min). The abscissa is the collected time course (min). ** $p < 0.01$, NS not significant.

B1: The intragastric administration of distilled water (3 mL) produced no significant change in the concentration of serotonin in rat mesenteric lymph collected during 0–45 min after water intake.

The ordinate is the concentration of serotonin (ng/mL). The abscissa shows the collected time

course (min). NS not significant.

B2: The intragastric administration of distilled water (3 mL) increased rapidly the lymph volume in the mesenteric lymph vessel during 0–15 min after water intake. The ordinate shows the lymph volume collected over the periods of 15 min (μL/15 min). The abscissa is the collected time course (min). ** $p < 0.01$, NS not significant.

B3: The intragastric administration of distilled water (3 mL) increased rapidly the total flux of serotonin through the mesenteric lymph vessel during 0–15 min after water intake. The ordinate shows the total flux of serotonin through the mesenteric lymph vessel (x 10^{-3} ng/15 min). The abscissa is the collected time course (min). ** $p < 0.01$, NS not significant.

Figure 3

A1: Representative recordings for the effect of intravenous one-shot administration of 10^{-5} M serotonin on the mesenteric lymph volume collected over the periods of 60 min.

A2: Summarised data for intravenous one-shot administration of 10^{-5} M serotonin on the mesenteric lymph volume collected over the periods of 60 min. The ordinate shows the normalized lymph volume. (100 % = 155.0 $\pm$ 42.5 μL/60 min). The abscissa is the collected time. * $p < 0.05$, NS not significant.

B: Dose-response relationship between the concentration of serotonin (10^{-5} M – 10^{-8} M) and the mesenteric lymph volume collected over the periods of 60 min (100 % = 155.0 $\pm$ 42.5 μL/60 min, n = 4). The ordinate and abscissa are the same items as those in the panel A2, respectively. * $p < 0.05$.

C: The effect of intravenous administration of serotonin (10^{-5} M, 0.3 mL, in one-time) on the concentration of albumin in the mesenteric lymph collected over the periods of 60 min. (100% = 15.9 $\pm$ 2.0 mg/mL, n = 4). The ordinate and abscissa show the same items as those in the panel A2. ** $p < 0.01$, NS not significant.

D: The effect of intravenous administration of serotonin (10^{-5} M, 0.3 mL, in one-time) on the concentration of IL-22 in the mesenteric lymph collected over the periods of 60 min (100% = 447.5 $\pm$ 60.5 pg/mL, n = 4). The ordinate and abscissa show the same items as those in the panel A2. * $p < 0.05$, NS significant.

Figure 4

A1: Representative recordings for the effect of intravenous one-shot administration of 10^{-6} M ketanserin (0.3 mL) on the mesenteric lymph volume collected over the periods of 60 min.

A2: Summarised data for intravenous one-shot administration of 10^{-6} M ketanserin (0.3 mL) on the mesenteric lymph volume collected over the periods of 60 min. The ordinate shows the normalized lymph volume. (100 % = 178.7 $\pm$ 33.3 μL/60 min). The abscissa is the collected time.

** $p < 0.01$.

B: The effect of intravenous one-shot administration of ketanserin (10^{-6} M, 0.3 mL) on the concentration of albumin in the mesenteric lymph collected over the periods of 60 min. (100 % = 15.7 $\pm$ 0.5 mg/mL, n = 4). The ordinate and abscissa show the same items as those in the panel A2. * $p < 0.05$.

C: The effect of intravenous one-shot administration of ketanserin (10^{-6} M, 0.3 mL) on the concentration of IL-22 in the mesenteric lymph collected over the periods of 60 min (100 % = 407.5 $\pm$ 60.5 pg/mL, n = 4). The ordinate and abscissa show the same items as those in the panel A2. NS not significant.

Figure 5

A1: Representative recordings for the effect of intravenous one-time administration of serotonin (10^{-5} M, 0.3 mL) under the continuous intravenous treatment of ketanserin (10^{-6} M).

A2: Summarised data for intravenous one-time administration of 10^{-5} M serotonin on the normalized mesenteric lymph volume collected over the periods of 15 min. The ordinate shows the normalized lymph volume. (100 % = 45.8 $\pm$ 4.6 µL/15 min, n =4). NS not significant.

B: Summarised data for intravenous one-time administration of 10^{-5} M serotonin (0.3 mL) on the normalized concentration of albumin in the lymph collected over the periods of 15 min. The ordinate shows the normalised concentration of albumin. (100 % = 7.4 $\pm$ 1.6 mg/mL, n =4).

** $p < 0.01$, NS not significant.

C: Summarised data for intravenous one-time administration of 10^{-5} M serotonin (0.3 mL) on the normalized concentration of IL-22 in the lymph collected over the periods of 15 minutes. The ordinate shows the normalised concentration of IL-22 (100% = 232.5 $\pm$ 18.0 pg/mL, n = 4).

NS not significant.

Figure 6

A1: Representative flow cytometry recording of non-T, non-B, and RORrt-positive cells (blue points) in the cells isolated from the lamina propria of jejunal villi.

A2: Representative flow cytometry data for the presence of CD127 on CD-45 and RORrt-positive cells in the jejunal villi.

B: The effect of serotonin (10^{-6} M) on the IL-22 mRNA expression in the non-T and non-B cells isolated from the jejunal lamina propria was analyzed in *in vitro* experiment with RT-PCR.

** $p < 0.01$.

C1: Representative photomicrographs of water intake (3mL, 45 min)-mediated upregulation of IL-22 immunohistochemical expression in the jejunal villi with or without intravenous pretreatment with 10^{-6} M methysergide (45 min). a; the control without water intake, b; water

intake and c; water intake with the pretreatment 10^{-6} methysergide. The cellular nuclei (blue color) were stained with DAPI. Each scale bar is 50 μm.

C2: Summarised data (n = 10) with density analysis for IL-22 immunohistochemical expression in the jejunal villi with water intake with or without the pretreatment with 10^{-6} M methysergide, **p < 0.01, *p < 0.05.

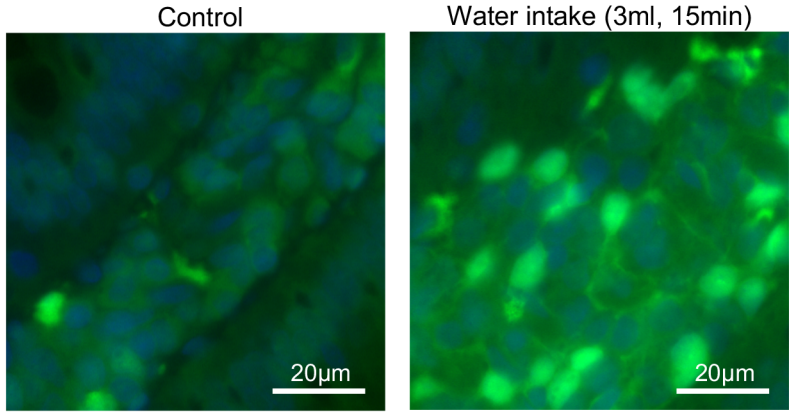
D1: Representative photomicrographs of water intake (3 mL, 45 min)-mediated upregulation of IL-22 immunohistochemical expression in the jejunal villi with or without the pretreatment with 10^{-6} M ketanserin (45 min). a; the control without water intake, b; water intake and c; water intake with the pretreatment with 10^{-6} M ketanserin. The cellular nuclei (blue color)were stained with DAPI. Each scale bar is 50 μm.

D2: Summarised data with density analysis for IL-22 immunohistochemical expression in the jejunal villi with water intake with or without the pretreatment with 10^{-6} M ketanserin (n = 10). ** p < 0.01, NS not significant.

Fig1

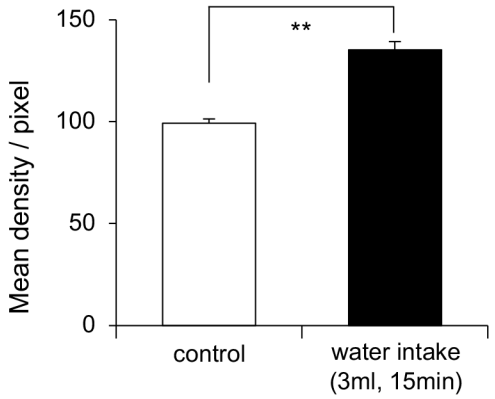
A1

Serotonin expression in rat jejunal villi



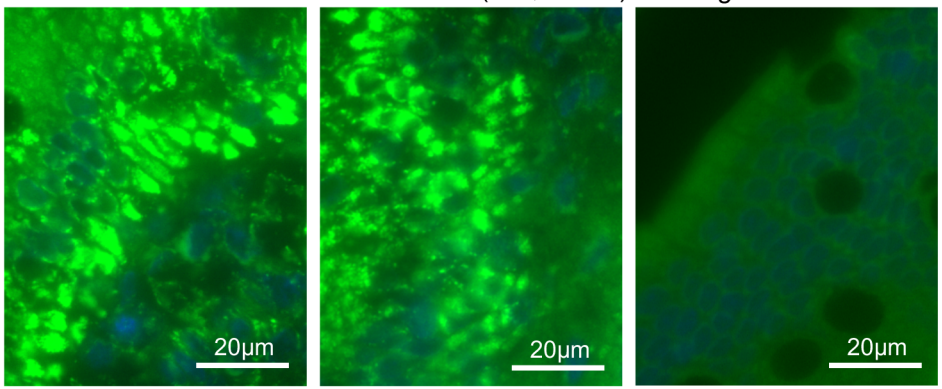
A2

Density analysis for serotonin expression



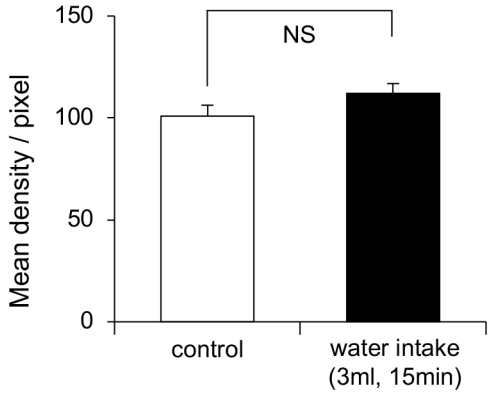
B1

Tryptophan hydroxylase (Tph) -1 expression in rat jejunal villi



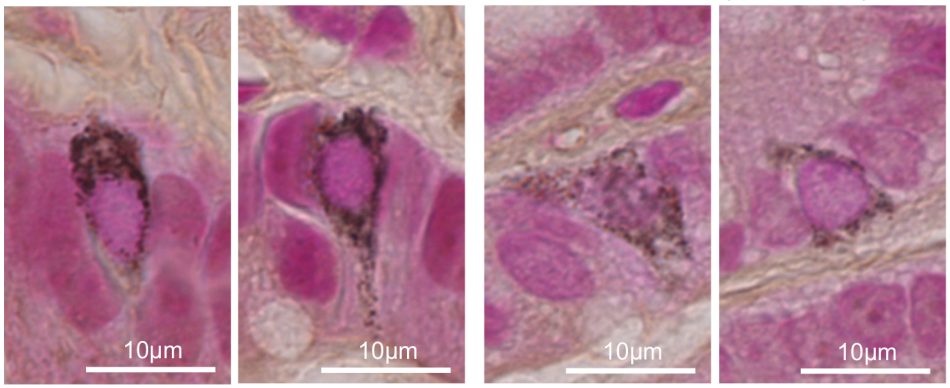
B2

Density analysis for Tph-1 expression



C1

Ag-sensitive granules in enterochromaffin cells in rat jejunal villi



C2

Density analysis for Ag-sensitive granules in enterochromaffin cells