Protective Effect of TRPM8 against Indomethacin-Induced Small Intestinal Injury via the Release of Calcitonin Gene-Related Peptide in Mice

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INTRODUCTION

Transient receptor potential melastatin 8 (TRPM8) is a non-selective cation channel activated by mild cooling and chemical agents including menthol. Nonsteroidal anti-inflammatory drugs have antipyretic, analgesic effects, and they can cause stomach and small intestinal injury. The current study investigated the role of TRPM8 in the pathogenesis of indomethacin-induced small intestinal injury. In male TRPM8-deficient (TRPM8KO) and wild-type (WT) mice, intestinal injury was induced via the subcutaneous administration of indomethacin. In addition, the effect of WS-12, a specific TRPM8 agonist, was examined in TRPM8KO and WT mice with indomethacin-induced intestinal injury. TRPM8KO mice had a significantly higher intestinal ulcerogenic response to indomethacin than WT mice. The repeated administration of WS-12 significantly attenuated the severity of intestinal injury in WT mice. However, this response was abrogated in TRPM8KO mice. Furthermore, in TRPM8-enhanced green fluorescent protein (EGFP) transgenic mice, which express EGFP under the direction of TRPM8 promoter, the EGFP signals in the indomethacin-treated intestinal mucosa were upregulated. Further, the EGFP signals were commonly found in calcitonin gene-related peptide (CGRP)-positive sensory afferent neurons and partly colocalized with substance P (SP)-positive neurons in the small intestine. The intestinal CGRP-positive neurons were significantly upregulated after the administration of indomethacin in WT mice. Nevertheless, this response was abrogated in TRPM8KO mice. In contrast, indomethacin increased the expression of intestinal SP-positive neurons in not only WT mice but also TRPM8KO mice. Thus, TRPM8 has a protective effect against indomethacin-induced small intestinal injury. This response may be mediated by the upregulation of CGRP, rather than SP.

Key words transient receptor potential melastatin 8; small intestinal injury; indomethacin; calcitonin gene-related peptide (CGRP); sensory afferent neuron

MATERIALS AND METHODS

Animals In this study, 8–10-week-old male C57BL/6

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mice weighing 22–27 g were purchased from Japan SLC (Shizuoka, Japan). TRPM8-deficient mice were established using C57BL/6J as described in a previous report.25 TRPM8-EGFP transgenic mice, which express EGFP under the direction of TRPM8 promoter, were established, as described previously.26 All mice were housed in plastic cages with free access to food and water, and the temperature was maintained at 22 ± 1°C under a 12-h light–dark cycle. All animal experimental procedures were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (Permit Nos. 18-004 and 19-004). Animal suffering was kept to a minimum whenever possible, and the smallest number of animals required for significant data interpretation was used.

**Drugs and Reagents** Indomethacin (Sigma-Aldrich, St. Louis, MO, U.S.A.) was suspended in normal saline with a drop of Tween 80 (Wako Pure Chemical Corporation, Osaka, Japan), and WS-12 (Tocris Bioscience, Bristol, U.K.) was dissolved in 0.5% carboxymethylcellulose (CMC, Nacalai Tesque Inc., Kyoto, Japan) via sonication. These drugs were prepared immediately before use and were administered subcutaneously and intraperitoneally at a volume of 0.1 mL/10g body weight.

**Induction of Intestinal Injury** The animals were subjected to fasting for 18 h, provided with free access to food for 1 h and then subcutaneously injected with indomethacin at a single dose of 8 mg/kg. The normal animals received vehicle only (saline with a drop of Tween 80). In some cases, WS-12 (3 and 10 mg/kg), a specific TRPM8 agonist, was administered intraperitoneally 30 min before and 8, 24, and 32 h after indomethacin administration. Control animals received CMC alone. Then, they were sacrificed 48 h after indomethacin administration, and each mouse was intravenously injected with 100 µL of 1% (w/v) Evans blue solution 30 min before sacrifice to examine for intestinal injury.24

**Macroscopic and Histological Observations** The small intestine was excised, washed with saline, fixed in 10% (v/v) neutralized formalin, embedded in paraffin, sectioned at 4-µm thickness, and stained with hematoxylin and eosin. Histological injury was observed under a microscope (BX51, Olympus, Tokyo, Japan) at a magnification of 100×.

**Determination of Myeloperoxidase Activity** The animals were sacrificed 48 h after indomethacin administration, and the small intestines were excised. Myeloperoxidase (MPO) activity was determined, as previously described.13 The MPO activity was presented as micromoles H₂O₂/mg protein.

**Determination of Cytokine Expression** The animals were sacrificed 48 h after indomethacin administration. Next, small intestinal tissues were removed, washed with cold phosphate-buffered saline (PBS), and stored in RNAlater (Ambion, Austin, TX, U.S.A.) at 4°C until use. The expression of interleukin (IL)-1β and tumor necrosis factor alpha (TNF-α) were determined, as previously described.13 Predesigned primer sets for mouse IL-1β (MA025939) and TNF-α (MA097070) were obtained from the Perfect Real-Time Supporting System (TaKaRa Bio Inc., Kusatsu, Shiga, Japan). The mRNA expression level was standardized to that of TATA-binding protein.

**Determination of CGRP Levels** The CGRP content in ileum homogenates was analyzed using enzyme immunoassay (EIA) (Bertin Bioreagent, France). Tissue preparation and analysis were performed based on the manufacturer's instructions. Data were presented as picogram per milligram (pg/mg) tissue weight.

**Western Blot Analysis** Tissue preparation was performed, as described by Eijkelkamp et al.25 The proteins were separated using 10% -sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred into polyvinylidene fluoride or polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.) via electroblotting. The membranes were blocked with 5% (w/v) skim milk dissolved in Tween-PBS. The membranes were stained with rabbit anti-TRPM8 (1:500; Alomone Labs, Jerusalem, Israel) and rabbit anti-β-actin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) antibodies and were incubated overnight at 4°C. Then, they were stained with horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Immunoreactivity was detected via enhanced chemiluminescence (PerkinElmer, Inc. Life Sciences, Boston, MA, U.S.A.), and the dominant band density was determined using the FUSION Solo 5 software (Vilber Lourmat, Marne-la-Vallée, France). The expression levels were normalized to that of β-actin.

**Retrograde Labeling** The origin of the primary afferent innervation to the mouse ileum was determined via retrograde tracing using fluorescent fluorogold dye (FG; Fluorochrome, Denver, CO, U.S.A.). Approximately 5µL of 4% FG was injected circumferentially into the ileum at five sites under 2% isoflurane anesthesia. Tissue recovery occurred 4 d after FG administration.

**Immunohistochemistry** Small intestine and dorsal root ganglion (DRG) segments were collected, fixed for 2 h at 4°C via immersion in fresh 4% paraformaldehyde in 0.1 M phosphate buffer and washed three times with PBS. Immunohistochemical procedures were performed by Matsumoto et al., as previously described.26 The frozen sections were double stained and probed overnight at room temperature with sheep anti-CGRP (1:2000, Enzo Life Sciences, Inc., Lausen, Switzerland). Subsequently, the sections were labeled for 3 h at room temperature with Alexa Fluor 488 donkey anti-chicken IgG (1:800, Thermo Fisher Scientific Inc.) and were probed overnight at room temperature with Alexa Fluor 594 donkey anti-rabbit IgG (1:800, Thermo Fisher Scientific Inc.).

In another experiment, the sections were double stained and probed overnight at room temperature with chicken anti-GFP antibody (1:5000, Cosmo Bio, LTD., Tokyo, Japan). Subsequently, the sections were labeled for 3 h at room temperature with Alexa Fluor 488 donkey anti-chicken IgG (1:800, Thermo Fisher Scientific Inc.) and were probed overnight at room temperature with either sheep anti-CGRP, rabbit anti-SP (1:1000, ImmunoStar Hudson, WI, U.S.A.), or rabbit anti-PGP9.5. Then, the sections were labeled for 3 h at room temperature with either Alexa Fluor 594 donkey anti-sheep IgG or Alexa Fluor 594 donkey anti-rabbit IgG (1:800, Thermo Fisher Scientific Inc.).

The sections were evaluated using a confocal microscope (AIR²; Nikon, Tokyo, Japan) at a magnification of 200× or 100×. The Nikon NIS-Elements AR 4.2.00 software was used to capture images. The ImageJ software was utilized to
quantify the positive signals for CGRP, SP, and TRPM8 expression. The positive areas of CGRP, SP, and TRPM8 were evaluated and divided by the total intestinal villi areas. Next, the results were presented as percentage of the total villi area.

Statistical Analyses Data were presented as means ± standard error of the mean. Statistical analyses were performed using the GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, CA, U.S.A.). Multiple groups were compared using one- or two-way ANOVA, followed by the Holm–Sidak’s multiple comparison test. Two group data were compared using the student’s t-test. A p value of <0.05 was considered statistically significant.

RESULTS

Effects of TRPM8 Deficiency on Indomethacin-Induced Intestinal Injury Indomethacin at a single dose caused injury along the small intestine, from the distal jejunum to the ileum, after 48 h. The size of hemorrhagic injury stained with Evans blue significantly increased in TRPM8KO mice compared with WT mice (Figs. 1A, B). Based on histological assessment, indomethacin-induced intestinal injury was larger in TRPM8KO mice than in WT mice. That is, it had reached the muscularis mucosa in TRPM8KO mice compared with WT mice (Figs. 1A, B). Based on histological assessment, indomethacin-induced intestinal injury was larger in TRPM8KO mice than in WT mice. That is, it had reached the muscularis mucosa in TRPM8KO mice (Fig. 1C). Furthermore, the effects of TRPM8 deficiency on MPO activity and inflammatory cytokine expressions in the intestinal mucosa were investigated (Fig. 2). Indomethacin administration increased intestinal MPO activity as well as IL-1β and TNF-α mRNA expressions in WT mice. MPO activity was further enhanced in TRPM8KO mice (Fig. 2A). In contrast, further enhancement of IL-1β and TNF-α mRNA expressions was not detected in TRPM8KO mice. However, the expression levels were similar in both mice (Figs. 2B, C). Taken together, TRPM8 deficiency significantly increased intestinal ulcerogenic response to indomethacin in TRPM8KO mice compared with WT mice.

Effects of TRPM8 Agonist on Indomethacin-Induced Intestinal Injury To further validate the role of TRPM8 in indomethacin-induced intestinal injury, we examined the effect of WS-12, a specific TRPM8 agonist (Fig. 3). The repeated administration of WS-12 (3 and 10 mg/kg) dose-dependently reduced the severity of macroscopic intestinal injury in WT mice (Figs. 3A, B). A significant protective effect was observed at a dose of 10 mg/kg. Further, indomethacin-induced histological intestinal injury and intestinal MPO activity upregulation in WT mice were attenuated by WS-12 (10 mg/kg) (Figs. 3C, D). In contrast, the protective effects of WS-12 (10 mg/kg) against indomethacin-induced intestinal injury and MPO upregulation were completely abrogated in TRPM8KO mice. There was no significant difference in the severity of intestinal lesions and MPO activity between the control (CMC alone) and WS-12-treated groups in TRPM8KO mice.

Changes in TRPM8 Expression in Indomethacin-Induced Intestinal Injury Next, we investigated the expression of TRPM8 in the intestinal mucosa via immunohistochemical examination in TRPM8-EGFP transgenic mice and
We detected EGFP signals representing TRPM8 expression in the mucosa of controls (Fig. 4). This expression significantly increased 48 h after indomethacin administration (Fig. 4B). Similarly, Western blot analyses showed that the protein expression of TRPM8 increased in the small intestine after indomethacin treatment.

Characterization of TRPM8 Immunoreactivities in the Small Intestine We characterized the immunoreactivities of TRPM8 in the small intestine via immunohistochemistry double staining using TRPM8-EGFP mice and several neural markers such as pan neuronal marker anti-PGP9.5 antibody, sensory neural marker anti-CGRP, and anti-SP antibodies (Fig. 5A). TRPM8-EGFP signals were co-localized with PGP9.5-immunopositive nerve fibers, indicating the presence of TRPM8-expressing neurons in the small intestine. Furthermore, TRPM8-EGFP signals were highly co-localized with CGRP immunopositive neurons and partially co-localized with SP immunoreactivities in the small intestine. Thus, TRPM8-immunopositive neurons expressed mainly CGRP-positive neurons and partly SP-positive sensory neurons.

Next, we investigated the origin of the primary afferent innervation of the small intestine using retrograde fluorescent tracer fluorogold dye (Fig. 5B). Most TRPM8-immunoreactive cell bodies were found in the DRG, and they were totally double-labeled with fluorogold retrogradely transported from the small intestine. We further observed the co-localization of TRPM8 with CGRP and SP in the DRG. Thus, TRPM8 was expressed in CGRP- and/or SP-positive primary afferent neurons originating from the DRG.

Changes in CGRP and SP Expressions in the Mucosa in WT and TRPM8KO Mice with Intestinal Injury To better understand the role of CGRP and SP in the pathogenesis of indomethacin-induced intestinal injury, we investigated changes in CGRP and SP expressions immunohistochemically in WT and TRPM8KO mice (Fig. 6). The expression of CGRP in the intestinal mucosa was significantly upregulated after indomethacin administration in WT mice. However, this response was completely abrogated in TRPM8KO mice (Figs. 6A, B). To validate these results, the amount of CGRP in the intestinal tissues was assessed using EIA (Fig. 6C). Indomethacin significantly increased the amount of CGRP in WT mice, but not in TRPM8KO mice. In contrast, the expression of SP in the intestinal mucosa was significantly upregulated after indomethacin administration in both WT and TRPM8KO mice. The expression levels in WT and TRPM8KO mice were almost comparable (Figs. 7A, B).

DISCUSSION

Several reports showed the roles of TRP channels and the sensory afferent neurons in the regulation of inflammation in the GI tract.21,27,28) TRPM8 has an anti-inflammatory effect in mice with experimentally induced colitis.8,9) The current study found that indomethacin-induced small intestinal injury was significantly enhanced via the genetic deletion of TRPM8 but was remarkably inhibited via the pharmacological activation of TRPM8 in mice. Therefore, TRPM8 plays a protective role against indomethacin-induced small intestinal injury.

NSAIDs cause injury in not only the stomach but also the small intestine in humans and experimental animals.12–14) In terms of pathophysiological similarity, indomethacin-induced small intestinal injury in mice may be an useful animal model for assessing intestinal inflammation in Crohn’s disease among humans.15) Several inflammatory responses such as infiltration of neutrophils and upregulation of inflammatory cytokines are involved in the occurrence of indomethacin-induced small intestinal injury.12,29,30) In the current study, we observed that indomethacin-induced small intestinal injury accompanied by an increase in mucosal MPO activity, an index of neutrophil infiltration, was significantly enhanced in TRPM8KO mice.
mice compared with WT mice. As previously reported, there was an upregulation of inflammatory cytokines such as TNF-α and IL-1β in the small intestine after indomethacin administration. However, the cytokine expressions in TRPM8KO mice compared with WT mice was not further assessed. This notion should also be considered because the expression of these cytokines may be maximally upregulated to cause severe intestinal injury even in WT mice.

Similar to the results of the current study, Jong et al. previously reported that TRPM8KO mice treated with dextran sulfate sodium (DSS) were more susceptible to experimentally induced colitis than WT mice. In contrast, Ramachandran et al. showed that the pharmacological activation of TRPM8 by icilin, a TRPM8 agonist, has anti-colitis effects. However, TRPM8KO did not enhance the severity of colitis in experimental colitis models induced by DSS and trinitrobenzene sulfonic acid (TNBS). To validate these differences, the current study examined the effect of WS-12, a significantly more selective agonist menthol derivative, on indomethacin-induced small intestinal injury. The repeated administration of WS-12 dose-dependently attenuated intestinal injury accompanied by increased MPO activity in WT mice. Further, the protective effect of WS-12 was completely abrogated in TRPM8KO mice. These findings strongly indicate the anti-inflammatory role of TRPM8 in indomethacin-induced small intestinal injury. Since a specific anti-mouse TRPM8 antibody is not avail-

![Effect of WS-12 on Indomethacin-Induced Intestinal Injury in WT and TRPM8-Deficient (TRPM8KO) Mice](image)

Indomethacin (8 mg/kg) was administered subcutaneously, and intestinal injury was examined after 48h. WS-12 (3 and 10 mg/kg) was intraperitoneally administered 30 min before and 8, 24, and 32 h after indomethacin administration. (A) The area of intestinal injury, (B) typical macroscopic, (C) histological observations, and MPO activity in controls (vehicle alone for WS-12) and WS-12-treated WT and TRPM8KO mice. Scale bars = 100 µm. Data were presented as means ± standard error of the mean (n = 4–6); *p < 0.05 relative to control; *p < 0.05 relative to vehicle- (control) or WS-12-treated WT mice. (Color figure can be accessed in the online version.)
able for immunohistochemical analyses, we investigated the localization of TRPM8 in the small intestine immunohistochemically using TRPM8-EGFP transgenic mice, which express EGFP under the direction of TRPM8 promoter. TRPM8-promoting EGFP signals were commonly found in neural fibers and were significantly upregulated after indomethacin administration. The protein expression of TRPM8 determined via Western blot analyses showed an increase after indomethacin administration. A previous study revealed that TRPM8 was upregulated in the murine asthma model. The current study revealed that TRPM8-promoting EGFP signals were co-localized mainly with CGRP and partly with SP in the sensory neurons of the small intestine. Further, retrograde tracing using fluorescent fluorogold dye showed that the extrinsic primary afferent innervation expressing CGRP and SP in the small intestine originated from the DRG.

Several studies showed that TRPM8 was expressed in mucosal sensory neurons for the regulation of several functions such as inflammation and visceral nociception. These functions are caused by the local release of neuropeptides such as CGRP and SP from sensory neurons. The current study revealed that TRPM8-promoting EGFP signals were co-localized mainly with CGRP and partly with SP in the sensory neurons of the small intestine. Further, retrograde tracing using fluorescent fluorogold dye showed that the extrinsic primary afferent innervation expressing CGRP and SP in the small intestine originated from the DRG.

Several studies reported that the number of CGRP- and SP-positive sensory neurons increased in the inflamed mucosa in experimentally induced colitis. The current study showed that both CGRP- and SP-positive sensory neurons were upregulated around the injured area in the small intestine treated with indomethacin. Interestingly, the upregulation of CGRP-positive sensory neurons was completely abrogated in TRPM8KO mice. However, the upregulation of SP-positive sensory neurons was not affected in TRPM8KO mice. Thus, TRPM8 may contribute to the production of CGRP, but not SP, in injured intestinal mucosa. CGRP has protective effects against experimentally induced colitis by DSS and TNBS. In contrast, the deleterious effects of SP on the pathogenesis of colitis have been described. Thus, TRPM8-mediated CGRP release from the sensory afferent neurons may have a protective effect against indomethacin-induced small intestinal injury. In contrast, SP independent of TRPM8 on the sensory afferent neurons may play a role in the progression of small intestinal injury.

We previously reported the role of TRPV1 and TRPA1 in sensory afferent neurons in the progression of DSS-induced colitis in relation to CGRP and SP in contrast to TRPM8. The severity of colitis was attenuated in mice deficient of either TRPV1 or TRPA1. Further, TRPV1- and TRPA1-deficiencies prevented the upregulation of SP, but not CGRP, in DSS-induced inflamed colon. Thus, the release of SP from the sensory afferent neurons expressing TRPV1 and TRPA1 may
be involved in the progression of DSS-induced colitis. And CGRP may have protective effects against colitis, independent of TRPV1 and TRPA1, on the sensory afferent neurons. We hypothesize that the different influence of these channels may be accounted for by the correlation of neuropeptides, such as CGRP and SP. Based on these observations, CGRP may be upregulated by TRPM8 and SP by TRPV1 and TRPA1 in the sensory afferent neurons during inflammation. By contrast, TRPM8 was localized in high-threshold visceral afferent neurons and may also colocalize with TRPV1 and TRPA1.
to restrain their downstream chemosensory and mechanosensory actions. TRPM8 is present on colonic sensory neurons, which may couple with TRPV1 and TRPA1 to also inhibit their downstream chemosensory and mechanosensory actions. Thus, it is possible that these channels that are colocalized in sensory neurons may interregulate the downstream neural and sensory responses.

In the GI tract, CGRP is well established as a protective neuropeptide released from the sensory afferent neurons via vasodilation and mucus secretion. The CGRP-mediated vasodilative hyperemic response and increased mucus secretion are well-known mucosal defense mechanisms against various noxious stimulants in the gastrointestinal lumen. Furthermore, CGRP has been reported to exert anti-inflammatory effect via various effector functions of immune cells, including inhibiting inflammatory cytokines and upregulating anti-inflammatory cytokines. In contrast, SP plays an important role in the pathogenesis of inflammation by increasing vascular permeability, leukocyte activation and mast cell degradation. To validate the association among these neuro-responses and the protective and deleterious effect of these neuropeptides, further studies should be conducted.

Recent advances have described the importance of gut microbiota in influencing the brain–gut axis that comprises bidirectional communication between the central and enteric nervous system, thereby linking emotional behavior, metabolism, and immune regulations. Thermosensitive TRP channels, including TRPM8 and TRPA1, and microbiota have been suggested to be involved in thermogenesis and energy metabolism. Moreover, we previously showed the involvement of intestinal microbiota in the development of indomethacin-induced intestinal injury. Thus, it is possible that alteration in intestinal microbiota via TRPM8 may be, in part, involved in the pathogenesis of intestinal injury.

In conclusion, TRPM8 expressed in the sensory afferent neurons plays a protective role against indomethacin-induced small intestinal injury. We hypothesized that the protective effect of TRPM8 is mediated by the upregulation of CGRP

Fig. 6. Changes in Calcitonin Gene-Related Peptide (CGRP) Expression in the Intestinal Mucosa with Indomethacin-Induced Intestinal Injury in WT and TRPM8-Deficient (TRPM8KO) Mice

(A) Typical images, (B) area of CGRP-positive neurons presented as percentage of the total villi area and (C) CGRP concentration (pg/mg in tissues) in intestinal homogenates determined using enzyme immunoassay in controls (vehicle alone) and indomethacin-injected WT and TRPM8KO mice. Scale bars = 100 µm. Data were presented as means ± standard errors of the mean (n = 5); *p < 0.05 relative to the WT normal. (Color figure can be accessed in the online version.)
rather than SP. Therefore, TRPM8 may be a potential target for the treatment of NSAID-induced enteropathy and inflammatory bowel diseases.

Conflict of Interest  The authors declare no conflict of interest.

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Fig. 7. Changes in Substance P (SP) Expression in the Intestinal Mucosa with Indomethacin-Induced Intestinal Injury in WT and TRPM8-Deficient (TRPM8KO) Mice

(A) Typical images and (B) area of SP-positive neuron presented as percentage of the total villi area in normal (vehicle alone) and indomethacin-treated WT and TRPM8KO mice. Scale bars = 100 µm. Data were presented as means ± standard error of the mean (n = 5). *p < 0.05 relative to the corresponding normal. (Color figure can be accessed in the online version.)


