Role of transient receptor potential vanilloid 4 activation in indomethacin-induced intestinal damage

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NONSTEROIDAL ANTI-INFLAMMATORY drugs (NSAID) can cause damage not only to the stomach, but also to the intestine. Among NSAID users, ~70% exhibit small degrees of gastrointestinal (GI) symptoms (2) as assessed by recent advances in modalities (e.g., capsule endoscopy, double-balloon enteroscopy) that enable detection of intestinal abnormalities (15, 16). Gastric acid also contributes to NSAID-induced gastropathy, and widely used proton pump inhibitors (PPIs), which significantly inhibit gastric acid production, can be effective in preventing NSAID-induced gastropathy (4). On the other hand, in the intestine the effects of gastric acid are less significant, and PPIs may worsen NSAID-induced enteropathy due to changes in the intestinal flora (39). To date, prostaglandin E derivatives and some mucosal protective drugs are known to have partially protective effects against NSAID-induced lesions (10, 11).

The pathogenic mechanisms of NSAID gastropathy and enteropathy appear to be distinct (38). Germ-free rats are resistant to intestinal damage induced by NSAIDs, suggesting that microorganisms are one of the possible causative factors in this damage (1, 28, 37). In addition, ampicillin administration inhibits bacterial invasion and prevents formation of intestinal lesions induced in response to the NSAID indomethacin (37). Together, these reports strongly suggest that microorganisms are involved in indomethacin-induced intestinal lesions. Epithelial hyperpermeability was also reported to be a cause of indomethacin-induced intestinal damage that occurs through a mechanism that remains unclear, although the arachidonic acid (AA) metabolite eicosanoids may contribute to this phenomenon (21).

Transient receptor potential vanilloid 4 (TRPV4) is a non-selective cation channel that is activated by temperature, extension, and chemicals such as 5,6-epoxyeicosatrienoic acid (5,6-EET). The aim of this study was to investigate the possible role of TRPV4 in NSAID-induced intestinal damage. TRPV4 mRNA and protein expression was confirmed by RT-PCR and immunohistochemistry, respectively, in mouse and human tissues while TRPV4 channel activity of the intestinal cell line IEC-6 was assessed by Ca2+ imaging. TRPV4 activators or the NSAID indomethacin significantly decreased transepithelial resistance (TER) in IEC-6 cells, and indomethacin-induced TER decreases were inhibited by specific TRPV4 inhibitors or small-interfering RNA TRPV4 knockdown, as well as by the epoxyenase inhibitor N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide, which decreased 5,6-EET levels. In TRPV4 knockdown mice, indomethacin-induced intestinal damage was significantly reduced compared with WT mice. Taken together, these results show that TRPV4 activation in the intestinal epithelium caused epithelial hyperpermeability in response to NSAID-induced arachidonic acid metabolites and contributed to NSAID-induced intestinal damage. Thus, TRPV4 could be a promising new therapeutic target for the prevention of NSAID-induced intestinal damage.
Materials and Methods

Animals. Male C57BL/6Ncr mice [8 wk old, purchased from SLC (Hamamatsu, Japan); wild-type (WT)] were used as a control. TRPV4 knockout (TRPV4KO) mice (23) were backcrossed on the same background. The mice were housed in a controlled environment (12:12-h light-dark cycle; room temperature, 22–24°C; 50–60% relative humidity) with free access to food and water. All procedures involving the care and use of animals were approved by The Institutional Animal Care and Use Committee of the University of Toyama and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell lines. We used intestinal epithelial cells-6 (IEC-6) (RCB0993, obtained from the Riken Cell Bank) that originated from nontransfected rat intestinal crypt cells (27). The cells were maintained in stock cultures in Dulbecco’s modified Eagle’s medium (DMEM; Nacalai Tesque) containing 5% fetal bovine serum (FBS; GIBCO) and 4 μg/ml insulin (Wako Pure Chemical Industries) in a humidified atmosphere of 5% CO2 at 37°C.

Human intestinal biopsy samples. We obtained intestinal biopsy samples from three healthy individuals aged 37–77 who underwent colonoscopy at Toyama University Hospital in Japan. Written informed consent was obtained from all individuals before obtaining the samples.

Reverse transcription-PCR analysis. To examine TRPV4 channel expression in mouse and human intestines and IEC-6 cells, total RNA (1 μg) was isolated from the samples using an RNasy Mini Kit (QIAGEN). PCR was performed using AmpliTaq Gold (Applied Biosystems) for mouse intestine tissues and IEC-6 cells or KOD FX neo (TOYOBO, Osaka, Japan) for human intestine in a TaKaRa PCR Biosystems) for mouse intestine (Int) and a rat intestinal cell line [intestinal epithelial cells-6 (IEC-6)]. TRPV4 and GAPDH mRNAs were examined with primer sets (Table 1) for TRPV4 and GAPDH as a positive control. PCR conditions used for AmpliTaq Gold were as follows: 1 cycle at 94°C for 2 min, 40 cycles at 94°C for 30 s, 55°C for 30 s followed by 1 cycle at 72°C for 2 min; and for KOD FX neo were as follows: 1 cycle at 94°C for 2 min, 40 cycles at 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s, 68°C for 1 min followed by 1 cycle at 72°C for 2 min. For KOD FX neo, we used similar conditions.

Immunohistochemistry. Methods were previously described (19, 20), and antibody information is summarized in Table 2. For section preparation, intestines isolated from WT and TRPV4KO mice were fixed with 4% paraformaldehyde in PBS at 4°C for 6 h, washed in PBS, and placed in PBS-sucrose for storage at 4°C overnight. Next, the tissues were embedded in OCT compound (Tissue-Tek; Sakura Finetec), and 10-μm-thick sections were collected on slides and cleared with PBS plus 0.3% Triton X-100 (PBS-T 0.3%) three times for 5 min each. Nonspecific antibody binding was reduced by incubation in BlockAce (Yukijirushi, Sapporo, Japan) in PBS-T 0.3% for 1 h at room temperature before antibody exposure. Preparations were analyzed using a fluorescence microscope (BZ-8000; Keyence) or a confocal laser scanning microscope (LSM 700; Carl Zeiss). For immunohistochemistry, IEC-6 cells cultured on cover glasses for 7 days were fixed with 4% paraformaldehyde in PBS at 4°C for 20 min. Samples were then cleared with PBS-T 0.1% three times for 5 min each and blocked as described above. Preparations were analyzed similarly to the intestinal sections.

Ca2+ imaging. Methods used were as previously described (19, 20). Fura 2 fluorescence was measured in IEC-6 cells in a standard bath solution containing (in mM) 140 NaCl, 5 KCl, 2 MgCl2, 2 CaCl2, 10 HEPES, and 10 glucose at pH 7.4 (adjusted with NaOH). The ratios of fluorescence intensities obtained with fura 2 emissions at 340 and 380 nm are shown. GSK (43) and 5,6-EET (40), RN-1734 (RN)
and HC-067047 (HC) (7), or ionomycin were used as TRPV4 agonists, antagonists, or a positive control, respectively (all were from Sigma except for 5,6-EET methyl ester and HC, which were from Cayman and Tocris Bioscience, respectively). Standard bath solution was used as a control. All experiments were performed at room temperature (25°C). The ratio of fluorescence at 340 to 380 nm was calculated and acquired with an imaging processing system (IP-Lab; Scanalytics, Rockville, MD) and ImageJ software (http://rsb.info.nih.gov/ij/). Changes in ratio were calculated by subtracting mean basal values from peak values.

Transepithelial resistance measurement in IEC-6 cells. Transepithelial resistance (TER) was determined with a Millicell-ERS voltohmeter (Millipore) (5). Cells (2–4 × 10⁵) were applied to 12-well Millicell inserts and maintained for 7 days to form a monolayer. Each chemical was added to the basolateral side. TRPV4 activators were applied 5 min after each inhibitor application. TRPV4 antagonists and N-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH; from Cayman Chemical) were applied 5 min before each basal TER measurement (0 min). Indomethacin and celecoxib (a selective COX-2 inhibitor) were obtained from Sigma, and SC-560 (a selective COX-1 inhibitor) was obtained from Cayman Chemical.

Small-interfering RNA and transfection. The method used was modified from that which was previously described (3). In brief, Stealth RNAi siRNA (ID RSS330373, designated “number 9”) targeting nucleotides 1201–1225 of rat TRPV4 (GenBank accession no. NM_023970.1) was purchased from Invitrogen. IEC-6 cells cultured on Transwells for 4 days were transfected using Lipofectamine RNAiMAX and Opti-MEM I reduced serum medium and a method that was slightly modified from the manufacturer’s instructions (Invitrogen). The final concentration for the selected RNAi or negative control was 10 nM with 1.7 μl of Lipofectamine RNAiMAX added to each insert. To assess transfection efficiency, we used the BLOCK-iT AlexaFluor Red Fluorescent Oligo (Invitrogen) that is not homologous to any known genes. Stealth RNAi negative control duplexes were used as a negative control. TER experiments were performed on day 3 after transfection.

NSAID-induced intestinal damage. The method used was modified from that which was previously reported (42). In brief, mice were treated with subcutaneous indomethacin (10 mg/kg; Sigma) two times daily at 8-h intervals for 1 day. The selected indomethacin dose was shown in previous studies to stably induce enteropathy. The specific TRPV4 antagonist HC (10 mg/kg) (7) or the epoxygenase inhibitor MS-PPOH (0.5 mg/body) (45) was administered subcutaneously concurrently with indomethacin two times a day. Sixteen hours after the final indomethacin administration, the extent of intestinal damage in the small intestine was measured (cumulative dimensions, in mm² of all lesions) by two independent observers using a VH analyzer (VH-H1A5; Keyence).

Data analysis. Values for Ca²⁺ imaging, TER, and in vivo experiments are presented as means ± SE from three or more independent experiments. A Student’s t-test or nonparametric Bonferroni-type multiple comparison was used. Significance was accepted for P < 0.05.

RESULTS

TRPV4 mRNA and protein expression in intestinal epithelia and a rat intestinal epithelial cell line (IEC-6). Because TRPV4 was shown to be expressed in the epithelium of the esophagus and intestine (6, 9, 19, 43), we first examined

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Fig. 2. TRPV4 immunoreactivities in mouse intestine and IEC-6 cells. A: TRPV4 was observed in intestinal epithelium (simple columnar) from wild-type (WT) but not TRPV4 knockout (TRPV4KO) mice (green, TRPV4; blue, DAPI). B: TRPV4 was also observed in the rat intestinal cell line IEC-6 (left). The immunoreactivity was diminished without anti-TRPV4 antibody (right). C: TRPV4 is shown to be localized on the basal side with an xz-scan through IEC-6 monolayer culture.
mRNA expression of TRPV4 in mouse and human intestines and the rat intestinal epithelial cell line (IEC-6). TRPV4 mRNAs were detected in all samples (Fig. 1).

TRPV4 was observed in WT mouse intestinal epithelium (simple columnar) but not in tissues from TRPV4KO mice (Fig. 2A). TRPV4 was also observed in the IEC-6 cell line, and an xz-scan through a cell monolayer indicated TRPV4 localization at the basal rather than apical side (Fig. 2C).

TRPV4-mediated cytosolic Ca\(^{2+}\) increases in IEC-6 cells. To confirm functional TRPV4 expression in IEC-6 cells, we examined cellular responses to the reported TRPV4 activator 5,6-EET (500 nM) (40) using a fluorescent Ca\(^{2+}\)-imaging system (5 μM fura-2-AM). Response traces to GSK in IEC-6 cells are shown in Fig. 3A. GSK- and 5,6-EET-induced cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) increases were significantly inhibited by pretreatment with the TRPV4-specific inhibitor RN (100 μM) (35) or in the absence of extracellular Ca\(^{2+}\) (Fig. 3B). We next examined the effect of indomethacin (2 μM) on [Ca\(^{2+}\)]\(_i\) increase. Although indomethacin had no effect on [Ca\(^{2+}\)]\(_i\) in IEC-6 cells, indomethacin pretreatment for 18 h significantly enhanced 5,6-EET-induced [Ca\(^{2+}\)]\(_i\) increases (Fig. 3B).

TRPV4-mediated reduction of TER in IEC-6 cells. The capacity of IEC-6 cells cultured on a transmembrane filter to differentiate and form epithelial cell layers as well as TER was monitored. To study whether TRPV4 modulates epithelial permeability, the TRPV4 activators GSK (100 nM) and 5,6-EET (500 nM) were compared with indomethacin (2 μM) in time-resolved TER measurements. We first observed TER changes in response to GSK, 5,6-EET, or indomethacin 10, 20, 30, 45, 60, 120, and 180 min after application. Application of the TRPV4 activator GSK or indomethacin to the basolateral side resulted in a maximal TER decrease at 30 min, and this decrease was reversed by 60 min. Meanwhile, the endogenous TRPV4 activator 5,6-EET gradually reduced TER for up to 60 min (Fig. 4A). GSK-induced TER reductions were significantly inhibited by pretreatment with the specific TRPV4 antagonists HC or RN (Fig. 4B), suggesting that TRPV4 activation reduced TER in IEC-6 cells. 5,6-EET-induced TER reductions were also significantly inhibited by pretreatment with RN (Fig. 4B), suggesting that 5,6-EET-induced TER reductions were mediated by TRPV4 activation. Application of HC or RN alone had no effect on TER (data not shown). Indomethacin-induced TER reductions were significantly inhibited by pretreatment with not only HC or RN, but also the microsomal CYP450 epoxygenase inhibitor MS-PPOH (20 μM) (Fig. 4C), suggesting that TRPV4 and AA metabolites are involved in indomethacin-induced TER reductions. To further confirm the involvement of TRPV4 in indomethacin-induced TER reduction, we next performed knockdown experiments using small-interfering RNA (siRNA). Effective transfection of siRNA was confirmed using a Fluorescent Oligo (Fig. 4D). TRPV4 knockdown completely inhibited GSK- and indomethacin-induced TER decreases (Fig. 4E), which directly demonstrates TRPV4 involvement in indomethacin-induced TER reductions. We compared the effects of selective COX1 (SC-560, 300 nM) and COX2 inhibitors (celecoxib, 0.1 μM) either alone or together on the TER. Whereas the application of celecoxib alone produced a slight decrease in the TER and SC-560 alone had little effect, the addition of both inhibitors significantly reduced TER 30 min after application (Fig. 4F). This result supports the hypothesis that inhibition of both COX1 and COX2 is necessary to increase 5,6-EET levels to those sufficient to decrease TER (Fig. 4F).

NSAID-induced intestinal damage. In mice, administration of two 10 mg/kg indomethacin doses spaced 8 h apart provoked ulcerous lesions in the small intestine that were predominantly located in the distal intestine along the mesenteric side. Compared with WT mice, TRPV4KO mice had fewer ulcerous intestinal lesions, the lesions had an overall smaller size, and total area was reduced by about one-half (Fig. 5, A and B). To further examine the involvement of TRPV4 and eicosanoids in indomethacin-induced intestinal damage, the specific TRPV4 antagonist HC or the epoxygenase inhibitor MS-PPOH was subcutaneously administered concurrently with indomethacin. The intestinal damage score was significantly reduced in WT mice treated with these agents individually compared with mice that received no treatment (Fig. 5B). These results suggest that TRPV4 and eicosanoids are involved in indomethacin-induced enteropathy in vivo.
In the present study, we evaluated TRPV4 expression in mouse and human small intestinal epithelia as well as IEC-6 cells. The homogenous expression pattern in mouse epithelial cells suggested that absorptive epithelial cells expressed TRPV4. The TRPV4 agonists GSK and 5,6-EET increased \([\text{Ca}^{2+}]_i\) in IEC-6 cells, and the responses were significantly inhibited by the TRPV4 antagonist RN. These results indicated that TRPV4 was functionally expressed in IEC-6 cells.

We showed that TRPV4 activation and indomethacin decreased the TER of IEC-6 cells. TRPV4 was shown to be expressed on the basolateral side in the colon cancer cell line Caco-2 and the mammary cell line HC11 (6, 28). The results of immunohistochemical study indicated TRPV4 localization on the basal side of IEC-6 cells (Fig. 2C). In addition, TER study using IEC-6 cell monolayers showed that TRPV4 on the basal side of IEC-6 cells was functional (Fig. 4). Indomethacin did not increase \([\text{Ca}^{2+}]_i\) (Fig. 3B), suggesting that it has no direct
effect on TRPV4 ion channels. To our knowledge, there are no reports of NSAIDs activating TRPV4 directly. We also showed that pretreatment with indomethacin for 18 h enhanced 5,6-EET-induced [Ca\(^{2+}\)] increases, which could be attributable to the ability of indomethacin to reduce 5,6-EET hydrolysis to subthreshold levels and promote additional 5,6-EET-enhanced [Ca\(^{2+}\)] increases. A similar phenomenon was reported for mouse aortic endothelial cells in which indomethacin-induced suppression of 5,6-EET hydrolysis enhanced TRPV4-dependent responses to AA or 5,6-EET (36). The discrepancy between Ca\(^{2+}\) imaging (Fig. 3B) and TER (Fig. 4C) results could be attributable to cell conditions. Whereas IEC-6 cells were isolated in bath solution for Ca\(^{2+}\) experiments, they were present as monolayers in TER experiments, which were performed in the culture medium. As such, the cells in the two assays may have had different metabolic statuses. We showed that decreases in TER produced by treatment with the TRPV4 activators GSK and 5,6-EET for 30 min were inhibited by TRPV4 antagonists (HC and RN) or TRPV4 knockdown. GSK-induced TER decreases eventually returned to basal levels, suggesting that cell viability was normal and the decreases were likely mediated by rapid TRPV4 desensitization and downregulation of channel expression on the plasma membrane (17). In the mouse mammary cell line HC11, TRPV4 activation increases epithelial permeability due to the endocytosis of TJ proteins, especially claudin4 (28), so that the observed transient TER reduction might be accompanied by the morphological changes in TJ proteins. Indomethacin also decreased TER while the TRPV4 antagonists, epoxygenase inhibitor MS-PPOH, and TRPV4 knockdown inhibited this effect, suggesting that it was mediated by TRPV4 and microsomal CYP450 epoxygenase activity because indomethacin has no direct effects on the TRPV4 ion channel. These data indicate that endogenously produced eicosanoids activate TRPV4, which is in agreement with the previous finding that the eicosanoid 5,6-EET opens TRPV4 channels directly (40). Although 5,6-EET is rapidly metabolized and therefore difficult to detect, indirect detection methods have been reported (12). We established an AA metabolite detection system using liquid chromatography-mass spectrometry to detect endogenous TRPV4 activators (5,6-EET and 8,9-EET) from indomethacin-applied IEC-6 cell supernatants. However, we did not detect a significant increase of 5,6-EET and 8,9-EET in cell supernatants. Because we could not detect all prostaglandins within the cells using an established protocol (24), sample preparation methods will be modified in future experiments. We also showed that the selective COX1 and COX2 inhibitors must be applied concurrently to reduce TER, suggesting that both enzymes participate in increasing eicosanoids to levels sufficient to reduce TER. This result is consistent with previous findings that COX1 inhibitors alone do not induce enteropathy (33).

We showed that indomethacin-induced intestinal injury in WT mice was partially reduced with the specific TRPV4 antagonist HC or the epoxygenase inhibitor MS-PPOH, suggesting that TRPV4 and eicosanoids are involved in indomethacin-induced intestinal damage in vivo (Fig. 6). Bacterial invasion is the major cause of NSAID-induced enteropathy (1, 28, 37). The open space between the cell membranes in the disruption of TJs and the diameter of enteric bacteria are ~15–20 nm and 1 μm, respectively. Therefore, some bacteria-derived factors (endotoxin, etc.) may be invasive via disrupted permeability in the intestinal tissues and be following the aggravation of the damage, and then bacteria itself might be invasive and aggravate it. Therefore, the enteric bacteria is, of course, a very essential factor to NSAID-induced enteropathy.
Intravenous administration of GSK to rats was previously shown to induce intestinal hemorrhage (43). As such, whether excessive activation of intestinal epithelial TRPV4 solely induces enteropathy or is merely an exacerbating factor should be explored by further investigation. Because it has been reported that NO or iNOS is important in the pathogenesis of indomethacin-induced small intestinal lesions (22) and mediates TRPV4 activation by S-nitrosylation on a cysteine residue of TRPV4 (44), NO or iNOS could further enhance the TRPV4 activation and epithelial permeability by AA metabolites.

TRPV4 is expressed throughout the body, and thus adverse effects of antagonists are a concern, as evidenced by the finding that mice lacking TRPV4 display water intake-related behavioral abnormalities, pressure reception dysfunction, hearing impairment, and dysuria (14, 23, 31, 32). Indeed, clinical trials examining the use of TRPV1 antagonists were halted because of hyperthermia effects (13). However, there is a growing body of evidence to support the effectiveness of TRPV4 antagonists for treating enteritis (9), cystitis (7), and pulmonary edema (34), and these antagonists produced no serious adverse effects in rodents. These results suggest that suppression of excessive TRPV4 activation may not inhibit the physiological function of TRPV4, thereby making it a promising therapeutic strategy for treating NSAID-induced intestinal damage.

In conclusion, TRPV4 is expressed in the intestine, and its activation causes epithelial hyperpermeability in response to NSAID-induced AA metabolites. This activity contributes to NSAID-induced intestinal damage, suggesting that TRPV4 could be a promising therapeutic target for preventing NSAID-induced intestinal damage.

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DISCLOSURES

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS


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