Epithelial transient receptor potential ankyrin 1 (TRPA1)-dependent adrenomedullin upregulates blood flow in rat small intestine

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Kono T, Kaneko A, Omiya Y, Ohbuchi K, Ohno N, Yamamoto M. Epithelial transient receptor potential ankyrin 1 (TRPA1)-dependent adrenomedullin upregulates blood flow in rat small intestine. Am J Physiol Gastrointest Liver Physiol 304: G428–G436, 2013. First published December 28, 2012; doi:10.1152/ajpgi.00356.2012.—The functional roles of transient receptor potential (TRP) channels in the gastrointestinal tract have garnered considerable attention in recent years. We previously reported that daikenchuto (TU-100), a traditional Japanese herbal medicine, increased intestinal blood flow (IBF) via adrenomedullin (ADM) release from intestinal epithelial (IE) cells (Kono T et al. J Crohns Colitis 4: 161–170, 2010). TU-100 contains multiple TRP activators. In the present study, therefore, we examined the involvement of TRP channels in the ADM-mediated vasodilatory effect of TU-100. Rats were treated intraduodenally with the TRP vanilloid type 1 (TRPV1) agonist capsaicin (CAP), the TRP ankyrin 1 (TRPA1) agonist allyl-isothiocyanate (AITC), or TU-100, and jejunum IBF was evaluated using laser-Doppler blood flowmetry. All three compounds resulted in vasodilatation, and the vasodilatory effect of TU-100 was abolished by a TRPA1 antagonist but not by a TRPV1 antagonist. Vasodilatation induced by AITC and TU-100 was abrogated by anti-ADM antibody treatment. RT-PCR and flow cytometry revealed that an IEC-6 cell line originated from the small intestine and purified IE cells expressed ADM and TRPA1 but not TRPV1. AITC increased ADM release in IEC cells remarkably, while CAP had no effect. TU-100 and its ingredient 6-shogaol (6SG) increased ADM release dose-dependently, and the effects were abrogated by a TRPA1 antagonist. 6SG showed similar TRPA1-dependent vasodilatation in vivo. These results indicate that TRPA1 in IE cells may play an important role in controlling bowel microcirculation via ADM release. Epithelial TRPA1 appears to be a promising target for the development of novel strategies for the treatment of various gastrointestinal disorders.

daikenchuto; TU-100; vasodilatation; 6-shogaol; inflammatory bowel diseases

Transient receptor potential (TRP) channels are nonselective calcium ion channels ubiquitously expressed in many tissues and are known to participate in a broad range of physical, chemical, and environmental stimuli such as taste, temperature, changes in osmolarity, pressure, stretch, and light. TRP channels are divided into seven subfamilies with 27 different channel types present in humans. Natural products, especially medicinal and culinary herbs such as chili pepper, mustard oil, and menthol, are known to stimulate some of these TRP channels. In recent years there has been a growing interest in elucidating the role of TRP channels in gastrointestinal physiology, including intestinal motility, secretion, and visceral sensation (23, 24, 39, 53). However, the physiological implications of TRP channels in intestinal blood flow (IBF) remain largely unexplored.

Daikenchuto (TU-100), a traditional Japanese herbal medicine (Kampo), is a mixture of extract powders from dried Japanese pepper, processed ginger, ginseng radix, and maltose powder. TU-100 is the most frequently prescribed Kampo medicine in Japan, especially for the treatment of postoperative paralytic and adhesive ileus and ischemic intestinal disorders (28). Basic studies have demonstrated the effect of TU-100 on intestinal motility, adhesion, vasodilatation, inflammation, and bacterial translocation (15, 22, 25, 27, 29, 30, 38, 44–47, 51, 52, 58). In a previous study, we demonstrated that TU-100 increases IBF via enhancement of adrenomedullin (ADM) release from the intestinal epithelial (IE) cells (27). However, the mechanism by which TU-100 enhances ADM release has not been elucidated.

Because some of the major ingredients of TU-100, such as 6-shogaol (6SG) and hydroxy-α-sanshool (HAS), are regarded as TRP vanilloid type 1 (TRPV1)/TRP ankyrin 1 (TRPA1) agonists (21, 31), we hypothesized that TRPV1/TRPA1 stimulation increases IBF via enhancement of ADM release from IE cells, and that the beneficial effect of TU-100 on IBF is mediated by this mechanism. Our results strongly suggest that TRPA1 present in IE cells controls IBF via ADM release and, therefore, the stimulation of intraluminal TRPA1 may be a promising approach for the relief of abdominal symptoms in various intestinal disorders associated with impaired IBF.

Materials and Methods

Test sample and reagents. TU-100 is an aqueous extract containing processed ginger, ginseng radix, and Japanese pepper in a ratio of 5:3:2. The dried powdered extract form of TU-100 was obtained from Tsumura and Co. (Tokyo, Japan). The yield of the extract was 12.5%. TU-100 is prepared by mixing TU-100 extract powder and maltose syrup powder (Tsumura and Co.) at a ratio of 1:8. Although the doses of TU-100 in the present study (270–2,700 mg/kg body wt) are higher than the clinical doses used in humans, previous studies in animals have shown that the relevant pharmacological effects occur only in the experimental doses. Furthermore, treatment of rodents with TU-100 at this higher dose range results in blood concentrations of major TU-100 constituents that are similar to those detected in human volunteers treated with TU-100 at clinical dose range (18, 37).

Ginsenoside Rb1, ginsenoside Rg1, ginsenoside Rd, protopanaxadiol, 6SG, 6-gingerol, 10-gingerol, maltose, allyl-isothiocyanate (AITC), and capsaicin (CAP) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Urethane, α-chloralose, cinnamalde-
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To investigate functional expression of TRPA1 in IEC-6 cells, the cell was exposed to the TRPA1-selective antagonist HC-030031 (100 µmol/l) 30 min before addition of TRPA1 activators.

Preparation of IE cells from small intestine. Segments of the small intestine were everted, end-ligated, and preincubated in HBSS containing 1 mmol/l DTT and 10% FBS to remove mucus. The sacs were then incubated for 10 min at 37°C in chelating digestive buffer (70 mmol/l NaCl, 5 mmol/l KCl, 20 mmol/l NaHCO3, 0.5 mmol/l NaH2PO4, 1 mmol/l NaHPO4, 50 mmol/l HEPES, 11 mmol/l glucose, 1 mmol/l EDTA, 0.5% BSA, and 0.05 mmol/l DTTO), followed by collection of the supernatant. The incubation was repeated twice, and the supernatants of each were pooled. The cell pellets obtained by centrifugation at 300 g for 10 min were suspended in 0.1% BSA HBBS and passed through a nylon mesh filter. The cell suspension was applied to a 25% gradient of Percoll (GE Healthcare, Piscataway, NJ). After centrifugation at 710 g for 30 min, the interface containing enriched IE cells was collected. IE cells were separated into negative fractions using a BI Mag cell separation system (BD Biosciences, San Jose, CA) with rabbit anti-nerve growth factor receptor p75 antibody (Millipore, Bedford, MA), followed by biotinylated anti-rabbit IgG (BD Bioscience) and biotinylated anti-CD45 antibody (clone, Ox-1; BD Bioscience), and thereafter incubated with streptavidin-labeled magnetic beads. Further, purified IE cells were stained with various cell-marker antibodies following a cytospin. Antibodies and positive cell percentages were wide cross-reactivity anti-cytokeratin (DAKO, Carpinteria, CA) at >90%, and anti-E-cadherin (clone, 36/E-cadherin; BD Bioscience) at >95%. Positive staining with anti-CD45 (clone, Ox-1; BD Bioscience), anti-PP9.5 (clone, 13C4/13C4; Abcam), or anti-GFP (clone, GF12.24; Progen, Heidelberg, Germany) was not detected.

Gene expression. The pellets of IEC-6 cells, IE cells obtained from the small intestines, and L1 to L6 dorsal root ganglia (DRG) isolated from normal rats were homogenized in QIAzol reagent (Qiagen, Valencia, CA), and total RNA was isolated using an RNaseasy kit (Qiagen) according to the manufacturer’s recommendations. The respective cDNA was prepared using a high-capacity RT kit (Applied Biosystems, Warrington, UK). The sequences of the sense and antisense primers for rat TRPA1 were 5’-TTTGGCCGCGATGATGGCCG-3’ and 5’-TGGTGC- CAGATGGAGAGGGTGT-3’ to obtain a 117-bp product. Those for rat TRPV1 were 5’-GGTGTGAGTTGGCCTCACTGCAG-3’ and 5’-CTCTG- TGAGTGGGACATC3’ to obtain a 107-bp product. Those for rat TRPV3 were 5’-CTGGTGATCTGGCTCACTGCAG-3’ and 5’-CTCTGTG- AGAGTGGTGGTGTGCTG-3’ to obtain a 446-bp product. Those for rat β-actin were 5’-CCCTGGGATATGGAAATTCCTGTGGCAT-3’ and 5’- GGAGCAATGATCTTGATCTTC-3’ to obtain a 117-bp product. An aliquot of the RT reaction product served as a template in 30 cycles with 10 s of denaturation at 98°C, 30 s of annealing at 60°C, and 30 s of extension at 68°C using the DNA polymerase KOD FX (TOYOBO, Osaka, Japan). A portion of the PCR mixture was electrophoresed on 2% agarose gel in Tris-acetate-EDTA buffer (pH 8.0), and the gel was stained with ethidium bromide and imaged on a Typhoon 9410 imager (GE Healthcare). Sample-to-sample variation in RNA loading was controlled by comparison with β-actin.

Flow cytometry. Single cells were suspended in Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4°C, washed, and then preincubated for 5 min at 4°C with goat polyclonal IgG antibody (Abcam) to reduce nonspecific binding of antibodies. Next, cells were incubated for 20 min at 4°C with rabbit polyclonal IgG antibody (4 µg/ml) against rat ADM (Abcam), TRPV1 (Alomone Labs, Jerusalem, Israel), or isotype control IgG (Abcam). Cells were washed, incubated for 20 min with the Alexa Fluor 488-labeled goat polyclonal antibody against rabbit IgG (Invitrogen, Carlsbad, CA), and subjected to flow cytometry analysis using a FACS calibur analyzer and CellQuest Pro software (BD Biosciences). In some experiments, a control peptide for TRPA1 or TRPV1 (Abcam) was added at 4 µg/ml with antigen-specific antibody.

Measurement of intestinal blood flow. Jejunal blood flow was measured by a laser-Doppler flowmeter (ALF21N, Advance, Tokyo) as previously described (30). Brieﬂy, rats were anesthetized with urethane (900 mg/kg ip), α-cloralose (45 mg/kg ip), and butorphanol (1 mg/kg im). A tracheotomy was performed and the rats were artiﬁcially ventilated. The left cervical artery was cannulated and connected to a transducer (P23XL, Nihon Kohden, Tokyo) to monitor systemic arterial blood pressure (AP) and heart rate (HR). Body temperature was maintained at 37 ± 0.5°C by a heating pad. After exposing the small intestine by a midline laparotomy, a cannula was inserted into the duodenum to facilitate injection of the test sample. A fiber optic probe was positioned 4 mm above the surface of the midjejunum. Vascular conductance (VC), calculated as the quotient of mean blood flow divided by mean AP, was used as an index of IBF.

Animals. Seven-week-old male Sprague-Dawley rats weighing 210–230 g were purchased from Japan SLC (Shizuoka, Japan). The animals were allowed free access to water and standard laboratory food, and housed at a temperature of 23 ± 2°C with relative humidity of 55 ± 10%, and a 12:12-h light/dark cycle with lights on from 0700–1900 daily. All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Asahikawa Medical University or Tsumura and Co. Ethical approval for the experimental procedures used in this study was obtained from the Laboratory Animal Committee of Asahikawa Medical University or Tsumura and Co. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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APB), 4-phorbol 12,13-didecanoate (4-PDD), H-89, calphostatin C, LY294002, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Aldrich (St. Louis, MO). HAS and hydroxy-β-sanshoo (HBS) were extracted from Japanese pepper at Tsumura and Co. with purities greater than 97.9%. Xanthoxyline (Tokyo Chemical Industry, Tokyo), butorphanol (Bristol-Myers Squibb, New York), HC-030031 (Biomol International, Plymouth Meeting, PA), and N-(4-tert-arybutylphenyl)-4-(3-chloropyridin-2-yl)-tetrahydropyr- azine-1(2H) carboxamide (BCTC; Biomol International) purchased for the study as well the other reagents used for analysis were the highest purity commercially available.

Gene expression. The pellets of IEC-6 cells, IE cells obtained from the small intestines, and L1 to L6 dorsal root ganglia (DRG) isolated from normal rats were homogenized in QIAzol reagent (Qiagen, Valencia, CA), and total RNA was isolated using an RNaseasy kit (Qiagen) according to the manufacturer’s recommendations. The respective cDNA was prepared using a high-capacity RT kit (Applied Biosystems, Warrington, UK). The sequences of the sense and antisense primers for rat TRPA1 were 5’-TTTGGCCGCGATGATGGCCG-3’ and 5’-TGGTGC- CAGATGGAGAGGGTGT-3’ to obtain a 117-bp product. Those for rat TRPV1 were 5’-GGTGTGAGTTGGCCTCACTGCAG-3’ and 5’-CTCTGTG- AGAGTGGTGGTGTGCTG-3’ to obtain a 446-bp product. Those for rat TRPV3 were 5’-CTGGTGATCTGGCTCACTGCAG-3’ and 5’- GGAGCAATGATCTTGATCTTC-3’ to obtain a 107-bp product. Those for rat β-actin were 5’-CCCTGGGATATGGAAATTCCTGTGGCAT-3’ and 5’- GGAGCAATGATCTTGATCTTC-3’ to obtain a 107-bp product. An aliquot of the RT reaction product served as a template in 30 cycles with 10 s of denaturation at 98°C, 30 s of annealing at 60°C, and 30 s of extension at 68°C using the DNA polymerase KOD FX (TOYOBO, Osaka, Japan). A portion of the PCR mixture was electrophoresed on 2% agarose gel in Tris-acetate-EDTA buffer (pH 8.0), and the gel was stained with ethidium bromide and imaged on a Typhoon 9410 imager (GE Healthcare). Sample-to-sample variation in RNA loading was controlled by comparison with β-actin.

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Calcium influx in rat TRPA1-transfected cells. A rat TRPA1-expressing cell line was generated using a tetracycline-inducible T-Rex expression system (Life Technologies, Grand Island, NY). T-Rex293 cell (Life Technologies) was transfected stably with pcDNA3/TO vector alone. Intracellular calcium was measured 1 day after induction with tetracycline (1 μg/ml). Cells were washed with an assay buffer (115 mmol/l NaCl, 5.4 mmol/l KCl, 13.8 mmol/l glucose, 2.5 mmol/l probenecid, 20 mmol/l HEPES, pH 7.6) and then loaded with Fluo-4 dye (Dojindo, Kumamoto, Japan). After 30 min incubation, cells were washed with the assay buffer. Then the test compound was added to each well. Fluorescence intensity was measured by FlexStation3 (Molecular Devices, Sunnyvale, CA). Concentration-response curves were fitted using Prism 3.0 with a Hill equation model.

Statistical analysis. All values are expressed as means ± SE. The statistical significance was evaluated by one- or two-way analysis of variance (ANOVA) followed by Dunnett’s test or Student’s t-test. A probability of less than 0.05 was considered significant.

RESULTS

Upregulation of IBF by TRPV1 and TRPA1 stimulation. We first investigated the vasoactive effect of TRPV1 and TRPA1 agonists administered into the lumen of the small intestine. The TRPV1 agonist CAP (3 mg/kg) caused a rapid increase in IBF, which peaked 15 min after administration and remained at high levels throughout data acquisition (Fig. 1A). The TRPA1 agonist AITC (0.002 mg/kg) produced a gradual increase in vasodilatation which peaked at 120 min or later (Fig. 1B). Neither of the agonists influenced systemic circulation (data not shown), and therefore, the effects were limited to the local microcirculation. The TRPV1-selective antagonist BCTC and the TRPA1-selective antagonist HC-03031 diminished the vasodilatory effect of CAP and AITC, respectively. Both agonists had no effect by themselves (data not shown).

Involvement of TRPA1 and ADM in the vasodilatory effect of TU-100. Past studies have shown that TU-100 increases blood flow in the small intestine of normal rats (38) and potentiates the production of vasoactive ADM by IE cells (27, 30). Here we examined the expression of TRPA1 and TRPV1 mRNAs in IEC-6 cells and purified IE cells obtained from the intestines. The expression of TRPA1 mRNA was clearly detected in these cells, as was DRG (Fig. 3A), while gene expression of TRPV1 was below the detection limit. TRPA1 protein levels in these cells were evaluated by flow cytometric analysis. As shown in Fig. 3B, the fluorescence intensities for anti-TRPA1 and anti-ADM antibody were higher than those of the subtype-control antibody. Marked reduction of fluorescence intensity by coexistence of the epitope peptide of TRPA1 antigen indicated that both of these cells types expressed TRPA1 protein.

ADM releasing activity of TRPA1 agonists and TU-100. Considering the expression of TRPA1 and ADM in IE cells, we investigated the ability of TRP channel agonists to release ADM. Samples tested were CAP, AITC, and CNA (TRPA1 agonists), 2-APB (agonist of TRPV1, TRPV2, and TRPV3), and 4o-PDD (TRPV4 agonist). As shown in Fig. 4A, the ADM concentrations in the culture fluids from rat IEC-6 cells treated with AITC (3–30 μmol/l) or CNA (100 μmol/l) were several times greater than control. On the other hand, CAP, 2-APB, and 4o-PDD were inactive in the test. As for TU-100 (Fig. 4B), the ADM concentrations in the culture fluids from IEC-6 cells with 270, 900, or 2,700 μg/ml of TU-100 were 16 ± 1, 17 ± 1, and 19 ± 1 pg/ml, respectively. These concentrations were 1.44, 1.60, and 1.74 times greater than control (11 ± 1), respectively. We then sought to identify the active ingredients responsible for the enhancement of ADM release. Twelve main ingredients were tested (Fig. 4, C–E). 6SG at concentrations of 10 and 30 μmol/l dramatically increased ADM release (2.27 and 8.30 times greater than control, respectively) with no cytotoxic effects. HAS significantly enhanced ADM release at concentrations of 30 and 100 μmol/l (1.49 and 1.83 times, respectively), although its activity was weaker than that of 6SG. 6-Gingerol was inactive in this test. Considering the intensity of ADM release activity and the high 6SG content in TU-100, 6SG appears to be the main active ingredient responsible for the vasodilatory effect of TU-100.

![Fig. 1. Intraluminal transient receptor potential (TRP) vaniloid type 1 (TRPV1) and TRP ankyrin 1 (TRPA1) agonists increase blood flow in the small intestine. Capsaicin (CAP, 3 mg/kg body wt) or allyl isothiocyanate (AITC, 0.002 mg/kg body wt) was administered intraduodenally, and vascular conductance (VC) in the midjejunum was monitored. A: the TRPV1 antagonist N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide (BCTC) (10 mg/kg) was given intravenously 15 min before CAP administration; N = 3; B: TRPA1 antagonist HC-03031 (1 mg/kg) was administered intraluminally together with AITC; N = 5–6. *P < 0.05, **P < 0.01 vs. water + vehicle (A) or vehicle (B). #P < 0.05, ##P < 0.01 vs. agonist alone, respectively.](http://ajpgi.physiology.org/)
Investigation of signal pathways linking TRPA1 to ADM release. The functional interaction of TRPA1 activators intrinsic to TU-100 with the TRPA1 molecule was investigated in two assays: blockage of ADM release using HC-030031 in IEC-6 cells and calcium influx in TRPA1-transfected cells. The influence of coaddition of HC-030031 was first examined with respect to ADM-releasing activity of TU-100, AITC, and 6SG. As shown in Fig. 5A, ADM release by these activators was significantly abolished by HC-030031. In addition, the ADM-releasing activity of these activators was not detected in calcium-free buffer (data not shown). T-Rex293 cells stably expressing rat TRPA1 were incubated with various concentrations of AITC and 6SG (Fig. 5B). Calcium influx was clearly evoked after their addition, while mock-transfected cells showed no response (data not shown).

Finally, the involvement of the kinase pathway in ADM release by TRPA1 activators was examined. This was accomplished by evaluating the effects of the cAMP-dependent protein kinase (PKA) inhibitor H-89, the protein kinase C (PKC) inhibitor calphostin C, and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 in an ADM release test of AITC and 6SG. As shown in Fig. 5C, ADM-releasing activity of AITC and 6SG was reduced by the addition of calphostin C. On the other hand, the activity of 6SG but not AITC was enhanced by the addition of H-89, while LY294002 had no effect. Moreover, the PKC-specific activator PMA significantly augmented ADM release (Fig. 5D).

Vasodilatory effect of 6SG. After confirming that 6SG was the main active ingredient of TU-100 that stimulates TRPA1 and ADM release, we evaluated its effect on IBF. As shown in Fig. 6A, the dose-dependent vasodilatory effect by 6SG was quantified using the area under curve of vascular conductance from 0 to 120 min. The effect of 6SG was completely abolished by pretreatment with HC-030031 (Fig. 6B).

DISCUSSION

In this study we demonstrated that J) freshly purified rat IE cells and the rat intestinal epithelial cell line IEC-6 expressed...
mRNAs and proteins of ADM and TRPA1, 2) TU-100 increased IBF via ADM release, 3) AITC, TU-100, and 6SG increased IBF in a TRPA1-dependent manner, and 4) AITC, TU-100, and 6SG stimulated ADM release/production in IE cells via stimulation of TRPA1. These data that suggest the activation of the epithelial TRPA1-ADM system in the small intestine as a potent factor in regulating IBF are a novel and important finding to understand intestinal physiology, and
pathobiology of various intestinal disorders with impaired intestinal microcirculation.

In the gastrointestinal tract, TRPA1 is predominantly expressed in a subset of TRPV1-expressing extrinsic sensory nerves, especially the DRG neurons (6, 32). The distribution of TRPA1 appears related to its physiological and pathophysiological roles such as mechanosensation (6, 9, 59), chemosensation (9) and inflammatory hyperalgesia (7, 36, 57). In addition, a recent study has also verified the presence of TRPA1 in several types of enteric nerves including inhibitory motoneurons, descending interneurons, and intrinsic primary afferent neurons (43). Furthermore, a subtype of enteroendocrine cells has been shown to express abundant TRPA1 whose stimulation induces 5-HT release that can activate intrinsic nerves and vagal endings (39). These reports strongly suggest that TRPA1 may play a role in the regulation of gut motility as confirmed by several motility studies of experimental animals using TRPA1 ligands and gene-manipulation (11, 12, 26, 42, 43).

More recently, considerable attention has been given to the presence of TRPA1 in IE cells. Kaji et al. (23, 24) detected TRPA1 mRNA and protein by RT-PCR and immunohistochemistry in human and rat epithelium isolated from intestinal mucosa, and Pool et al. (43) reported on TRPA1 immunosignals in mouse IE cells. The former study showed that AITC and an herbal ingredient, thymol, evoked electrogenic anion secretion from colonic epithelium segments in a TRPA1-dependent manner, although it is still unclear which cell types were stimulated by TRPA1 agonists because the study used unpurified epithelial preparations. The latter study did not examine the biological effect of TRPA1 in IE cells. In contrast, our study clearly showed that the stimulation of epithelial TRPA1 induces endogenous ADM release, which in turn participates in the regulation of IBF. Determining the specificity and mechanistic pathways of the epithelial TRPA1-ADM axis is an important area for further investigation, and studies using siRNA and knockout approaches remain to be done.

We also found that 6SG was the main active ingredient in TU-100 with ADM-releasing activity on that basis that 1) 6SG
potently induced vasodilatation in vivo, ADM release in vitro, and calcium influx in vitro in a TRPA1-dependent manner, and 2) the amount of 6SG in TU-100 was sufficient to explain most of the vasodilatation produced by TU-100 (a 2,700 mg/kg dose of TU-100 contains about 0.6 mg/kg of 6SG). Yet given that a small amount of 6SG in TU-100 has been reported to enter systemic circulation (48), our concern was whether TU-100 may affect systemic blood flow. A recent examiner-blinded randomized crossover trial investigating the effects of TU-100 on cardiac output and blood flow volume in the superior mesenteric artery in humans showed that a significant increase in blood flow in this artery occurred after TU-100 administration without any increase in systemic circulation (48). Another study indicated that TU-100 administration increased portal blood flow in healthy volunteers, cirrhotic patients, and liver-transplant patients without any significant changes in the systemic blood pressure and heart rate (40). These clinical findings are in good agreement with those of other basic studies using experimental animals. Thus TU-100 was surmised to increase IBF by affecting the regulatory mechanism of local blood circulation and thereby alleviate the detrimental effects of intestinal ischemia without causing cardiovascular complications.

ADM is known to have anti-inflammatory and vasodilatory effects, which have been confirmed by multiple colitis models induced by trinitrobenzenesulfonic acid and dextran sulfate sodium, the commonly used experimental models of inflammatory bowel diseases (4, 13). Some of the reported activities of ADM include suppression of certain proinflammatory cytokine production and release (16), antimicrobial effects (56), and enforcement of endothelial barrier function (49). These lines of evidence are consistent with the conjecture that intestinal ADM release via epithelial TRPA1 stimulation is involved in the maintenance and protection of gut functions. Although the results of these studies collectively suggest a novel approach to the treatment of colitis with ADM, exogenous administration of ADM is impractical because of its rapid clearance and potential systemic effects (35, 55). Meanwhile, as described in the previous paragraph, TU-100 appears to affect endogenous ADM system and IBF only locally and not systemically. In fact, we previously demonstrated that oral administration of TU-100 exerted an anti-colitis effect in trinitrobenzenesulfonic acid-induced colitis model via upregulation of intestinal ADM. Such localized increase in endogenous ADM by TU-100 may be advantageous because the potent biological effect of ADM is more or less confined to the diseased sites. On the basis of a number of reports indicating the ameliorating effect of TU-100 in various animal GI disease models (1, 8, 17, 20, 27, 30, 51), several double-blind, placebo-controlled, randomized trials in patients with postoperative paralytic ileus, refractory functional constipation, irritable bowel syndrome, and Crohn’s disease are currently being conducted in Japan (JFMC39–0902, JFMC40–1001 and JFMC42–1002 funded by the Japanese Foundation For Multidisciplinary Treatment of Cancer) and the United States (NCT00871325, NCT01139216, NCT01389933, and NCT01348152). Among these studies, one recent study reported that TU-100 has a prokinetic effect in healthy volunteers (33).

The present study has addressed the possibility that PKC and/or PKA/cAMP may play a role in TRPA1-related ADM release. This was of interest because a role of these molecules in TRPA1 signaling has not been reported except for the sensitization of TRPA1, an event that occurs upstream of TRPA1 signaling (2, 34, 54). Clarifying the PKC isoform(s) and molecular pathways involved in the effect is a priority for future research. As to the possible involvement of PKA/cAMP, it should be noted that H89 affected only 6SG-induced ADM release. Furthermore, there was no detectable change in cAMP levels in AITC- and 6SG-treated IEC6 cells (unpublished observations). These results suggest that PKA/cAMP may not be involved in vasodilatation induced by either 6SG or AITC. However, the enhancement of effect of 6SG by H89 suggests the possible involvement of mitogen-activated kinases (MAPKs): i.e., H89 inhibits not only PKA but also mitogen- and stress-activated kinase 1 (MSK1), which plays a critical role in NFκB-related inflammatory responses including production of prostaglandins, interleukin(IL)-8, and IL-10 (3, 10, 50). Multiple studies have shown that 6SG inhibits inflammatory responses (e.g., prostaglandin E2 synthesis) concomitant with potent suppression of the activation of certain mitogen-activated kinases (MAPKs) (5, 14, 41) including ERK1/2, which is typically located upstream of MSK1. Although the effect of 6SG on MSK has not been reported, it would be worthwhile to determine whether the MAPK system plays a role in the stimulation by 6SG.

In conclusion, our study revealed that epithelial TRPA1-ADM axis constitutes a possible regulatory system of IBF. In the gastrointestinal tract, TRPA1 appears to modulate digestive functions in at least three ways: induction of nociception via neuropeptide release from sensory neurons, facilitation of motility via 5-HT release from enterochromaffin cells, and promotion of vasodilatation via ADM release from IE cells (Fig. 7). Emerging physiological implications of TRPA1, especially its activity on the intestinal epithelium, identify TRPA1 ligands as promising drug targets for the management of gastrointestinal disorders with aberrant microcirculation.
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AUTHOR CONTRIBUTIONS

Author contributions: T.K. conception and design of research; T.K., A.K., Y.O., K.O., and N.O. prepared figures; M.Y. drafted manuscript.

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