千葉大学学位申請論文

Pharmacological Studies on the effect of a TRPA1 activator on gastric mucosal blood flow

（温度感受性 TRPA1 チャネル活性化薬の胃粘膜血流に関する実験薬理学的研究）

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Contents

Chemical receptors and substance

TRPA1: Transient receptor potential ankyrin-1

TRPV1: Transient receptor potential vanilloid-1

NO: nitric oxide

CGRP: Calcitonin-gene-related peptide

Medications and agents

AITC: allyl isothiocyanate

Others

GMBF: gastric mucosal blood flow

S.E.: standard error

Introductory Remarks

I entered Chiba University Graduate School to understand the effects of Kampo medicine from the basics. Kampo medicine can treat diseases for which Western medicine is sometimes inadequate; however, its mechanisms of action remain unclear in many aspects. Western medicine has no drugs that can improve colds whereas Kampo medicines are remarkably effective against them. Therefore, clarifying the exact mechanism of action for these medicines is highly desirable. The mechanism of action for Daikenchuto, a drug used to treat abdominal pain, bloating, cold hands, feet and stomach, loss of appetite, and malaise in people with cold stomach pain, has been partially elucidated. Daikenchuto induces a local mucosal hyper-perfusion reaction when applied to the stomach and intestines of rats. Its mechanism of action is reported to involve release of neurotransmitters such as adrenomedullin in the intestine via transient receptor potential (TRP) channels, resulting in increased blood flow [1]. Nitric oxide (NO) and calcitonin gene-related peptide (CGRP) are also reported as neurotransmitters for TRP channels [2].

TRP channels are diverse cation channels composed of homo- or hetero-tetramers of TRP proteins with six transmembrane domains. The activation opening of TRP

channels is induced by various physicochemical stimuli, including temperature, mechanical stimuli, pain, and acid-base balance; these channels are distributed in various tissues, but are highly expressed in the central and peripheral nerves. Their dysfunction has been reported to be involved in various diseases [3].

Nitric oxide is known to relax the smooth muscles of the tunica media in blood vessels, causing vasodilation and subsequently increased blood flow [4].

CGRP, a peptide consisting of 37 amino acids, is a neurotransmitter widely distributed from the central to peripheral nerves, but is rarely present in endocrine cells, and is a typical neuropeptide. CGRP has a vasodilating effect, and is highly expressed in the trigeminal nerve; further, the blood concentration of CGRP is increased in patients with migraine [5].

Adrenomedullin, a peptide comprising 52 amino acids, is a bioactive substance with strong vasodilatory action. It was first discovered in human pheochromocytoma tissue. Adrenomedullin production is increased in cardiovascular and inflammatory diseases; it exerts various physiological effects, including cardiovascular protective, angiogenic, and anti-inflammatory effects [6]. It is ubiquitous in the gastrointestinal (GI) tract and plays an important role in regulating microcirculation [7].

The mechanism by which TRP channel activators induce gastric mucosal hyperperfusion remains unclear [8]. Based on these gaps, I first attempted to elucidate the exact mechanism by which TRP channel activators induce gastric mucosal blood flow-enhancing reactions, thereby clarifying the pharmacological and physiological responses of the body. I would like to further clarify this aspect and connect it to elucidate the mechanism of action of Kampo medicines. Clarifying some of the functions of the body can improve our understanding regarding the mechanism of Kampo medicines and facilitate their future development.

Aim and Scope

The aim of this study was to clarify the mediators and TRPA1-expressing nerves involved in inducing vasodilation related to acute inflammation in response to TRPA1 agonist application to the gastric mucosa.

Ethics

Animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, approved by the Japanese Pharmacological Society and the guidelines approved by the Ethical Committee on Animal Care and Animal Experimentation of Josai International University (Protocol number: 2000073). The number of animals used was kept to the minimum necessary for meaningful data interpretation.

Introduction

Transient receptor potential ankyrin 1 (TRPA1) channel is the lone member of the mammalian ankyrin TRP subfamily. TRPA1 is reported to be a cold receptor activated by cold stimuli below 17 °C [9] and can be activated by various substances such as cinnamaldehyde [10], allyl isothiocyanate (AITC) [11], menthol [12], and prostaglandins [13,14]. TRPA1 is expressed in extrinsic primary afferent nerve cells and intrinsic enteric neurons in the mammalian gastrointestinal tract [15,16]. In addition, TRPA1 is predominantly co-expressed with TRPV1 in primary afferent neurons [17,18]. In our previous study, we found that TRPA1 is expressed in TRPV1-expressing sensory nerves in the rat stomach using immunohistochemistry [1].

TRPA1 plays an important role in gastrointestinal functions such as motility [15,19,20,21], epithelial barrier function [22,23], and colonic mucosal ion secretion [24,25]. Interestingly, Kono et al. reported that TRPA1 is expressed in rat intestinal epithelial cells by using reverse transcriptase-polymerase chain reaction and flow cytometric analysis, and the activation of epithelial TRPA1 with AITC or the traditional Japanese herbal medicine daikenchuto facilitates the release of adrenomedullin, resulting in increased intestinal mucosal blood flow in rats [7]. However, the physiological

implications of neuronal and epithelial TRPA1 in gastric mucosal blood flow (GMBF) remain unclear.

Nitric oxide (NO) and calcitonin gene-related peptide (CGRP) are released as neurotransmitters from TRPV1-expressing sensory nervous system [2]. AITC-induced increase in small intestinal mucosal blood flow is mediated by adrenomedullin [7]. Based on these findings, we decided to examine NO, CGRP, and adrenomedullin [26] as mediators of increased GMBF response induced by AITC.

In this study, we investigated the mechanism involved in TRPA1-mediated vasodilation using the selective TRPA1 activator AITC in a rat gastric mucosal model ex vivo using laser doppler flowmetry. A pharmacological approach enabled us to examine the contribution of NO, CGRP, and adrenomedullin in AITC-induced GMBF. This study provides valuable insights regarding the physiological function of TRPA1 in gastric microcirculation and suggests potential therapeutic targets for future drug discovery.

Materials and Methods

Animals

Male Sprague-Dawley rats (SLC, Hamamatsu, Japan) weighing 160–320 g were used. The animals were housed under controlled environmental conditions (24 ± 2℃, 12 h day/night cycle [lights on from 7.00 a.m. to 7.00 p.m.]) and fed commercial rat chow MF (Oriental Yeast, Tokyo, Japan). The animals were kept in individual cages with raised mesh bottoms to prevent coprophagy; they were deprived of food but allowed free access to tap water for 18 h before the experiments. Animal experiments were performed in compliance with the Guiding Principles for the Care and Use of Laboratory Animals, approved by the Japanese Pharmacological Society and the guidelines approved by the Ethical Committee on Animal Care and Animal Experimentation of Josai International University (Protocol number: 2000073). The number of animals used was kept to the minimum necessary for meaningful data interpretation.

Experimental Procedures

The animals were anesthetized with urethane (1.25 g/kg, i.p.) or isoflurane (1.5 ml/min, inhalation). The stomach was exposed through a midline incision and delivered onto the abdominal surface by gentle traction on the spleen; the pylorus was ligated. A 2-part lucite chamber was used for maintaining the gastric mucosal conditions *ex vivo*. One part is a lucite base, and the other is a plastic rim with two holes on the side wall, which are cannulated for perfusing the mucosa with saline (154 mM NaCl, 37℃) at a flow rate

of 1 ml/min. The lucite base was lowered over the animal, and the stomach was drawn though the central hole using forceps applied only to the forestomach. The stomach was then opened along the greater curvature from the middle part of the forestomach to the area where the epiploic artery terminates, and the edges were expanded by gently stretching the glandular mucosa. The plastic rim was then applied and pressed down on the mucosa. Under these conditions, only the glandular mucosal area, comprising mainly the corpus region, was exposed. The chamber was set at the level of the abdominal wall so that the external wall of the stomach remained inside the abdominal cavity. The body temperature was maintained at a rectal temperature of approximately 37℃ using a small animal warmer equipped with a thermometer (Bio Research Center, Model BWT-100, Nagoya, Japan). GMBF was measured using laser Doppler flowmetry (Advance, Model ALF-21N, Tokyo, Japan) with a non-touching probe (1 mm in diameter) on the corpus mucosal surface. After GMBF was stabilized, perfusion was discontinued, the luminal solution was removed, and the mucosa was exposed to 2 ml of capsaicin (3.3 mM) or AITC (0.33-10 mM) for 10 min. After application of capsaicin or AITC, the mucosa was rinsed with saline, another 2 ml of saline was instilled, and perfusion was resumed. Changes in GMBF were continuously monitored and recorded for 2 h-test periods using a PowerLab system (Model ML845; AD Instruments, Bella Vista, N.S.W., Australia). A

nonselective NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg, i.v.); or calcitonin gene-related peptide receptor antagonist, BIBN 4096 (10 mg/kg, i.p.) was administered 30 min before exposing the stomach to 2 ml AITC for 10 min or a TRPA1-selective blocker, HC-030031 (14.1 mM, i.g.) or AP-18 (10 mM, i.g.) was administered 20 min before exposing the stomach to 2 ml AITC for 10 min. In one group, an adrenomedullin receptor antagonist, AGP-8412 (10 μg/kg, i.v.), was administered 10 min before AITC application.

Sensory deafferentation of animals

Chemical deafferentation was performed 2 weeks before the experiment by successive subcutaneous injections of capsaicin, once daily for 3 days (20, 30, and 50 mg/kg) in rats. All capsaicin injections were performed under isoflurane anesthesia, and the rats were pretreated with the β-adrenergic receptor agonist isoproterenol (0.01 mg/kg, i.m.) and the selective β1-adrenergic antagonist atenolol (0.01 mg/kg, i.m.) to counteract the respiratory impairment associated with capsaicin injection. To verify the effectiveness of the treatment, a drop of 0.1 mg/ml capsaicin in 0.5% carboxymethyl cellulose solution (CMC) was instilled into one eye of each rat, and the protective wiping movements were counted.

Preparations and Drugs Used

AITC, atenolol, capsaicin, CMC, dimethyl sulfoxide (DMSO), DL-isoproterenol, and urethane (ethyl carbamate) were obtained from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). Carbamyl-β-methylcholine chloride (bethanechol chloride), L-arginine, L-NAME, and omeprazole were from Sigma-Aldrich (St. Louis, Mo., USA), isoflurane was from Mylan Seiyaku Ltd. (Tokyo, Japan). HC-030031, AP-18, and BIBN 4096 were purchased from Tocris Bioscience (Bristol, UK). AGP-8412 was from ANYGEN Co. Ltd. (Gwangji, Republic of Korea). Capsaicin was dissolved in Tween 80-ethanol solution (10% ethanol, 10% Tween 80, and 80% saline) for subcutaneous injections or suspended in 0.5% CMC for mucosal application. The other drugs were dissolved in saline with no organic solvent or detergent. Each drug was prepared immediately before use and was administered at 0.5 ml/100 g of body weight for intraperitoneal and subcutaneous administration or at 0.1 ml/100 g of body weight for intravenous administration. Control animals received only the vehicle.

Regarding TRPA1 antagonists, HC-030031 [7,27,28] and AP-18 [29] have been developed as selective TRPA1 antagonists. Therefore, HC-030031 and AP-18 were selected in this study; they were applied to the gastric mucosa. Indeed, Kono et al. have

shown that application of HC-030031 from the luminal side inhibits rat colonic mucosal blood flow in response to AITC [7]. In separate experiments, we investigated the potency of inhibition of TRPA1 in isolated mouse distal colon using Magnus apparatus. We found that the antagonistic effect of AP-18 was 1.4–3-times higher than that HC-030031; therefore, we determined the concentration for mucosal application to be 10 mM for AP-18 and 14.1 mM for HC-030031 (unpublished data).

Regarding the non-peptide BIBN 4096, which is a calcitonin gene-related peptide receptor antagonist, its affinity for the human CGRP receptor is higher than that of the endogenous ligand CGRP and 150-fold higher than that of the peptidic antagonist $CGRP_{8-37}$ [30]. BIBN 4096 proved to be a competitive antagonist without any intrinsic agonistic effect; moreover, it had no affinity for different receptors including the adrenomedullin receptor [30]. Warwick et al. reported that BIBN 4096 (10 mg/kg, ip) inhibited the proinflammatory fragment C5a-induced mechanosensitivity in rodents [31].

Regarding AGP-8412, which is an adrenomedullin receptor antagonist "$adrenomedullin_{22-52}$", it has been shown that $adrenomedullin_{22-52}$ (5 μg/kg, i.p.) significantly inhibited rat gastric acid secretion [32]. Juhl et al. reported that intravenous injection of $adrenomedullin_{22-52}$ (5 μg/kg) significantly inhibited vasodilation in response to exogenous adrenomedullin in the rat dural and pial artery [33]. In our experiment,

adrenomedullin$_{22-52}$ (10 μg/kg, i.v.) inhibited GMBF in response to exogenous adrenomedullin in this experiment.

Statistical Analyses

The data are presented as the means ± standard error (SE) from 3–9 rats per group. The statistical significance of differences between two groups was assessed using Student's *t*-test. Multiple comparisons against a single control group were made using a one-way analysis of variance with Bonferroni's multiple comparison test. The level of significance was set at 0.05. Sigma Stat 3.1 software (Jandel Scientific Software, San Rafael, Calif., USA) was applied for all statistical analyses.

Results

Effects of intragastric AITC on GMBF in *ex vivo* stomachs of anesthetized rats

Intragastric administration of AITC (0.33, 1, 3.3, and 10 mM) induced gastric hyperemic responses in a concentration-dependent manner; a significant effect was observed at concentrations greater than 3.3 mM (Fig. 1A, B). The response of GMBF to AITC (3.3, and 10 mM) during intragastric application for 10 min was 178.6 ± 12.9%

and 251.8 ± 7.0%, respectively. GMBF in response to 10 mM AITC decreased after AITC removal from the chamber (Fig. 1A, B). In addition, it was not observed hemorrhagic damage after the mucosal application of 10 mM AITC for 10 min in the rat stomach (data not shown). Mucosal application of the control solution (saline with 0.1% Tween) did not increase GMBF (Fig. 1A, B). As the effect of AITC on GMBF reached the maximal value upon application at 10 mM, this concentration was used in subsequent experiments to examine the effects of various agents on GMBF in response to AITC.

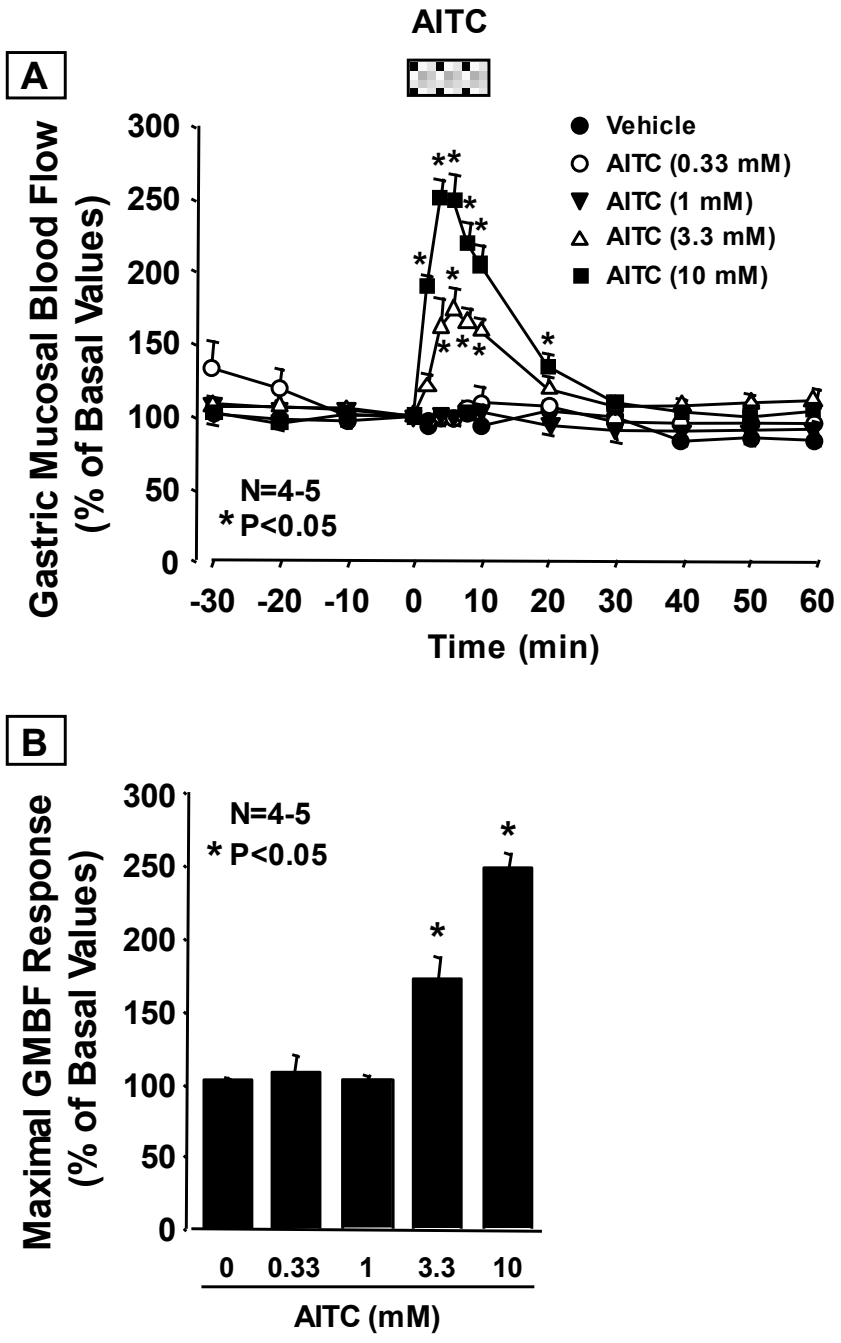

AITC
A
Gastric Mucosal Blood Flow
(% of Basal Values)
300
250
200
150
100
50
0
Vehicle
AITC (0.33 mM)
AITC (1 mM)
AITC (3.3 mM)
AITC (10 mM)
N=4-5
* P<0.05
-30 -20 -10 0 10 20 30 40 50 60
Time (min)
B
Maximal GMBF Response
(% of Basal Values)
300
250
200
150
100
50
0
N=4-5
* P<0.05
0 0.33 1 3.3 10
AITC (mM)

Fig. 1

Fig. 1. Effect of allyl isothiocyanate (AITC) mucosal application on gastric mucosal blood flow (GMBF) in the *ex-vivo* stomachs of anesthetized rats. AITC (0.33–10 mM) was topically applied to the mucosa for 10 min and the stomach was perfused with saline, before and after the application. **A**: The data are expressed as % increase in basal GMBF and represent the means ± standard error (S.E.) of values obtained from 4–5 rats at every 2 or 10 min. **B**: The maximal GMBF response induced by mucosal application of AITC (0.33–10 mM) is shown. The GMBF data are expressed as % increase in basal values and represent the means ± S.E. from 4–5 rats. *$P < 0.05$ compared with vehicle (0.1% Tween-saline) by analysis of variance (ANOVA) with Bonferroni's multiple comparison test. Notably, mucosal application of AITC increased GMBF in a concentration-dependent manner.

Effects of repeated intragastric AITC application on GMBF in *ex vivo* stomachs of anesthetized rats

The gastric hyperemic response to the first AITC application decreased to the baseline level after AITC removal from the chamber. The response to the second AITC application was about one-sixth of that of the first application (Fig. 2A, B). A similar phenomenon was reported upon repeated application of the TRPV1 activator, capsaicin, to the gastric mucosa, indicating that capsaicin stimulates a TRPV1 channel as only one site [1]. Therefore, these data suggested that the AITC-induced increase in GMBF was also attributed to TRPA1 channels alone.

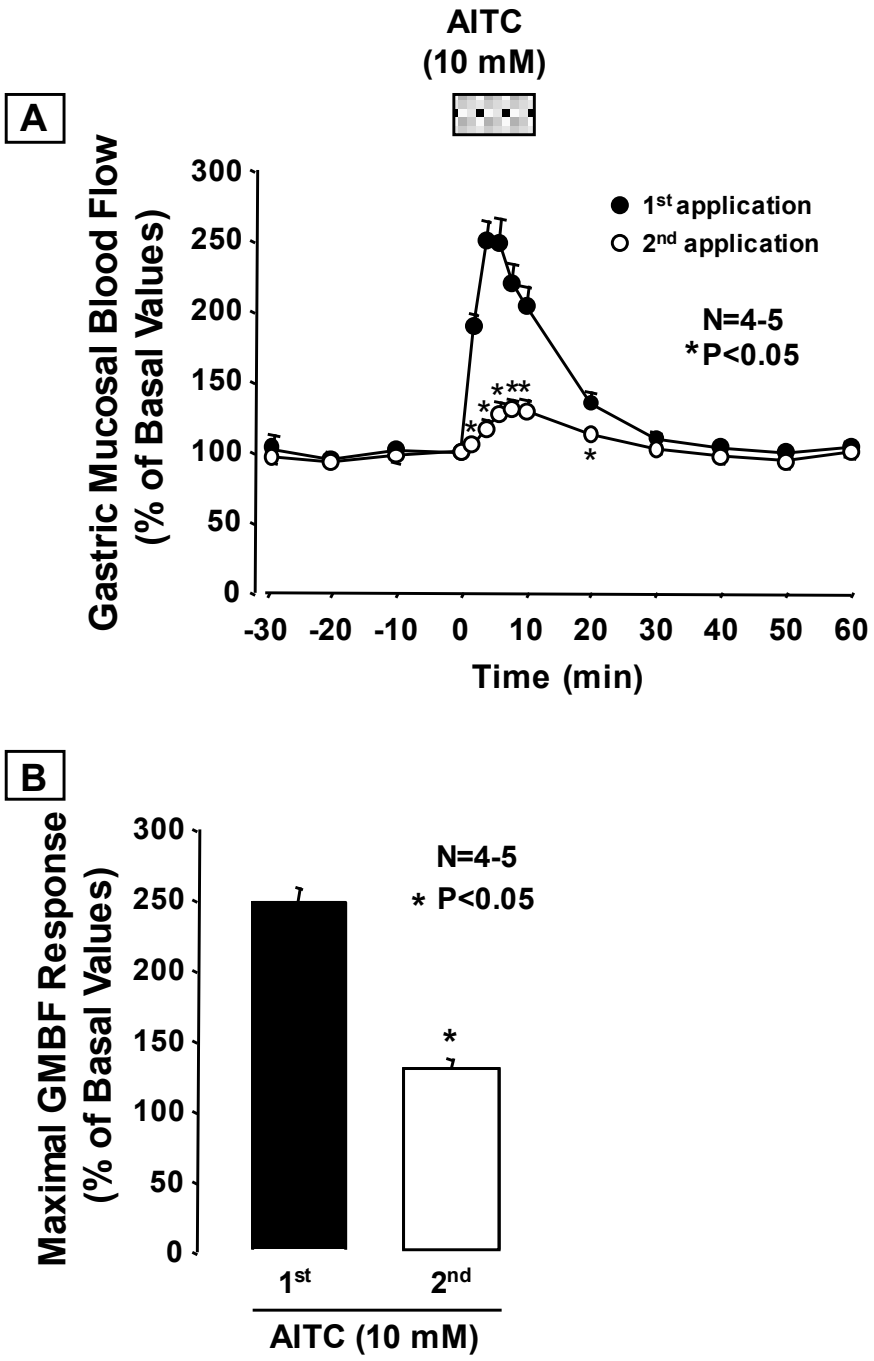

A
AITC
(10 mM)
Gastric Mucosal Blood Flow
(% of Basal Values)
300
250
200
150
100
50
0
1st application
2nd application
N=4-5
*P<0.05
-30 -20 -10 0 10 20 30 40 50 60
Time (min)
B
Maximal GMBF Response
(% of Basal Values)
300
250
200
150
100
50
0
N=4-5
* P<0.05
1st
2nd
AITC (10 mM)

Fig. 2

Fig. 2. Effects of repeated allyl isothiocyanate (AITC) application on gastric mucosal blood flow (GMBF) in the *ex-vivo* stomachs of anesthetized rats. The gastric mucosa was exposed to AITC (10 mM) for 10 min, and this was repeated after a 60 min-interval in the same animal. **A**: Data are expressed as % increase in basal GMBF and represent the means ± standard error (S.E.) of values obtained from 4–5 rats at every 2 or 10 min. Figure **B**: The maximal GMBF response induced by the mucosal application of AITC (10 mM) was shown. The GMBF data are expressed as % increase in basal values and represent the means ± S.E. from 4–5 rats. *$P < 0.05$ compared with the values observed after the first application, using Student's *t*-test.

Effects of intragastric application of TRPA1 blocker HC-030031 and AP-18 on GMBF in response to AITC in *ex vivo* stomachs of anesthetized rats

Increased GMBF in response to AITC (10 mM) was almost completely abolished in animals when the mucosa was exposed to a TRPA1 blocker HC 030031 (14.1 mM) (Fig. 3A). The maximal response of GMBF induced by 10 mM AITC in the presence of HC 030031 was 113.5 ± 8.9% (Fig. 3B). A similar phenomenon was observed in the gastric mucosa of animals treated with other TRPA1 blockers and AP-18 (10 mM). The increased GMBF in response to AITC (10 mM) was significantly attenuated by AP-18 during its application (Fig. 3A). The maximum response of GMBF in response to AITC in the control group and in the animals treated with AP-18 (10 mM) was 207.2 ± 26.2% and 146.1 ± 12.6%, respectively (Fig. 3B). These data indicated that gastric hyperemia in response to AITC was attributable to TRPA1 upon intragastric application of AITC in anesthetized rats.

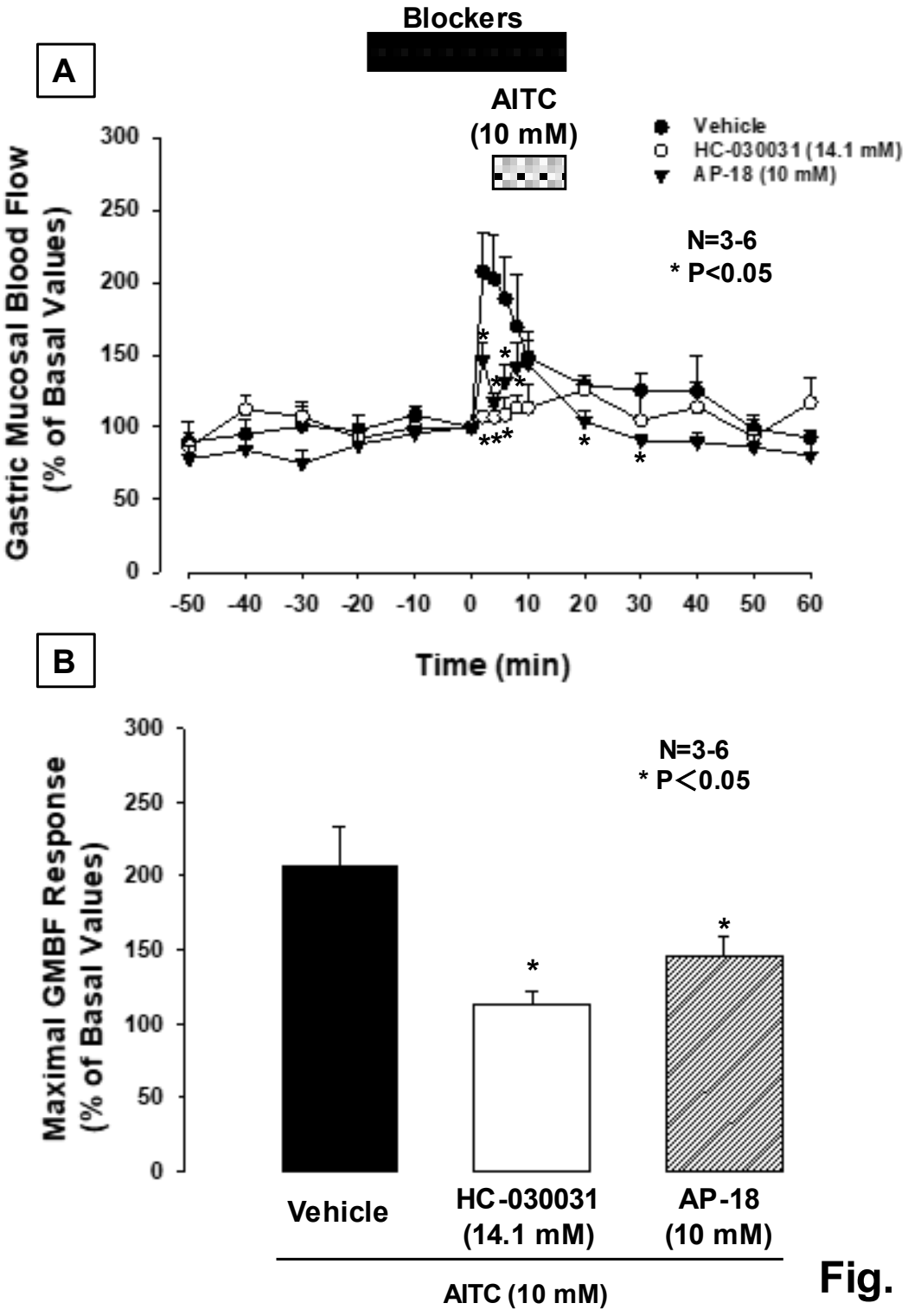

A
Blockers
AITC
(10 mM)
Vehicle
HC-030031 (14.1 mM)
AP-18 (10 mM)
N=3-6
* P<0.05
Gastric Mucosal Blood Flow
(% of Basal Values)
300
250
200
150
100
50
0
-50 -40 -30 -20 -10 0 10 20 30 40 50 60
Time (min)
B
N=3-6
* P<0.05
Maximal GMBF Response
(% of Basal Values)
300
250
200
150
100
50
0
Vehicle
HC-030031
(14.1 mM)
AP-18
(10 mM)
AITC (10 mM)

Fig. 3

Fig. 3. Effects of TRPA1 channel blockers HC 030031 and AP-18 on gastric mucosal blood flow (GMBF) induced by allyl isothiocyanate (AITC) in the *ex-vivo* stomachs of anesthetized rats. Either HC 030031 (14.1 mM), or AP-18 (10 mM) was applied to the chamber for 30 min, starting at 20 min before AITC (10 mM) application. **A**: The data are expressed as % increase in basal GMBF and represent the means ± standard error (S.E.) of values obtained from 3–6 rats at every 2 or 10 min. **B**: The maximal GMBF response induced by mucosal application of AITC (10 mM) was shown. The GMBF data are expressed as % increase in basal values and represent the means ± S.E. from 3–6 rats. *P < 0.05 compared with vehicle, using Student's *t*-test. Notably, increased GMBF in response to AITC was markedly inhibited by the TRPA1 channel blockers HC 030031 and AP-18 in the *ex-vivo* stomachs of anesthetized rats.

Effect of sensory deafferentation on GMBF in response to AITC in *ex-vivo* stomachs of anesthetized rats

Next, we investigated whether TRPA1-expressing sensory nerves are similar to capsaicin-sensitive sensory nerves. Rats with sensory deafferentation were developed via consecutive injections of capsaicin (total 100 mg/kg, s.c.) 2 weeks before the experiment. In these rats, although the GMBF remained unchanged during saline perfusion, we did not observe an increase in GMBF in response to the mucosal application of capsaicin (3.3 mM), and the maximal response was considerably less (103.1% ± 4.2%) than that (163.1% ± 6.2%) in the control rats (Fig. 4A and C). Accordingly, the neurotoxic dose of capsaicin induced the degeneration of TRPV1-expressing primary afferent neurons [1]. Interestingly, here, intragastric application of AITC induced a considerably lower increase in GMBF than that in the control rats. The GMBF was not completely suppressed (Fig. 4B and C). The enhanced GMBF in response to AITC could be attributed to TRPA1, which is not expressed in TRPV1-expressing nerve fibers or gastric epithelial cells.

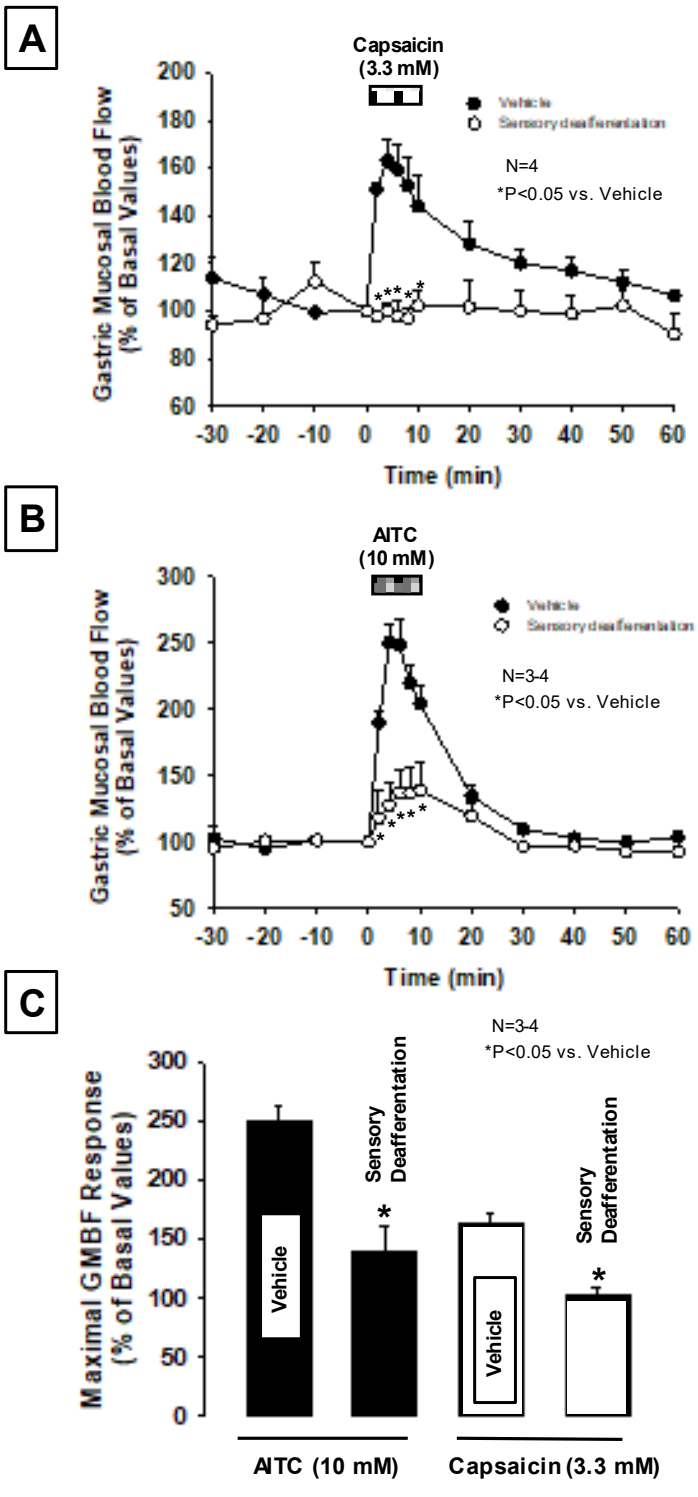
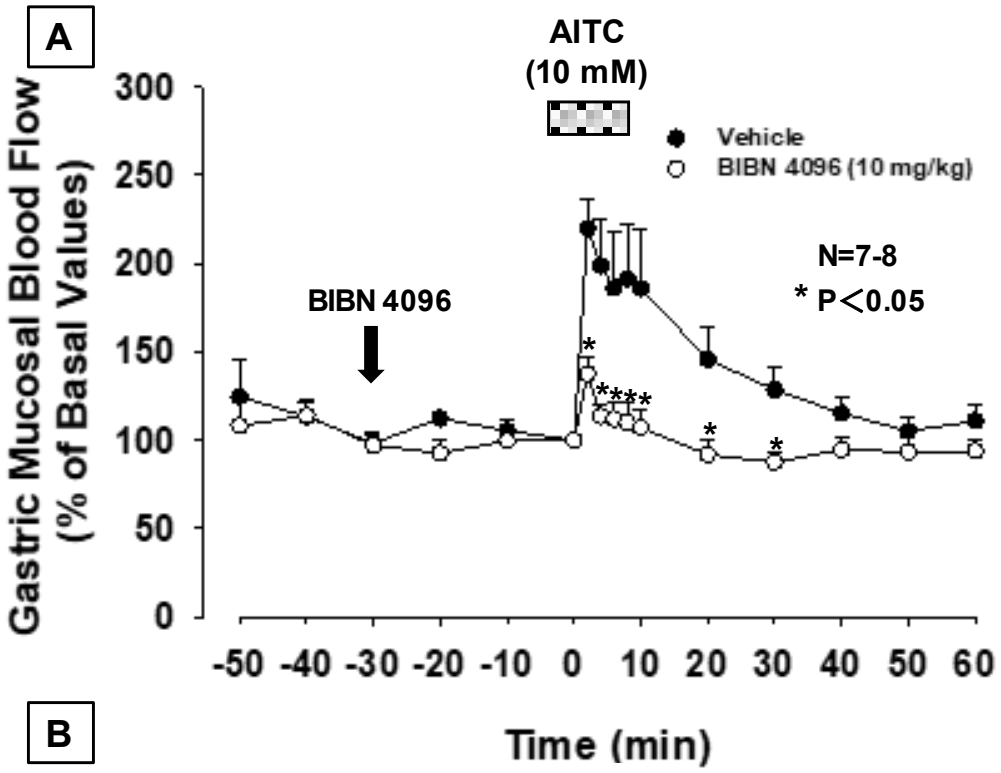

A
Capsaicin
(3.3 mM)
Vehicle
Sensory deafferentation
N=4
*P<0.05 vs. Vehicle
Gastric Mucosal Blood Flow
(% of Basal Values)
Time (min)
B
AITC
(10 mM)
Vehicle
Sensory deafferentation
N=3-4
*P<0.05 vs. Vehicle
Gastric Mucosal Blood Flow
(% of Basal Values)
Time (min)
C
N=3-4
*P<0.05 vs. Vehicle
Maximal GMBF Response
(% of Basal Values)
Vehicle
Sensory Deafferentation
Vehicle
Sensory Deafferentation
AITC (10 mM)
Capsaicin (3.3 mM)

Fig. 4

Fig. 4. Effect of sensory deafferentation on the increased gastric mucosal blood flow (GMBF) induced by mucosal application of allyl isothiocyanate (AITC) and capsaicin in the *ex-vivo* stomachs of anesthetized rats. The stomach was exposed to either AITC (10 mM) or capsaicin (3.3 mM) for 10 min and perfused with saline before and after the exposure. Sensory deafferentation was performed by repeated subcutaneous (s.c.) administration of capsaicin (total 100 mg/kg) 2 weeks before the experiment. **A**: The data are expressed as % increase in basal GMBF and represent the means ± standard error (S.E.) of values obtained from 3–4 rats at every 2 or 10 min. **B**: The data of AITC-induced GMBF responses are expressed as percent increase in basal GMBF and represent mean ± SE of values obtained from 4 rats at every 2 or 10 min. **C**: The maximal GMBF response induced by AITC (10 mM) or capsaicin (3.3 mM) in normal and sensory deafferentation rats was shown. Values represent the % increase in GMBF (% of basal values) and are presented as the mean ± S.E. from 3–4 rats. *$P < 0.05$ compared with the control, using Student's *t*-test. Notably, the increased GMBF in response to AITC was apparently attenuated, but not completely abolished, in sensory deafferentation rats.

Effect of BIBN 4096, a CGRP receptor antagonist, on GMBF in response to AITC in *ex-vivo* stomachs of anesthetized rats

GMBF did not change when BIBN 4096 (10 mg/kg) was injected alone (Fig. 5A). The increase in GMBF in response to AITC (10 mM), as observed in control rats, was remarkably inhibited by BIBN 4096. The maximum response of GMBF in response to AITC in the control group and animals treated with BIBN 4096 (10 mg/kg) was 207.8 ± 24.0% and 137.6 ± 9.6%, respectively (Fig. 5B). These data indicate that gastric hyperemia in response to AITC is attributable to CGRP during AITC application in anesthetized rats.


A
AITC
(10 mM)
Vehicle
BIBN 4096 (10 mg/kg)
N=7-8
* P＜0.05
BIBN 4096
Gastric Mucosal Blood Flow
(% of Basal Values)
300
250
200
150
100
50
0
-50 -40 -30 -20 -10 0 10 20 30 40 50 60
Time (min)

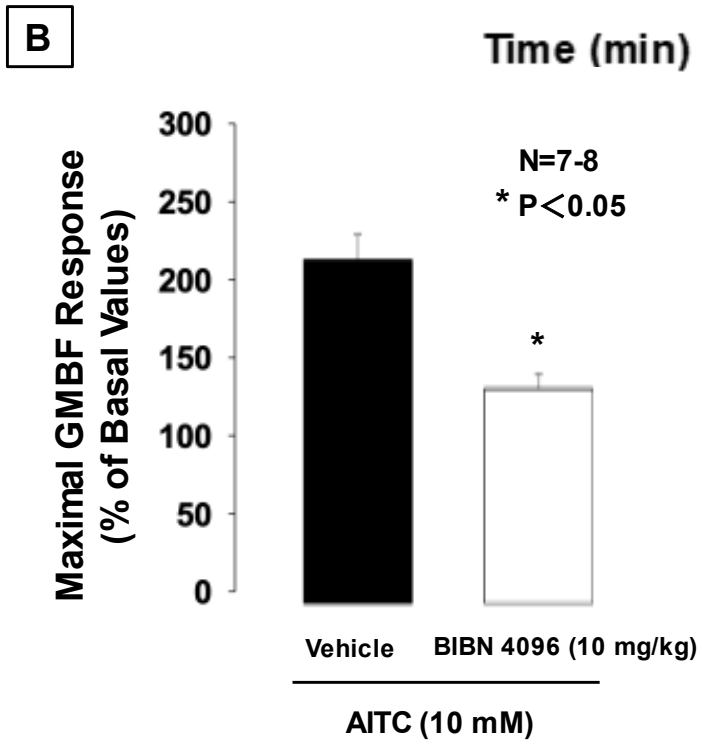

B
N=7-8
* P＜0.05
Maximal GMBF Response
(% of Basal Values)
300
250
200
150
100
50
0
Vehicle
BIBN 4096 (10 mg/kg)
AITC (10 mM)

Fig. 5

Fig. 5. Effect of BIBN 4096, a selective calcitonin gene-related peptide (CGRP) receptor antagonist, on gastric mucosal blood flow (GMBF) induced by mucosal application of allyl isothiocyanate (AITC) in the *ex-vivo* stomach of anesthetized rats. **A**: Time course of analysis for the GMBF response to AITC (10 mM) in anesthetized rats pretreated with BIBN 4096. The stomach was perfused with saline before application. BIBN 4096 (10 mg/kg) was administered by an intraperitoneal injection, and 30 min later, AITC (10 mM) was topically applied to the mucosa for 10 min. The data are expressed as the % increase in basal values and represent the means ± standard error (S.E.) of values from 7–8 rats at every 2 or 10 min. * $P < 0.05$ compared with vehicle (saline), using Student's *t*-test. **B**: Maximal response of GMBF during AITC (10 mM) application in animals treated with BIBN 4096 (10 mg/kg). Data are expressed as percent increase in basal values and represent the mean ± S.E. from 7–8 rats. Notably, increased GMBF in response to AITC application was significantly inhibited by BIBN 4096 (10 mg/kg), but no effect was found on basal GMBF before AITC application.

Effect of AGP-8412, an adrenomedullin receptor antagonist, on GMBF in response to AITC in *ex-vivo* stomachs of anesthetized rats

GMBF did not change when AGP-8412 (10 μg/kg) was injected alone (Fig. 6A). A significant increase in GMBF in response to AITC (10 mM) was observed in control rats, but this was not completely inhibited by AGP-8412 (10 μg/kg). The maximum response of GMBF in response to AITC in the control group and in animals treated with AGP-8412 (10 μg/kg) was 304.2 ± 30.0% and 158.0 ± 6.9%, respectively (Fig. 6B). These data suggest that gastric hyperemia in response to AITC is partly attributable to adrenomedullin.

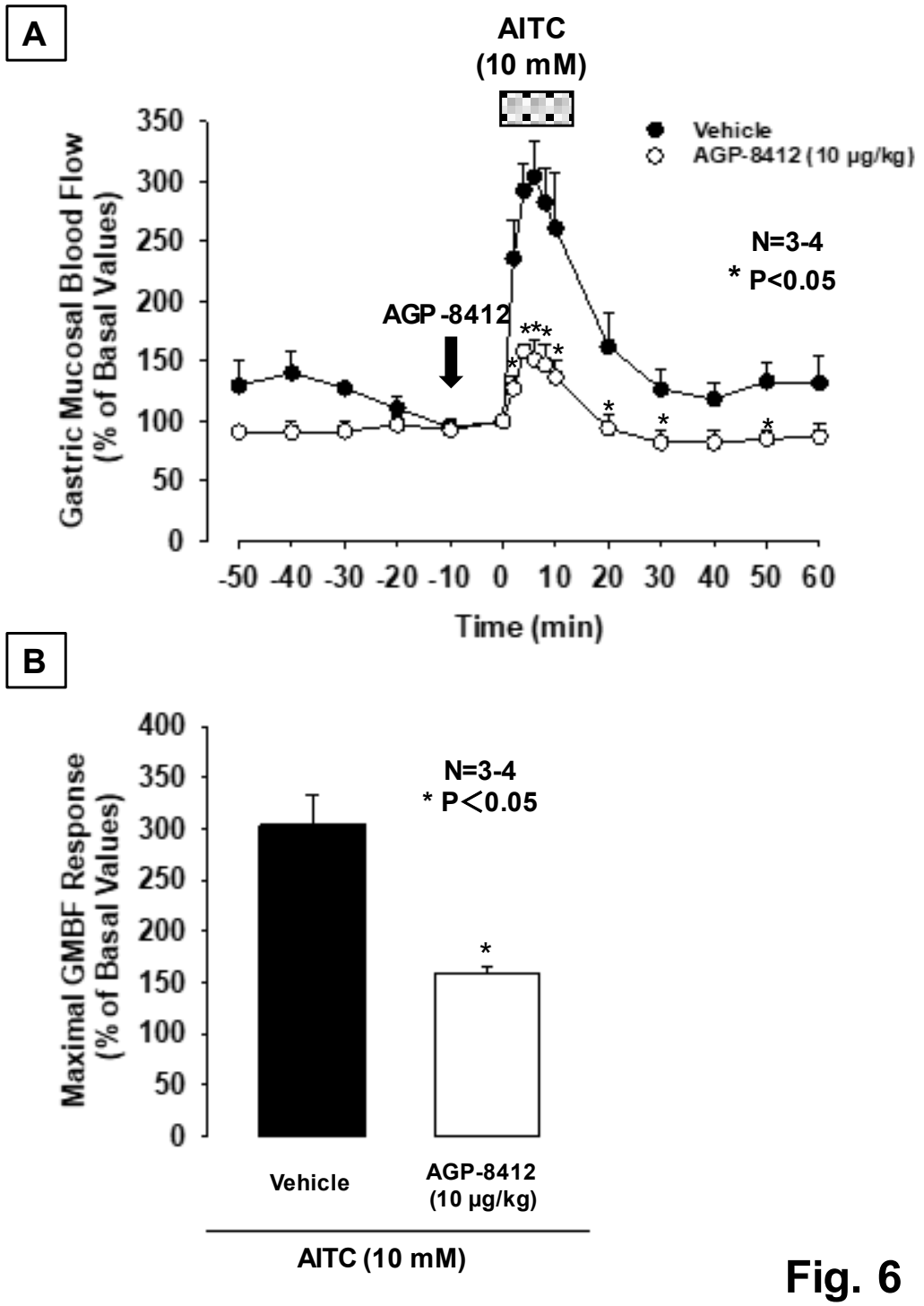

A
AITC
(10 mM)
Vehicle
AGP-8412 (10 μg/kg)
N=3-4
* P<0.05
AGP-8412
Gastric Mucosal Blood Flow
(% of Basal Values)
350
300
250
200
150
100
50
0
-50 -40 -30 -20 -10 0 10 20 30 40 50 60
Time (min)
B
Maximal GMBF Response
(% of Basal Values)
400
350
300
250
200
150
100
50
0
N=3-4
* P<0.05
Vehicle
AGP-8412
(10 μg/kg)
AITC (10 mM)

Fig. 6

Fig. 6. Effect of AGP-8412, an adrenomedullin receptor antagonist, on gastric mucosal blood flow (GMBF) induced by mucosal application of allyl isothiocyanate (AITC) in the *ex-vivo* stomach of anesthetized rats. **A**: Time course analysis of GMBF response to AITC (10 mM) in anesthetized rats pretreated with AGP-8412. The stomach was perfused with saline before application, AGP-8412 (10 μg/kg) was administered via an intravenous injection, and 20 min later, AITC (10 mM) was topically applied to the mucosa for 10 min. The data are expressed as the % increase in basal values and represent the means ± standard error (S.E.) of values from 3–4 rats every 2 or 10 min. * $P < 0.05$ compared with vehicle (saline) values after the AGP-8412 addition, using Student's *t*-test. **B**: Maximal response of GMBF during AITC (10 mM) application in animals treated with AGP-8412 (10 μg/kg). The data are expressed as the % increase in basal values and represent the mean ± S.E. from 3–4 rats. Notably, increased GMBF in response to AITC application was significantly inhibited by AGP-8412 (10 μg/kg), but no effect was found on basal GMBF before AITC application.

Effects of L-NAME, a nonselective NOS inhibitor, on GMBF in response to AITC in *ex-vivo* stomachs of anesthetized rats

The increased GMBF in response to AITC (10 mM) seen in control rats was not attenuated by L-NAME (Fig. 7A). The maximal response of GMBF in response to AITC in the control group and in animals treated with L-NAME (10 mg/kg) was 242.1 ± 38.5% and 194.5±29.1%, respectively (Fig. 7B). These data suggested that increased GMBF in response to AITC was not attributed to the endogenous vasodilator NO.

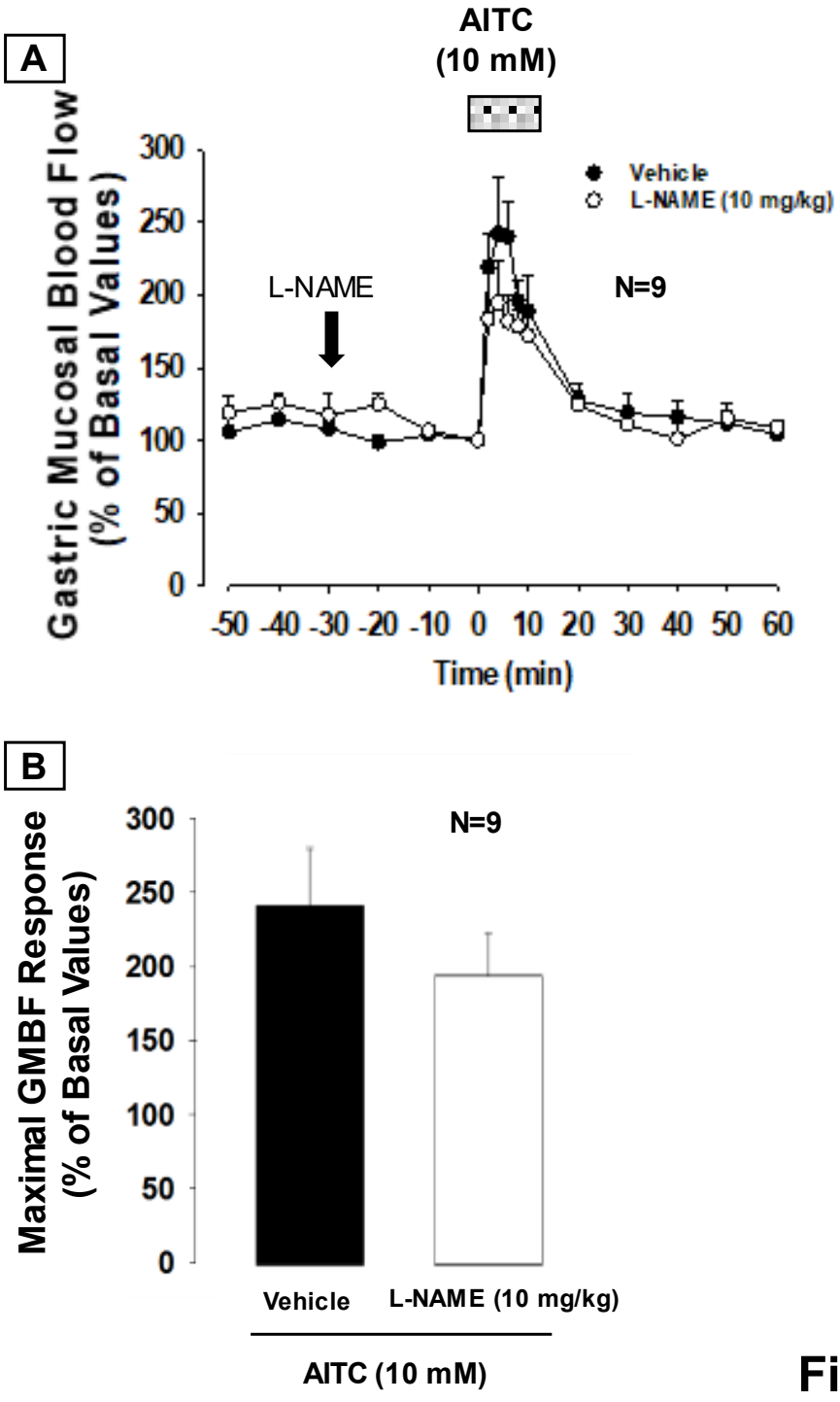

A
AITC
(10 mM)
Gastric Mucosal Blood Flow
(% of Basal Values)
300
250
200
150
100
50
0
Vehicle
L-NAME (10 mg/kg)
L-NAME
N=9
-50 -40 -30 -20 -10 0 10 20 30 40 50 60
Time (min)
B
Maximal GMBF Response
(% of Basal Values)
300
250
200
150
100
50
0
N=9
Vehicle
L-NAME (10 mg/kg)
AITC (10 mM)

Fig. 7

Fig. 7. Effect of L-NAME, a nonselective NOS inhibitor, on gastric mucosal blood flow (GMBF) induced by mucosal application of allyl isothiocyanate (AITC) in the *ex-vivo* stomach of anesthetized rats. **A**: Time course analysis of GMBF response to AITC (10 mM) in anesthetized rats pretreated with L-NAME. The stomach was perfused with saline before application, L-NAME (10 mg/kg) was administered via an intravenous injection, and 30 min later, AITC (10 mM) was topically applied to the mucosa for 10 min. L-NAME by itself produced a temporary increase in GMBF upon injection, but the GMBF immediately returned to basal values. The data are expressed as the % increase in basal values and represent the means ± standard error (S.E.) of values from 5–6 rats at every 2 or 10 min. * $P < 0.05$ compared with vehicle (saline), using Student's *t*-test. Figure **B**: Maximal response of GMBF during AITC (10 mM) application in animals treated with L-NAME (10 mg/kg) is shown. The data are expressed as the % increase in basal values and represent the mean ± S.E. from 5–6 rats. Notably, there was no significant difference in the group treated with L-NAME (10 mg/kg) compared with the corresponding values in the vehicle.

Discussion

In this study, we found that intragastric application AITC facilitates an increase in GMBF via CGRP and adrenomedullin after stimulating TRPA1-expressing sensory neuron and epithelial cells in rat stomachs. To investigate the mechanism underlying increased blood flow in response to intragastric AITC application in rats, we focused on NO, CGRP, and adrenomedullin as mediators, and investigated the effects of the corresponding blockers on AITC-induced increased gastric blood flow. The degree of mediator involvement in the flow-increasing reaction was also examined. Further, different types of TRPA1 blockers were pre-administered to confirm the changes in the blood flow increase response. The results suggested that the AITC-induced increase in mucosal blood flow is mediated by CGRP and adrenomedullin, whereas NO is not significantly involved as a mediator.

Mucosal blood flow increase induced by TRPA1 activation

AITC, an activator of TRPA1 [34], increased GMBF in a dose-dependent manner in our study. TRPA1 response was inhibited by selective TRPA1 blockers [27,28,29]. We previously examined the effect of the concentration-dependent response of AITC (0.3–

300 mM) on GMBF in the rat stomach. Especially, luminal acid loss, which reflects irritated mucosa, was measured every 15 min before and after the mucosal application of AITC. It was observed that the mucosal application of AITC, at 30–300 mM, induced luminal acid loss, followed by an increase in GMBF, resulting in no hemorrhagic damage (unpublished data). However, mucosal application of AITC, at less than 10 mM, did not induce enhanced luminal acid loss. Therefore, AITC (10 mM)-induced increased mucosal blood flow might not have involved the effect of AITC as a mild irritant in this study." Therefore, it is suggested that the mucosal blood flow increase induced by AITC was mediated by TRPA1.

TRPA1 activation mediates neurogenic vasodilation, and TRPA1 agonist-induced vasodilation requires NO derived from neuronal NOS [35]. In addition, TRPA1 activation in sensory neurons causes release of the potent vasodilator CGRP [36] and adrenomedullin, which induces a vasodilatory effect [26]. A previous study have also reported the involvement of some TRPV1-mediated responses [37]. In this study, we observed that AITC increased GMBF in a dose-dependent manner; this effect was markedly attenuated by the repeated application of AITC.

MacNaughton et al. reported that exogenous vasoconstrictive endothelin i.v. injection augmented gastric mucosal damages in rats; the damages were prevented by exogenous

prostaglandin I_2 [38]. Therefore, we considered the effect of vasoconstrictive endothelin on GMBF in this study. On the contrary, it has been reported that adrenomedullin inhibits the release of endothelin in response to thrombin or platelet-derived growth factor (PDGF), but adrenomedullin did not inhibit the spontaneous release of endothelin in rat vascular smooth muscle cell [39]. Based on these findings, it was suggested that adrenomedullin did not affect the spontaneous release and production of endothelin in response to mucosal application of AITC, because AITC (10 mM) did not induce hemorrhagic mucosal damages without the need to produce thrombin and PDGF. Furthermore, endothelin facilitates not only the contraction of vascular smooth muscle through the ET_A receptor, but also the production of vasodilator substances including NO and adrenomedullin from vascular endothelium through the ET_B receptor to regulate vasocontraction [40]. Therefore, it is unlikely that endothelin affects increased gastric mucosal blood flow in response to AITC. Indeed, there is no report on the effects of both endothelin and TRPA1 activator AITC on mucosal blood flow. Then, we focused on the involvement of CGRP, adrenomedullin, NO, and capsaicin-sensitive sensory nerves in the increased GMBF in response to by AITC.

Data from animals with degenerated sensory nerves

When AITC was administered after the pretreatment with a neurotoxic dose of capsaicin, a much smaller increase of GMBF by AITC was observed compared to that in normal animals, whereas the increase by capsaicin was abolished. Additionally, it was assessed GMBF in response to the nitric oxide (NO) donor in sensory deafferented rats compared with that in normal rats as a pilot study. It was not observed any changes in gastric hyperemia between the groups. Thus, it was suggested that the neurotoxic dose of capsaicin does not affect GMBF in response to exogenous and endogenous vasodilators.

TRPV1 and TRPA1 are co-expressed on primary sensory nerves [17,41]. Immunostaining results revealed that TRPA1-expressing extrinsic nerve fibers were localized around the blood vessels. As TRPA1-expressing neurons have their cell bodies in the myenteric plexus, TRPA1 is expressed on intrinsic and extrinsic sensory neurons. TRPV1 immunoreactivities were not detected in cell bodies in the submucosal or muscular layers. Thus, the TRPV1 may be expressed on extrinsic sensory nerve fibers [1]. It is considered that TRPV1-expressing extrinsic nerve fibers express TRPA1; a previous report indicated that the co-existence ratio is approximately 80% [42]. The extrinsic TRPV1 sensory nerve fibers contain CGRP and substance P as neurotransmitters [2]. In animals pre-treated with the neurotoxic dose of capsaicin, the nerve fibers in which TRPV1 and TRPA1 were co-expressed were destroyed, but the only TRPA1-expressing

nerve fibers remained. AITC acts on the only TRPA1-expressing nerve fibers, leading to a low AITC response in the sensory deafferentated rat stomach. Furthermore, the activation of TRPA1, which is expressed on gastrointestinal epithelial cells, facilitates adrenomedullin release, inducing vasodilation in the intestinal mucosa [7]. Thus, adrenomedullin released from gastric mucosa may also be involved in the low AITC response in the sensory deafferentated rats.

AITC-induced increase in mucosal blood flow (vasodilation) is mediated by CGRP and adrenomedullin, but this effect is not observed with L-NAME treatment

In the present study, the nonselective NOS inhibitor L-NAME did not affect the increased GMBF. Aubdool et al. showed that TRPA1 activation with cinnamaldehyde causes a vasodilatory effect mediated by NO in ear blood flow [35]. The discrepancy may be due to the difference in blood circulation mechanism in both tissues. It is probably attributed to the blood flow-increasing effect of prostaglandins (PGs) despite NO suppression by L-NAME. TRPA1 activators such as AITC have also been reported to exert a prostaglandin E_2 (PGE_2)-release effect [25,43], which is not observed with TRPV1 agonists. A GMBF-increasing effect has also been reported for PGE_2 [44]. Furthermore, in this study, we measured the GMBF in rats, whereas in previous studies, skin blood flow

in the ears of mice was measured, which may also explain this difference. Further studies are required to investigate this discrepancy.

As no decrease in the basal blood flow was observed following BIBN 4096 administration alone, CGRP was not considered to be involved in regulating basal blood flow in the steady state. On the other hand, the GMBF response to AITC was markedly inhibited by the CGRP receptor antagonist BIBN 4096. It is suggested that CGRP is mainly involved in these increased responses to AITC. CGRP may play an important role in the vasodilation induced by the activation of TRPA1 due to acute inflammation.

Activation of TRPA1, which is expressed on gastrointestinal epithelial cells, facilitates adrenomedullin release for inducing vasodilation in the mucosa [7]; therefore, we examined whether adrenomedullin is involved in the increased GMBF in response to AITC application. As no reduction in basal blood flow was observed after administration of the adrenomedullin receptor antagonist AGP-8412 alone, adrenomedullin is not considered to be involved in the steady-state regulation of basal blood flow. The GMBF response to AITC was markedly inhibited by AGP-8412, indicating that adrenomedullin is also mainly involved in these increased responses.

Taken together, CGRP and adrenomedullin play an important role in the increased GMBF by the activation of TRPA1 due to the stimulation of AITC.

Summary

In conclusion, the AITC-induced augmentation of GMBF response in rats under anesthesia was clarified to be mediated by activation of TRPA1 channels, which are expressed in capsaicin-sensitive sensory nerves. This response was suggested to be caused by CGRP and adrenomedullin.

Perspective

The enhanced GMBF through the activation of TRPA1 helps maintain mucosal integrity when the gastric mucosa is exposed to stimuli such as low temperatures, reactive oxygen species, and inflammatory mediators. This study is of physiological significance as it clarifies the mechanism of gastric mucosal protection by increasing mucosal blood flow under conditions such as inflammation. In addition, if medications that inhibit or activate the TRPA1 channels are discovered, we could moderate TRPA1 activation in the body.

Concluding Remarks

I elucidated the mediators involved in increasing the blood flow in gastric mucosa via TRPA1 channel-expressing nerves, and established an experimental mechanism for pharmacological analysis. Based on the results of this research, I intend to further elucidate the mechanism of action of Kampo medicines considering their pre-administration, and link the obtained results with future development of Kampo medicines.

List of publication

Noriyuki Ohashi, Kimihito Tashima, Takao Namiki, Syunji Horie: Allyl isothiocyanate, an activator of TRPA1, increases gastric mucosal blood flow through calcitonin gene-related peptide and adrenomedullin in anesthetized rats

Journal of pharmacological sciences, received for production

Acknowledgements

I would like to express my gratitude to Associate Professor Takao Namiki of the Department of Japanese Oriental Medicine, Graduate School of Chiba University for his supervision of my research, his support and continuous encouragement.

I also express my sincere thanks to Professor Syunji Horie of the Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Josai International University for his invitation to basic research, supervision, and continuous encouragement.

I am deeply grateful to Kimihito Tashima, Professor of Laboratory of pharmacology, Faculty of Pharmaceutical Sciences, Josai International University, for his advice about operating procedures and support with plenty of discussions. I am indebted to Yoshiro Hirasaki, Associate Professor of the Department of Japanese Oriental Medicine, Graduate School of Chiba University for his useful and logical advice and continuous encouragement. I am deeply grateful to all stuff members of the Department of Japanese Oriental Medicine, Chiba University and students at the Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Josai International University.

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