Pharmacological studies on the effect of transient receptor potential vanilloid-1-related agonists on gastric mucosal blood flow

（バニロイド受容体TRPV1作動性関連薬の胃粘膜血流に関する薬理学的解析）
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Chemical receptors and substance

TRPV1: transient receptor potential vanilloid-1
CGRP: calcitonin gene-related peptide

Medications and agents

DKT: daikenchuto
BCTC: N-(4-t-butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide

Others

S.E.M.: standard error of mean
ANOVA: analysis of variance
Introductory Remarks

Transient receptor potential channels

Transient receptor potential (TRP) channels are nonselective calcium ion channels ubiquitously expressed in many tissues and respond to a broad range of physical, chemical, and environmental stimuli such as taste, temperature, change in osmolarity, pressure, stretch, and light. TRP channels are divided into seven subfamilies, and different types of TRP channels are present in humans. Natural products, especially medicinal and culinary herbs such as chili pepper, mustard oil, and menthol, stimulate some of these TRP channels. In recent years, elucidation of the role of TRP channels in gastrointestinal physiology, including intestinal motility, secretion, and visceral sensation, has attracted a lot of attention [1,2,3,4]. However, the physiological implications of TRP channels in gastrointestinal blood flow remain to be completely elucidated.

Capsaicin, a selective transient receptor potential vanilloid-1 agonist, in chili pepper

Transient receptor potential vanilloid-1 (TRPV1) was discovered as a thermosensor, which reacts to temperatures greater than 43°C. TRPV1 senses spicy tastes in free sensory nerve terminals, is activated by acids and pungent substances like capsaicin, and is involved in inflammatory thermal hyperalgesia. Sensory nerve fibers expressing TRPV1 are polymodal nociceptors that are also sensitive to chemical irritants such as gastric acid and noxious heat [5] and a mechanical target for pain [6]. Red hot chili peppers are a species of the genus Capsicum, and they have been used in foods as a spice for their pungency since 7000 B.C. In folk medicine, capsicum extract is used for appetite stimulation, relief for toothache, treatment for gastric ulcers, and rheumatism [5]. Capsaicin, a pungent ingredient of the chili pepper, is the most typical selective TRPV1 agonist. Capsaicin exerts a gastroprotective effect against experimental gastric injury induced by activation
of TRPV1 [7]. However, some studies show that capsaicin alleviates gastrointestinal disease, and that this effect is associated with the activation of TRPV1.

**Kampo formula for gastrointestinal disorders**

Traditional Japanese medicines, including Kampo medicines have been used for 1500 years [8]. Traditional Kampo medicine has been practiced widely and has been integrated into Western medicine in Japan. Kampo formulas are prescribed for various gastrointestinal disorders. These formulas are intended not only to restore homeostasis in the human body [8], but also to treat disorders, especially indigestion. In addition, some textbooks on Oriental medicine state that some formulas have clinical effects on problems of the upper gastrointestinal tract, such as epigastralgia and abdominal distress.

**Daikenchuto and gastrointestinal disorders**

Daikenchuto (DKT) is one of the most frequently prescribed Kampo formulas in Japan, and it consists of the following four crude drugs: processed ginger, ginseng, zanthoxylum fruit, and malt sugar in the ratio of 5:3:2:80. DKT is used for the treatment of a cold sensation in the abdomen and intestinal disorders of motility and inflammation such as postoperative bowel ileus [8]. Additionally, textbooks on Oriental medicine indicate that DKT has been used not only for intestinal disorders but also for problems of the upper gastrointestinal tract such as epigastric pain and bloating. Further, DKT ameliorates the delayed gastric motility induced by morphine and chlorpromazine in mice [9,10].

A previous study showed that DKT increases blood flow in the small intestine of rats [11]. However, the effect of DKT on gastric mucosal blood flow has not been investigated thus far.
Involvement of nitric oxide synthase isoforms in increased gastric mucosal blood flow by capsaicin

We have reported that capsaicin stimulates the primary afferent nerves through activation of TRPV1, which results in gastric mucosal protection [12] and gastric hyperemic response [13], which are mediated by calcitonin gene-related peptide (CGRP) and nitric oxide (NO) [14,15]. Vasodilation of the gastric mucosa induced by intragastric application of capsaicin decreased after treatment with an inhibitor of NO synthase (NOS), NG-nitro-L-arginine methyl ester (L-NAME) [16]. NO affects gastric mucosal blood flow (GMBF) [17]. In mammals, three isoforms of NOS encoded by different genes have been identified [18]. Neuronal NOS (nNOS) and the isoform present in the endothelium lining the vasculature, endothelial NOS (eNOS), are constitutively expressed in humans. Inducible NOS (iNOS) requires a stimulus (cytokines and lipopolysaccharides) for expression in specific cell types such as macrophages, neutrophils, and epithelial cells. Therefore, eNOS-derived NO is assumed to contribute to the gastric hyperemic response to capsaicin although the gastric mucosa has been shown to contain not only eNOS [19,20] but also nNOS [19] and iNOS [21]. Interestingly, Chen et al. [22] speculated that NO is produced not only from the endothelium but also from nitroxidergic nerves in the submucosa in gastric vasodilation response to capsaicin. However, which isoform of NOS contributes to gastric hyperemic responses to capsaicin has not been determined thus far.
Aim and Scope

The aim of this study was to clarify whether the TRPV1 agonist capsaicin and the relevant Kampo formula DKT increases gastric mucosal blood flow through activation of TRPV1 and nNOS.

The specific aims of the proposal studies were:

**Part 1 Capsaicin**

1. To clarify the roles of NOS isoforms on the gastric hyperemic response to capsaicin in urethane-anesthetized rats by using pharmacological tools, including N5-[imino(propylamino) methyl]-L-ornithine (L-NPLA; a selective nNOS inhibitor), N5-(l-iminoethyl)-L-ornithine (L-NIO; a selective eNOS inhibitor), and 1400W (a selective iNOS inhibitor).

2. To verify the interaction of nNOS and TRPV1-expressing nerves in the stomach of rats by using immunohistochemical analysis.

**Part 2 Kampo formulas**

1. To clarify whether DKT increases gastric mucosal blood flow through activation of TRPV1 in rats.

2. To verify whether nNOS-derived NO is involved in the gastric mucosal hyperemic response to DKT in rats.
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 (#52). The number of animals used was kept to the minimum necessary for meaningful interpretation of the data.
Part 1 Effects of the TRPV1 agonist capsaicin on gastric mucosal blood flow in the stomach of anesthetized rats ex vivo

Introduction

Capsaicin-sensitive afferent nerves play an important role in maintaining the integrity of the gastric mucosa. Stimulation of these nerves by capsaicin protects the gastric mucosa from a variety of noxious stimuli through increased gastric mucosal blood flow (GMBF) [23,24]. The binding site of capsaicin has been cloned and is known as the transient receptor potential vanilloid-1 (TRPV-1), a nonselective cationic channel [25]. Previously, we reported that capsaicin stimulates these afferent nerves through activation of TRPV1, which results in gastric mucosal protection [12] and gastric hyperemic response [13], which are mediated by calcitonin gene-related peptide (CGRP) and nitric oxide (NO) [14,15]. NO affects GMBF [17].

In mammals, three isoforms of NOS encoded by different genes have been identified [18]. The constitutively expressed isoforms include neuronal NOS (nNOS) and endothelial NOS (eNOS) present in the endothelium lining the vasculature. Inducible NOS (iNOS) requires a stimulus (cytokines and lipopolysaccharides) for expression in specific cell types such as macrophages, neutrophils, and epithelial cells. Therefore, eNOS-derived NO is assumed to contribute to the gastric hyperemic response to capsaicin although the gastric mucosa has been shown to contain not only eNOS [19,20] but also nNOS [19] and iNOS [21]. However, which isoform of NOS contributes to gastric hyperemic responses to capsaicin has not been determined thus far.

In the present study, we examined the roles of NOS isoforms on the gastric hyperemic response to capsaicin in urethane-anesthetized rats by using pharmacological tools, including N5-[imino (propylamino) methyl]-L-ornithine (NPLA; a selective nNOS inhibitor), N5-(l-iminoethyl)-L-ornithine (l-NIO; a selective eNOS inhibitor), and 1400W (a selective iNOS inhibitor). In addition,
we investigated the interaction of nNOS and TRPV1-expressing nerves in the stomach of rats by using immunohistochemical analysis.

Material and Methods

Animals
Male Sprague–Dawley strain rats (SLC, Hamamatsu, Japan) weighing 180–220 g were used. Animals were housed under controlled environmental conditions (temperature, 24 ± 2°C and lights on 7:00 AM to 7:00 PM) and fed commercial rat chow MF (Oriental Yeast, Tokyo, Japan). The animals were kept in individual cages with raised mesh bottoms to prevent coprophagy, and they were deprived of food but allowed free access to tap water for 18 h before the experiments. Animal experiments were performed according to 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 (#52). The number of animals used was kept to the minimum necessary for meaningful interpretation of the data.

Experimental procedures
Chemical deafferentation was performed two weeks before the experiment by successive subcutaneous injections of capsaicin once daily for 3 days (20, 30, and 50 mg/kg) [26]. All capsaicin injections were performed under ether anesthesia, and the rats were pretreated with the beta-adrenergic receptor agonist isoproterenol (0.01 mg/kg, intramuscular [i.m.]) and the selective beta 1-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 a 0.1 mg/mL solution of capsaicin in 0.5% carboxymethyl cellulose solution (CMC) was instilled into one
eye of each rat, and the protective wiping movements were counted [27]. The animals were anesthetized with urethane (1.25 g/kg, intraperitoneal [i.p.]) and pretreated with omeprazole (60 mg/kg, i.p.) to exclude the possibility that activation of TRPV1 by H+ facilitates the increased GMBF responses [28]. The stomach was exposed through a midline incision, delivered onto the abdominal surface by gentle traction on the spleen, and the pylorus was ligated. A two-part lucite chamber was used for maintaining ex vivo conditions of the gastric mucosa. One part is a lucite base, and the other is a plastic rim, which has two holes on the side wall. The two holes are cannulated for perfusing the mucosa with saline (154 mmol/L NaCl, 37°C) at a flow rate of 1 mL/min. The lucite base was lowered over the animal, and the stomach was drawn through the center hole with the 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 area of the glandular mucosa, which consists mostly of 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 temperature similar to that of the rectum at around 37°C by using a small animal warmer and thermometer (Model BWT-100; Bio Research Center, Nagoya, Japan) [29]. GMBF was measured using laser-Doppler flowmetry (Model ALF-21N; Advance, Tokyo, Japan) and a non-touching probe (diameter, 1 mm) on the surface of the corpus mucosa. Arterial pressure (AP) was monitored via the femoral artery by using a blood pressure transducer (DX-100; Nihon Kohden, Tokyo, Japan) and amplifier system (Nihon Kohden AP-601G & AP-611). After GMBF and AP were well stabilized, the perfusion was discontinued, the luminal solution was removed, and the mucosa was exposed to 2 mL of capsaicin (0.33–3.3 mmol/L) for 10 min. After application of capsaicin, the mucosa was rinsed with saline, another 2 mL of saline was instilled, and the perfusion was resumed. Changes in the GMBF were

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continuously monitored and recorded for 2-h test periods by using a PowerLab system (Model ML845; AD Instruments, Bella Vista, NSW, Australia). A TRPV1 antagonist N-(4-<i>t</i>-butylphenyl)-4-(3-chlopyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide (BCTC) (0.8 mmol/L, i.g.) [30,31], a nonselective NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 5 and 10 mg/kg, i.v.), a nNOS selective inhibitor NPLA (0.02 and 0.2 mg/kg, i.v.), an eNOS selective inhibitor L-NIO (3 and 10 mg/kg, i.v.), or an iNOS selective inhibitor 1400W (3 and 10 mg/kg, i.v.) were administered 20 min before exposing the stomach to 2 mL of capsaicin for 10 min. In one group, L-arginine (300 mg/kg, i.v.) was administered twice 40 min and 60 min before the application of capsaicin.

**Preparations and drugs used**

Allyl isothiocyanate, atenolol, capsaicin, CMC, dimethyl sulfoxide (DMSO), dl-isoproterenol, and urethane (ethyl carbamate) were obtained from 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). NPLA and L-NIO were purchased from Tocris Cookson (Ellisville, MO, USA). BCTC was purchased from BIOMOL (Plymouth Meeting, PA, USA). Capsaicin was dissolved in Tween 80-ethanol solution (10% ethanol, 10% Tween 80, and 80% saline) [26] for subcutaneous (s.c.) injection or suspended in 0.5% CMC for mucosal application. Indomethacin was suspended in 1% Tween 80 in saline for s.c. injection. Omeprazole was suspended in 0.5% CMC for i.p. injection. BCTC was dissolved in DMSO before in saline, and the final concentration of DMSO was less than 1.0%. Other drugs were dissolved in saline with no organic solvent or detergent. Each drug was prepared immediately before use and was administered at a volume of 0.5 mL/100 g of body weight for i.p. and s.c. administration or at a volume of 0.1 mL/100 g of body weight for intravenous (i.v.) administration. Control animals received the vehicle alone.
Statistics

The data are presented as the mean ± standard error of mean (S.E.M.) of 3 to 7 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 one-way analysis of variance (ANOVA) with Bonferroni correction. The level of significance was set at 0.05. Sigma Stat 3.1 software (Jandel Scientific Software, San Rafael, CA, USA) was used for statistical analysis.

Results

Effects of intragastric capsaicin on GMBF in the stomach of anesthetized rats ex vivo

Intragastric administration of capsaicin (0.33, 1, and 3.3 mmol/L) induced gastric hyperemic responses in a concentration-dependent manner; a significant effect was observed at concentrations greater than 1 mmol/L (Fig. 1B). The GMBF after intragastric application of 0.33, 1, and 3.3 mmol/L of capsaicin for 10 min was 121.5 ± 2.6%, 146.4 ± 8.9%, and 178.6 ± 12.9%, respectively. Interestingly, the GMBF in response to 3.3 mmol/L capsaicin remained significantly high despite the removal of capsaicin from the chamber (data not shown). Mucosal application of the control solution (0.5% CMC) did not increase the GMBF (Fig. 1A). GMBF reached the maximal value after treatment with 3.3 mmol/L of capsaicin; therefore, this concentration was used in the subsequent experiments to examine the effects of various agents on the GMBF in response to capsaicin. The increased GMBF in animals in response to capsaicin (3.3 mmol/L) was totally abolished when the gastric mucosa was exposed to a TRPV1 antagonist BCTC (0.8 mmol/L; Fig. 2A, B). The maximum GMBF induced by 3.3 mmol/L capsaicin in the presence of BCTC was 102.4 ± 6.1% throughout the experiment. A similar phenomenon was observed in the animals after chemical deafferentation by consecutive injections of capsaicin (total 100 mg/kg, s.c.) two weeks
before the experiment (Fig. 2A, B). In these animals, the GMBF remained unchanged during exposure of the mucosa to capsaicin (3.3 mmol/L), and the maximum GMBF was 103.1 ± 4.2%. However, intragastric capsaicin significantly increased the GMBF via activation of TRPV1 expressed in capsaicin-sensitive sensory nerves without deafferentation in anesthetized rats.
Fig. 1

A

Gastric Mucosal Blood Flow (% of Basal Values)

N=4-7
* P<0.05

Time (min)

Control
○ Capsaicin (3.3 mM)

Capsaicin
Fig. 1 Effect of mucosal application of capsaicin on gastric mucosal blood flow (GMBF) in the stomach of anesthetized rats ex vivo. Figure A shows the time course of GMBF response to capsaicin (3.3 mmol/L) in anesthetized rats. The stomach was perfused with saline, and capsaicin was topically applied to the mucosa for 10 min from time 0. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M) of values obtained every 2 or 10 min from 4 to 7 rats. Statistically significant difference at P < 0.05: *from the corresponding values in the group treated with control (0.5% carboxymethyl cellulose [CMC]). Figure B shows the maximal GMBF response after treatment with capsaicin (0.33–3.3 mmol/L). The data are expressed as a % increase in baseline values and represent the mean ± S.E.M of 4 to 7 rats. * indicates statistically significant difference at P < 0.05 compared to the group treated with control (0.5% CMC).
Fig. 2

A

Gastric Mucosal Blood Flow (% of Basal Values)

Time (min)

-40 -30 -20 -10 0 10

Capsaicin (3.3 mmol/l)

N=3-4

* P<0.05

- Vehicle
- BCTC (0.8 mmol/l)
- Sensory deafferentation
Fig. 2 Effects of \(N\)-(4-\(t\)-butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide (BCTC), a transient receptor potential vanilloid-1 (TRPV1) antagonist, and sensory deafferentation on gastric mucosal blood flow (GMBF) induced by mucosal application of capsaicin to the stomach of anesthetized rats ex vivo. Chemical deafferentation (capsaicin pretreatment) was performed 2 weeks before the experiment by consecutive subcutaneous (s.c.) injections of capsaicin once daily for 3 days (total dose, 100 mg/kg). Figure A shows the time course of GMBF response to capsaicin (3.3 mmol/L) in anesthetized rats treated with BCTC or sensory deafferentation. The stomach was perfused with saline, BCTC (0.8 mmol/L) was applied to the chamber for 30 min, starting at 20 min before the capsaicin application. Capsaicin was topically applied to the mucosa for 10 min. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M.) of values obtained every 2 or 10 min from 3 to 4 rats. Statistically significant difference at \(P < 0.05\): *from the corresponding values in the group treated with vehicle. Figure B shows a maximal GMBF response induced by mucosal application of capsaicin (3.3 mmol/L). The data are expressed as a % increase of baseline values and represent the mean ± S.E.M. of 3–4 rats. * indicates statistically significant difference at \(P < 0.05\) compared to the group treated with vehicle. Note that increased GMBF in response to capsaicin was completely abolished by BCTC and sensory deafferentation.
Effects of L-NAME, a non-selective NOS inhibitor, and combination treatment with L-NAME and L-arginine (300 mg/kg, ×2) on GMBF in response to capsaicin in the stomach of anesthetized rats ex vivo

GMBF temporarily increased after injection of L-NAME but immediately returned to the baseline value (Fig. 3A). The baseline value of arterial pressure (AP) in urethane-anesthetized rats was about 50–100 mmHg, and the AP did not change after intravenous injection of the vehicle (saline) 20 min before mucosal application of capsaicin (Fig. 4A). However, the AP increased immediately from about 75–100 mmHg to about 120–150 mmHg after the administration of L-NAME (10 mg/kg, iv), and the increase in AP induced by L-NAME was retained throughout the experiment (Fig. 4B). The increase in GMBF in response to capsaicin (3.3 mmol/L) observed in control rats was apparently decreased by L-NAME in a dose-dependent manner. The maximum GMBF in response to capsaicin in animals treated with L-NAME (5 and 10 mg/kg) was 138.8 ± 11.6% and 125.6 ± 11.6% (Fig. 3B).

In contrast, the substrate for NOS L-arginine (300 mg/kg) which was pretreated twice, reversed the inhibitory effects of L-NAME (10 mg/kg) on gastric hyperemic response during capsaicin application (Fig. 3A, B). Thus, gastric hyperemia in response to capsaicin was attributable to both NO-dependent and NO-independent hyperemia during capsaicin application in anesthetized rats.
Fig. 3

A

Gastric Mucosal Blood Flow (% of Basal Values)

Time (min)

Capsaicin (3.3 mmol/l)

N=4-5

* # P<0.05

Vehicle
L-NAME (10 mg/kg)
L-NAME (10 mg/kg) + L-Arg (300 mg/kg, x2)
Fig. 3 Effects of NG-nitro-L-arginine methyl ester (L-NAME), a non-selective nitric oxide synthase (NOS) inhibitor and combination treatment with L-NAME and L-arginine (300 mg/kg, ×2) on gastric mucosal blood flow (GMBF) induced by mucosal application of capsaicin in the stomach of anesthetized rats ex vivo. Figure A shows the time course of analysis for GMBF response to capsaicin (3.3 mmol/L) in anesthetized rats pretreated with L-NAME and L-arginine. The stomach was perfused with saline before the application, L-NAME (5 and 10 mg/kg) was given via intravenous injection, and 20 min later, capsaicin (3.3 mmol/L) was topically applied to the mucosa for 10 min. Injection of L-NAME alone produced a temporary increase in GMBF, but the GMBF immediately returned to the baseline value. L-arginine (300 mg/kg) was administered via intravenous injection 40 and 60 min before capsaicin application. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M.) of values obtained every 2 or 10 min from 4 to 5 rats. * indicates statistically significant difference at P < 0.05 compared to the corresponding values in the group treated with vehicle (saline); #, compared to the corresponding values in the group treated with L-NAME (10 mg/kg). Figure B shows the maximum GMBF during capsaicin (3.3 mmol/L) application in animals treated with L-NAME (5 and 10 mg/kg) or L-NAME (10 mg/kg) plus L-arginine (300 mg/kg, ×2). The data are expressed as a % increase in baseline values and represent the mean ± S.E.M. of 4–5 rats. * indicates statistically significant difference at P < 0.05 compared to the group treated with vehicle (saline); #, compared to the corresponding values in the group treated with L-NAME (10 mg/kg). Note that increased GMBF during capsaicin application was significantly inhibited by L-NAME (10 mg/kg), and the response was prevented by pretreatment with L-arginine (300 mg/kg, ×2).
Fig. 4

A

Gastric Mucosal Blood Flow (Arbitrary Unit: mV)

Vehicle (Saline, iv)

Capsaicin (3.3 mmol/L, ig)

Arterial

Gastric Mucosal Blood Flow (Arbitrary Unit: mV)

Vehicle (Saline, iv)

Capsaicin (3.3 mmol/L, ig)

B

Gastric Mucosal Blood

L-NAME (10 mg/kg, iv)

Arterial

L-NAME (10 mg/kg, iv)
Fig. 4. Representative effects of NG-nitro-L-arginine methyl ester (L-NAME) on gastric mucosal blood flow (GMBF) and arterial pressure (AP) in urethane-anesthetized rats. Vehicle (saline; A) and L-NAME (10 mg/kg; B) were intravenously injected 20 min before capsaicin application. The AP and GMBF increased immediately after the administration of L-NAME (10 mg/kg) unlike that in the vehicle group; subsequently, AP, but not GMBF, remained at high levels throughout the experiment.
Effect of NPLA, a selective nNOS inhibitor, on GMBF in response to capsaicin in the stomach of anesthetized rats ex vivo

The roles of different isoforms of NOS in the gastric hyperemia induced by capsaicin were investigated by using selective NOS inhibitors, including the nNOS inhibitor NPLA, the eNOS inhibitor L-NIO, and the iNOS inhibitor 1400W. Administration of the nNOS specific inhibitor NPLA alone did not significantly affect the GMBF (Fig. 5A). Interestingly, the increase in GMBF in response to capsaicin (3.3 mmol/L) during the application was apparently decreased by administration of 0.2 mg/kg NPLA. The maximum GMBF during capsaicin application in animals treated with NPLA (0.02 and 0.2 mg/kg) were 182.1 ± 26.6% and 141.9 ± 8.9% (Fig. 5B).

Preliminary experiments showed that AP in rats treated with NPLA (2 mg/kg, iv) returned to the baseline value, which was about 55–80 mmHg although the AP increased temporarily after the administration of NPLA (Fig. 6A).
Fig. 5

A

Capsaicin (3.3 mmol/l)

Capsaicin (3.3 mmol/l)

Gastric Mucosal Blood Flow (% of Basal Values)

Time (min)

N=4

*P<0.05

Vehicle

NPLA (0.2 mg/kg)
B

Maximal GMBF Response (% of Basal Values)

Vehicle 0.02 0.2

NPLA (mg/kg)

Capsaicin (3.3 mmol/l)

N=4-5
P<0.05

*
Fig. 5 Effect of N5-[imino (propylamino) methyl]-l-ornithine (NPLA), a selective neuronal nitric oxide synthase (nNOS) inhibitor, on gastric mucosal blood flow (GMBF) induced by capsaicin in the stomach of anesthetized rats ex vivo. Figure A shows the time course of the effect of NPLA (0.2 mg/kg) on GMBF in response to capsaicin (3.3 mmol/L) in anesthetized rats. The stomach was perfused with saline, NPLA (0.2 mg/kg) was administered via intravenous injection, and after 20 min, capsaicin (3.3 mmol/L) was topically applied to the mucosa for 10 min. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M.) of values obtained every 2 or 10 min from 4 rats. Statistically significant difference at P < 0.05; * indicates statistically significant difference compared to the group treated with vehicle (saline). Figure B shows the maximum GMBF during capsaicin (3.3 mmol/L) application in animals treated with NPLA (0.02 and 0.2 mg/kg). The data are expressed as a % increase in baseline values and represent the mean ± S.E.M. of 4–5 rats. Statistically significant difference at P < 0.05; * indicates statistically significant difference compared to the group treated with vehicle (saline). Note that the increased GMBF during capsaicin application was inhibited by NPLA.
Fig. 6

A

Gastric Mucosal Blood Flow

Arterial Pressure

B

Gastric Mucosal Blood Flow

Arterial Pressure

NPLA (2 mg/kg, iv)

Capsaicin (3.3 mmol/L, ig)

Bethanechol (5 µg/kg/min, iv)

L-NIO (10 mg/kg, iv)
Fig. 6 Representative effects of N5-[imino (propylamino) methyl]-L-ornithine (NPLA), and N5-(l-iminoethyl)-L-ornithine (L-NIO) on gastric mucosal blood flow (GMBF) and arterial pressure (AP) in urethane-anesthetized rats. NPLA (2 mg/kg; A) and L-NIO (10 mg/kg; B) were intravenously injected 20 min before capsaicin application. AP in the NPLA-treated rats (2 mg/kg) returned to the baseline, while AP, but not GMBF, increased temporarily after the administration of NPLA. AP in rats treated with L-NIO (10 mg/kg) was maintained at a level slightly above the baseline although AP and GMBF transiently increased after the administration of L-NIO.
Effect of L-NIO, a selective eNOS inhibitor, on GMBF in response to capsaicin in the stomach of anesthetized rats ex vivo

Administration of the selective eNOS inhibitor L-NIO (10 mg/kg) alone temporarily increased the GMBF, but the GMBF immediately returned to the baseline value (Fig. 7A). The gastric hyperemic response after treatment with capsaicin was not significantly affected by L-NIO (3 and 10 mg/kg), and the maximum GMBF was 158.7 ± 8.4% and 160.0 ± 9.6% (Fig. 7B). Preliminary experiments showed that the AP in rats treated with L-NIO (10 mg/kg, iv) was maintained at a slightly higher level than that at the baseline, which was about 50–60 mmHg to about 65–75 mmHg (Fig. 6B) although the AP transiently increased after the administration of L-NIO.
Fig. 7 Effect of L-NIO, a selective endothelial nitric oxide synthase (eNOS) inhibitor, on gastric mucosal blood flow (GMBF) induced by capsaicin in the stomach of anesthetized rats ex vivo. Figure A shows the time course of analysis of the effect of L-NIO (10 mg/kg) on the GMBF in response to capsaicin (3.3 mmol/L) in anesthetized rats. L-NIO (10 mg/kg) was injected intravenously 20 min before capsaicin application, the stomach was perfused with saline, and capsaicin (3.3 mmol/L) was applied topically to the mucosa for 10 min. Injection of L-NIO alone induced a temporary increase in the GMBF, but the GMBF immediately returned to the baseline value. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M.) of values every 2 or 10 min from 4 rats. Statistically significant difference at P < 0.05; * indicates statistical difference compared to corresponding values in the group treated with vehicle (saline). Figure B shows the maximum GMBF during capsaicin (3.3 mmol/L) application in animals treated with L-NIO (3 and 10 mg/kg). The data are expressed as a % increase in baseline values and represent the mean ± S.E.M. of 4–5 rats. Statistically significant difference at P < 0.05; * indicates statistical difference compared to the group treated with the vehicle (saline). Note that GMBF response during capsaicin application was not affected by L-NIO.
Effect of 1400W, a selective iNOS inhibitor, on GMBF in response to capsaicin in the stomach of anesthetized rats ex vivo

GMBF was unchanged by the administration of the iNOS selective inhibitor 1400W (10 mg/kg) (Fig. 8A). In addition, the increased GMBF in response to capsaicin was not affected by 1400W (3 and 10 mg/kg, Fig. 8B).
Fig. 8 Effect of 1400W, a selective inducible nitric oxide synthase (iNOS) inhibitor, on gastric mucosal blood flow (GMBF) induced by capsaicin in the stomach of anesthetized rats ex vivo. Figure A shows the time course of analysis for the effect of 1400W (10 mg/kg) on GMBF response to capsaicin (3.3 mmol/L) in anesthetized rats. The stomach was perfused with saline before the application, 1400W (3 and 10 mg/kg) was administered via an intravenous injection 20 min before capsaicin application, and capsaicin (3.3 mmol/L) was topically applied to the mucosa for 10 min. The data are expressed as a % increase in baseline values and represent the mean ± standard error (S.E.) of values every 2 or 10 min from 4 rats. Figure B shows the maximum GMBF during capsaicin (3.3 mmol/L) application in animals treated with 1400W (3 and 10 mg/kg). The data are expressed as a % increase of baseline values and represent the mean ± S.E. of 4–5 rats. Note that the increased GMBF in response to capsaicin was not affected by 1400W.
Distribution of TRPV1 immunoreactivity in the rat stomach

Numerous TRPV1-immunoreactive axons were found around in arterioles in the mucosal and submucosal layer of the stomach (Fig. 9A, D). In addition, many TRPV1 axons were observed to be immunoreactive for nNOS (Fig. 9C, F). A few axons of this type were present in the mucosa (Fig. 9C), but they were particularly abundant in the vicinity of blood vessels in the submucosa (Fig. 9F).
Fig. 9 Confocal images showing co-localization of transient receptor potential vanilloid-1 (TRPV1) and neuronal nitric oxide synthase (nNOS) immunoreactivities in the corpus mucosa (A, B, C) and submucosa (D, E, F). Labeling for TRPV1 (green) and nNOS (red) are shown separately (A, D) and (B, E), respectively, and merged (C, F). Double immunolabeling for TRPV1 and nNOS showed colocalization in many but not in all axons. This photo shows abundant TRPV1 axons containing nNOS in the submucosa, with a high density
of immunoreactive axons around the blood vessels. CM, circular muscle; A, arteriole; and V, venule. Scale bars are 50 μm.
Discussion

In this chapter, we found that intragastric application of capsaicin facilitates an initial increase in GMBF by NO mainly derived from nNOS after stimulating capsaicin-sensitive sensory nerves, and that the sustained hyperemic response after removal of capsaicin might be attributed to eNOS/NO in the rat stomach. In addition, capsaicin-sensitive sensory nerves are involved in maintenance of the gastric mucosal integrity against irritants [14,23,28]. Topical application of capsaicin causes dilatation of the submucosal and mucosal arterioles in the rat stomach [32,33]. Our findings were consistent with those reported previously in that mucosal application of capsaicin increased GMBF in the rat stomach, and this effect was totally abolished by a TRPV1 antagonist, BCTC, and chemical deafferentation by capsaicin pretreatment, which suggested that this action is mediated by capsaicin-sensitive sensory nerves expressing TRPV1. In addition, the GMBF responses to capsaicin were markedly inhibited by a non-selective NOS inhibitor L-NAME, which indicated that mainly NO is involved in those hyperemic responses. However, L-NAME did not inhibit the increases in GMBF as much as BCTC. This result suggests that gastric mucosal hyperemia in response to capsaicin is attributable not only to NO but also to other mediators, including prostaglandins (PGs) and CGRP. Several studies have shown that endogenous prostaglandins (PGs), especially PGI₂, contributes to gastric hyperemic responses to capsaicin by sensitizing TRPV1-expressing afferent nerves, which was revealed by the treatment of indomethacin (5 mg/kg, s.c.) and the animals lacking IP receptors [24,34,35]. In addition, Chen et al. reported the interaction of NO and CGRP in gastric vasodilation through sensory nerves [22]. These results can be explained by the previous findings that BCTC functionally antagonizes the action of capsaicin at the peripheral terminals of sensory nerves and inhibits the release of some transmitters from capsaicin-sensitive afferent nerves [30,31]. Further, Chen et al. reported that submucosal
application of CGRP induced dose-dependent dilation of gastric submucosal arterioles, which was significantly decreased by L-NAME [33]. However, the dilation induced by submucosal CGRP was decreased to a much lesser degree by inhibition of NO synthesis than that induced with intragastric capsaicin. This indicates that the NO released by CGRP was not the only source of submucosal NO in capsaicin-induced dilation, and that there may be another source of submucosal NO such as nitroxidergic nerves that has not been determined thus far [22]. NO is synthesized from L-arginine by NOS in various cells. NOS is present not only in vascular endothelial cells (eNOS) but also in perivascular nerves (nNOS) [18,34]. It is well known that NO is a vasodilator that increases GMBF, and this mediator is important in the modulation of gastric mucosal integrity through interaction with sensory nerves [14]. The increase in GMBF in response to capsaicin is mitigated by NG-monomethyl L-arginine, and the gastric cytoprotection induced by capsaicin is also decreased by the NOS inhibitor [32,33]. Previous studies have shown that gastric hyperemia caused by acid back-diffusion or exogenous CGRP is mediated partly by NO, but which NOS isoform is involved in those hyperemic responses has not been investigated in any of the studies thus far. In addition, the source of the NO involved in the actions of capsaicin was not identified [14]. That is because highly selective inhibitors of NOS isoforms had not been developed at that time. We investigated which NOS isoform mediates the increased GMBF in response to capsaicin. In this study, NPLA and L-NIO were adopted as selective nNOS and eNOS inhibitors, respectively. NPLA rapidly binds to nNOS and is slowly dissociated from nNOS in vitro followed by inhibition of NO production [36], and NPLA exerts potent inhibition of nNOS with an observed IC50 value of 57 nM, a value 149-fold and 3,158-fold lower than the concentration of NPLA required to inhibit the eNOS and iNOS isoforms, respectively [38,39]. However, an in vivo pharmacokinetic analysis of the half-life of NPLA has not been performed thus far. However, recent studies showed that administration of NPLA at a dose of 0.5–2 mg/kg (i.p.) for 90 min and 2 μg/kg (i.c.v) for 120 min to
different animal models showed a persistent inhibition of nNOS [38,39]. These findings are consistent with our results that NPLA inhibits nNOS activity throughout the experiment.

In addition, L-NIO was the most potent inhibitor of the eNOS isoform; the IC50 value of L-NIO was 0.08 µM, which was approximately 4-fold lower than that observed for iNOS (IC50 = 0.3 µM) and nNOS (IC50 = 0.3 µM) isoforms [40]. However, the selectivity of L-NIO is not very high, and this agent at a high dose (>30 mg/kg) also suppresses the iNOS isoform [20,41]. Further, L-NIO inhibits eNOS activity in vivo for 4 h [20].

NPLA significantly decreased the maximal GMBF response during the application of capsaicin. In contrast, L-NIO did not affect the maximal GMBF response induced by capsaicin. These results suggest that nNOS/NO, which might be released from TRPV1-expressing nerves stimulated by capsaicin, plays an important role in gastric hyperemia during the luminal application of capsaicin. On the other hand, intravenous injection of 1400W did not affect the resting GMBF or the increase in GMBF in response to capsaicin. Our results were consistent with those reported previously that eNOS was expressed mostly in the vasculature throughout the gastric mucosa in rats, but the expression of iNOS was hardly observed in the gastric mucosa of normal rats by using immunohistochemical analysis [19,20]. These findings support our hypothesis that eNOS/NO plays a role in the resting state responses of GMBF, while iNOS/NO plays no role in the increased GMBF in response to capsaicin.

In this study, TRPV1 immunoreactivity was detected in the mucosal and submucosal layers of the corpus of the rat stomach. Many TRPV1 nerve fibers contain nNOS, but not all axons do. In addition, numerous TRPV1-immunoreactive axons were found around arterioles in the submucosal layer. These data led us to speculate that the capsaicin-induced increase in GMBF could be attributed to nNOS/NO in that capsaicin stimulates TRPV1 nerves and releases CGRP and NO, and NO is immediately provided by nNOS and dilates the blood vessels. Stimulation of sensory nerves leads to gastric hyperemic response, which is mediated partly by NO formation, but it is not clear
whether the NO is derived solely from endothelial cells or is also released directly by extrinsic and/or intrinsic nerves [14,22]. To our knowledge, this is the first study in which abundant TRPV1 axons containing nNOS in the submucosa with a high density of immunoreactive axons around blood vessels have been reported. Therefore, our study suggests that capsaicin and/or nerve activation is likely to cause vasodilatation by inducing NO release from TRPV1-expressing extrinsic sensory fibers. In the corpus, TRPV1/nNOS immunoreactive axons were also present in the mucosa.

**Summary**

In conclusion, our results showed that mucosal application of capsaicin increased GMBF by releasing NO derived from nNOS after stimulation of capsaicin-sensitive sensory nerves. To our knowledge, this is the first study in which the contribution of nNOS-derived NO in gastric hyperemic responses to the activation of TRPV1 by capsaicin has been reported using not only pharmacological but also immunohistological techniques.
Part 2 Effects of Daikenchuto on gastric mucosal blood flow in rats

Introduction

Kampo formulas are prescribed for various gastrointestinal disorders [8]. Daikenchuto (DKT), which is used for the treatment of a cold sensation in the abdomen (mainly umbilical portions) and decreasing intestinal dysmotility and inflammation, is one of the most frequently prescribed kampo formulas in Japan. DKT consists of four crude drugs as follows: processed ginger, ginseng, zanthoxylum fruit, and malt sugar. DKT increases the intestinal blood flow in rats and improves the small intestinal movement in guinea pigs [11, 43], and a previous study has shown that DKT increases the levels of gastrointestinal hormones and neuropeptides such as motilin, vasoactive intestinal peptide (VIP), and calcitonin gene-related peptide (CGRP) [8]. In addition, Kono et al. reported that DKT induces an increase in mucosal blood flow in rat colon via CGRP but not via VIP and nitric oxide (NO) [44]. These results support the finding that the increase in blood flow in the gastrointestinal tract lead to the clinical effect of DKT against the cold feeling and dysmotility in the abdomen. However, the mechanism underlying the increase in the gastric mucosal blood flow induced by DKT remains to be clarified. The aim of this study was to determine whether DKT facilitates an increase in the mucosal blood flow in the stomach of rats through activation of transient receptor potential vanilloid-1 (TRPV1), which is expressed in nerve fibers containing the vasodilator CGRP and NO.

Material and Methods

Animals
Male Sprague–Dawley strain rats (SLC, Hamamatsu, Japan) weighing 180–220 g were used. Animals were housed under controlled environmental conditions (temperature, 24 ± 2°C and lights on 7:00 AM to 7:00 PM) and fed commercial rat chow MF (Oriental Yeast, Tokyo, Japan). The animals were kept in individual cages with raised mesh bottoms to prevent coprophagy, and 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 (#52). The number of animals used was kept to the minimum necessary for a meaningful interpretation of the data.

**Experimental procedures**

The animals were anesthetized with urethane (1.25 g/kg, i.p.). The stomach was exposed through a midline incision, delivered onto the abdominal surface by gentle traction on the spleen, and the pylorus was ligated. A two-part lucite chamber was used for maintaining ex vivo conditions of the gastric mucosa. One part is a lucite base and the other is a plastic rim, which has two holes on the side wall. The two holes are cannulated for perfusing the mucosa with saline (NaCl, 154 mmol/L at 37°C) at a flow rate of 1 mL/min. The lucite base was lowered over the animal, and the stomach was drawn though the center hole with the forceps applied only to the forestomach. Then, the stomach was 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 mucosa area, which consists mostly of 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
temperature similar to that of the rectum at around 37°C by using an incandescent lamp [29]. Gastric mucosal blood flow (GMBF) was measured using laser-Doppler flowmetry (Model ALF-21N; Advance, Tokyo, Japan) and a touching probe (diameter, 1 mm) on the surface of the corpus mucosa. After the GMBF was well-stabilized, the perfusion was discontinued, the luminal solution was removed, and then the mucosa was exposed to 2 mL of DKT for 10 min or 30 min. After the application of DKT, the mucosa was rinsed with saline, another 2 mL of saline instilled, and the perfusion resumed. Changes in the GMBF were continuously monitored and recorded for 2-h test periods by using a PowerLab system (Model ML845; AD Instruments, Bella Vista, NSW, Australia). A TRPV1 antagonist \( N\-(4-t\)-butylphenyl\)-4-(3-chlopyridin-2-yl) tetrahydroxyprazine-1(2H)-carboxamide (BCTC) (0.8 mmol/L, intragastric [i.g.]) was administered for 50 min, starting at 40 min before the DKT application [30,31]. Either a nonselective NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg, i.v.) or a nNOS-selective inhibitor NPLA (0.2 mg/kg, i.v.) was administered 20 min before exposing the stomach to 2 mL of DKT for 10 min. In some animals treated with L-NAME, L-arginine (300 mg/kg, i.v.) was administered twice 40 min and 60 min before the application of DKT.

**Preparations and drugs used**

Capsaicin, CMC, and urethane (ethyl carbamate) were obtained from Wako Pure Chemical Industries (Osaka, Japan). L-arginine and L-NAME were from Sigma–Aldrich (St. Louis, MO, USA). NPLA was from Tocris Cookson (Ellisville, MO, USA). BCTC was purchased from BIOMOL (Plymouth Meeting, PA, USA). BCTC was suspended in 10% 2-hydroxypropyl-\(\beta\)-cycloxydextrin (Enzo Life Science, Farmingdale, NY, USA) and distilled water for i.g. application. DKT extract (Tsumura & Co., Tokyo, Japan, lot No. 2100100010) and maltose syrup powder (Tsumura & Co. Lot No. 3020168) were used. DKT extract was obtained in the form of a spray-
dried powder. The extract solution was separated from the non-soluble waste and concentrated by removing water under reduced pressure. DKT extract powder is manufactured as an aqueous extract that contains processed ginger, ginseng, and zanthoxylum fruit in the ratio of 5:3:2. DKT is prepared by mixing DKT extract powder and maltose syrup powder at the ratio of 1:8. DKT extract was suspended in distilled water for i.g. application. Although the doses of TJ-100 in the present study (1440 mg/kg) 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. Other drugs were dissolved in saline with no organic solvent or detergent. Each drug was prepared immediately before use and was administered at a volume of 0.5 mL/100 g of body weight in the case of i.p. and s.c. administration or at a volume of 0.1 mL/100 g of body weight in the case of i.v. administration. Control animals received the vehicle alone.

Statistics

The data are presented as the mean ± standard error of mean (S.E.M.) of 4–7 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 one-way analysis of variance (ANOVA) with Bonferroni correction. The level of significance was set at 0.05. Sigma Stat 3.1 software (Jandel Scientific Software, San Rafael, CA, USA) procedure was applied for statistical analysis.

Results

Effects of intragastric DKT on GMBF in the stomach of anesthetized rats ex vivo

Intragastric administration of DKT (360, 720, 1440 mg/mL) induced gastric hyperemic responses in a concentration-dependent manner; a significant effect was observed at concentrations greater than
360 mg/mL (Fig. 10A). GMBF remained high despite removal of DKT (over 720 mg/mL) from the chamber. The maximal responses of GMBF to DKT (360, 720, 1440 mg/mL) during intragastric application for 10 min were 128.9 ± 6.2%, 136.0 ± 10.4%, and 162.9 ± 8.5% (Fig. 10B). Mucosal application of the vehicle (distilled water) did not increase the GMBF (Fig. 10A, B). Interestingly, the maximum GMBF after administration of 1440 mg/mL of DKT was similar to that observed after administration of 0.3 mg/mL of capsaicin to the rat stomach (Fig. 11A). The gastric hyperemic response to the 1st application of capsaicin decreased to the baseline after removal of capsaicin from the chamber. The response to the 2nd application of capsaicin was about one-third of that to the 1st application. On the other hand, the response to the 2nd application of DKT was almost as strong as that to the 1st application (Fig. 11B). The above finding may be because, the GMBF reached its maximum value after administration of 1440 mg/mL of DKT; thus, this concentration was used in the subsequent experiments to examine the effects of various agents on the GMBF in response to DKT.
Fig. 10

A

Gastric Mucosal Blood Flow (% of Basal Values)

Time (min)

-50 -40 -30 -20 -10 0 10 20 30 40 50 60 70

Vehicle
DKT (360 mg/ml)
DKT (720 mg/ml)
DKT (1440 mg/ml)

N=4.6

*P<0.05

B
Fig. 10 Effect of mucosal application of daikenchuto (DKT) on gastric mucosal blood flow (GMBF) in the stomach of anesthetized rats ex vivo. Figure A shows the time course of GMBF response to DKT (360, 720, and 1440 mg/mL) in anesthetized rats. The stomach was perfused with saline, and DKT was topically applied to the mucosa for 10 min from time 0. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M.) of values obtained every 2 or 10 min from 4 to 6 rats. Statistically significant difference at P < 0.05; *indicates significant difference compared to the corresponding values in the group treated with the vehicle (no DKT in distilled water). Figure B shows the maximal GMBF response during DKT (360–1440 mg/mL) application. The data are expressed as a % increase in baseline values, and represent the mean ± S.E.M. of 4–6 rats. Statistically significant difference at P < 0.05; *, significantly different from the group treated with the vehicle.
Fig. 11

A

![Graph showing gastric mucosal blood flow as a function of time for DKT and capsaicin treatments. The graph includes error bars and indicates statistical significance with *P<0.05. The x-axis represents time in minutes, and the y-axis represents gastric mucosal blood flow as a percentage of basal values. N=6.7 for both treatments.]

B

![Bar graph comparing maximal GMBF response to DKT and capsaicin treatments for the 1st and 2nd trials. The bars are labeled for DKT (1440 mg/ml) and capsaicin (0.3 mg/ml) with error bars. The x-axis represents the trials (1st and 2nd), and the y-axis represents maximal GMBF response as a percentage of basal values. N=6.7 for both treatments. *P<0.05 for the 2nd trial compared to the 1st trial.]
Fig. 11  Effect of mucosal application of either capsaicin or daikenchuto (DKT) on gastric mucosal blood flow (GMBF) in the stomach of anesthetized rats ex vivo. Figure A shows the time course of GMBF response to capsaicin (0.3 mg/mL) and DKT (1440 mg/mL) in anesthetized rats. The stomach was perfused with saline, and capsaicin was topically applied to the mucosa for 10 min from time 0. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M.) of values obtained every 2 or 10 min from 6 to 7 rats. Statistically significant difference at P < 0.05; * indicates from the group treated with capsaicin (0.3 mg/mL). Figure B shows the effect of repeated application of capsaicin and DKT on the peak responses of GMBF (% increase from baseline values) in each group, and values are the mean ± S.E.M. of 6–7 rats. * indicates statistically significant difference at P < 0.05 compared to the group treated one application.
Effect of BCTC, a TRPV1 antagonist, on GMBF in response to DKT in the stomach of anesthetized rats ex vivo

The increase in GMBF in response to DKT (1440 mg/mL) was almost abolished in the animals when the mucosa was exposed to a TRPV1 antagonist BCTC (0.8 mmol/L) (Fig. 12). The maximum response of GMBF induced by 1440 mg/mL DKT in the presence of BCTC was 122.5 ± 10.1% throughout the experiment. These findings suggested that intragastric DKT produced a significant increase in GMBF via activation of TRPV1, which is expressed in capsaicin-sensitive sensory nerves in anesthetized rats.
Fig. 12  Effects of N-(4-t-butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide (BCTC), a transient receptor potential vanilloid-1 (TRPV1) antagonist, on gastric mucosal blood flow (GMBF) induced by mucosal application of daikenchuto (DKT) in the stomach of anesthetized rats ex vivo. This figure shows a maximal GMBF response induced by mucosal application of DKT (1440 mg/mL). The stomach was perfused with saline, and BCTC (0.8 mmol/L) was applied to the chamber for 50 min starting at 40 min before the DKT application. DKT was topically applied to the mucosa for 10 min. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M.) of values obtained every 2 or 10 min from 4 to 6 rats. Statistically significant difference at P < 0.05; * indicates significant difference compared to the corresponding values in the group treated with vehicle. Note that increased GMBF in response to DKT was only partly abolished by BCTC.
Effects of L-NAME, a non-selective NOS inhibitor, and treatment with a combination of L-arginine (300 mg/kg, ×2) and L-NAME on GMBF in response to capsaicin in the stomach of anesthetized rats \textit{ex vivo}

The increase in GMBF in response to DKT (1440 mg/mL) in control rats was decreased by L-NAME. The maximum GMBF in response to DKT in animals treated with L-NAME (10 mg/kg) was 102.4 ± 6.1% (Fig. 13). In contrast, administration of 300 mg/kg of L-arginine, a substrate for NOS, twice reversed the inhibitory effects of L-NAME (10 mg/kg) on gastric hyperemic response during DKT application (Fig. 13). Thus, gastric hyperemia in response to DKT might be attributed to NO-dependent hyperemia in anesthetized rats.
Fig. 13. Effects of NG-nitro-L-arginine methyl ester (L-NAME), a non-selective nitric oxide synthase (NOS) inhibitor, and treatment with a combination of L-arginine (300 mg/kg, ×2) and L-NAME on gastric mucosal blood flow (GMBF) induced by mucosal application of daikenchuto (DKT) in the stomach of anesthetized rats ex vivo. This figure shows the maximum GMBF during DKT (1440 mg/mL) application for 10 min in animals treated with L-NAME (10 mg/kg) or L-NAME (10 mg/kg) plus L-arginine (300 mg/kg, ×2). The stomach was perfused with saline before the application, L-NAME (10 mg/kg) was administered via intravenous injection, and after 20 min, DKT (1440 mg/mL) was topically applied to the mucosa for 10 min. L-arginine (300 mg/kg) was
administered via an intravenous injection 40 and 60 min before DKT application. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M.) of values obtained every 2 or 10 min from 4 to 5 rats. Note that the increased GMBF during DKT application was slightly inhibited by l-NAME (10 mg/kg), and the response was prevented by pretreatment with l-arginine (300 mg/kg, ×2).
Effect of NPLA, a selective nNOS inhibitor, on GMBF in response to DKT in the stomach of anesthetized rats ex vivo

The roles of different isoforms of NOS in gastric hyperemia induced by DKT were investigated by using the selective nNOS inhibitor NPLA. The increase in GMBF in response to DKT (1440 mg/mL) administration was not decreased by administration of 0.2 mg/kg NPLA. The maximum GMBF during DKT application in animals treated with NPLA (0.2 mg/kg) was 147.7 ± 26.6% (Fig. 14).
Fig. 14  Effect of N5-[imino (propylamino) methyl]- L-ornithine (NPLA), a selective neuronal nitric oxide synthase (nNOS) inhibitor, on gastric mucosal blood flow (GMBF) induced by daikenchuto (DKT) in the stomach of anesthetized rats ex vivo. This figure shows the maximum GMBF response during DKT (1440 mg/mL) application for 10 min in animals treated with NPLA (0.2 mg/kg). The stomach was perfused with saline, NPLA (0.2 mg/kg) was administered via an intravenous injection, and after 20 min, DKT (1440 mg/mL) was topically applied to the mucosa for 10 min. The data are expressed as a % increase in baseline values and represent the mean ± standard error of mean (S.E.M.) of 4–5 rats. Note that the increased GMBF during DKT application was not inhibited by NPLA.
In this study, we found that mucosal application of DKT increased the GMBF in the stomach of rats, and this effect was partly blocked by a TRPV1 antagonist BCTC. These findings suggest that this effect is mainly mediated by capsaicin-sensitive sensory nerves expressing TRPV1.

NO is a well-known vasodilator that increases the GMBF, and this gastric mediator is important in the modulation of gastric mucosal integrity through interaction with sensory nerves. Similar to the capsaicin-induced increase in the GMBF, the responses to DKT were also partly inhibited by a non-selective NOS inhibitor L-NAME, which indicated a partial involvement of NO in those hyperemic responses. The gastric hyperemia in response to DKT can be attributed to both NO-dependent and NO-independent hyperemia during capsaicin application in anesthetized rats. Adrenomedullin and CGRP contribute to hyperemic responses to DKT in the small intestine of rats [11]. Gastric mucosal hyperemia in response to DKT may be attributable not only to NO but also to other gastric mediators, including adrenomedullin and CGRP. On the other hand, NPLA, an inhibitor of nNOS, did not decrease the maximal GMBF response during the application of DKT. The result suggests that nNOS/NO does not play an important role in the gastric hyperemia during the luminal application of DKT. Further, eNOS is expressed mostly in the vasculature throughout the gastric mucosa in rats. It is speculated that eNOS/NO may play an important role in the increased GMBF response to DKT.

Further studies are required to elucidate these unknown vasorelaxant mediators to clarify the mechanism underlying the increased GMBF induced by DKT. Kampo medicines are used for various diseases in Japan. DKT is one of the most frequently used Kampo medicine clinically effective for a cold sensation and dysmotility in the abdomen. Although previous studies have shown the effect of DKT in improving these symptoms [42,43], our results indicate that DKT
stimulates TRPV1 on sensory nerves to induce the gastric circulation through NO release from TRPV1-expressing nerve fibers.

**Summary**

I performed pharmacological studies and observed that DKT increases the GMBF through the activation of TRPV1. TRPV1 stimulation by DKT in turn induced the release of NO derived from an NOS isozyme other than nNOS.
Concluding Remarks

I studied whether capsaicin and DKT increase the gastric mucosal blood flow through activation of TRPV1.

**Part 1 Effects of the TRPV1 agonist capsaicin on gastric mucosal blood flow in rats**

Mucosal application of capsaicin increased the GMBF by releasing NO derived from nNOS after stimulation of capsaicin-sensitive sensory nerves. We showed the contribution of nNOS-derived NO in gastric hyperemic responses to the activation of TRPV1 by capsaicin not only using pharmacological tools but also using immunohistological techniques.

**Part 2 Effects of DKT on gastric mucosal blood flow in rats**

Pharmacological studies showed that DKT increases the GMBF through the activation of TRPV1. TRPV1 stimulation by DKT in turn induced the release of NO derived from an NOS isozyme other than nNOS.

**Conclusion**

Capsaicin increases the GMBF in rats by stimulating the TRPV1 on sensory nerves and nNOS/NO. DKT increased the GMBF in rats by stimulating the TRPV1 on sensory nerves.
List of publications

Chapter 1:
Raimura Masaki, Tashima Kimihito, Matsumoto Kenjiro, Tobe Sinya, Chino Atsushi, Namiki Takao, Terasawa Katsutoshi, Horie Syunji: Neuronal nitric oxide synthase-derived nitric oxide is involved in gastric mucosal hyperemic response to capsaicin in rats
Pharmacology 92 (1-2) 60-70, 2013.

Chapter 2:
To be prepared
Acknowledgements

I want to express my gratitude to Professor Katsutoshi Terasawa a former professor at the Department of Japanese Oriental Medicine, Graduate School of Chiba University for his support and continuous encouragement. I would also like to thank Associate Professor Takao Namiki at the Department of Japanese Oriental Medicine, Graduate School of Chiba University for his supervision of my research.

I express my sincere gratitude 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, Associate 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 thankful to Kenjiro Matsumoto, Assistant Professor of the Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Josai International University for his useful and logical advice and continuous encouragement. I am grateful to Hirokuni Okumi for his/her inputs in this study. I would also like to thank Tsumura & Co. for supplying Japanese Oriental herbs as Kampo formulas and relevant crude drugs. I am deeply grateful to all staff 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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