Title
Inhibitory effect of the selective serotonin 5-HT$_3$ receptor antagonist ramosetron on duodenal acidification-induced gastric hypersensitivity in rats

Author names and affiliations
Mari Nakata-Fukuda$^{a,b}$, Takuya Hirata$^{a}$, Yoshihiro Keto$^{a}$, Mayumi Yamano$^{a}$, Toshihide Yokoyama$^{a}$, and Yasuo Uchiyama$^{b}$
$^a$Pharmacology Research Labs., Drug Discovery Research, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan
$^b$Department of Cell Biology and Neuroscience, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

Corresponding author
Mari Nakata-Fukuda
21 Miyukigaoka
Tsukuba, Ibaraki 305-8585, Japan
Phone: +81-29-862-5111
Fax: +81-29-852-2965
E-mail: mari.nakata@astellas.com
Abstract

Irritable bowel syndrome (IBS) and functional dyspepsia (FD) are both functional gastrointestinal disorders and frequently co-occur in patients. While one cause of FD appears to be gastric hypersensitivity, whether the hypersensitivity is affected by IBS treatments remains unclear, given the lack of appropriate animal models for testing. Here, we established an experimental model of duodenal acidification-induced gastric hypersensitivity in conscious rats. The model involved duodenal acidification induced by the infusion of hydrochloric acid into the proximal duodenum, with the nociceptive response being determined as the change in mean arterial pressure (MAP) during gastric distension via an indwelling latex balloon. Using our model we evaluated the effects of duodenal acidification, increased distension pressure, and orally administered therapeutic agents for IBS with diarrhea (IBS-D). Duodenal acidification enhanced the pressor response during gastric distension, and pretreatment with the opioid κ-receptor agonist fedotozine (10 mg/kg, intra-arterial) inhibited the pressor response. Pressure levels of 15-60 mmHg increased MAP in response to gastric distension. The serotonin 5-HT3 receptor antagonist ramosetron (30 μg/kg) inhibited MAP increase induced by duodenal acidification, with no other IBS-D therapeutic agents showing any effect. In contrast, the serotonin 5-HT3 receptor agonist m-chlorophenylbiguanide (1 mg/kg) significantly enhanced the pressor response during gastric distension. These findings indicate that the serotonin 5-HT3 receptor plays a key role in duodenal acidification-induced gastric hypersensitivity in rats, suggesting that ramosetron may reduce FD symptoms by ameliorating sensitized gastric perception.

Keywords

Duodenal acidification, gastric hypersensitivity, serotonin 5-HT3 receptor, ramosetron, rat
**Abbreviations**

FD, functional dyspepsia; HCl, hydrochloric acid; IBS, irritable bowel syndrome; IBS-D, irritable bowel syndrome with diarrhea; MAP, mean arterial blood pressure; MC, methylcellulose; mCPBG, $m$-chlorophenylbiguanide
1. INTRODUCTION

Functional dyspepsia (FD) is a common functional disorder of the upper gastrointestinal tract associated with postprandial fullness, early satiety, and epigastric pain (Tack et al., 2006). Hypersensitivity in gastric perception, delay in gastric emptying, and abnormal gastric accommodation are thought to play a role in the pathogenesis of FD (Tack et al., 2006). Several studies have shown that duodenal acidification increases gastric sensitivity to distension (Ishii et al., 2010; Lee et al., 2004), positioning this acidification as a recent focus of investigation regarding the cause of FD. However, no animal model able to accurately reflect the clinical findings of gastric hypersensitivity induced by duodenal acidification has yet been reported.

Irritable bowel syndrome (IBS) is one of functional diseases in the lower gastrointestinal tract, with symptoms of persistent abdominal pain or discomfort, and abnormal defecation (Longstreth et al., 2006). Although several recent studies have shown that the symptoms of FD are strongly associated with IBS (Corsetti et al., 2004; Hori et al., 2009; Talley et al., 2003), no reports have yet referenced the outcome of IBS therapeutic agents on the pathophysiology of FD, such as gastric hypersensitivity.

Here, in order to evaluate the effect of IBS therapeutic agents on gastric hypersensitivity, we established an experimental rat model involving duodenal acidification in which the gastric nociceptive response during gastric distension could be evaluated in conscious rats. Further we investigated the effects of several drugs commonly used to treat IBS with diarrhea (IBS-D) in this model, namely a serotonin 5-HT3 receptor antagonist ramosetron (Hirata et al., 2007), opioid µ-receptor agonists, loperamide and trimebutine (Awouters et al., 1993; Roman et al., 1987), and a non-selective muscarinic receptor antagonist tiquizium (Moriya et al., 1999).
2. MATERIALS AND METHODS

2.1. Animals

Male Sprague Dawley rats (9 weeks) were housed in a temperature-controlled environment (22 ± 2 °C) under a 12-h light-dark cycle with food and water available ad libitum. All experimental procedures involving rats were approved by the institutional Animal Care and Use Committee of Astellas Pharma Inc., Tsukuba Research Center, which is AAALAC accredited.

2.2. Drugs

Ramosetron hydrochloride, fedotozine (Astellas Pharma Inc., Ibaraki, Japan), loperamide hydrochloride, trimebutine maleate, m-chlorophenylbiguanide (mCPBG, Sigma-Aldrich Japan, Tokyo, Japan), and tiquizium bromide (Permachem Asia, Ltd., Tokyo, Japan) were used in this study. Ramosetron was dissolved in distilled water and diluted with 0.5% methylcellulose (MC) solution. Loperamide, trimebutine, and tiquizium were suspended in and diluted with 0.5% MC solution. Fedotozine and mCPBG were dissolved in and diluted with saline. Dose levels of all test compounds in the present study were expressed as their salt forms.

2.3. Surgery

After fasting overnight, rats were anesthetized with isoflurane, and a mid-line abdominal incision was made to expose the stomach and duodenum. A latex balloon (4 cm × 2 cm) attached to a polyethylene cannula was inserted into the stomach from a small incision on the forestomach and fixed in place. A second polyethylene cannula was inserted into the proximal duodenum and secured. The gastrointestinal tract was then returned to the abdominal cavity, and the abdominal muscle was sutured. The tail ends of the cannulae from both the stomach and duodenum were passed subcutaneously to the back of neck, and all incisions were sutured.
After recovery from anesthesia, rats were housed individually. The following day, a polyethylene cannula filled with saline/heparin was fixed in the left carotid artery under isoflurane anesthesia. The cannula was passed subcutaneously to the back of the neck, and the incision was sutured. The gastric nociceptive response of the rats was measured 4-6 days after surgery.

2.4. Measurement of the gastric nociceptive response

The gastric nociceptive response in 96 rats was measured as the change in mean arterial pressure (MAP) (Rossi et al., 1998; Van Orshoven et al., 2004) during duodenal acidification-induced gastric hypersensitivity. After fasting overnight, rats were lightly anesthetized with isoflurane. The stomach cannula was then connected with three-way taps to a carrier amplifier (AP-601G; Nihon Koden, Tokyo, Japan) via a pressure transducer (P23XL-1; Nihon Becton Dickinson, Tokyo, Japan) to measure intragastric pressure, and to a pressure loading system to inflate the gastric balloon. The custom-made pressure-loading system consisted of a flask, plastic bottle, pressure transducer, and metal bars. A water-filled plastic bottle was suspended on the bars at heights predetermined to produce the appropriate distension pressure, and then connected hermetically to a water-filled flask with a silicon tube. The flask was in turn similarly connected to a pressure transducer. The duodenal cannula was connected to a 50-mL syringe fixed to an infusion pump (Pump 22; Harvard Apparatus, Holliston, MA, USA) for infusion of either saline or hydrochloric acid (HCl) solution, and the carotid-artery cannula was connected to a blood pressure amplifier (AP-641G; Nihon Koden) via a pressure transducer for systemic blood pressure measurement. After recovery from anesthesia, measurements were taken while rats were unrestrained, and the signals of intragastric pressure (mmHg) and MAP were quantified using a recording interface.
(PowerLab 4/26; AD Instruments, Bella Vista, NSW, Australia) and analyzed using LabChart version 6.1.2 (AD Instruments).

2.5. **Experimental procedures**

Duodenal acidification was induced as previously reported (Hirata et al., 1997) with modification; briefly, 0.01 mol/L HCl was infused into the proximal duodenum at a rate of 0.1 mL/min, 15 min prior to balloon inflation, and continued during gastric distension. The intra-gastric balloon was inflated sequentially to 15, 30, 45, and 60 mmHg for 3 min each, and the change in MAP was determined. Positive controls received an intra-arterial injection of the opioid κ-receptor agonist fedotozine, which increases nociceptive threshold in the gastrointestinal tract (Riviere, 2004), at a dose of 10 mg/kg, 10 min before the initiation of gastric distension.

Ramosetron (3 and 30 μg/kg), loperamide (30 mg/kg), trimebutine (1000 mg/kg), tiquizium bromide (100 mg/kg), or 0.5% MC were orally administered 45 min before HCl infusion and 1 h before the initiation of gastric distension. The serotonin 5-HT₃ receptor agonist mCPBG at 1 mg/kg or saline was administered intraperitoneally 15 min before the initiation of gastric distension. The dose of mCPBG was chosen according to the previous study which showed that mCPBG at 1 mg/kg inhibited gastric emptying in rats (Hirata et al., 2012). In this experiment, mice did not receive HCl infusion. Eight rats were used in each group.

2.6. **Statistical analysis**

Results were statistically analyzed using Statistical Analysis System software (SAS Institute Japan Ltd., Tokyo, Japan). The maximum percentage change in the MAP during distension for 3 min at each pressure levels from the baseline (pre-distension value) was calculated for each
animal, as was the mean ± standard error of the mean (S.E.M.) of each treatment group. Student’s t-test was performed to compare the maximum percentage change in the MAP at each pressure level between pairs of treatment groups, and Dunnett’s multiple comparison test was performed to compare the maximum percentage change in the MAP at each pressure level among multiple treatment groups. Statistical significance was set at 1.25% ($P < 0.05/4$; consideration of multiplicity by Bonferroni’s correction).

3. RESULTS

3.1. Influence of duodenal acidification on the gastric nociceptive response in rats

Gastric distention in rats received duodenal infusion of saline resulted in percentage increases of MAP as follows: 15 mmHg (5.1% ± 1.5%), 30 mmHg (10.5% ± 1.0%), 45 mmHg (16.6% ± 2.6%), and 60 mmHg (20.8% ± 4.7%) (Figs 1A and 2). Duodenal infusion of 0.01 mol/L HCl solution enhanced the pressor response induced by gastric distension (Figs 1B and 2), and gastric distension resulted in the percentage increase of MAP as follows: 15 mmHg (13.6% ± 2.9%), 30 mmHg (21.8% ± 3.9%), 45 mmHg (32.5% ± 4.5%), and 60 mmHg (45.8% ± 5.5%). These increases were statistically significant at 45 and 60 mmHg (Fig. 2). In contrast, the intra-arterial injection of fetodozine abrogated the duodenal acidification-induced enhancement of the pressor response (Fig. 2).

3.2. Effects of IBS-D therapeutic agents on duodenal acidification-induced gastric hypersensitivity in rats

The enhanced pressor response observed during gastric distension after duodenal HCl infusion was inhibited by the oral administration of the serotonin 5-HT$_3$ receptor antagonist ramosetron (30 μg/kg). Statistically significant decreases in MAP following the administration of 30 μg/kg of ramosetron were observed at 45 mmHg (12.7% ± 3.2%) and
60 mmHg (17.7% ± 2.7%), compared to 0.5% MC at 45 mmHg (31.8% ± 4.7%) and 60 mmHg (43.8% ± 6.7%) (Fig. 3). In contrast, neither oral administration of loperamide (30 mg/kg), trimebutine (1000 mg/kg), nor tiquizium (100 mg/kg) affected the duodenal acidification-induced enhancement of the pressor response in rats (Fig. 3).

3.3. Effect of mCPBG on the gastric nociceptive response in rats

Intraperitoneal administration of the serotonin 5-HT₃ receptor agonist mCPBG at 1 mg/kg significantly enhanced the pressor response by gastric distension at all pressure levels, resulting in the following percentage increases of MAP: 15 mmHg (25.7% ± 3.1%), 30 mmHg (23.6% ± 3.1%), 45 mmHg (31.8% ± 3.8%), and 60 mmHg (40.2% ± 3.6%). In contrast, saline slightly enhanced the pressor response by gastric distension at increasing pressure levels, resulting in reduced percentage increases of MAP as follows: 15 mmHg (7.9% ± 2.1%), 30 mmHg (11.8% ± 1.0%), 45 mmHg (17.4% ± 1.8%), and 60 mmHg (20.9% ± 3.3%) (Fig. 4).

4. DISCUSSION

Duodenal infusion of HCl enhanced the gastric nociceptive response in conscious rats. The selective serotonin 5-HT₃ receptor antagonist ramosetron significantly inhibited the duodenal acidification-induced gastric hypersensitivity, with no other IBS-D therapeutic agent showing any effect. Further, the serotonin 5-HT₃ receptor agonist mCPBG induced gastric hypersensitivity. These results suggest that the serotonin 5-HT₃ receptor is closely involved in duodenal acidification-induced gastric hypersensitivity and that ramosetron, which ameliorates sensitized gastric perception, may in turn reduce the symptoms of FD.

Although several clinical studies have shown the involvement of duodenal acidification-induced gastric hypersensitivity in the pathogenesis of FD (Ishii et al., 2010; Lee
et al., 2004), no animal models have been developed which exhibit such clinical findings. Our results show that duodenal acidification enhanced the nociceptive pressor response during gastric distension in conscious rats, representing the first experimental animal model for duodenal acidification-induced gastric hypersensitivity. We measured the change in MAP as an indicator of the nociceptive response in rats. This approach has been used, as well as measurement of changes in electromyographic activity of abdominal muscle, to assess the nociceptive response of hollow organs (Ness and Gebhart, 1990). The pressure-dependent increases in MAP we observed during gastric distension in this study are comparable to those found in clinical studies as Rossi et al. (1998) and Van Orshoven et al. (2004) have previously reported that gastric distension increased systemic blood pressure as well as the feeling of fullness in healthy volunteers. Thus, the increase in MAP during gastric distension in rats corresponds to the nociceptive response to gastric distension observed in humans. We also confirmed that pretreatment with fedotozine, which increases gastric perception thresholds in response to gastric distension in IBS patients (Riviere, 2004), abolished the duodenal acidification-induced gastric hypersensitivity in rats. Together, these findings suggest that our experimental rat model exhibits validated FD pathophysiology, is clinically predictive, and will enable the screening of drugs with potential to relieve gastric hypersensitivity caused by duodenal acidification.

Lee et al. (2004) reported that duodenal acidification induced proximal gastric relaxation and inhibited gastric accommodation to meals in humans, suggesting that gastric accommodation in rats may change in response to the duodenal acidification in our model. Although we did not measure gastric accommodation in the present study, gastric nociceptive response was induced by predetermined pressure loaded with intra-gastric pressure monitoring, but not by volume. Therefore the result and conclusion of this study are considered to be consistent, regardless of the possible change in the gastric accommodation.
Although FD symptoms have been reported to be frequently associated with IBS (Corsetti et al., 2004; Hori et al., 2009; Talley et al., 2003), few animal or clinical studies have evaluated the effect of IBS therapeutic agents on the pathophysiology of FD. We evaluated the effects of these therapeutic agents on duodenal acidification-induced gastric hypersensitivity in our experimental rat model at doses previously reported to inhibit stress-induced diarrhea in rats (Hirata et al., 2008a, 2008b). We observed that ramosetron, which has already been proven effective in treating IBS-D (Hirata et al., 2008b; Matsueda et al., 2008), inhibited gastric hypersensitivity in our experimental rat model. In addition, Vanuytsel et al. (2011) showed that intravenous administration of another serotonin 5-HT3 receptor antagonist, ondansetron, inhibited duodenal acidification-induced gastric sensitization to distension in humans. Together, these findings suggest that serotonin 5-HT3 receptor antagonists, including ramosetron, might ameliorate the FD symptoms in IBS-D patients.

Chemical stimulants, such as hyper-osmotic solution, carbohydrates, and HCl, are known to enhance the release of endogenous serotonin from intestinal enterochromaffin cells (Resnick and Gray, 1962; Zhu et al., 2001). Raybould et al. (2003) showed that activation of serotonin 5-HT3 receptors, which located on the duodenal vagus and spinal nerves, inhibits gastric emptying in rats, indicating the existence of a duodeno-gastric neural reflex mediated by serotonin 5-HT3 receptors. Our results provide further evidence for such a reflex and highlight the importance of the serotonin 5-HT3 receptor in regulating gastric perception. Ramosetron has also been reported to have a high affinity and selectivity for serotonin 5-HT3 receptor (Hirata et al., 2007) and to act only in peripheral tissue (Yamamoto et al., 2002). Consistent with these reports, our results demonstrate that ramosetron clearly inhibits hypersensitivity induced by duodenal acidification. Further, the commonly used selective serotonin 5-HT3 receptor agonist mCPBG (Fakhfouri et al., 2010; Ito et al., 1996; Yamano et
al., 1995) caused gastric hypersensitivity that closely mimicked the effects of HCl in our experimental rat model. Together, these data suggest that endogenous serotonin released from intestinal enterochromaffin cells in response to duodenal acidification activates peripheral serotonin 5-HT\textsubscript{3} receptors, which in turn sensitize the stomach to distension. Although the precise mechanism by which duodenal acidification induced gastric hypersensitivity via the activation of serotonin 5-HT\textsubscript{3} receptor remains unknown, the serotonin 5-HT\textsubscript{3} receptor has been reported to regulate the release of various neurotransmitters (Faerber et al., 2007) and activity of sensory nerves (Moalem et al., 2005). The duodenal acidification-induced activation of serotonin 5-HT\textsubscript{3} receptors may have therefore directly sensitized the gastric nociceptive process. Further study will be needed to evaluate the neuronal and molecular mechanisms in greater detail.

In contrast to ramosetron, other IBS-D therapeutic agents such as the opioid μ-receptor agonists loperamide and trimebutine (Awouters et al., 1993; Roman et al., 1987) and the non-selective muscarinic receptor antagonist tiquizium (Moriya et al., 1999) did not inhibit duodenal acidification-induced gastric hypersensitivity. The results concerning fedotozine, loperamide, and trimebutine are consistent with those of Sengupta et al. (1996), which indicated that stimulation of peripheral κ-opioid receptors, but not μ- or δ-opioid receptors, attenuated visceral nociception in rats. Tiquizium is a non-selective muscarinic receptor antagonist prescribed as a spasmolytic agent for several gastrointestinal diseases. As acetylcholine is not considered to be involved in the transmission of visceral nociceptive signals (Blackshaw and Gebhart, 2002), these findings indicate that anti-muscarinic agents are not effective against gastric hypersensitivity.

To our knowledge, the present study is the first report of an experimental animal model of duodenal acidification-induced gastric hypersensitivity. The selective serotonin 5-HT\textsubscript{3} receptor antagonist ramosetron was the only IBS-D therapeutic agent that significantly
inhibited gastric hypersensitivity caused by duodenal acidification. Our findings further revealed the importance of the serotonin 5-HT3 receptor in duodenal acidification-induced gastric hypersensitivity, suggesting that ramosetron may be effective in treating FD symptoms in IBS patients.

**Conflict of Interest Disclosure**

Astellas Pharma Inc. financially supported this study. All authors except Yasuo Uchiyama are employees of Astellas Pharma Inc.
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FIGURE LEGENDS

Fig. 1. MAP in conscious rats with gastric distension and duodenal infusions of saline (A) and 0.01 mol/L HCl solution (B). Intragastric balloons were inflated at 15, 30, 45, and 60 mmHg for 3 min each, and the change in MAP was determined. Infusion of saline or 0.01 mol/L HCl into the duodenum was initiated 15 min prior to gastric distension.

Fig. 2. Effect of duodenal acidification on gastric nociception and effect of fedotozin on duodenal acidification-induced gastric hypersensitivity in rats. Each column with a vertical bar represents the mean ± S.E.M. for eight rats. # P<0.0125 in comparison to rats with duodenal infusion of saline (open column), and * P<0.0125 in comparison to rats with duodenal infusion of 0.01 mol/L HCl (black column) using Student’s t-test with Bonferroni’s correction.

Fig. 3. Effect of IBS-D therapeutic agents on duodenal acidification-induced gastric hypersensitivity in rats. Each column with a vertical bar represents the mean ± S.E.M. for eight rats. # P<0.0125 and ## P<0.0025 in comparison to rats with duodenal infusion of saline (open column) using Student’s t-test, and * P<0.0125 and ** P<0.0025 in comparison to rats with duodenal infusion of 0.01 mol/L HCl (black column) using Dunnett’s multiple comparison test, with Bonferroni’s correction.

Fig. 4. Effect of mCPBG on duodenal acidification-induced gastric hypersensitivity in rats. Each column with a vertical bar represents the mean ± S.E.M. for eight rats. ## P<0.0025, ### P<0.00025 in comparison to rats treated with saline (open column) using Student’s t-test with Bonferroni’s correction.
Figure 1

(A) Intra-gastric pressure (mmHg)

(B) Blood pressure (mmHg)

MAP (mmHg)
Figure 2

% increase in MAP

- Saline
- 0.01 mol/L HCl
- Fedotozine 10 mg/kg+HCl

Intra-gastric pressure (mmHg)

- 15
- 30
- 45
- 60

* denotes significance compared to control
# denotes significance compared to 0.01 mol/L HCl
Figure 3

- 0.5% MC+Saline
- 0.5% MC+0.01 mol/L HCl
- Ramosetron 3 μg/kg+HCl
- Ramosetron 30 μg/kg+HCl
- Loperamide 30 mg/kg+HCl
- Trimebutine 1000 mg/kg+HCl
- Tiquizium 100 mg/kg+HCl
- Ramosetron 30 mg/kg+HCl

% increase in MAP

Intra-gastric pressure (mmHg)
Figure 4

Intra-gastric pressure (mmHg)

% increase in MAP

Saline
mCPBG 1 mg/kg

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