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Decreased Motility of the Lower Esophageal Sphincter in a Rat Model of Gastroesophageal Reflux Disease May Be Mediated by Reductions of Serotonin and Acetylcholine Signaling

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To elucidate the altered function of the lower esophageal sphincter (LES) in gastroesophageal reflux disease (GERD), we evaluated the motility proximal to LES using force transducers, contraction and relaxation responses to neurotransmitters in LES strips, and gene expression of neurotransmitter receptors in GERD rats. Force transducers were applied to the proximal LES, and contraction of the LES was monitored during free moving. In addition, LES was isolated from sham-operated and GERD rats to investigate the LES function in an organ bath, and to determine gene expression. The *in vivo* motility proximal to LES (% motility index) in conscious rats was decreased by atropine treatment and increased by cisapride (5-HT₄ receptor agonist) treatment. Acetylcholine- and serotonin (5-HT)-induced LES contraction and sodium nitroprusside-induced relaxation in LES strips of GERD rats markedly decreased compared to sham-operated rats. The mRNA expressions of 5-HT₄ and muscarinic acetylcholine 3 receptors were significantly reduced in esophageal LES strips of GERD rats compared with sham-operated rats. Intraperitoneal administration of cisapride improves the erosive damage in the esophagus in GERD rats. It is suggested that the reduction of 5-HT-induced contraction in LES strips in GERD rats may be partly due to the decrease in 5-HT₄-receptor activation. The reduction of LES function may be due to the decrease in neurotransmitters signal transduction, leading to the deterioration of histopathological damage in GERD.

Key words acetylcholine; gastroesophageal reflux disease; lower esophageal sphincter; motility; rat; serotonin

The pathogenesis of gastroesophageal reflux disease (GERD) is multifactorial but the main factor is extended exposure to gastric acid. The extent of esophageal mucosal injury is determined by the degree and duration of esophageal exposure to acid. Patients with GERD have been found to have delayed acid clearance times that are 2–3 times longer than those without GERD.¹⁾ Indeed, the process of esophageal acid clearance is an important factor in the worsening of esophageal mucosal injury.²⁾ Impaired esophageal clearance can be partly caused by peristaltic dysfunction²⁾ and lower esophageal sphincter (LES) functioning.³⁾ Esophageal motility is also regulated by the esophageal vagal nerve at the interneurons of the central subnucleus of the solitary tract complex.⁴⁾ A recent study demonstrated the effect of baclofen, a gamma aminobutyric acid B receptor agonist, on esophageal motility and transient LES relaxation in GERD patients.⁵⁾ Although esophageal acid clearance is regulated by neurotransmitters both peripherally and centrally, the local responsiveness of the muscle is still unknown.

The motor innervations of LES and the proximal stomach are known to be under cholinergic control.^{6–8)} A recent study demonstrated that the serotonin-4 (5-hydroxytryptamine 4; 5-HT₄) receptor is localized in LES and regulates LES tone.⁹⁾ Thus, we hypothesized that a neurotransmitter-induced LES response in GERD rats may be impaired at the esophageal-tissue level, leading to the development of GERD pathology. In the present study, we examined this hypothesis by measuring LES motility in GERD rats and the effects of neurotrans-

mitters on contraction and relaxation using isolated LES strips in an *in vitro* study. In addition, we investigated the effects of activation of the 5-HT₄ receptor on esophageal erosion in GERD rats.

MATERIALS AND METHODS

Test Substances Acetylcholine (ACh), atropine, 5-HT, sodium nitroprusside (SNP), α -methylserotonin (α -Me-5-HT: a 5-HT₂ receptor agonist), 5-methoxytryptamine (5-MeOT: a nonspecific 5-HT receptor agonist), 1-(3-chlorophenyl)biguanide HCl (a 5-HT₃ receptor agonist), BW723 C86HCl (a 5-HT_{2B} receptor agonist), *m*-chlorophenyl-piperazine HCl (mCPP: a 5-HT_{2C} receptor agonist), or SB204070 (a 5-HT₄ receptor antagonist) were all purchased from Sigma Aldrich Corp. U.S.A. Cisapride and mosapride (5-HT₄ receptor agonists) was purchased from Tocris Bioscience. Ondansetron (a 5-HT₃ receptor antagonist) was purchased from GlaxoSmithKline.

Test Animals Male Wistar rats aged 8 weeks (CLEA Japan Inc., Tokyo, Japan) were used for all experiments. During testing, 4–5 animals were housed in each cage and were allowed free access to food and water in a room that was illuminated between 07:00 and 19:00 h. Temperature and humidity were maintained at constant levels. For tests that evaluated the motility of the proximal LES, animals were housed singly in cages and deprived of food for 24 h after surgery, after which they were given free access to food. Animals that

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did not experience a sudden reduction in body weight were selected for testing. All tests were performed between 09:00 and 18:00 h according to the guidelines set forth by the Experimental Animal Ethics Committee of Tsumura & Co., Japan.

Study of LES Responsiveness *in Vitro* As reported previously,¹⁰⁾ rats were rendered unconscious by a blow to the head and exsanguinated by severing the carotid artery. The abdomen was opened, and the area between the point of contact between the esophagus, diaphragm, and gastric cardia was isolated and placed in a petri dish filled with Krebs–Henseleit liquid. Unwanted materials, including fat and blood vessels, were removed. Approximately 3 mm of the cardia was excised and opened with scissors. After LES was longitudinally suspended in a 10 ml organ bath for 1 h under a 1 g load, the test substance (ACh) was administered to LES strips at a dose of 10 $\mu\text{mol/l}$ until contraction of the specimens became constant (Fig. 1). Only specimens exhibiting contraction of 0.5 g or greater in response to 10 $\mu\text{mol/l}$ ACh were used for the study. The test substances were administered in 100 μl increments to the water bath holding the prepared suspended specimen as described above, and the resulting contraction and relaxation were recorded. Response to the test substance was recorded for 10 min just after its administration, and the sample was washed 3 times between each administration of the test substance. Specimens were also allowed to stand for at least 15 min after being washed before administration of the test substance. The organ bath was maintained at a temperature of 37°C, saturated with Krebs–Henseleit liquid, and aerated with 95% O₂ and 5% CO₂ during all recordings. Contraction and relaxation were measured using isometric transducers (type TB-611T; Nihon Kohden Corp. and type 45196A; NEC San-ei Instruments, Ltd., Japan) connected to a pressure amplifier (type AP-621G; Nihon Kohden Corp., Japan). Recordings were made with either an MP100 or MP150 instrument (Biopac Systems, Inc., U.S.A.), and a pen recorder (type R-62; Rikadenki Electronics Corp., Japan). The actual evaluation of response

by addition of ACh was expressed as “g” after stability of the wave, and the evaluation of 5-HT or SNP was expressed as “%” of ACh contraction.

To clarify the role of 5-HT-receptor subtypes in the LES contraction response, we evaluated the antagonist on 5-HT-induced contraction in LES. The antagonist was first added to the organ bath, the bath was then allowed to stand for 10 min, and 5-HT (10 $\mu\text{mol/l}$) was administered. The response was calculated by the following formula:

$$\text{response} = 100 \times (\text{5-HT, or 5-HT plus 5-HT antagonist-induced wave length}) / (\text{acetylcholine-induced wave length})$$

Preparation of the Reflux Esophagitis Model and Fixature of the Strain Gauge Force Transducer The GERD model was prepared based on the method of Omura *et al.*¹⁰⁾ Rats deprived of food for 24 h were anesthetized with an intraperitoneal (i.p.) infusion of pentobarbital sodium (Nembutal; Dainippon Sumitomo Pharma Co., Ltd., Japan). The abdomen was opened using a 2-cm upper median abdominal incision. The stomach and duodenum were exteriorized, and the transitional region between the forestomach and glandular stomach (limiting ridge) was ligated to make it easier to raise the contents of the stomach to the esophagus. A pre-cut 2-mm wide 18-Fr Nelaton catheter was used to cover an area proximal to the pylorus on the duodenal side. The serous membrane of the area proximal to the pylorus was then sutured and fixed to delay gastric emptying (Fig. 2). Subsequently, a strain gauge force transducer (F-04IS, F-08IS; Starmedical Co., Japan) was fixed to the serous membrane surface approximately 5 mm above LES with a surgical square needle and a gently-curved tapered needle for blood vessels to allow contraction to be measured in the annular plane of the circular muscle. Finally, after confirmation of complete hemostasis, the abdominal wall was sutured with an atraumatic needle and a sharply curved cutting needle for external surgery, and the skin of the abdomen was closed with a rapid continuous suture device. During surgery, a catheter was placed in the jugular vein and heparin-contain-

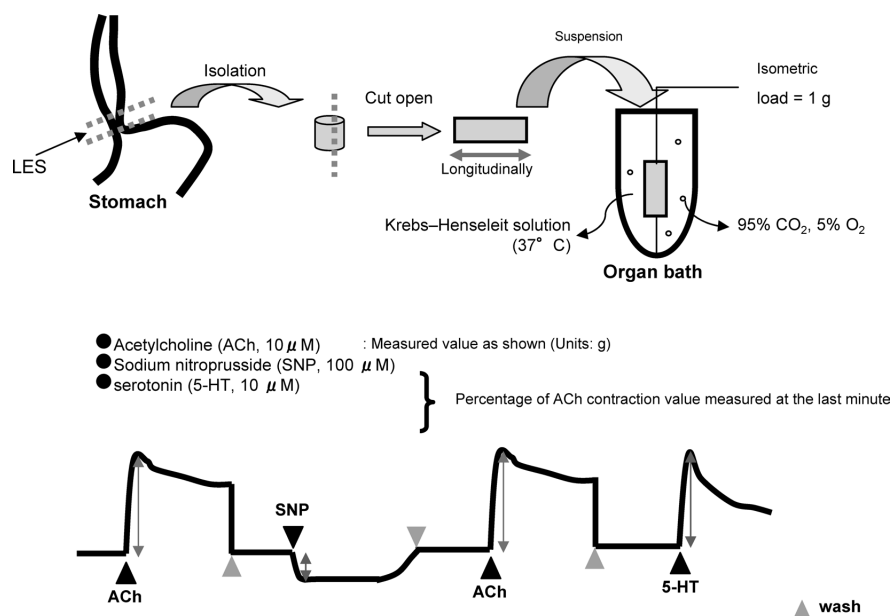


Fig. 1. Schematic of the Method for Neurotransmitters-Induced Response in LES Strips

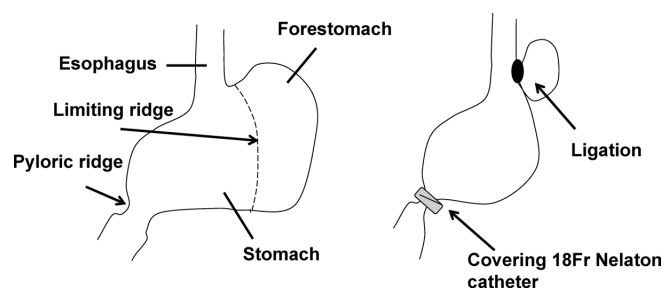


Fig. 2. Preparation of the Rat Model of GERD

ing physiological saline was infused. After surgery, the animals were deprived of food for 24 h and housed with a device allowing free movement.

Effect of Cisapride on Esophageal Erosion in GERD Rats To clarify the role of the 5-HT₄ receptor in LES of GERD rats, the effects of cisapride on esophageal erosion in GERD rats was evaluated. From the next day (day 1) to day 10 after GERD induction, rats i.p. administered test drug at a dosage of 1 mg/kg×2/d. The sham-operated and control rats were administered 0.1% Tween80 saline, as vehicle, instead of test drug. The esophagus in each rat was isolated on day 10, and histopathological determination was performed. The number of esophageal erosive sites of GERD rats was evaluated.

Analysis of Gastrointestinal Tract Motility We examined the effects of atropine at a dosage of 1 mg/kg i.p. and cisapride at a dosage of 1 mg/kg i.p. on LES motility in normal rats to determine the roles of acetylcholine neurons or the 5-HT₄ receptor. The area under the wave (motility index; MI) per minute in the proximal LES was measured and is shown as a percentage (MI%) relative to preadministration data. The rats were deprived of food and water for 24 h and an in-place strain gauge force transducer was connected to a special preamplifier (type FS-04M; Starmedical Co.) through a bridge box (type FB-01; Starmedical Co.) to measure motility of the LES proximal to the esophagus. Data were recorded using an MP150 instrument. MI for LES was used to compare the data obtained from the sham-operated and GERD-rat groups. MI was calculated from the waveforms with the longest duration during consistent contractions of the empty stomach and was reported as the area under the curve (AUC) per minute. Frequency was defined as the rate of occurrence of forceful contractions during the time period used to calculate MI.

Extraction of Total RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis LES in sham-operated or GERD rats on days 2 and 10 after induction were rapidly removed and immediately frozen by placing in a tube on dry ice. In GERD rats, isolated LES was discriminated between erosive and non-erosive esophagus in GERD rats. Once completely frozen, samples were stored at -80 °C. Subsequently, homogenization of the isolated tissue and total RNA extraction were performed according to the protocol from the RNeasy Universal Tissue Kit (Qiagen, Valencia, CA, U.S.A.). Subsequently, each sample was diluted to 100 ng/μl. The diluted total RNA was incubated at 70 °C for 5 min and then cooled on ice. Total RNA (1000 ng) was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, U.S.A.) ac-

cording to the manufacturer's protocol. Quantitative PCR assays were performed using TaqMan Universal PCR Master Mix (Applied Biosystems) using a Prism 7900HT Sequence Detection System (Applied Biosystems). Expression of mRNA was normalized using β-actin as an endogenous control to correct the differences in the amount of total RNA added to each reaction. These differences were expressed as the dC_t value using the following equation: $dC_t = 2^{-(A-B)}$, where A was the number of cycles needed to reach the threshold for the housekeeping gene (C_t : threshold cycle) and B was the number of cycles needed for the target gene. All oligonucleotide primers and fluorogenic probe sets for TaqMan real-time PCR were manufactured by Applied Biosystems (*Actb*: Rn006678691_m1, *Htr2a*: Rn00568473_m, *Htr2b*: Rn00568450_m1, *Htr2c*: Rn00562748_m1, *Htr4*: Rn00563402_m1, *Chrm1*: Rn00589936_s1, *Chrm2*: Rn02532311_s1, *Chrm3*: Rn00560986_s1, and *Chrm4*: Rn01512605_s1).

Statistical Analysis Statistical significance was analyzed by the Student's *t*-test and the one-way analysis of variance, followed by Dunnett analysis. Data were expressed as the mean±standard error for each group, and a *p* value less than 0.05 was considered significant.

RESULTS

Motility of the Proximal LES from GERD Rats As shown in Fig. 3, contraction of the area proximal to the LES was clear and occurred at constant intervals. MI% of the area proximal to LES after intravenous administration of atropine decreased by 45% (Fig. 3A). In contrast, MI% of the proximal LES contractions showed a 181% increase after cisapride treatment (Fig. 3B). On the other hand, clear and forceful contractions were not seen in the proximal LES from GERD rats. The percentages of MI for the proximal LES contractions were compared between the sham-operated and GERD rats. MI% for contractions was significantly lower in the proximal LES of GERD rats than that of sham-operated rats (Figs. 3C, D).

Reactivity of LES Strips from Normal Rats This experiment was performed to clarify the 5-HT₄ receptor that significantly contributed significantly to 5-HT-induced contraction in LES strips. The LES contraction dose-dependently increased in response to the addition of the nonspecific 5-HT receptor agonist 5-MeOT (Fig. 4A). The LES contraction response to added 5-HT was dose-dependently inhibited by the addition of the 5-HT₄ receptor antagonist SB204070 (Fig. 4B). The LES contraction response induced by 5-HT was also significantly inhibited by the addition of 5-HT_{2B} receptor antagonist SB204741 or 5-HT_{2C} receptor antagonist SB242084, although 5-HT₃ receptor antagonist ondansetron failed to inhibit it (Figs. 5A—C). Of 5-HT receptor agonists, the addition of 5-HT₂ receptor agonist α-methylserotonin only induced LES contraction in sham-operated rats (Figs. 5D—G).

Reactivity of LES Strips from GERD Rats in Response to Neurotransmitters As shown in Fig. 6, reduction in the contraction response to 5-HT (Fig. 6A) and Ach (Fig. 6B) and the relaxation response to SNP (Fig. 6C) was significantly lower in LES strips from the GERD group than that from the sham-operated group on day 10 after GERD induc-

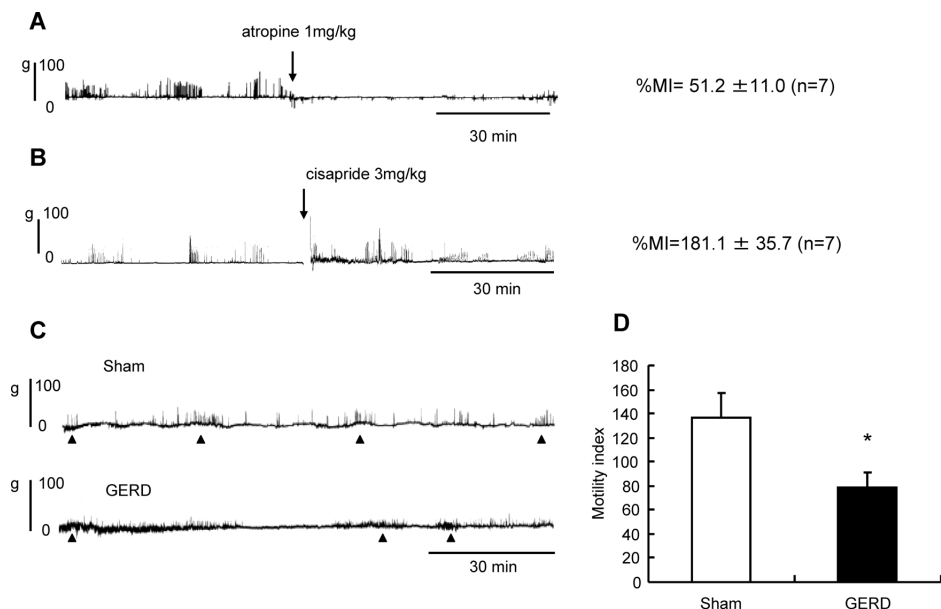


Fig. 3. Effects of Atropine and Cisapride on the LES % Motility Index in Sham-Operated Rats, and Changes in GERD Rats and Sham-Operated Rats

The contraction of the area proximal to LES was obstructed completely in the upper motility wave by intravenous administration of atropine. The contraction of the area proximal to LES was increased by cisapride (second motility wave). Although contraction of the area proximal to LES in sham-operated rats was clear and occurred at constant intervals (third motility wave), LES contraction in GERD rats failed to occur at constant intervals (fourth motility wave). (A) Effect of atropine; (B) effect of cisapride; (C, D) changes in GERD and sham-operated rats. * $p < 0.05$ vs. sham-operated rats (vehicle-treated rats) by Student's *t*-test. The motility of the LES was determined on day 10 after induction of the GERD model. $n = 10$.

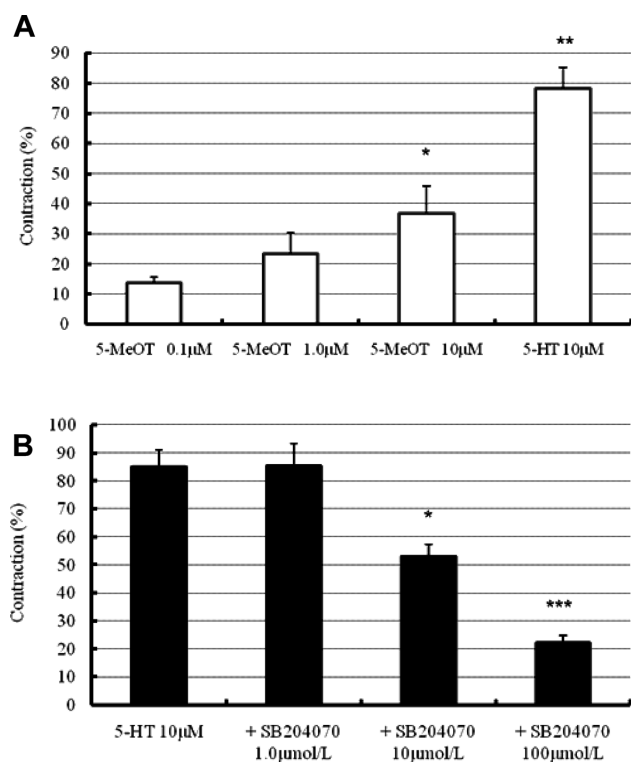


Fig. 4. Effects of the Nonspecific 5-HT Receptor Agonist 5-HT_{2A} on LES Contraction and Effect of 5-HT_{2A} Receptor Antagonist SB204070 on 5-HT-Induced Contraction in LES

(A) Effect of 5-HT_{2A}; * $p < 0.05$, ** $p < 0.01$ vs. saline-treated contraction by Dunnett's *t*-test. (B) Effect of SB204070; * $p < 0.05$, *** $p < 0.001$ vs. 5-HT-induced contraction by Dunnett's *t*-test. SB204070 was first added to the organ bath, the bath was allowed to stand for 10 min, and then 5-HT administered. 5-HT, serotonin 10 μmol/L.

tion. The contraction response to 5-HT was significantly lower in LES strips taken from GERD rats on day 2 than in sham-operated rats, though SNP-induced relaxation in LES

from GERD rats showed a tendency to decrease. On the other hand, LES strips from GERD rats failed to show a lower contraction response by the addition of Ach, in comparison with sham-operated rats.

Changes in the Expression of Neurotransmitter Receptor mRNA in LES Strips from GERD Rats The 5-HT₄- and M3-receptor mRNA expression on day 10 after induction was significantly reduced in LES of GERD rats (Figs. 7A, B), although on day 2, these expressions showed a tendency to be inhibited. As shown in Fig. 7C, the 5-HT_{2A}- or 5-HT_{2B}-receptor mRNA expression in LES of GERD rats showed a tendency to increase compared with sham-operated rats. The M1-, 2-, and 4-receptor mRNA expressions in LES of GERD rats at day 10 after induction were not significantly decreased compared with the sham group. The 5-HT_{2C}-receptor mRNA expression could not be detected in LES of both GERD and sham-operated rats.

Effect of Cisapride on the Number of Erosive Sites in the Esophagus of GERD Rats In GERD rats with vehicle treatment, the number of erosion sites in the esophagus was 2.9 ± 0.7 at day 10 after the induction (Fig. 8A). Compared with vehicle-treated rats, cisapride i.p. administration significantly reduced it in the esophagus (1.3 ± 0.2) (Fig. 8A). Photographs of esophagus in GERD rats are shown as Fig. 8B. In comparison with sham-operated group, the esophageal erosion in GERD rats increased at day 10. In contrast, cisapride treatment attenuated the number of erosion sites in esophagus.

DISCUSSION

We first demonstrated that the comparison of the *in vivo* motility of LES showed a markedly lower motility proximal to LES in the GERD group than in the sham-operated group. The most noteworthy results of this study include the obser-

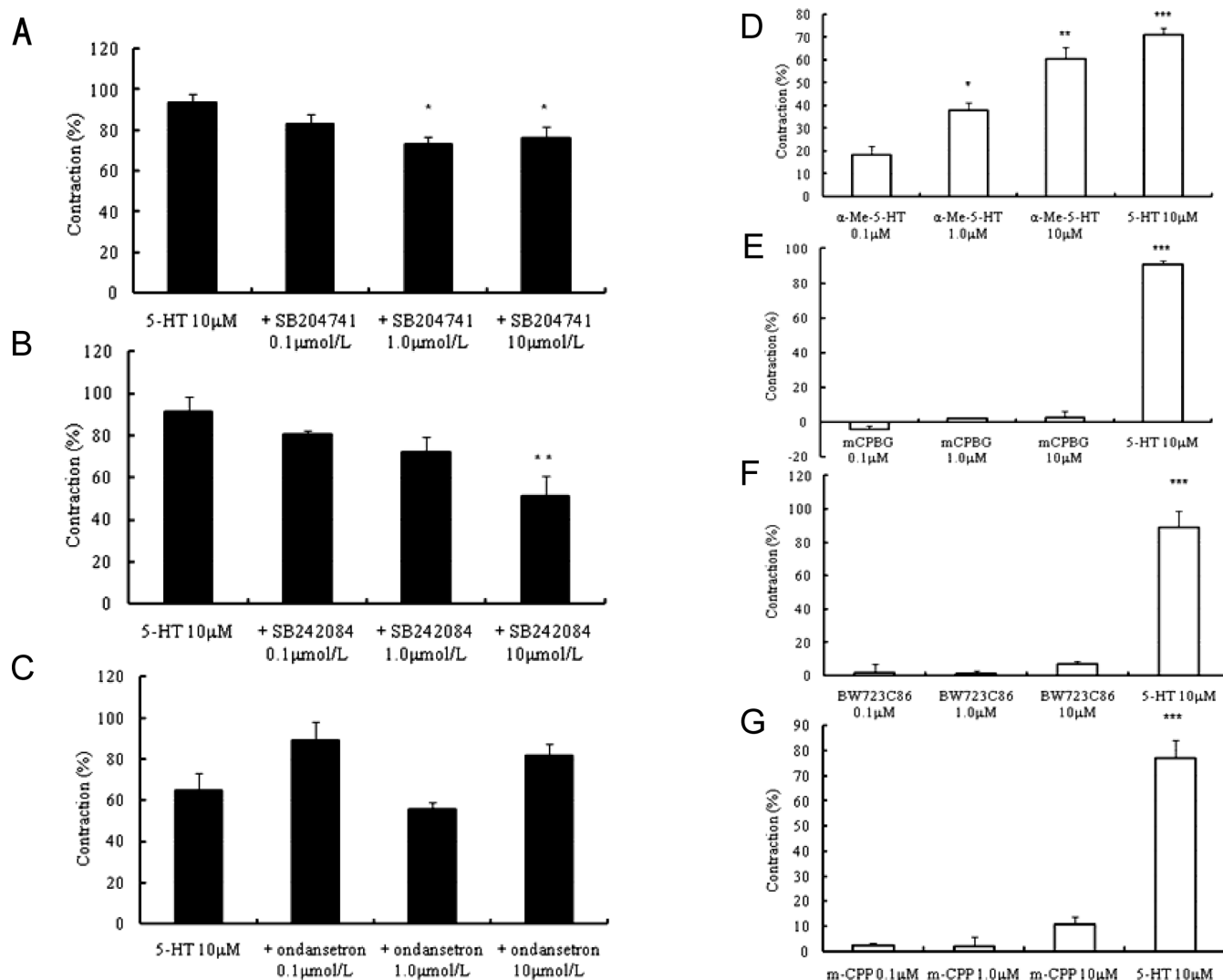


Fig. 5. Effects of 5-HT_{2B}, 5-HT_{2C} or 5-HT₃ Receptor Antagonist on 5-HT-Induced LES Contraction and Difference between the Effects of 5-HT₂ and 5-HT₃ Receptor Agonists on LES Contraction

Effects of the 5-HT_{2B} receptor antagonist, SB204741, 5-HT_{2C} receptor antagonist, SB242084(B), 5-HT₃ receptor antagonist, ondansetron (c) on 5-HT-induced LES contraction. * p <0.05, ** p <0.01 vs. non-treated contraction by Dunnett's test. n =6–10, Effects of the 5-HT₂ receptor agonist α -methylserotonin (D), 5-HT₃ receptor agonist, 1-(3-chlorophenyl)biguanide HCl (E), 5-HT_{2B} receptor agonist BW723C86HCl (F), 5-HT_{2C} receptor agonist, m -chlorophenyl piperazine HCl (G) on LES contraction. * p <0.05, ** p <0.01, *** p <0.001 vs. non-treated contraction by Dunnett's test. α -Me-5-HT; α -methyl serotonin, mCPBG; 1-(3-chlorophenyl)biguanide HCl, mCPP; m -chlorophenyl piperazine HCl n =6–10.

vation of reductions in Ach- or 5-HT-induced contraction and relaxation as well as SNP-induced relaxation in LES of GERD rats. In addition, we found that the administration of cisapride was effective for the representative histopathological parameters in GERD. This supports the possibility that deterioration of the esophageal mucosal injury in GERD rats may be due to the reduction of 5-HT₄-receptor signal transduction.

It has been believed that the LES pressure in GERD patients is generally reduced. However, no study that directly evaluated the motility of the esophagus has been previously conducted to clarify the mechanisms responsible for impairment of LES functioning in GERD subjects. Previously, in the GERD rat that was used in the present study, esophagitis was noted in all rats until 1–3 weeks after induction.¹⁰ Esophagitis was found 2 or 3 cm above the esophagogastric junction in most cases, and at 4.0 ± 2.3 sites per animal. Histopathologically, there was increased thickness of the esophageal epithelium, elongation of the lamina propria

papillae, which extended upward into the epithelium, marked inflammatory cell infiltration, interruption of the lamina muscularis mucosae, and increase of collagen fibers in the lamina propria and submucosa. We evaluated contractility of the area proximal to LES using a strain-gauge force transducer under free-moving conditions. Forceful contractions were seen in sham-operated rats, in which motility was consistent and regular in the proximal LES. On the other hand, the contraction frequency (data not shown in Figures and Tables) and motility index of the area proximal to LES was significantly lower in GERD rats than in sham-operated rats. This result suggested a reduction in motility in the proximal LES from GERD rats. It is likely that a reduction in contractile activity in this area may be a reflection of LES pressure or esophageal motility, which can be used for evaluation of LES functioning in a clinical setting.

In the current study, we first demonstrated in an *in vivo* experiment that motility of the proximal LES area in normal rats was abolished by intravenous administration of atropine.

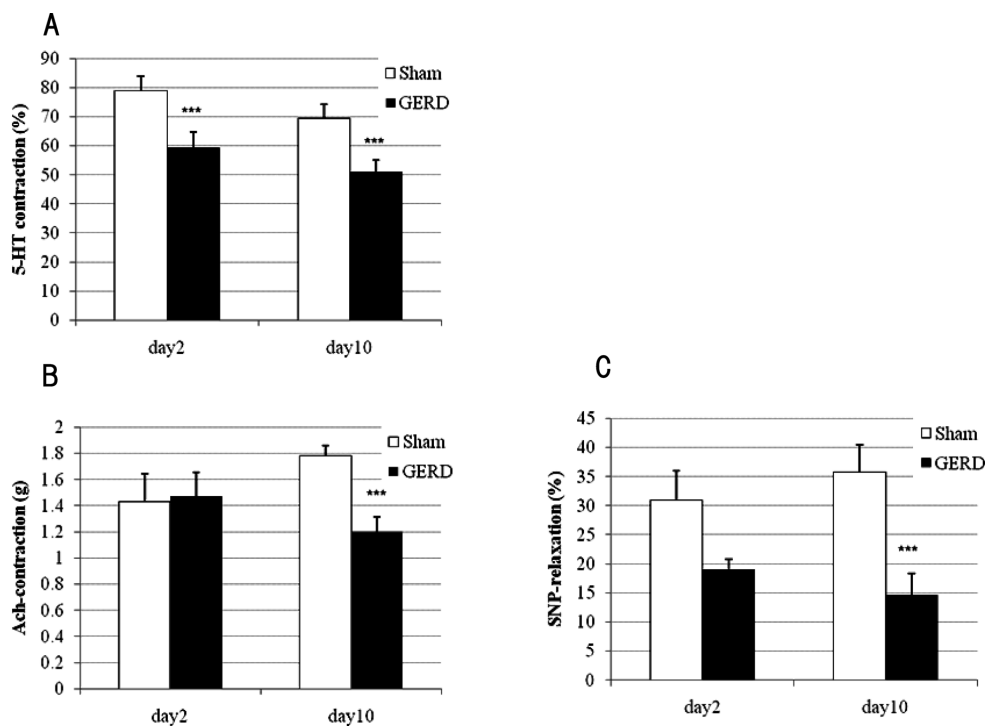


Fig. 6. Changes in Neurotransmitters-Induced Contraction or Relaxation of LES from GERD and Sham-Operated Rats

(A) 5-HT, serotonin 10 $\mu\text{mol/l}$; (B) Ach, acetylcholine 10 $\mu\text{mol/l}$; (C) SNP: sodium nitroprusside 100 $\mu\text{mol/l}$. *** $p < 0.001$ vs. sham-operated rats by Dunnett's *t*-test. $n = 6-18$.

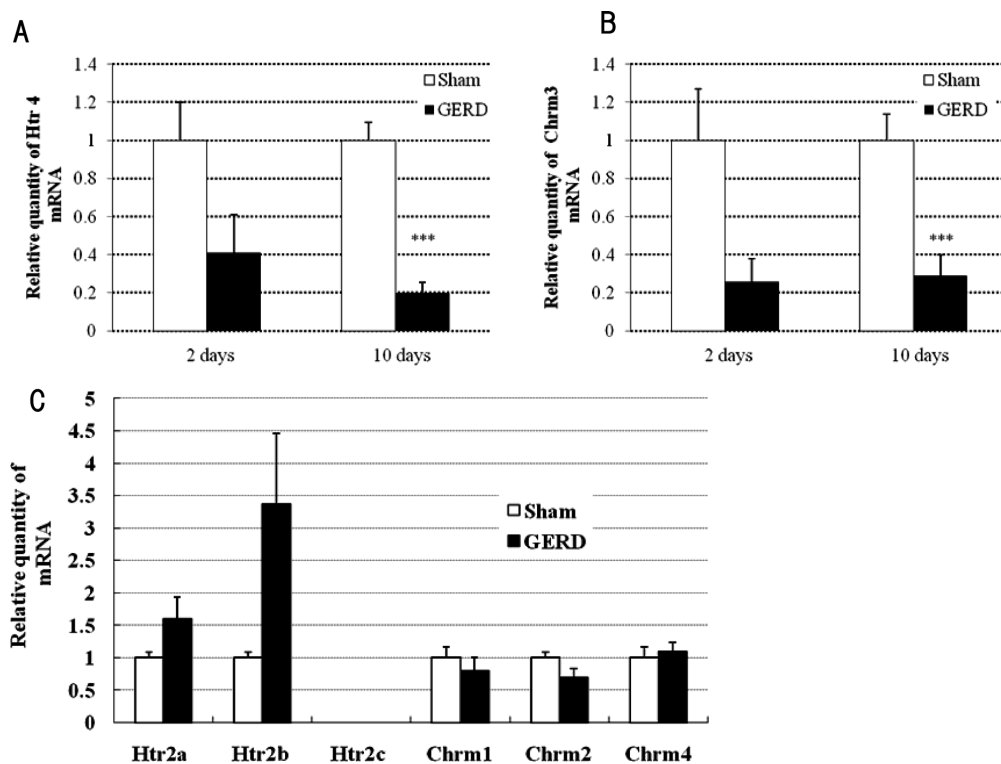


Fig. 7. Changes in 5-HT_{2A}, 2B, 2C, 4- and Muscarinic 3 Receptor-mRNA Expression of LES from GERD Rats and Sham-Operated Rats

The LES sample was collected at 2 or 10 d after induction of GERD models. (A) Changes in 5-HT₄ receptor at 2 and 10 d, (B) changes in muscarinic acetylcholine 3 receptor at 2 and 10 d, (C) changes in 5-HT_{2A}, 2B, 2C, and M1, M2, and M4 receptor at 10 d. *** $p < 0.001$ vs. sham-operated rats by Student's *t*-test. $n = 5$. 5-Htr2a: 5-HT_{2A} receptor, 5-Htr2b: 5-HT_{2B} receptor, 5-Htr2c: 5-HT_{2C} receptor, 5-Htr4: 5-HT₄ receptor, chrm 1: muscarinic acetylcholine 1 receptor, chrm 2: muscarinic acetylcholine 2 receptor, chrm 3: muscarinic acetylcholine 3 receptor, chrm 4: muscarinic acetylcholine 4 receptor.

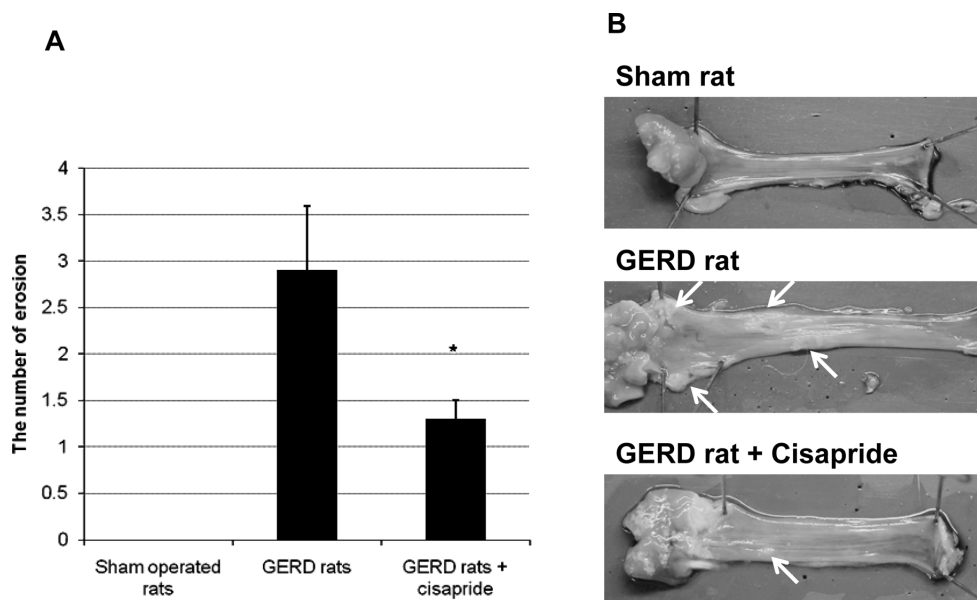


Fig. 8. Effects of Cisapride on Esophagus Erosion in GERD Rats

Test drug i.p. administration was performed at the next day to day 10 after GERD induction. (A) Effect of cisapride on the number of erosion sites in the esophagus, * $p < 0.05$ vs. sham-operated rats by Student's *t*-test. (B) Typical photographs of sham-operated, GERD+vehicle and GERD+cisapride.

Our study also showed that LES strips from sham-operated rats exhibited contraction with Ach. The motor innervations of LES and proximal stomach are known to be under cholinergic control.^{6–8)} Takeuchi *et al.*⁶⁾ demonstrated contraction using Ach and interruption using atropine on LES strips isolated from rats. In contrast, it has been demonstrated that the main neurotransmitter released by the inhibitory neurons is nitric oxide.⁷⁾ The relaxation response was induced in LES strips by SNP, a donor of nitrous oxide groups. These findings from other studies^{8,11)} are consistent with our *in vitro* results, suggesting that LES is simultaneously controlled by the cholinergic system and nitric oxide. On the other hand, localization of 5-HT-receptor subtypes in LES remains unclear. It has been demonstrated that 5-HT₄-receptor activation may be associated with esophageal peristalsis and LES tone.^{9,12)} Of the 5-HT receptor antagonists tested, contraction by 5-HT was significantly reduced by adding the 5-HT₄ receptor antagonist, but not by the 5-HT₃ receptor antagonist. Although, the 5-HT_{2B} receptor antagonist SB204741 and 5-HT_{2C} receptor antagonist SB242084 partly attenuated 5-HT-induced contraction in LES strips, LES contraction was not induced by the 5-HT_{2A} receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (data not shown in Figs.), the 5-HT_{2B} receptor agonist BW723C86, and the 5-HT_{2C} receptor agonist mCPP up to the concentration of 100 μ mol/l. It is well known that the 5-HT_{2C} receptor is localized in the central nervous system. In addition, we did not observe expression of the 5-HT_{2C}-receptor mRNA in LES derived from both sham-operated and GERD rats. In contrast, expression of 5-HT_{2A/2B}-receptor mRNA in LES of sham-operated and GERD rats showed a tendency to increase in the current study. Thus, we speculated that activations of 5-HT₂-receptor subtypes could not mainly mediate LES contraction, and the localized 5-HT_{2A/2B} receptor in the LES may play other roles in LES function.

The 5-HT₄ receptors are localized on cholinergic neurons in the gut, and prokinesia results from increased release of

Ach from excitatory neurons. In our present study, i.p. administration of cisapride to rats induced an increase in LES motility. The fact that adding the 5-HT₄ receptor antagonist inhibited the 5-HT-induced LES contraction suggests that the 5-HT₄ receptor is partially involved in the LES contraction response *via* an increased release of Ach. In addition, we have obtained preliminary results which show that adding mosapride to LES on day 10 after GERD induction could not induce LES contraction. However, since we have had several experimental problems that have prevented successful investigation of the effects of the 5-HT₄ receptor agonist on LES motility in GERD rats, further study will be necessary to determine if the 5-HT₄ receptor is a factor in decreased LES motility of GERD rats.

After 2 d of induction in the GERD model, contraction by 5-HT was significantly reduced in LES from GERD rats (Fig. 5A). In addition to 5-HT, contraction induced by Ach and SNP was reduced at day 10 after induction. At day 10 after GERD induction, erosion and thickening of the esophageal mucosa were observed in the middle to lower esophagus in rats.¹²⁾ It is likely that histological damage in LES may contribute to reduction in LES responses by the neurotransmitters. However, by microscopic observation in a preliminary study, we found that severe mucosal injury in the esophagus could not be observed until day 5 after induction in the GERD model, it seems likely that a reduction in 5-HT-induced LES contraction probably occurs before tissue damage is induced. In addition, we demonstrated that no difference in LES responsiveness 10 d after GERD induction was found by microscopic observation between rats with and without erosion (Ach: erosive LES, 1.11 ± 0.11 g and non-erosive LES, 1.30 ± 0.2 g, $p = 0.42$ by Student's *t*-test; 5-HT: erosive LES, 20.54 ± 0.10 g and non-erosive LES, 0.76 ± 0.17 g, $p = 0.3$ by Student's *t*-test, data not shown in Figures and Tables). Thus, this indicates that the reduction of LES responsiveness induced by neurotransmitters is not caused by tissue injury in GERD rats. However, we could not exclude the possibility

that the decreased LES function in GERD rats is mediated by ultra-microstructural tissue damage, because the intercellular epithelium space in the non-erosive esophagus up to 10 d after GERD induction has been observed by electron microscopy.^{13,14)} Therefore, it appears that the depression of LES reactivity by Ach and SNP at 10 d after GERD induction may be influenced by microstructural damage in LES.

To elucidate a possible mechanism for the decrease in the LES-contracted reaction by neurotransmitters, the receptor mRNA expression was evaluated. In GERD rats, the 5-HT₄-receptor and M3-mRNA expression in LES at day 2 after induction showed a decreasing trend, and at day 10 these gene expressions were significantly reduced. Since the 5-HT-induced contraction in LES of GERD rats was already decreased by day 2, our present data on the 5-HT₄-receptor gene expression and 5-HT-induced LES contraction in GERD rats are partially conflicting. Because M1-, M2-, and M4-receptor mRNA expression in LES from GERD rats at day 2 did not significantly decrease compared with sham-operated rats (data not shown in Figures and Tables), Ach-induced LES contraction in GERD rats may have been retained until day 2 after operation. Thus, the decrease in LES function induced by neurotransmitters may be partially mediated by the decrease in gene expression of the 5-HT₄. However, we did not evaluate the effect on neurotransmitter signaling parameters in LES of GERD rats. The 5-HT₄ receptor can be transactivated by G protein-coupling to Gs and elevate intracellular cyclic AMP, leading to activation of MAP kinase.¹⁵⁾ We could not exclude the possibility that reduction of the LES contraction in GERD rats may be not only due to the effects of expression of these receptors but also due to the decrease in signal factors in neurotransmitters downstream.

It has been demonstrated that administration of 5-HT₄ receptor agonists for GERD patients significantly decreased the frequency of transient LES relaxation and increased lower LES pressure.¹⁶⁾ However, whether 5-HT₄ receptor agonists can show effectiveness for esophageal damage in GERD is still controversial. To clarify the roles of the 5-HT₄ receptor on development of erosive injury in the esophagus of GERD rats, we investigated the effects of cisapride on ero-

sive injury in the esophagus of GERD rats at day 10 after induction. Cisapride i.p. administration attenuated the increased number of erosive sites. In addition, our preliminary results indicated that by an administration of GR-125487, the blockade of the 5-HT₄ receptor in GERD rats leads to a tendency to increase in the number of erosion sites in the esophagus (2.9 ± 0.7 vs. 4.0 ± 0.9 , $p = 0.32$ by Student's *t*-test). Thus, we suggest that 5-HT₄-receptor activation may protect from excessive gastric acid exposure in the esophagus *via* LES contraction.

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