MATERIALS AND METHODS

All experiments were performed in accordance with the protocols approved by the Animal Experiments Review Board of Tsumura (approved protocol No. 09-22, 10-047, 11-010).

Animals. Eight-week-old male Wistar rats were obtained from CLEA Japan (Tokyo, Japan) and maintained under stable conditions of temperature and humidity and a 12-h:12-h dark/light cycle (7 AM to 7 PM). Rats had access to food and water ad libitum and were housed in groups of four or five. All animal experiments were performed between 9 AM and 6 PM. To avoid the influence of diurnal variations, feeding was stopped 24 h before the experiment, and blood and tissue samples were collected between 1 PM and 4 PM. During intravenous administration through the tail vein, we held the conscious rat lightly with a rat holder and warmed the tail in hot water. The test substance was administered slowly using 26-gauge needles to cause less stress. This procedure was concluded within 1 min.

Chemicals. Rat ghrelin was obtained from the Peptide Institute (Osaka, Japan) and was dissolved in 0.9% sterilized physiological saline (Otsuka Pharmaceutical, Tokyo, Japan). Cisapride (an agonist of the 5-HT_4 receptor) was obtained from Sigma-Aldrich (St. Louis, MO) and was suspended in 0.1% carboxymethyl cellulose (Maruishi Pharmaceutical, Osaka, Japan). Rikkunshito (Tsumura, Tokyo, Japan) was made from a hot-water extract of a mixture of eight varieties of crude drugs: sojutsu (*Atractylodis lanceae rhizoma*), ninjin (*Ginseng radix*), hange (*Pinelliae tuber*), bukuryo (*Hoelen*), taiso (*Zizyphi fructus*), chinpi (*Aurantii nobilis pericarpium*), kanzo (*Glycyrrhizae radix*), and shokyo (*Zingiberis rhizoma*), which was then spray dried. Rikkunshito was suspended in distilled water (DW) at a concentration of 1.2% wt/vol and ingested ad libitum by GERD rats for 10 days (32). The approximate daily intake of rikkunshito was 1,000 mg/kg.

Preparation of the GERD model. GERD was surgically induced in rats according to the method of Omura et al. (36, 40). In brief, rats were first anesthetized with ether after 24 h of fasting, and then laparotomy was then performed at the midline. The stomach and duodenum were exposed extracorporeally, and a transitional (boundary) section from the forestomach to the glandular stomach was ligated using 1–0 silk thread (Natsume Seisakusho, Tokyo, Japan). The duodenal side of the pylorus was covered with a 2-mm-wide 18-Fr nelaton catheter (Terumo, Tokyo, Japan) that was sutured and fixed to the surface of the duodenal serous membrane using a 5–0 nylon yarn. The stomach and duodenum were returned into the abdominal cavity, which was then closed. Sham-operated rats were first laparotomized to expose their stomach and duodenum for about 1 min, after which their abdominal cavities were closed. After surgery, rats were fasted for a further 24 h (resulting in a total fasting of 48 h). Sham-operated and GERD rats were given food ad libitum from the day after surgery, and the pair-fed sham-operated rats were given the same amount of food as that consumed by GERD rats on the day before to examine the influence of nutritional decline.

Measurement of gastric emptying. This experiment was performed 10 days after GERD induction (10 AM to 3 PM). Gastric emptying was assessed according to a previously reported method (33). Sham-operated and GERD rats were orally administered 0.5 ml of the test meal prepared by mixing standard powdered chow (Oriental Yeast, Tokyo, Japan) and glass beads (0.2-mm diameter, BZ-02; AS ONE, Osaka, Japan) using Teflon tubes (internal diameter, 1.68 mm) connected to a 2.5-ml syringe with a 10-Fr nelaton catheter 24 h after fasting. The test meal was prepared by mixing 16 g of powdered chow, 20 g of glass beads, and 40 ml of DW. The glass beads were added to enhance the solidity of the meal. The rats were decapitated 2 h or immediately after administration of the test meal to measure gastric emptying. The stomach was removed after incising the abdomen along the median line and ligating the pylorus and cardia. The stomach contents were collected in a 50-ml tube with DW and then centrifuged. We measured the weight of the sediments after drying overnight at 45°C. Gastric emptying was calculated according to the following formula: gastric emptying (%) = (1 − A/B) × 100, where A represents the dry weight of the test meal recovered from the stomach 2 h after its administration and B represents the average value of the dry weight of the test meal recovered from the stomach immediately after its administration. Cisapride (20 mg/kg) or vehicle was administered orally 1 h before test meal administration to elucidate the effects of prokinetic agents in GERD rats.

To examine the effects of exogenous ghrelin on gastric emptying in sham-operated and GERD rats, rat ghrelin (3 or 10 nmol/rat) or saline was injected intravenously via the tail vein immediately after test meal administration and gastric emptying was measured 1 h later. In addition, GERD rats were administered DW containing rikkunshito (1,000 mg/kg) ad libitum for 10 days after GERD induction, whereas the control group was administered DW alone. Next, rats were intravenously administered 1 nmol/rat ghrelin or saline, and gastric emptying was measured 1 h later.

Measurement of food intake. Rats were housed separately after GERD-inducing surgery, and 24-h cumulative food intake was measured on *day 10*. To examine the effects of exogenous ghrelin on food intake of sham-operated and GERD rats, rat ghrelin (3 nmol/rat) or saline was injected intravenously via the tail vein and food intake was measured 1 h later. Food intake was calculated as the difference between the preprandial and postprandial weight of the food. The experiment was conducted between 10 AM and 12 PM under fed conditions 10 days after GERD induction.

Fixation of a strain gauge force transducer. After 24 h of fasting, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan). During GERD-inducing surgery, laparotomy was performed at the midline. Thereafter, a strain gauge force transducer (F-08IS; Star Medical, Tokyo, Japan) was sutured to the surface of the antral and duodenal serous membrane to measure circular muscle contractions. In addition, a catheter filled with heparin in physiological saline was fixed to the jugular vein. After surgery, rats were housed separately and fasted for a further 24 h (resulting in a total fasting of 48 h).

Analysis of gastroduodenal motility. After GERD induction, rikkunshito (1,000 mg/kg) was administered to rats ad libitum for 10 days. On *day 10* (9 AM to 3 PM), after 24 h of fasting, gastroduodenal motility was measured in conscious, freely moving rats. The control groups of sham-operated and GERD rats were given DW only. The transducer was connected to a preamplifier (FS-04M; Star Medical) via a bridge box (FB-01; Star Medical). Data were recorded using an MP150 (Biopac Systems, Aero Camino Goleta, CA) and analyzed using AcqKnowledge (Biopac Systems). To observe phase III-like contractions, gastroduodenal motility was monitored for 2–3 h.

To examine the effects of exogenous ghrelin on gastroduodenal motility in sham-operated and GERD rats, rat ghrelin (3 nmol/rat) or saline was administered through the jugular vein catheter. The effects of the drug were evaluated by frequency of phase III-like contractions and changes in MI before and after its administration.

Phase III-like contractions were defined as the existence of at least three potent contractions (>1/3 of maximum contraction magnitude) of short duration (<5 min). MI was defined as the mean of the area under the contractility recording curve per minute. The definition was slightly modified from a previous report (46).

Measurement of plasma ghrelin and GH levels. Ten days after GERD induction (1 PM to 4 PM), blood was taken after decapitation in a tube containing aprotinin (Wako Pure Chemical Industries, Osaka, Japan) and EDTA-2Na (Dojindo Laboratories, Kumamoto, Japan) to obtain plasma samples as reported previously (44). Blood samples were immediately centrifuged at 4°C. Plasma was acidified with 1 mol/l HCl (1/10 volume) and stored at −80°C until measurement. Plasma ghrelin levels were measured using the Active- and Desacyl-Ghrelin ELISA Kit (Mitsubishi Chemical Medience, Tokyo, Japan). Plasma GH levels were measured using the Rat/Mouse GH ELISA Kit (Millipore, Billerica, MA) using nonacidified plasma samples. To examine the effects of exogenous ghrelin on GH release

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in sham-operated and GERD rats, rat ghrelin (3 nmol/rat) or saline was injected intravenously via the tail vein, and plasma GH levels were measured at 0, 10, 30, and 60 min.

Immunohistochemical studies. The stomachs of 24-h-fasted rats were excised 10 days after GERD induction (1 PM to 4 PM). A part of the gastric body was fixed for 2 days in a 10% formalin/phosphoric acid buffer solution. A 1.5-cm section was excised along the proximal to distal stomach, paraffin embedded, and immunohistochemically stained. Methanol containing 3% hydrogen peroxide was used to inhibit endogenous peroxidase activity. After wash with PBS, paraffin sections were incubated with polyclonal anti-ghrelin antibody (Trans Genic, Kumamoto, Japan) diluted to 1:500 for 90 min. The sections were then washed with PBS and incubated with the Immunohistochemical Staining Kit (MAX-PO; Nichirei Biosciences, Tokyo, Japan) for 20 min, washed again with PBS, and then incubated with a 3,3′-diaminobenzidine solution. The total number of ghrelin-positive cells in this section of the gastric body was counted and expressed as the number of ghrelin-positive cells per millimeter of mucosa in longitudinal sections.

RNA extraction, reverse transcription, and qPCR analysis. Gastric body and hypothalamus were excised 10 days after GERD induction (1 PM to 4 PM) and placed in a tube on dry ice for freezing. Once completely frozen, tubes were stored at −80°C. The tissue was homogenized, and total RNA was extracted using the RNeasy Universal Tissue Kit (Qiagen, Valencia, CA). Total RNA from each sample was diluted to 100 ng/μl, allowed to react for 5 min at 70°C, and finally cooled on ice. An aliquot of 1 μg of total RNA was reverse transcribed using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. qPCR analysis was performed with the PRISM 7900HT Sequence Detection System (Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems). To compensate for the differences in the amount of total RNA added to each reaction, mRNA expression was normalized with β-actin as an endogenous control and expressed by the Δ threshold cycle (ΔC_t) value: $\Delta C_t = 2^{(-[A-B])}$, where A is the number of cycles that reached the β-actin gene threshold and B is the number of cycles that reached the target gene threshold. The set of oligonucleotide primers and fluorescent probes used in the TaqMan quantitative PCR were provide by Applied Biosystems: cytoplasmic β-actin: Rn00667869_m1; preproghrelin: Rn00572319_m1; GHS-R: Rn00821417_m1; neuropeptide Y (NPY): Rn00561681_m1; and agouti-related protein (AgRP): Rn01431703_g1.

Statistical analysis. Statistical significance was examined using the Student's *t*-test or Aspin-Welch *t*-test after the *F* test or by Dunnett's analysis after one-way ANOVA. Data are expressed as the means ± SE of each group, and $P < 0.05$ was considered to be significant.

RESULTS

Gastric emptying was delayed and food intake was decreased in GERD rats. The body weight of GERD rats after 24 h of fasting decreased significantly compared with that of sham-operated rats (sham-operated, 247.3 ± 2.0 vs. GERD, 202.4 ± 4.7 g; $P < 0.001$). Gastric emptying in vehicle-treated GERD rats was significantly delayed compared with that in vehicle-treated sham-operated rats 2 h after test meal administration (Fig. 1*A*). The prokinetic agent cisapride (20 mg/kg) significantly accelerated gastric emptying in sham-operated rats. In addition, cisapride improved the delayed gastric emptying in GERD rats. Twenty-four-hour cumulative food intake of GERD rats was significantly decreased compared with sham-operated rats (Fig. 1*B*).

Interdigestive antral motility was decreased in GERD rats. Contractions were observed in the antrum and duodenum at regular intervals in sham-operated and GERD rats (Fig. 2, *A* and *B*)

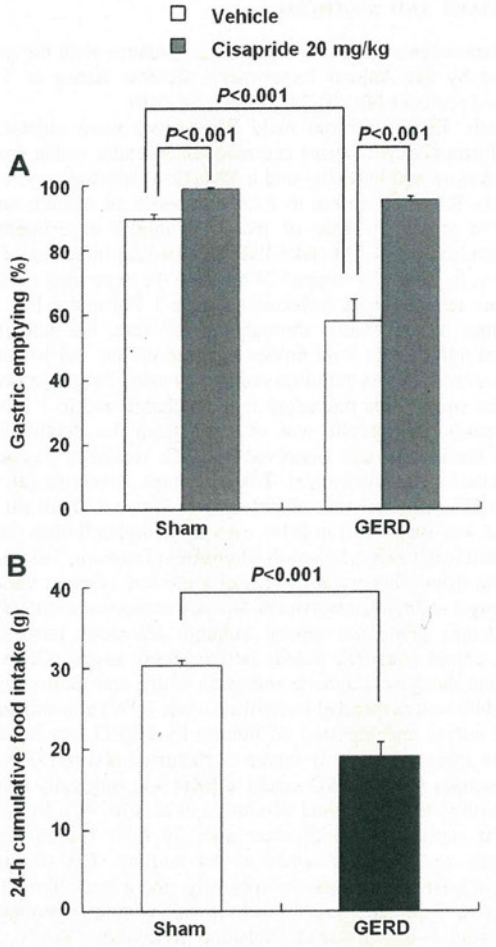

Fig. 1. Gastric emptying and food intake in sham-operated and gastroesophageal reflux disease(GERD) rats. *A*: gastric emptying measured 2 h after administration of 0.5-ml test meal. Cisapride (20 mg/kg) or vehicle was administered orally 1 h before test meal administration. *B*: 24-h cumulative food intake under free-feeding conditions. Results are expressed as means ± SE (n = 6–16).

although the frequency of phase III-like contractions in the antrum was significantly decreased in GERD rats compared with that in sham-operated rats (Fig. 2*C*). However, no difference in phase III-like contractions was observed in the duodenum between both groups (Fig. 2*D*).

Plasma ghrelin levels increased and the number of gastric ghrelin-positive cells decreased in GERD rats. Plasma acyl and desacyl ghrelin levels significantly increased in GERD rats compared with those in sham-operated rats (Fig. 3, *A* and *B*). We examined the influence of nutritional decline on plasma ghrelin levels. Although the body weight was approximately the same (P = 0.19) between GERD (202.4 ± 4.7 g) and pair-fed sham-operated rats (209.6 ± 2.1 g), plasma ghrelin levels in GERD rats (acyl ghrelin, 133.8 ± 9.0 fmol/ml; desacyl ghrelin, 709.0 ± 49.5 fmol/ml) increased significantly compared with those in pair-fed sham-operated rats (acyl ghrelin, 86.5 ± 7.7 fmol/ml; P = 0.001; desacyl ghrelin, 423.5 ± 40.7 fmol/ml, $P < 0.001$). No difference in plasma GH levels was observed (sham-operated, 2.4 ± 1.2 vs. GERD, 4.7 ± 2.8 ng/ml; P = 0.54). The number of ghrelin-positive

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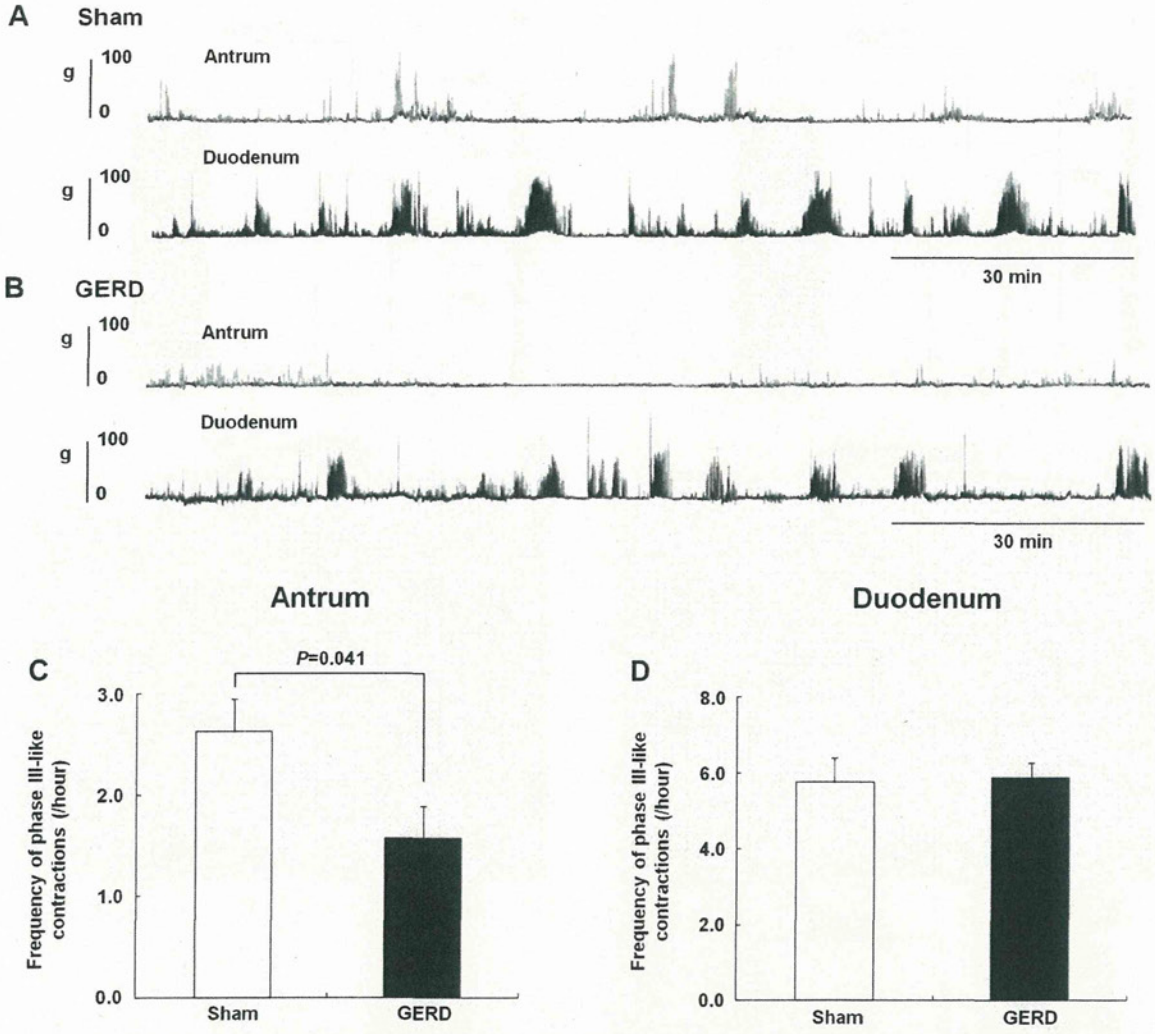

Fig. 2. Fasted gastroduodenal motility in sham-operated and GERD rats. *A* and *B*: antral and duodenal motor patterns detected by a strain gauge force transducer in sham-operated and GERD rats, respectively. *C* and *D*: frequency of phase III-like contractions in the antrum and duodenum. Results are expressed as means ± SE (n = 6–8).

cells in the gastric body in GERD rats significantly decreased compared with that in sham-operated rats (Fig. 3, *C–E*).

Expression of orexigenic genes in the hypothalamus increased in GERD rats. The expression of preproghrelin and GHS-R genes in the stomach of GERD rats did not differ from that of sham-operated rats (Fig. 4, *A* and *B*). In contrast, the expression of NPY and AgRP genes, which are known to be orexigenic in the hypothalamus, significantly increased in GERD rats compared with that in sham-operated rats (Fig. 4, *C* and *D*).

Ghrelin administration did not improve gastric emptying, food intake, and GH release in GERD rats. Ghrelin administration significantly accelerated gastric emptying in sham-operated rats 1 h after test meal administration in a dose-dependent manner (Fig. 5*A*). Gastric emptying in GERD rats 1 h after test meal administration showed a trend toward acceleration following administration of 3 or 10 nmol/rat ghrelin. Food intake in sham-operated rats after ghrelin administration (3 nmol/rat) was significantly higher than that after saline administration, but no significant difference was observed between GERD rats administered saline and ghrelin (3 nmol/rat) (Fig. 5*B*). In addition, plasma GH levels reached a peak 10 min after ghrelin administration (3 nmol/rat) in both sham-operated and GERD rats, but the peak level of GH in GERD rats was significantly lower than that in sham-operated rats (Fig. 5*C*).

Ghrelin administration did not affect gastroduodenal motility in GERD rats. Figure 6, *A* and *B*, shows gastroduodenal motility patterns in sham-operated and GERD rats administered ghrelin (3 nmol/rat). In sham-operated rats, the change in MI in both the antrum and duodenum significantly increased after ghrelin administration (Fig. 6, *C* and *D*). However, in GERD rats, the change in MI in the antrum and duodenum showed no difference after ghrelin and saline administration. In the antrum, the frequency of phase III-like contractions did not differ between sham-operated and GERD rats (Fig. 6*E*). In the duodenum, the frequency of phase III-like contractions significantly increased after ghrelin administration in sham-operated rats, but not in GERD rats (Fig. 6*F*).

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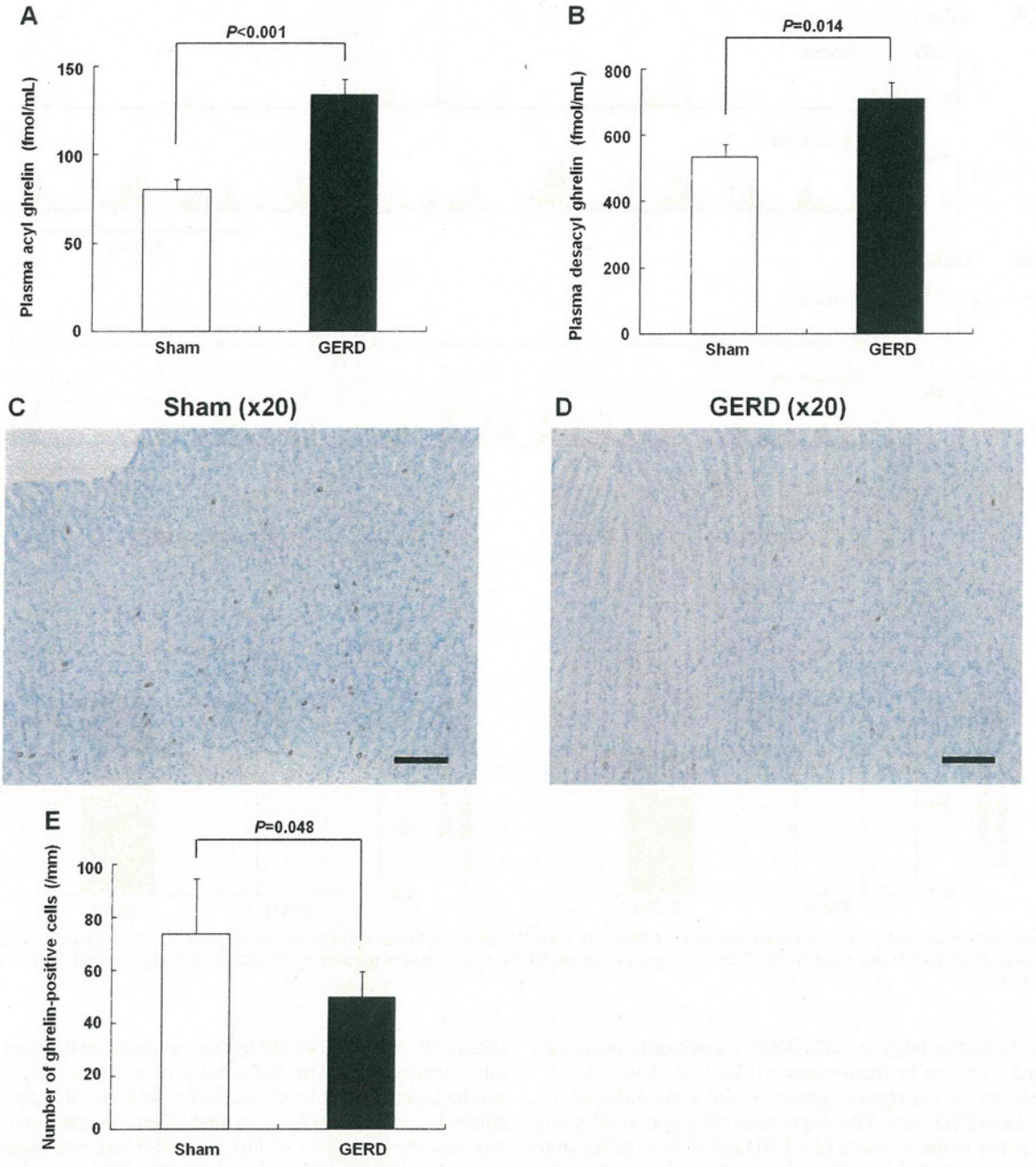

Fig. 3. Plasma ghrelin levels and number of gastric ghrelin-positive cells in sham-operated and GERD rats. *A* and *B*: plasma acyl and desacyl ghrelin levels. *C* and *D*: ghrelin-positive cells in the gastric body of sham-operated and GERD rats (scale bars = 100 μm). *E*: number of ghrelin-positive cells per millimeter of mucosa in longitudinal sections. Results are expressed as means ± SE (n = 5–8).

Rikkunshito administration restored impaired ghrelin response on gastroduodenal motility in GERD rats. Figure 7, *A* and *B* show gastroduodenal motility patterns in GERD rats administered DW or rikkunshito for 10 days after GERD induction and intravenous injection of ghrelin via the jugular vein catheter (3 nmol/rat). Before ghrelin administration, the frequency of phase III-like contractions in the antrum of GERD rats administered rikkunshito significantly increased compared with that in the antrum of GERD rats administered DW (DW administration, 1.6 ± 0.1 vs. rikkunshito administration, 2.1 ± 0.2 count/h; P = 0.035). There was no difference in the frequency in the duodenum between both groups (DW administration, 5.4 ± 0.5 vs. rikkunshito administration, 5.7 ± 1.0 count/h; P = 0.78). After ghrelin administration, there was no change in MI in the antrum and duodenum of GERD rats administered DW. However, a significant increase in the change in MI in the antrum (Fig. 7*C*) and an increasing trend in the same in the duodenum of GERD rats administered

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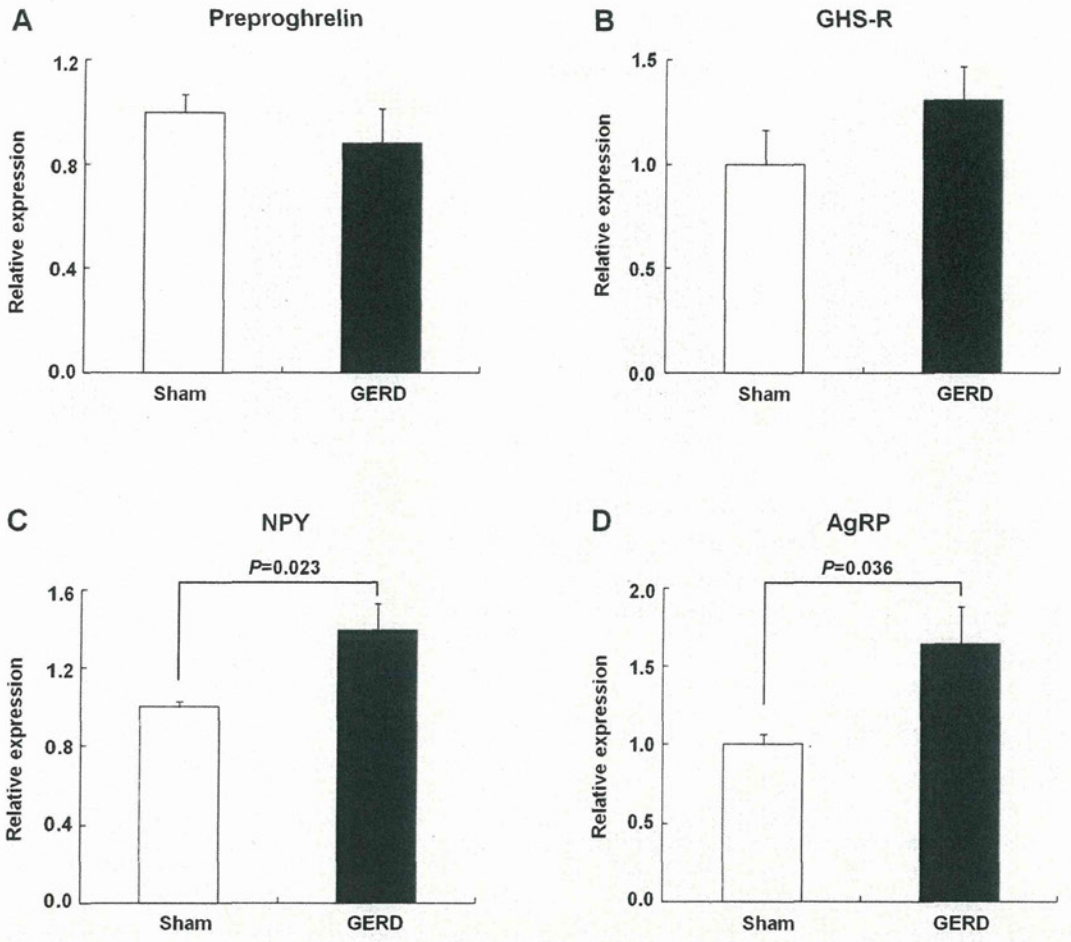

Fig. 4. Gastric and hypothalamic gene expression in sham-operated and GERD rats. *A* and *B*: expression of gastric preproghrelin and growth hormone secretagogue receptor (GHS-R) mRNA. *C* and *D*: expression of hypothalamic neuropeptide Y (NPY) and agouti-related protein (AgRP) mRNA. Results are expressed as means ± SE (n = 4–8).

rikkunshito (P = 0.074 vs. saline administration; Fig. 7*D*). Similarly, there was a significant increase in the frequency of phase III-like contractions in the antrum (Fig. 7*E*) and an increasing trend in the same in the duodenum of GERD rats administered rikkunshito (P = 0.074 vs. saline administration; Fig. 7*F*).

The food intake of GERD rats administered rikkunshito did not differ from that of GERD rats administered DW (9 days after GERD induction: DW administration, 19.9 ± 1.2 vs. rikkunshito administration, 17.9 ± 0.9 g/day; P = 0.24). Ten days after GERD induction, the effect of ghrelin administration on gastric emptying in GERD rats administered rikkunshito was examined. In GERD rats administered DW, ghrelin administration had no effect on gastric emptying (Fig. 7*G*). However, ghrelin administration significantly increased gastric emptying compared with saline administration in GERD rats administered rikkunshito.

DISCUSSION

In this study, we focused on the association between peripheral ghrelin and GERD. We demonstrated that *1*) GERD rats showed decreased gastric emptying, food intake, and antral motility and increased peripheral ghrelin levels and *2*) ghrelin administration to GERD rats did not improve decreased gastric emptying, food intake, and antral motility, whereas rikkunshito administration improved decreased antral motility.

Although decreased gastric emptying has already been reported in patients with GERD (3, 31), its mechanism remains unclear. However, treatment with prokinetic agents has been shown to improve several symptoms in patients with GERD (16, 39), and we have recently demonstrated that cisapride improves the motility proximal to the lower esophageal sphincter in GERD rats (40). These findings suggest that gastrointestinal dysmotility is involved in GERD progression. In the present study, cisapride administration had a significant inhibitory effect on delayed gastric emptying in GERD rats, suggesting that motility in the upper gastrointestinal tract in such rats is regulated by 5-HT_4 signaling. At present, no study on changes in antral motility of GERD rats has been reported. We found that antral motility significantly decreased in GERD rats; however, no difference was observed in duodenal motility. These results suggest that decreased antral motility is one of the causes of delayed gastric emptying in GERD rats.

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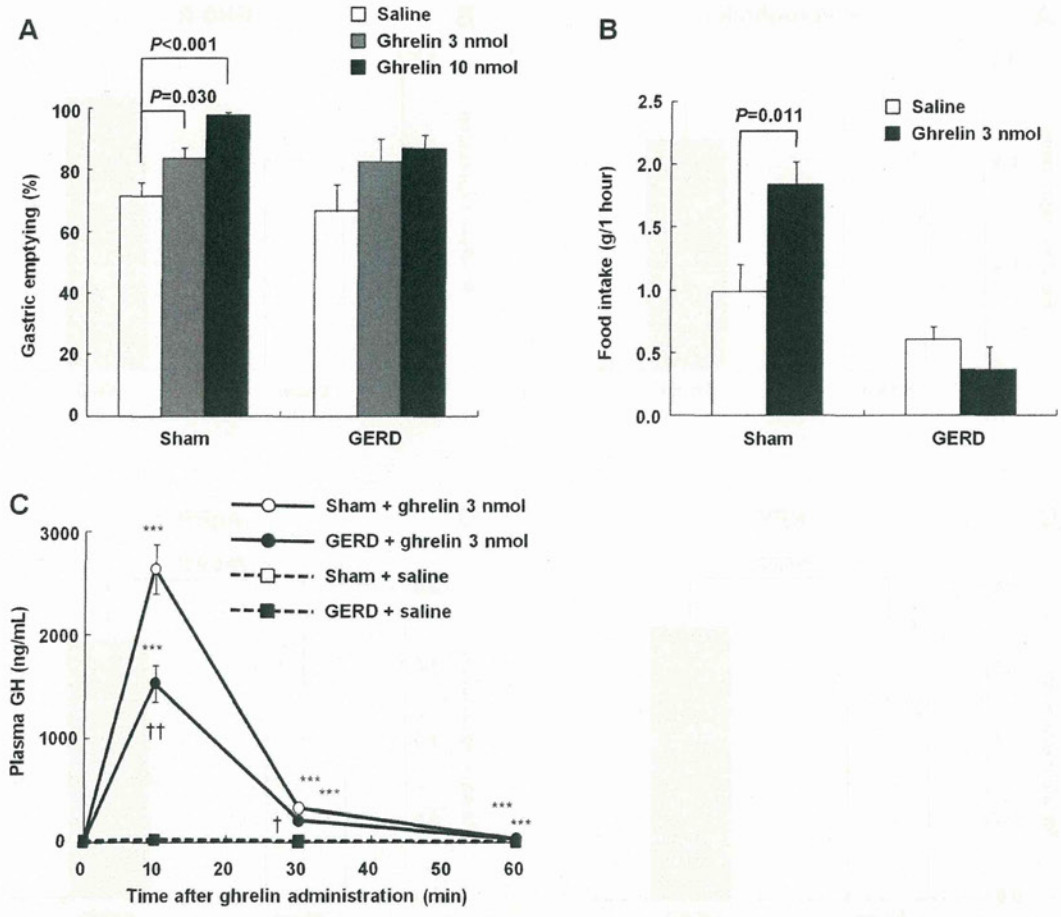

Fig. 5. The effects of exogenous ghrelin on gastric emptying, food intake, and GH release in sham-operated and GERD rats. *A*: effects of exogenous ghrelin administration (3 or 10 nmol/rat, intravenously) on gastric emptying 1 h after test meal administration. *B*: effects of exogenous ghrelin administration (3 nmol/rat, intravenously) on food intake under free-feeding conditions. *C*: effects of exogenous ghrelin administration (3 nmol/rat, intravenously) on GH release. Results are expressed as means ± SE (n = 5–8). ***$P < 0.001$ vs. each group of rats administered saline. †,††$P < 0.05$, 0.01 vs. sham-operated rats administered ghrelin.

Interdigestive contractions in the antrum are regulated in part by peripheral acyl ghrelin (13). Increased circulating acyl ghrelin levels have been shown to induce phase III-like contractions in the antrum. In the present study, plasma acyl and desacyl ghrelin levels significantly increased in GERD rats; however, the number of ghrelin-positive cells in the stomach significantly decreased. Moreover, the expression of the preproghrelin gene in the stomach of GERD rats was not affected. These results suggest that higher plasma ghrelin levels observed in GERD rats is not due to an increase in the synthesis of ghrelin in the stomach but due to enhanced secretion of ghrelin into the circulation from the stomach, resulting in a decrease in stored ghrelin in X/A-like cells, which is consistent with our observation that the number of ghrelin-positive cells decreased in GERD rats.

In rats, serum ghrelin levels increased with fasting and decreased with refeeding and administration of glucose (48). Furthermore, serum or plasma ghrelin levels are inversely proportional to body mass index in humans (19, 35). In this study, the body weight of GERD rats significantly decreased compared with that of sham-operated rats. Therefore, it was suggested that nutritional status may have influenced plasma ghrelin levels. However, the pair-fed sham-operated rats that were controlled in order that they would consume the same amount of food as that consumed by GERD rats showed plasma ghrelin levels similar to those in sham-operated rats. It has been reported that plasma ghrelin levels in rats with food intake restricted by 25% and 50% that were measured 15 days after the initiation of restriction did not differ from rats with ad libitum food intake (27). Therefore, it has been suggested that the increased plasma ghrelin levels in GERD rats may have not resulted solely from reduced food intake.

In the present study, although plasma ghrelin levels increased in GERD rats, decreased phase III-like contractions were observed in the antrum due of delayed gastric emptying and decreased food intake. Ghrelin administration increased gastric emptying, food intake, and the frequency of phase III-like contractions in the duodenum in sham-operated rats but did not improve delayed gastric emptying and decreased food intake in GERD rats. In this study, sham-operated and GERD rats were exogenously administered 3 nmol/rat ghrelin. Gastric emptying in the vehicle group of GERD rats varied significantly. The number of

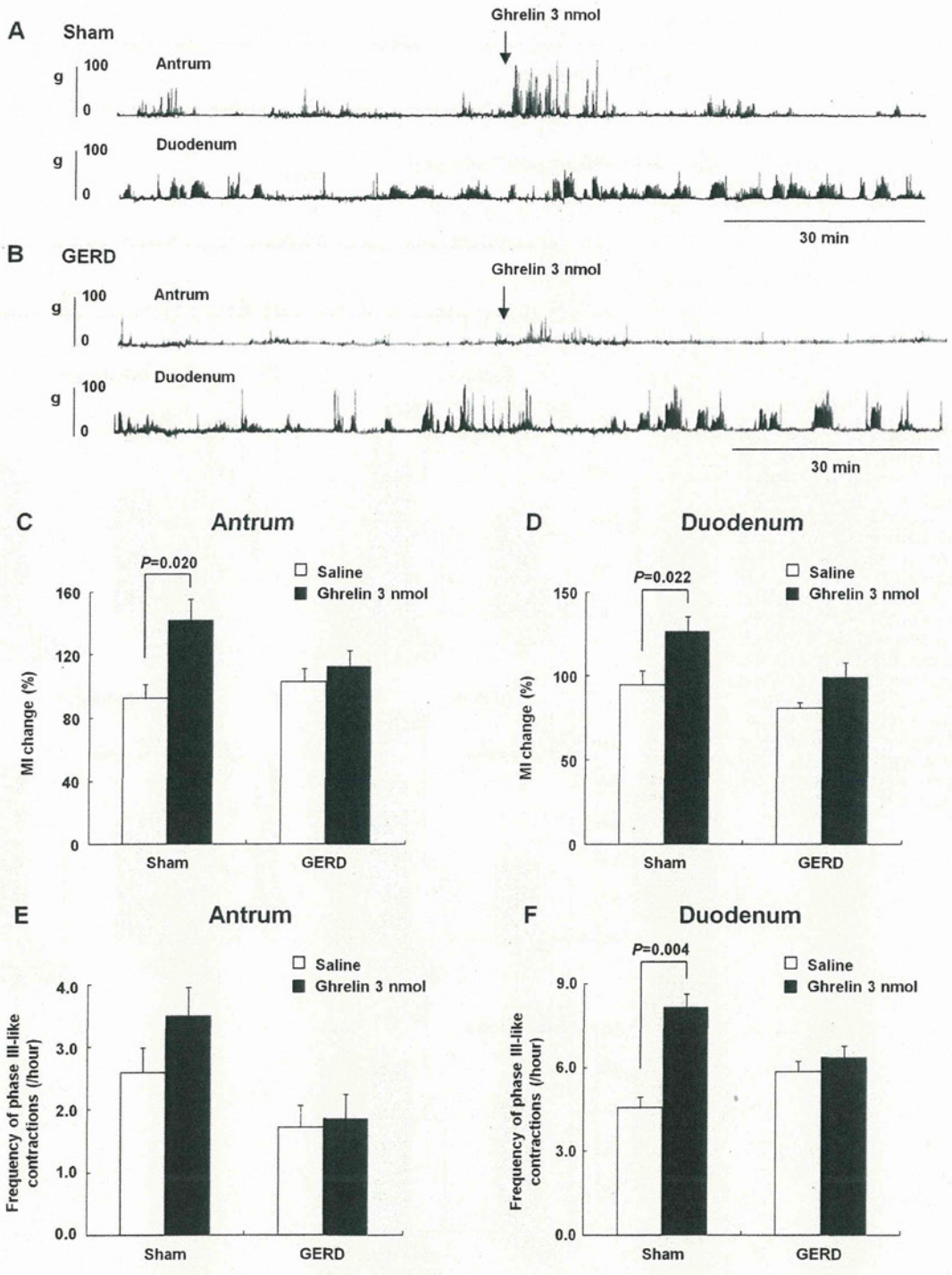

Fig. 6. Effects of exogenous ghrelin on fasted gastroduodenal motility in sham-operated and GERD rats. *A* and *B*: antral and duodenal motor patterns detected by a strain gauge force transducer in sham-operated and GERD rats. *C* and *D*: effects of exogenous ghrelin administration (3 nmol/rat, intravenously) on the change in motility index (MI) in the antrum and duodenum. *E* and *F*: effects of exogenous ghrelin administration (3 nmol/rat, intravenously) on the frequency of phase III-like contractions in the antrum and duodenum. Results are expressed as means ± SE (n = 5–10).

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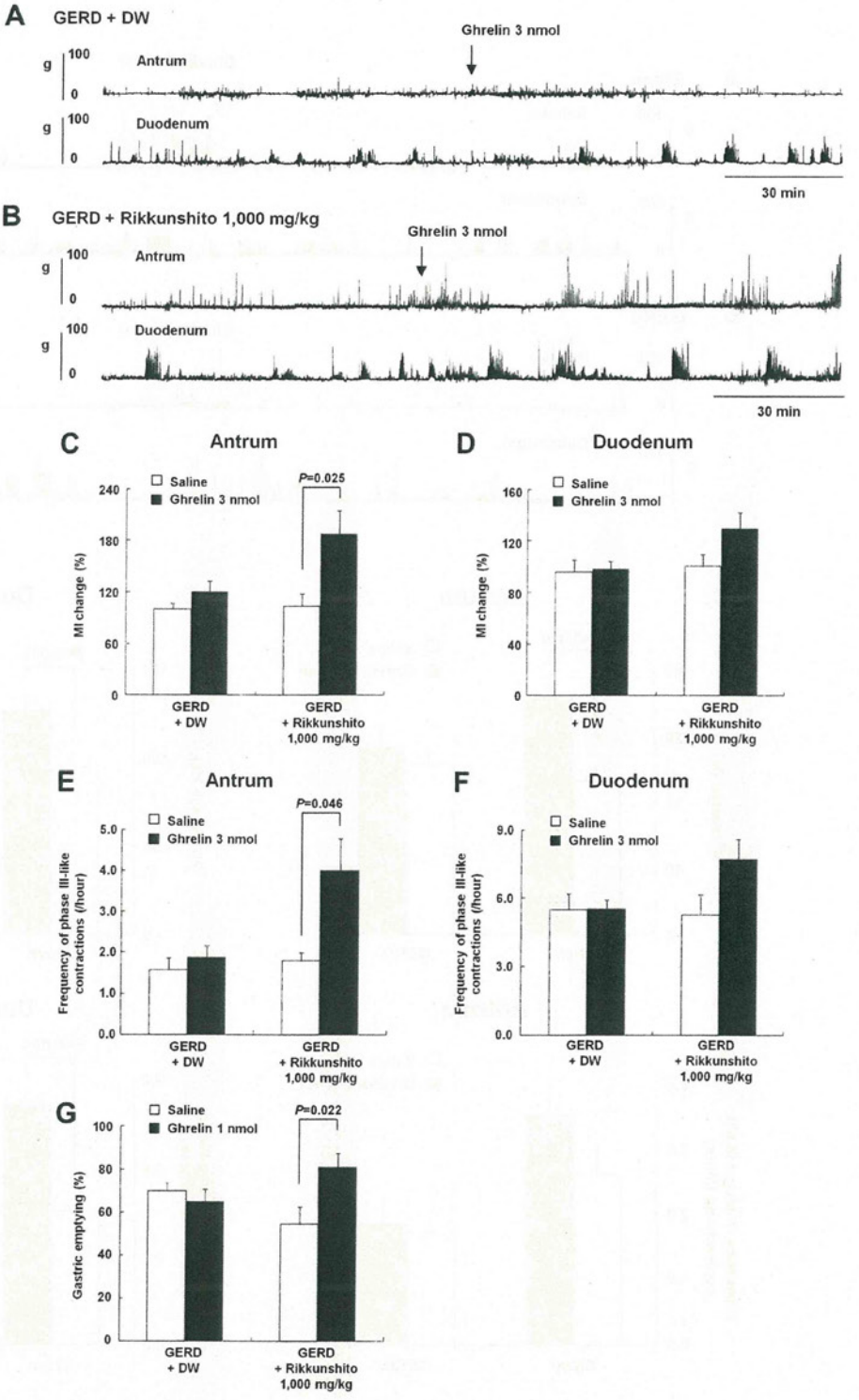

Fig. 7. The effects of exogenous ghrelin on fasted gastroduodenal motility in GERD rats administered distilled water (DW) or rikkunshito (1,000 mg/kg) for 10 days. *A* and *B*: antral and duodenal motor patterns detected by a strain gauge force transducer in GERD rats administered DW or rikkunshito. *C* and *D*: combined effects of exogenous ghrelin administration (3 nmol/rat, intravenously) and rikkunshito on the change in MI in the antrum and duodenum. *E* and *F*: combined effects of exogenous ghrelin administration (3 nmol/rat, intravenously) and rikkunshito on the frequency of phase III-like contractions in the antrum and duodenum. *G*: combined effects of exogenous ghrelin administration (1 nmol/rat, intravenously) and rikkunshito on gastric emptying. Results are expressed as means ± SE (n = 5–12).

animals in each group was seven. Variations in the pathological conditions may have had an effect on gastric emptying. Although GERD rats were administered a higher dosage of ghrelin than sham-operated rats when converted into kilogram of body weigh (sham-operated, 12.1 nmol/kg; GERD, 14.8 nmol/kg), no significant improvements were observed in gastric emptying. In addition, although the frequency of phase III-like contractions in the duodenum of GERD rats was similar to that in the duodenum of sham-operated rats, ghrelin administration did not increase their

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frequency in the duodenum. These results suggest that ghrelin response is attenuated in GERD rats.

The expression of genes that encode the orexigenic hypothalamic neuropeptides NPY and AgRP significantly increased in GERD rats than that in sham-operated rats, whereas that of GHS-R in the stomach remained unchanged. Peripheral ghrelin is known to transmit signals by binding to receptors on the vagus nerve that are axonally transported to the gastric mucosa and activate the NPY/AgRP neurons of the arcuate nucleus of the hypothalamus, and thus increase feeding behavior (1, 34) and gastrointestinal motility (12, 20). However, upregulation of these orexigenic neuropeptides did not increase appetite in GERD rats, suggesting that their downstream orexigenic signaling was impaired. NPY and AgRP synthesis or release in the hypothalamus may have been involved, as demonstrated by Scarlett et al. (41). In rat models of colitis and obstructive cholestasis, decreased feeding response to orexigenic peptides was observed even though NPY release was normal (2, 38). These reports indicate that the orexigenic neuronal pathway may have been impaired or that anorexigenic factors, such as interleukin-1β, may have been involved, but further investigations are needed.

In the present study, GH release was attenuated after ghrelin administration in GERD rats. In ghrelin transgenic mice, which exhibit constant high peripheral ghrelin levels, and in patients with anorexia nervosa, who exhibit high plasma ghrelin levels, ghrelin had a decreased effect on GH release (5, 50). The precise mechanism of the decreased response to ghrelin caused by hyperghrelinemia is unknown. Recently, it has been reported that stress responses inhibit the effects of ghrelin (6). We measured plasma corticosterone levels as an indicator of stress (data not shown) and found no difference between sham-operated and GERD rats. Therefore, ghrelin function may have been attenuated in GERD rats not because of stress hormones (e.g., corticotropin-releasing factor, urocortin). Although the attenuation of ghrelin function needs to be studied further, we suggest that it may be due to the desensitization of ghrelin receptors.

In GERD rats administered rikkunshito for 10 days, a significant increase in MI and frequency of phase III-like contractions in the antrum was observed compared with that in GERD rats administered DW. Rikkunshito has been shown to increase esophageal clearance in patients with GERD and improve their symptoms (22). It also significantly mitigates decreased voluntary movement, which is an index of pain, in GERD rats (32). In an in vitro study using GHS-R1a-overexpressing cells or NPY neurons, rikkunshito was reported to increase [I^{125}]ghrelin binding and elevate the influx of Ca^{2+} continuously after ghrelin addition (14). From these findings, it appears that rikkunshito may improve endogenous ghrelin signal transduction in GERD rats.

In this study, rikkunshito treatment enhanced the decreased response to ghrelin in MI and frequency of phase III-like contractions in the antrum of GERD rats. Furthermore, although rikkunshito alone had no effect on decreased food intake and gastric emptying, delayed gastric emptying in GERD rats was significantly improved with the combined administration of rikkunshito and ghrelin (1 nmol/rat). These results suggest that rikkunshito may improve defective signaling of ghrelin and suppress the delay in gastric emptying.

It is known that ghrelin transmits signals to the afferent vagus nerve and passes through the central nervous system to induce gastrointestinal contractions through the efferent vagus nerve. In GERD rats, we speculate that the vagus nerves are impaired and may lead to decreased ghrelin response. Therefore, to determine whether the decreased response to ghrelin in GERD rats is due to decreased functionality of the ghrelin receptors, we investigated the effects of concomitant administration of rikkunshito and ghrelin on the antral and duodenal motility and gastric emptying. Ghrelin receptors are G protein-coupled receptors and mediate Ca^{2+} influx through the phospholipase C/protein kinase C/inositol triphosphate pathway (18) and adenylate cyclase/cyclic AMP/protein kinase A pathway (25). In AgRP/NPY neurons in the arcuate nucleus, ghrelin signaling is blocked by the action of phosphodiesterase type III and phosphatidylinositol 3-kinase, which decrease cyclic AMP (24). Rikkunshito contains heptamethoxyflavone and nobiletin, which have been shown to inhibit phosphodiesterase type III activity in vitro (43). From those findings, it was speculated that the increase in the frequency of spontaneous phase III-like contraction in the antrum and ghrelin response in GERD rats administered rikkunshito was due to the stimulation of ghrelin signaling by protein kinase A because of the prevention of cyclic AMP decomposition by rikkunshito, which inhibits phosphodiesterase type III activity. Therefore, it is possible that factors that decrease cyclic AMP may increase in the vagal nerve endings with ghrelin receptors in our GERD model.

In conclusion, impaired ghrelin signaling is involved in gastrointestinal dysmotility in GERD rats. Moreover, rikkunshito improves gastrointestinal motility by enhancing the decreased response to ghrelin. Improvement of ghrelin signaling may provide a new therapeutic approach for GERD.

GRANTS

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: M.N., S.M., S.O., K.N., C.S., Y.S., and T.H. performed experiments; M.N., S.M., S.O., K.N., C.S., Y.S., and T.H. analyzed data; M.N., S.M., S.O., K.N., C.S., Y.S., and T.H. interpreted results of experiments; M.N. prepared figures; M.N., S.O., C.S., Y.S., and T.H. drafted manuscript; M.N., S.M., N.O., S.O., K.N., C.S., Y.S., T.H., M.A., and H.T. approved final version of manuscript; N.O., M.A., and H.T. conception and design of research; H.T. edited and revised manuscript.

REFERENCES

1. **Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M.** Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology* 120: 337–345, 2001.
2. **Ballinger AB, Williams G, Corder R, El-Haj T, Farthing MJ.** Role of hypothalamic neuropeptide Y and orexigenic peptides in anorexia associated with experimental colitis in the rat. *Clin Sci (Lond)* 100: 221–229, 2001.
3. **Benini L, Sembenini C, Castellani G, Caliari S, Fioretta A, Vantini I.** Gastric emptying and dyspeptic symptoms in patients with gastroesophageal reflux. *Am J Gastroenterol* 91: 1351–1354, 1996.

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4. **Boeckxstaens GE.** Review article: the pathophysiology of gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 26: 149–160, 2007.
5. **Broglio F, Gianotti L, Destefanis S, Fassino S, Abbate Daga G, Mondelli V, Lanfranco F, Gottero C, Gauna C, Hofland L, Van der Lely AJ, Ghigo E.** The endocrine response to acute ghrelin administration is blunted in patients with anorexia nervosa, a ghrelin hypersecretory state. *Clin Endocrinol (Oxf)* 60: 592–599, 2004.
6. **Currie PJ, Coiro CD, Duenas R, Guss JL, Mirza A, Tal N.** Urocortin I inhibits the effects of ghrelin and neuropeptide Y on feeding and energy substrate utilization. *Brain Res* 1385: 127–134, 2011.
7. **Dass NB, Munonyara M, Bassil AK, Hervieu GJ, Osbourne S, Corcoran S, Morgan M, Sanger GJ.** Growth hormone secretagogue receptors in rat and human gastrointestinal tract and the effects of ghrelin. *Neuroscience* 120: 443–453, 2003.
8. **Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M.** Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* 141: 4255–4261, 2000.
9. **Date Y, Murakami N, Toshinai K, Matsukura S, Niijima A, Matsuo H, Kangawa K, Nakazato M.** The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology* 123: 1120–1128, 2002.
10. **Emerenziani S, Sifrim D.** Gastroesophageal reflux and gastric emptying, revisited. *Curr Gastroenterol Rep* 7: 190–195, 2005.
11. **Fujimiya M, Asakawa A, Ataka K, Kato I, Inui A.** Different effects of ghrelin, des-acyl ghrelin and obestatin on gastroduodenal motility in conscious rats. *World J Gastroenterol* 14: 6318–6326, 2008.
12. **Fujino K, Inui A, Asakawa A, Kihara N, Fujimura M, Fujimiya M.** Ghrelin induces fasted motor activity of the gastrointestinal tract in conscious fed rats. *J Physiol* 550: 227–240, 2003.
13. **Fujitsuka N, Asakawa A, Hayashi M, Sameshima M, Amitani H, Kojima S, Fujimiya M, Inui A.** Selective serotonin reuptake inhibitors modify physiological gastrointestinal motor activities via 5-HT2c receptor and acyl ghrelin. *Biol Psychiatry* 65: 748–759, 2009.
14. **Fujitsuka N, Asakawa A, Uezono Y, Minami K, Yamaguchi T, Niijima A, Yada T, Maejima Y, Sedbazar U, Sakai T, Hattori T, Kase Y, Inui A.** Potentiation of ghrelin signaling attenuates cancer anorexia-cachexia and prolongs survival. *Transl Psychiatry* 1: e23, 2011.
15. **Fukuda H, Mizuta Y, Isomoto H, Takeshima F, Ohnita K, Ohba K, Omagari K, Taniyama K, Kohno S.** Ghrelin enhances gastric motility through direct stimulation of intrinsic neural pathways and capsaicin-sensitive afferent neurones in rats. *Scand J Gastroenterol* 39: 1209–1214, 2004.
16. **Gardner JD, Rodriguez-Stanley S, Robinson M, Miner PB Jr.** Cisapride inhibits meal-stimulated gastric acid secretion and postprandial gastric acidity in subjects with gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 16: 1819–1829, 2002.
17. **Hattori T, Fujitsuka N, Asakawa A, Inui A.** A new strategy using Rikkunshito (Liu-Jun-Zi-Tang), a Japanese traditional medicine, to treat gastrointestinal disease. In: *Basics of Evidences-Based Herbal Medicine*, edited by Satoh H. Kerala: Research Signpost, 2010, p. 149–160.
18. **Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LH.** A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 273: 974–977, 1996.
19. **Ingelsson E, Larson MG, Yin X, Wang TJ, Meigs JB, Lipinska I, Benjamin EJ, Keaney JF Jr, Vasan RS.** Circulating ghrelin, leptin, and soluble leptin receptor concentrations and cardiometabolic risk factors in a community-based sample. *J Clin Endocrinol Metab* 93: 3149–3157, 2008.
20. **Inui A, Asakawa A, Bowers CY, Mantovani G, Laviano A, Meguid MM, Fujimiya M.** Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. *FASEB J* 18: 439–456, 2004.
21. **Johnson DA, Levy BH 3rd.** Evolving drugs in gastroesophageal reflux disease: pharmacologic treatment beyond proton pump inhibitors. *Expert Opin Pharmacother* 11: 1541–1548, 2010.
22. **Kawahara H, Kubota A, Hasegawa T, Okuyama H, Ueno T, Ida S, Fukuzawa M.** Effects of rikkunshito on the clinical symptoms and esophageal acid exposure in children with symptomatic gastroesophageal reflux. *Pediatr Surg Int* 23: 1001–1005, 2007.
23. **Kawahara H, Mitani Y, Nomura M, Nose K, Yoneda A, Hasegawa T, Kubota A, Fukuzawa M.** Impact of rikkunshito, an herbal medicine, on delayed gastric emptying in profoundly handicapped patients. *Pediatr Surg Int* 25: 987–990, 2009.
24. **Kohno D, Nakata M, Maekawa F, Fujiwara K, Maejima Y, Kuramochi M, Shimazaki T, Okano H, Onaka T, Yada T.** Leptin suppresses ghrelin-induced activation of neuropeptide Y neurons in the arcuate nucleus via phosphatidylinositol 3-kinase- and phosphodiesterase 3-mediated pathway. *Endocrinology* 148: 2251–2263, 2007.
25. **Kohno D, Sone H, Minokoshi Y, Yada T.** Ghrelin raises [Ca2+]i via AMPK in hypothalamic arcuate nucleus NPY neurons. *Biochem Biophys Res Commun* 366: 388–392, 2008.
26. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K.** Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 402: 656–660, 1999.
27. **Ling PR, Bistrian BR.** Comparison of the effects of food versus protein restriction on selected nutritional and inflammatory markers in rats. *Metabolism* 58: 835–842, 2009.
28. **Liu YL, Malik NM, Sanger GJ, Andrews PL.** Ghrelin alleviates cancer chemotherapy-associated dyspepsia in rodents. *Cancer Chemother Pharmacol* 58: 326–333, 2006.
29. **Maddern GJ, Chatterton BE, Collins PJ, Horowitz M, Shearman DJ, Jamieson GG.** Solid and liquid gastric emptying in patients with gastro-oesophageal reflux. *Br J Surg* 72: 344–347, 1985.
30. **Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, Kangawa K.** Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun* 276: 905–908, 2000.
31. **McCallum RW, Berkowitz DM, Lerner E.** Gastric emptying in patients with gastroesophageal reflux. *Gastroenterology* 80: 285–291, 1981.
32. **Miwa H, Koseki J, Oshima T, Kondo T, Tomita T, Watari J, Matsumoto T, Hattori T, Kubota K, Iizuka S.** Rikkunshito, a traditional Japanese medicine, may relieve abdominal symptoms in rats with experimental esophagitis by improving the barrier function of epithelial cells in esophageal mucosa. *J Gastroenterol* 45: 478–487, 2010.
33. **Mogami S, Suzuki H, Fukuhara S, Matsuzaki J, Kangawa K, Hibi T.** Reduced ghrelin production induced anorexia after rat gastric ischemia and reperfusion. *Am J Physiol Gastrointest Liver Physiol* 302: G359–G364, 2012.
34. **Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S.** A role for ghrelin in the central regulation of feeding. *Nature* 409: 194–198, 2001.
35. **Nanjo Y, Adachi H, Hirai Y, Enomoto M, Fukami A, Otsuka M, Yoshikawa K, Yokoi K, Ogata K, Tsukagawa E, Kasahara A, Murayama K, Yasukawa H, Kojima M, Imaizumi T.** Factors associated with plasma ghrelin level in Japanese general population. *Clin Endocrinol (Oxf)* 74: 453–458, 2011.
36. **Omura N, Kashiwagi H, Chen G, Suzuki Y, Yano F, Aoki T.** Establishment of surgically induced chronic acid reflux esophagitis in rats. *Scand J Gastroenterol* 34: 948–953, 1999.
37. **Qiu WC, Wang ZG, Wang WG, Yan J, Zheng Q.** Gastric motor effects of ghrelin and growth hormone releasing peptide 6 in diabetic mice with gastroparesis. *World J Gastroenterol* 14: 1419–1424, 2008.
38. **Rioux KP, Le T, Swain MG.** Decreased orexigenic response to neuropeptide Y in rats with obstructive cholestasis. *Am J Physiol Gastrointest Liver Physiol* 280: G449–G456, 2001.
39. **Ruth M, Hamelin B, Rohss K, Lundell L.** The effect of mosapride, a novel prokinetic, on acid reflux variables in patients with gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 12: 35–40, 1998.
40. **Saegusa Y, Takeda H, Muto S, Oridate N, Nakagawa K, Sadakane C, Nahata M, Harada Y, Iizuka M, Hattori T, Asaka M.** 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. *Biol Pharm Bull* 34: 704–711, 2011.
41. **Scarlett JM, Zhu X, Enriori PJ, Bowe DD, Batra AK, Levasseur PR, Grant WF, Meguid MM, Cowley MA, Marks DL.** Regulation of agouti-related protein messenger ribonucleic acid transcription and peptide secretion by acute and chronic inflammation. *Endocrinology* 149: 4837–4845, 2008.
42. **Tack J, Depoortere I, Bisschops R, Delporte C, Coulie B, Meulemans A, Janssens J, Peeters T.** Influence of ghrelin on interdigestive gastrointestinal motility in humans. *Gut* 55: 327–333, 2006.

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43. **Takeda H, Muto S, Hattori T, Sadakane C, Tsuchiya K, Katsurada T, Ohkawara T, Oridate N, Asaka M.** Rikkunshito ameliorates the aging-associated decrease in ghrelin receptor reactivity via phosphodiesterase III inhibition. *Endocrinology* 151: 244–252, 2010.
44. **Takeda H, Sadakane C, Hattori T, Katsurada T, Ohkawara T, Nagai K, Asaka M.** Rikkunshito, an herbal medicine, suppresses cisplatin-induced anorexia in rats via 5-HT2 receptor antagonism. *Gastroenterology* 134: 2004–2013, 2008.
45. **Taniguchi H, Ariga H, Zheng J, Ludwig K, Takahashi T.** Effects of ghrelin on interdigestive contractions of the rat gastrointestinal tract. *World J Gastroenterol* 14: 6299–6302, 2008.
46. **Tatewaki M, Harris M, Uemura K, Ueno T, Hoshino E, Shiotani A, Pappas TN, Takahashi T.** Dual effects of acupuncture on gastric motility in conscious rats. *Am J Physiol Regul Integr Comp Physiol* 285: R862–R872, 2003.
47. **Tatsuta M, Iishi H.** Effect of treatment with liu-jun-zi-tang (TJ-43) on gastric emptying and gastrointestinal symptoms in dyspeptic patients. *Aliment Pharmacol Ther* 7: 459–462, 1993.
48. **Tschop M, Smiley DL, Heiman ML.** Ghrelin induces adiposity in rodents. *Nature* 407: 908–913, 2000.
49. **Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R.** The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. *Am J Gastroenterol* 101: 1900–1920; quiz 1943, 2006.
50. **Wei W, Qi X, Reed J, Ceci J, Wang HQ, Wang G, Englander EW, Greeley GH Jr.** Effect of chronic hyperghrelinemia on ingestive action of ghrelin. *Am J Physiol Regul Integr Comp Physiol* 290: R803–R808, 2006.
51. **Yagi M, Homma S, Kubota M, Iinuma Y, Kanada S, Kinoshita Y, Ohtaki M, Yamazaki S, Murata H.** The herbal medicine Rikkunshi-to stimulates and coordinates the gastric myoelectric activity in post-operative dyspeptic children after gastrointestinal surgery. *Pediatr Surg Int* 19: 760–765, 2004.
52. **Zheng J, Ariga H, Taniguchi H, Ludwig K, Takahashi T.** Ghrelin regulates gastric phase III-like contractions in freely moving conscious mice. *Neurogastroenterol Motil* 21: 78–84, 2009.

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Rikkunshito and Ghrelin Secretion

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Abstract: Rikkunshito is a kampo herbal medicine which is widely used in Japan for the treatment of the upper gastrointestinal symptoms of patients with functional dyspepsia (FD), gastroesophageal reflux disease (GERD), dyspeptic symptoms of postgastrointestinal surgery patients, and chemotherapy-induced dyspepsia in cancer patients. Recently, very unique characteristics of rikkunshito have been unveiled; oral administration of rikkunshito potentiates orexigenic action of ghrelin through several different mechanisms. In addition, several lines of evidence obtained from both animal and human studies indicate that rikkunshito can be an attractive and promising therapeutic option for the anorectic conditions including cisplatin-induced dyspepsia, anorexia of aging, stress-induced hypophagia, cancer cachexia-anorexia syndrome. In this review, we will highlight what is known about the orexigenic effect of rikkunshito with a special focus on an interaction with ghrelin signaling system.

Keywords: Rikkunshito, ghrelin, GHSR1a, serotonin 2B-receptor, serotonin 2C-receptor, phosphodiesterase 3, carboxylesterase, butyrylcholinesterase.

1. INTRODUCTION

Rikkunshito is a kampo herbal medicine which is prepared by compounding eight herbal medicines: *Atractylodis Lanceae Rhizoma, Ginseng Radix, Pinelliae Tuber, Hoelen, Zizyphi Fructus, Aurantii Nobilis Pericarpium, Glycyrrhizae Radix* and *Zingiberis Rhizoma* [1,2]. Rikkunshitois widely used in Japan for the treatment of the upper gastrointestinal symptoms of patients with functional dyspepsia (FD)[3-5] and gastroesophageal reflux disease (GERD)[6-8], chemotherapy-induced dyspepsia in cancer patients [9,10], and dyspeptic symptoms of postgastrointestinal surgery patients [11] (Table 1).

Recently, it was shown that oral administration of rikkunshito stimulates secretion of the orexigenic peptide, ghrelin, in rodents and human. [12-14]. More recent evidence suggests that rikkunshito enhances ghrelin's orexigenic effect by several additional mechanisms [15-17]. In this review, we will discuss the currently available literature on the orexigenic effect of rikkunshito with a special attention to an interaction with ghrelin signaling system.

2. CLINICAL APPLICATION OF RIKKUNSHITO

Rikkunshito is used to treat various gastrointestinal tract disorders, such as FD, GERD, dyspeptic symptoms of postgastrointestinal surgery, and chemotherapy-induced nausea.

2.1. Functional Dyspepsia

In a double-blinded, randomized, placebo-controlled trial of rikkunshito, gastric emptying and gastrointestinal symptoms were evaluated in 42 patients with FD [3]. Subjects were randomized to receive either oral treatment with 2.5 g rikkunshito three times daily or placebo. Gastric emptying was measured by the acetaminophen absorption method. After 7 days of treatment, gastric emptying was significantly accelerated and gastrointestinal symptoms were significantly reduced in patients treated with rikkunshito, indicating that rikkunshito has a prokinetic action on gastric emptying and may be useful in treating FD. Recently, Arai *et al.* [4] conducted a randomized controlled study to compare the effects of rikkunshito and domperidone on upper gastrointestinal symptoms as well as plasma acylated ghrelin levels in patients with FD. Although the changes in dyspeptic symptoms scores were comparative in both groups, elevation of plasma acylated ghrelin level was noted only in rikkunshito-treated group. Of particular interest, the improvement of dyspeptic symptoms by rikkunshito was correlated with an increase of plasma ghrelin level.

2.2. Gastroesophageal Reflux Disease

In recent years, rikkunshito has been used to treat symptoms of gastroesophageal reflux disease (GERD), and reports of its efficacy have been published [6,7]. In children with symptomatic GERD, rikkunshito relieved symptoms and reduced distal esophageal acid exposure through improved esophageal acid clearance. Although rikkunshito did not change the number of acid reflux events, it reduced the esophageal acid clearance time. The mechanism underlying the improved esophageal clearance capacity with rikkunshito remains unknown.

Recently, Tominaga *et al.* [8] compared the efficacies of rikkunshito combined with rabeprazole (RPZ) and a double dose of RPZ in a prospective randomized multicenter trial in PPI-refractory GERD patients. One hundred and four patients with GERD symptoms remaining after 4-week treatment with RPZ were enrolled. Four-week treatment with rikkunshito combined with RPZ significantly decreased FSSG (the frequency scale for the symptoms of GERD) score similar to that seen on treatment with a double dose of RPZ. In the subgroup analysis, the improvement rate of male non-erosive GERD patients in the rikkunshito group was significantly greater than that of such patients in the other group. From these results, it was concluded that rikkunshito combined with standard-dose RPZ therapy may be a useful new strategy for PPI refractory GERD patients.

2.3. Cancer Patients Treated with Chemotherapy

It was reported that rikkunshito administered in combination with granisetron, an anti-emetic drug, was useful in alleviating the anorexia and vomiting occurring as adverse reactions to anticancer drugs in patients with advanced breast cancer [9].

Very recently, Ohno *et al.* [10] conducted a randomized crossover study to investigate the effects of rikkunshito on ghrelin secretion and on cisplatin-induced anorexia in humans. Ten unresectable or relapsed gastric cancer patients receiving combined chemotherapy with S-1 plus cisplatin were analyzed. The primary endpoint was the amount of oral intake, and the categories of scales of ano-

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Table 1. Therapeutic Potential of Rikkunshito for Various Diseases Associated with Anorexia

	References
Human studies	
Functional dyspepsia (7.5 g/day, P.O.)	[3], [4], [5]
Gastroesophageal reflux disease (7.5 g/day, P.O.)	[6], [7], [8]
Chemotherapy-induced anorexia (7.5 g/day, P.O.)	[9], [10], [16]
Postoperative dyspepsia (7.5 g/day, P.O.)	[11], [21]
Anorexia of aged patients (7.5 g/day, P.O.)	[22]
Depression (7.5 g/day, P.O.)	[23]
SSRI-induced dyspepsia (7.5 g/day, P.O.)	[24]
Animal studies	
Cisplatin-induced anorexia (rats, 500, 1000mg/Kg, P.O.)	[13], [15], [72]
Anorexia of aging (mice, 1000mg/Kg, P.O.)	[102]
Novelty stress (mice, 250, 500mg/Kg, P.O.)	[139]
Cancer anorexia–cachexia syndrome (rats, 1000mg/Kg, P.O.)	[16]
SSRI-induced dyspepsia (rats, 250, 1000mg/Kg, P.O.)	[164]

rexia, nausea, and vomiting; secondary endpoints included the plasma concentration of acyl-ghrelin. In the rikkunshito-on period, no decrease of the plasma concentration of acyl-ghrelin induced by cisplatin was observed. The average oral intake in the rikkunshito-on period was significantly larger than that in the rikkunshito-off period, and the grade of anorexia was significantly lower in the rikkunshito-on period than in the Rikkunshito-off period. These results suggest that rikkunshito prevented the chemotherapy-related anorexia by sustaining the blood level of acyl-ghrelin.

In a recent study, Fujituska *et al.* [16] reported that the median survival of pancreatic cancer patients treated with gemcitabine was prolonged by the addition of rikkunshito, particularly for those with ascites. This suggests that rikkunshito may be useful in clinical practice for cachectic cancer patients.

2.4. Postoperative Dyspepsia

Yagi *et al.* [11] evaluated the effect of rikkunshito on symptoms and gastric myoelectric activity in dyspeptic pediatric patients whose symptoms persisted for over 1 year after gastrointestinal surgery. With the administration of rikkunshito, all patients exhibited symptomatic relief and a significant decrease in mean symptom scores that were sustained over a 1-month period. The coordinating and stimulating effect of rikkunshito on the gastric myoelectric activity therefore seems to play an important role in the reduction of dyspeptic symptoms.

Pylorus preserving gastrectomy (PPG) is now being applied to early gastric cancer to avoid the dumping syndrome and to reduce bile reflux and maintain normal mucosal integrity of the remnant stomach [18]. The patients with PPG had been reported to have better QOL than those with distal gastrectomy because of a decrease in dumping related symptoms. However, some patients still suffer from dyspeptic symptoms such as epigastric fullness, nausea, and vomiting due to delayed gastric emptying [19]. Although prokinetic agents including erythromycin, cisapride, metoclopramide, and domperidone, theoretically accelerate gastric emptying and alleviate symptoms after PPG [20], these drugs lack supporting evidence of positive effect. Takahashi *et al.* [21] examined the clinical effects of rikkunshito on patients who were to undergo PPG. The results indicated that rikkunshito accelerated emptying of solid meals from the remnant stomach and decreased postoperative stasis-related symptoms. Thus, rikkunshito may improve the postoperative QOL of patients undergoing PPG.

2.5. Anorexia of Aged Patients

Utumi *et al.* [22] carried out oral administration of rikkunshito to elderly dementia patients with appetite loss, and examined its effects on food intake. Food intake, weight, total protein, albumin and potassium in plasma were examined before and after the administration of rikkunshito for 4 weeks. Food intake was significantly improved after the administration of rikkunshito in the six elderly dementia patients. Other parameters, such as body weight and albumin in plasma, did not change significantly during the examination, although they slightly increased in some patients.

Profoundly handicapped patients with delayed gastric emptying.

2.6. Depression

Rikkunshito, a gastrointestinal function regulatory kampo medicine, has recently been evaluated for its clinical usefulness in stress and depression [23]. This medicine has modulatory effects on the hypothalamo-pituitary-adrenal (HPA) axis and autonomic nervous function. It regulates adrenocorticotropic hormone (ACTH) and cortisol levels in plasma to normal ranges in FD [23]. Some abnormalities of gastrointestinal function are presumed to result from changes in hormone levels. Moreover, the herbal components of rikkunshito, *P. tuber* and *Zingiberis rhizome*, have modulatory effects on human plasma adrenocorticotropic hormone (ACTH) and cortisol levels with continual stress exposure [23].

2.7. Selective Serotonin Reuptake Inhibitor-induced Dyspepsia

Upper gastrointestinal symptoms such as nausea and vomiting are common adverse events associated with selective serotonin reuptake inhibitors (SSRIs), and may result in discontinuation of drug therapy in patients with depressive disorders. Oka *et al.* [24] conducted a randomized controlled study to determine if rikkun-

shito reduces gastrointestinal symptoms in depressed patients treated with fluvoxamine. Fifty patients with depressive disorder were randomly assigned for the treatment with fluvoxamine alone or fluvoxamine in combination with rikkunshito for eight weeks. The number of patients who complained of gastrointestinal adverse events was significantly lower in the fluvoxamine plus rikkunshito group than that in the control group. This suggests that rikkunshito reduces fluvoxamine-induced adverse events, especially nausea, and improves quality of life (QOL) of the patients.

3. MECHANISM OF ACTION OF RIKKUNSHITO

In addition to the enhancement of ghrelin secretion, there are several other mechanisms responsible for the effects of rikkunshito on gastrointestinal function (Table 2). Rikkunshito was reported to promote gastric emptying in both animal and human studies [3,5,25-27]. Hesperidin and L-arginine were identified as two of the active ingredients contributing to facilitate gastric emptying [25]. Rikkunshito stimulates gastric myoelectric activity [11], inhibits stress-induced decreases in gastric accommodation in humans [28], and promotes gastric adaptive relaxation in isolated guinea pig stomachs [29]. Direct effects of rikkunshito on gastrointestinal smooth muscle cells were also reported [30-32]. Rikkunshito was reported to inhibit gastric mucosal damage caused by absolute ethanol [33], to protect small intestinal cells by inducing HSP-60 [34], and to improve the barrier function of esophageal mucosa [35]. Moreover, rikkunshito reportedly enhances insulin secretion and reduces the plasma levels of free fatty acids after taking solid meals [36]. Given that there is an intimate relation between ghrelin and insulin secretion [37], further studies are warranted to examine the effect of rikkunshito on glucose and/or lipid metabolism in more detail.

4. GHRELIN AS AN OREXIGENIC HORMONE

Ghrelin is a peripherally active orexigenic gut hormone. In 1999, ghrelin was identified in gastric extracts as an endogenous ligand of the orphan growth hormone secretagogue receptor type 1a (GHS-R1a) [38-40]. Several studies have provided evidence that ghrelin is involved in the hypothalamic regulation of energy homeostasis by increasing food intake and reducing fat utilization [41-43]. Plasma levels of ghrelin rise during fasting, and fall upon eating, which has led to the suggestion that ghrelin is a meal-initiating hormone [44]. Plasma levels of ghrelin are inversely correlated with body weight in humans and rise after weight loss [45]. Besides to the regulation of energy homeostasis, ghrelin mediates an increase in gastric motility, induces a positive inotropic effect on the heart and causes vasodilatation [46,47].

Ghrelin is consisting of 28 amino acids, and the third N-terminal amino-acid serine (Ser) residue is octanoylated. This modification is essential for interacting with the GHS-R receptor, which is important for molecular recognition [48]. Furthermore, this n-octanoyl group may confer hydrophobicity upon the N terminus of ghrelin allowing it to enter the brain barrier to target the hypothalamus/pituitary system [49]. Recently, two different research groups identified the enzyme responsible for catalyzing the addition of the octanoyl group to ghrelin. The Ghrelin O-Acyltransferase (GOAT) is one of 16 members of the family of membrane-bound O-acyl transferases (MBOAT) that specifically octanoylates Ser of the ghrelin peptide [50,51].

Ghrelin receptor, or GHS-R, is a typical G-protein coupled receptor with seven transmembrane domains (7-TM) and coupled to a Gq/11α subunit. [52]. Ghrelin acts on the GHS-R and activates phospholipase C to generate inositol trisphosphate (IP3) and diacylglycerol, resulting in an increase of intracellular Ca^{2+}. Recent experiments in GHS-R1a knockout mice showed that GHS-R1a is essential for ghrelin-mediated stimulation of growth hormone (GH) secretion and orexigenic effects [53]. It has been demonstrated that GHS-R1a is also expressed in other central nervous system (CNS) areas and peripheral tissues, where ghrelin is also expressed, suggesting that ghrelin possesses many others effects besides the release of GH and the stimulation of food intake. In fact, numerous

Table 2. Putative Mechanisms for Orexigenic Action of Rikkunshito

	References
Human studies	
Accelerate gastric emptying	[3], [5], [27]
Promote gastric myoelectric activity	[7]
Inhibit stress-induced decreases in gastric accommodation	[28]
Elevate plasma level of acylated ghrelin	[4], [10], [12]
Animal studies	
Accelerate gastric emptying	[25], [26]
Promote gastric adaptive relaxation	[29]
Relax gastrointestinal smooth muscle	[30], [31]
Elevate plasma level of acylated ghrelin	[12], [13], [17]
Enhance hypothalamic ghrelin secretion	[72]
Increase hypothalamic GHS-R1a gene expression	[15]
Antagonize 5-HT2B and 5-HT2C receptors	[13]
Inhibit PDE3	[102]
Inhibit CES, BuChE	[17]
Enhance GHSR1a signaling	[16]

studies have shown that ghrelin also affects glucose homeostasis, gastrointestinal, cardiovascular, pulmonary and immune function, cell proliferation and differentiation [39,40,47,54].

About two-thirds of circulating ghrelin are produced by X/A-like cells in the oxyntic mucosa of the stomach, while most of the remainder originates in X/A-like cells of the small intestine [55]. Lower amounts of ghrelin are also produced in other organs, such as pancreas, kidney, placenta, lymphatic tissue, gonads, thyroid, adrenal, heart, lung, eye, pituitary and hypothalamus, and in various neoplastic tissues and cancer-cell lines [39.40,47,56]. In the central nervous system, ghrelin and its receptor are mainly expressed in the hypothalamus as well as in many other areas [57,58]. Peripheral ghrelin binds to its specific growth hormone secretagogue receptor (GHS-R) at the end of the vagus nerve around the stomach. The ghrelin signal is transmitted to the nucleus tractus solitarius (NTS) via the vagus nerve and stimulates neuropeptide Y (NPY)/agouti-related protein (AGRP) neurons of the arcuate nucleus (ARC) via the noradrenaline nerve, which increases appetite [42, 59].

Multiple studies have shown that intravenous infusion of ghrelin in humans increases food intake [60-62], thus supporting its relevance in food intake behaviour. In addition, several studies reported an increase in the palatability of the food after receiving ghrelin infusion [61,63]. Infusion of ghrelin into subjects with appetite loss due to cancer [64] increased energy intake by 31% at a subsequent buffet meal. These observations have led to the suggestion that ghrelin or ghrelin receptor agonists might become a useful human therapeutic for those disorders.

5. CISPLATIN-INDUCED ANOREXIA

5.1. Cisplatin-induced Anorexia and Ghrelin

Patients with cancer being treated with cytotoxic drugs such as cisplatin often experience a number of undesirable side-effects which include acute and delayed nausea, vomiting, anorexia, dyspepsia, and disrupted gastrointestinal function [65,66]. While several antiemetics including serotonin (5-HT) 3–receptor antagonist and neurokinin-1 (NK-1) receptor antagonists have been introduced for treating gastrointestinal symptoms associated with cisplatin use [65,66], these symptoms continue to be major contributors to the reduced patients' quality of life.

Recent evidence has demonstrated the relationship between chemotherapy-induced gastrointestinal disorders and ghrelin in both clinical [10,67-69] and animal studies [13,15,70-72]. Shimizu *et al.* [67] found that an increase in plasma ghrelin concentrations in patients with a reduction of food intake after the start of anti-cancer chemotherapy, while there was no change in the plasma concentrations of ghrelin in patients without a reduction of food intake. On the other hand, recent study conducted by Ohno *et al.* [10] showed that the plasma concentration of acylated ghrelin was decreased in patients with gastric cancer receiving combined chemotherapy with S-1 plus cisplatin. Similar results were obtained in a study where patients with esophageal cancer were treated with cisplatin-based neoadjuvant chemotherapy [68]. Plasma total ghrelin significantly decreased at days 3 and 8 of chemotherapy, and the adverse events including neutropenia and anorexia were associated with low plasma ghrelin levels [68]. More recently, the same study group conducted a prospective, randomized trial to evaluate the effects of exogenous ghrelin during cisplatin-based chemotherapy for patients with esophageal cancer, showing that food intake and appetite scores were significantly higher in the ghrelin-treated group than in the placebo group [69].

In animal studies, we and others reported that circulating ghrelin concentrations are reduced in cisplatin-treated rats until 6 h during the early stage of anorexia [13,72]. However, in another study, it was reported that the plasma level of acylated ghrelin returned to a normal level 24h after a single administration of cisplatin, although the decrease in food intake lasted more than 48 h [15,72]. In line with this, Malik *et al.* [73] reported an increase in the plasma level of acylated but not des-acylated ghrelin in rats treated with cisplatin. They suggested that an increase in circulating ghrelin in cisplatin-treated rats may be an adaptive response to protect them against a toxic challenges to the gut.

Intraperitoneal injection of 5-HT decreased the 24-hour food intake and the plasma acylated-ghrelin level in a dose-dependent manner [13]. This result suggests that the cisplatin-induced reduction in the plasma level of acylated-ghrelin may be mediated via a release of 5-HT from the gastrointestinal tract mucosa triggered by cisplatin. Indeed, a 5-HT2B–receptor agonist BW723C86, and a 5-HT2C agonist m-chlorophenylpiperazine HCl (mCPP) markedly decreased plasma acylated-ghrelin levels and increased intragastric ghrelin content suggesting that 5-HT2B/2C–receptor stimulation inhibits the release of gastric ghrelin into the circulation [13]. In contrast, 5-HT3 and 5-HT4 agonists had no effect on ghrelin dynamics. 5-HT2B and 5-HT2C antagonists suppressed the cisplatin-induced decrease of plasma acylated-ghrelin level and food intake [13]. These results strongly imply that activation of 5-HT2B and 5-HT2C–receptors, but not 5-HT3 and 5-HT4 receptors, play an important role in the decrease in plasma ghrelin level in cisplatin-induced anorexia. Of note, granisetron used in this study clearly inhibited delayed gastric emptying after cisplatin treatment, but it failed to improve cisplatin-induced anorexia [13,72]. The results of our study also suggested that cisplatin-induced emesis and anorexia may develop by different mechanisms, where 5-HT3 receptor may be involved in cisplatin-induced emesis, whereas 5-HT2B/2C receptors may be involved in anorexia. Interestingly, administration of 5-HT2B or 5-HT2C receptor antagonists had no effect on plasma acyl- or desacyl-ghrelin levels in normal rats (our unpublished observation), suggesting that the regulation of ghrelin secretion under normal conditions is unaffected by 5-HT2B or 5-HT2C activation.

The 5-HT2B receptors mainly are distributed peripherally [74], while the expression of 5-HT2C receptors is restricted to the central nervous system [75]. When the connection between the peripheral and central regions was dissociated by vagotomy, intraperitoneal injection of cisplatin still caused a significant reduction in the plasma acylated-ghrelin level suggesting that the control of the plasma acylated-ghrelin level may occur within the peripheral tissues in the rats administered cisplatin [13].

Peripheral administration of exogenous ghrelin ameliorates anorexia [13,70] and vomiting [71] induced by cisplatin. Administration of exogenous ghrelin has been shown to have the potential to reduce each of these symptoms in relevant animal models treated with cisplatin as an exemplar cytotoxic agent: emesis in the ferret [71]; anorexia in the rat and mouse [70]; delayed gastric emptying in the mouse [70]. Studies in cancer patients with impaired appetites have shown that ghrelin administration can increase energy intake and meal appreciation of a buffet meal [76].

Yakabi *et al.* [72] examined the changes of hypothalamic ghrelin secretion in cisplatin-treated rats to elucidate the mechanism underlying chemotherapy-induced delayed anorexia. Although ghrelin secretion in the hypothalamus did not decrease within 24 h of cisplatin administration, it started to decline significantly after 24 h and continued to decrease at least until 48 h, while their plasma ghrelin levels were comparable [13,72].

Given that 5-HT concentrations in the hypothalamus increases in cisplatin-treated rats [77], and that 5-HT2C receptor is reportedly expressed in proopiomelanocortin neurons in the hypothalamus [78] which is the major site of its anorexigenic action, it should be reasonable to assume that the activation of 5-HT2C receptor is involved in cisplatin-induced anorexia. Yakabi *et al.* [72] showed that hypothalamic 5-HT2C receptor gene expression in cisplatin-treated rats increased significantly, whereas that of 5-HT2A, 5-HT2B, and 5-HT3 receptors did not. Administration of mCPP, a 5-HT2C receptor agonist, inhibited hypothalamic ghrelin secretion. Intracere-

broventricular (i.c.v.)-administered 5-HT2C antagonist SB242084 prevented a decrease in secretion of hypothalamic ghrelin in cisplatin-treated rats, but granisetron, a 5-HT3 antagonist, or WIN51708 hydrate, a nonpeptide NK-1 antagonist, did not [72]. These results indicate that the reduced ghrelin secretion in the hypothalamus secondary to 5-HT2C receptor activation may be involved in cisplatin-induced anorexia.

In another study, Yakabi *et al.* [15] demonstrated that hypothalamic GHS-R1a gene expression was significantly reduced after cisplatin or mCPP treatment and this change was reversed by the treatment with 5-HT2C receptor antagonist, SB242084, but not with 5-HT3 receptor antagonists. 5-HT2C receptor antagonist also suppressed cisplatin-induced delayed anorexia. I.c.v. injection of GHS-R1a antagonist to saline or cisplatin-treated rats significantly reduced food intake compared with those injected with saline alone, and this inhibitory effect was abolished by the coadministration of 5-HT2C receptor antagonist. From these results, it was suggested that delayed-onset anorexia induced by cisplatin may be partly mediated by the activation of the hypothalamic 5-HT2C receptor and the resultant suppression of hypothalamic GHS-R1a gene expression as well as decreased ghrelin secretion in the hypothalamus.

5.2. Effect of Rikkunshito on Cisplatin-induced Anorexia

Rikkunshito ameliorated the decrease in circulating ghrelin concentration and this effect was abolished by coadministration of a GHS-R1a antagonist, [D-Lys^3]-GHRP-6 [13]. This finding suggests that the mechanism of improvement of anorexia by rikkunshito may involve ghrelin receptor activation. Moreover, Yakabi *et al.* [72] found that rikkunshito reversed the decrease in hypothalamic ghrelin secretion and the decrease in GHS-R1a gene expression 24h after cisplatin treatment. I.c.v. injection of the GHS-R1a antagonist impedes the rikkunshito-mediated improvement in cisplatin-induced anorexia [72]. Hence, it seems likely that rikkunshito ameliorates cisplatin-induced anorexia by restoring ghrelin secretion and GHS-R1a expression in the hypothalamus. Collectively, rikkunshito suppressed cisplatin-induced anorexia by improving ghrelin signal transduction system by both the peripheral and the central mechanisms.

The induction of ghrelin secretion by rikkunshito is supposed to be based on the 5-HT2B/2C–receptor antagonism owing to multiple active ingredients. We screened 33 compounds contained in rikkunshito and found that 13 out of 33 compounds showed antagonistic activity against binding to any of 5-HT 1A, 1B/D, 2A, 2B, 2C, 3, 4, 6, and 7 receptors [13]. Among them, heptamethoxyflavone, nobiletin, and tangeretin contained in *Aurantii nobilis pericarpium* had potent 5-HT2B–receptor antagonistic activity. The inhibitory activity of hesperidin against the 5-HT2B receptor was weak, but the concentration of hesperidin in rikkunshito is the highest among the ingredients tested. In addition, isoliquiritigenin, which is an ingredient of *Glycyrrhizae radix,* had the most potent activity against the 5-HT2C receptor binding. Our study indicated that the administration of heptamethoxyflavone, isoliquiritigenin, and hesperidin attenuated the decrease in plasma ghrelin level, while tangeretin, nobiletin, and 8-shogaol did not. This suggested that the ingredients that inhibit 5-HT2B /5-HT2C–receptor binding are likely to be effective *in vivo.*

6. ANOREXIA OF AGING

6.1. Anorexia of Aging and Ghrelin

In the elderly subjects, the reduction in energy intake often exceeds energy expenditure resulting in weight loss and protein energy malnutrition [79-83]. Protein energy malnutrition in the elderly is a frequent and clinically important problem, which leads to increased morbidity, mortality, disability and health costs in this growing population. One of the most important causes of the reduction in energy intake is anorexia [80,82]. The causes of the anorexia of aging have not yet been fully defined, they are probably multifactorial and include sensory impairment, social isolation, psychological and physiologic factors, in addition to the presence of disease [79-83]. To date, effective therapy for anorexia in older people has not been established.

Food intake is regulated by numerous factors of both central and peripheral origin which interact to determine a feeding response. In animal studies, levels of NPY and AGRP in the hypothalamus are altered with aging [84-87], while no change or decrease in hypothalamic proopiomelanocortin (POMC) and a decrease in cocaine- and amphetamine regulated transcript (CART) with aging have been observed [86,88-90].

Although many peripheral anorexigenic hormones including cholecystokinin, leptin, and insulin have been found to rise with increased age [91], findings for ghrelin are controversial [92–95]. Most of the human studies indicated that ghrelin secretion and ghrelin-induced GH secretion decreased in elderly people compared to younger subjects [92,96]. In a recent study, Schneider *et al.* [97] found no increase in ghrelin levels in malnourished elderly subjects compared to well-nourished elderly ones, which suggests that hunger may be suppressed during the postprandial period in aged population. In another study, it was found that fasting acylated ghrelin is lower in elderly subjects and the postprandial acylated ghrelin curve remains low and flat after a meal [98]. Moreover, Serra-Prat *et al.* [99] found that advanced age determines a poorer ghrelin postprandial recuperation phase, a reduced cholecystokinin (CCK) postprandial response, and an exaggerated postprandial insulin release. Of note, a loss of ghrelin prandial rhythm was present in old frail persons [99]. All of these findings suggest that disturbance of regulation of ghrelin secretion and reduced production during hunger and satiety may cause "anorexia of aging" in elderly people.

In contrast to human data, several lines of animal studies have revealed that plasma ghrelin concentrations and stomach ghrelin contents in aged rats are significantly higher than in young rats [100]. Sun *et al.* [95] found a gradual increase in plasma acylated ghrelin in older mice, although the plasma acylated/deacylated ratio showed a tendency to decline in these mice compared to younger ones. Contrary to these findings, however, Wolden-Hanson [101] reported that fasting failed to induce increased ghrelin in aged animals. The reason for these conflicting data seems to be owing to the differences in their experimental conditions (fasting or freely fed, daytime or night) under which the plasma ghrelin concentration was measured. Indeed, our group found that plasma ghrelin in aged C57BL/6 mice does not increase under fasted conditions, but is higher than that in young mice under freely fed conditions [102]. This suggests that regulation of ghrelin secretion from the stomach may be disturbed in older mice.

In a previous study, Ariyasu *et al.* [103] reported that subcutaneous injection of ghrelin (360 μg/kg twice a day) enhanced food intake in 72-hour fasted and aged mice and restored the decrease in body weight caused by fasting. Contrary to their data, we recently found that much lower dose of ghrelin (33 μg/kg) failed to increase food intake in 75-wk-old mice, whereas the same dose of ghrelin had an orexigenic effect in young mice [102], suggesting that aging is associated with attenuation of endogenous ghrelin signaling. Collectively, it seems that dysregulation of ghrelin secretion as well as ghrelin resistance in the appetite control system is occurring in aged mice. Although the detailed mechanisms of disturbed ghrelin dynamics remain unclear, one of the possible causes seems to be leptin.

It was reported that fasting leptin or insulin is higher in elderly people than in the young ones. The detailed mechanisms of disturbed regulation of ghrelin secretion remain unclear, but it has been shown that unbalanced serum leptin and ghrelin dynamics prolong postprandial satiety in the elderly [98].

We have found that plasma leptin and insulin levels in aged mice are significantly higher compared to those in young ones

[102]. Leptin and insulin are reported to inhibit ghrelin secretion from the stomach into the circulation [104], hence elevated leptin and insulin in the elderly may contribute to inhibition of secretion of ghrelin during fasting, resulting in prolonged satiety and inhibition of hunger sensation.

It has been reported that leptin suppresses the ghrelin-induced activation of NPY neurons [105]. Moreover, leptin activates the phosphoinositide 3-kinase (PI3K) - phosphodiesterase 3 (PDE3) pathway in NPY neurons [106-108]. The activation of PI3K-PDE3 pathway by leptin was recently proposed as a mechanism by which leptin blocks the activity of ghrelin, and it may counteract the adenylate cyclase-cAMP-protein kinase A system implicated in the effect of ghrelin [105]. Other studies showed that the effect of leptin was abolished by the administration of either PDE3 inhibitor [106] or PI3K inhibitor [107]. We demonstrated that the plasma leptin level in aged mice was greatly increased under both feeding and fasting conditions [102]. Furthermore, we found that administration of either a PI3K inhibitor LY-294002 or the PDE3 inhibitor cilostamide improved anorexia in aged mice [102]. These results suggest that plasma leptin, which increases with age, may induce resistance to ghrelin reactivity via cAMP down-regulation.

6.2. Effect of Rikkunshito on Hypophagia of Aged Mice

Recently, we demonstrated that the administration of rikkunshito improve anorexia of aging [102]. In addition, we found that rikkunshito increased the reactivity of ghrelin by inhibiting PDE3 activity. The components of rikkunshito (nobiletin, isoliquiritigenin, and heptamethoxyflavone) had inhibitory effects against PDE3 activity [102]. These results suggest that dysregulation of ghrelin secretion and ghrelin resistance in the appetite control system occurred in aged mice and that rikkunshito ameliorated aging-associated anorexia via inhibition of PDE3.

7. STRESS

7.1. Stress and Ghrelin

Stress and negative emotions have been associated with both increased and decreased food intake [109]. The mechanism underlying this opposed behavioral responses to similar stressors has not been determined, but high stress levels appear to lead to decreased eating [110].

Conflicting data are available regarding the effect of stress on ghrelin secretion. In animal studies, elevations in plasma ghrelin have been observed in response to various psychological/environmental stressors, including a tail pinch stress, a water avoidance stress, chronic exposure to cold, repeated restraint stress and chronic social defeat stress [111-115]. In contrast, exposure to immune, visceral or strenuous physical stressors causes reduction of plasma ghrelin level. For example, intraperitoneally administered LPS has been reported to decrease circulating ghrelin [116-118], which is mediated by IL-1β, prostaglandin and 5-HT2C receptor dependent mechanisms [119,120]. In another model, abdominal surgery induced a rapid and long-lasting decrease in fasted plasma acyl and desacyl ghrelin levels [121].

In humans, acute psychosocial stress [122,123] or cold exposure [124] increased plasma ghrelin levels. However, there are several reports showing that plasma ghrelin level did not change or even decreased by an exposure to stresses. For example, recent study by Zimmerman and colleagues [125] revealed that plasma ghrelin levels of normal weight men subjected to the Trier Social Stress Test did not change when cortisol levels increased. Moreover, ghrelin levels decreased after drinking alcohol. Another recent study showed that strenuous physical cycling exercise in healthy subjects results in a decline in fasted levels of acyl-ghrelin while the change of desacyl and total ghrelin plasma levels did not [126].

In a study by Ochi *et al.* [112], active ghrelin levels in plasma were not increased in the initial phase (until 24 h) of stress loading, although they were significantly higher on day 3 than that in the control group. Zheng *et al.* [113] revealed that the delayed gastric emptying observed in acute stress loading was completely restored following repeated chronic stress in rats. They concluded that the homeostatic adaptation mechanism may develop in response to repeated stress involving upregulation of gastric ghrelin secretion and attenuation of the HPA axis. Collectively, these findings support the idea that acute or severe stress causes a reduction of circulating ghrelin level resulting in the suppression of appetite, whereas mild or chronic repeated stress causes an upregulation of ghrelin secretion as an adaptation to stress. In support of this notion, Lutter *et al.* [111] found that increased ghrelin levels produced anxiolytic and antidepressant responses in mice suggesting that increased ghrelin in response to stress protects against depressive reactions to stress and helps them cope with stress.

Corticotropin-releasing factor (CRF) and its family peptides, urocortin1 (Ucn1), urocortin2 (Ucn2) and urocortin3 (Ucn3), play an important role in the control of food intake [127]. Among the CRF family peptides, Ucn1 was shown to have the most potent inhibitory effect on the food intake in mice. Ucn1 has been identified in the brain and has a higher affinity for CRF2 receptors (CRFR2) than for CRF1 receptors (CRFR1) [128], hence it is believed that CRFR2 plays the major role in satiety [129,130].

Activation of CRFR1 in the brain can suppress feeding independently of CRFR2-mediated mechanisms. For example, selective CRFR1 antagonists reverse at least some forms of stress-induced anorexia [131]. CRF1 and CRF2 receptor-mediated anorexia appear to exhibit different time-courses; in rats, i.c.v. administration of CRFR1 agonists elicited rapid-onset anorexia with short duration, while CRFR2 agonists caused delayed-onset, prolonged anorexia [132, 133].

There are several reports showing that administration of Ucn1 to humans and rodents reduces plasma ghrelin concentrations [134-136]. In addition, Ucn1-induced reduction of plasma ghrelin and food intake were restored by CRFR2 but not CRFR1 [136]. However, much less information is available on the relationship between ghrelin and CRFR1.

Novelty stress model, where animals are removed from their home cage and placed somewhere they have never been before, has been used to estimate the levels of anxiety and depression [137,138]. Using this stress model, we found that three hours after the novelty stress, appetite reduction was associated with a decrease in plasma ghrelin level, reduced levels of neuropeptide Y/agouti-related peptide mRNA and increased levels of proopiomelanocortin mRNA in the hypothalamus [139]. Administering a CRFR1 selective antagonist, but not a CRFR2 antagonist, resolved the reduction in food intake 3 h after the novelty stress by enhancing circulating ghrelin concentrations. Interestingly, 5-HT1B/2CR antagonist and melanocortin-4 receptor (MC4R) antagonist alleviated the novelty stress-induced hypophagia and the reduction in circulating ghrelin level [139]. We hypothesized that acute appetite suppression due to CRFR1 activation after a novelty stress is caused by a chain reaction of appetite control mechanisms mediated by 5-HT1B/2CR in ARC to MC4R system in paraventricular nucleus (PVN), causing lowered peripheral ghrelin secretion.

7.2. Effect of Rikkunshito on Novelty Stress Model

Oral administration of rikkunshito inhibited the reduction of food intake at 1 and 3 h in mice exposed to the novelty stress, and coadministration of the ghrelin receptor antagonist [D-Lys^3]GHRP-6 with rikkunshito abolished this effect [139]. Rikkunshito also increased plasma acyl-ghrelin concentrations at 1 and 3 h after the novelty stress, suggesting that blocking the decrease in endogenous peripheral ghrelin in mice exposed to the novelty stress also acts to sustain feeding behavior. We found that the oral administration of glycycoumarin and isoliquiritigenin inhibited the reduction in food intake in mice exposed to the novelty stress [139]. We have previ-

ously shown that glycycoumarin and isoliquiritigenin potently inhibit 5-HT2C receptor ligand binding and that orally administering rikkunshito abolishes the decrease in food intake in mCPP-treated rats [13]. These findings support the notion that rikkunshito improved hypophagia and decreased plasma ghrelin levels via 5-HT2C receptor antagonism-like action in mice exposed to the novelty stress.

8. CANCER ANOREXIA–CACHEXIA SYNDROME

8. 1. Cancer Anorexia–cachexia Syndrome and Ghrelin

Cancer anorexia–cachexia syndrome is characterized by decreased food intake, weight loss, muscle tissue wasting and psychological distress and a lower quality of life [140,141]. In advanced-stage cancer, up to 85% of patients experience this syndrome which contributes to at least 20% of cancer deaths overall [142]. The weight loss experienced by patients can be severe and is associated with a worsened prognosis, poorer response to chemotherapy and increased morbidity [143]. In addition to metabolic changes, cachexia is often associated with anorexia. But the lack of nutrients alone cannot explain the metabolic changes seen in cachexia. In clinical trials, nutritional supplementation and dietary counseling failed to increase body weight [144]. Only limited treatment options exist for patients with clinical cancer cachexia. Corticosteroids improve the sensation of well-being and leads to increased food intake, but this effect lasts only a few weeks [140]. Progesterone such as megestrol acetate cause weight gain [142, 145], but this gain is resulted only from increased body fat and fluid, with no change in lean body mass [144]. Moreover, therapy with progesterone increases the frequency of thromboembolic events [141, 146]. Hence a better understanding of the underlying mechanisms of this syndrome should be very important in the development of new therapies to improve quality of life and potentially to prolong survival in patients with cancer-induced anorexia–cachexia.

Accumulating evidence suggests that anorexia–cachexia is caused predominantly by cytokines that are either produced by cancer cells or released by the host immune system in response to the cancer. [140-142]. Pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α can be produced by tumor cells, as well as from the host response to tumor [140-142]. Up to 50% of cancer patients exhibit evidence of elevated inflammation at diagnosis and the associated increase in cytokines is strongly implicated in producing anorexia, at least partly due to action at the central melanocortin system [147].

CRF is a mediator of the endocrine, autonomic and immune responses to stress, including anorexia and anxiety-related behavior [148]. The central 5-HT system has been implicated in the processes of meal satiation and satiety and hypothalamic 5-HT and CRF activities are stimulated by proinflammatory cytokines [149]. On the basis of these findings, it is probable that 5-HT and CRF might have a role in the pathogenesis of cancer anorexia–cachexia by modulating central and peripheral mechanisms as part of the stress response.

Although one recent study reported reduced plasma ghrelin concentrations in cancer patients [150], cachexia is more frequently associated with increased circulating ghrelin levels compared to healthy control subjects or non-cachectic patients with the same underlying diseases [151, 152]. Increased plasma ghrelin concentrations in cachectic patients could be a compensatory response to tissue wasting. Alternatively, it might reflect a state of ghrelin resistance.

Recently, Fujitsuka *et al.* [16] found that plasma acyl-ghrelin concentrations in tumor-bearing rats were higher than that in free-fed normal rats, but lower than that in pair-fed normal rats. This indicates that the compensatory responses to cachectic state including upregulation of ghrelin secretion are attenuated in tumor-bearing rats.

Use of ghrelin and other GHSR-1a agonists has been tested in animal models of cancer cachexia [147] and has demonstrated an increase in food intake, weight gain, and reversal of lean and fat mass losses [153 -155]. Early trials in cancer patients demonstrated that administering ghrelin increased appetite [156,157]. Phase I and II clinical trial with the orally available, synthetic ghrelin mimetic RC-1291 have demonstrated increases in body weight and lean body mass in healthy subjects [158] and in cancer patients [159], without any dose limiting side effects.

Recent studies have demonstrated that CRF decreased the plasma level of acyl-ghrelin [16, 139]. Fujituska *et al.* [16] found that CRF receptor antagonist, a-helical CRF improved cancer anorexia-cachexia syndrome in tumor-bearing rats. They also found that the administration of 5-HT2C receptor antagonist SB242084 decreased hypothalamic CRF level and improved anorexia, gastrointestinal dysmotility and body weight loss in tumor-bearing rats with cachexia [16]. In earlier studies, 5-HT concentration in the hypothalamus was reported to be increased in humans and animals with cancer [160,161]. CRF neurons are involved in 5-HT-regulated ghrelin secretion and this pathway has a major role in cancer anorexia–cachexia.

Peripheral ghrelin administration stimulates food intake in cancer patients [142] in the short term as well as in healthy subjects [162].

8.2. Cancer Anorexia–cachexia Syndrome and Rikkunshito

In animal model of cancer cachexia, rikkunshito was found to reduce hypothalamic CRF levels and improve anorexia, gastrointestinal dysmotility, muscle wasting, and anxiety-related behavior [16]. It was also shown that rikkunshito activated the efferent vagus nerve, which may be mediated by both the vagal afferent nerve and the direct central action. Of particular interest, rikkunshito and its active component, atractylodin, prolonged survival in these animals [16]. *In vitro*, ghrelin-induced cellular signaling in GHS-R-expressing cells was enhanced by pretreatment with rikkunshito and atractylodin which enhance ghrelin/GHS-R binding activity. In contrast to the effect of rikkunshito, either 5-HT2C receptor antagonist or exogenous ghrelin failed to prolong survival [16]. This suggests that the sensitizing effect on ghrelin signaling pathway may be essential for ameliorating anorexia–cachexia and the prolongation of survival. These findings are compatible with the idea that the physiological functions of endogenous ghrelin are enhanced by the dual actions of rikkunshito, which involve the stimulation of ghrelin secretion and the activation of GHS-R activity.

9. SSRI-INDUCED DYSPEPSIA

9.1. SSRI-induced Dyspepsia and Ghrelin

Selective serotonin reuptake inhibitors (SSRIs) are commonly prescribed to patients with major depression. Upper gastrointestinal symptoms such as nausea and vomiting are one of the most common adverse events, and for some patients, dyspeptic symptoms become critical issues that impair their quality of life and may result in discontinuation of SSRI therapy [163].

By using a freely moving conscious rat model equipped with force transducers, Fujitsuka *et al.* [150] found that intraperitoneal administration of SSRIs, fenfluramine or fluvoxamine decreased plasma level of acyl-ghrelin and changed upper gastrointestinal motility from a fasted-like pattern to fed-like motor activities similar to those seen after feeding. These effects on ghrelin and gastrointestinal motility were blocked by 5-HT2C receptor antagonist. Neither melanocortin 4 nor the 3/4 receptor antagonists blocked this motor effect, although they restored the anorexia induced by SSRIs suggesting that SSRI-induced anorexia is dependent on a melanocortin system [164]. From these results it was concluded that SSRIs have inhibitory effects on acyl-ghrelin and gastrointestinal motor activities via an activation of 5-HT2C receptors.

9.2. SSRI-induced Dyspepsia and Rikkunshito

Concomitant oral administration of rikkunshito with an SSRI suppressed the decrease in plasma acylated ghrelin, changed the fed like motor activity to fasted activity, improved anorexia, and enhanced gastric emptying [164]. These effects of rikkunshito were abolished by coadministration of a ghrelin receptor antagonist and were mimicked by its active ingredient hesperidin [164]. Given that hesperidin was shown to interact with 5-HT2C and 5-HT2B receptors [13]. It makes sense that appetite-stimulating effect of rikkunshito may be attributed to its 5-HT2C receptor antagonism.

10. GHRELIN DEGRADATING ENZYME AND RIKKUNSHITO

Plasma acylated ghrelin level is regulated by both the secretion from stomach and the elimination from circulation which includes degradation of acylghrelin by deacylating enzymes. Plasma acylated-ghrelin is rapidly deacylated in a process that is believed to be primarily mediated by carboxylesterase (CES) in rodents and butyrylcholinesterase (BuChE) in humans [165].

Rikkunshito administration has been shown to stimulate food intake and peripheral and central ghrelin secretion in rodents [12,13,72,139] and humans [12]. Interestingly, it was shown that oral administration of rikkunshito enhanced circulating acyl-ghrelin level without a significant effect on the plasma level of desacyl-ghrelin in both normal-fed and cisplatin-treated rats, leading to the increase in the acyl- to desacyl-ghrelin (A/D) ratio [13]. Furthermore, in human study, it was demonstrated that plasma acyl-ghrelin level and A/D ratio increased significantly after rikkunshito administration, whereas desacyl-ghrelin level showed a decreasing trend [12]. These results raise the possibility that rikkunshito increases circulating acyl-ghrelin level by inhibiting its degradation.

To test the hypothesis that some components of rikkunshito contribute to the inhibition of ghrelin degradation, 48 components in rikkunshito were screened for inhibitory activity against ghrelin degrading enzymes such as CES and BuChE [17]. It was found that eight compounds exhibited inhibitory activity against CES or BuChE. Among them, 10-gingerol exhibited the highest inhibitory activity against CES (5.2 μM inhibition constant) in a competitive manner [17]. In addition, pachymic acid and glycycoumarin were shown to be competitive inhibitors of BuChE. Furthermore, rikkunshito and its component 10-gingerol inhibited the decrease in plasma acyl-ghrelin level of exogenously administered ghrelin, and the CES inhibitor BNPP inhibited cisplatin-induced decreases in food intake [17]. On the basis of these findings, it is conceivable that rikkunshito may enhance plasma acyl-ghrelin level, at least in part, by inhibiting the circulating ghrelin degrading enzyme.

11. SUMMARY

Because kampo (Japanese herbal) medicines contain multiple active ingredients, it is usually difficult to disclose its precise mechanism of action. However, recent progress in our knowledge about the unique characteristics of rikkunshito as a powerful orexigenic drug, prompted us to investigate the potential mechanism action much further. From the clinical point of view, currently available clinical evidence showing the efficacy of kampo medicine is very limited. Well-designed randomized, placebo-controlled studies are warranted to disclose the merit for the usage of kampo medicine in a clinical setting.

CONFLICT OF INTEREST

H.T. received grant support from Tsumura & Co. S.M., K.N., S.O., and M.A. have no conflict to declare.

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REFERENCES

[1] Mochiki E, Yanai M, Ohno T, Kuwano H. The effect of traditional Japanese medicine (Kampo) on gastrointestinal function. Surg Today 2010; 40: 1105-11.

[2] Suzuki H, Inadomi JM, Hibi T. Japanese herbal medicine in functional gastrointestinal disorders. Neurogastroenterol Motil 2009; 21: 688-96.

[3] Tatsuta M, Iishi H. Effect of treatment with liu-jun-zi-tang (TJ-43) on gastric emptying and gastrointestinal symptoms in dyspeptic patients. Aliment Pharmacol Ther 1993; 7: 459-62.

[4] Arai M, Matsumura T, Tsuchiya N, *et al* Rikkunshito improves the symptoms in patients with functional dyspepsia, accompanied by an increase in the level of plasma ghrelin. Hepatogastroenterology 2012; 59: 62-6.

[5] Kusunoki H, Haruma K, Hata J, *et al.* Efficacy of rikkunshito, a traditional Japanese medicine (Kampo), in treating functional dyspepsia. Intern Med 2010; 49: 2195-202.

[6] Kawahara H, Kubota A, Hasegawa T, *et al.* Effects of rikkunshito on the clinical symptoms and esophageal acid exposure in children with symptomatic gastroesophageal reflux. Pediatr Surg Int 2007; 23: 1001-5.

[7] Kawahara H, Okuyama H, Nose K, *et al.* Physiological and clinical characteristics of gastroesophageal reflux after congenital diaphragmatic hernia repair. J Pediatr Surg 2010; 45: 2346-50.

[8] Tominaga K, Iwakiri R, Fujimoto K, *et al.* GERD 4 Study Group. Rikkunshito improves symptoms in PPI-refractory GERD patients: a prospective, randomized, multicenter trial in Japan. J Gastroenterol 2012; 47: 284-92.

[9] Tomono H, Ito Y, Watanabe T. Successful antiemetic treatment of Tsumura rikkunshi-to extract granules for ethical use in addition to other antiemetic agents in neoadjuvant chemotherapy for an advanced breast cancer patient. Jpn J Cancer Chemother 2006; 33: 1129-31.

[10] Ohno T, Yanai M, Ando H, *et al.* Rikkunshito, a traditional Japanese medicine, suppresses cisplatin-induced anorexia in humans. Clin Exp Gastroenterol 2011; 4: 291-6.

[11] Yagi M, Homma S, Kubota M, *et al.* The herbal medicine Rikkunshi-to stimulates and coordinates the gastric myoelectric activity in post-operative dyspeptic children after gastrointestinal surgery. Pediatr Surg Int 2004; 19: 760-5.

[12] Matsumura T, Arai M, Yonemitsu Y, *et al.* The traditional Japanese medicine Rikkunshito increases the plasma level of ghrelin in humans and mice. J Gastroenterol 2010; 45: 300-7.

[13] Takeda H, Sadakane C, Hattori T, *et al.* Rikkunshito, an herbal medicine, suppresses cisplatin-induced anorexia in rats via 5-HT2 receptor antagonism. Gastroenterology 2008; 134: 2004-13.

[14] Hattori T. Rikkunshito and ghrelin. Int J Pept 2010; 2010. *pii*: 283549.

[15] Yakabi K, Kurosawa S, Tamai M, *et al.* Rikkunshito and 5-HT2C receptor antagonist improve cisplatin-induced anorexia via hypothalamic ghrelin interaction. Regul Pept 2010; 161: 97-105.

[16] Fujitsuka N, Asakawa A, UezonoY, *et al.* Potentiation of ghrelin signaling attenuates cancer anorexia-cachexia and prolongs survival. Trans Psychiatry 2011; 1: e23.

[17] Sadakane C, Muto S, Nakagawa K, *et al.* 10-Gingerol, a component of rikkunshito, improves cisplatin-induced anorexia by inhibiting acylated ghrelin degradation. Biochem Biophys Res Commun 2011; 412: 506-11.

[18] Imada T, Rino Y, Takahashi M, *et al.* Postoperative functional evaluation of pylorus-preserving gastrectomy for early gastric cancer compared with conventional distal gastrectomy. Surgery 1998; 123: 165-70.

[19] Nishikawa K, Kawahara H, Yumiba T, *et al.* Functional characteristics of the pylorus in patients undergoing pyloruspreserving gastrectomy for early gastric cancer. Surgery 12002; 31: 613-24.

[20] Nakabayashi T, Mochiki E, Kamiyama Y, *et al.* Erythromycin induces pyloric relaxation accompanied by a contraction of the gastric body after pylorus-preserving gastrectomy. Surgery 2003; 133: 647-55.

[21] Takahashi T, Endo S, Nakajima K, Souma Y, Nishida T. Effect of rikkunshito, a chinese herbal medicine, on stasis in patients after pylorus-preserving gastrectomy. World J Surg 2009; 33: 296-302.
[22] Utumi Y, Iseki E, Murayama N, *et al.* Effect of Rikkunshi-to on appetite loss found in elderly dementia patients: a preliminary study. Psychogeriatrics 2011; 11: 34-9.
[23] Naito T, Itoh H, Takeyama M. Some gastrointestinal function regulatory kampo medicines have modulatory effects on human plasma adrenocorticotropic hormone and cortisol levels with continual stress exposure. Biol Pharm Bull 2003; 26: 101-4.
[24] Oka T, Tamagawa Y, Hayashida S, Kaneda Y, Kodama N, Tsuji S. Rikkunshi-to attenuates adverse gastrointestinal symptoms induced by fluvoxamine. Biopsychosoc Med 2007; 1: 21.
[25] Kido T, Nakai Y, Kase Y, *et al.* Effects of Rikkunshi-to, a traditional Japanese medicine, on the delay of gastric emptying induced by N(G)-nitro-L-arginine. J Pharmacol Sci 2005; 98: 161-7.
[26] Tominaga K, Kido T, Ochi M, *et al.* The Traditional Japanese Medicine Rikkunshito Promotes Gastric Emptying via the Antagonistic Action of the 5-HT3 Receptor Pathway in Rats. Evid Based Complement Alternat Med 2011; 2011: 248481.
[27] Kawahara H, Mitani Y, Nomura M, *et al.* Impact of rikkunshito, an herbal medicine, on delayed gastric emptying in profoundly handicapped patients. Pediatr Surg Int 2009; 25: 987-90.
[28] Shiratori M, Shoji T, Kanazawa M, Hongo M, Fukudo S. Effect of rikkunshito on gastric sensorimotor function under distention. Neurogastroenterol Motil 2011; 23: 323-9.
[29] Hayakawa T, Arakawa T, Kase Y, *et al.* Liu-Jun-Zi-Tang, a Kampo medicine, promotes adaptive relaxation in isolated guinea pig stomachs. Drugs Exp Clin Res 1999; 25: 211-8.
[30] Sakai Y, Nobe K, Maruyama Y, Momose K, Homma I. A traditional herbal medicine, rikkunshi-to (TJ-43), prevents intracellular signaling disorders in gastric smooth muscle of diabetic rats. Am J Chin Med 2004; 32: 245-56.
[31] Ozaki M, Nagatomo T, Maeda T, Kishioka S, Yamamoto H. Pharmacological differences between Liu-Jun-Zi-Tang, a traditional Chinese herbal medicine, and domperidone on isolated guinea-pig ileum. Biol Pharm Bull 2006; 29: 1349-54.
[32] Kito Y, Suzuki H. Properties of Rikkunshi-to (TJ-43)-induced relaxation of rat gastric fundus smooth muscles. Am J Physiol Gastrointest Liver Physiol 2010; 298: G755-63.
[33] Arakawa T, Higuchi K, Fujiwara Y, *et al.* Gastroprotection by Liu-Jun-Zi-Tang (TJ-43): possible mediation of nitric oxide but not prostaglandins or sulfhydryls. Drugs Exp Clin Res 1999; 25: 207-10.
[34] Tamaki K, Otaka M, Shibuya T, *et al.* Traditional Herbal Medicine, Rikkunshito, Induces HSP60 and Enhances Cytoprotection of Small Intestinal Mucosal Cells as a Nontoxic Chaperone Inducer. Evid Based Complement Alternat Med 2012; 2012: 278958.
[35] Miwa H, Koseki J, Oshima T, *et al.* Rikkunshito, a traditional Japanese medicine, may relieve abdominal symptoms in rats with experimental esophagitis by improving the barrier function of epithelial cells in esophageal mucosa. J Gastroenterol 201; 45: 478-87.
[36] Tanaka K, Urita Y, Nara K, Miura O, Sugimoto M. Effects of the traditional Japanese medicine Rikkunshito on postprandial glucose and lipid metabolism. Hepatogastroenterology 2011; 58: 1112-8.
[37] Chen CY, Fujimiya M, Laviano A, Chang FY, Lin HC, Lee SD. Modulation of ingestive behavior and gastrointestinal motility by ghrelin in diabetic animals and humans. J Chin Med Assoc 2010; 73: 225-9.
[38] Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999; 402: 656-60.
[39] Kojima M, Kangawa K. Ghrelin: structure and function. Physiol Rev 2005; 85: 495-522.
[40] Chen CY, Asakawa A, Fujimiya M, Lee SD, Inui A. Ghrelin gene products and the regulation of food intake and gut motility. Pharmacol Rev 2009; 61: 430-81.
[41] Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000; 407: 908-13.
[42] Nakazato M, Murakami N, Date Y, *et al.* A role for ghrelin in the central regulation of feeding. Nature 2001; 409: 194-8.
[43] Wren AM, Seal LJ, Cohen MA, *et al.* Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 2001; 86: 5992.
[44] Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 2001; 50: 1714-9.
[45] Cummings DE, Weigle DS, Frayo RS, *et al.* Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. N Engl J Med 2002; 346: 1623-30.
[46] Korbonits M, Goldstone AP, Gueorguiev M, Grossman AB. Ghrelin—a hormone with multiple functions. Front Neuroendocrinol 2004; 25: 27-68.
[47] van der Lely AJ, Tschop M, Heiman ML, Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev 2004; 25: 426-57.
[48] Bednarek MA, Feighner SD, Pong SS, *et al.* Structure and function studies on the new growth hormone releasing peptide, ghrelin: minimal sequence of Ghrelin necessary for activation of growth hormone secretagogue receptor 1a. J Med Chem 2000; 43: 4370-6.
[49] Banks WA, Tschop M, Robinson SM, Heiman ML. Extent and direction of Ghrelin transport across the blood-brain barrier is determined by its unique primary structure. J Pharmacol Exp Ther 2002; 302: 822-7.
[50] Gutierrez JA, Solenberg PJ, Perkins DR, *et al.* Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci USA 2008; 105: 6320-5.
[51] Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 2008; 132: 387-96.
[52] Wettschureck N, Moers A, Wallenwein B, Parlow AF, Maser-Gluth C, Offermanns S. Loss of Gq/11 family G proteins in the nervous system causes pituitary somatotroph hypoplasia and dwarfism in mice. Mol Cell Biol 2005; 25: 1942-8.
[53] Sun Y, Wang P, Zheng H, Smith RG. Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc Natl Acad Sci USA 2004; 101: 4679-84.
[54] Leite-Moreira AF, Soares JB. Physiological, pathological and potential therapeutic roles of ghrelin. Drug Discov Today 2007; 12: 276-88.
[55] Date Y, Kojima M, Hosoda H, *et al.* Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 2000; 141: 4255-61.
[56] Gnanapavan S, Kola B, Bustin SA, *et al.* The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab 2002; 87: 2988-91.
[57] Cowley MA, Smith RG, Diano S, *et al.* The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 2003; 37: 649-61.
[58] Korbonits M, Bustin SA, Kojima M, *et al.* The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. J Clin Endocrinol Metab 2001; 86: 881-7.
[59] Date Y, Murakami N, Toshinai K, *et al.* The role of the gastric afferent vagal nerve in ghrelin induced feeding and growth hormone secretion in rats. Gastroenterology 2002; 123: 1120-8.
[60] Akamizu T, Takaya K, Irako T, *et al.* Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects. Eur J Endocrinol 2004; 150: 447-55.
[61] Druce MR, Wren AM, Park AJ, *et al.* Ghrelin increases food intake in obese as well as lean subjects. Int J Obes (Lond) 2005; 29: 1130-6.
[62] Druce MR, Neary NM, Small C, *et al.* Subcutaneous administration of ghrelin stimulates energy intake in healthy lean human volunteers. Int J Obes (Lond) 2006; 30: 293-6.
[63] Hansen TK, Dall R, Hosoda H, *et al.* Weight loss increases circulating levels of ghrelin in human obesity. Clin Endocrinol (Oxf) 2002; 56: 203-6.
[64] Neary NM, Small CJ, Wren AM, *et al.* Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. J Clin Endocrinol Metab 2004; 89: 2832-6.
[65] Hesketh PJ. Chemotherapy-induced nausea and vomiting. N Engl J Med 2008; 358: 2482-94.
[66] Holmes AM, Rudd JA, Tattersall FD, Aziz Q, Andrews PL. Opportunities for the replacement of animals in the study of nausea and vomiting. Br J Pharmacol 2009; 157: 865-80.