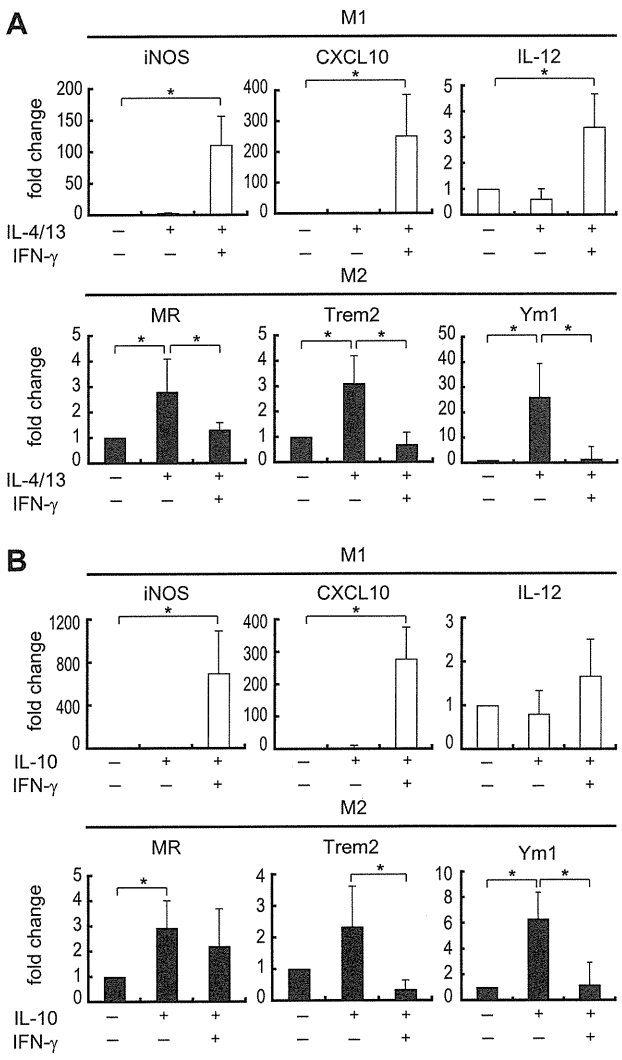

Fig. 6. IFN-γ had sufficient capacity to alter the phenotype of mouse peritoneal macrophages from M2 to M1 even in the presence of IL-4, IL-13 and IL-10. (**A** and **B**) mRNA expression of M1 and M2 genes was evaluated by qRT–PCR in freshly isolated mouse peritoneal macrophages. (**A**) Together with 20 ng/ml of IL-4 and IL-13, 20 ng/ml of IFN-γ was added to M2-polarized macrophages. qRT–PCR showed significant upregulation of all M1 markers and downregulation of all M2 markers by administration of IFN-γ, even in the presence of IL-4 and IL-13. Representative data from at least four independent experiments are shown. $^{*}P < 0.05$. (B) Together with 20 ng/ml of IL-10, 20 ng/ml of IFN-γ was added to M2-polarized macrophages. qRT–PCR showed upregulation of M1 markers and downregulation of all M2 markers by administration of IFN-γ, even in the presence of IL-10. Representative data from at least four independent experiments are shown. $^{*}P < 0.05$.

that accumulating M2 TAMs play a role in intestinal tumor progression that is similar to that in other organs.

Subsequently, we investigated the effect of COX-2 inhibition on TAM activation in $Apc^{Min/+}$ mouse polyps. As previously reported (9,10,23), COX-2 inhibitor reduced intestinal polyps in $Apc^{Min/+}$ mice. Although it is generally accepted that COX-2 inhibition induces tumor reduction partly through the regulation of cancer-related immune responses (20–23), few papers have directly demonstrated the effect of COX-2 inhibition on TAM phenotypes. In the present study, we showed that COX-2 inhibition skewed M2 TAMs in $Apc^{Min/+}$ mouse polyps to M1 with enhanced IFN-γ production.

Although IFN-γ is known to be one of the key inducers of M1 polarization (4,5), the superiority between Th1 and Th2 cytokines to regulate the activation of TAM remains to be elucidated. Therefore, to determine this, we used mouse peritoneal macrophages and demonstrated that COX-2 inhibition alone was not sufficient to alter M2 markers of macrophages in the presence of IL-4, IL-13 and IL-10. These data suggest that additional factors, secreted from other cell types, are required to alter TAM phenotypes in $Apc^{Min/+}$ mouse polyps, particularly in response to administration of a COX-2 inhibitor. Notably, we observed that the administration of IFN-γ altered macrophage phenotypes even in the presence of IL-4, IL-13 and IL-10. Taken together with our data in $Apc^{Min/+}$ mice, COX-2 inhibition appeared to change TAM phenotypes not directly but through IFN-γ induction in T cells and natural killer cells. These results are consistent with a pivotal role for IFN-γ in altering the tumor microenvironments (33,42,43). Therefore, it can be suggested that the skewing of TAM phenotypes by IFN-γ contributes to COX-2–dependent reduction of $Apc^{Min/+}$ mouse polyps.

In conclusion, we demonstrated that COX-2 inhibition altered TAM phenotypes, possibly in an IFN-γ-dependent manner, in the polyps of $Apc^{Min/+}$ mouse. The study provides a new insight into the regulation of TAM phenotypes, and bridges the immune network with the antitumor properties of COX-2 inhibitor. These findings may support the development of novel therapeutic strategies in colorectal cancer patients through the skewing of TAM phenotypes.

Funding

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Review

Ghrelin and cardiovascular diseases

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Sympathetic nerve

Summary In 1999, a peptide from the stomach called ghrelin was discovered, which exerts potent growth hormone releasing powers. Subsequent studies revealed that it exerts a potent orexigenic action. In addition, the beneficial effects of ghrelin in cardiovascular diseases have been recently suggested. In humans as well as in animals, administration of ghrelin improves cardiac function and remodeling in chronic heart failure. In an animal model for myocardial infarction, ghrelin treatment early after coronary ligation effectively reduces fatal arrhythmia and, consequently, mortality, suggesting the potential therapeutic role of the peptide in acute myocardial infarction. Although how ghrelin may influence the cardiovascular system is not fully understood, the cardiovascular beneficial effects are mediated possibly through a combination of various actions, such as an increase in growth hormone level, an improvement in energy balance, direct actions to the cardiovascular cells, and regulation of the autonomic nervous activity. Of note, current experimental evidence suggests that ghrelin may act centrally to decrease sympathetic nervous system activity through peripheral afferent nerve. Thus, administration of ghrelin might become a unique new therapy for cardiovascular diseases.

Contents

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Introduction

Small synthetic compounds called growth hormone secretagogues (GHS) stimulate the release of growth hormone (GH) from the pituitary [1]. In 1996, a receptor for GHS (GHS-R) was cloned from swine and human pituitary gland, and it was revealed that GHS-R is one of the G-protein coupled receptors with seven membrane spanning domains [2]. Since the discovery of the receptor, the quest for searching the endogenous ligand for GHS-R started by many groups worldwide. Most of them thought that the endogenous ligand should be found in the brain and selected the brain as a starting material for the purification of the ligand, based on the assumption that pituitary hormones are regulated by midbrain-pituitary axis. However, although studied thoroughly and extensively, all purification attempts using the brain as a source ended unsuccessfully. Thus, the endogenous ligand was not found until 1999, when it was finally identified from the stomach, the tissue that almost nobody had expected as a source. Using a reverse pharmacology approach, a 28 amino-acid peptide was isolated from the rat stomach and named "Ghrelin" derived both from the word root "ghre" in Proto-Indo-European languages meaning "grow", and from the abbreviation for "GH-release," a characteristic feature of the peptide [3]. Since the discovery of ghrelin, a number of unique features have been identified. First, the discovery of ghrelin from the stomach indicates that the release of GH from the pituitary is regulated not only by the hypothalamus but also by the digestive tract. Second, ghrelin has a unique structural property that is an acylation in its third residue, usually serine. This is the first peptide hormone with acyl modification. Interestingly, the acylation is essential for the ghrelin's ability for the binding to and the activation of its receptor, GHS-R. Third, subsequent studies revealed that exogenously administered ghrelin potently stimulates appetite in humans and in rodents [4,5]. Fourth, not only in the release of GH and in the stimulation of appetite, the roles of ghrelin have also been implicated in the cardiovascular, bone, gastrointestinal, and immune systems [6]. In the present review, we will discuss some of these characteristic features of ghrelin and its possible therapeutic roles in cardiovascular diseases.

Ghrelin is a potent GH secretagogue

Ghrelin was originally discovered as an endogenous ligand for GHS-R and, in fact, has potent GH releasing activity. Intravenous ghrelin administration markedly increases plasma GH levels in humans and in rats. After a bolus ghrelin injection, the level of GH peaks at 15–20 min and the elevation lasts longer than 60 min thereafter [7]. Since the effect of ghrelin on the release of GH is not observed after resection of the gastric branch of the vagus nerve, the vagal afferent nerve is supposed to mediate the effect [8]. The hypothesis is supported by the fact that GHS-R is synthesized in vagal afferent neurons and transported to the afferent nerve terminals [8,9].

Ghrelin as a gastrointestinal hormone

X/A-like cells are among four types of endocrine cells in the oxyntic mucosa of the stomach, and so named as their function had been undefined until recently and as their morphology is similar to pancreatic alpha cells. In situ analysis revealed that ghrelin and its mRNA are mainly localized in X/A-like cells. In the stomach, the 28 amino acids of mature ghrelin are cleaved off from its precursor preproghrelin which is composed of 117 amino acids in rats or in humans. From the submucosal layer of the stomach, ghrelin is secreted into the blood stream (not into the gastrointestinal tract). From plasma ghrelin levels in patients with gastrectomy or gastric bypass surgery, it is demonstrated that the stomach is a major organ secreting the circulating ghrelin [10]. Although the contents are much less, cells producing ghrelin are also found in the intestines and in specific regions of the brain such as the arcuate nucleus.

Octanoyl modification

The distinguished structural feature of ghrelin is its fatty acid modification at the third residue (serine in most species including humans) [3,11] (Fig. 1). Interestingly, the acylation, particularly n-octanoyl modification, is conserved among many species including mammals, fish, birds, and

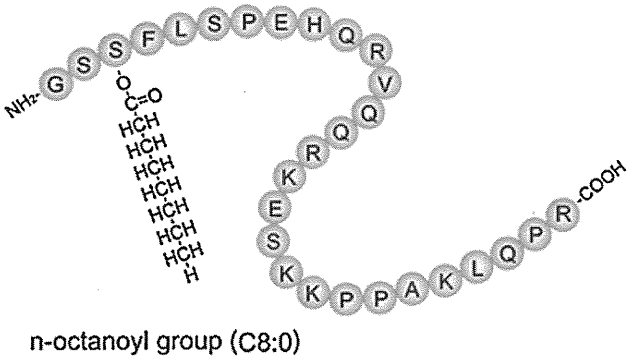

Figure 1 The structure of human ghrelin. Ghrelin is a 28 amino acid peptide discovered from the stomach. A distinguished structural feature of ghrelin is its n-octanoylation at the third serine residue, which is necessary for its receptor binding and function.

amphibians and is necessary for full binding of ghrelin to its receptor, GHS-R, and thus for expressing the biological function. The attachment of octanoate to the third serine residue of ghrelin is catalyzed by a membrane-bound enzyme, named ghrelin O-acyltransferase (GOAT) [12].

Localization of ghrelin receptor, GHS-R

In the brain, gene expression of the receptor for ghrelin, GHS-R, is detected predominantly in the arcuate nuclei, in the ventromedial nuclei, and in the hypocampus [2]. To a lesser extent, it is also detected in pituitary and in detate gyrus. Outside of the brain, various organs including lung, liver, kidney, pancreas, and gastrointestinal tract expressed GHS-R gene. In the cardiovascular system, GHS-R is expressed in the heart and in the aorta. It is also reported that, GHS-R gene can be detected in cultured cardiomyocyte cell line and in human vascular endothelial cells.

Ghrelin as a hunger hormone

Exogenously administered ghrelin has a potent appetite-stimulating effect [4]. Since the orexigenic effect of ghrelin can be observed in GH-deficient dwarf rats, the appetite-promoting effect is independent of GH release. The plasma level of ghrelin and mRNA level in the stomach are increased by fasting and decreased by feeding [10,13]. Oral or intravenous administration of glucose decreases plasma ghrelin level. Since ghrelin has a potent orexigenic action, ghrelin can serve as a ''hunger hormone.'' In addition, fasting plasma ghrelin level is low in obese people [14] and high in lean people and in patients with anorexia nervosa. Since ghrelin induces weight gain by promoting appetite and by reducing fat utilization [15], the nutritional state seems to be a major determinant of release of ghrelin from the stomach.

Multiple actions of ghrelin

Since its discovery, many studies were conducted and it has been demonstrated that ghrelin has multiple biological actions, all of which could affect the cardiovascular system.

Activation of GH/IGF-1 pathway

Ghrelin activates the pathway of GH and its mediator, insulin-like growth factor-1 (IGF-1), both of which are anabolic hormones necessary for skeletal and myocardial growth and for metabolic homeostasis. Since GH/IGF-1 exerts effects on cardiac structure and function, ghrelin can affect the cardiovascular system through the elevation of plasma GH levels.

Stimulation of appetite

Endogenous ghrelin and its receptor are involved in the regulation of food intake and adiposity. Intravenous infusion of ghrelin is reported to increase food intake and body weight in healthy subjects [16—18] and to stimulate appetite and food intake in patients with congestive heart failure [19], chronic obstructive pulmonary disease [20], cancer [21], functional dyspepsia [22], and anorexia nervosa [23]. Recently, in a prospective randomized, placebo-controlled, clinical trial, it was suggested that administration of ghrelin after esophagectomy increased oral food intake, attenuated weight loss, and improved decreased lean body weight after operation [24]. Cachexia, which is a catabolic state characterized by weight loss and muscle wasting, is associated with hormonal changes and cytokine activation in severely sick patients. Since ghrelin causes a positive energy balance through GH-dependent and independent mechanisms, it could improve cachexia due to severe pathological conditions as seen in many end-stage diseases. In fact, in ghrelin-treated cachectic patients with congestive heart failure, increases in body weight, in lean body mass, and in muscle strength are reported [19]. Therefore, it is conceivable that ghrelin administration can be a novel therapeutic approach for cachexia in humans.

Direct cardiovascular action

Ghrelin is demonstrated to dilate human artery [25] and the action is endothelium-independent. In addition, ghrelin inhibits apoptosis of cultured cardiomyocytes and endothelial cells possibly through activation of extracellular signal-regulated kinase-1/2 and Akt serine kinases [26]. Together with the localization of GHS-R in the cardiovascular system, these results suggest that ghrelin may act directly on the cardiovascular system.

Anti-inflammatory action

Ghrelin suppresses the production of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α. In addition, ghrelin inhibits the activation of nuclear factor-κB (NF-κB), a transcriptional factor regulating the gene expression of pro-inflammatory cytokines [27]. In patients with pulmonary infections, it is reported that chronic administration of ghrelin is reported to decrease inflammatory cytokine levels. The inhibition of the release of proinflammatory

cytokines could be mediated at least partly by the ghrelin-induced activation of the vagus nerve. Since cardiovascular diseases are often accompanied by an augmented inflammatory response, ghrelin may exert its protective actions through these anti-inflammatory potentials.

Suppression of sympathetic nerve

As described below, ghrelin potently inhibits sympathetic nerve which is often over-activated in cardiac diseases.

Cardiovascular actions of ghrelin

Since the administration of ghrelin has been demonstrated to decrease blood pressure, reduce cardiac afterload, and increase cardiac output without affecting heart rate in humans and in animals, the therapeutic potentials of ghrelin in cardiac diseases have been speculated. In addition, ghrelin potently stimulates GH release from the pituitary gland, improves energy balance, and modulates the autonomic nervous system, all of which could have beneficial effects on the cardiovascular system. Moreover, the receptor for ghrelin, GHS-R, can be demonstrated in the cardiac ventricles and in the blood vessels, suggesting that ghrelin might have direct cardiovascular actions.

Ghrelin in cardiovascular diseases

Heart failure

In rats with heart failure, chronic ghrelin treatment improved cardiac systolic dysfunction [28]. In addition, in patients with congestive heart failure, intravenous administration of ghrelin (2 μg/kg, twice a day) for three weeks significantly improved left ventricular ejection fraction (from 27% to 31%; $p < 0.05$), and increased peak workload and peak oxygen consumption during exercise, which was accompanied by a dramatic decrease in plasma norepinephrine (from 1132 to 655 pg/mL; $p < 0.001$) [19]. The therapeutic potential of ghrelin is, therefore, suggested in heart failure patients.

Myocardial infarction

Left ventricular remodeling after myocardial infarction is often associated with subsequent heart failure, which could lead to a fatal outcome. In a rat model of experimental myocardial infarction, peripheral ghrelin administration attenuated left ventricular dysfunction and remodeling was examined as described below.

Chronic treatment: subcutaneous administration of ghrelin at a dose of 100 μg/kg twice a day for two weeks significantly improved left ventricular enlargement induced by myocardial infarction. In addition, there was a substantial improvement in cardiac function parameters in ghrelin-treated rats compared with saline-treated controls. Furthermore, ghrelin attenuated an increase in interstitial fibrosis in the non-infarct region. Importantly, the infarction-induced increase of heart rate was suppressed completely in ghrelin-treated animals [29].

Acute treatment: whether one bolus subcutaneous injection of ghrelin 1 min after the coronary ligation leads to a beneficial effect during the acute phase was next examined using a rat model of myocardial infarction [30]. Surprisingly, the high mortality rate after myocardial infarction was significantly reduced by the early bolus of ghrelin administration [61% in saline-treated rats vs 23% in ghrelin-treated rats ($p < 0.05$)]. In addition, mortality due to fatal arrhythmias was also improved by the ghrelin treatment. Furthermore, the ghrelin-treated group had significantly fewer arrhythmic insults by the second to third hour after myocardial infarction [30]. The results show that one bolus of ghrelin treatment early after myocardial infarction improves survival after myocardial infarction by preventing the increase in frequency of ventricular arrhythmias.

Myocardial ischemia/reperfusion injury

It is reported that administration of ghrelin protects the heart against ischemia/reperfusion injury [31]. The cardioprotective effects of ghrelin are independent of GH release and likely involve binding of the peptide to receptors in the heart. The anti-apoptotic effect of ghrelin via the ERK 1/2 and PI3K/Akt-dependent pathway could potentially contribute to the beneficial effect of ghrelin infusion on myocardial ischemia/reperfusion injury.

Pulmonary hypertension

Whether ghrelin would impede pulmonary arterial hypertension during chronic hypoxia has been examined [32]. Conscious male Sprague Dawley rats were housed in a hypoxic chamber (10% oxygen) and received daily subcutaneous injection of ghrelin. While, in saline-treated rats, chronic hypoxia significantly elevated pulmonary arterial pressure and increased wall thickness of peripheral pulmonary arteries, the hypoxia-induced development of pulmonary arterial hypertension (110% increase in control vs 48% increase in ghrelin group), pulmonary vascular remodeling was significantly attenuated in ghrelin-treated animals. Therefore, the therapeutic benefits of ghrelin for pulmonary hypertension are suggested, particularly in subjects prone to chronic hypoxia.

Sympathetic inhibitory action of ghrelin

Recently, the effects of ghrelin on blood pressure, sympathetic nervous system activity, and mental stress responses were investigated in lean and overweight or obese individuals and it was found that stress-induced significant increase in these parameters were significantly reduced by 1 h intravenous infusion of ghrelin irrespective of obese phenotype [33]. In addition, administration of ghrelin significantly suppressed heart rate increase and ghrelin significantly suppressed plasma norepinephrine level in both humans and animals. Furthermore, it has been reported that the intracerebroventricular administration of ghrelin inhibited the sympathetic nerve activity [34].

Using a rat model of myocardial infarction, we investigated the beneficial effect of peripheral subcutaneous

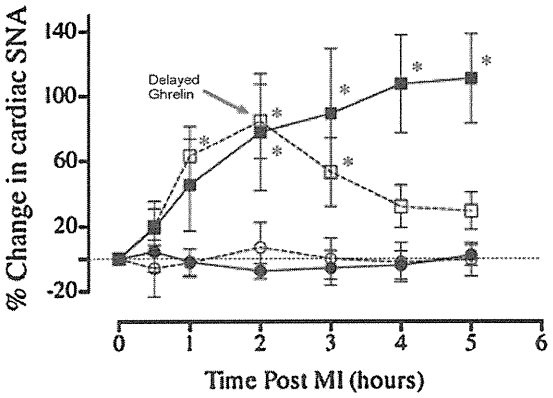

Figure 2 The sympathetic inhibitory effect of ghrelin after myocardial infarction. Transient responses in cardiac sympathetic nervous activity (SNA; percent increase in cardiac SNA of integrated area of the raw nerve signal) in sham rats (closed circle) and three groups of myocardial infarction (MI) rats: untreated (closed square); ghrelin treated immediately after myocardial infarction (open circle); and ghrelin treated 2 h after MI (open square). Either immediately after MI or 2 h after MI, ghrelin treatment effectively reduces the up-regulated SNA. *Significantly different from before MI (time ''0'') ($p < 0.05$).
Adapted from Schwenke et al. [30].

ghrelin administration. Direct recording of cardiac sympathetic nerve activity revealed that ghrelin administration prevents an increase in cardiac sympathetic nerve activity as shown in Fig. 2. Importantly, the effect of ghrelin was accompanied by a reduction in mortality [30]. In acute myocardial infarction, the initial increase in the cardiac sympathetic nervous activity often leads to the fatal ventricular arrhythmia. The results, therefore, suggest that ghrelin-induced attenuation of the early increase in cardiac sympathetic nerve activity could potentially improve cardiac prognosis.

Ghrelin signaling through cardiac vagal afferent pathway

Interestingly, the orexigenic effect of peripherally administered ghrelin was suppressed by ligation of the gastric branch of the vagal nerve [8] or by pre-treatment with capsaicin, a neurotoxin specific for sensory afferent, indicating that vagal sensory afferent mediates the appetite promoting effect of peripherally administered ghrelin [8]. Furthermore, when ghrelin was microinjected into the nucleus of the solitary tract, the brain region important for controlling the autonomic nervous system, there was observed significant decreases in heart rate and mean arterial pressure [35]. In addition, GHS-R is shown to localize on the nerve terminals within the heart [9]. Furthermore, the sympathoinhibitory effect of intravenous administration of ghrelin was abolished in post-gastrectomy vagotomized patients, suggesting the vagus nerve is important for the effects of peripheral ghrelin [36]. Taken together, by acting on the vagal afferent nerve, which sends signals to the vasomotor center of the medulla via the nucleus of the solitary tract, ghrelin might exert its potent sympathetic inhibitory action

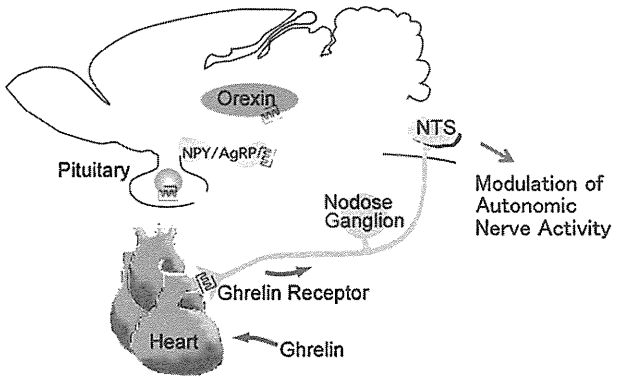

Figure 3 The signaling pathway in autonomic modulatory actions of ghrelin. Ghrelin acts on the cardiac vagal afferent nerve terminals, which send signals to the vasomotor center of the medulla through the nucleus of the solitary tract (NTS), which inhibits the sympathetic nerve activity and protects the heart from excessive damage.
Adapted from Kishimoto et al. [9,37].

resulting in decreases in sympathetic activity and in heart rate elevated after myocardial infarction (Fig. 3).

Conclusion

As described above, ghrelin has potent cardioprotective actions in diseases such as heart failure, myocardial infarction, pulmonary hypertension, and fatal arrhythmias through various mechanisms including GH release, direct actions on cardiovascular cells and inhibition of the sympathetic nervous activity. Since ghrelin is an endogenous hormone, it has advantages over other medications. It is, therefore, suggested that ghrelin can be a promising new treatment for cardiac diseases.

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ORIGINAL ARTICLE

Enhanced Gastric Ghrelin Production and Secretion in Rats with Gastric Outlet Obstruction

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Abstract
Background and Aim Ghrelin has distinct effects on gastrointestinal motility through the vagus nerve and gastric excitatory neural plexus. The objectives of this study were to investigate the dynamics of ghrelin and expression of neuromuscular markers in a newly established surgically manipulated rat model of gastric outlet obstruction (GOO), akin to the pyloric stricture associated with duodenal ulcer, advanced gastric cancer, and other conditions, in the clinical setting.
Material and Methods The rats were divided into two groups, a control group (sham operation) and the GOO group (proximal duodenal stricture). The animals were sacrificed 2 weeks after the operation. Plasma and gastric ghrelin were measured by radioimmunoassay. mRNA expression in the stomach of neural choline acetyltransferase (ChAT), c-kit, and membrane-bound stem cell factor (SCF) were analyzed by quantitative RT-PCR. In addition, gastric mRNA expression of the aforementioned were also evaluated 60 min after intraperitoneal administration of a synthetic GHS-R1a antagonist ([D-Lys3] GHRP-6 6.0 mg/kg).
Results Mechanical GOO induced increases of fasting plasma ghrelin levels and hyperplasia of the gastric muscle layers, with enhanced expression of the gastric neuromuscular markers. Administration of [D-Lys3] GHRP-6 normalized the enhanced expression of c-kit and SCF.
Conclusion GOO stimulates ghrelin dynamics and then enhances the mechanistic expression of gastric cellular communication network molecules between nerves and smooth muscle cells.

Keywords Ghrelin · Gastric emptying · Motility · Gastric outlet obstruction

Introduction

Ghrelin, a 28-amino-acid motilin-related peptide, was first purified from the rat stomach as a natural ligand for the growth hormone secretagogue receptor (GHSR) [1]. It has also been shown to stimulate food intake, induce body weight gain, and enhance gastric motility. Recent animal studies have shown that ghrelin has distinct effects on gastrointestinal motility, which may be mediated through the GHSR expressed on the vagus nerve and enteric nerve endings [2, 3]. Ghrelin has been reported to enhance gastric motility and accelerate gastric emptying in rats and mice and to stimulate small intestinal transit [2, 4]. Fujino et al. reported that ghrelin induces accelerated motor activity of the gastrointestinal tract via ghrelin receptors expressed on vagal afferent nerve terminals and activated neuropeptide Y neurons in the brain [5].

Chasen et al. reported that abnormal electrogastrography diagnosis and increased levels of plasma ghrelin were

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found in most patients with advanced cancer [6]. We have previously shown increased fasting plasma levels of ghrelin in patients with functional dyspepsia (FD), especially those with dysmotility-like FD, possibly originating from gastric motility disorders, including delayed gastric emptying [7]. Although the precise molecular mechanisms are not yet clear, such clinical manifestations suggest that ghrelin production might be increased by impaired gastric motility.

The objectives of this study were to investigate the dynamics of ghrelin and expression of neuromuscular markers in a newly established surgically manipulated rat model of gastric outlet obstruction (GOO), akin to the pyloric stricture associated with duodenal ulcer, advanced gastric cancer, and other clinical conditions.

Methods

Animal Procedures

This study was conducted with the approval of Keio University Animal Research Committee (no. 056188). Seven-week-old male specific-pathogen-free (SPF) Sprague–Dawley (SD) rats (Sankyo Laboratory Service, Tokyo, Japan) were used for the study after acclimatization for 7 days in an animal room at a controlled temperature (24 ± 2°C). The rats were fed standard chow and deprived of food for 24 h before the operation. After induction of anesthesia by intraperitoneal injection of 50 mg/kg sodium pentobarbital, the hair was shaved off the upper abdomen of the animals. The abdomen was opened via a 25-mm-long median incision, and the stomach was exposed. The proximal duodenum was then carefully covered with an 18-Fr Nelaton catheter (diameter, 4.0 mm; Nippon Sherwood, Tokyo, Japan) and sutured with a 5–0 nylon thread (Fig. 1). The width of the catheter was 2.0 mm. This surgical duodenal stricture induced incomplete gastric outlet obstruction with gastric retention; this animal group was named the GOO group. After the operation, the animals were deprived of food for 24 h, but allowed free access to water. A sham operation, involving only abdominal incision, was also undertaken on the same number of rats of the control group.

Because weight loss is known to increase circulating levels of ghrelin, we designed a pair-feeding method to evaluate the dynamics of ghrelin without the confounding effect of body weight changes. The results of our preliminary experiments revealed that food intake in the GOO group was lower than that in the control group. We measured daily food intake in the GOO group, and the control group of rats received the same amount of food on the following day. After 2 weeks, after food deprivation for 24 h, the rats were sacrificed under ether anesthesia and the gastric wet weight and intraluminal pH were measured. The thicknesses of the gastric antral mucosal layer and muscular layer were measured by light-microscopic examination of hematoxylin–eosin-stained sections. The average thicknesses 1, 1.5, and 2.0 mm from the pylorus were measured.

Evaluation of Gastric Emptying

Our surgical procedure is the first reported method for establishing an animal model of GOO. We compared the gastric emptying rate in this model with that in another group of 7-week-old SPF male SD rats. The animals were divided into a control group and GOO group as previously described. Two weeks after the operation, the rats were deprived of food, but allowed free access to water for 24 h before the start of the subsequent experiment. The gastric emptying rate was measured by the phenol red (PR) method reported by Ohnishi et al. [8]. One milliliter of PR (100 μg/ml) was administered orally to the rats, and the rats were sacrificed by cervical dislocation either immediately (Control group, $n = 4$) or 15 min (Control group, $n = 4$; GOO group, $n = 6$) after administration of the PR solution. The standard stomachs (0 min) and test stomachs (15 min) were exposed and ligated at the cardia and pylorus. Each stomach was removed and placed in 10 ml 0.1 M Na_2HPO_4 solution, and the contents of the stomach were washed out. The absorbance of the supernatant was measured at 570 nm with a spectrometer (Shimadzu, Kyoto, Japan). The gastric emptying rate for each rat was calculated as described previously [8].

Measurement of the Ghrelin Dynamics

The rats were divided into two groups, the control group ($n = 14$) and the GOO group ($n = 14$). After 2 weeks, following food deprivation for 24 h, the rats were sacrificed under ether anesthesia. The abdomen and chest were opened via a median incision. Blood was collected from the heart and centrifuged at 3,000 rpm for 10 min to obtain plasma and serum samples for assay. After collection of the blood, the stomachs of the animals were excised and the intraluminal pH was examined by use of a pH meter (Horiba Techno Service, Kyoto, Japan). The stomachs were then cut along the greater curvature and rinsed with isotonic saline. The two radioimmunoassays (RIAs) for measurement of the gastric and plasma ghrelin levels were performed as described previously [9, 10]. Two polyclonal rabbit antibodies were raised against the N-terminal [1–11] (Gly1-Lys11) and C-terminal [13–28] (Gln13-Arg28) fragments of rat ghrelin. [Cys12]-rat ghrelin [1–11] (4 mg) and [Cys0]-rat ghrelin [13–28] (10 mg) were separately conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (mcKLH, Pierce, Rockford, IL, USA; 6 mg) in conjugation buffer (Pierce). Each conjugate was

emulsified with an equal volume of Freund's complete adjuvant. Two corresponding batches of antiserum were obtained by immunization of New Zealand white rabbits by subcutaneous injection. Using these antibodies, two types of RIAs to measure the plasma and gastric ghrelin levels were performed as described previously [11].

Immunohistochemistry for Ghrelin

Stomach samples were fixed in 10% formaldehyde neutral buffer solution for 24 h, then embedded in paraffin. Tissue sections were deparaffinized and hydrated, and endogenous peroxidase was quenched by treatment with 0.3% hydrogen peroxide for 20 min. Nonspecific binding was blocked by use of a blocking reagent (BlockAce; Dainippon Pharmaceuticals, Osaka, Japan). After washing with TBS-T, the tissue slices were incubated for 60 min at 4°C with anti-ghrelin antiserum (final dilution, 1:10,000). Then, after washing again with TBS-T, the slides were incubated with EnVision + Peroxidase rabbit (DAKO Japan, Kyoto, Japan) for 30 min at room temperature, and then visualized after color development with 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution for 1 min. The sections were then counterstained with hematoxylin. The stained sections were examined under high-power magnification (objective lens ×40) by light microscopy equipped with a 3CCD digital camera (C7780; Hamamatsu Photonics, Hamamatsu, Japan). The nuclei were counted using Image-J software (National Institutes of Health, Bethesda, Maryland, USA). The density of the ghrelin-immunoreactive cells was computed by use of the equation: Dghrelin = (Ng/Nt) × 100 (%), where Ng and Nt represent the number of ghrelin-immunoreactive cells and the total cell number, respectively, in the three mucosal regions of the stomach.

Preparation of Total RNA and Quantitative RT-PCR Analysis

Total mRNA was extracted from the stomach tissue by use of the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and DNase treatment was performed with an RNase-free DNase set (Qiagen). RNA was converted into cDNA by use of the PrimeScript RT reagent kit (Takara, Ohtsu, Japan). Cyber green quantitative real-time RT-PCR was performed to detect the mRNA using the Thermal Cycler Dice Real Time System (Takara Bio, Otsu, Japan). The primers used to amplify the target mRNA were: c-kit mRNA 5′-ATC CAG CCC CAC ACC CTG TT-3′, and 5′-TGT AGG CAA GAA CCA TCA CAA TGA-3′, SCF (membrane-bound isoform) mRNA: 5′-TGA GAA AGG GAA AGC CGC-3′, and 5′-TAA GGC TCC AAA AGC AAA GC-3′, choline acetyltransferase (ChAT) mRNA: 5′-CAA CCA TCT TCT GGC ACT GA-3′, and 5′-TAG CAG GCT CCA TAG CCA TT-3′, glyceraldehyde-3-phosphate (GAPDH) mRNA: 5′-GGC ACA GTC AAG GCT GAG AAT G-3′, and 5′-ATG GTG GTG AAG ACG CCA GTA-3′. The target mRNA expression levels were normalized to the GAPDH mRNA expression levels.

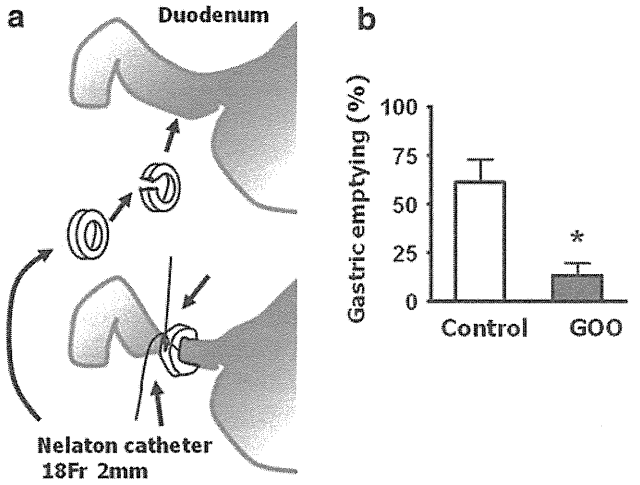

Fig. 1 **a** Surgical manipulation for induction of duodenal stricture. The proximal duodenum was covered with a small piece of an 18Fr-Nelaton catheter. **b** Liquid gastric emptying rates of control rats ($n = 4$, *open bar*) and GOO rats ($n = 6$, *filled bar*) 2 weeks after the operation (mean ± S.E.M. $^*P < 0.05$ compared with control)

RT-PCR Analysis for Interstitial Cells of the Cajal (ICC) Network Using a GHSR1a Antagonist

In a separate experiment, to examine the relationship between ghrelin and neuromuscular marker expression, the rats were administered an intraperitoneal injection of [D-Lys3] GHRP-6, a GHSR antagonist, (6.0 mg/kg; Bachem, King of Prussia, PA, USA) in 1 ml saline ($n = 8$), or saline alone ($n = 8$) and then euthanized 60 min after administration. Then, the mRNA expression levels of c-kit, SCF, and ChAT were analyzed by quantitative RT-PCR, as previously described.

Statistical Analysis

All results are expressed as mean ± SE, and the statistical analysis was performed using the Student t test (two-tailed test) with Stat Mate III (Atoms, Tokyo, Japan). P values less than 0.05 were considered to be statistically significant.

Results

Gastric Emptying Rate in this Rat Model

The gastric emptying rate after 15 min as measured by the PR method was significantly lower in the GOO group than

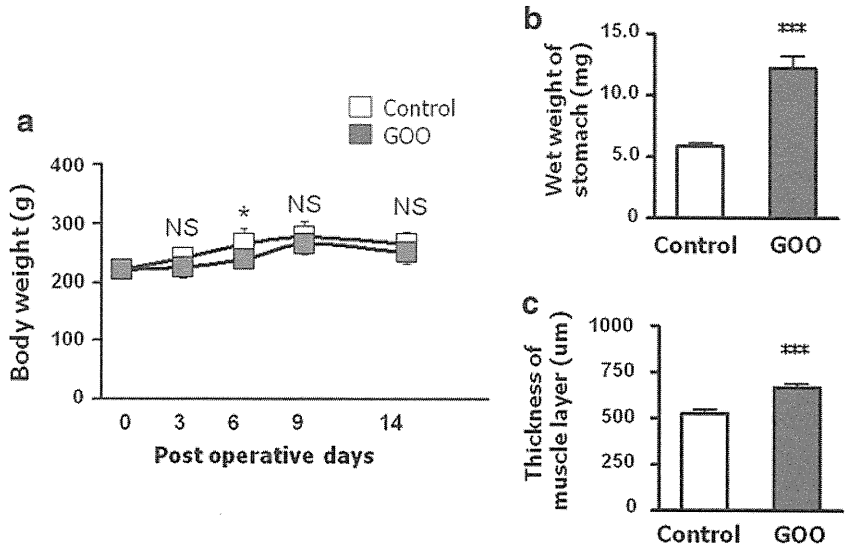

Fig. 2 **a** Body weight was measured 0, 3, 6, 9, and 14 days after the operation. On days 9 and 14 after the operation, no significant differences in the changes of the body weights were observed between the two groups. The mean weight of the rats on the operation day was 220.2 ± 4.5 g in the control group and 218.2 ± 2.0 g in the GOO group. **b** Wet weight of the removed stomach 2 weeks after operation. **c** Thickness of the muscle layer at the antrum using HE stain. (mean ± S.E.M.; $n = 13$ in each group.*$P < 0.05$, ***$P < 0.001$ compared with control.)

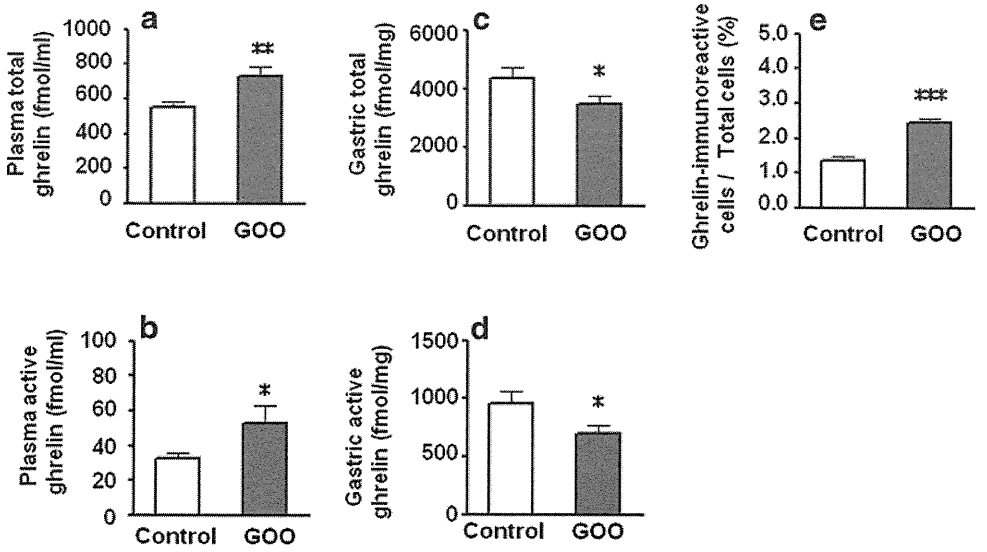

Fig. 3 Fasting levels of total (**a**) and active (**b**) plasma ghrelin concentrations and of the total (**c**) and active (**d**) gastric ghrelin contents were measured (**a–d**). The plasma ghrelin levels were increased in the GOO group, whereas gastric ghrelin levels decreased. **e** Immunohistochemistry for ghrelin. The density of the ghrelin-immunoreactive cells in the gastric corpus (mean ± S.E.M.; $n = 13$ in each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control.)

in the control group (21.8 ± 7.8% vs. 61.0 ± 12.0%, $P = 0.011$; Fig. 1b).

Gastric Wet Weight and Thickness After Outlet Obstruction

In this model of GOO, 2-weeks survival in the GOO group was 92.9% ($n = 13/14$); survival in the control group was 92.9% ($n = 13/14$). The changes in the mean weights of the rats are shown in Fig. 2a. After 14 days of obstruction, the gastric wet weight in the GOO group was increased compared with that in the control group (12.2 ± 3.3 g vs. 5.8 ± 1.1 g, $P < 0.001$; Fig. 2b). The thickness of the gastric antral muscle layer was significantly higher in the GOO group than in the control group (675.4 ± 24.6 μm vs. 558.5 ± 20.8 μm, $P < 0.005$; Fig. 2c). Similarly, the thickness of the gastric antral mucosal layer was also significantly increased in the GOO group compared with that in the control group (322.0 ± 26.4 μm vs. 196.8 ± 7.9 μm, $P < 0.001$). In contrast, there was no significant difference in the fasting intraluminal pH of the stomach between the two groups (control group, pH 1.70 ± 0.13 vs. GOO group, pH 1.70 ± 0.26).

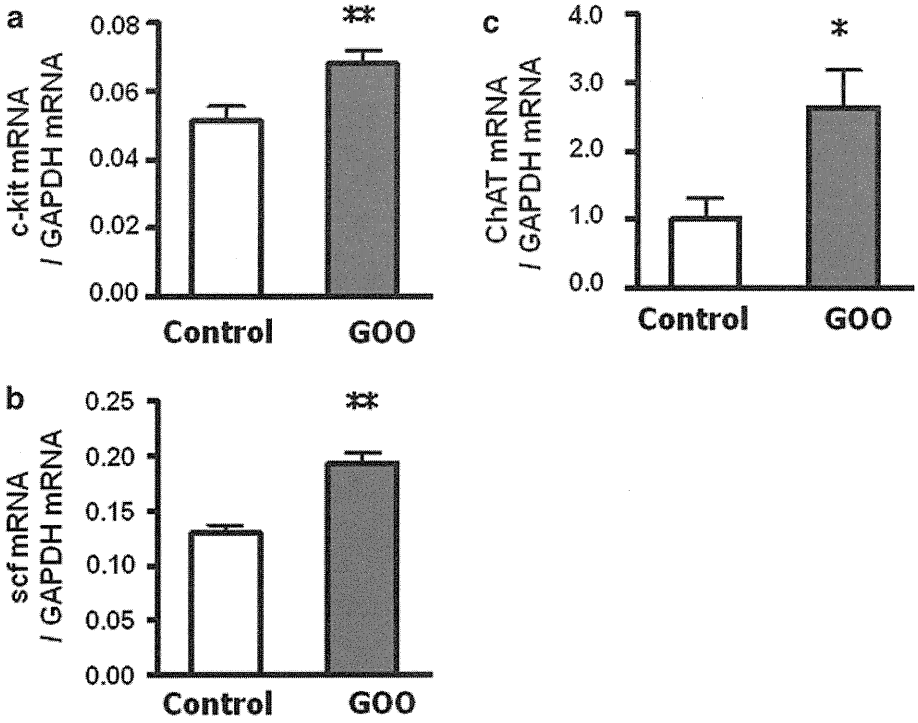

Fig. 4 Gastric c-kit, membrane-bound SCF, ChAT mRNA expression was measured by RT-PCR. **a** gastric c-kit mRNA; **b** membrane-bound SCF mRNA; **c** ChAT mRNA. (mean ± S.E.M.; $n = 13$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control)

Ghrelin Dynamics

The ghrelin dynamics 2 weeks after the operation are shown in Fig. 3. The plasma total and active ghrelin levels were higher in the GOO group than in the control group (plasma total ghrelin; $P = 0.002$, plasma active ghrelin; $P = 0.024$; Fig. 3a, b). In contrast, the gastric total and active ghrelin levels were lower in the GOO group than in the control group (gastric total ghrelin; $P < 0.001$, gastric active ghrelin; $P < 0.001$; Fig. 3c, d). The results of the immunohistochemical analysis to determine the density of the ghrelin-immunoreactive cells in the gastric corpus are shown in Fig. 3e. Increase in the cell count ratio in the GOO group compared with that in the control group was observed ($P < 0.001$).

Gastric Neuromuscular Marker Expression

The mRNA expression levels of these markers as assessed by quantitative RT-PCR analysis are shown in Fig. 4. Significant increases of the expression levels of ChAT mRNA (263.9 ± 54.0% compared with control $P = 0.019$), c-kit mRNA (132.5 ± 7.2% compared with control, $P = 0.008$), and SCF mRNA (149.4 ± 7.8% compared with control, $P < 0.001$) were observed in the GOO group compared with expression levels in the control group.

Effects of [D-Lys3] GHRP-6 on c-Kit and SCF Expression

Significant decreases of expression of c-kit mRNA (54.8 ± 10.3%, $P = 0.024$) and membrane-bound SCF mRNA (51.8 ± 7.5%, $P = 0.009$) were observed in the GOO group after pretreatment with [D-Lys3] GHRP-6 compared with those in the control group (Fig. 5a, b).The expression of ChAT mRNA also tended to decrease to the control level.

Discussion

This study showed experimentally that GOO induced an increase of the fasting plasma ghrelin levels and hyperplasia of the gastric muscle layers. Associated with these pathological processes, expression of ChAT, a marker of vagal efferent fibers in the stomach, c-kit, a marker of the interstitial cells of Cajal (ICC), and SCF, a c-kit ligand on the gastric muscles, were all significantly enhanced.

Two signal transmission pathways from secreted ghrelin to the myenteric plexus in the stomach have been reported [12, 13]. One is a direct route in which ghrelin directly stimulates the GHSR on the surface of the myenteric neurons [12]; the other is an indirect route in which ghrelin signaling stimulates GHSR on the vagal afferent fibers, with the vagal signal traveling through the central nervous system and then to the vagal efferent nerve fibers, finally

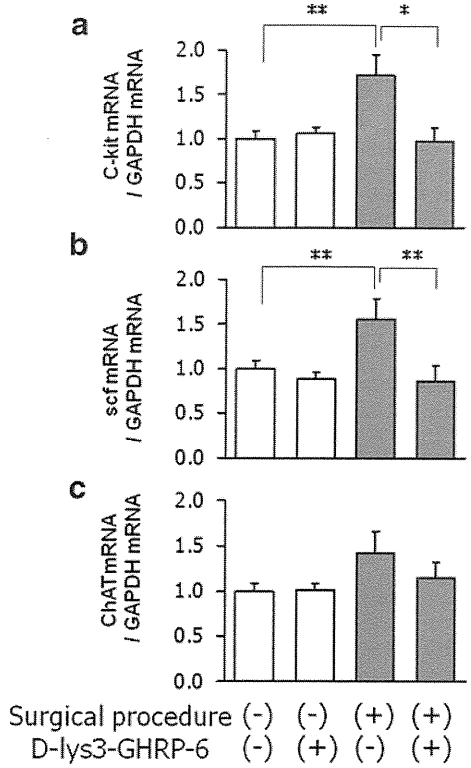

Fig. 5 Effect of synthetic GHS-R1a antagonist ([D-Lys3]-GHRP-6 6.0 mg/kg) on mRNA expression of gastric c-kit (**a**), membrane-bound SCF (**b**) and ChAT (**c**) at 2 weeks after the operation. (mean ± S.E.M.; $n = 8$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control)

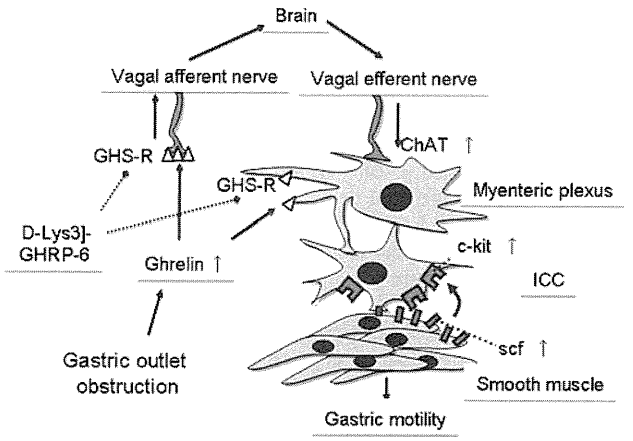

Fig. 6 Model of the association of ghrelin with the ICC network in the present GOO model. Signal of enhanced levels of plasma ghrelin in the GOO model is transmitted to the brain via vagal afferent nerves. Enhancement of ChAT mRNA might be induced via vagal efferent nerves from the central nervous system. Sustained enhanced ghrelin secretion might be associated with the activated ICC network in this animal model. The compensative ghrelin secretion and production are enhanced by gastric outlet obstruction, and enhanced ghrelin activates the ICC network either through the vagal nerve or the direct effect of ghrelin

activating the myenteric plexus [13]. In this study, because not only c-kit and SCF expression but also expression of ChAT were enhanced (Fig. 4), the ghrelin signal might be transmitted not only through the direct route, but also via the indirect route (Fig. 6).

Because [D-Lys3] GHRP-6, a receptor antagonist of GHSR1a, normalized the enhanced expression levels of c-kit and SCF (Fig. 5), it seems that ghrelin signaling might be an upstream event in relation to other neuromuscular activation markers, for example vagal efferent choline acetyl transferase (ChAT), c-kit (ICC), and SCF (gastric smooth muscle).

Both the increase in the plasma ghrelin levels and in the number of ghrelin-immunoreactive cells in the gastric corpus clearly indicates the activation of ghrelin production under the state of GOO (Fig. 3a, b, e). On the other hand, the decreased gastric ghrelin content in GOO (Fig. 3c, d) might be because of emptying (degranulation) of ghrelin from the A-like cells of the stomach in response to fasting. A similar phenomenon has already been reported in the Mongolian gerbil model of *Helicobacter pylori* infection [14].

Whereas the rat model of diabetic gastroparesis induced by STZ showed vagal denervation [15] and the rat model of ischemia–reperfusion induced transient gastroparesis showed vagal and c-kit damage [16], this method of induction of mechanical GOO was superior to the above-mentioned methods, because it involved simple obstruction of gastric outflow without vagal denervation or drug administration. Therefore, we could observe the neurological or hormonal feedback in simple outlet obstruction under the condition of intact gastric mucosa, vagal nerve, and gastric nerve plexus.

In conclusion, this study provides the first evidence to suggest that the production and secretion of gastric ghrelin is increased in rats with GOO, implying that dysregulation of gastric motility may alter the ghrelin dynamics, as reported in clinical settings [7, 17]. This experimental rat model is not only useful for study of GOO, but also for that of chronic gastric emptying disorders, for example gastric paresis or FD, especially postprandial distress syndrome.

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Effects of calcium salts of long-chain fatty acids and rumen-protected methionine on plasma concentrations of ghrelin, glucagon-like peptide-1 (7 to 36) amide and pancreatic hormones in lactating cows

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Abstract

Our objective was to determine the effects of calcium salts of long-chain fatty acids (CLFAs) and rumen-protected methionine (RPM) on plasma concentrations of ghrelin, glucagon-like peptide-1 (7 to 36) amide, and pancreatic hormones in lactating cows. Four Holstein cows in midlactation were used in a 4 by 4 Latin square experiment in each 2-wk period. Cows were fed corn silage-based diets with supplements of CLFAs (1.5% added on dry matter basis), RPM (20 g/d), CLFAs plus RPM, and without supplement. Jugular blood samples were taken from 1 h before to 2 h after morning feeding at 10-min intervals on day 12 of each period. CLFAs decreased dry matter intake, but RPM did not affect dry matter intake. Both supplements of CLFAs and RPM did not affect metabolizable energy intake and milk yield and composition. Plasma concentrations of NEFAs, triglyceride (TG), and total cholesterol (T-Cho) were increased with CLFAs alone, but increases of plasma concentrations of TG and T-Cho were moderated by CLFAs plus RPM. Calcium salts of long-chain fatty acids increased plasma ghrelin concentration, and the ghrelin concentration with CLFAs plus RPM was the highest among the treatments. Plasma concentrations of glucagon-like peptide-1, glucagon, and insulin were decreased with CLFAs, whereas adding RPM moderated the decrease of plasma glucagon concentration by CLFAs. These results indicate that the addition of methionine to cows given CLFAs increases plasma concentrations of ghrelin and glucagon associated with the decrease in plasma concentrations of TG and T-Cho.

Keywords: Dairy cow; Fatty acid; Ghrelin; Glucagon-like peptide-1 (7 to 36) amide; Methionine

1. Introduction

Fat supplementation in diets for milk production is valid to increase dietary energy density [1]. However, increasing the dietary fat content often depresses dry matter intake (DMI) of lactating cows [2]. The mech-

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anism of hypophagia induced by fat supplementation is incompletely understood and assumed to be affected by various factors that involve the absorption process and metabolism of fatty acids. Methionine (Met) improves lipid metabolism [3] partially by altering the activities of hormone-sensitive lipase and lipoprotein lipase [4]. In dairy cows, Met is the first limiting amino acid for milk production [5]. Supplementation of Met hydroxyl analog increases milk fat synthesis with hypertriglycemia [6]. Conversely, an insufficiency of Met during the periparturient period results in the development of hepatic lipidosis [7]. These reports suggest that Met may affect lipid metabolism in dairy cows. In lactating ewes, supplementation of rumen-protected Met (RPM) combined with fat increased DMI, milk yield, and milk fat secretion compared with the supplementation of fat only [8], but the effects of Met on plasma metabolite and hormone concentrations were unknown. Recently, Relling and Reynolds [9,10] reported changes in plasma concentrations of some gut hormones, such as glucagon-like peptide-1 (7 to 36) amide (GLP-1) and ghrelin, caused by several nutrients in lactating cows, partly because of their role in the regulation of feed intake. In addition, these gut hormones were reported to influence insulin and glucagon secretion [11,12]. Therefore, in this study we evaluated the effects of adding calcium salts of long-chain fatty acids (CLFAs) and RPM on plasma metabolite and hormone concentrations in lactating cows.

2. Materials and methods

The procedures used in the present study were performed in accordance with the principles and guidelines for animal use issued by the National Institute of Livestock and Grassland Science Animal Care Committee and were formulated to comply with Japanese regulations.

2.1. Animals and management

One primiparous and three multiparous Holstein cows (143.5 ± 3.9 d in milk; calving number, 1.75 ± 0.25; initial body weight, 547.3 ± 14.7 kg) were fed four diets formulated to meet the nutrient requirements according to the Japanese Feeding Standard for Dairy Cattle [13]. The ingredients and composition of the diets are presented in Table 1. The cows were managed in individual tie stalls, allowed free access to water, and provided experimental diets twice daily at 9:00 AM and 6:00 PM. They were milked twice daily before each feeding (8:40 AM and 5:40 PM), and weighed every week.

Table 1
Ingredient and chemical composition of the diets.

Item	Non-CLFAs	CLFAs
Ingredient (DM basis)		
Corn silage (%)	42.2	41.6
Alfalfa hay cubes (%)	7.60	7.48
Sudan grass hay (%)	5.68	5.60
Concentrate[a] (%)	43.9	43.2
Calcium salts of fatty acids[b] (%)	0.00	1.50
Calcium carbonate (%)	0.60	0.60
Salt (%)	0.01	0.01
Vitamin premix (%)	0.01	0.01
Chemical composition (DM basis)		
OM (%)	93.0	92.7
CP (%)	14.1	13.9
NDF (%)	32.2	31.7
Crude fat (%)	3.24	4.45
Me[c] (Mcal/kg)	2.42	2.48

Abbreviations: CLFAs, calcium salts of long-chain fatty acids; DM, dry matter; ME, metabolizable energy; OM, organic matter.
[a] Concentrate contained 39% corn grain, 17% corn gluten feed, and 16% beet pulp pellets.
[b] Megalac; declared fatty acids contained 26% palmitic acid, 4% stearic acid, 33% oleic acid, 32% linoleic acid, and 5% linolenic acid.
[c] Estimated value was from the National Research Council [1].

2.2. Treatments and experimental design

The cows were used in a 4 by 4 Latin square design in each 2-wk period. Four treatments consist of basal diet only (without supplement), supplemented with CLFAs made from palm and soybean oil (Megalac R; Church & Dwight, Co, Inc, Princeton, NJ, USA), with RPM (Lactet SP; Nippon Soda, Co, Ltd, Tokyo, Japan), and with CLFAs plus RPM. The CLFAs containing 85% fatty acids were added to 1.5% of the diet dry matter, following a previous study [14]. The RPM containing 67% of D-, L-Met was supplemented at 20 g/d for RPM or CLFAs plus RPM treatment. Cows were offered each diet ad libitum, allowing for 15% refusal for the first 10 d for each treatment. For the last 4 d of each period, the cows were fed 95% of ad libitum intake for estimating metabolizable energy intake. Refusals were weighed daily before each morning feeding (8:00 AM).

2.3. Sampling

Samples of the diets and the refusals were collected and pooled for the last 4 d of each period. Milk samples were collected for the last 4 d of each period, added with sodium azide as preservative, and stored at 4°C until analysis. Blood samples were taken at 12 d from

the jugular vein catheter (Argyle 14 G CV catheter kit; Nippon Sherwood Medical Industries, Ltd, Tokyo, Japan) inserted on 10 d of each period. Blood samples (8 mL) were taken at 10-min intervals from 8:00 AM to 11:00 AM. Cows were milked during bleeding period (−20 and −10 min sampling time), and they were fed just after 0 min sampling. Blood samples were collected into heparinized tubes with aprotinin [500 kilo inhibitor unit/mL of blood; Trasylol; Bayer, Leverkusen, Germany], and centrifuged at 1,500 × *g* for 20 min at 4°C. Harvested plasma samples were stored at −80°C until assay.

2.4. Sample analysis

The diet samples and refusals were analyzed for dry matter, CP, NDF, crude fat, and crude ash contents according to the procedures of the Association of Official Analytical Chemists [15]. Metabolizable energy contents in the treatment diets were calculated according to requirements from the National Research Council [1].

Milk samples were measured for fat, protein, lactose, total solid (TS), and solids not fat (SNF) by infrared analysis [Milko-Scan (1344) A/BN; Foss Electric Company, Inc, Hillerod, Denmark].

Plasma concentrations of ghrelin, insulin, and GLP-1 were measured every 10 min by time-resolved fluoroimmunoassay.

Assay for bioactive ghrelin was conducted as described previously [16]. Ghrelin concentration was measured by competitive solid-phase immunoassay that used europium-labeled synthetic bovine ghrelin and polystyrene microtiter strips (Nalge Nunc Int, Tokyo, Japan) coated with anti-rabbit γ-globulin. Intra- and interassay CVs were 1.3% and 1.5%, respectively. Least detectable dose and 50% inhibitory concentration in this assay system were 0.025 and 0.831 ng/mL, respectively.

Insulin assay was conducted as described previously [17]. Insulin concentration was measured by competitive solid-phase immunoassay with the use of europium-labeled synthetic bovine insulin and polystyrene microtiter strips coated with anti-guinea pig γ-globulin. Intra- and interassay CVs were 2.2% and 1.8%, respectively. Least detectable dose and 50% inhibitory concentration in this assay system were 0.016 and 1.073 ng/mL, respectively.

Glucagon-like peptide-1 concentration was measured by competitive solid-phase immunoassay according to the method described by Sugino et al [16] with the use of rat GLP-1 (Peptide Institute, Inc, Osaka, Japan), europium-labeled rat GLP-1, polystyrene microtiter strips coated with anti-rabbit γ-globulin, and anti-human GLP-1 rabbit serum (1:20,000; Yanaihara Institute, Inc, Shizuoka, Japan). Intra- and interassay of CVs were 1.7% and 4.8%, respectively. Least detectable dose and 50% inhibitory concentration in this assay system were 0.024 and 0.172 ng/mL, respectively.

Plasma glucagon levels were measured every 20 min with the use of a commercially available RIA kit (glucagon assay kit;, Daiichi;, Radioisotope Co Ltd, Tokyo, Japan). Glucagon concentrations were measured in the same assay, and the intra-assay CV was 3.4%. Least detectable dose and 50% inhibitory concentration in this assay system were 15.6 and 275 pg/mL, respectively.

Plasma glucose concentrations were determined every 10 min with the use of a glucose analyzer (GA-1151; Arkray, Co, Ltd, Kyoto, Japan). Plasma concentrations of β-hydroxy butyrate (BHBA), NEFA, triglyceride (TG), total-cholesterol (T-Cho) and urea nitrogen (UN) were determined every 20 min with the use of an automated biochemistry analyzer (Beckman Coulter, Inc, Tokyo, Japan).

2.5. Statistics

Data for feed intake, milk yield, milk composition, and plasma amino acid concentrations were analyzed with the MIXED procedure of SAS (SAS Institute, Inc, Cary, NC, USA). The mixed model included treatment as a fixed effect and cow and period as random effects. For the statistical analysis of plasma hormone and metabolite concentrations, sampling time and sampling time by treatment were added to the model. Factorial contrasts were used to test the main effects of CLFA supplementation (CLFAs vs non-CLFA), RPM supplementation (RPM vs non-RPM), and their interaction. Results are reported as least squares means and SEM. Significant differences were set at $P < 0.05$.

3. Results

3.1. Feed intake, milk yield, and milk composition

Dry matter intake, milk yield, and milk composition are presented in Table 2. Dry matter intake in cows fed CLFA diets compared with cows fed non-CLFA diets was lower ($P = 0.025$), whereas metabolizable energy intake was not affected. No significant effect of RPM was observed on DMI and metabolizable energy intake. Milk yield and composition, except for total solid con-

Table 2
Feed intake, milk production, and composition during experimental period in lactating cows.

	Treatment							
	Non-CLFAs		CLFAs			Contrast P^a		
Item	Non-RPM	RPM	Non-RPM	RPM	SEM	CLFA	RPM	CLFAs × RPM
DMI (kg/d)	22.4	22.2	21.3	21.7	0.675	0.025	0.803	0.322
MEI (Mcal/d)	56.7	56.2	55.4	56.4	1.72	0.463	0.794	0.325
Milk yield (kg/d)	27.2	27.1	27.3	27.5	1.83	0.508	0.982	0.655
4% FCM[b] (kg/d)	29.9	28.8	30.0	30.3	1.63	0.505	0.766	0.574
Milk composition								
Fat (%)	4.46	4.35	4.48	4.42	0.44	0.825	0.685	0.909
Protein (%)	3.51	3.56	3.47	3.45	0.132	0.177	0.729	0.508
Lactose (%)	4.62	4.59	4.61	4.61	0.059	0.736	0.743	0.662
Total solid (%)	13.6	13.5	12.7	12.5	1.01	0.107	0.759	0.966
Solid nonfat (%)	9.13	9.11	9.08	9.11	0.124	0.736	0.973	0.710
Body weight changes (kg)	14.3	18.5	13.5	12.7	4.60	0.359	0.612	0.474

Abbreviations: CLFAs, calcium salts of long-chain fatty acids; DMI, dry matter intake; FCM, fat-corrected milk; MEI, metabolizable energy intake; RPM, rumen-protected methionine.
Note: Data are shown by least-squares means and SEMs.
[a] P value for factorial contrasts: CLFAs, RPM, and the interaction between CLFAs and RPM.
[b] 4% FCM (kg/d) = 0.4 × milk yield (kg/d) + 15 × Milk yield (kg/d) × milk fat (%).

tents, were unaffected by CLFAs and RPM. Milk total solid contents tended to be lower in cows fed CLFA diets than in cows fed non-CLFA diets ($P = 0.107$).

3.2. Plasma concentration of hormones and metabolites

The changes in plasma concentration of hormones and metabolites are presented in Figs. 1 and 2, respectively. Plasma concentrations of ghrelin, insulin, glucose, BHBA, NEFA, TG, and UN varied largely through time ($P < 0.002$; Figs. 1 and 2), and no significance of the interaction time by treatment was observed ($P > 0.192$). The time effect for plasma concentrations of ghrelin, glucose, and NEFAs was due to a postprandial decrease. Plasma concentrations of insulin, BHBA, TG, and UN increased after feeding.

The means of plasma ghrelin concentration were higher ($P < 0.001$; Table 3) in cows fed CLFAs than in cows not fed CLFAs. Compared with cows fed non-CLFAs, plasma concentrations of glucagon and insulin in cows fed CLFAs were lower ($P = 0.002$ and $P = 0.012$, respectively), and plasma GLP-1 concentration tended to be lower ($P = 0.061$). Plasma insulin concentration tended to be higher ($P = 0.092$) in cows supplemented with RPM than those without RPM. There were interactions between CLFAs and RPM for ghrelin and glucagon ($P = 0.002$ and $P = 0.041$, respectively): CLFAs plus RPM increased plasma ghrelin and glucagon, but RPM alone did not show such effects.

Compared with cows without CLFAs, plasma concentrations of NEFA, TG, T-Cho, and UN in cows fed CLFAs were higher ($P < 0.001$, $P = 0.029$, $P < 0.001$, and $P < 0.001$, respectively) but with lower plasma BHBA concentration ($P = 0.006$; Table 3). In cows fed RPM diets, plasma glucose concentration was higher ($P = 0.001$), plasma T-Cho concentration was lower ($P = 0.009$), and plasma NEFA and UN concentrations tended to be lower ($P = 0.15$ and $P = 0.121$, respectively). Interactions between CLFAs and RPM were observed for TG and T-Cho ($P = 0.049$ and $P = 0.003$, respectively): RPM decreased plasma concentrations of TG and T-Cho in cows fed CLFA but did not affect the cows not fed with CLFAs.

4. Discussion

High-fat inclusion in diets reduces fiber digestion, increases fatty acid absorption, inhibits abomasal motility [18], and increases gut hormone secretion [19,20]. Consequently, DMI tends to be depressed. CLFAs could prevent such negative effects on ruminal fermentation and fiber digestibility in lactating cows [21,22]. However, DMI was decreased by CLFAs in this study, consistent with other reports [23,24]. Rumen-protected Met did not improve such DMI depression by CLFAs contrary to results found by Goulas et al [8] who used lactating ewes. In addition, Chillard and Doreau [25] observed no improvement effect of RPM on DMI of cows fed a fish oil–supplemented diet during midlacta-

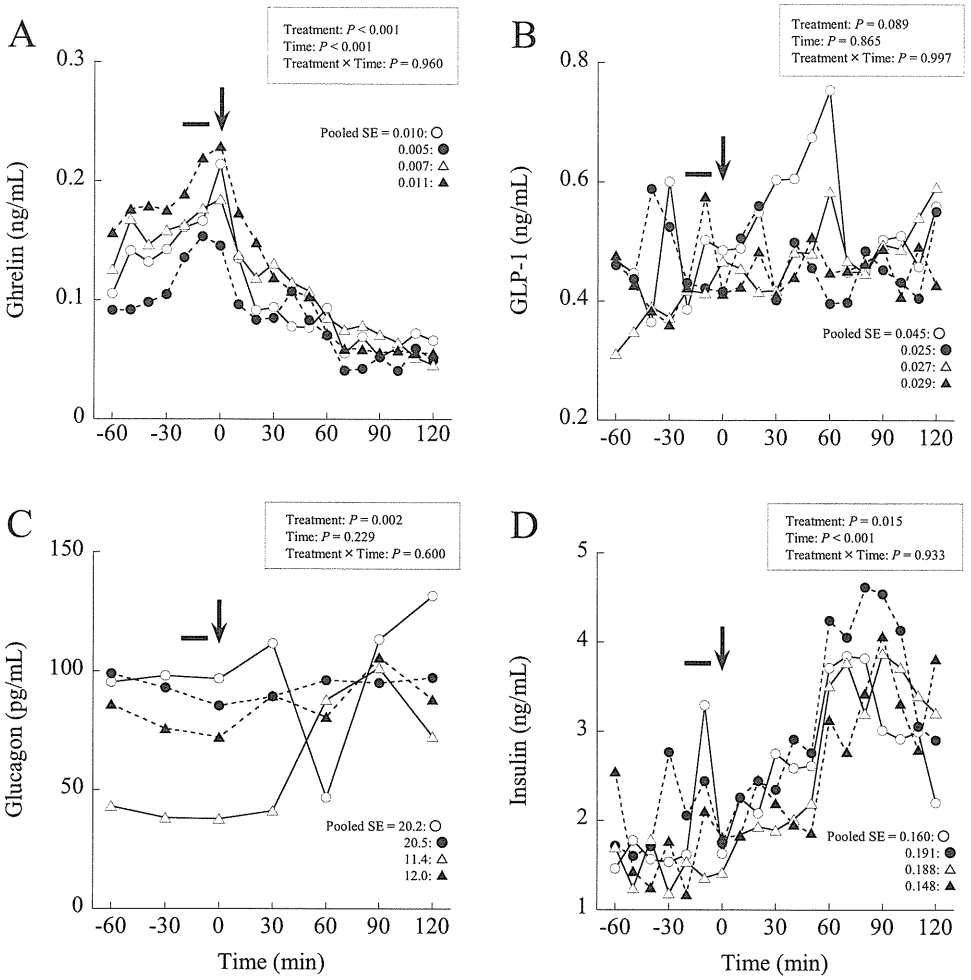

Fig. 1. Plasma concentrations of hormone [ghrelin, A; glucagon-like peptide-1 glucagon-like peptide-1 (7 to 36) amide (GLP-1), B, glucagon, C; and insulin, D] in cows fed each treatment diet: non–calcium salts of long-chain fatty acids (CLFAs) + non–rumen-protected methionine (RPM; ○ with a solid line), non-CLFAs + RPM (● with a dotted line), CLFAs + non-RPM (Δ with a solid line), and CLFAs + RPM (▲ with a dotted line). Values are expressed as least squares means (n = 4). Probability values are for effects of treatment, time, and the interaction between treatment and time. The horizontal bar and arrow show the milking period and feeding time, respectively.

tion, although such nonprotected fat, including high polyunsaturated fatty acids, could largely inhibit dietary fiber digestion and depress DMI.

The increase in plasma glucose concentration of cows fed RPM was consistent with Berthiaume et al [26] and inconsistent with Bertics and Grummer [27]. Such discrepancies might be explained by the differences in the physiological state of cows (mid vs early lactation) and energy balance. Plasma concentrations of NEFAs, T-Cho, and TG increased by CLFAs in the current study have been previously observed in cows [19,28]. By contrast, RPM in cows fed CLFAs decreased plasma T-Cho and TG concentrations and tended to decrease plasma NEFA concentration. Methionine is a methyl group donor for phosphatidylcholine in dairy cows [29] to enhance plasma lipoprotein. In laboratory animals, casein (a Met-rich protein) or L-Met induced hypercholesterolemia in rabbits and rats [30,31]. In calves, however, high-fat diet supplemented with L-Met did not increase plasma concentration of very low density lipoproteins [32], and Met hydroxyl analog did not affect hepatic TG accumulation in cows [27]. Whether Met enhanced very low density lipoproteins that were not measured in this study is unknown. By contrast, because Met is converted to taurine in dairy cows, plasma taurine concentration increased linearly with an increase of postruminal Met infusion [33]. Taurine conjugates with bile acids to become taurocholate in the liver and promotes lipid absorption and cholesterol consumption [34]. Yagasaki et al [3]