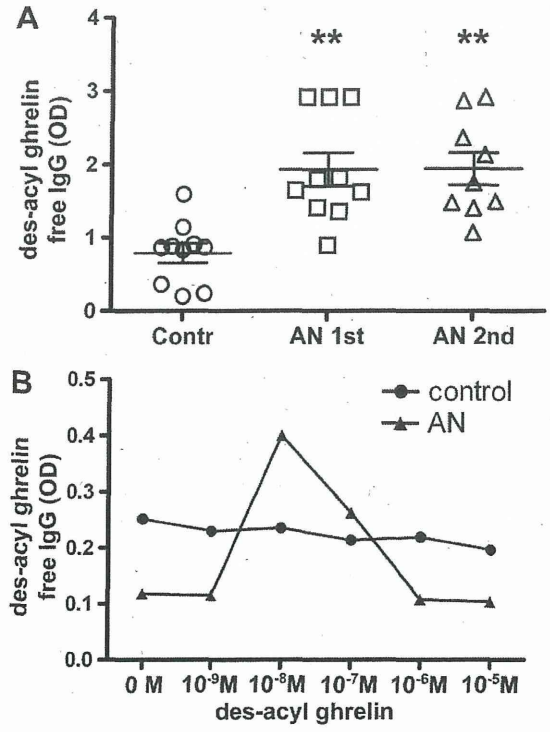

Fig. 4. (A) Plasma levels (mean ± SEM) of des-acyl ghrelin free IgG autoantibodies after absorption of sera with 10^{-8} M des-acyl ghrelin in control subjects ($n = 10$) and in patients with anorexia nervosa before ($n = 10$) and after refeeding ($n = 9$). (B) Effect of absorption of sera from one control and one patient with anorexia nervosa with different concentrations of des-acyl ghrelin on plasma levels of des-acyl ghrelin free IgG autoantibodies. ** $P < 0.01$, Tukey test versus Contr ($P = 0.0003$, analysis of variance). AN 1st, patients with anorexia nervosa at admission; AN 2nd, patients with anorexia nervosa 1 mo after refeeding; Contr, control subjects; IgG, immunoglobulin G; OD, optical density.

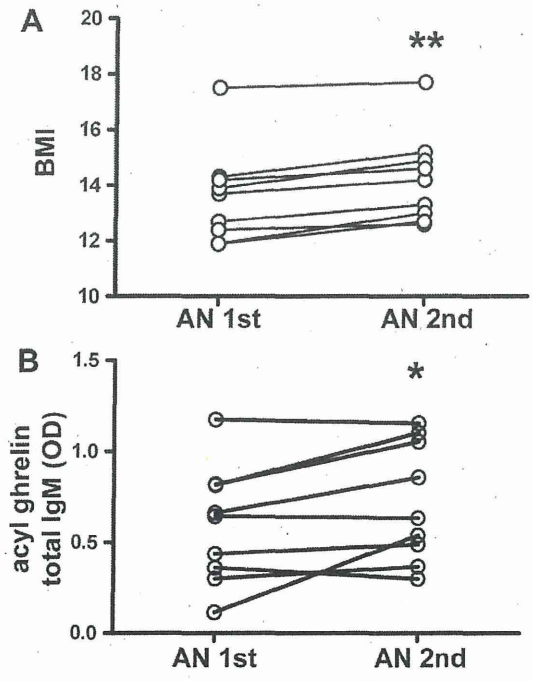

Fig. 5. Dynamics of changes in patients with anorexia nervosa ($n = 9$) before and after refeeding with respect to (A) BMI and (B) plasma levels of acyl ghrelin total IgM autoantibodies. ** $P < 0.01$, * $P < 0.05$, paired t tests. AN 1st, patients with anorexia nervosa at admission; AN 2nd, patients with anorexia nervosa 1 mo after refeeding; BMI, body mass index; IgM, immunoglobulin M; OD, optical density.

neuropeptide autoAbs has been reported for autoAbs reactive with α-melanocyte–stimulating hormone by potentiation of α-melanocyte–stimulating hormone-induced behavioral responses after acute stress [37]. Thus, it cannot be excluded that low levels of ghrelin autoAbs in AN may cause a deficit in ghrelin transport and decreased biological effects, i.e., an apparent ghrelin-resistance state.

Involvement of antihormone antibodies in hormonal resistance has been extensively studied as a putative mechanism underlying insulin resistance after exogenous insulin administration [38]. Insulin antibodies have been found to have blocking or transporting properties in different patients [39,40], probably as a reflection of their binding affinities [41]. The reason for such a dual response is not certain, but a role of insulin autoAbs before insulin therapy cannot be excluded [42]. Because a therapeutic use of synthetic ghrelin and its analogs for anorexia–cachexia treatment is forthcoming [43,44], the antighrelin antibody response is inevitable, and it is important to understand what will be the contribution of existing ghrelin autoAbs and of exogenous ghrelin-induced Abs in the functional activity of the ghrelin system.

In conclusion, our study showed that patients with AN display low levels of autoAbs reactive with acyl ghrelin and higher levels of autoAbs reactive with des-acyl ghrelin present as immune complexes. These data and the negative correlations found between plasma levels of ghrelin autoAbs and ghrelin peptides suggest that altered production of ghrelin reactive autoAbs is

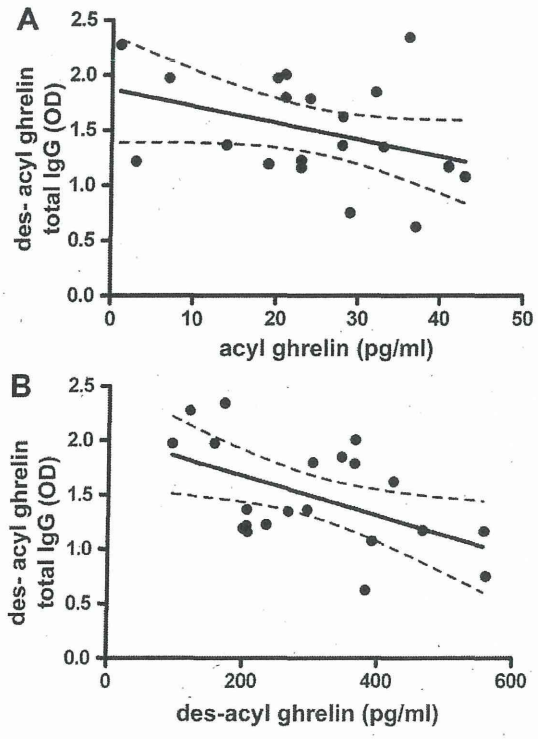

Fig. 6. Negative correlations between plasma levels of des-acyl ghrelin total IgG autoantibodies and plasma concentration of (A) acyl-ghrelin (Spearman $r = -0.46$, $P < 0.05$) and (B) des-acyl ghrelin (Spearman $r = -0.55$, $P = 0.01$) in study subjects. IgG, immunoglobulin G; OD, optical density.

associated with persistently elevated plasma ghrelin and eventually ghrelin resistance in AN.

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GASTROENTEROLOGY

Ghrelin family of peptides and gut motility

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Key words
corticotropin-releasing factor, gastroduodenal motility, ghrelin, obestatin, rat.

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Abstract

Acyl ghrelin, des-acyl ghrelin, and obestatin are three peptides isolated from the gastrointestinal tract and encoded by the same preproghrelin gene. Three ghrelin gene products participate in modulating appetite, adipogenesis, glucose metabolism, cell proliferation, immune, sleep, memory, anxiety, cognition, and stress. We have investigated the effects of ghrelin family of peptides on fed and fasted motor activities in the stomach and duodenum of freely moving conscious rats by manometric method. Intracerebroventricular (ICV) and intravenous (IV) administration of acyl ghrelin induced fasted motor activity in the duodenum in fed rats. ICV and IV administration of des-acyl ghrelin disrupted fasted motor activity in the antrum. Changes in gastric motility induced by IV administration of des-acyl ghrelin were antagonized by ICV administration of a corticotropin-releasing factor (CRF) 2 receptor antagonist. IV administration of obestatin decreased the percentage motor index in the antrum and prolonged the time taken to return to fasted motility in the duodenum in fed rats. ICV administration of CRF 1 and 2 receptor antagonists prevented the effects of obestatin on gastroduodenal motility. Ghrelin gene products regulate feeding-associated gastroduodenal motility. Stomach may regulate various functions including gastrointestinal motility *via* acyl ghrelin, des-acyl ghrelin and obestatin as an endocrine organ. Increasing knowledge of the effects of ghrelin family of peptides on gastrointestinal motility could lead to innovative new therapies for functional gastrointestinal disorders.

Introduction

Ghrelin, a 28-amino acid peptide with structural resemblance to motilin, was identified in the stomach as an endogenous ligand for growth-hormone secretagogue receptor (GHS-R).[1,2] The ghrelin gene is predominantly expressed in the stomach and ghrelin is secreted into the circulatory system. Two major molecular forms of ghrelin are found in the stomach and plasma: acyl ghrelin, which has n-octanoylated serine in position 3; and des-acyl ghrelin. The third ghrelin gene product, obestatin, a novel 23-amino acid peptide identified from rat stomach, was found by comparative genomic analysis.[1,2] Three ghrelin gene products actively participate in modulating appetite, adipogenesis, glucose metabolism, cell proliferation, immune, sleep, memory, anxiety, cognition, and stress.[1,2] We have investigated the effects of ghrelin family of peptides on fed and fasted motor activities in the stomach and duodenum of freely moving conscious rats by manometric method.[3–5]

Acyl ghrelin

Intracerebroventricular (ICV) (0.1 and 1 ug/rat) and intravenous (IV) (1 and 10 ug/rat) administration of acyl ghrelin induced phase III like contraction in the duodenum and increased the percentage motor index (%MI) in the antrum in fed rats. The results indicate that acyl ghrelin is involved in regulation of motor activity in the stomach and duodenum. Truncal vagotomy blocked the effects of ICV administration of acyl ghrelin on antral and duodenal motility, suggesting that vagal pathway may mediate the action of centrally administered ghrelin on gastroduodenal motility. IV administration of acyl ghrelin induced fasted motor activity in both the stomach and duodenum in vagotomized rats. The effects of ICV and IV injected acyl ghrelin were blocked by GHS-R antagonist, (D-Lys3) GHRP-6 (ICV: 1 nmol/rat, IV: 100 nmol/rat), given by the same route and also blocked by immunoneutralization of neuropeptide Y (NPY) (5 ul/rat anti-NPY antiserum) in the brain. The effects of IV injected acyl ghrelin were not altered by ICV administration of GHS-R antagonist in vagotomized rats. Administration of GHS-R antagonist blocked the fasted motor activity in both the stomach and duodenum in vagotomized rats but did not affect the fasted motor activity in normal rats. Peripheral acyl ghrelin may induce the fasted motor activity by activating the NPY neurons in the brain, probably through acyl ghrelin receptors on vagal afferent neurons.[3]

Des-acyl ghrelin

ICV (0.03 and 0.3 nmol/rat) and IV (0.3 and 3 nmol/rat) administration of des-acyl ghrelin disrupted fasted motor activity in the

Table 1 Effects of Ghrelin family of peptides on gastroduodenal motility

Acyl ghrelin	↑ In fed state	Frequency of phase III like contraction (duodenum) % motor index (antrum)
Des-acyl ghrelin	↓ In fasted state	Frequency of phase III like contraction (antrum)
Obestatin	↓ In fed state	Initiation of phase III like contraction (duodenum) % motor index (antrum)

↑, stimulation; ↓, inhibition.

antrum but not in the duodenum in freely moving conscious rats. The results indicate that des-acyl ghrelin is involved in regulation of motor activity in the stomach. Changes in gastric motility induced by IV administration of des-acyl ghrelin were completely antagonized by ICV administration of a selective CRF 2 receptor antagonist antisauvagine-30 (6 nmol/rat); however, the CRF 1 receptor antagonist NBI 27914 (50 ug/rat) had no effects. Intraperitoneal administration of des-acyl ghrelin (5 nmol/rat) enhanced c-fos expression in the arcuate and paraventricular nucleus but not in the nucleus of the solitary tract. Peripheral des-acyl ghrelin may induce this function by direct activation of brain receptor by crossing the blood-brain barrier but not by the activation of vagal afferent pathways. In the brain, CRF 2 receptor, but not CRF 1 receptor, is involved in this action.[4] We have recently reported the differential localization of acyl ghrelin and des-acyl ghrelin in the stomach.[6] Immunofluorescence double staining showed that acyl ghrelin- and des-acyl ghrelin-positive reactions overlapped in closed-type round cells, whereas des-acyl ghrelin-positive reaction was found in open-type cells in which acyl ghrelin was negative. In addition, des-acyl ghrelin has been shown to counteract the orexigenic effect of acyl ghrelin.[7,8] Moreover, Qader *et al.* reported that the effects of acyl ghrelin on the secretion of insulin, glucagon, pancreatic polypeptide, and somatostatin are reduced by des-acyl ghrelin.[9]

Obestatin

After IV administration, obestatin (15 and 30 nmol/rat) decreased the %MI in the antrum and prolonged the time taken to return to fasted motility in the duodenum in fed rats. Immunohistochemical analysis demonstrated that CRF- and urocortin-2-containing neurons in the paraventricular nucleus in the hypothalamus were activated by IV administration of obestatin (30 nmol/rat). ICV administration of CRF 1 (NBI 27914: 100 nmol/rat) and 2 (antisauvagine-30: 5 nmol/rat) receptor antagonists prevented the effects of obestatin (15 nmol/rat) on gastroduodenal motility. Capsaicin treatment blocked the effects of obestatin (15 nmol/rat) on duodenal motility but not on antral motility. Obestatin failed to antagonize acyl ghrelin (0.3 nmol/rat) -induced stimulation of gastroduodenal motility. These results suggest that, in the fed state, obestatin inhibits motor activity in the antrum and duodenum and that CRF 1 and 2 receptors in the brain might be involved in these effects of obestatin on gastroduodenal motility.[5]

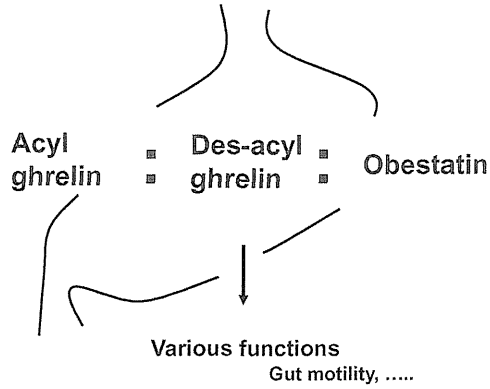

Figure 1 Stomach regulates various functions via ghrelin family of peptides.

Conclusion

Ghrelin gene products regulate feeding-associated gastrointestinal motility (Table 1). Stomach may regulate various functions including gastrointestinal motility *via* acyl ghrelin, des-acyl ghrelin and obestatin as an endocrine organ (Fig. 1). Increasing knowledge of the effects of ghrelin family of peptides on gastroduodenal motility could lead to innovative new therapies for functional gastrointestinal disorders.

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Potentiation of ghrelin signaling attenuates cancer anorexia–cachexia and prolongs survival

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Cancer anorexia–cachexia syndrome is characterized by decreased food intake, weight loss, muscle tissue wasting and psychological distress, and this syndrome is a major source of increased morbidity and mortality in cancer patients. This study aimed to clarify the gut–brain peptides involved in the pathogenesis of the syndrome and determine effective treatment for cancer anorexia–cachexia. We show that both ghrelin insufficiency and resistance were observed in tumor-bearing rats. Corticotropin-releasing factor (CRF) decreased the plasma level of acyl ghrelin, and its receptor antagonist, α-helical CRF, increased food intake of these rats. The serotonin 2c receptor (5-HT2cR) antagonist SB242084 decreased hypothalamic CRF level and improved anorexia, gastrointestinal (GI) dysmotility and body weight loss. The ghrelin receptor antagonist (D-Lys3)-GHRP-6 worsened anorexia and hastened death in tumor-bearing rats. Ghrelin attenuated anorexia–cachexia in the short term, but failed to prolong survival, as did SB242084 administration. In addition, the herbal medicine rikkunshito improved anorexia, GI dysmotility, muscle wasting, and anxiety-related behavior and prolonged survival in animals and patients with cancer. The appetite-stimulating effect of rikkunshito was blocked by (D-Lys3)-GHRP-6. Active components of rikkunshito, hesperidin and atractylodin, potentiated ghrelin secretion and receptor signaling, respectively, and atractylodin prolonged survival in tumor-bearing rats. Our study demonstrates that the integrated mechanism underlying cancer anorexia–cachexia involves lowered ghrelin signaling due to excessive hypothalamic interactions of 5-HT with CRF through the 5-HT2cR. Potentiation of ghrelin receptor signaling may be an attractive treatment for anorexia, muscle wasting and prolong survival in patients with cancer anorexia–cachexia.
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Introduction

Cachexia is characterized by weight loss, fat and muscle tissue wasting, psychological distress and a lower quality of life. In cancer patients, anorexia development is frequently associated with the presence of cachexia, resulting in the so-called cancer anorexia–cachexia syndrome.[1] This syndrome is observed in 80% of patients with advanced-stage cancer and is a frequent cause of death.[2] Recent reports have indicated that an imbalance between anorexigenic and orexigenic peptides leads to appetite suppression.[3–5] 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,[6] but the neurochemical mechanisms responsible for cancer anorexia–cachexia remain uncertain. The two major options for pharmacological therapy are megestrol acetate and glucocorticoids,[7,8] but both have limited effectiveness. A better understanding of the underlying mechanisms of this syndrome will help in the development of new therapies to improve quality of life and potentially to prolong survival in patients with cancer-induced anorexia–cachexia.

Anxiety and depressive symptoms are associated with various gastrointestinal (GI) disorders, including cancers,[6] chronic liver diseases, inflammatory bowel diseases and functional GI diseases.[9,10] Corticotropin-releasing factor (CRF) is a mediator of the endocrine, autonomic and immune responses to stress, including anorexia and anxiety-related behavior.[11] The central serotonin (5-HT) system has also been implicated in the processes of meal satiation and satiety. Hypothalamic 5-HT and CRF activities are stimulated by proinflammatory cytokines in the circulation and the hypothalamus.[12] Therefore, we hypothesized 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.

Ghrelin system is involved in eliciting feeding, inducing adiposity, and regulating glucose metabolism and body weight.[13] Ghrelin has an important role in triggering the adaptive response to starvation. In this study, we demonstrate

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that cancer anorexia–cachexia is mediated by decreased ghrelin signaling due to excessive hypothalamic interactions of 5-HT and CRF through the 5-HT 2c receptor (5-HT2cR) in a tumor-bearing rat model.

Materials and methods

Male Wistar rats were intraperitoneally (i.p.) inoculated with AH-130 ascites hepatoma cells (Tohoku University, Sendai, Japan). The effects of α-helical CRF, 5-HT2cR antagonist, ghrelin, ghrelin receptor (GHS-R) antagonist, and rikkunshito on food intake, weight and GI motility were examined in the tumor-bearing rats. Anxiety-related behavior was estimated using the open-field test. Plasma levels of peptides were determined by enzyme immunoassay. Ca^{2+} imaging and radioligand binding were performed using GHS-R-expressing cells and rat single neurons isolated from the arcuate nucleus (ARC) or paraventricular nucleus (PVN). In all, 39 patients who had pathologically proven stage III/IV pancreatic cancer with ascites were eligible candidates for rikkunshito, as suggested from clinical experiences of this drug in Japan. The patients were retrospectively analyzed from 2004 to 2009 in Chiba Cancer Center (Chiba, Japan). To assess the difference among groups, a Student *t*-test or a multi-group Dunnett test was performed. Mortality data were compared using Kaplan–Meier plots and Gehan–Breslow–Wilcoxon tests (see Supplementary Materials).

Results

Ghrelin and cancer anorexia–cachexia. Decreased food intake, low rectal temperature, weight loss and wasting of muscle and fat mass were observed after tumor injection in rats (Figure 1a). Plasma concentrations of cytokines and c-reactive protein (CRP) were elevated (Figure 1b). Plasma acyl ghrelin concentrations were higher in tumor-bearing rats than in free-fed normal rats, but were significantly lower than in pair-fed normal rats and had an inverse relationship with plasma leptin concentrations (Figure 1c). Significant decreases in the hypothalamic expression of appetite-regulating peptides, neuropeptide Y (NPY), agouti-related peptide, proopiomelanocortin (POMC), urocortin-2,3 and CRF, were observed in tumor-bearing rats compared to pair-fed controls (Figure 1d). This indicates a pathogenetic role of orexigenic peptides in cancer anorexia–cachexia.

Intravenous administration of ghrelin increased food intake for 2 h, but not 6 h in normal and tumor-bearing rats on day 5 (Figure 1e). These responses were attenuated in tumor-bearing rats compared with normal rats owing to ghrelin resistance. In contrast, i.p. administration of the GHS-R antagonist (D-Lys3)-GHRP-6 (4 μmol kg^{-1}; data not shown) worsened anorexia in tumor-bearing rats. Oral (per os) administration of a 5-HT2cR antagonist, SB242084 (5 mg kg^{-1}), increased food intake in tumor-bearing rats (Figure 1f). The traditional herbal medicine rikkunshito, which stimulates the secretion of endogenous acyl ghrelin by blocking 5-HT2 receptors in rats,[14] also increased food intake in tumor-bearing rats (Figure 1g). The effect of rikkunshito was inhibited by intravenous administration of (D-Lys3)-GHRP-6 (2 μmol kg^{-1}), suggesting mediation by endogenous acyl ghrelin. Daily administration of SB242084 or rikkunshito in tumor-bearing rats inhibited weight loss without affecting ascites volume (Supplementary Figure S1).

Phase III-like contractions in the antrum and duodenum of normal fasted rats are mediated by orexigenic signaling from ghrelin.[15] Tumor-bearing rats exhibited fed-like motor activities in the antrum and duodenum, and the frequency of their phase III-like contractions significantly decreased (Supplementary Figure S2). Intravenous administration of ghrelin (3 nmol) to tumor-bearing rats on day 5 immediately potentiated the fasted motor activity and increased the frequency of the phase III-like contractions (Figure 1h). Oral administration of SB242084 (1 mg kg^{-1}) or rikkunshito (1000 mg kg^{-1}) gradually restored the fasted motor patterns (Supplementary Figure S2).

Involvement of CRF in cancer anorexia. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in single neurons isolated from the PVN of rats was measured by fura-2 microfluorometry. Administration of 10^{-5} mol l^{-1} 5-HT for 10–15 min into superfusion solutions increased the $[Ca^{2+}]_i$ in a continuous oscillatory manner. The 5-HT-induced $[Ca^{2+}]_i$ increase was inhibited by administration of 100 μg ml^{-1} rikkunshito to the PVN neurons; 83% of which subsequently demonstrated immunoreactivity to CRF (Figure 2a). In contrast, rikkunshito had little inhibitory effect on 30 mmol l^{-1} potassium chloride-induced increases in $[Ca^{2+}]_i$ (data not shown).

A significant decrease in the plasma concentration of acyl ghrelin was observed 3 h after intracerebroventricular administration of CRF (1.5 nmol) to fasted rats (Figure 2b), suggesting that endogenous ghrelin secretion is regulated by central CRF neurons. The electrophysiological study demonstrated that ghrelin and rikkunshito influenced CRF-regulated adrenal function by decreased adrenal sympathetic nerve activity (Figure 2c).

Administration of a CRF antagonist, α-helical CRF (50 μg, intracerebroventricular), increased food intake in tumor-bearing rats (Figure 2d), suggesting that the hypothalamic CRF system is activated in tumor-bearing rats, despite the overall reduction in CRF expression due to negative feedback inhibition resulting from increased corticosterone secretion (Figure 2e). CRF levels in the hypothalamus of tumor-bearing rats were significantly decreased by SB242084 and rikkunshito (Figure 2f). CRF-treated animals are known to display anxiety-related responses with decreased exploratory behavior.[16] Tumor-bearing rats showed a significant decrease in rearing in the open-field test and increased fecal pellet output. Oral administration of rikkunshito to these rats recovered rearing and reversed fecal pellet output (Figure 2g).

Ghrelin signaling and rikkunshito. The afferent activity of the gastric vagus nerve decreased with intravenous administration of ghrelin (Figure 3a), as we have reported previously.[17] In contrast, the efferent activities of the gastric (Figure 3b) and celiac (data not shown) branches of the vagus nerve increased with intravenous administration of ghrelin (10 ng). Similar effects were observed with intraduodenal, but not intragastric, administration of

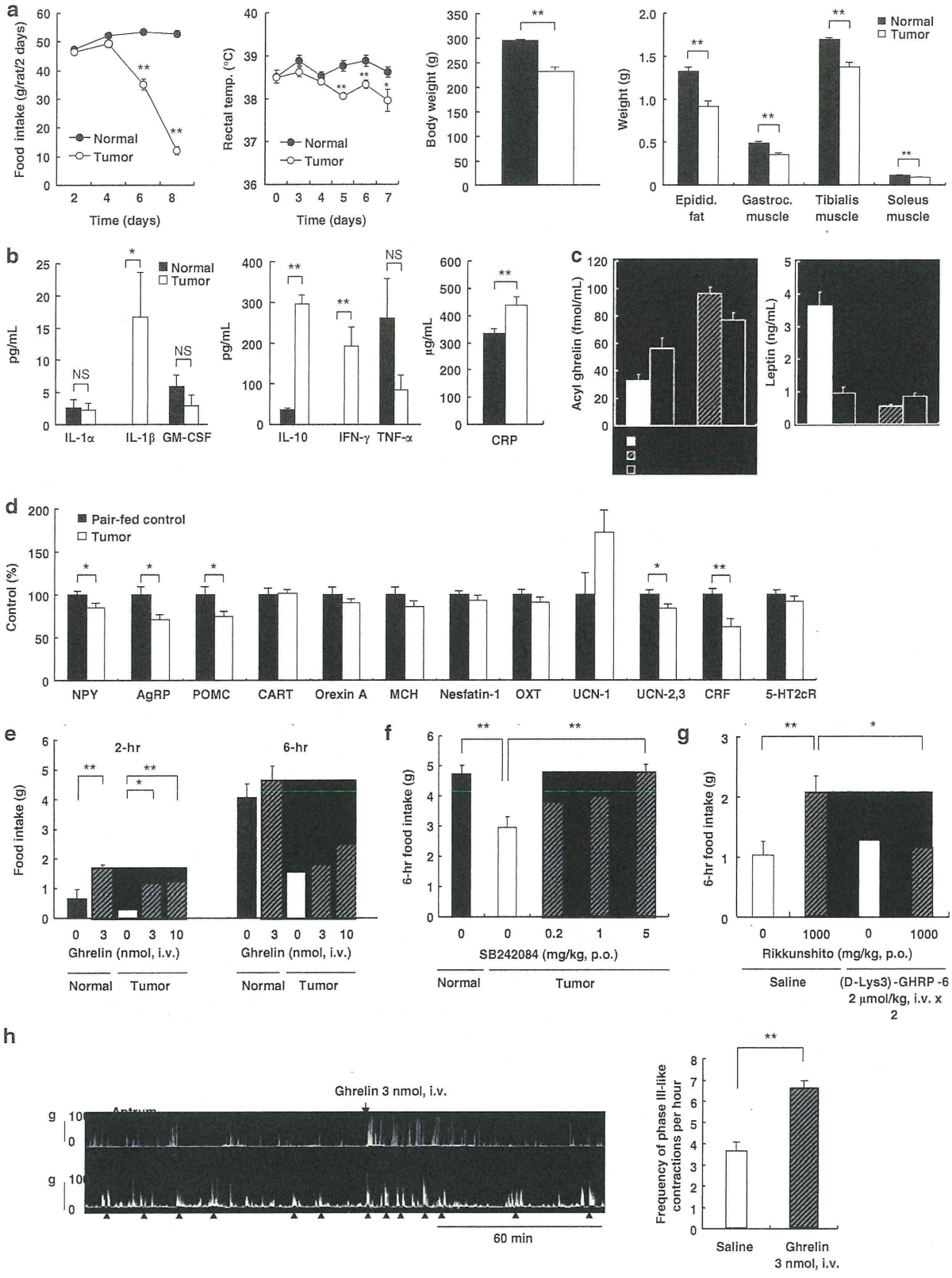

Figure 1 Cancer anorexia–cachexia. (**a**) Food intake, rectal temperatures and weights of tumor-bearing rats ($n = 6$–8). (**b**–**d**) Plasma and hypothalamic appetite-regulating peptides ($n = 8$–10). (**e**–**g**) Effects of ghrelin, the serotonin 2c receptor (5-HT2cR) antagonist SB242084 and rikkunshito on food intake of tumor-bearing rats and blockade by the ghrelin receptor (GHS-R) antagonist (D-Lys3)-GHRP-6 ($n = 8$–10). (**h**) Fasted gastrointestinal (GI) motor activity in tumor-bearing rats on day 5. Ghrelin increased the frequency of phase III-like contractions (▲) in the duodenum ($n = 8$). $^*P < 0.05$; $^{**}P < 0.01$. AgRP: agouti-related peptide; CART: cocaine- and amphetamine-regulated transcript; MCH: melanin-concentrating hormone; OXT: oxytocin; UCN, urocortin.

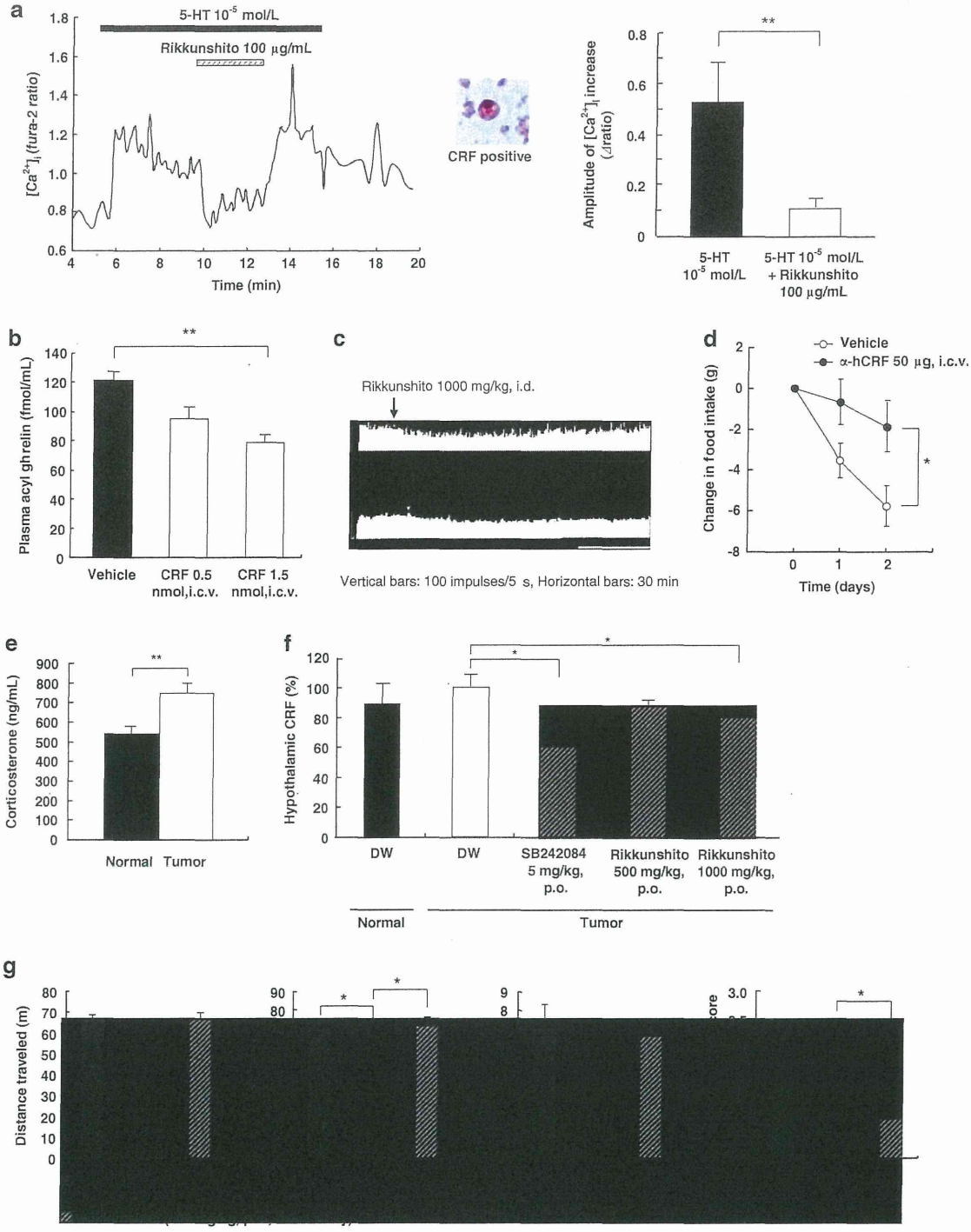

Figure 2 Involvement of corticotropin-releasing factor (CRF) in cancer anorexia. (**a**) The serotonin (5-HT)-induced cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) increase was suppressed by rikkunshito in single CRF neurons isolated from the paraventricular nucleus (PVN). (**b**) Inhibitory effect of CRF on plasma acyl ghrelin concentrations in fasted rats ($n=8$–9). (**c**) Inhibitory effects of ghrelin and rikkunshito on the efferent activity of the adrenal sympathetic nerve in rats. (**d**) Food intake was increased in tumor-bearing rats by daily administration of the CRF receptor antagonist α-helical CRF ($n=9$–10). (**e**) Plasma corticosterone concentration of tumor-bearing rats ($n=12$). (**f**) Hypothalamic CRF levels in tumor-bearing rats were decreased by SB242084 or rikkunshito ($n=10$). (**g**) Daily administration of rikkunshito in tumor-bearing rats improved rearing and decreased fecal pellet output score in an open-field test ($n=9$–10). *$P<0.05$; **$P<0.01$. DW: distilled water.

rikkunshito (1000 mg kg^{-1}). Gastric vagotomy eliminated the stimulatory effect of ghrelin (10 ng, intravenous) on the efferent activities of the gastric vagus nerve, but did not influence the effect of rikkunshito (1000 mg kg^{-1}, intraduodenal) or a 100-fold higher dose of ghrelin (1000 ng, intravenous) (Supplementary Figure S3).

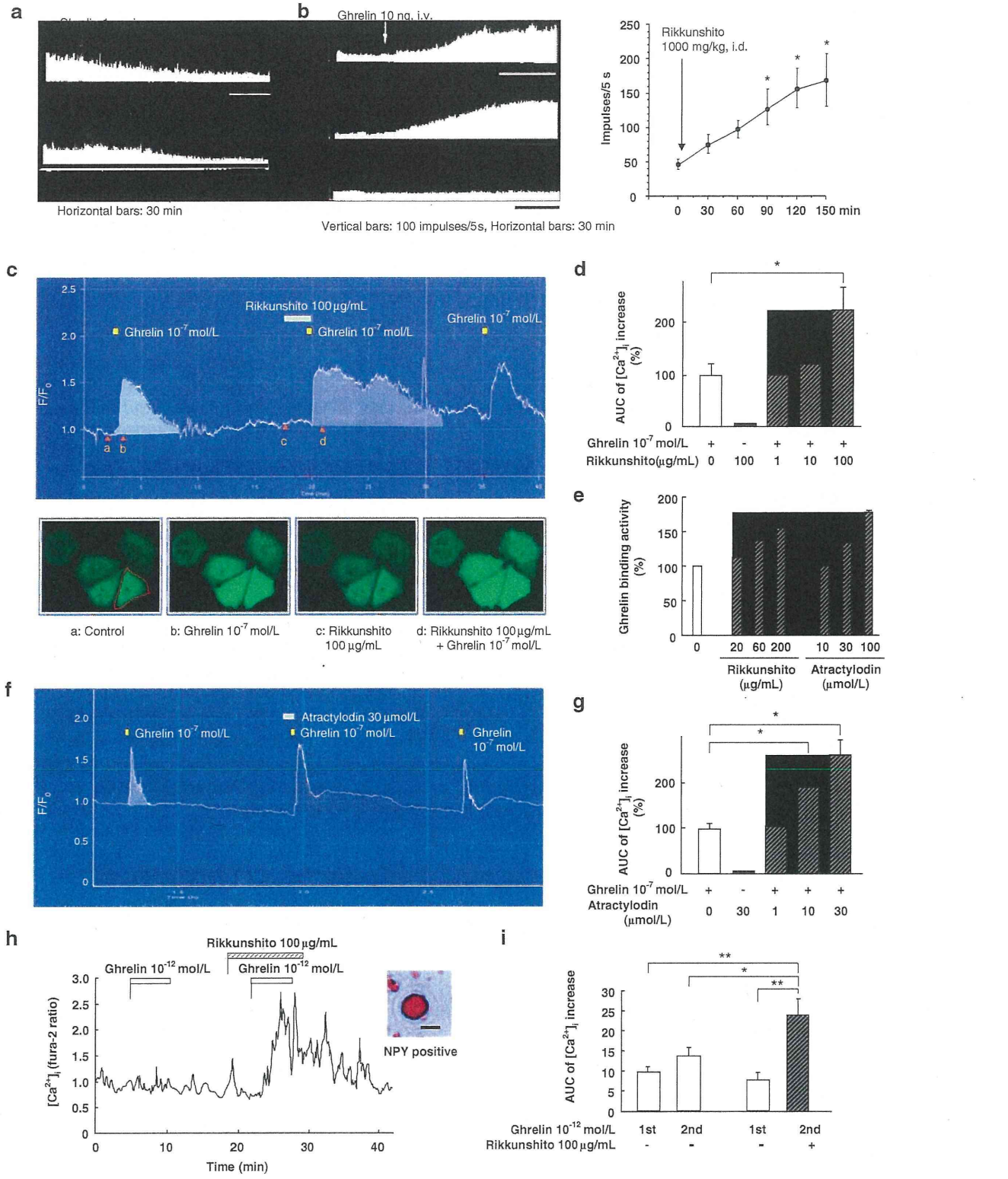

Figure 3 Ghrelin signaling and rikkunshito. (**a**, **b**) Effects of ghrelin and rikkunshito on the afferent (**a**) and efferent (**b**, $n=6$) activities of the gastric vagus nerve in rats. (**c**, **d**) Changes in F/F_0 fluorescence evoked by ghrelin in ghrelin receptor (GHS-R)-expressing cells. Representative Ca^{2+}-imaging figures were taken as indicated by the arrowheads in the figures (**a**–**d**) and the intensities within the area of a cell (red line). $[Ca^{2+}]_i$ increase in the area under the curve (AUC) was evaluated ($n=6$–9). (**e**) Effect of rikkunshito and atractylodin on ghrelin/GHS-R binding activity. (**f**, **g**) Atractylodin enhanced the ghrelin-induced $[Ca^{2+}]_i$ increase in GHS-R-expressing cells ($n=8$–12). (**h**, i) Ghrelin (10^{-12} mol l^{-1}) increased the $[Ca^{2+}]_i$ in single neuropeptide Y (NPY) neurons isolated from the arcuate nucleus (ARC). The increase in AUC of the $[Ca^{2+}]_i$ in response to secondary ghrelin with rikkunshito ($n=22$) was significantly greater than the response to primary or secondary ghrelin without rikkunshito ($n=32$). $^*P<0.05$; $^{**}P<0.01$.

Ghrelin is predominantly produced in gastric X/A-like cells. Rikkunshito elevated the gene expression of gastric ghrelin and hypothalamic NPY genes in rats (Supplementary Figure S4a). Rikkunshito did not acutely stimulate ghrelin secretion from minced stomach tissues (data not shown), but normalized the fenfluramine-induced decrease in ghrelin secretion mediated by the 5-HT2cR in the brain (Supplementary Figure S4b), suggesting that this is the centrally predominant site of action.

Ghrelin (10^{-7} mol l^{-1}) elicited an increase in $[Ca^{2+}]_i$ of GHS-R-expressing COS cells. Rikkunshito had no effect on the $[Ca^{2+}]_i$ in these cells. However, the ghrelin-induced $[Ca^{2+}]_i$ increase was enhanced by a 2-min pretreatment with rikkunshito in a concentration-dependent manner, and 100 μg ml^{-1} of rikkunshito significantly enhanced the duration of the $[Ca^{2+}]_i$ increase induced by ghrelin (Figure 3c and d).

Rikkunshito enhanced the binding activity of [^{125}I]ghrelin to the GHS-R (Figure 3e). We screened the 43 compounds (100 μmol l^{-1}) contained in rikkunshito. Two of these compounds, atractylodin and atractylodinol, showed a marked increase in ghrelin/GHS-R binding activity. Atractylodin also sustained the ghrelin-induced $[Ca^{2+}]_i$ increase in GHS-R-expressing cells (Figure 3f and g).

Ghrelin increases the $[Ca^{2+}]_i$ in the NPY neurons of the hypothalamic ARC,[18] and this effect is linked to stimulation of feeding.[19] Ghrelin, at a submaximal concentration of 10^{-12} mol l^{-1}, increased $[Ca^{2+}]_i$ levels in acutely isolated fura-2-loaded rat ARC neurons, which were subsequently shown to be NPY neurons by immunocytochemistry. Pretreatment with rikkunshito enhanced the ghrelin-induced increase in $[Ca^{2+}]_i$ compared with first ghrelin or second ghrelin administration without rikkunshito (Figure 3h and i). These data indicate that rikkunshito potentiates the action of ghrelin to increase the $[Ca^{2+}]_i$ in NPY neurons in the ARC.

Survival. Daily administration of (D-Lys3)-GHRP-6 (4 μmol kg^{-1}, i.p.; Figure 4a) decreased median survival in AH-130 tumor-bearing rats, demonstrating the importance of ghrelin signaling in cancer anorexia–cachexia. In contrast, median survival in AH-130 tumor-bearing rats was significantly increased by the daily administration of rikkunshito (250 and 500 mg kg^{-1}, per os; Figure 4b) and atractylodin (1 mg kg^{-1}, per os; Figure 4c), but not SB242084 (5 mg kg^{-1}, per os) or ghrelin (3 nmol, i.p.) (Supplementary Figure S5). Rikkunshito also exhibited a positive effect on survival in CT-26 colon carcinoma-bearing mice (Supplementary Figure S6). Survival in tumor-bearing rats was also increased by administration of cisplatin (CDDP; 1 mg kg^{-1}, i.p.), and 6 of 27 rats survived until the end of the experimental period. Administration of rikkunshito further prolonged survival in CDDP-treated tumor-bearing rats (Figure 4d).

The beneficial effect of rikkunshito on survival was also demonstrated in human patients. Pancreatic cancer patients with ascites received gemcitabine or gemcitabine plus rikkunshito. There was no significant difference between the two groups in baseline data with respect to stage and age. Median survival of pancreatic cancer patients with ascites who were treated with gemcitabine was significantly prolonged by administration of rikkunshito (Figure 4e).

Discussion

Weight loss is a potent stimulus of food intake in healthy humans and animals, and ghrelin secretion increases under conditions of negative energy balance such as starvation. Increased concentrations of acyl ghrelin have been found in patients[20,21] and mice[22] with various cancer diagnoses and staging. These findings imply that the persistence of anorexia in cancer patients is due to a failure of the adaptive feeding response by ghrelin, which is robust in normal animals and subjects.[23–25] We 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, and had an inverse relationship with plasma leptin concentrations. These results indicate that changes in ghrelin and leptin secretion in pair-fed animals represent a compensatory mechanism in a persistent catabolic state and that these responses are attenuated in tumor-bearing rats. The hypothermia in tumor-bearing rats may be due to a state of negative energy balance or a decrease in the threshold for the activation of thermogenesis, which is involved in starvation-induced hypothermia.[26] Interleukin-1β[17] and leptin[27] decrease the expression of ghrelin mRNA in the stomach, whereas interleukin-6 produced in various cells, including adipocytes, regulates leptin production.[28] These findings suggest that cytokines have an important role in energy balance through the persistent activation of the leptin system and the inhibition of the ghrelin-NPY/agouti-related peptide orexigenic network in tumor-bearing rats. In addition to NPY and agouti-related peptide, the level of POMC mRNA was also decreased in the hypothalamus of the tumor-bearing rats. Synaptic input organization and mRNA expression of POMC neuron have been shown to be increased in adrenalectomized animals and restored by corticosterone replacement.[29] Thus, activity of hypothalamic POMC neuron may be affected by changes in circulating levels of corticosterone and a state of negative energy balance.

Peripheral ghrelin administration stimulates food intake in melanoma cell-bearing mice and cancer patients[30] in the short term as well as in lean, healthy men and women.[31] In this study, we found similar therapeutic effects of ghrelin on anorexia and GI dysmotility in cachectic animal models, suggesting that high plasma concentrations of ghrelin may overcome resistance to the appetite-stimulating effects of the endogenous peptide in the short term. Rikkunshito, which mimics these ghrelin effects, effectively improved food intake and GI motor activities in this study. Rikkunshito is a traditional herbal medicine used to treat GI tract disorders such as functional dyspepsia[32–36] and gastroesophageal reflux.[37] Oral administration of rikkunshito increases plasma acyl ghrelin levels in humans, mice,[38] rats[14,15] and dogs (data not shown). Rikkunshito stimulates ghrelin secretion through 5-HT2b/2c receptor antagonism, and its active flavonoid ingredients such as hesperidin that antagonize 5-HT2b/2c receptor binding have been identified.[14] In addition, rikkunshito and 5-HT2cR antagonist suppress cisplatin-induced anorexia by inhibiting reduction of GHS-R1a gene expression in the hypothalamus.[39]

The central 5-HT system has been implicated in the processes of meal satiation and satiety. 5-HT reuptake