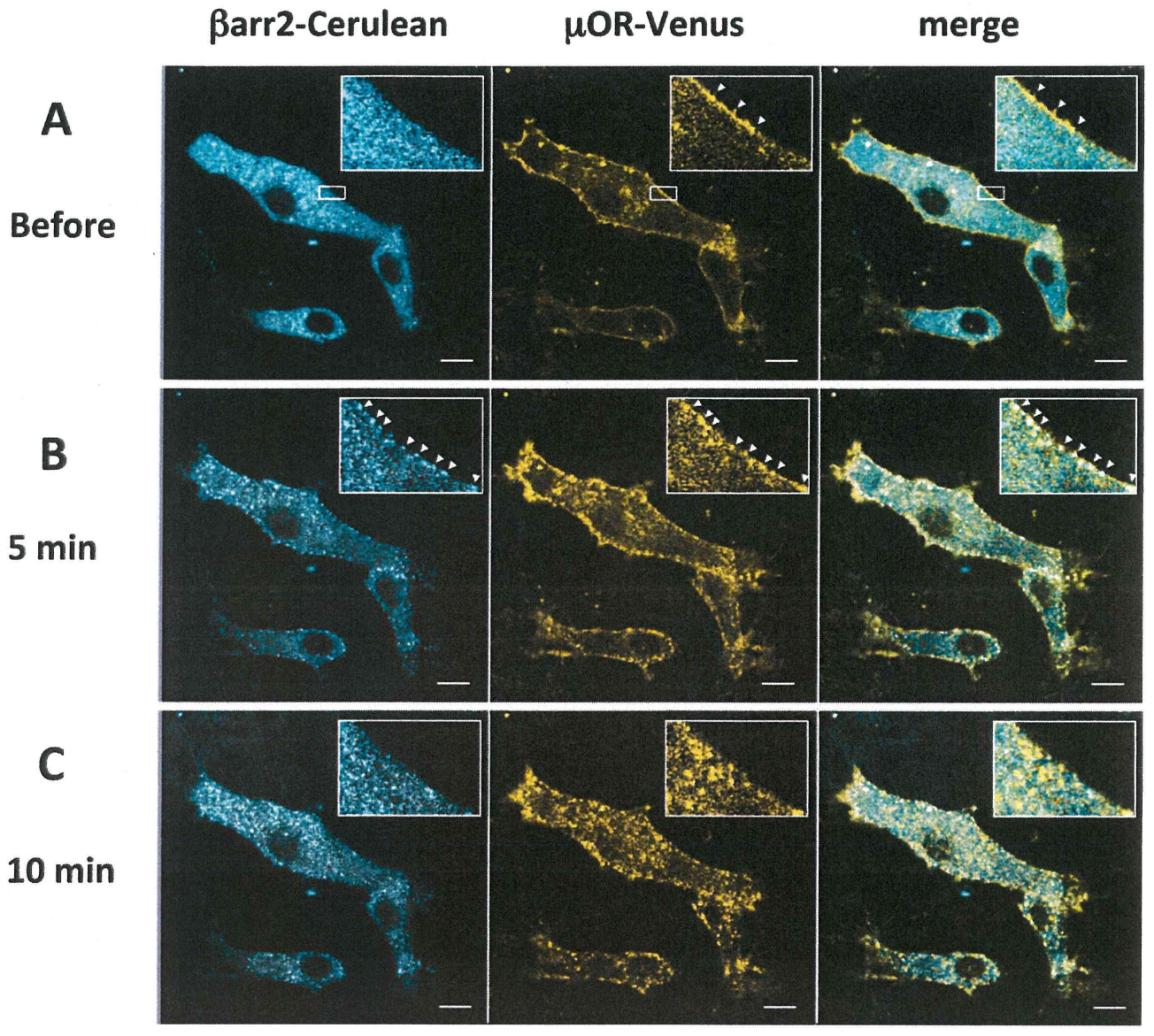

Fig. 5. Confocal imaging of the translocation of μOR-V and βarr2-C in BHK cells expressing μOR-V, βarr2-C, and GRK2. Visualization of μOR-V and βarr2-C in BHK cells before (**A**) and 5 min (**B**) and 10 min (**C**) after stimulation with 10^{-7} M DAMGO. Arrowheads show μOR and βarr2-C on the plasma membranes. Similar results were obtained in at least six independent experiments. Calibration bar = 10 μm.

reports that $GABA_BR$ does not internalize on stimulation with $GABA_BR$ agonists (Fairfax et al., 2004; Grampp et al., 2007; Laffray et al., 2007; Perroy et al., 2003). One early study, by contrast, showed that $GABA_{B1}R$ tagged with cyan fluorescent protein and $GABA_{B2}R$ tagged with yellow fluorescent protein were both internalized after treatment with baclofen. However, this happened at only one time point (2 h after stimulation of GABA at 10^{-4} M), and the study did not monitor the intensities of the fluorescence in both the plasma membrane and cytosol (González-Maeso et al., 2003). The discrepancy of this result compared with other studies probably results from the different cell types and experimental designs used.

We also studied the role of phosphorylation in these processes. Specifically, phosphorylation of GPCRs by several protein kinases, such as the GRKs, plays a role in the desensitization and internalization of most of these receptors (Kelly et al., 2008; Luttrell and Lefkowitz, 2002). $GABA_BR$ phosphorylation is unique in that, though some GRKs are involved in GABA- or baclofen-mediated $GABA_BR$ desensitization (especially GRK4 and 5 but not GRK2, 3, or 6), these kinases do not phosphorylate the receptors (Kanaide et al., 2007; Perroy et al., 2003). These results suggest that GRK4 and GRK5 may behave as anchoring proteins instead of as kinases (Kanaide et al., 2007; Perroy et al., 2003; Terunuma et al., 2010).

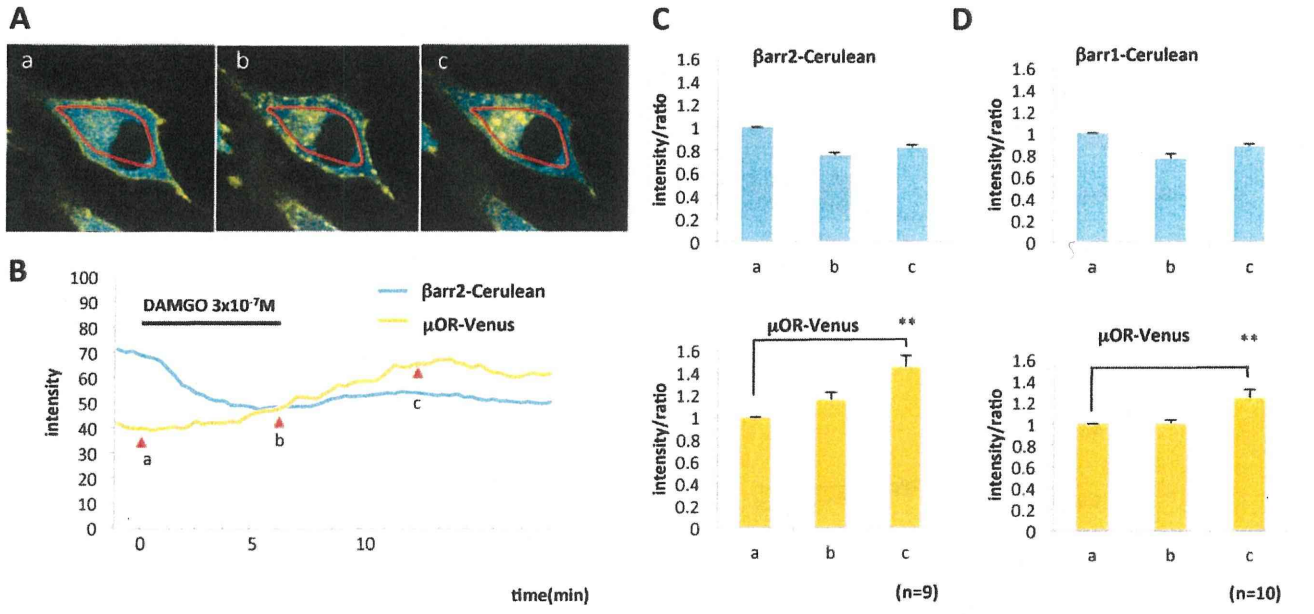

Fig. 6. Time courses of changes in intensities of μOR-V, βarr2-C, or βarr1-C in BHK cells. **A**: Confocal imaging of the BHK cells expressing μOR-V, GRK2, and βarr2-C or βarr1-C. For calculation, intensities of the areas within the red line (cytosol) were measured. **B**: Changes in intensities before (a) and 5 min (b) and 10 min (c) after stimulation of DAMGO (10^{-7} M) in real time. **C**: Intensity ratio of βarr2-C and μOR-V at the indicated points as in (B). **D**: Intensity ratio of βarr1-C and μOR-V at the indicated points as in (B). Intensity ratio were expressed as ratio of the level at "b" or "c"/the level at "a."

It is also known that β-arrestins are involved in the internalization steps (Gainetdinov et al., 2004). Once receptors are phosphorylated by several kinases, βarr-1 or βarr-2 binds to the receptors, followed by internalization of the receptor/β-arrestin complex (Gainetdinov et al., 2004; Luttrell and Lefkowitz, 2002). Previous reports have shown that baclofen failed to recruit βarr-1 or βarr-2 to the plasma membrane (Fairfax et al., 2004; Perroy et al., 2003). In this study, with BHK cells coexpressing $GB_{1a}R$, GB_2R-V, β-arrestins-C, and GRK4, we investigated the mobility of both GB_2R-V and β-arrestins-C on stimulation by baclofen. This agonist was administered at concentrations and durations at which GRK4 translocated to the plasma membranes, formed GB_2R/GRK4 complex, and consequently desensitized the receptor functions. Our results were in accordance with previous studies showing that GABA$_B$Rs were not internalized and β-arrestins were not mobilized on baclofen stimulation, despite their desensitization under those conditions (Fairfax et al., 2004; Perroy et al., 2003). In the clinical therapy, intrathecal baclofen therapy (ITB) is an established treatment for severe spasticity (Slonimski et al., 2004). Tolerance to ITB for treatment of spasticity is produced by desensitization of the GABA$_B$R (Kanaide et al., 2007; Nielsen et al., 2002). Desensitization of GABA$_B$R by baclofen was mediated by protein complex formation of GABA$_B$R with GRK4 or GRK5 (Ando et al., 2011; Kanaide et al., 2007; Perroy et al., 2003). In such situation, baclofen did not internalize GABA$_B$R as shown this study, suggesting that GABA$_B$R internalization process by itself may not be involved in the development of tolerance to ITB by baclofen.

Recent reports have shown that distinct phosphorylation sites on G protein-coupled adrenergic β_2 receptors act as a "barcode" for the differential functions for β-arrestin, including its receptor-internalization profiles (Nobles et al., 2011). The authors indicated that the specific and distinct patterns of receptor phosphorylation by individual GRKs correlate with different β-arrestin functions. They thus proposed that these distinct phosphorylation patterns create a "barcode" that imparts distinct conformations to the recruited β-arrestin, thus regulating its functional activities. Another report has shown that GABA$_B$R is internalized by *N*-methyl-D-asparate (NMDA) receptor stimulation (but not GABA$_B$R activation itself) due to site-specific phosphorylation of the receptor (serine 867 in $GB_{B1}R$) by calmodulin-dependent protein Kinase II (Guetg et al., 2010). In previous studies, including our own, GABA$_B$R was not phosphorylated by GRK4 or GRK5, even if they induced GABA$_B$R desensitization (Kanaide et al., 2007; Perroy et al., 2003). Collectively, these results seem to suggest that agonist stimulation caused little or no phosphorylation of GABA$_B$R. Thus, internalization of the receptor due to mobilization of β-arrestins or complex formation with β-arrestins was not caused by baclofen.

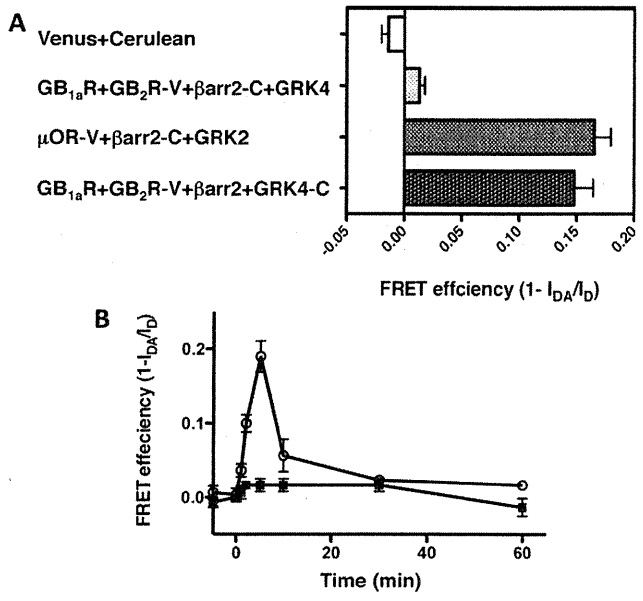

Fig. 7. **A**: Comparison of FRET efficiency on the plasma membranes in BHK cells expressing $GB_{1a}R$, GB_2R-V, and GRK4 with βarr2-C; or μOR-V and GRK2 with βarr2-C on the plasma membranes, with or without stimulation of baclofen or DAMGO for 5 min, respectively. FRET efficiency was calculated from emission spectra. Note the increase of the Cerulean peak emission (488 nm) following photobleaching of Venus (528 nm). I_{DA} = peak of donor emission in the presence of acceptor. I_D = peak of donor emission in the presence of sensitized acceptor. The combination of Venus + Cerulean or $GB_{1a}R$ + GB_2R-V + GRK4-C pairs were used as negative and positive controls for protein–protein interaction, respectively. Each bar represents mean ± SEM of FRET efficiency in independent experiments using six cells with three regions of interest per BHK cell (n = 18). **B**: Changes in the FRET ratio on the plasma membranes in BHK cells coexpressing $GB_{1a}R$, GB_2R-V, and GRK4 with βarr2-C; or μOR-V and GRK2 with βarr2-C. Photobleaching assay was performed for the periods indicated, and then FRET efficiencies were calculated (n = 3 at each point). The open circles (○) or the closed squares (■) represent data obtained from μOR- or $GABA_BR$-expressing cells, respectively.

On the other hand, some reports with different experimental methods from our own have shown that $GABA_BR$ was constitutively (without agonist stimulation) internalized into the cytosol (Grampp et al., 2007). In our study, stimulation by baclofen for up to 90 min failed to cause any detectable, spontaneous internalization, as determined by laser microscopy. However, in our experimental system, we are not aware of the functions of $GABA_BR$. Specifically, we do not know if these receptors elicit acute internalization followed by quick (for example, within 1 min) recycling to the plasma membranes. We were unable to detect basal internalization of GB_2R-Venus or recycling of the receptors, as shown by the lack of change in the intensity of GB_2R-V at the plasma membranes. Another report, however, has shown that heterodimeric $GABA_BR$ internalizes basally and recycles back to plasma membranes (Vargas et al., 2008). The discrepancies between their results and ours are probably due to the different experimental approaches used. The receptor might internalize to the superficial sites and then recycle quickly to the plasma membrane, and our experimental system may not be able to detect such movements due to the limitation of resolution ability of our laser confocal microscopy.

Further study will be necessary to determine the involvement of β-arrestins in such quick internalization and recycling. Although unknown, it is important to understand their involvement in such short periods of internalization as well as agonist-induced internalization. Such short-term trafficking might play roles in fundamental functions of $GABA_BR$ by activating of multifunctioning proteins β-arrestins (Nobles et al., 2011).

In conclusion, these findings suggest that $GABA_BR$ fails to undergo agonist-induced and spontaneous internalization in part because of their failure to interact with β-arrestins.

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Nutrition 27 (2011) 407–413

Contents lists available at ScienceDirect

Nutrition

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Applied nutritional investigation

Ghrelin reactive autoantibodies in restrictive anorexia nervosa

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ARTICLE INFO

Article history:
Received 8 December 2010
Accepted 11 January 2011

Keywords:
Appetite
Eating disorders
Malnutrition
Peptide hormones
Autoimmunity

ABSTRACT

Objective: Subjects with restrictive anorexia nervosa (AN) display increased basal plasma levels of ghrelin that normalize after refeeding. The mechanism responsible for increased ghrelin levels in AN is unknown. We studied if changes of ghrelin reactive autoantibodies (autoAbs) could explain elevated plasma ghrelin in AN.
Methods: Plasma levels of autoAbs reactive with ghrelin and des-acyl ghrelin were measured by enzyme-linked immunosorbent assay in subjects with AN before and 1 mo after hospitalization (refeeding) and compared with healthy controls and with plasma levels of ghrelin peptides.
Results: Decreased levels of immunoglobulin (Ig) G, IgM, and IgA classes of autoAbs reacting with acyl ghrelin were found in patients with AN. Addition of des-acyl ghrelin but not of acyl ghrelin peptides at 10^{-8} M to plasma before enzyme-linked immunosorbent assay showed in patients with AN but not in controls high levels of IgG autoAbs reacting with des-acyl ghrelin as a result of dissociation of des-acyl ghrelin autoAbs in immune complexes. Plasma levels of acyl and des-acyl ghrelin peptides correlated negatively with des-acyl ghrelin IgG autoAbs. Body mass index, which improved after refeeding, correlated with an increase of acyl ghrelin IgM autoAbs.
Conclusion: These results show that in patients with AN, ghrelin IgG autoAbs exist mainly as immune complexes with des-acyl ghrelin accompanied by a decrease of a free fraction of these autoAbs binding acylated and des-acyl ghrelin. This decrease of bioavailable ghrelin autoAbs may underlie a long-term elevation of plasma ghrelin levels and the resulting phenomenon of ghrelin resistance in malnourished patients with AN.

Introduction

Ghrelin is a 28-amino acid peptide hormone, produced mainly in the stomach [1], and is involved in a variety of physiologic functions [2]. Orexigenic and growth hormone–stimulating effects of ghrelin depend on its acylation with an octanoyl fatty acid residue at the serine-3 position [1]. In contrast, des-acyl ghrelin, the main form of circulating ghrelin, is devoid of many biological activities but may inhibit food intake [3,4]. The physiologic role of ghrelin in food intake regulation is manifested by an increase in plasma levels before meal initiation and its decrease after the meal [5].

A paradoxical situation of increased basal plasma ghrelin concentrations exists in subjects with long-term energy deficit and decreased food intake such as in restrictive anorexia nervosa (AN-R) [6]. This finding has been repeatedly shown and a meta-analysis study has confirmed increased ghrelin levels in AN-R [7]. Most studies in AN-R have reported increased plasma total or des-acyl ghrelin [8,9], but increased levels of active acyl ghrelin have also been found [10–12] with the exception of one study [13]. Refeeding and weight gain in patients with AN-R lead to normalization of basal plasma ghrelin [6,9] suggesting that elevated ghrelin levels are associated with starvation and malnutrition. Increased plasma ghrelin levels should result in increased appetite and food intake, which are not observed in AN-R, pointing to a functional ghrelin resistance that may be relevant to the decreased effects of ghrelin to promote a positive energy balance [14]. The existence of pharmacologic ghrelin resistance has been demonstrated in patients with AN-R who did

Mutsumi Terashi was supported by a fellowship from Kagoshima University, Kagoshima, Japan.
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doi:10.1016/j.nut.2011.01.002

not respond to ghrelin administration by increasing appetite and food intake as in healthy controls [15]. Increases in circulating ghrelin levels in AN-R are also paradoxical considering the atrophic changes of gastric and intestinal mucosa associated with malnutrition [16], suggesting that this increase is related rather to an increased post-translational stability than to an increased production of ghrelin per se, but the underlying mechanisms of this phenomenon are not known.

A factor thus far unexplored that may interfere with the regulation of plasma ghrelin levels is represented by naturally occurring ghrelin-reactive autoantibodies (autoAbs). The presence of autoAbs reactive with ghrelin, among other appetite-regulating peptide hormones, has been shown in healthy subjects and rats [17]. Furthermore, the functional relevance of circulating antighrelin antibodies to ghrelin levels and appetite control has been shown in rats immunized with ghrelin peptide fragments [18]. It is, therefore, possible that physiologic autoAbs reactive with ghrelin may regulate its plasma levels; accordingly, their decrease may result in elevated ghrelin levels, as observed in subjects with AN-R.

Thus, in the present study, to investigate the possible link between ghrelin autoAbs and increased levels of ghrelin in AN, we measured plasma levels of autoAbs reactive with acyl ghrelin and des-acyl ghrelin in patients with AN compared with healthy controls and with plasma levels of ghrelin peptides.

Materials and methods

Study subjects

The study was approved by the ethical committee of Kagoshima University, Kagoshima, Japan. Patients with AN were diagnosed according to criteria of the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* [19] and were admitted to Kagoshima University Hospital. Plasma from 10 female patients with AN including seven with a restrictive type, one with a binge-purging type, and two with eating disorders not otherwise specified close to the restrictive type and 10 healthy female subjects were analyzed. Body mass indices (BMIs) of patients and controls are listed in Table 1. Mean ages in the AN and control groups were 21.9 ± 1.8 and 23.5 ± 0.8 y, respectively. Routine venous blood samples were taken at admission (AN-1; $n = 10$) and 1 mo after refeeding (AN-2; $n = 9$ because one patient dropped out from the study). Blood samples were collected at 08:00 h from all subjects after an overnight fast. Blood samples were collected into tubes containing ethylenediaminetetraacetic acid (1 mg/mL) and aprotinin (500 U/mL) and plasma was separated by centrifugation at 4°C and stored at −80°C until assayed. Plasma levels of ghrelin autoAbs were measured by an enzyme-linked immunosorbent assay (ELISA) technique as described below from aliquots of the same blood samples used in a previous study [11] for the assay of acyl ghrelin and des-acyl ghrelin peptides by ELISA kits (Mitsubishi Kagaku Iatron, Tokyo, Japan). Values of plasma levels of acyl- and des-acyl ghrelin were available for the present study.

Ghrelin autoantibody assay

Plasma levels of autoAbs reacting with ghrelin peptides were measured using ELISA. Acyl- and des-acyl ghrelin peptides (Peptide Institute, Inc., Osaka, Japan) were coated on Maxisorp plates (Nunc, Rochester, NY, USA) using 100 μL and a concentration of 2 μg/mL in 100 mM $NaHCO_3$ buffer (pH 9.6) for 72 h at 4°C. Plates were washed (three times) in phosphate buffered saline with 0.05% Tween 200 (pH 7.4) and then incubated overnight at 4°C with 100 μL of patient or control subject sera diluted 1:400 in phosphate buffered saline to determine free autoAb levels or diluted 1:400 in dissociative 3 M NaCl and 1.5 M glycine buffer (pH 8.9) to determine total autoAb levels. The plates were washed (three times), and for the detection of immunoglobulin (Ig) G, IgM, or IgA, classes of autoAbs were incubated for 3 h at room temperature with 100 μl (1:1000) of rabbit anti-human IgG, anti-human IgM or anti-human IgA Abs, respectively, all conjugated with alkaline phosphatase (Sigma, St. Louis, MO, USA). After washing (three times), 100 μL of *p*-nitrophenyl phosphate solution (Sigma) was added as an alkaline phosphatase substrate. After 40 min of incubation at room temperature, the reaction was stopped by adding 3 N NaOH. The optical density (OD) was determined at 405 nm using a microplate reader. Blank OD values resulting from the reading of plates without the addition of rat sera were subtracted from the sample OD values. Each determination was done in duplicate. The variation between duplicate values was less than 5%.

Absorptions with ghrelin peptides

Before ELISA, to measure plasma levels of acyl- and des-acyl ghrelin free IgG autoAbs as described earlier, plasma samples were incubated overnight at 4°C with acyl ghrelin or des-acyl ghrelin peptides diluted at 10^{-8} M. In addition, for the detection of des-acyl ghrelin IgG autoAbs, sera from one control subject and from one patient with AN-1 were preincubated with serial dilutions of des-acyl ghrelin peptide ranging from 10^{-9} to 10^{-5} M.

Statistical analysis

Data were analyzed and graphs were plotted using GraphPad Prism 5.02 (GraphPad Software, Inc., San Diego, CA, USA). Levels of autoAbs were compared using analysis of variance (ANOVA) or Kruskal-Wallis test according to normality tests and post hoc Tukey or Dunn test was correspondingly performed. The AN groups were also compared with the control group using Student's *t* test or the Mann-Whitney test according to normality tests. The effect of absorptions was analyzed using the paired *t* test. Correlations between ghrelin autoAb levels and BMI or ghrelin plasma levels in patients with AN-1 and controls were calculated using the Pearson or Spearman two-tail test, respectively, according to the normality test. In all cases, $P < 0.05$ was considered statistically significant.

Table 1
Body mass index and plasma levels of ghrelin reactive autoantibodies and ghrelin peptides*

	Controls ($n = 10$)	Subjects with AN at admission ($n = 10$)	Subjects with AN at 1 mo after refeeding ($n = 9$)
Body mass index (kg/m^2)	21.6 ± 1.2	13.6 ± 0.6†	14.2 ± 0.5†
Des-acyl ghrelin free IgG autoAbs	0.68 ± 0.09	0.62 ± 0.1	0.66 ± 0.1
Des-acyl ghrelin total IgG autoAbs	1.4 ± 0.15	1.36 ± 0.16	1.35 ± 0.15
Acyl ghrelin free IgM autoAbs	0.67 ± 0.1	0.42 ± 0.07‡	0.44 ± 0.06‡
Acyl ghrelin total IgM autoAbs	0.96 ± 0.09	0.61 ± 0.09‡	0.72 ± 0.1
Acyl ghrelin free IgA autoAbs	0.73 ± 0.06	0.54 ± 0.09§	0.49 ± 0.09§
Acyl ghrelin total IgA autoAbs	2.0 ± 0.09	1.46 ± 0.14‖	1.36 ± 0.18‖
Plasma acyl ghrelin (pg/mL)	22.3 ± 2.16	28.6 ± 2.16†	NA
Plasma des-acyl ghrelin (pg/mL)	230.8 ± 20.01	340.1 ± 38.76‡	NA

AN, anorexia nervosa; autoAbs, autoantibodies; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; NA, not available

* Body mass index (mean ± SEM) and plasma levels of free and dissociated (total) ghrelin autoAbs (mean optical density ± SEM) were measured in control subjects and in patients with AN at admission and 1 mo after refeeding. Plasma concentrations of ghrelin peptides from the same blood samples used for autoAbs assay are listed for control subjects and in patients with AN at admission as previously reported [11].

† $P < 0.01$, Tukey test versus controls ($P = 0.009$, analysis of variance).

‡ $P < 0.05$, *t* test versus controls.

§ $P < 0.05$, Mann-Whitney test versus controls ($P = 0.04$, Kruskal-Wallis test).

‖ $P < 0.05$, Dunn test versus controls ($P = 0.007$, Kruskal-Wallis test).

Results

Plasma ghrelin autoantibodies

Mean plasma levels of acyl ghrelin free IgG autoAbs were lower in patients with AN compared with controls before and after refeeding (Fig. 1A). In the AN-1 group was one outlier value with an OD of 2.5, which was omitted from the statistical analysis of group differences. Mean plasma levels of acyl ghrelin dissociated (total) IgG autoAbs were also lower in patients with AN, particularly before refeeding (Fig. 1B). Mean plasma levels of free and total acyl ghrelin autoAbs of IgM and IgA classes were also lower in patients with AN than in controls at admission (Table 1). After refeeding, a significant difference was lost for total acyl ghrelin IgM autoAb levels but remained for IgM free and IgA free and total acyl ghrelin autoAbs. Mean plasma levels of des-acyl ghrelin free and total IgG autoAbs did not differ significantly between patients with AN and controls (Table 1).

Ghrelin autoantibodies after absorption with ghrelin peptides

Plasma levels of acyl ghrelin free IgG autoAbs changed after absorption with acyl ghrelin or des-acyl ghrelin 10^{-8} M peptides differently in patients with AN and controls (Fig. 2). In controls, absorption with acylated and des-acyl ghrelin peptides resulted in a decrease of signal (Fig. 2A,B). In patients with AN, absorption of plasma with acyl ghrelin did not significantly change the levels of acyl ghrelin autoAbs (Fig. 2C,E), whereas absorption with des-acyl ghrelin increased the signal for acyl ghrelin in the AN-1 group but decreased the signal in the AN-2 group (Fig. 2D versus 2F). No significant differences between group means were found after absorption with acyl ghrelin ($P = 0.5$, ANOVA) or des-acyl ghrelin ($P = 0.2$, ANOVA).

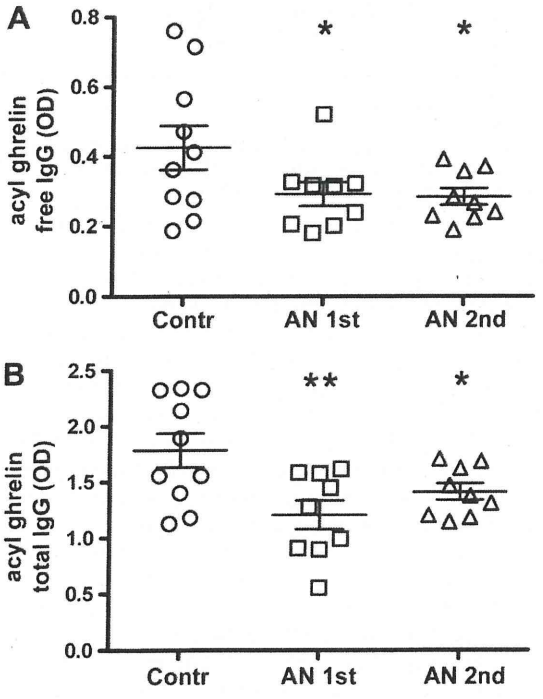

Fig. 1. Plasma levels (mean ± SEM) of (A) free and (B) dissociated (total) acyl ghrelin IgG autoantibodies in control subjects ($n = 10$) and in patients with anorexia nervosa at admission ($n = 9$) and 1 mo after refeeding ($n = 9$). * $P < 0.05$, t test versus Contr; ** $P < 0.01$, Tukey test versus Contr ($P = 0.009$, 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.

Plasma levels of des-acyl ghrelin free IgG autoAbs was decreased by absorption with acyl ghrelin in controls and patients (Fig. 3A,C,E). No significant differences between group means for des-acyl ghrelin IgG autoAbs were found after absorption with acyl ghrelin ($P = 0.29$, ANOVA). After absorption with des-acyl ghrelin, no significant change in signal intensity was found in controls (Fig. 3B). In contrast, a strong increase of the signal was observed in the AN-1 and AN-2 groups (Fig. 3D,E), resulting in higher mean levels of des-acyl ghrelin IgG autoAbs in patients with AN ($P = 0.0003$, ANOVA; Fig. 4A).

By absorption of sera from one control and one patient with AN-1 with increasing concentrations of des-acyl ghrelin peptide, an increase versus baseline of des-acyl ghrelin IgG autoAb levels was observed only in the patient with AN and at peptide concentrations of 10^{-8} M and to a lesser extent at 10^{-7} M (Fig. 4B).

Correlations of ghrelin autoantibodies with BMI and plasma ghrelin

Refeeding resulted in a slight but significant increase of BMI in patients with AN (Fig. 5A). The only significant dynamic of changes of ghrelin autoAb levels before and after refeeding was a slight increase of IgM class of acyl ghrelin total autoAbs (Fig. 5B). A positive correlation between BMI and acyl ghrelin total IgM autoAbs was also found (Pearson $r = 0.47$, $P < 0.05$).

Plasma levels of acyl ghrelin and des-acyl ghrelin peptides were higher in patients with AN-1 than in controls (Table 1), as reported in a previous study [11]. There were no data available on plasma ghrelin in patients with AN after refeeding. Plasma levels of des-acyl ghrelin total IgG autoAbs correlated negatively with plasma acyl ghrelin (Spearman $r = -0.46$, $P < 0.05$; Fig. 6A) and with plasma des-acyl ghrelin (Spearman $r = -0.55$, $P = 0.01$; Fig. 6B). Acyl ghrelin total IgA autoAbs also showed negative correlations with plasma des-acyl ghrelin levels (Spearman $r = -0.44$, $P < 0.05$).

Discussion

The present study confirmed earlier data showing ghrelin reactive IgG autoAbs in healthy subjects [17]. Accordingly to our hypothesis, we found that in patients with AN, plasma levels of acyl ghrelin IgG autoAbs present as free or total immunoglobulins were significantly lower with an exception of one patient with AN who showed at admission very high level of these autoAbs. A decrease of acyl ghrelin autoAbs in AN was also found for the IgM and IgA classes, suggesting a deficit in the initial antigenic stimulation to produce acyl ghrelin reactive autoAbs, but not in the alteration of a class switch from IgM. Because no decrease in humoral immunity is normally found in patients with AN [20] and even an increase in total IgM has been reported in the acute stage of AN [21], lower levels of acyl ghrelin autoAbs appears to be a specific change. The persistence of low levels of acyl ghrelin autoAbs after 1 mo of refeeding in patients with AN shows the association of slow nutritional recovery with physiologic parameters [22].

Considering the elevated levels of acyl- and des-acyl ghrelin peptides in patients with AN, our finding of low levels of acyl ghrelin autoAbs and unchanged levels of des-acyl ghrelin autoAbs suggest that insufficient plasma binding by these

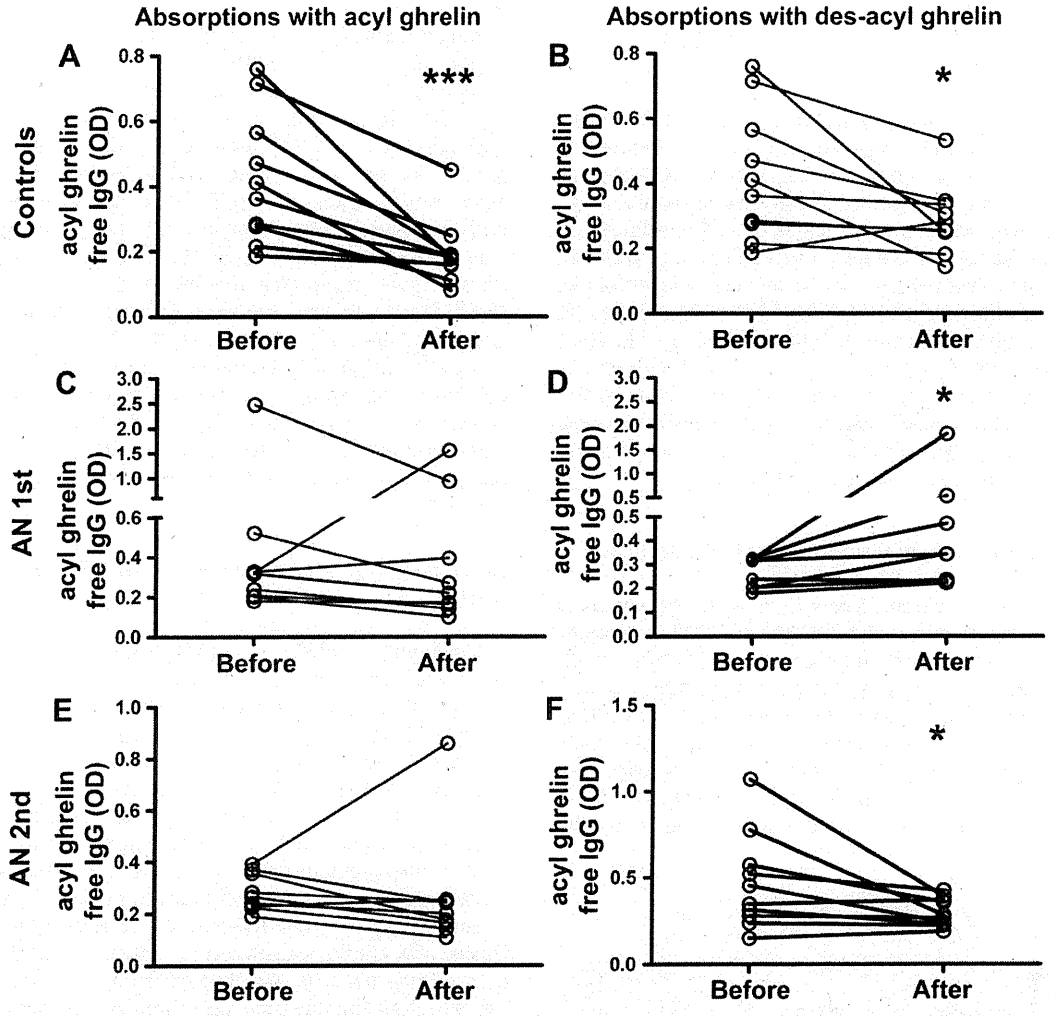

Fig. 2. Plasma levels of acyl ghrelin free IgG autoantibodies before and after absorption of sera with (A, C, E) 10^{-8} M acyl ghrelin or (B, D, F) 10^{-8} M des-acyl ghrelin in (A, B) control subjects ($n = 10$) and in patients with anorexia nervosa (C, D) before ($n = 10$) and (E, F) after refeeding ($n = 9$). $^{***}P < 0.001$, $^{*}P < 0.05$, paired t test. AN 1st, patients with anorexia nervosa at admission; AN 2nd, patients with anorexia nervosa 1 mo after refeeding; IgG, immunoglobulin G; OD, optical density.

autoAbs of these two forms of ghrelin may explain their persistently elevated levels in AN. The negative correlations between levels of ghrelin autoAbs and ghrelin peptides found in this study further support this possibility. We, however, acknowledge that such an interpretation is limited to the single, although representative for increased ghrelin levels, time point of the day when blood samples were taken.

Furthermore, from the absorption experiments it may be deduced that ghrelin reactive autoAbs may bind to both forms of ghrelin but in healthy subjects they are reactive mainly with acyl ghrelin and in AN patients with des-acyl ghrelin. These results support data of lower levels of acyl ghrelin autoAbs in AN. Another interesting finding in patients with AN was the presence of immune complexes of ghrelin IgG autoAbs with des-acyl ghrelin. Detection of these complexes was not possible by dissociation in NaCl-glycine buffer but was disclosed in absorption experiments after adding 10^{-8} M des-acyl ghrelin. In fact, adding des-acyl ghrelin to sera from patients but not from controls increased dramatically the detectable levels of des-acyl ghrelin autoAbs. Such a phenomenon may be explained as a new equilibrium between free and bound ghrelin autoAbs at certain peptide concentrations. This was also illustrated in a plasma sample from a patient with AN incubated with different concentrations of des-acyl ghrelin, revealing the dissociating effect of des-acyl ghrelin at 10^{-8} M but not at lower or higher concentrations. Altogether, these results suggest that in patients with AN ghrelin autoAbs may bind des-acyl ghrelin close to nanomolar affinity, resulting in relatively stable immune complexes. Such complexes may be also responsible for insufficient availability of autoAbs to reversibly bind ghrelin peptides.

If the increase and normalization of plasma ghrelin during starvation and refeeding, respectively, in AN is related to nutritional status, then we can assume that it may also underlie changes of ghrelin autoAbs, which also are reversible. The following possibilities can be considered to explain a decrease in the levels of acyl ghrelin autoAbs during AN-associated starvation. The physiologic production of ghrelin autoAbs can be modulated by gut microflora known to display a molecular mimicry to ghrelin [17], and, hence, starvation-induced changes in gut content and decreased gut-barrier permeability [23,24] may decrease ghrelin autoAb production. We found that refeeding in patients with AN was accompanied by an increase of an IgM class of acyl ghrelin autoAbs, which may indicate new antigenic stimulation. Another possibility is that

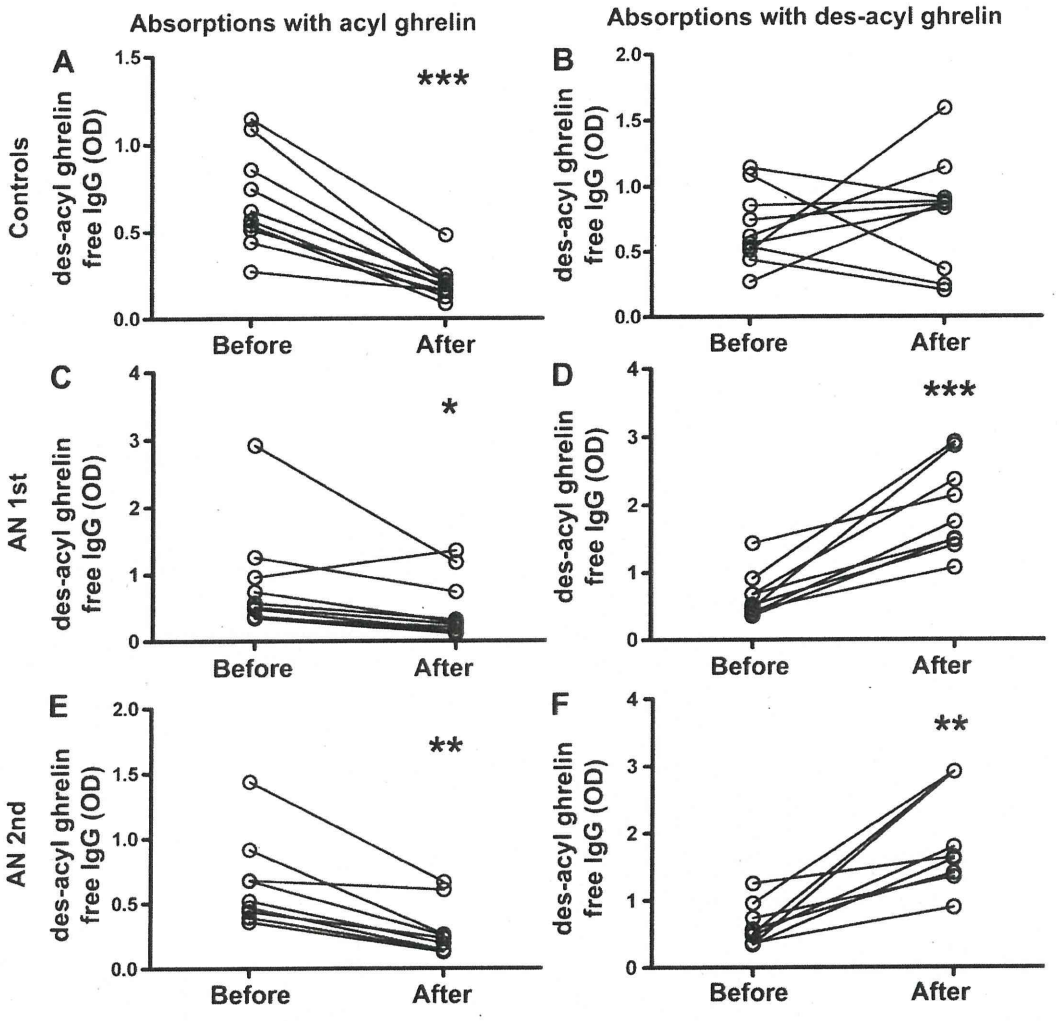

Fig. 3. Plasma levels of des-acyl ghrelin free IgG autoantibodies before and after absorptions of sera with (A, C, E) 10^{-8} M acyl ghrelin or (B, D, F) 10^{-8} M des-acyl ghrelin in (A, B) control subjects ($n = 10$) and in patients with anorexia nervosa (C, D) before ($n = 10$) and (E, F) after refeeding ($n = 9$). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, paired t tests. AN 1st, patients with anorexia nervosa at admission; AN 2nd, patients with anorexia nervosa 1 mo after refeeding; IgG, immunoglobulin G; OD, optical density.

a starvation-induced deficit in alimentary sources of octanoic acid [25] will decrease the level of acylated ghrelin but will not decrease the production of des-acyl ghrelin, resulting in an epitope switch of ghrelin autoAbs from acyl- to des-acyl ghrelin. This is supported by the presence of relatively high-affinity des-acyl ghrelin autoAbs in AN. It is also known that calorie restriction is associated with the activation of some deacylation enzymes [26]. These two mechanisms may contribute to the observed shift from acylated to des-acyl ghrelin reactive autoAbs in healthy subjects versus patients with AN. Notably, as revealed by the absorption experiments, the inverse tendency was observed and more autoAbs reactive with acylated ghrelin were present after refeeding.

Can these data be relevant to the phenomenon of "ghrelin resistance" initially postulated to be present in AN [6]? Whether true functional ghrelin resistance exists in AN resulting in decreased hunger signaling and other ghrelin-mediated functions, e.g., anxiety [27], is not certain. Pharmacologic doses of ghrelin in patients with AN have not shown consistent results in significantly improving appetite and food intake, supporting the existence of ghrelin resistance [15,28,29]. However, one cannot exclude that some ghrelin downstream pathways involved in appetite control override the ghrelin orexigenic effect in AN [30], e.g., enhanced α-melanocyte–stimulating hormone signaling [31]. Moreover, meal- and glucose-induced decreases of ghrelin release are preserved in AN [11,13,32]. If ghrelin reactive autoAbs are increased in AN, then these could produce ghrelin resistance by ghrelin neutralization [33]; however, ghrelin autoAbs were decreased, which intuitively indicates relevance to elevated ghrelin levels but not to ghrelin resistance. Elevated hormone levels can trigger resistance by receptor desensitization. However, no evidence for desensitization of ghrelin orexigenic properties in chronic hyperghrelinemia has been found in ghrelin-overexpressing mice [34]. Although increased ghrelin secretion has been shown to desensitize the growth hormone–stimulatory effect of ghrelin [35], it is not clear if this occurs in AN, because patients with AN display high plasma growth hormone but low insulin-like growth factor-1 levels [36].

Alternatively, ghrelin reactive autoAbs may be relevant not only to neutralization of ghrelin peptides but also to their transportation. Accordingly, lower levels of ghrelin in healthy subjects compared with patients AN can be explained by a fraction of bioavailable ghrelin reversibly bound to autoAbs before it is released toward ghrelin receptors. Such a role for

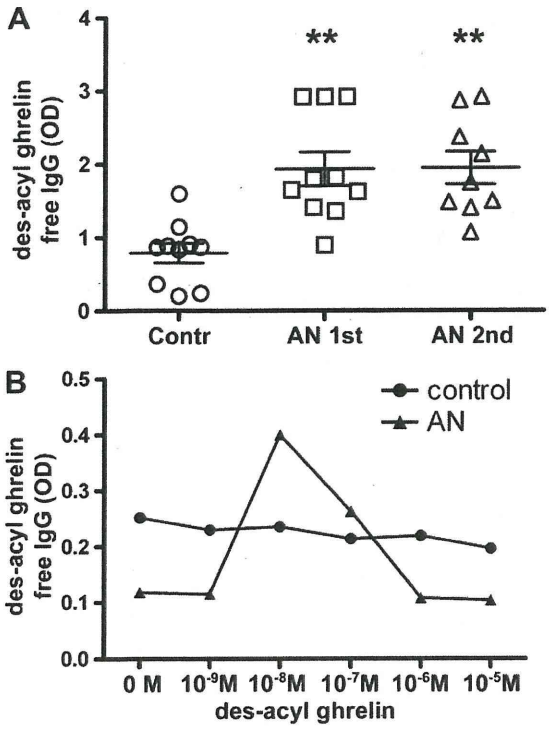

Fig. 4. (A) Plasma levels (mean ± SEM) of des-acyl ghrelin free IgG autoantibodies after absorption of sera with 10^{-8} M des-acyl ghrelin in control subjects ($n = 10$) and in patients with anorexia nervosa before ($n = 10$) and after refeeding ($n = 9$). (B) Effect of absorption of sera from one control 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.

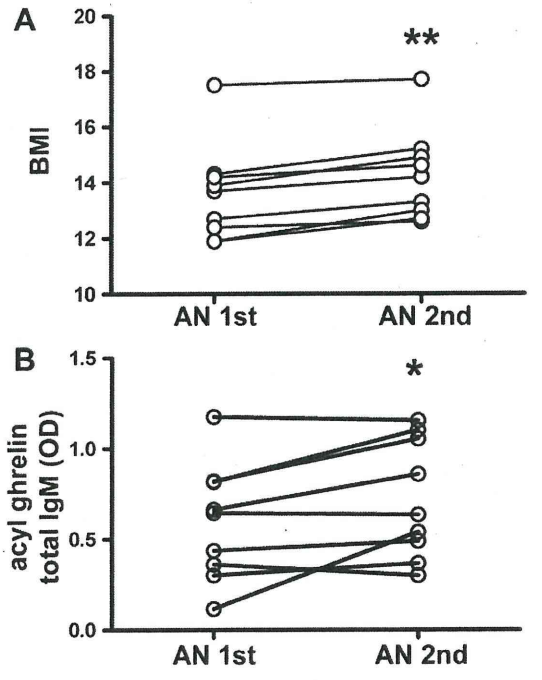

Fig. 5. Dynamics of changes in patients with anorexia nervosa ($n = 9$) before and after refeeding with respect to (A) BMI and (B) plasma levels of acyl ghrelin total IgM autoantibodies. ** $P < 0.01$, * $P < 0.05$, paired t tests. AN 1st, patients with anorexia nervosa at admission; AN 2nd, patients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

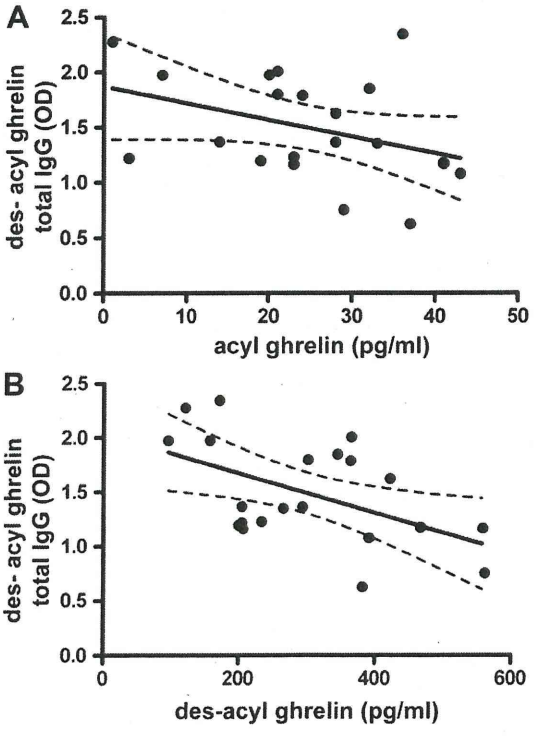

Fig. 6. Negative correlations between plasma levels of des-acyl ghrelin total IgG autoantibodies and plasma concentration of (A) acyl-ghrelin (Spearman $r = -0.46$, $P < 0.05$) and (B) des-acyl ghrelin (Spearman $r = -0.55$, $P = 0.01$) in study subjects. IgG, immunoglobulin G; OD, optical density.

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

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JGH Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/j.1440-1746.2011.06638.x

GASTROENTEROLOGY

Ghrelin family of peptides and gut motility

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

Accepted for publication 24 January 2011.

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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]

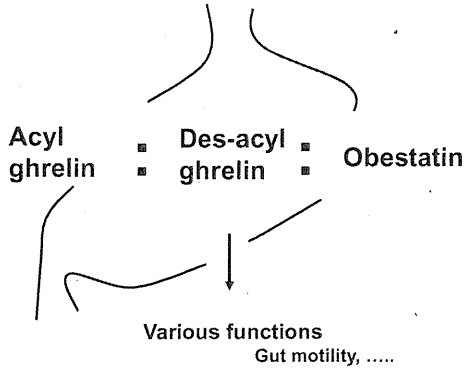

Figure 1 Stomach regulates various functions via ghrelin family of peptides.

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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Citation: Transl Psychiatry (2011) 1, e23, doi:10.1038/tp.2011.25

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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.
Translational Psychiatry (2011) **1**, e23; doi:10.1038/tp.2011.25; published online 26 July 2011

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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Keywords: anorexia–cachexia; CRF; ghrelin; GHS-R; neuropeptide Y
Received 21 April 2011; revised 23 May 2011; accepted 16 June 2011

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 $\mu mol\,kg^{-1}$; 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 $\mu mol\,kg^{-1}$), 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 $\mu g\,ml^{-1}$ 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

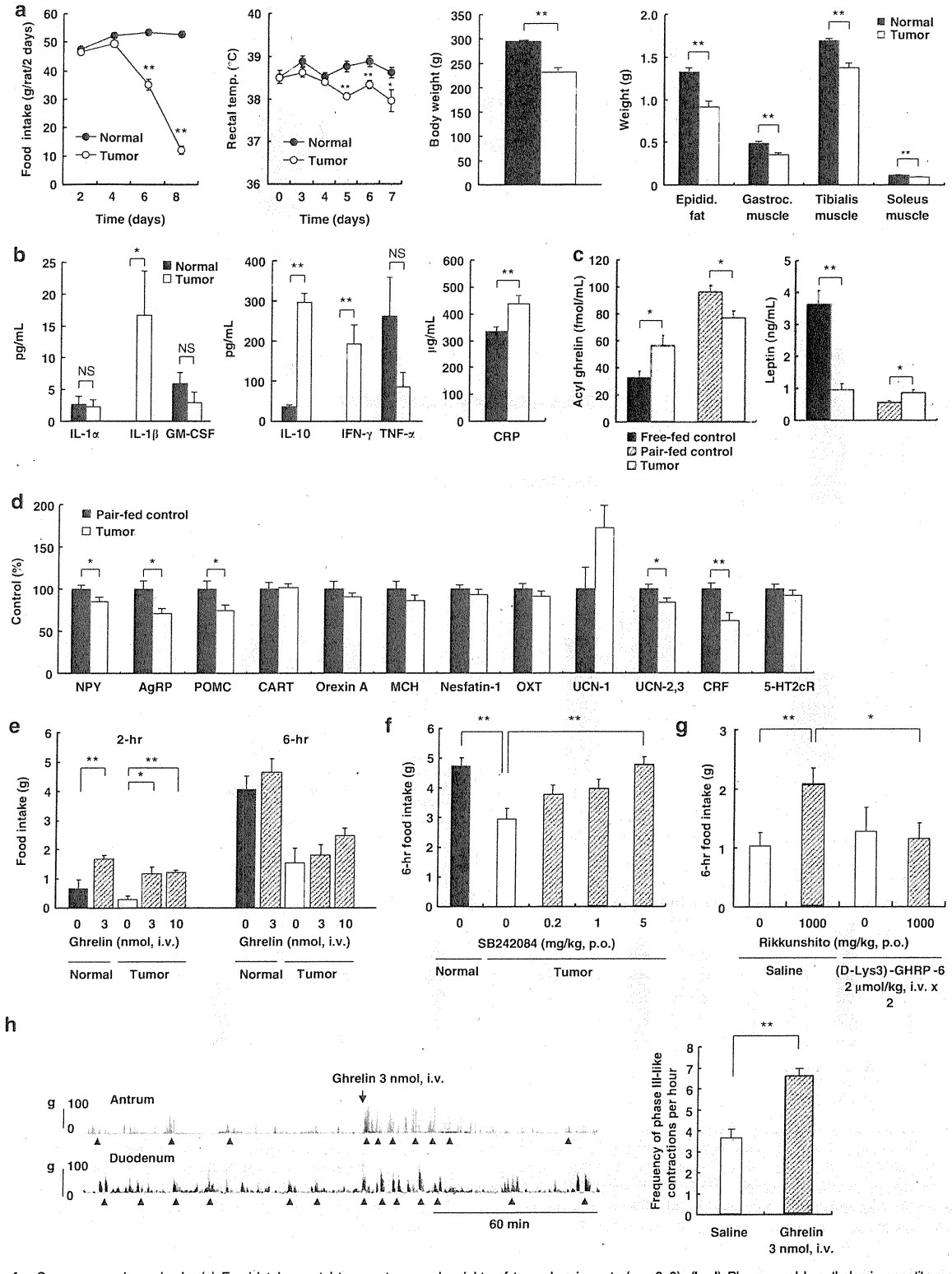

Figure 1 Cancer anorexia–cachexia. (**a**) Food intake, rectal temperatures and weights of tumor-bearing rats ($n=6$–8). (**b–d**) Plasma and hypothalamic appetite-regulating peptides ($n=8$–10). (**e–g**) Effects of ghrelin, the serotonin 2c receptor (5-HT2cR) antagonist SB242084 and rikkunshito on 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.

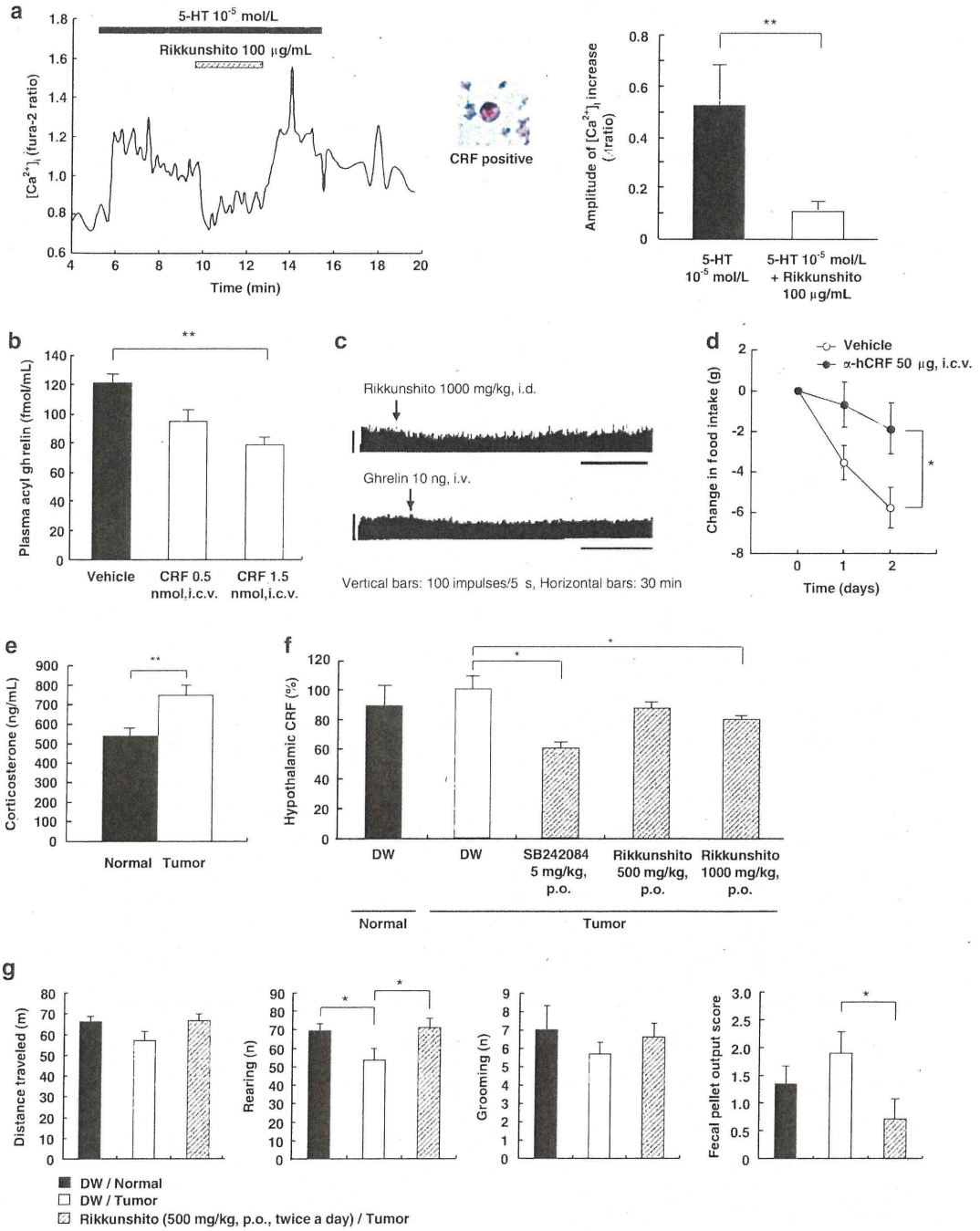

Figure 2 Involvement of corticotropin-releasing factor (CRF) in cancer anorexia. (**a**) The serotonin (5-HT)-induced cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) increase was suppressed by rikkunshito in single CRF neurons isolated from the paraventricular nucleus (PVN). (**b**) Inhibitory effect 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).

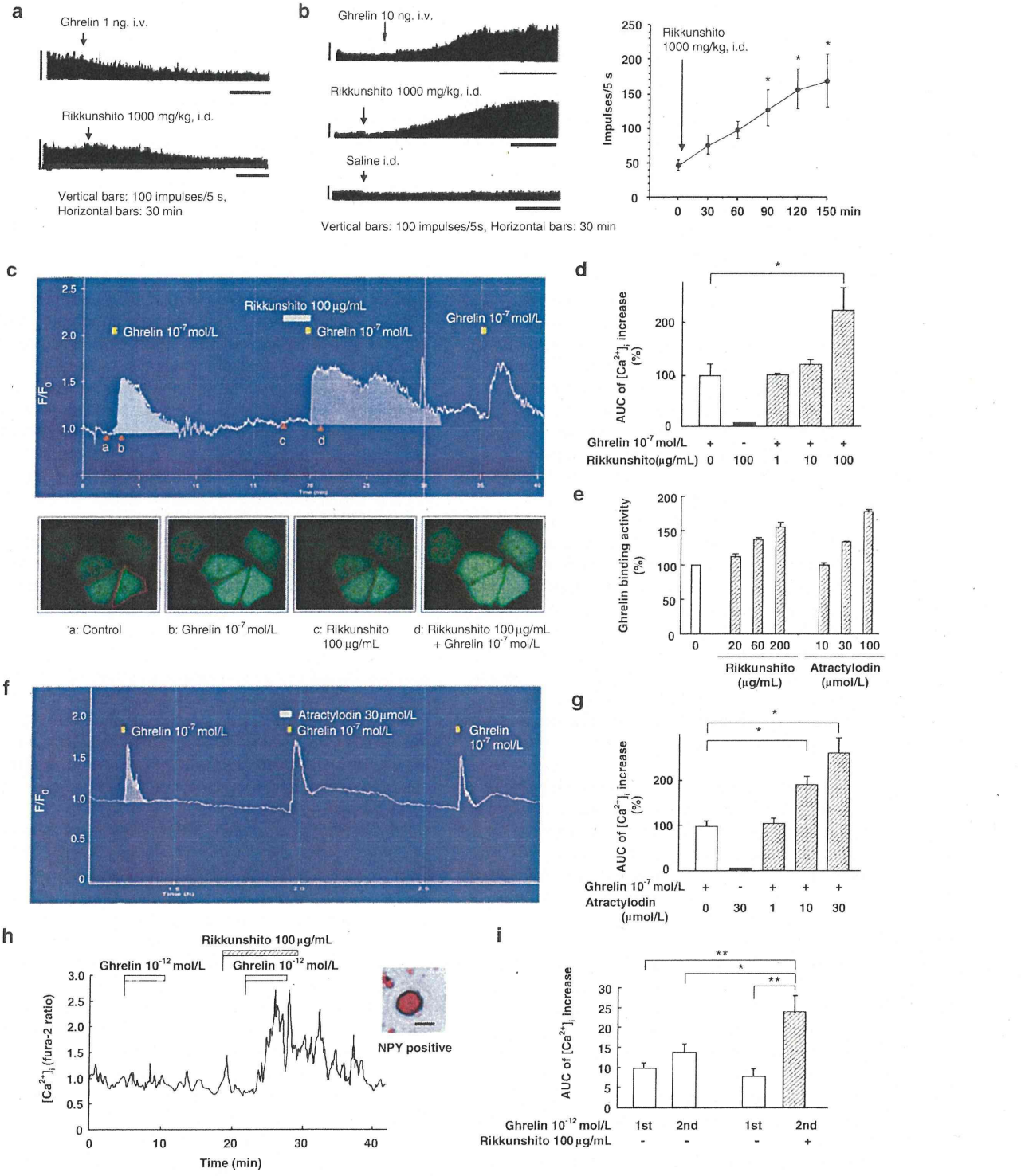

Figure 3 Ghrelin signaling and rikkunshito. (**a**, **b**) Effects of ghrelin and rikkunshito on the afferent (**a**) and efferent (**b**, $n=6$) activities of the gastric vagus nerve in rats. (**c**, **d**) Changes in F/F_0 fluorescence evoked by ghrelin in ghrelin receptor (GHS-R)-expressing cells. 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

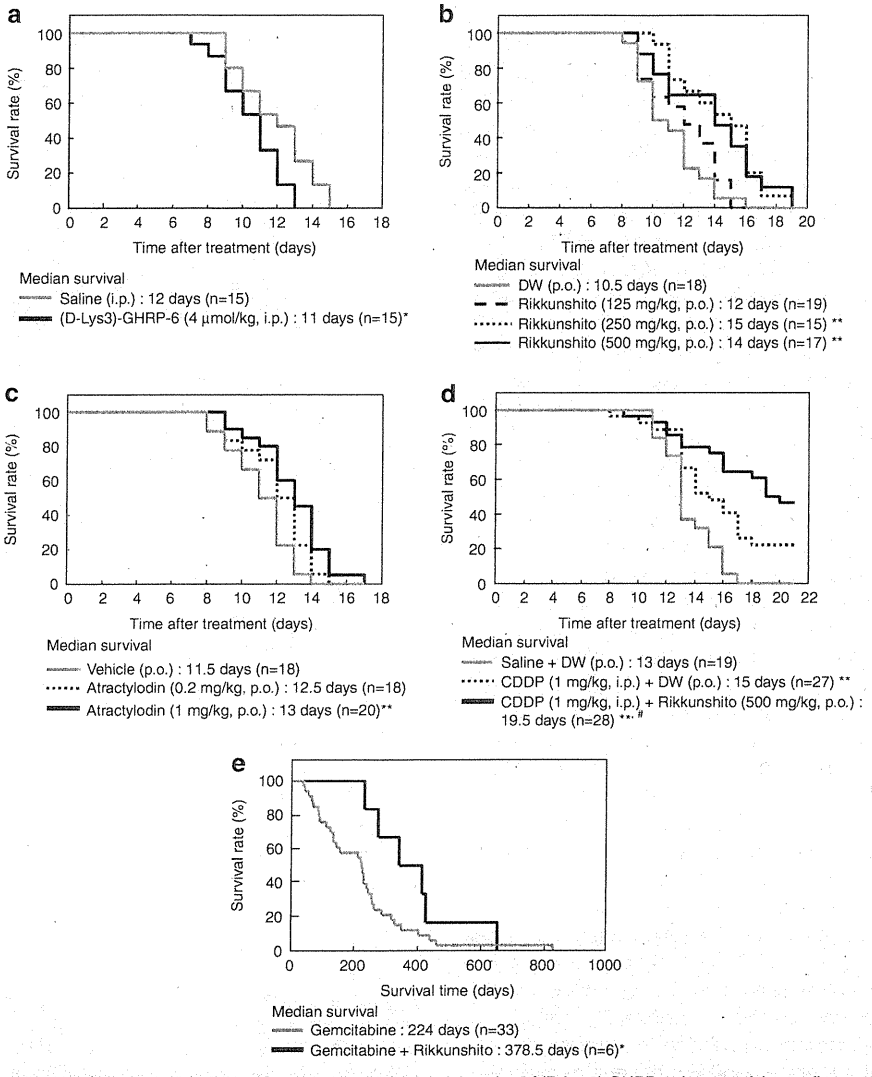

Figure 4 Survival of tumor-bearing rats and patients with pancreatic cancer. (**a**) Administration of (D-Lys3)-GHRP-6 decreased the median survival of tumor-bearing rats. (**b**, **c**) Administration of rikkunshito and atractylodin increased the median survival of tumor-bearing rats. (**d**) Survival of tumor-bearing rats was increased by intraperitoneal (i.p.) administration of cisplatin (CDDP) and further prolonged by co-administration of rikkunshito. (**e**) Rikkunshito prolonged the median survival of pancreatic cancer patients with ascites who were treated with gemcitabine. $^{*}P<0.05$; $^{**}P<0.01$ vs control group; $^{\#}P<0.05$ vs CDDP + DW. DW: distilled water.

inhibitors such as fenfluramine and 5-HT2cR agonists attenuate food intake and weight gain in rodents and humans,[40–42] with the involvement of potentiated MC signaling and decreased ghrelin secretion. 5-HT also inhibits NPY/agouti-related peptide neurons by activating the 5-HT1bR, leading to decreased orexigenic signaling and an inhibitory drive onto POMC cells. However, our previous study suggested that the 5-HT2cR has a major role in the regulation of physiological fasted and fed motor activities in addition to feeding through changes in endogenous ghrelin.[15] In this study, we found that the decreases in food intake and GI motor activities in tumor-bearing rats were recovered after administration of either a 5-HT2cR antagonist or ghrelin. The 5-HT concentration in the hypothalamus is increased in humans and animals with cancer;[43,44] in addition, NPY and dopamine concentrations decrease simultaneously, while 5-HT concentration increases in the PVN at the onset of anorexia in tumor-bearing rats.[45] These findings suggest that 5-HT2cR activation in tumor-bearing rats induces anorexia in part via decreased ghrelin secretion.

We have previously shown that a central 5-HT2cR pathway regulates ghrelin secretion without downstream activation of melanocortin 3/4 receptors.[15] The 5-HT2cR is expressed in many brain regions and its expression is restricted to the central nervous system.[46] Dual-neurohistochemical labeling has revealed that approximately one-half of PVN CRF-containing neurons co-express 5-HT2cR mRNA.[47] In this study, we found that 5-HT activated single CRF neurons isolated from the PVN, and the activities of the CRF neurons were blocked by simultaneous administration of rikkunshito.