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Ghrelin Treatment Causes Increased Food Intake and Retention of Lean Body Mass in a Rat Model of Cancer Cachexia

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Cancer cachexia is a debilitating syndrome of anorexia and loss of lean body mass that accompanies many malignancies. Ghrelin is an orexigenic hormone with a short half-life that has been shown to improve food intake and weight gain in human and animal subjects with cancer cachexia. We used a rat model of cancer cachexia and administered human ghrelin and a synthetic ghrelin analog BIM-28131 via continuous infusion using sc osmotic minipumps. Tumor-implanted rats receiving human ghrelin or BIM-28131 exhibited a significant increase in food consumption and weight gain *vs.* saline-treated animals. We used dual-energy x-ray absorptiometry scans to show that the increased weight was due to maintenance of lean mass *vs.* a loss of lean mass in saline-treated animals. Also, BIM-28131 significantly limited the loss of fat mass normally observed in tumor-implanted rats. We further performed real-time PCR analysis of the hypothalami and brainstems and found that ghrelin-treated animals exhibited a significant increase in expression of orexigenic peptides agouti-related peptide and neuropeptide Y in the hypothalamus and a significant decrease in the expression of IL-1 receptor-I transcript in the hypothalamus and brainstem. We conclude that ghrelin and a synthetic ghrelin receptor agonist improve weight gain and lean body mass retention via effects involving orexigenic neuropeptides and antiinflammatory changes. (*Endocrinology* 148: 3004–3012, 2007)

CACHEXIA IS A metabolic disturbance consisting of anorexia and loss of lean body and fat mass associated with a paradoxical increase in energy expenditure that accompanies a variety of conditions, including cancer, AIDS, heart failure, renal failure, rheumatoid arthritis, and cystic fibrosis and as part of the catabolic mechanism accompanying weight loss after gastric bypass for morbid obesity (1, 2). Paradoxically, the comorbidities of these catabolic conditions and also of obesity are mediated by inflammatory cytokines and neuropeptides, which are associated with the inflammation that is produced by the immune system's response to these underlying disease states (3–7). Cachexia is a major source of morbidity and mortality related to the associated diseases, and thus far no therapy has proven to be satisfactorily effective in the treatment of these metabolic derangement (8).

One potential therapeutic agent in the treatment for cachexia is ghrelin. Because its discovery as the endogenous ligand for the GH secretagogue (GHS)-1a receptor, ghrelin has been well established as the only known circulating orexigenic hormone (9, 10). It is produced primarily in the fundus of the stomach in response to fasting and exhibits actions in a wide variety of areas in which its receptor is expressed, including appetite centers in the hypothalamus (11–13). When given in experimental conditions, ghrelin increases food intake in rodents and humans (14–16).

Patients with cancer and cardiac cachexia have been shown to have increased circulating concentrations of ghrelin, perhaps related to their prolonged state of negative energy balance or body composition changes (17–20). Nevertheless, short-term administration of supraphysiological doses of ghrelin in these conditions still results in an increase in food intake (14, 21, 22). This was verified in longer-term administration in cardiac cachexia, although widespread application is limited because of the short half-life of ghrelin and previous applications using intravenous administration (23).

In addition to its appetite-stimulating properties, ghrelin has also been noted to have antiinflammatory effects. The receptor for ghrelin is expressed on lymphocytes and administration of the ghrelin receptor agonist GH-releasing peptide-2 in a rat model of arthritis has been shown to decrease serum levels of IL-6 and reduce signs of joint inflammation (24). Additionally, when pretreated with ghrelin or GH-releasing peptide-2, cultured macrophages decrease lipopolysaccharide-induced IL-6 production. Because of the

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Abbreviations: AgRP, Agouti gene-related peptide; DEXA, dual-energy x-ray absorptiometry; GHS, GH secretagogue; h, human; IL-1RI, type 1 IL-1 receptor I; NPY, neuropeptide Y; POMC, proopiomelanocortin; RQ, relative quantity; RT, reverse transcription.

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central role of inflammation in cachexia, these antiinflammatory effects of ghrelin may represent another mechanism of action by which ghrelin could improve cachexia (3, 7).

Given the limitations on the use of ghrelin due to its short half-life, an alternative would be to find a GHS-1a receptor agonist with sufficient selectivity, potency, and plasma stability to be used in a clinical setting. Because of the interest in ghrelin as a GHS, many such compounds have been developed. The data presented below provide evidence that a synthetic GHS-1 receptor agonist can provide protection against the development of cachexia in a cancer model and that its mechanism of action is likely to be similar to native ghrelin.

In this paper we describe the effects of ghrelin and the synthetic ghrelin receptor agonist BIM-28131 on food intake, body composition, and gene expression in a rat model of cancer anorexia leading to cachexia (25–27). We administered ghrelin and BIM-28131 via sc osmotic minipumps to rats that had been implanted with a robust cachexigenic sarcoma and measured the effect these compounds on food intake and body composition parameters including lean body mass. Additionally, we harvested the hypothalami and brainstems of these animals to investigate the effect of ghrelin on the expression of genes related to appetite and inflammation. This is the first demonstration of the potential for the use of a synthetic GHS-1 agonist in cancer cachexia and suggests two mechanisms whereby the anticachectic properties of ghrelin may be mediated.

Materials and Methods

Compounds

BIM-28131 and human (h)-ghrelin were provided by IPSEN (Milford, MA). BIM-28131 is a pentapeptide analog that binds to the known ghrelin receptor (GHS-1a) with subnanomolar affinity (inhibitory constant, 0.42 ± 0.063 nM) [~3 times greater affinity than native ghrelin (inhibitory constant, 1. 12 ± 0.17 nM)] (25–27). BIM-28131 is 6 times more potent (EC_{50}, 0.71 ± 0.09 nM) in activating the GHS-1a receptor than native ghrelin (EC_{50}, 4.2 ± 1.2 nM) as assessed *in vitro* by calcium mobilization. BIM-28131 has greater enzymatic stability in plasma than native ghrelin (half-time rat plasma 24 *vs.* 1.9 h, respectively) and, when injected iv, is observed to have a 10-fold greater circulating half-life as compared with native ghrelin.

Experimental animals

Studies were approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University and conducted according to National Institutes of Health Guide for the Care and Use of Laboratory Animals. F344/NTacfBR male rats (Taconic Farms, Inc., Germantown, Hudson, NY) were housed two per cage, fed rat chow (Diet 5001; Purina Mills, Inc., St. Louis, MO) and acclimated for at least 3 d before use. Before tumor implantation, rats were weighed and evenly divided into five groups stratified according to body weight to ensure that the mean weight of each group was similar before the surgical procedures: controls and sham-operated rats received vehicle, whereas tumor-bearing rats received one of two treatment compounds: BIM-28131 or h-ghrelin. A subset of sham-operated animals were pair fed with the saline-treated, tumor-bearing rats such that these pair-fed rats were given a quantity of food each day equal to the average amount eaten by the tumor-bearing rats from the previous day. The pair-fed rats were then used as a control to compare relative gene expression between groups that had consumed similar amounts of food.

Tumor tissue preparation

The tumor is a methylcholanthrene sarcoma that does not metastasize. Its characteristic growth curve *vs.* time is curvilinear and was previously documented (28). Rats were between 10 and 12 wk of age and weighed more than 200 g at time of tumor implantation. Tumor implantation was performed 8 d before compound treatment to allow time for adequate tumor growth.

Fresh tumor tissue (0.2–0.3 g) from a rat donor was implanted sc into the flank of a rat, after anesthesia (55.5 mg ketamine, 5.5 mg xylazine, and 1.1 mg acepromazine per milliliter; 1.0 ml/kg body weight ip) as previously described (29).

Rats were individually housed and daily body weight and food intake were measured. Tumors became palpable 6–7 d after implantation and tumor size measured daily thereafter. Tumor volume was calculated from the formula for a prolate sphere ($V = \frac{1}{2} ab^2$), where a is the longer and b the shorter dimension. By d 13, the tumor burden had fallen into the end points of the tumor growth studies, according to Oregon Health and Science University Institutional Animal Care and Use Committee Policy on Tumor Burden. After the animals were killed, the tumor was dissected free and weighed.

Compound administration

A continuous sterile infusion of either BIM-28131 or h-ghrelin or saline was administered at a rate of 0.5 μl/h for 5 d sc using Alzet miniosmotic pumps (model 2002; Durect Corp., Cupertino, CA). The day before implantation of the pumps, the mean body weight of each group was determined. To calculate the concentration required for the treatment group, we considered the molecular weight of each compound, the dose (50 or 500 nmol/kg·d), and the pump delivery rate. Each compound was dissolved in vehicle solution (2% inactivated rat serum saline, 5% Tween 80) sonicated, and filtered through a 0.2-μl syringe filter.

On d 8 after tumor implantation, the diameter of the tumors was equal to or greater than 1 cm. Rat were anesthetized and the primed pumps were placed sc via a small intrascapular incision into the various treatment groups. The skin incision was closed with sutures. Sham rats received anesthesia for both the sham tumor implantation and placement of the saline minipump.

Body composition

Body composition was determined before tumor implantation or sham operation under anesthesia by dual-energy x-ray absorptiometry (DEXA; Discovery A-QDR series; Hologic Corp., Waltham, MA) and on the final day after the animals were killed with CO_2 and tumor resection.

Tissue collection

On d 13 after tumor implantation (and d 5 after minipump placement), tumor growth and overall condition of the tumor-bearing animals had fallen within the predetermined end points of the study, with particular attention paid to the volume of the tumor and the overall health of the experimental animal. After the animals were killed, blood, brain, stomach, and muscle were collected. The tumors were dissected away from surrounding tissue and weighed. A subset of the rats had their hypothalami and brainstems dissected out, preserved in RNAlater solution, and stored at −70 C for extraction of RNA and RT-PCR analysis. Hypothalamic blocks were dissected by making coronal transections at the optic chiasm and at the intersection between the hypothalamus and the mammillary bodies and sagittal transections along the optic tracks. Cortex was then removed at the level of the corpus callosum. Brainstem blocks were dissected by removal of the cerebellum and coronal transections at the rostral border of the pons and at the spinal cord.

Because final DEXA scanning required tumor removal and thus postmortem tissue (*i.e.* not a good source of mRNA), the rats used for RNA extraction did not undergo DEXA scan and included only saline- and ghrelin-treated tumor bearing animals, sham, and pair-fed sham animals.

RNA preparation and RT-PCR

Total RNA was extracted using RNeasy kits (QIAGEN, Inc., Valencia, CA), and DNA was removed from total RNA, using RNase-Free DNase

(QIAGEN). Reverse transcription (RT) reactions were prepared using a TaqMan reverse transcription kit (Applied Biosystems, Inc., Foster City, CA). For each reaction cDNA synthesis was prepared using 500 ng of RNA in a reaction containing 4 μl 10× RT buffer, 9 μl 25 mM $MgCl_2$, 8 μl 10 mM deoxynucleotide triphosphates, 1.5 μl 50 μM random hexamers, 1 μl RNase inhibitor, 1.5 μl MulitScribe reverse transcriptase, quantity sufficient to 40 μl with nuclease-free water. RT reactions were performed on a Mastercycler (Eppendorf AG, Hamburg, Germany) programmed for 25 C for 10 min, 37 C for 1 h, and 95 C for 5 min. Samples were diluted with 40 μl nuclease-free water stored at 4 C until RT-PCR was performed.

RT-PCR was performed on an ABI 7300 real-time PCR system using rat-specific primer probe sets obtained from Applied Biosystems. Each RT-PCR contained 10 μl TaqMan universal PCR master mix, 1 μl Assays-on-Demand gene expression assay mix (Applied Biosystems), 4 μl nuclease-free water, and 5 μl cDNA. Samples and endogenous controls (eukaryotic 18s rRNA) were run in duplicate to assure repeatability. Auto cycle threshold values were calculated using 7300 RQ study software (version 1.3; Applied Biosystems) and verified. Gene expression values are expressed fold change relative to sham/normal-fed mean.

GH and IGF-I assays

Serum collected at the time the animals were killed was tested for GH levels using a RIA. A separate aliquot underwent an ethanol/HCl extraction procedure to remove binding proteins and was tested for IGF-I levels, also using a RIA.

Cytokine measurement

Rat serum samples were tested simultaneously for cytokines IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, granulocyte macrophage colony-stimulating factor GM-CSF, interferon-γ, and TNF-α using a rat cytokine 9-Plex assay (Bio-Plex; Bio-Rad, Hercules, CA). The assay was run according to the manufacturer's instructions. In brief, the premixed standards were reconstituted in 0.5 ml of a Bio-Plex human serum standard diluent, generating a stock concentration of 50,000 pg/ml for each cytokine. The standard stock was serially diluted in the Bio-Plex rat serum standard diluent to generate eight points for the standard curve. The assay was performed in a 96-well filtration plate supplied with the assay kit. Premixed beads (50 μl) coated with target capture antibodies were transferred to each well of the filtration plate and washed twice with Bio-Plex wash buffer. The samples were diluted 1:4 in the Bio-Plex serum sample diluent. Premixed standards or diluted samples (50 μl) were added to each well containing washed beads. The plate was shaken and incubated at room temperature for 30 min at low speed (300 rpm). After incubation and washing, premixed biotin conjugated detection antibodies were added to each well. Then the plate was incubated for 30 min with shaker at low speed (300 rpm). After incubation and washing, streptavidin-phycoerythrin was added to each well. The incubation was terminated after shaking for 10 min at room temperature. After washing, the beads were resuspended in 125 μl of Bio-Plex assay buffer. Beads were read on the Bio-Plex suspension array system (Bio-Rad), and the data were analyzed using Bio-Plex Manager software version 3.0 with 5PL curve fitting.

Results

Food intake, body weight, and tumor mass

As shown in Fig. 1, administration of ghrelin and a ghrelin receptor agonist resulted in an increase in food intake [tumor/saline 41.4 ± 3.3 g, tumor/ghrelin 66.0 ± 3.0 ($P < 0.001$), tumor/BIM-28131 72.5 ± 3.0 g ($P < 0.001$), n = 11 for each group] and weight gain [tumor/saline −10.3% ± 2.90, tumor/ghrelin +13.0% ± 4.3 ($P < 0.001$), tumor/BIM-28131 +19.5% ± 2.80 ($P < 0.001$)]. For ghrelin and BIM-28131, these effects were noted only at relatively high-dose delivery (500 nmol/kg·d). These effects were not observed at low-dose delivery (50 nmol/kg·d, data not shown), nor were they observed when ghrelin was given by twice-daily injection rather than continuous infusion (200 nmol/kg·dose, data not shown).

Body composition

DEXA scan (Fig. 2) revealed decreased loss of body mass *vs.* control following treatment with high-dose ghrelin and BIM-28131 [tumor/saline −17.5% ± 1.30, tumor/ghrelin −6.10% ± 1.00 ($P < 0.001$), tumor/BIM-28131 −6.10 ± 1.30 ($P < 0.001$), all n = 11]. Accompanying these changes in body mass, there was no difference in percent change in fat mass *vs.* control after treatment with ghrelin at either high dose or low dose (low dose data not shown). Animals treated with BIM-28131 at both high and low doses lost less fat mass than controls [tumor/saline −50.8% ± 4.90, tumor/BIM-28131 low dose −31.5% ± 4.3 ($P < 0.05$), tumor/BIM 28131 high dose −31.5 ± 4.30 ($P < 0.05$), all n = 11].

High-dose treatment with ghrelin and BIM-28131 resulted in a near maintenance of lean body mass [tumor/saline −12.6% ± 1.60, tumor/ghrelin −1% ± 1.90 ($P < 0.001$), tumor/BIM-28131 −2.70% ± 1.20 ($P < 0.001$), all n = 11]. Low-dose treatment with both ghrelin and BIM-28131, however, failed to improve loss of LBM (data not shown).

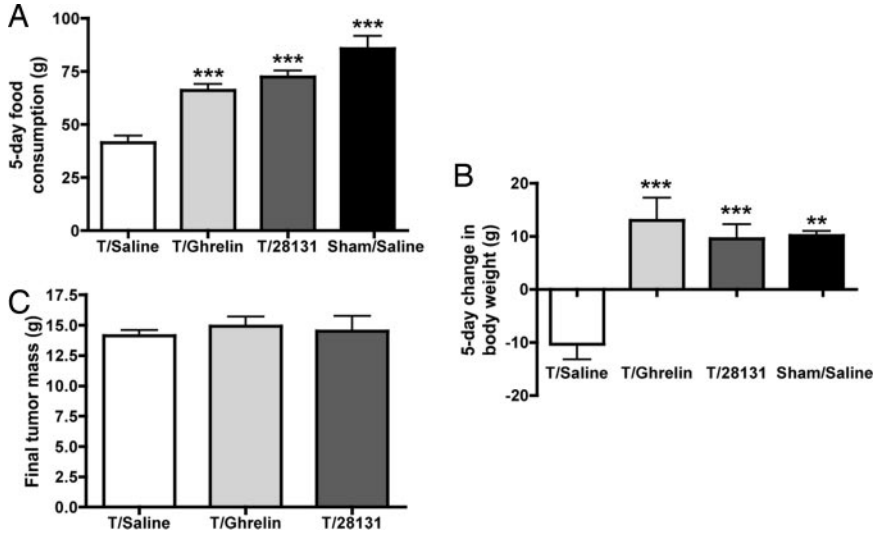

FIG. 1. Food intake and body weight changes. Food consumption (A), body weight change (B), and final tumor mass (C) after 5 d of treatment as follows: T/saline, Tumor-bearing, saline-treated animals; T/ghrelin, tumor-bearing, ghrelin-treated animals; T/28131, tumor-bearing, BIM-28131-treated animals; sham/saline, sham-surgery, saline-treated animals. Compounds were administered at high dose (500 nmol/kg·d). Significance is shown compared with tumor-bearing, saline-treated control (**, $P < 0.01$; ***, $P < 0.001$).

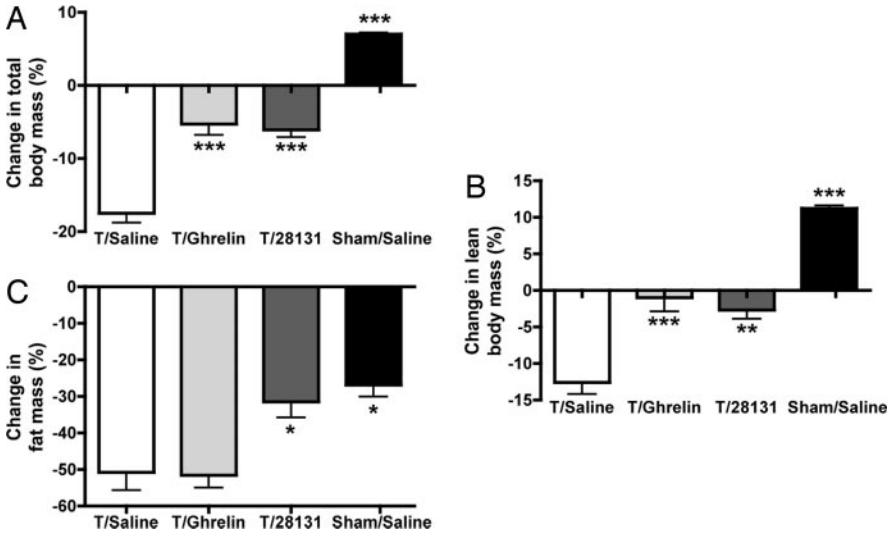

FIG. 2. Body composition changes. Changes in total body mass (A), lean body mass (B), and fat mass (C) as determined by DEXA before and after 14 d of tumor burden. Treatment groups are as follows: T/saline, Tumor-bearing, saline-treated animals; T/ghrelin, tumor-bearing, ghrelin-treated animals; T/28131, tumor-bearing, BIM-28131-treated animals; sham/saline, sham-surgery, saline-treated animals. Treatment was given for the final 5 d of tumor burden and compounds were administered at high dose (500 nmol/kg·d). Significance is shown compared with tumor-bearing, saline-treated control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

There was no change in tumor mass among any of the treatment groups [tumor/saline 14.1 g ± 0.45, tumor/ghrelin 14.9 ± 0.79, tumor/BIM-28131 14.5 ± 1.25 ($P > 0.05$), all n = 11] (Table 1).

Sham-treated animals exhibited a body mass gain, a decrease in fat mass, and an increase in LBM during both phases of the experiment (Table 1).

Gene expression

The 5 d of ghrelin treatment resulted in increased hypothalamic expression of the transcripts for the orexigenic peptides agouti gene-related peptide (AgRP) and neuropeptide Y (NPY) relative to both tumor-bearing/saline-treated and sham/pair-fed animals [AgRP: relative quantity (RQ) *vs.* sham/normal-fed: tumor/saline 2.52 ± 0.31 (n = 14), tumor/ghrelin 4.50 ± 0.51 (n = 8), $P < 0.01$ *vs.* tumor/saline; sham/pair-fed 1.93 ± 0.10 (n = 9), $P < 0.01$ *vs.* tumor/ghrelin; NPY: RQ *vs.* sham: tumor/saline 1.04 ± 0.57 (n = 12), tumor/ghrelin 1.36 ± 0.072 (n = 13), $P < 0.01$ *vs.* tumor/saline; sham/pair-fed 1.08 ± 0.019 (n = 9), $P < 0.01$ *vs.* tumor/ghrelin] (Fig. 3, A and B). There was no change in expression of proopiomelanocortin (POMC) in the hypothalamus or brainstem after ghrelin treatment, although both tumor-bearing groups showed a decrease in hypothalamic POMC expression relative to the sham/pair-fed animals [POMC hypothalamus: RQ *vs.* sham/normal-fed: tumor/saline 0.53 ± 0.051 (n = 13), tumor/ghrelin 0.53 ± 0.020 (n = 11), sham/pair-fed 0.91 ± 0.054 (n = 9), $P < 0.001$ *vs.* tumor/saline, $P < 0.001$ *vs.* tumor/ghrelin; POMC brainstem: RQ *vs.* sham/normal-fed tumor/saline 0.380 ± 0.027 (n = 6), tumor/ghrelin 0.471 ± 0.058 (n = 9), sham/pair-fed 0.778 ± 0.197 (n = 7)] (Fig. 3, C and D). Ghrelin treatment resulted in a decrease

TABLE 1. Weight changes, food intake, and body composition data for tumor-bearing treatment groups and sham-treated animals

	Tumor/saline (n = 11)	Tumor/ghrelin (n = 11)	Tumor/BIM-28131 (n = 11)	Sham (n = 5)
A. Weight changes, food intake, and tumor weights (g)				
Weight d 0	229.7 ± 3.72	228.64 ± 4.05	228.91 ± 3.94	231.0 ± 3.89
Weight d 10	243.4 ± 5.44	244.5 ± 4.36	248.8 ± 4.14	249.1 ± 3.46
Weight d 14	233.1 ± 7.38	257.8 ± 4.12[a]	259.5 ± 12.8[a]	259.2 ± 4.07[a]
Change in weight d 10–14	−10.3 ± 2.92	13.0 ± 4.26[a]	9.46 ± 2.78[a]	10.1 ± 0.901[a]
Food intake d 10–14	41.4 ± 3.34	66.0 ± 3.04[a]	72.5 ± 2.95[a]	85.7 ± 6.1[b]
Tumor mass	14.1 ± 0.45	14.9 ± 0.79	14.5 ± 1.25	NA
	Tumor/saline (n = 5)	Tumor/ghrelin (n = 5)	Tumor/BIM-28131 (n = 5)	Sham (n = 3)
B. Body composition changes as measured by DEXA (mass = g)				
Total mass (DEXA, d 0)	228.7 ± 4.7	229.5 ± 4.9	227.6 ± 5.2	230.8 ± 7.4
Total mass (without tumor, d 14)	188.9 ± 7.7	217.3 ± 6.2[a]	213.6 ± 4.6[a]	247.0 ± 7.6[a]
% Change in mass	−17.5 ± 1.3	−5.3 ± 1.5[a]	−6.1 ± 1.0[a]	7 ± 0.2[a]
Lean mass (d 0)	200.6 ± 3.7	204.0 ± 5.2	201.9 ± 5.6	203.3 ± 8.2
Lean mass (d 14)	175.6 ± 7.2	201.9 ± 7.0[a]	196.3 ± 4.3[b]	225.8 ± 7.4[a]
% Change in lean mass	−12.6 ± 1.6	−1.0 ± 1.9[a]	−2.7 ± 1.2[b]	11.2 ± 0.40[a]
Fat mass (d 0)	28.1 ± 1.4	25.5 ± 0.7	25.7 ± 0.5	27.5 ± 1.6
Fat mass (d 14)	13.3 ± 2.2	15.5 ± 1.2	17.3 ± 0.8[c]	21.1 ± 1.8[c]
% Change in fat mass	−50.8 ± 4.9	−51.6 ± 3.4	−31.5 ± 4.3[c]	−26.9 ± 3.2[c]

NA, Not applicable.
Significance shown is for tumor/ghrelin, tumor/BIM-28131, and sham *vs.* tumor/saline ([a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.05$).

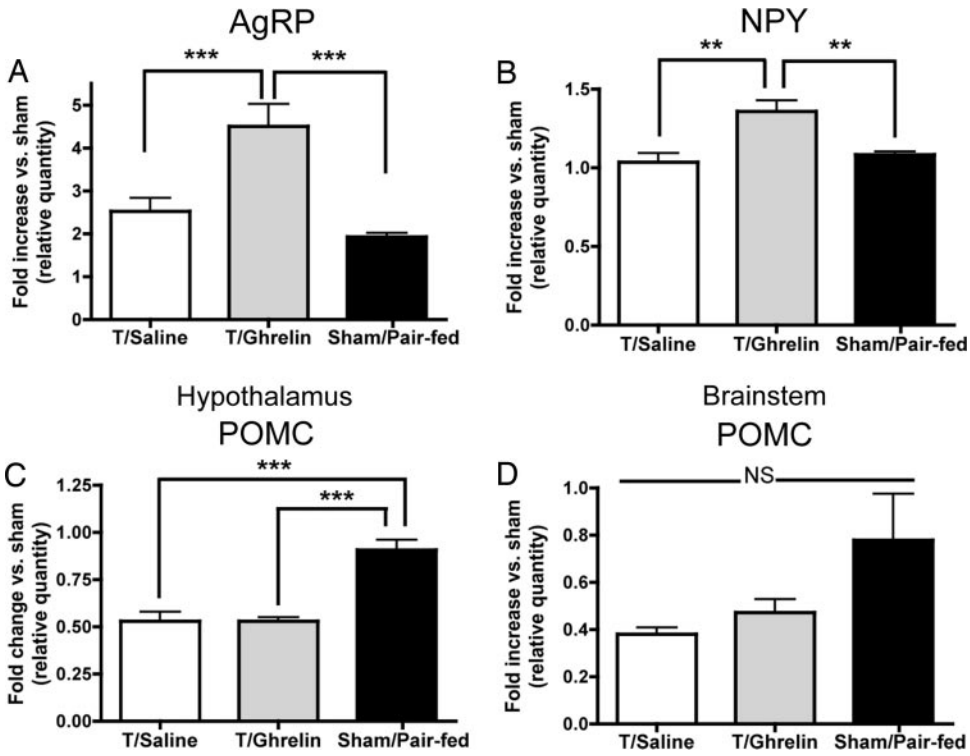

FIG. 3. Appetite-regulating neuropeptide gene expression. Change in expression of neuropeptide transcript as determined by real-time PCR relative to sham normal-fed rats. Data are reported as fold change for tumor-bearing rats treated with saline (T/saline) or ghrelin (T/ghrelin) and after pair-feeding sham rats the quantity of food eaten by tumor/saline rats (sham/pair-fed). Expression levels are shown in the hypothalamus for AgRP (A), NPY (B), POMC (C), and in the brainstem for POMC (D). Significant differences: **, $P < 0.01$; ***, $P < 0.001$. NS, Not significant.

in expression of the type 1 IL-1 receptor I (IL-1RI) mRNA in the hypothalamus and brainstem [IL-1RI hypothalamus: RQ *vs.* sham/normal fed: tumor/saline 1.83 ± 0.14 (n = 14), tumor/ghrelin 1.43 ± 0.088 (n = 15), $P < 0.05$ *vs.* tumor/saline; sham/pair-fed 0.92 ± 0.098 (n = 9), $P < 0.05$ *vs.* tumor/ghrelin, $P < 0.001$ *vs.* tumor/saline; IL-1RI brainstem: RQ *vs.* sham/normal fed: tumor/saline 1.26 ± 0.127 (n = 6), tumor/ghrelin 0.837 ± 0.053 (n = 9), $P < 0.05$ *vs.* tumor/saline; sham/pair-fed 0.616 ± 0.073 (n = 5), $P < 0.05$ *vs.* tumor/ghrelin, $P < 0.001$ *vs.* tumor/saline] but no significant decrease in expression of IL1-β [IL-1β hypothalamus: RQ *vs.* sham: tumor/saline 1.21 ± 0.11 (n = 14), tumor/ghrelin 1.04 ± 0.10 (n = 14), sham/pair-fed 0.91 ± 0.79 (n = 9); IL-1β brainstem: RQ *vs.* sham: tumor/saline 1.08 ± 0.13 (n = 6), tumor/ghrelin 0.727 ± 0.10 (n = 9), sham/pair-fed 1.09 ± 0.35 (n = 4)] (Fig. 4, A–D).

GH and IGF-I levels

There was no significant change in GH levels after treatment with ghrelin or BIM-28131 (tumor/saline 15 ± 6.10, n = 10; tumor/ghrelin 20.1 ± 2.73, n = 11; tumor/BIM-28131 28.36 ± 4.38, n = 11, sham/saline 12.7 ± 4.42, n = 6, all $P > 0.05$, all nanograms per milliliter) (Fig. 5A). There was a significant decrease in IGF-I levels in tumor-bearing animals relative to sham-treated animals (tumor/saline 81.6 ± 7.35, n = 10, $P < 0.001$ *vs.* sham; tumor/ghrelin 97.7 ± 4.04, n = 11, $P < 0.001$ *vs.* sham; tumor/BIM-28131 101.6 ± 7.33, n = 11, $P < 0.001$ *vs.* sham; sham/saline 227.8 ± 12.9, n = 6, all nanograms per milliliter) (Fig. 5B). There was, however, no significant difference in IGF-I levels between any of the tumor-bearing groups ($P > 0.05$).

Cytokine levels

There was no difference in circulating levels of proinflammatory cytokines among tumor/saline, tumor/ghrelin, and sham animals (see the supplemental figure published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Discussion

These data provide the first demonstration of an improvement in food intake and lean body mass after treatment with ghrelin and a synthetic ghrelin receptor agonist in a rodent model of cancer cachexia. These improvements were accompanied by an increase in expression of orexigenic genes and a decrease in the expression of the IL-1 receptor in the hypothalamus and brainstem during ghrelin treatment.

The improvement in lean body mass during treatment is likely to be significant in that the loss of lean body mass is felt to be a proximal cause of the morbidity and mortality associated with cachexia (30). Although prior studies demonstrated increased food intake and weight gain with ghrelin treatment, we do not know of any reports on the effect of ghrelin treatment on lean body mass in the setting of cancer cachexia (14, 21, 22). As was seen with the progestational agent megestrol acetate, which increased water weight but did not increase lean body mass, weight gain without increased lean body mass may not improve disease outcome (31, 32). Because of ethical considerations and the increasingly morbid nature of the tumor-bearing animals, we had to kill these animals 2 wk after tumor implantation and were not able to carry out these experiments to a point that would give information on long-term survival. However, human studies have shown that patients with improved lean body

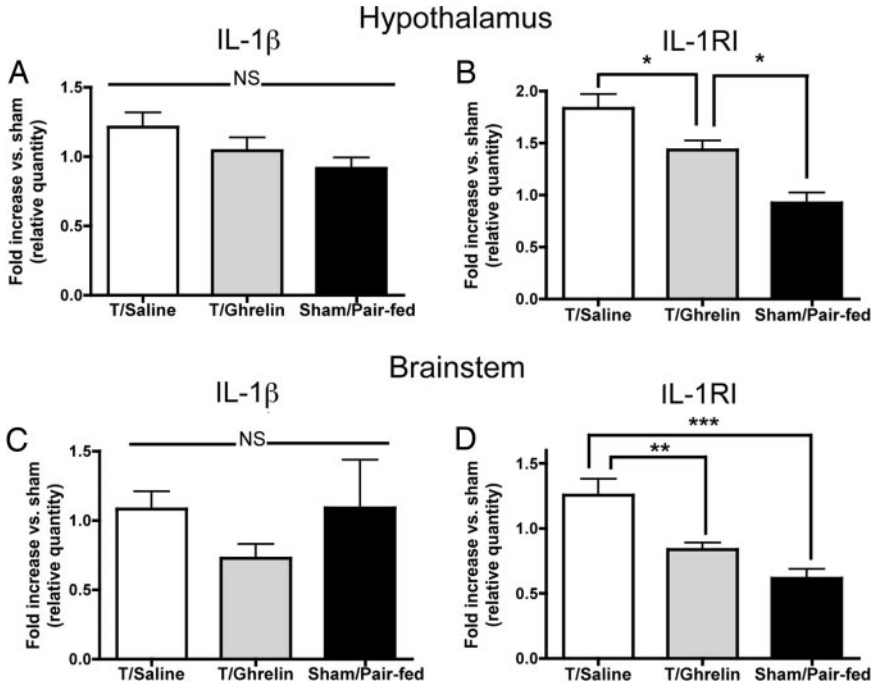

FIG. 4. IL-1β and IL-1RI gene expression. Change in expression of IL-1β and IL-1RI transcript as determined by real-time PCR relative to sham normal-fed rats. Data are reported as fold change for tumor-bearing rats treated with saline (T/saline) or ghrelin (T/ghrelin) and after pair-feeding sham rats the quantity of food eaten by tumor/saline rats (S/pair-fed). Expression levels are shown for IL-1β and IL-1RI in the hypothalamus (A and B) and brainstem (C and D). Significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, Not significant.

mass have improved survival (33–35). Interestingly, our observations of increased lean body mass may have understated the true effect of treatment, given that ghrelin has also been shown to increase levels of glucocorticoids, which themselves cause a wasting of lean tissue (36). Although ghrelin was not observed to cause a decrease in the loss of fat mass, the ghrelin receptor agonist BIM-28131 did result in an improved retention of fat mass, which has also been linked to increased survival (33).

One aspect of this study that improves the potential for future human use is the demonstration of efficacy of a novel ghrelin receptor agonist, BIM-28131, in reversing cancer cachexia. This is significant because ghrelin itself has a half-life estimated to be on the order of 30 min in humans, and past trials have used iv administration, limiting ghrelin's clinical application (37). Although ghrelin can also be administered by sc injection, causing a short-term increase in food intake, our prior studies and those of other investigators demonstrated that twice-daily injections of ghrelin in rodents do not improve cachexia or normalize overall food intake, emphasizing the need for continuous infusion of ghrelin or the use of compounds with longer half-lives in the circulation (14). Due to its smaller size, higher potency at the ghrelin receptor, and increased plasma stability, BIM-28131 has emerged as a promising candidate for therapeutic intervention in cancer cachexia. Given its longer half-life, one may have expected BIM-28131 to be effective in smaller concentrations than ghrelin. In this study we noted efficacy of BIM-28131 at only 500 nmol/kg·d and not at 50 nmol/kg·d. It may be that BIM-28131 is also effective at an intermediate dose or that higher doses are needed to be above some minimal threshold. A more rigorous dose-response study for BIM-28131 is needed to determine efficacy at intermediate doses and verify that there is no toxic effect of ghrelin or BIM-28131 at the doses used in this study.

The receptor for ghrelin is expressed in many brain regions (13). Given the important role of the hypothalamus in the regulation of appetite and metabolism, we elected to investigate the effect of ghrelin administration on hypothalamic expression of genes known to be involved in appetite regulation. Under normal conditions, release of POMC (cleaved to α-MSH) from the arcuate nucleus causes a decrease in feeding behavior, whereas release of AgRP (an endogenous antagonist of central melanocortin receptors) or NPY causes an increase in feeding behavior (38). Symptoms of cancer cachexia have been shown to be ameliorated by administration of AgRP (39). Our experiments here have shown that one effect of ghrelin administration in the setting of cancer ca-

FIG. 5. GH and IGF-I levels. GH and IGF-I levels at the time the animals were killed for tumor-bearing rats treated with saline, ghrelin, and BIM-28131 as well as for sham-operated rats treated with saline. Significance for tumor-bearing groups relative to sham: ***, $P < 0.001$. NS, Not significant.

chexia is to increase expression of the orexigenic genes. We did not note a difference in POMC gene expression after 5 d of treatment, although there may have been a change in the amount of α-MSH released from POMC-expressing neurons. One important consideration with regard to expression of appetite-effecting neuropeptides is the feeding history of the animal. We noted the same changes on gene expression when ghrelin-treated animals were compared with sham animals that had been pair fed the same amount of food consumed by saline-treated tumor-bearing animals with cachexia. This indicates that the gene expression differences observed were due to not merely food intake differences but specifically ghrelin administration.

Multiple studies reported that elevated circulating levels of ghrelin are already elevated in the setting of cancer cachexia, likely indicating that our model represents supraphysiological levels to achieve these improvements (17–20). It may be that the increased physiological levels of ghrelin normally seen in cachexia are still not adequate to produce improvements in appetite because of an excess of negative regulatory effects of factors such as inflammatory cytokines. With treatment doses, however, ghrelin is able to overcome anorexic effects on appetite centers, producing increased orexigenic effects such as we observed.

Inflammation is postulated to play a key role in the initiation and maintenance of cachexia (3, 7, 40). Evidence for this hypothesis is found in previous studies demonstrating increased circulating levels of inflammatory cytokines in a variety of disease states and in the ability to recapitulate the symptoms of cachexia via treatment with inflammatory cytokines alone (30, 41–48). Neuropeptide-releasing neurons in the arcuate nucleus, including those expressing POMC, express IL-1 receptor and increase transcriptional and neuronal activity in the presence of IL-1β (49). Thus, the observations of antiinflammatory effects of ghrelin *in vitro* and *in vivo* raised an interesting further mechanism for ghrelin action.

We observed a decrease in IL-1 receptor expression in the hypothalamus and brainstem during long-term ghrelin administration. Although the source of inflammatory cytokines in cancer is likely peripheral, similar peripheral immune response, such as that seen after lipopolysaccharide administration, leads to increased levels in the central nervous system, partially due to increased central expression of cytokines (50–52). Additionally, the presence of cytokines causes an acute increase in cytokine receptor expression (53). The decrease in expression of IL-1 receptor during ghrelin administration would be expected to decrease the downstream activity of cytokine receptor stimulation, thereby leading to a decrease in physiological variables (including decreased appetite, loss of lean body mass, and increased energy expenditure) that are seen with both cancer cachexia and isolated central IL-1β administration. This suggests a further mechanism whereby ghrelin exerts its anticatabolic activity. Alternatively, it may also be that ghrelin's primary action is on appetite regulating centers and that the outputs from these systems, including improved lean body mass and decreased energy expenditure, causes an improvement in overall health of the animal including a change in the immune response to the tumor. However, we also measured levels of circulating inflammatory cytokines and found that these were unchanged between tumor/saline and tumor/ghrelin animals (see supplemental data), suggesting that ghrelin does not decrease IL-1RI levels by causing decreased levels of peripheral cytokines.

An important consideration in the use of ghrelin in the setting of cancer relates to possible effects on the cancer cells themselves. Ghrelin is the endogenous agonist of the GHS receptor, and although ghrelin knockout mice do not exhibit a decrease in IGF-I levels relative to wild-type mice, exogenous ghrelin administration increases levels of GH and IGF-I in humans (23, 54). The potential increase in IGF-I levels is an additional mechanism whereby ghrelin may produce its effects on lean body mass. However, the possibility for increased IGF-I levels has also raised concerns about the possible effects on tumor growth. Although not ruling out the potential for increased tumor growth after longer-term treatment with ghrelin, we did not observe increases in tumor size after up to 5 d of ghrelin administration. Moreover, enhanced growth of these cachexigenic tumors would have likely led to further worsening of appetite, and we instead observed increases in food intake and lean body mass.

We noted nonsignificant increases in random GH levels among ghrelin- and BIM-28131-treated animals, although given that GH is secreted in an episodic pattern (brief bursts of secretion separated by relatively low levels), there may have been differences in overall GH secretion that were not picked up by testing at one single time point. GH causes the liver and other tissues to produce IGF-I. It has been well documented that many disease states cause a resistance to the effects of GH, including decreased IGF-I expression (55). We found a significant decrease in IGF-I levels among all tumor-bearing groups relative to sham/saline animals, with no significant increase due to treatment with ghrelin or BIM-28131. This may be due to the relatively short duration of ghrelin treatment in these experiments (5 d) or the severity of the effects of this tumor. Further experiments will be needed to study the effects of longer-term treatment on tumor size and IGF-I levels.

Conclusion

We have used a rodent model with robust cancer cachexia characteristics to show that both ghrelin and a ghrelin receptor agonist improve food intake and lean body mass in tumor-bearing rats. We have also shown that these improvements in appetite and body composition are accompanied by increased hypothalamic expression of orexigenic genes and decreased expression of the transcript for IL1-receptor, possibly implicating both appetite-regulating and antiinflammatory mechanisms. Further experiments will be needed to define these mechanisms and investigate for safety and efficacy in long-term human application.

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