Continuous Intravenous Infusion of Ghrelin Does Not Stimulate Feeding in Tumor-Bearing Rats

William T. Chance
Research Service, Veterans Affairs Medical Center, and Department of Surgery, University of Cincinnati Medical Center, Cincinnati, Ohio, USA

Ramesh Dayal
Research Service, Veterans Affairs Medical Center, Cincinnati, Ohio, USA

Lou Ann Friend, Ingrid Thomas, and Sulaiman Sheriff
Department of Surgery, University of Cincinnati Medical Center, Cincinnati, Ohio, USA

The development of anorexia continues to be a serious treatment issue for cancer patients. Because the orexigenic peptide, ghrelin, is active through systemic routes and activates hypothalamic neuropeptide systems known to be refractory in anorectic tumor-bearing (TB) rats, we investigated whether it would prevent the development of cancer anorexia when infused continuously intravenously. The 24-h food intake was increased in nontumor-bearing (NTB) rats at a dose of 288 µg/day ghrelin. However, no tested dose of ghrelin, up to 576 µg/day, elicited increased feeding in TB rats prior to or subsequent to the development of anorexia. In hypothalamus, ghrelin-infused TB rats exhibited significantly increased concentration of neuropeptide Y (NPY) as compared to saline-infused TB rats. Hypothalamic expression of NPY and agouti-related protein (AgRP) messenger RNA were elevated in ghrelin-infused TB rats as compared to NTB rats, but saline-infused TB rats also exhibited increased expression of AgRP. Proopiomelanocortin message was reduced in ghrelin-infused and saline-infused TB rats as compared to noninfused TB control rats. Although ghrelin infusion did not preserve muscle protein, a significant saving in body fat was observed in TB rats. Thus, the adiposity effects of ghrelin did not require an orexigenic response to the peptide. These results suggest that continuous ghrelin infusion may not be an effective treatment for cancer anorexia.

INTRODUCTION

Anorexia accompanies many diseases, including cancer (1), congestive heart failure (2), pneumonia (3), renal failure (4), sepsis (5), and AIDS (6). Because energy expenditure may actually be elevated in many of these diseases, this uncoupling of caloric intake from metabolic demand complicates clinical management of these patients. Thus, ensuring adequate nutrition may be prognostic of decreased morbidity and mortality especially in more chronic diseases such as cancer (7,8). The clinical significance of anorexia and cachexia is emphasized by estimates that at least two-thirds of all cancer patients are anorectic at death (1). As early as 1930, anorexia was suggested as the leading immediate cause of cancer deaths (9). The presence of cachexia also limits surgery as well as the aggressive use of chemotherapy and radiation (10). Although several factors—including cytokines, serotonin, neuropeptides and tumor-secreted toxins—have been implicated in cancer anorexia, correcting the nutritional imbalance has proven very difficult. Simply supplying calories and protein as supplemental nutrition does not appear to adequately replete lean body mass and usually also fails to significantly improve quality of life for the cancer patient (11). In addition, few appetite stimulant treatments for anorexia are available. Megestrol acetate is a progestational agent that has been used most often to treat cancer anorexia. Although there is some improvement in appetite and small weight gain has been reported (12) with megestrol acetate, correction of the anorexia and cachexia of cancer to normal clearly does not occur.

A particular problem in treating anorexia is that the biochemical mechanisms that control feeding are located primarily within hypothalamic and brain stem nuclei. Thus, they are relatively protected from circulating biochemistry by the blood-brain barrier. Recent investigations, however, have revealed a circulating peptide named ghrelin that has potent appetite-stimulating properties following systemic injection. Thus, in both animal (13,14) and human (15) studies, ghrelin has been shown to increase feeding following peritoneal or intravenous injections, respectively. Because ghrelin has been shown to stimulate feeding through the hypothalamic release of the orexigenic peptides (16), neuropeptide Y (NPY), and agouti-related protein (AgRP), which...
appear to be dysfunctional in cancer anorexia (17,18), it might be a particularly effective treatment for cancer anorexia. Additionally, ghrelin treatment has been reported to preserve fat in mice (19), which might be beneficial for cachectic patients.

Ghrelin has been investigated as an anti-cachectic agent in nude mice bearing human melanoma tumors (20) as well as in anorectic tumor-bearing (TB) patients (21). In both studies, positive effects of ghrelin treatment were noted; however, in the mouse study, the orexigenic effect of ghrelin was slight, with ghrelin-treated TB mice still exhibiting nearly a 50% reduction in food intake. The clinical study reported increased intake of a buffet meal by anorectic cancer patients following a 90-min intravenous (iv) ghrelin infusion. Although there was also a 35% increase in 24-h intake by the cancer patients, only 2 of 6 patients exhibited what appeared to be significantly increased 24-h elevation in energy intake. Therefore, the usefulness of ghrelin as a longer term antianorectic agent in TB organisms is still open to question. In order to answer that question, we infused ghrelin, iv, into non-TB rats and rats bearing methacholanthrene sarcomas for 8 days. In the first experiment, we infused increasing doses of ghrelin, iv, into non-TB rats in order to determine the threshold dose for elicitation of feeding. In the second study, ghrelin was infused in TB rats across these doses to determine whether the orexigenic potency of ghrelin was altered as these rats developed anorexia. We also measured the expression and levels of several neuropeptides known to control food intake in the hypothalamus of TB and control rats and assessed several nutrition-related physiological variables.

MATERIALS AND METHODS

Subjects and Procedures

Fifty-two male Fischer 344 rats, weighing between 225 and 250 g, were purchased from Charles River Laboratories (Wilmington, MA). These rats were housed individually in shoe-box cages located in a temperature- and humidity-controlled vivarium under a 12-h light–dark cycle (lights on at 7:00 AM) for at least 2 wk prior to experimental manipulation. The rats were maintained ad libitum on rat chow and tap water throughout the studies. Two experiments were conducted using these animals. Both studies included appropriate sham-operated noninfused control rats.

Experiment 1

Following anesthetization [ketamine/xyazine: 80/15 mg/kg, intramuscular injection (im)], silastic catheters (Dow Corning No. 602–155, Midland, MI) were surgically implanted into the external jugular veins of 16 adult male Fischer 344 rats (Charles River Laboratories, Wilmington, MA). Additionally, 8 rats received sham operations, which involved occluding the external jugular vein, unilaterally. These operations were conducted aseptically according to our previously published report (22).

Following the surgeries, the rats were transferred to stainless-steel metabolism cages, and the catheters were connected to syringe pumps (Harvard Apparatus, Holliston, MA) by 22-gauge feed-through swivels (Harvard Apparatus) that allowed free movement of the rats. In the first experiment, normal saline was infused through the catheters for the first 2 days following surgery at a rate of 1.5 ml/h. On Day 3, rat ghrelin trifluoroacetate (serine (n-octanoate; American Peptide Co., Sunnyvale, CA) was added to the saline infusate of 8 rats at an initial concentration of 1 µg/ml (36 µg/day). This concentration was doubled every other day to a maximum of 8 µg/ml (288 µg/day) for infusion on Days 7 and 8. The syringes were refilled with fresh ghrelin preparations each day. On the following day, all rats were euthanized by decapitation. Blood was collected into tubes containing aprotinin (0.5 trypsin inhibitor unit) and potassium ethylenediamine tetracetic acid, as an anticoagulant. These tubes were centrifuged (2,500 g, 4°C) for 20 min, and the plasma was stored at −80°C to await assay. The brains were removed rapidly from the skulls, with the hypothalamus being dissected free as described previously (23) and sectioned into halves prior to freezing in liquid nitrogen. Gastrocnemius, soleus, and extensor digitorum longus (EDL) muscles were also taken and frozen in liquid nitrogen prior to assay for protein and uncoupling protein messenger RNA (mRNA). The liver, heart, stomach, and epididymal fat were removed and weighed.

Experiment 2

The rats were anesthetized (Halothane; Halocarbon, Inc., Rivers Edge, NJ) and received either methylcholanthrene (MCA) sarcomas or sham inoculations, subcutaneously, in the midscapular dorsum. These inoculations employed a 4-mm diameter trocar to transplant approximately 50 mg of fresh MCA sarcoma, which was harvested from a donor animal in our tumor colony. As described in our previous report (24), this tumor produces significant anorexia within 3 wk of inoculation while not metastasizing to any organs during the 5 wk that the host can tolerate its growth.

Thirteen days after tumor inoculation, the rats were anesthetized (ketamine/xyazine: 80/15 mg/kg, im) prior to the aseptic insertion of catheters into the external jugular veins of 16 TB rats. Eight additional TB and non-TB rats underwent sham operations. As in the previous study, these rats were begun on saline infusion at a rate of 1.5 ml/h. After 3 days of infusion, 1 group was switched to receive ghrelin at an initial concentration of 2 µg/ml (72 µg/day). This dose of peptide was doubled every other day until a final concentration of 16 µg/ml (576 µg/day) was achieved on infusion Days 7 and 8. On the following day, the rats were euthanized, and all of the tissue taken in the first study were removed and preserved. In addition, the tumors were removed and weighed. Body composition was also measured using nuclear magnetic resonance, which quantified fat, lean body mass, and water content of the rats postmortem. Because two of
the TB rats died shortly after initiating the infusions, the results are based on the 14 surviving rats.

**Biochemical Assays**

*Radioimmunoassay.* Levels of NPY and α-melanocyte stimulating hormone (α-MSH) were determined in hypothalamic halves by radioimmunoassay (RIA) according to our previously published methods (25). Samples of hypothalami were extracted in 10 volumes of 0.2 N hydrochloric acid. After homogenization over ice and centrifugation, the acid extracts were lyophilized, with the residues being resuspended in 1 ml of assay mixture. The assay mixture consisted of 100 µl sample or standard, 100 µl assay buffer or NPY/α-MSH -free plasma, and 100 µl NPY/α-MSH antiserum, which was incubated overnight at 4°C. Next, 100 µl of 125I-NPY or α-MSH tracer was added, and the mixture was incubated overnight again at 4°C. Then, 100 µl of anti-rabbit gamma globulin and 100 µl 10% polyethylene glycol were added, with the mixture being incubated for 2 h prior to the addition of 500 µl 1% bovine serum albumin assay buffer. Bound and free peptides were separated by centrifugation (20 min), with the supernatant being discarded and the residue counted for 1 min. Concentrations of total ghrelin were also determined in plasma by RIA (Phoenix Pharmaceuticals, Burlingame, CA).

**Quantitative Real-Time Reverse Transcription (RT) Polymerase Chain Reaction (PCR).** Hypothalami halves were used for the determination of peptide and peptide receptor mRNA by RT-PCR. Total RNA was isolated using Tri reagentR (Molecular Research Center, Inc., Cincinnati, OH) as described by supplier’s protocol. The yield and the purity of the RNA was determined by absorbance at 260 nM and 260:280 ratio, respectively. Complementary DNA (cDNA) was prepared using Super Script First-Strand synthesis for RT-PCR from Invitrogen respectively. Complementary DNA (cDNA) was prepared using the supplier’s protocol. The yield and the purity of the RNA (Molecular Research Center, Inc., Cincinnati, OH) as described in Table 1. Fluorescence intensity was monitored during the annealing-extension steps. The threshold cycle (Ct), a cycle at which the PCR reaction emits a fluorescence signal greater than background, was used for the quantification of mRNA. Cyclophilin mRNA expression was used to normalize the RNA input. Relative quantities of peptide or receptor mRNA were determined utilizing the formula fold change = $2^{-\Delta\Delta Ct}$ Ct = [Ct target gene, where (experimental sample)-Ct GAPDH gene (experimental sample)]-[Ct target gene (calibrator, control sample)-Ct cyclophilin gene (calibrator, control sample)]. The 2 in the formula refers to the 100% efficiency of PCR for target genes and cyclophilin (26,27). PCR efficiency, Ct value analysis, linearity, slopes of the standard curve, relative quantity of fluorescence, and dissociation curve analysis were calculated by Stratagene software program (Mx 3000p, La Jolla, CA).

**Statistical and Procedural Evaluations**

Data generated by these experiments were evaluated using analysis of variance (ANOVA) procedures. Comparisons of individual means, post hoc, were accomplished using Tukey’s conservative $t$ statistic. For analysis of real-time PCR data, the Ct value, PCR efficiency, linearity, slopes of the standard curve, relative quantity of fluorescence, and dissociation curve analysis were calculated by a Stratagene software program built in the Mx-3000p apparatus. All animal procedures were review and approved by institutional animal care and use committees at the respective institutions.

**RESULTS**

As illustrated in Fig. 1A, continuous iv infusion of increasing concentrations of ghrelin into non-TB rats elicited a significant elevation in 24-h feeding at 288 µg/day. In the second study, however, no concentration of ghrelin increased food intake in TB rats (Fig. 1B). Both saline- and ghrelin-infused groups of TB
rats tended to consume less food than did the noninfused TB rats across the second half of the experiment. As shown in Fig. 1B, noninfused TB rats first exhibited a significant reduction in 24-h food intake 21 days after tumor inoculation. Saline-infused TB rats exhibited reduced feeding earlier, with significantly reduced feeding first occurring 18 days after tumor inoculation. Food intake by ghrelin-infused TB rats was not different from that of the saline-infused TB rats, and increased doses of ghrelin actually appeared to reduce feeding at least as compared to noninfused TB rats.

None of the rats in either experiment exhibited any overt signs of distress or illness, such as diarrhea, piloerection, abnormal posture, or distressed breathing, during the infusion period. Although body weights were generally maintained, the saline-infused rats exhibited a trend toward weight loss, whereas the noninfused rats gained weight across the 8 ghrelin infusion days (Fig. 2A). Because there was a significant overall difference in body weights between groups, additional statistical analyses were conducted on body weight difference scores from baseline. For the first experiment, the ANOVA for difference scores indicated an overall gain of weight for the noninfused rats ($P < 0.01$), loss of weight for the saline-infused rats ($P < 0.01$), and no significant change in ghrelin-infused rats across the infusion days. No significant differences were found between groups in the second experiment (Fig. 2B). Although TB rats did not appear to lose weight, subtraction of tumor weights from the total body weights demonstrates the degree of the cachectic response. At sacrifice, there was no significant difference in tumor weight among the 3 groups of TB rats (noninfused = 43 ± 4 g; saline-infused = 51 ± 4 g; ghrelin-infused = 57 ± 7 g). Similarly, carcass (nontumor body) weight was not different in the TB groups, with carcass weight being reduced an average of 15% as compared to non-TB rats. Plasma concentrations of total ghrelin were not altered significantly in saline-infused rats (2,339 ± 146 pg/ml) as compared to noninfused rats (1,821 ± 224 pg/ml) but were above reliable detection limits (+6,000 pg/ml) in the ghrelin-infused group. In the second study, plasma concentrations of total ghrelin were elevated in the saline-infused TB rats (2,090 ± 146 pg/ml) as compared with noninfused TB rats (1,554 ± 45 pg/ml) or noninfused non-TB rats (1,555 ± 45 pg/ml). Similar to the results in the first study, ghrelin levels were highly elevated in the ghrelin-infused TB rats (+6,000 pg/ml).

Examination of tissue weights in the first study revealed no significant differences in gastrocnemius (GAST) muscle, liver, heart, or stomach across the groups (Table 2). Epididymal fat mass was not altered in saline-infused rats but tended to be increased in ghrelin-infused rats as compared to the saline-infused non-TB rats. In the second experiment (Table 2), the mass of stomach and GAST muscle were decreased significantly ($P < 0.01$) in TB groups, whereas epididymal fat mass was decreased in noninfused and saline-infused TB rats. In the ghrelin-infused TB group, the mass of the liver was increased as compared to non-TB rats. Plasma concentrations of total ghrelin were not altered significantly in saline-infused rats (2,339 ± 146 pg/ml) as compared to noninfused rats (1,821 ± 224 pg/ml) but were above reliable detection limits (+6,000 pg/ml) in the ghrelin-infused group. In the second study, plasma concentrations of total ghrelin were elevated in the saline-infused TB rats (2,090 ± 146 pg/ml) as compared with noninfused TB rats (1,554 ± 45 pg/ml) or noninfused non-TB rats (1,555 ± 45 pg/ml). Similar to the results in the first study, ghrelin levels were highly elevated in the ghrelin-infused TB rats (+6,000 pg/ml).

### Table 1
Assay conditions for real-time RT-PCR analysis of hypothalamic neuropeptides and receptors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP-3</td>
<td>10 min @ 95°C</td>
<td>1 min @ 94°C</td>
<td>90 s @ 58°C</td>
<td>90 s @ 72°C</td>
</tr>
<tr>
<td>AgRP</td>
<td>15 min @ 95°C</td>
<td>30 s @ 95°C</td>
<td>30 s @ 55°C</td>
<td>30 s @ 60°C</td>
</tr>
<tr>
<td>Orexin</td>
<td>10 min @ 95°C</td>
<td>30 s @ 95°C</td>
<td>1 min @ 58°C</td>
<td>30 s @ 72°C</td>
</tr>
<tr>
<td>NPY</td>
<td>15 min @ 95°C</td>
<td>30 s @ 95°C</td>
<td>45 s @ 53°C</td>
<td>30 s @ 72°C</td>
</tr>
<tr>
<td>NPY Y1R</td>
<td>15 min @ 95°C</td>
<td>30 s @ 95°C</td>
<td>30 s @ 55°C</td>
<td>30 s @ 72°C</td>
</tr>
<tr>
<td>POMC</td>
<td>15 min @ 95°C</td>
<td>30 s @ 95°C</td>
<td>30 s @ 55°C</td>
<td>30 s @ 72°C</td>
</tr>
<tr>
<td>MC-3R</td>
<td>15 min @ 95°C</td>
<td>30 s @ 95°C</td>
<td>30 s @ 55°C</td>
<td>30 s @ 72°C</td>
</tr>
<tr>
<td>MC-4R</td>
<td>15 min @ 95°C</td>
<td>30 s @ 95°C</td>
<td>30 s @ 55°C</td>
<td>30 s @ 72°C</td>
</tr>
<tr>
<td>CRF</td>
<td>15 min @ 95°C</td>
<td>1 min @ 94°C</td>
<td>40 s @ 56°C</td>
<td>40 s @ 72°C</td>
</tr>
<tr>
<td>UCN-1</td>
<td>10 min @ 95°C</td>
<td>30 s @ 94°C</td>
<td>30 s @ 58°C</td>
<td>2 min @ 72°C</td>
</tr>
<tr>
<td>UCN-2</td>
<td>15 min @ 95°C</td>
<td>30 s @ 95°C</td>
<td>30 s @ 55°C</td>
<td>30 s @ 72°C</td>
</tr>
<tr>
<td>CRF R-2</td>
<td>15 min @ 95°C</td>
<td>30 s @ 95°C</td>
<td>30 s @ 55°C</td>
<td>30 s @ 72°C</td>
</tr>
</tbody>
</table>

*Following the initial denaturation, 40 cycles of secondary denaturation, annealing, and extension were performed on the samples. At the conclusion of the extension period, one cycle (1 min @ 95°C, 30 s @ 55°C, 30 s @ 95°C) was performed to generate a dissociation curve for each compound. Abbreviations are as follows: RT-PCR, reverse transcription polymerase chain reaction; UCP, uncoupling protein; AgRP, agouti related protein; NPY, neuropeptide Y; Y1R, Y-1 receptor; POMC, proopiomelanocortin; MC-3R, melanocortin receptor-3; MC-4R, MC-receptor-4; CRF, corticotrophin releasing factor; UCN, urocortin; CRF R-2, CRF receptor-2.*
FIG. 1. Mean (± standard error of the mean) daily intake of rat chow by non-tumor-bearing (NTB; Panel A) and tumor-bearing (TB; Panel B) rats infused with saline or increasing doses of ghrelin. Ghrelin was infused continuously, iv, with the dose being doubled every other day. TB rats were infused with a higher dose of ghrelin to better assess their refractoriness to ghrelin-induced feeding. Numbers in parentheses refer to days after tumor inoculations. BW, body weight.

As shown in Table 3, protein content of GAST and EDL muscles was reduced in saline-infused and ghrelin-infused non-TB rats. Consistent with the NMR observations of lean body mass changes, all 3 TB groups exhibited significant ($P < 0.01$) reduction in protein concentration in GAST and EDL muscles (Table 3), with changes in the soleus muscle not reaching statistical significance. Determination of UCP-3 mRNA in muscle by quantitative real-time RT-PCR (Fig. 4A) revealed significant ($P <$
0.01) elevation in UCP-3 message in the GAST of both saline-infused and ghrelin-infused non-TB rats, whereas EDL UCP-3 message was elevated significantly only in saline-infused non-TB rats. Saline-infused TB rats exhibited significant elevation in UCP-3 mRNA in both GAST and EDL muscles as compared to either non-TB or TB control rats. Although UCP-3 mRNA was elevated in the GAST of ghrelin-infused TB rats, EDL UCP-3 message was significantly reduced in ghrelin-infused as compared to the saline-infused TB group (Fig. 4B).

In the hypothalamus, the RIA did not reveal any significant changes in the concentrations of NPY or α-MSH in saline-infused or ghrelin-infused non-TB groups (Fig. 5A). As
shown in Fig. 5B, however, the level of NPY was decreased in non-infused and saline-infused TB rats and elevated in the ghrelin-infused TB group. Concentrations of α-MSH were not altered significantly in any TB groups.

The expression of orexigenic peptides AgRP, orexin, and NPY and NPY Y-1 receptor as determined by real-time RT-PCR was not altered in hypothalamic tissue taken from either infusion group of non-TB rats (Fig. 6A). AgRP and NPY messages were elevated in all groups of TB rats (Fig. 6B), with AgRP mRNA being increased further in both infusion groups and NPY message being elevated further only in the ghrelin-infusion group. Expression of the Y-1 receptor was decreased in all TB groups, with ghrelin treatment having no effect on the NPY Y-1 receptor mRNA (Fig. 6B).

The expression of POMC mRNA tended to be reduced in ghrelin-infused non-TB rats but was not statistically significant (Fig. 7A). However, MC-3 receptor mRNA was reduced in ghrelin-infused non-TB rats (Fig. 7A) but only as compared to the noninfused group. MC-4 receptor mRNA was not altered in any non-TB infusion group (Fig. 7A). POMC expression was not altered in noninfused TB rats but was decreased significantly in both saline-infused and ghrelin-infused TB groups (Fig. 7B).

**TABLE 2**
Mean (± SEM) weights (g) of tissues taken from NTB (Experiment 1) and TB (Experiment 2) noninfused rats (C) and rats infused for 8 days with SAL or increasing concentrations of GHR

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Heart</th>
<th>Stomach</th>
<th>Gastrocnemius</th>
<th>Epididymal fat</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTB-C</td>
<td>8</td>
<td>0.81 ± 0.02</td>
<td>1.32 ± 0.03</td>
<td>1.37 ± 0.03</td>
<td>3.21 ± 0.01</td>
<td>10.00 ± 0.38</td>
</tr>
<tr>
<td>NTB-SAL</td>
<td>8</td>
<td>0.85 ± 0.03</td>
<td>1.20 ± 0.03</td>
<td>1.28 ± 0.02</td>
<td>3.04 ± 0.14</td>
<td>10.04 ± 0.34</td>
</tr>
<tr>
<td>NTB-GHR</td>
<td>8</td>
<td>0.77 ± 0.02</td>
<td>1.20 ± 0.03</td>
<td>1.23 ± 0.06</td>
<td>3.38 ± 0.14</td>
<td>9.70 ± 0.63</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTB-C</td>
<td>7</td>
<td>0.83 ± 0.02</td>
<td>1.32 ± 0.02</td>
<td>1.53 ± 0.04</td>
<td>4.11 ± 0.19</td>
<td>11.41 ± 0.40</td>
</tr>
<tr>
<td>TB-C</td>
<td>7</td>
<td>0.81 ± 0.03*</td>
<td>1.17 ± 0.03**</td>
<td>1.25 ± 0.04**</td>
<td>2.53 ± 0.25**</td>
<td>10.60 ± 0.36</td>
</tr>
<tr>
<td>TB-SAL</td>
<td>7</td>
<td>0.89 ± 0.01**</td>
<td>1.06 ± 0.03**</td>
<td>1.17 ± 0.07**</td>
<td>2.80 ± 0.24**</td>
<td>11.67 ± 0.37</td>
</tr>
<tr>
<td>TB-GHR</td>
<td>7</td>
<td>0.87 ± 0.04*</td>
<td>1.14 ± 0.04*</td>
<td>1.20 ± 0.06**</td>
<td>3.40 ± 0.33+</td>
<td>12.55 ± 0.33**</td>
</tr>
</tbody>
</table>

*Abbreviations are as follows: SEM, standard error of the mean; NTB, nontumor-bearing; TB, tumor-bearing; SAL, saline; GHR, ghrelin. **P < 0.01 vs. NTB-C; *P < 0.05 vs. NTB-C; +P < 0.05 vs. TB-C.
TABLE 3
Mean (± SEM) protein content (g/muscle) of muscles taken from NTB (Experiment 1) and TB (Experiment 2) noninfused rats (C), and rats infused for 8 days with SAL or increasing concentrations of GHR

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Gastrocnemius</th>
<th>Extensor digitorum longus</th>
<th>Soleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTB-C</td>
<td>8</td>
<td>246.4 ± 6.3</td>
<td>23.2 ± 0.8</td>
<td>18.0 ± 1.1</td>
</tr>
<tr>
<td>NTB-SAL</td>
<td>8</td>
<td>222.5 ± 5.8*</td>
<td>20.7 ± 0.7*</td>
<td>17.0 ± 0.6</td>
</tr>
<tr>
<td>NTB-GHR</td>
<td>8</td>
<td>228.8 ± 10.5</td>
<td>19.8 ± 0.7*</td>
<td>15.9 ± 0.9</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTB-C</td>
<td>7</td>
<td>287.4 ± 5.8</td>
<td>22.6 ± 0.7</td>
<td>16.3 ± 0.6</td>
</tr>
<tr>
<td>TB-C</td>
<td>7</td>
<td>244.3 ± 10.0*</td>
<td>19.2 ± 0.7*</td>
<td>14.9 ± 0.9</td>
</tr>
<tr>
<td>TB-SAL</td>
<td>7</td>
<td>227.6 ± 13.2*</td>
<td>17.8 ± 0.8*</td>
<td>13.6 ± 0.8</td>
</tr>
<tr>
<td>TB-GHR</td>
<td>7</td>
<td>230.0 ± 11.8*</td>
<td>18.7 ± 1.1*</td>
<td>14.2 ± 1.0</td>
</tr>
</tbody>
</table>

Abbreviations are as follows: SEM, standard error of the mean; NTB, nontumor-bearing; TB, tumor-bearing; SAL, saline; GHR, ghrelin.

*P < 0.05 vs. NTB-C.

MC-3 and MC-4 expression were not altered except for a significant increase in MC-4 message in saline-infused TB rats as compared to the noninfused TB group (Fig. 7B).

As illustrated in Fig. 8A, expression of UCN-2 was decreased in both infused groups, whereas CRF, UCN-1, and CRF receptor-2 (CRFR-2) messages were not changed. CRF expression was not altered in any TB groups (Fig. 8B), whereas UCN-1 mRNA was elevated in saline-infused TB rats and normalized with ghrelin infusion (Fig. 8B). Expression of UCN-2 was decreased in noninfused TB rats and was elevated to control level in both infusion groups. As compared to the noninfused non-TB group, ghrelin-infused TB rats exhibited nearly 50% reduction in CRFR-2 mRNA (Fig. 8B).

DISCUSSION

This study demonstrated that the continuous infusion of ghrelin into normal rats increased daily intake of chow, with a statistically significant effect being observed at a concentration of 8 µg/ml (288 µg/day). This increase in food intake indicates that the delivery system was effective and was well tolerated by the rats. Thus, even though there were indications of significant catabolism and alterations of metabolically important peptides in saline-infused rats, there were no obvious suggestions of infection such as diarrhea, piloerection, or abnormal breathing. These results are similar to those reported following repeated injections of 1 µg ghrelin into the lateral ventricle of rats every 12 h for 72 h (16). Across the 3 days of the cited study, total food intake by the ghrelin-treated rats increased by 19%, which compares favorably with the 20% increase in food intake in this study following the highest dose of the peptide infused into non-TB rats.

The failure of ghrelin-infused non-TB rats to gain body weight was surprising considering the reports of weight gain following systemic (28) or continuous infusions (29) of the peptide in rats. However, in the Thompson et al. (29) study, pulsed ghrelin (10 µg/3 h) was more effective than continuous infusion (80 µg/day) of the peptide for stimulating body weight gain. However, that study (29) was conducted in growth hormone-deficient rats, so the results are not directly comparable to this study. Still, continuous ghrelin infusion may lead to suppression of the hypothalamic-pituitary-growth hormone axis and reduced gain in body weight. Supporting this idea is the observed weight gain induced by discrete repeated intraventricular triamcinolone (ivt) (19) or intraperitoneal (28) ghrelin treatments. In addition, as suggested by skeletal muscle protein and UCP-3 results, the infusion procedure was somewhat catabolic. Furthermore, the ghrelin-infused non-TB rats did not increase their food intake until 2 days prior to sacrifice, leaving little time for feeding-induced stimulation of body weight.

The primary uniqueness of ghrelin is its ability to elicit feeding following systemic injection, which makes it an attractive candidate for treating anorexia. Unfortunately, in TB rats, ghrelin infusion did not increase food intake, even prior to the development of significant anorexia. Increasing the dose of the peptide by 2 times the dose that elicited feeding in non-TB rats still did not increase food intake in TB rats. These results suggest that the ghrelin feeding pathway is dysfunctional in anorectic TB rats. Rapid degradation of the octanoate to des-acyl-ghrelin could be a potential problem in this study. Although the syringes were refilled each day with fresh ghrelin solution, in vitro experiments suggest that ghrelin may degrade to des-acyl-ghrelin in culture medium within hours (30). Arguing against degradation of ghrelin as a cause for the lack of feeding response by the TB rats is its effectiveness in the non-TB group. In addition, 7 days of ivt infusion of ghrelin using minipumps elicited feeding, suggesting the peptide remained intact (19). Furthermore, we have assayed by high-performance liquid chromatography (HPLC) freshly prepared ghrelin and ghrelin solution that was stored at room temperature for 24 h and found no degradation.
in the peptide. It appears that the trifluoroacetate counter ion confers greater stability to ghrelin in solution (31). Therefore, it is unlikely that the TB rats did not eat to ghrelin infusion due to breakdown of the peptide.

Consistent with the inability of infused ghrelin to stimulate feeding in TB rats are reports of its elevation in plasma of cachectic but not in noncachectic cancer patients (32,33). In addition, plasma ghrelin was further increased in cancer patients following chemotherapy-induced anorexia (32). These results suggest that although ghrelin upregulation may be a response to cancer cachexia, this compensatory mechanism is ineffective in reversing cancer anorexia, which may be secondary to an alteration of the ghrelin receptor.

Ghrelin has been investigated as an anticachectic agent in methy cholanthrene sarcoma-bearing mice (34), in nude mice bearing human melanoma tumors (20), as well as in anorectic

FIG. 4. Real-time reverse transcription polymerase chain reaction determination of uncoupling protein-3 (UCP-3) messenger RNA (mRNA) in gastrocnemius and extensor digitorum longus (EDL) muscles taken from nontumor-bearing (NTB; Panel A) and tumor-bearing (TB; Panel B) rats infused with saline or increasing concentrations of ghrelin.
cancer patients (21). Although positive effects of ghrelin treatment were observed in each of these studies, in TB mice, the orexigenic effect was slight, with ghrelin-treated TB mice still exhibiting a highly significant reduction in food intake. As compared to the ghrelin-infused non-TB controls, the response of the ghrelin-infused TB rats appeared to be decreased by nearly 50%, suggesting that orexigenic signaling by ghrelin was significantly depressed in TB mice. This conclusion is similar to that drawn in the present study, with ghrelin being even less effective in the current study, perhaps due to altered receptor response in the presence of constant infusion. In the clinical study, 90 min of iv ghrelin infusion increased food intake in cancer patients. However, this study, which used a crossover design, did not use a simultaneous saline-infused control group. In addition, only 2 of 6 patients exhibited a 24-h increase in energy intake (21). Therefore, the usefulness of ghrelin as a longer term antianorectic agent in TB organisms is still open to question.

In the present experiments, we observed alterations in hypothalamic concentration and mRNA in ghrelin-infused rats of several peptides associated with feeding and satiety. In ghrelin-infused TB rats, the concentration of NPY in the hypothalamus
was elevated. This increase in peptide level is what one would expect from the literature because one mechanism of ghrelin-induced feeding is to increase synthesis and release of NPY and AgRP (35). Non-TB rats may not have exhibited such an increase in NPY level because they only received half as much ghrelin as did the TB rats during the 2 days prior to their sacrifice. Alternatively, NPY level may not have increased in ghrelin-infused non-TB rats because they ate in response to the ghrelin infusion. Thus, it is well known that hypothalamic NPY level of hungry rats returns to normal on feeding (36,37). Conversely, the TB rats did not feed in response to infused ghrelin and as shown in Fig. 5B, and NPY concentration in the hypothalamus remained elevated. This observation suggests that the anorectic dysfunction in TB rats may be at the level of the NPY nerve terminals in the hypothalamic paraventricular nucleus.

Although there was no alteration in mRNA for the orexigenic peptides or Y-1 receptor in the ghrelin-infused non-TB rats, the noninfused TB rats exhibited significant increases in both NPY and AGRP mRNA. The mRNA for AgRP was further
FIG. 7. Real-time reverse transcription polymerase chain reaction determination of proopiomelanocortin (POMC), melanocortin receptor-3 (MC-3), and receptor-4 (MC-4) messenger RNA (mRNA) in hypothalamus taken from nontumor-bearing (NTB; Panel A) and tumor-bearing (TB; Panel B) rats infused with saline or increasing concentrations of ghrelin.

increased in both infused groups, whereas NPY mRNA tended to be further elevated only in the ghrelin-infused group. Activation of NPY and AgRP systems has been reported following both central (16) and systemic (35) infusions of ghrelin. These elevations in AgRP and NPY mRNA suggest that the anorectic rats were attempting to upregulate peptide synthesis, perhaps in response to the degree of anorexia. Thus, both infused TB groups were generally significantly more anorectic than was the noninfused TB group up to the day of sacrifice. This upregulation of NPY synthesis may also have played a role in the decrease of Y-1 receptor mRNA because receptor density tends to decrease in the presence of excess ligand. Elevated NPY message has been reported in whole hypothalamus (38,39), arcuate (ARC) nucleus (40,41), and the ventromedial hypothalamic region (24,25) taken...
from anorectic TB rats and mice. In the studies in which pair-fed (PF) controls have been used, NPY message was also increased in basal-medial hypothalamus, suggesting similar NPY recruitment responses in anorectic and food-restricted rats. In the dorsomedial hypothalamic region, however, only the PF rats exhibited this elevation of NPY mRNA, indicating a problem with NPY translation or transport in TB rats (24,25).

We and others have observed reduced feeding responses to both NPY (17,42) and AgRP (18) in anorectic TB rats. Thus, even prior to the onset of significant anorexia, TB rats ate less chow than did non-TB controls following an intrahypothalamic injection of NPY. The feeding response of TB rats to NPY also continued to deteriorate as the rats become overtly anorectic. Although not universally observed in other models of cancer anorexia (17), MCA sarcoma-bearing rats exhibited less feeding than controls following the injection of AgRP into the 3rd ventricle. Our more recent observations suggest that both mRNA and immunostaining for NPY Y-1 receptor is reduced in the hypothalamus of anorectic TB rats (43). Therefore, the dysfunction of NPY feeding mechanisms in TB rats may be associated with
experiments, the procedure of infusing normal saline into non-TB rats appears to be distal to the NPY and AGRP cell bodies.

An effect of ghrelin infusion on anorectic peptides was also observed, with POMC mRNA being decreased in ghrelin-infused non-TB and in both groups of infused TB rats. Although it is difficult to specify why the saline-infused TB rats also exhibited decreased POMC mRNA, one should remember that these rats were also anorectic, and the infusion process was somewhat catabolic. Thus, these rats may have downregulated mRNA for this anorectic peptide. Therefore, it appears that the anorectic rats may have been attempting to upregulate orexigenic and downregulate anorexigenic peptides. Supporting this hypothesis is our previous observation (24) that the NPY message was upregulated in the gross ventromedial area of the hypothalamus, which contained the ARC nucleus. This change in POMC message in TB rats is consistent with reports that have suggested decreased POMC activity has been associated with ghrelin-induced feeding (16,35).

Alterations in peptides of the CRF family were also observed in both non-TB and TB rats. CRF family peptides are predominantly anorexia-producing compounds (44,45), which have been reported to modulate NPY-induced feeding (46). Both UCN-1 and UCN-2 mRNA tended to be decreased in both groups of infused non-TB rats. Although one would predict that these anorexia-producing peptides would be decreased during treatments with an orexigenic compound, their reduction in saline-infused non-TB rats is problematic. Clearly, iv saline-infusion is not an innocuous procedure and represents a metabolic stress to which the rats may react by reducing synthesis of potential anorectic peptides such as the UCNs. These peptides were also altered in TB rats, with UCN-2 message being decreased in noninfused TB rats. In addition, CRF-R2 mRNA was decreased significantly in ghrelin-infused TB rats, suggesting that the ghrelin treatment may have reduced the density of this anorexia-producing peptide receptor.

The various uncoupling proteins tend to have tissue-specific distributions, with UCP-3 being localized primarily to skeletal muscle and brown adipose tissue (47). These proteins have the capacity to uncouple mitochondrial respiration, resulting in the production of heat rather than adenosine triphosphate molecules (48). Upregulation of UCPs could be a source of energy depletion in TB organisms and contribute to the cachectic effect of cancer. Although UCP-3 has been reported to be elevated in muscles taken from TB rats (49) and mice (50) as well as in muscles taken from cachectic cancer patients (51), it was also elevated in PF controls (49,50), suggesting that UCP-3 upregulation may be secondary to malnutrition. In the present experiments, the procedure of infusing normal saline into non-TB rats induced weight loss, decreased muscle protein, and elevated muscle UCP-3, indicating that a significant degree of catabolism was associated with this route of delivery. As suggested by the elevated UCP-3 message, this catabolic effect may have resulted from the rats’ need to produce more body heat, perhaps due to the tendency of constant infusion of a relatively large volume of saline (36 ml/day) to reduce body temperature. Alternatively, it may represent a prolonged recovery from surgery and residual catabolic hormones. Ghrelin infusion did not prevent this loss of muscle protein in non-TB rats; however, UCP-3 mRNA expression in the EDL muscle was not elevated significantly in these rats, suggesting a possible attempted correction of energy loss for heat production. Cachectic responses were also observed in TB rats, most of which were not prevented or reversed by ghrelin infusion. As with the non-TB rats, infusion of saline exacerbated UCP-3 mRNA, with ghrelin infusion reducing this increase significantly in the EDL muscle. These effects of ghrelin on muscle UCP-3 are consistent with reports of both centrally administered (52) and systemically administered (53) ghrelin reducing UCP-1 in brown adipose tissue. Thus, one effect of ghrelin on metabolism may be to reduce the loss of energy dissipated as heat.

Ghrelin also had dramatic effects on adipose tissue, which was particularly evident in the TB rats. Although the noninfused TB rats had lost a third of their epididymal fat mass, ghrelin infusion increased this fat depot to where it was no longer significantly different from that found in the non-TB group. A trend toward elevated adiposity was also observed in the ghrelin-infused non-TB rats, which was not statistically significant. This lack of effect may have been due to the non-TB rats receiving only half the maximal dose given the TB rats. Alternatively, perhaps the fat preserving power of ghrelin is best observed in catabolic situations. Ghrelin appears to shift the metabolic balance from lipolysis to lipogenesis, with isoproterenol-induced lipolysis being reduced in adipocytes (54) and brown adipose tissue sympathetic activity being decreased (55) following administration of the peptide. These observations are consistent with reports of ghrelin inducing adiposity (19), elevating the respiratory quotient (19), and affecting enzymes toward lipogenesis and away from lipolysis. Systemically administered ghrelin may also reduce fatty acid oxidation in liver by inhibiting adenosine diphosphate-activated kinase (56). Many of these metabolic effects have been observed following injection of ghrelin into the third ventricle, suggesting that circulating ghrelin controls metabolic activity by elevating efferent sympathetic outflow from the hypothalamus to peripheral target organs including liver, muscle, and adipose tissue. The observation that ghrelin still preserved adipose tissue in TB rats even though it did not elicit increased feeding indicates separate mechanisms and perhaps separate receptors for these 2 activities of the peptide. This observation is consistent with reports of different receptors that have mediated the orexigenic and adiposity effects of ghrelin (54,57).
CONCLUSIONS

These results indicate that the continuous infusion of ghrelin does not stimulate feeding in anorectic TB rats. Although the possibility exists that more success may have been observed using pulsatile dosing, it appears that the ghrelin treatment did activate the downstream mediator of feeding: NPY. Therefore, it is suggested that ghrelin did not stimulate feeding in this animal model of cancer anorexia due to dysfunctional NPY feeding systems. The exact nature of this dysfunction cannot be specified at this time.

ACKNOWLEDGMENTS

Supported by VA Merit Review Grant (W. T. Chance), American Institute for Cancer Research Grant 03B056 (W. T. Chance) and NIH DK–53548 (S. Sheriff). Appreciation is expressed to Steve Woods for the HPLC analyses of ghrelin.

REFERENCES

37. Kalra SP, Dube MG, Sahu A, Phelps CP, and Kalra PS: Neuropeptide Y secretion increases in the paraventricular nucleus in association with...


