Effect of chronic hyperghrelinemia on ingestive action of ghrelin

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The stomach hormone ghrelin is the endogenous ligand for the growth hormone secretagogue receptor (GHS-R). Systemic administration of ghrelin will cause elevations in growth hormone (GH) secretion, food intake, adiposity, and body growth. Ghrelin also affects insulin secretion, gastric acid secretion, and gastric motility. Several reports indicate that repeated or continuous activation of GHS-R by exogenous GHSs or ghrelin results in a diminished GH secretory response. The purpose of this study was to examine the extent to which the acute stimulation of food intake by exogenous ghrelin is altered by chronic hyperghrelinemia in transgenic mice that overexpress the human ghrelin gene. The present findings show that the orexigenic action of exogenous ghrelin is not diminished by a chronic hyperghrelinemia and indicate that the food ingestive pathway of the GHS-R is not susceptible to desensitization. In contrast, the epididymal fat pad growth response, like the GH response, to exogenous ghrelin is blunted in ghrelin transgenic mice with chronic hyperghrelinemia.

GROWTH HORMONE SECRETAGOGUES (GHSs) are synthetic, nonnatural ligands that stimulate growth hormone (GH) release and food intake by activation of the GHS-receptor (GHS-R), a member of the G protein-linked receptor superfamily (8, 10, 18, 37, 40). GHSs were developed based on the structure of met-enkephalin and are structurally unrelated to GH-releasing hormone.

Ghrelin is a 28-amino acid peptide identified as the endogenous ligand for the GHS-R (23, 36). Ghrelin stimulates GH secretion, food intake, body growth, and adiposity (16, 23, 28, 36, 38, 40). Ghrelin also affects gastric acid secretion and motility (26). Ghrelin is a unique peptide in that it has an n-octanoyl modification at the 3Ser (23). The 3Ser acylation is thought to be essential for biological activity (6, 23); however, some reports indicate that the des-acyl variant, which lacks the fatty acid modification, influences cancer cell proliferation and cardiomyocyte apoptosis in vitro and the GH-IGF-I axis in vivo (1, 5, 11). The stomach is the predominant production site for ghrelin in the body (23); ghrelin is produced in gastric enteroendocrine cells called X/A cells (13). Ghrelin is also expressed in the intestine, kidney, hypothalamus, heart, and placenta (40). Gastrectomy will lower circulating ghrelin levels by ~85% in rats and humans (2, 19). plasma levels of ghrelin increase in response to fasting and decrease upon refeeding (19).

Several reports have shown that continuous activation of the GHS-R by GHSs or ghrelin can result in an attenuated GH secretory response (4, 7, 14, 21, 30, 31, 34) as well as a flat growth response (27, 33, 34, 43). Additionally, a blunted GH response to ghrelin challenge has been shown in patients with anorexia nervosa, a hyperghrelinemia condition (9). Our laboratory has generated a ghrelin transgenic mouse that has chronic “hyperghrelinemia.” The purpose of this study, in part, was to examine the extent to which the acute stimulatory action of exogenous ghrelin on short-term food intake is impaired by a chronic hyperghrelinemia in ghrelin transgenic mice. In addition, the ability of exogenous ghrelin to stimulate GH secretion and epididymal fat pad growth in the face of chronic hyperghrelinemia was tested.

MATERIALS AND METHODS

Animals

All animal experiments were conducted in accordance with mandated standards of humane care and were approved by the Institutional Animal Care and Use Committee. Mice were maintained in an air-conditioned and light-regulated room (lights on, 0600–1800) and given access to food and water ad libitum. The ghrelin transgenic mouse was generated by a standard protocol. The human ghrelin cDNA was inserted in BamHI and XhoI sites of pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) to make the pCMV-human ghrelin plasmid. The CMV-human ghrelin transgene (~1,800 bp) comprising a CMV promoter, the human ghrelin cDNA, and a bovine GH polyadenylation sequence were then released by digesting pCMV-human ghrelin with MfeI and Nael, purified using a Clontech Nucleosip spin kit (Clontech, Palo Alto, CA), and resuspended in 10 mM Tris·HCl (pH 8.0) and 0.1 mM EDTA. F1 female mice (C57BL/6 × C3H/He, 3 wk old) were superovulated by intraperitoneal injection with 5 units of pregnant mare serum (Calbiochem, San Diego, CA) and 48 h later with 5 units of human gonadotropin (Sigma, St. Louis, MO). Fertilized embryos were collected in Whitten’s 640 media ~19 h after mating with (C57BL/6 × C3H/He) F1 males. Each construct was microinjected into the male pronucleus as previously described (29). Microinjected embryos were incubated overnight to the two-cell stage and then transferred into Swiss Webster foster mothers.

Progeny were tested at 3 wk of age for the presence of the transgene either by Southern blot analysis of PvuII-digested mouse tail DNA or by PCR analyses of mouse tail DNA. The Southern blot probe was a 289-bp fragment that contains the bovine GH polyadenylation sequence. The primers used for PCR analysis of mouse tail DNA were as follows: 5’-GCAGGCCACCTGTTGCTGAC-3’ (sense) and 5’-AGCATGCCCTATTTGCTCTCCAA-3’ (anti-sense), which corresponded to +790 bp to +1,599 bp of the trans-
gene, respectively. Use of this primer combination resulted in amplification of a PCR product of ~809 bp that comprised the human ghrelin cDNA and a majority of the bovine GH polyadenylation sequence. Three founder mice were generated. One was infertile. Of two fertile females, one was selected for continued breeding based upon her elevated plasma ghrelin levels. Founder mice were backcrossed with C57BL/6 mice. F2-g generations were used in the present experiments.

For characterization of the ghrelin transgenic mouse, mRNA levels of the human ghrelin transgene in extracts of various tissues harvested from transgenic and wild-type (WT) mice were measured by the real-time RT-PCR protocol described earlier by our laboratory (39). The human ghrelin probe and primer sequences were as follows: probe, CACCTGCTGCAACCC; forward primer, GCCAGGAACTGCAGCAG; and reverse primer, GGGCATGGCCCTCAGCT. The sites of highest expression (mRNA levels) of the ghrelin transgene are the stomach fundus, skeletal muscle, kidney, lung, and heart (Fig. 1). Lower expression levels of the ghrelin transgene are found in the colon, pancreas, and brain.

Additionally, for characterization of the ghrelin transgenic mouse, plasma and tissue ghrelin levels were measured. Plasma levels of acyl-ghrelin (N-octanoylated ghrelin) were measured by an ELISA that recognizes the acyl-ghrelin form only (American Laboratory Products, Windham, NH). The assay measures mouse and human acyl-ghrelin equally well. Plasma acyl-ghrelin levels were ~14-fold higher in transgenic mice when compared with WT mice [WT: 29 pg/ml (SD 18) vs. transgenic: 402 pg/ml (SD 130); n = 9–10 mice/group]. Tissue ghrelin levels were measured by an immunoassay that detects both acyl- and nonacyl-ghrelin (24). The total ghrelin immunoreactivity detects mouse and human ghrelin equipotently, since minor differences exist in the sequences of mouse and human ghrelin (23). Therefore, for transgenic mice, tissue ghrelin peptide levels reflect a summation of ghrelin generated by the mouse and human genes. Pancreatic ghrelin levels were increased ~20-fold in ghrelin transgenic mice when compared with WT mice (Fig. 1). We also examined cellular localization of ghrelin production in the pancreas by immunohistochemistry (IHC; see Ref. 39). IHC showed no ghrelin immunostaining in the WT pancreas, whereas ghrelin immunostaining was observed in pancreatic islets and acini in the transgenic mice (Fig. 2). Together, the immunoassay and IHC findings suggest that the pancreatic islets represent the source of the increased levels of circulating ghrelin, since islets, but not pancreatic acini, express processing enzymes (prohormone convertases) essential for production of mature peptide hormones from precursor forms (17, 25, 42). The strong ghrelin immunostaining in the pancreatic acini of transgenic mice most likely represents ghrelin that has not been processed. When compared with WT mice, stomach levels of ghrelin peptide in ghrelin transgenic mice were unchanged in spite of the high expression of the human ghrelin transgene. IHC analysis of stomach sections supported the tissue immunoassay data and showed that the number of ghrelin-containing cells and their staining intensity in transgenic mice did not differ when compared with WT mice (data not shown). The finding that gastric ghrelin peptide levels are not increased in transgenic mice suggests that ghrelin exerts a local inhibitory feedback action on its own production in the stomach. Ghrelin peptide was not detected in skeletal muscle extracts and tissue sections by immunoassay and IHC, respectively. These findings are anticipated, since skeletal muscle does not express prohormone convertases.

Body weights of ghrelin transgenic male [22 g (SD 7)] and female [21 g (SD 2)] mice did not differ significantly when compared with body weights of WT male [26 g (SD 5)] and female [19 g (SD 3)] mice. Circulating GH levels were not significantly elevated when compared with WT mice [WT: 6 ng/ml (SD 5) vs. transgenic: 16 ng/ml (SD 7); P > 0.05], although GH levels tended to be higher in transgenic mice. Serum GH levels were measured by a mouse specific GH immunoassay. In ghrelin transgenic mice, food intake in response to an overnight fast was unchanged when compared with WT mice.

Fig. 1. Top: human ghrelin mRNA levels (means ± SD) in various tissues harvested from ghrelin transgenic mice (n = 6 mice). Tissue human ghrelin mRNA levels were measured by real-time RT-PCR. The sites of highest expression (mRNA levels) of the ghrelin transgene are the stomach fundus, skeletal muscle, kidney, lung, and heart. Lower levels of the transgene are expressed in the colon, pancreas, and brain. Bottom: ghrelin peptide levels (mean ± SD) in various tissues harvested from wild-type (WT) and ghrelin transgenic (tg) mice (n = 6 mice/group). Ghrelin peptide levels were measured by a ghrelin immunoassay that detects acyl- and nonacylated ghrelin as well as mouse and human ghrelin peptides. Compared with WT mice, stomach ghrelin peptide levels in transgenic mice were unchanged, whereas pancreatic ghrelin levels were increased ~20-fold in ghrelin transgenic mice. Compared with WT mice, ghrelin peptide levels were also increased in an extract prepared from a pool of pituitaries harvested from transgenic mice. No SD bar is shown for pituitary extracts, since single WT and tg pituitary pools were measured. Ghrelin peptide was not detected in skeletal muscle extracts.

Experiments

Experiment 1. Groups (n = 9–18/group) of ad libitum-fed male and female WT and ghrelin transgenic mice (1/cage) were kept overnight in an isolated, quiet room in standard plastic mouse cages (1 mouse/cage). In the morning, cages were fitted with a 1-in. raised wire screen floor insert. A paper insert was placed beneath the screen floor to catch food waste. Mice were allowed to acclimate to the wire floor for 1–2 h; food was withheld during this period. At ~1,000, a preweighed amount of food was placed in the cage, and mice were given either vehicle (controls) or one of several doses of synthetic mouse ghrelin (1.0, 3.0, and 10.0 nmol ip in 100 μl water containing 0.05% BSA; Bachem, Torrance, CA). Mice were allowed to eat for 1 h. The amounts of uneaten food and the food waste below the screen floor...
were weighed, and the amount of food consumed was calculated for each mouse. Mice were then returned to the mouse quarters and allowed to eat and drink ad libitum until the next trial. With the use of different groups of mice (generations 3–5), the food intake in response to human ghrelin was also tested in WT and ghrelin transgenic mice, as described above. There are minor amino acid sequence differences for mouse and human ghrelin; human ghrelin differs at positions 11 and 12 when compared with the sequence of mouse ghrelin (23). Selection of ghrelin doses was based on previously published reports (3, 41). Acute administration of acyl-ghrelin (1, 3, and 10 nmol ip) caused significant elevations in circulating acyl-ghrelin levels. Circulating levels of acyl-ghrelin were 2,667 pg/ml, 9,433 pg/ml, and 47,050 pg/ml in mice given 1, 3, and 10 nmol acyl-ghrelin, respectively. Plasma acyl-ghrelin levels did not differ significantly in WT and transgenic mice after intraperitoneal administration of acyl-ghrelin.

Experiment 2. Groups (n = 6–8 mice/group) of ad libitum-fed male WT and ghrelin transgenic mice (generations 8–9) were treated with 0.05% BSA in saline (vehicle control) or ghrelin (3 nmol, 3 times/day sc) for 7 days. Mice were killed 16–18 h after the last vehicle or ghrelin treatments. The epididymal fats pads were removed.

Experiment 3. To confirm earlier reports that a repeated or continuous ghrelin administration will blunt GH secretion to an acute ghrelin challenge (4, 7, 14, 21, 30, 31, 34), groups (n = 5–7 mice/group) of ad libitum-fed male WT and ghrelin transgenic mice (generations 8–9) were given ghrelin (3, 10 nmol ip) and killed 30 min later for collection of plasma. Plasma GH levels were measured in duplicate by means of a mouse specific GH immunoassay.

Statistics

Values are given as means with SDs. The effects of peptide dose on food intake were evaluated using an ANOVA followed by the Newman-Keul’s test where appropriate by means of Statview Software (Cary, NC). The effects of ghrelin on epididymal fat pad weights were examined by the Kruskal-Wallis test.

RESULTS

Experiment 1

In WT and ghrelin transgenic mice, food intake in response to the lowest dose of mouse ghrelin (1 nmol; Fig. 3) was significantly greater when compared with the food intake for 1 h without ghrelin treatment, i.e., vehicle [WT: 0.08 g/mouse (SD 0.05); transgenic: 0.09 g/mouse (SD 0.07)]. Administration of mouse ghrelin increased food intake in a dose-related manner in WT and ghrelin transgenic mice. In WT mice, food intake in response to 10 nmol was significantly greater when compared with the food intake response to 1 and 3 nmol ghrelin (P values: 1 nmol: 0.0002; 3 nmol: 0.004). In ghrelin transgenic mice, food intake in response to 10 nmol was significantly greater when compared with the food intake response to 1 and 3 nmol mouse ghrelin (P values: 1 nmol: 0.0002; 3 nmol: 0.0004). Food intake in WT and ghrelin transgenic mice in response to different doses of mouse ghrelin (1, 3, and 10 nmol) did not differ significantly (P > 0.05).

The orexigenic action of human ghrelin was also tested using separate groups of WT and ghrelin transgenic mice. The
rationale behind the testing of the orexigenic action of human ghrelin in the transgenic mice that overexpress human ghrelin was that the stimulatory action of human ghrelin on food intake may differ compared with mouse ghrelin. The primary sequences of mouse and human ghrelin differ only by two residues. Administration of human ghrelin increased food intake in a dose-related manner in WT and ghrelin transgenic mice (Fig. 4). The food intake responses to the different doses of ghrelin in the WT and ghrelin transgenic mice were significantly higher than the food intake responses to 1 and 3 nmol ghrelin (P values: 1 nmol: 0.0001; 3 nmol: 0.0459). In ghrelin transgenic mice, food intake in response to 10 nmol was significantly greater compared with the food intake responses to 1 and 3 nmol ghrelin (P values: 1 nmol: 0.0021; 3 nmol: 0.0491). Food intake in response to increasing doses of human ghrelin (1, 3, and 10 nmol/kg) in WT and ghrelin transgenic mice did not differ significantly (P > 0.05).

**Experiment 2**

Epididymal fat pad weights did not differ significantly (P > 0.05) in WT and ghrelin transgenic mice (Table 1). Treatment of WT mice with ghrelin for 7 days caused a significant increase in epididymal fat pad weights. Treatment of ghrelin transgenic mice with ghrelin did not alter epididymal fat pad weights. Epididymal fat pad weights of ghrelin-treated WT mice were significantly heavier than epididymal fat pad weights of ghrelin-treated transgenic mice (P < 0.05).

**Experiment 3**

Acute administration of ghrelin (3 and 10 nmol ip) caused significant elevations in circulating GH levels in both WT and ghrelin transgenic mice compared with basal GH levels; however, the GH response was significantly lower in the transgenic mice (Table 2).

### Table 1. Effect of ghrelin treatment on epididymal fat pad weights in wild-type and ghrelin transgenic mice

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>Vehicle</th>
<th>Ghrelin</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>284±62</td>
<td>620±219*†</td>
</tr>
<tr>
<td>tg</td>
<td>366±106</td>
<td>439±128</td>
</tr>
</tbody>
</table>

Values are means ± SD. Either vehicle (0.05% BSA in saline) or acyl-ghrelin (3 nmol, 3x/d, IP) was given to wild-type (WT) or transgenic (tg) mice. Mice were killed 18–20 h after the last dosing. P < 0.05, significantly different vs. vehicle-treated WT mice (*) and vs. ghrelin-treated tg mice (†).

### DISCUSSION

Ghrelin is a stomach hormone that stimulates food intake, GH secretion, body growth, and adiposity (23, 28, 36, 38, 40). Ghrelin activates the same receptor that is activated by GHSs, the GHS-R (23). Human and animal studies demonstrate that the GH secretory response can become attenuated during a continuous exposure to GHSs or ghrelin (4, 7, 14, 21, 30, 31, 34). The expected body growth response can also be absent during a continuous exposure to GHSs or ghrelin (27, 33, 34, 43). The present study confirms and extends these earlier reports by showing that basal serum GH levels and body weights in WT and ghrelin transgenic mice do not differ. The unchanged basal GH levels are in spite of a 14-fold elevation in plasma acyl-ghrelin levels in ghrelin transgenic mice. Furthermore, the present acute study confirms earlier reports by showing that the GH response to exogenous ghrelin challenge is blunted by a precedent hyperghrelinemia. The attenuated GH response observed with continuous activation of GHS-R is not associated with a downregulation of GHS-R expression or a desensitization of the pituitary to GHSs (34).

In contrast to the blunted GH response to GHSs or ghrelin, the present findings demonstrate that the food ingestive response to exogenous ghrelin challenge remains intact during a chronic hyperghrelinemia condition. Furthermore, the present study shows that the epididymal fat pad weights of ghrelin transgenic mice are not increased compared with WT mice and that exogenous ghrelin treatment fails to provoke an epididymal fat pad growth response in ghrelin transgenic mice. The absence of an adiposity response during hyperghrelinemia coincides with the lack of a weight gain in ghrelin transgenic mice and indicates that continuous ghrelin exposure will blunt the stimulatory actions of ghrelin on adipose tissue and GH secretion. The desensitization of fat depots to ghrelin exposure has not been described previously.

### Table 2. Blunted GH response to ghrelin in ghrelin transgenic mice

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>3 nmol Ghrelin</th>
<th>10 nmol Ghrelin</th>
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<tbody>
<tr>
<td>WT</td>
<td>49±18</td>
<td>97±33</td>
</tr>
<tr>
<td>tg</td>
<td>26±6*</td>
<td>35±9*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5–7 mice/group. GH, growth hormone. WT and ghrelin tg mice were given exogenous ghrelin (3 and 10 nmol iv). Plasma was harvested 10 min after ghrelin administration. Basal serum GH levels in WT and tg mice are 7 ± 3 and 8 ± 3 ng/ml, respectively. *P = 0.025, significantly different vs. WT mice.
In the present study, we tested the food ingestive action of both mouse and human ghrelin, since the ghrelin transgenic mouse overexpresses human ghrelin and the amino acid sequences of mouse and human ghrelin differ in two residues at positions 11 and 12. For mouse ghrelin, residues 11 and 12 are glycine and arginine, respectively, whereas in human ghrelin residues 11 and 12 are serine and glutamate. Because of these differences in the sequences of mouse ghrelin and human ghrelin, it is conceivable that the putative “desensitization” action of hyperghrelinemia in the mouse would influence food intake in response to human ghrelin selectively and not the response to mouse ghrelin challenge. However, our experimental findings clearly indicate that the orexigenic action of either mouse ghrelin or human ghrelin challenge is not altered by an ongoing human hyperghrelinemia. Furthermore, the food intake response to GH-releasing peptide-6 challenge, a synthetic GHS structurally unrelated to ghrelin, did not differ in ghrelin transgenic mice compared with WT mice (Greeley, et al., unpublished observations). Collectively, these data indicate that the food ingestive component of the GHS-R is not modified by a chronic hyperghrelinemia.

Fasting causes an elevation in ghrelin secretion (24). Consideration of the present findings along with earlier data showing a diminished GH secretory and body growth response to repeated ghrelin exposure suggest an interesting explanation of the physiological function of the increased ghrelin secretion normally seen in the fasted state. During an enteral nutrient-restricted condition, i.e., fasting, the increased ghrelin secretion may function primarily to activate food intake, whereas the GH secretory response becomes acutely refractory to the increased ghrelin secretion. The elevated ghrelin secretion during the fasting state resembles a continuous infusion of exogenous ghrelin (35) and would not result in a stimulation of GH secretion but will stimulate food intake only based on the present findings.

Because our findings show that the orexigenic action of ghrelin is maintained during a state of hyperghrelinemia, we would expect food intake, body growth, body weights, and fat depots to be larger in ghrelin transgenic mice; however, the present study clearly shows that they do not differ. In fact, our food intake and body growth findings agree with those of a recent report on a ghrelin transgenic mouse (22). The finding that adipose tissue growth (epididymal fat pad) is unresponsive to ghrelin challenge in the ghrelin transgenic mouse indicates that another target tissue of ghrelin, adipose tissue, can become densely sensitized to ghrelin with continuous ghrelin exposure.

Although speculative, the absence of a “large phenotype” (i.e., absence of hyperphagia and increased body growth) in the ghrelin transgenic mouse may be explained by the complex nature of regulation of food intake and body weight (44) and involvement of redundant stimulatory and inhibitory signals such that the stimulatory action of hyperghrelinemia is counterbalanced by inhibitory mechanisms.

In humans, the Prader-Willi syndrome (PWS) is a genetic obesity condition characterized in part by hyperphagia (20). These individuals show chronically elevated plasma ghrelin levels (12, 15). PWS patients have a three- to fourfold higher fasting ghrelin level compared with normal obese humans who have low fasting plasma ghrelin levels. It appears that PWS patients have a phenotype inconsistent with that of the ghrelin transgenic mice, since hyperghrelinemic mice are not hyperphagic. In PWS patients, however, the hyperphagia may not be related causally to hyperghrelinemia, since a reduction in plasma ghrelin levels by somatostatin infusion does not reduce appetite in PWS patients (32).

In summary, the present study shows that a chronic hyperghrelinemia does not decrease the orexigenic action of an acute ghrelin challenge; however, another target tissue of ghrelin, adipose tissue (epidymal fat pad), resembles the GH and body growth responses and is unresponsive to exogenous ghrelin stimulation in the ghrelin transgenic mouse.

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GRANTS

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