Vagotomy Dissociates Short- and Long-Term Controls of Circulating Ghrelin

DIANA L. WILLIAMS, HARVEY J. GRILL, DAVID E. CUMMINGS, AND JOEL M. KAPLAN

Plasma ghrelin levels are responsive to short- and long-term nutrient fluctuation, rapidly decreasing with food consumption and increasing with food deprivation or weight loss. We hypothesized a vagal contribution to both responses. Nutrient-related ghrelin suppression may be mediated by gastrointestinal load-related vagal afferent activity, or depend upon vagal efferent input to the foregut, where most ghrelin is produced. Similarly, the deprivation-induced ghrelin rise could require state-related vagal afferent or efferent activity. Here, we examined the role of the vagus nerve in the regulation of plasma ghrelin by sampling blood from rats with subdiaphragmatic vagotomy and from sham-operated controls over 48 h of food deprivation, and before and after gastric gavage of liquid diet. Vagotomy affected neither baseline ghrelin levels nor the suppression of ghrelin by a nutrient load. The food deprivation-induced elevation of plasma ghrelin levels (160% of baseline), however, was completely prevented by subdiaphragmatic vagotomy. In a separate experiment, the deprivation-related rise in plasma ghrelin was substantially reduced by atropine methyl nitrate treatment, indicating that the response to fasting is driven by increased vagal efferent tone. The dissociation between nutrient load- and deprivation-related ghrelin responses indicates that the regulation of circulating ghrelin levels involves separate mechanisms operating through anatomically distinct pathways. (Endocrinology 144: 5184–5187, 2003)

Ghrelin IS AN orexigenic peptide hormone produced primarily in the gastrointestinal tract (1–3). A role for ghrelin in the control of food intake has been widely hypothesized (4), and supported in part by the observed variation in plasma ghrelin levels in response to fluctuating nutritive status. Circulating ghrelin levels rise before meals (5), and rapidly decline after feeding or gastrointestinal nutrient infusion (1, 5–7). Ghrelin levels are also responsive to longer-term changes in metabolic state, the clearest examples of which are the elevations of plasma ghrelin after food deprivation and with chronic weight loss (8, 9). The mechanisms that underlie the nutrient-related reduction and the deprivation-related elevation of plasma ghrelin level have not been identified. The precedents for vagal involvement in the regulation of gut hormones (e.g. 10, 11), and the well-known sensitivity of vagal afferents to intestinal nutrients (12, 13), raise the possibility that the vagus nerve plays a role in the meal-related reduction of circulating ghrelin. A vagal contribution to the food deprivation-induced rise in ghrelin levels is also possible. The vagus nerve mediates behavioral responses to hepatic metabolic depletion (14), and food restriction alters the pattern of gene expression in the nodose ganglion (15). Here, we evaluate the role of the vagus nerve in the control of plasma ghrelin levels by examining the nutrient load- and food deprivation-related responses in rats with subdiaphragmatic vagotomy and in their sham-operated controls.

For the interpretation of any vagal contribution to the nutrient load- and deprivation-related ghrelin responses, the role of tonic vagal activity in the maintenance of baseline plasma ghrelin levels must be considered. One study showed that truncal vagotomy substantially elevated circulating ghrelin in the rat (16). This and other observations (17) may be taken to suggest that tonic vagal activity restrains ghrelin secretion, and present potential complications for the interpretation of altered ghrelin responses to nutrient load or to deprivation in vagotomized rats. The issue of vagal involvement in the control of circulating ghrelin, however, remains open. One might expect, based on the reported effect of truncal vagotomy mentioned above (16), that vagal stimulation would reduce plasma ghrelin secretion, but one group has shown that electrical stimulation of the left cervical vagus had no effect on circulating ghrelin (18). In addition, it is important to consider the issue of nutritional maintenance procedures in studies of vagotomized rats. Post-vagotomy syndrome (i.e. reduced gastric motility, hypophagia, hypodipsia, and body weight loss) is commonly observed when rats are maintained on solid food. An increase in plasma ghrelin under these circumstances could be more indicative of a response to weight loss than of a vagal contribution to the regulation of this hormone. If, however, vagotomized rats are maintained on a liquid diet, post-vagotomy syndrome can be avoided (19). In the present study, we maintained rats on a liquid diet, and evaluated the effect of vagotomy on ghrelin levels under baseline conditions, before and after nutrient loads, and during 48 h of food deprivation. We also determined whether vagal efferent blockade via atropine treatment mimics the primary effect of subdiaphragmatic vagotomy reported here—a disruption of the food deprivation-induced rise in plasma ghrelin.

Materials and Methods

Ghrelin assay

Total immunoreactive ghrelin levels were measured in EDTA-containing plasma with an RIA that uses a polyclonal antibody raised

Abbreviation: AUC, Area under the curve.
against acylated, human ghrelin, and I$^{131}$-labeled ghrelin as the tracer (Phoenix Pharmaceuticals, Belmont, CA) (5). This assay detects both acylated and des-acyl ghrelin. Although only acylated ghrelin is bioactive (2), levels of total ghrelin appear to be a good surrogate for those of acylated ghrelin because the ratio of the two remains constant under a wide variety of physiological manipulations that affect ghrelin (18, 20, 21).

**Experiment 1**

**Animals.** Naïve male Sprague Dawley rats (n = 16; Charles River, Wilmington, MA) weighing 275–325 g at the time of surgery were housed in hanging stainless steel cages in a vivarium under a 12-h light, 12-h dark cycle. Rats were maintained on a liquid diet (equal parts sweetened condensed milk and water, with iron and Poly-vi-sol vitamins; Ref. 19), and water was available ad libitum except where otherwise noted. All procedures conformed to institutional standards of animal care and use (University of Pennsylvania).

**Vagotomy.** Eight rats were anesthetized by im injection with ketamine (90 mg/kg), xylazine (2.7 mg/kg), and acepromazine (0.64 mg/kg), and the trunks of the subdiaphragmatic vagus were transected. Briefly, a midline abdominal incision was made, and the dorsal and ventral branches of the vagus nerve were dissected from the esophagus. Each branch of the nerve was tied with surgical suture at two points separated by approximately 1 cm, and then cauterized between the sutures. Sham surgeries were performed (n = 8), in which each trunk of the nerve was exposed but not tied or cauterized. The incision was then closed, and rats were allowed to recover for 1 wk, over which liquid diet intake stabilized.

**Experimental procedures.**

A. **Response to nutrient load.** Before the experiment, rats were habituated to gavage-feeding of 6 ml of liquid diet (maintenance diet). Rats were tested during the mid-light phase, with food removed 1 h before blood samples were taken (to ensure that all rats enter the test with stomachs empty). On separate test days, rats were gavage-fed 6 ml of liquid diet or water. Two hundred microliters of tail blood were collected for ghrelin assay (see Ghrelin assay methods above) before and 60 min after the gavage. This experiment was performed 3 d after the deprivation experiment described below, when rats had completely recovered to their predeprivation body weights.

B. **Response to deprivation.** Tail blood samples (200 μl in EDTA tubes for ghrelin assay) were taken daily during the mid-light phase over 5 d of the experiment: 2 predeprivation baseline days, 24 h of deprivation, 48 h of deprivation, and 24 h of refeeding. On baseline days, liquid diet was removed from rats’ cages 1 h before sampling, and was replaced shortly after blood was drawn. Rats had access to water throughout the experiment.

**Statistical analysis.** To evaluate the effect of subdiaphragmatic vagotomy on baseline plasma ghrelin levels, we took an average of each animal’s baseline ghrelin level on each of the 2 predeprivation days. These average values were subjected to a two-tailed Student’s t test comparing sham-operated and vagotomized rats.

The plasma ghrelin responses to nutrient or water loads were assessed by separate two-way ANOVA, with time (pre- and post-gavage) and group ( sham-operated and vagotomized) as factors. Post hoc comparisons were made using Bonferroni-adjusted two-tailed Student’s t tests.

The effects of food deprivation and vagotomy on plasma ghrelin levels were assessed by two-way mixed (repeated measures and between-groups) ANOVA [day of experiment (baseline, 24 h deprived, 48 h deprived, 24 h re-fed) × group ( sham-operated, vagotomized)]. Each group’s response to food deprivation was then analyzed in separate one-way repeated measures ANOVA. In addition, for each subject’s plasma ghrelin levels over the course of the experiment, area under the curve (AUC) was calculated using the linear trapezoidal rule. Group differences in AUC were evaluated with a two-tailed Student’s t test.

**Results**

**Experiment 2**

**Animals.** Twelve naïve male Sprague Dawley rats (Charles River, Wilmington, MA) weighing 375–425 g at the start of the experiment were housed in hanging stainless steel cages in a vivarium under a 12-h light, 12-h dark cycle. Animals were maintained on pellet food (Purina 5001, Purina Mills, St. Louis, MO) and water was available ad libitum, except where otherwise noted. All procedures conformed to institutional standards of animal care and use.

**Experimental procedures.** The effect of atropine methyl nitrate (an antagonist of all muscarinic receptor isomers that does not cross the blood–brain barrier) on the food-deprivation-induced rise in plasma ghrelin was evaluated. Rats were injected with either atropine (3 mg/kg ip, dissolved in sterile saline; Sigma; St. Louis, MO) or saline vehicle, after 48 h of food deprivation. Five tail blood samples (200 μl in EDTA tubes for ghrelin assay) were taken from each animal during the mid-light phase: predeprivation (1 h after food was removed from cages); after 48 h of deprivation (immediately before injections); and 1, 2, and 3 h after injections. After the last blood sample was taken, food was returned to the animals’ cages. Rats recovered for 1 wk, during which they returned to their predeprivation body weights. This testing protocol was then repeated, reversing the drug treatment condition for each animal (i.e. rats that had received atropine before were given saline, and vice versa).

**Statistical analysis.** The effects of food deprivation and atropine treatment on plasma ghrelin levels were evaluated with two-way repeated measures ANOVA [sample time (baseline, 48 h food-deprived, and three post-injection points) × drug treatment (saline or atropine)]. Post hoc comparisons were made using Tukey’s honest significant difference test.

**Figure 1.** Plasma ghrelin (ng/ml) in sham-operated and vagotomized rats under baseline, ad libitum-fed conditions.
both 24-h and 48-h sample points [one-way ANOVA: F(3, 21) = 34.19, \(P < 0.001\); post hoc \(P < 0.05\)], and they returned to baseline after 24 h of refeeding. The vagotomized rats, by contrast, showed no change in plasma ghrelin levels after 24 or 48 h of food deprivation [one-way ANOVA: F(3, 21) = 1.23, NS]. This group difference is captured in the AUC analysis; AUC for ghrelin levels (ng-d/ml) over the course of the experiment was significantly greater for sham-operated rats (mean: 74.04) compared with vagotomized rats (mean: 54.82; t(14) = 2.58, \(P < 0.05\)).

**Experiment 2**

Figure 4 shows the elevation of plasma ghrelin levels after 48 h of food deprivation, and the substantial reduction in plasma levels associated with atropine methyl nitrate treatment [main effect of time F(4, 44) = 46.24, \(P < 0.001\); main effect of drug F(1, 11) = 10.66, \(P < 0.01\); interaction between drug and time F(4, 44) = 14.57, \(P < 0.001\)]. Atropine injection reduced ghrelin levels significantly at all three post-injection sample points relative to preinjection levels (\(P < 0.001\)). Plasma ghrelin levels remained stable after saline injection, and the atropine-related ghrelin reduction was significant at all sample points relative to vehicle control values (\(P < 0.001\)).

**Discussion**

The results obtained here demonstrate a role for the vagus nerve in the plasma ghrelin response to long-term changes in energy stores, but not in the response to short-term fluctuation in nutritive status. We first showed that an intact vagus nerve is not required for the maintenance of baseline plasma ghrelin levels, in contrast with a previous report of a substantial rise in ghrelin levels after vagotomy (16). This contrast may relate to differences between the studies in the level of vagal transection, or perhaps more probably, to differences in subject diet and health (see Introduction). The lack of effect of subdiaphragmatic vagotomy on baseline ghrelin levels is interesting in its own right because it suggests that tonic vagal activity plays little role in ghrelin regulation under metabolically neutral conditions. Further evidence of a lack of vagal involvement in the short-term regulation of ghrelin is provided by the significant plasma ghrelin reduction observed in response to nutrient load in both control and vagotomized rats. This finding was not anticipated, given the established role of the vagus in gut hormone control (e.g. 10, 11), and the increase in vagal afferent activity in response to intestinal nutrients (12, 13). The results presented here indicate that such functions of the vagus, although important for the short-term control of food intake, apparently are not necessary for the ghrelin response to nutrients in the gut.

Vagal mediation is indicated, however, for the food deprivation-induced elevation of plasma ghrelin. Subdiaphragmatic vagotomy completely prevented the otherwise robust (~60% in sham-operated rats) deprivation-related increase in circulating ghrelin. This result is consistent with several other examples of vagal involvement in the control of long-term energy balance. Treatments that simulate food deprivation by reducing hepatic ATP production, such as 2,5-anhydro-D-mannitol, elicit hyperphagia and induce c-fos expression in a variety of sites in the central nervous system.
Both of these responses are eliminated by vagotomy (14). Further support for vagal afferent signaling in response to food deprivation is suggested by the up-regulation of mRNA for the long form of the leptin receptor (Ob-Rb) in the nodose ganglion (which contains vagal afferent nerve cell bodies) in response to food restriction (15). Vagal efferent contributions to adaptive responses to food deprivation have also been indicated, based on analysis of changes in heart-rate variability (22). The present results add weight to the suggestion that the vagus nerve is involved in the long-term regulation of energy balance.

Strong support for a vagal efferent contribution to the plasma ghrelin response to fasting was provided in experiment 2, where the increase in circulating ghrelin with food deprivation was substantially reduced by atropine methyl nitrate treatment. We can infer, given the rapid decrease in plasma ghrelin after atropine injection, that the deprivation-related response is driven by sustained elevation of vagal efferent activity. Our pharmacological result does not speak to the identity of the relevant efferent target(s). Possibilities include a direct action on or near the ghrelin-secreting cells themselves, or indirect action through humoral or enteric mechanisms.

Subdiaphragmatic vagotomy revealed a clear dissociation between the mechanisms governing the maintenance of baseline ghrelin levels and the nutrient load-induced ghrelin reduction, on the one hand, and the elevation of plasma ghrelin in response to food deprivation, on the other. Further work is required to clarify the nonvagal mechanisms involved in the regulation of baseline ghrelin and the response to nutrient load, and to pursue the sensory signals that drive the ghrelin response to food deprivation. Apart from the specific conclusions about vagal contributions, the present results indicate, most generally, that the ghrelin responses to food deprivation, on the other. Further work is required to clarify the nonvagal mechanisms involved in the regulation of baseline ghrelin and the response to nutrient load, and to pursue the sensory signals that drive the ghrelin response to food deprivation. Apart from the specific conclusions about vagal contributions, the present results indicate, most generally, that the ghrelin responses to short- and long-term changes in nutritive status are mediated through anatomically distinct pathways.

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Address all correspondence and requests for reprints to: Diana L. Williams, Veterans Affairs Puget Sound Health Care System, 1660 South Columbian Way, Mail Stop 111-ENDO, Seattle, Washington 98108. E-mail: dianalw@u.washington.edu.

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