GHRELIN SECRETION IS NOT REDUCED BY INCREASED FAT MASS DURING DIET-INDUCED OBESITY

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Running Title: Ghrelin Activity and Obesity

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ABSTRACT

Ghrelin is a stomach hormone that stimulates growth hormone (GH) secretion, adiposity and food intake. Gastric ghrelin production and secretion are regulated by caloric intake, ghrelin secretion increases during fasting, decreases with re-feeding and is reduced by diet-induced obesity. The aim of our study was to test the hypotheses that an increase in body adiposity 1) will exert an inhibitory role in the reduction of gastric ghrelin synthesis and secretion during a chronic high fat (HF) diet; and, 2) that a chronic HF diet will suppress the rise in circulating ghrelin levels in response to acute fasting. Adult male Sprague-Dawley rats were fed either a standard AIN-76A (~5-12% calories-fat) or a HF diet (~45% calories-fat). The effect of an increased adiposity on gastric ghrelin homeostasis was assessed by comparing stomach ghrelin production and plasma ghrelin levels in obese and non-obese rats fed a HF diet. HF diet fed, non-obese rats were generated by administration of triiodothyronine (T3) to lower body fat accumulation. Findings indicate that an increased fat mass per se does not exert an inhibitory action on ghrelin homeostasis during HF diets. Additionally, the magnitude of the plasma ghrelin increment to fasting was not blunted, indicating that a presumed, endogenous signal for activation of ingestive behavior remains intact in spite of excess stored calories in HF fed rats.

Key Words: obesity, gut hormones, regulation
INTRODUCTION

The stomach hormone, ghrelin, is a 28-amino acid peptide that was discovered in extracts of the rat stomach as the endogenous ligand for the growth hormone secretagogue-receptor (GHS-R) (31, 46). The search for ghrelin resulted directly from the development of synthetic substances called growth hormone secretagogues (GHSs) and the subsequent discovery of a natural receptor, the GHS-receptor (GHS-R) for these artificial ligands (8, 9, 21, 47). Systemic administration either ghrelin or of GHSs evokes GH secretion from the pituitary by activation of GHS-R. GHSs were synthesized based on the structure of enkephalins and do not resemble the hypothalamic hormone, GH-releasing hormone (GHRH) nor cause GH secretion by activation of GHRH-R.

Ghrelin exerts a variety of important metabolic actions. Ghrelin stimulates GH secretion, food intake, body growth and adiposity (17, 31, 42, 49, 53). Ghrelin also influences gastric acid secretion and exerts a prokinetic action on the stomach (38). Ghrelin is the first, endogenous peptide to be isolated having an n-octanol modification at the 3Ser (31). Much data indicate that both the acyl- and des-acyl forms of ghrelin exert biological activity (4, 6, 7, 10, 31). Although ghrelin is expressed in the pancreas, intestine, kidney, hypothalamus, heart and placenta (51), the stomach epithelium is the primary site for synthesis of ghrelin immunoreactivity measured in the general circulation. Ghrelin is produced in gastric enteroendocrine cells called X/A cells (14). Our laboratory and others have shown that gastrectomy in rats and humans will diminish plasma ghrelin levels approximately 85% (5, 22).

Regulation of ghrelin secretion from the stomach is unique when compared to other gastrointestinal hormones. In contrast to other gut hormones, plasma ghrelin levels increase in response to fasting and decrease upon refeeding (22). Furthermore, plasma ghrelin levels are
reduced by chronic intake of high-calorie diets and obesity in humans (50). In rodents, exposure to high-fat (HF) diets for prolonged periods will result in a positive energy balance, obesity and a reduction in stomach production and secretion of ghrelin (33), however little is known about the extent to which the increased adiposity itself exerts an inhibitory influence on stomach ghrelin production and secretion.

The purpose of this present study therefore, was to investigate the role of the increased body adiposity on ghrelin production and secretion during a HF diet. In order to diminish the influence of the increased adiposity during the high fat diet, rats fed high fat diets were given triiodothyronine (T3) to “clamp” their body weights and adiposity at levels comparable to those measured in control rats.

METHODS AND MATERIALS

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. Adult male Sprague-Dawley rats were maintained in an air-conditioned and light-regulated room (lights on, 0600–1800 h) and given access to food and water ad libitum.

Chemicals and peptides

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Synthetic peptides were purchased from Phoenix (Belmont, CA).

Experiments

Experiment 1. There are six groups of rats in this experiment (N = 8-10 rats/group). Two groups of rats (AIN + VEH; HF + VEH) were fed either a commercial AIN-76A (~5% calories-
fat by weight) or a HF diet (~45% calories-fat) for 8 weeks (BioServ, Frenchtown, NJ) and given vehicle treatment (VEH). Two groups of rats (AIN + T3; HF + T3) were fed the AIN-76 and HF diets and given concurrently triiodothyronine (T₃, 100 µg/kg, SC, every other day). T₃ treatment was given to avert body fat accumulation in rats fed a HF diet. Thyroid hormones (thyroxine [T₄] and triiodothyronine [T₃]) are the predominant regulators of basal metabolic rate and circulating levels of thyroid hormones have a direct correlation with energy expenditure and caloric loss (35). T₃ was prepared as described previously (23). Because T₃ administration has been reported to stimulate food intake (32, 36), and food intake affects stomach ghrelin homeostasis, a group of rats (HF_PFHF+VEH + T3) were T₃ treated and pair-fed a HF diet according to the daily caloric of rats fed the HF diet but not given T3. Another group of rats (HF_PFAIN+VEH + VEH) were pair-fed the HF diet according to the daily caloric intake of rats fed ad lib the AIN-76A diet in order to determine the extent to which a higher percentage of ingested dietary fat is a key signal to reducing stomach ghrelin production and blood ghrelin levels. Daily food intakes were monitored for AIN + VEH and HF + VEH rats during the entire experiment. Food intakes were measured in AIN + T3 and HF + T3 rats during the last two weeks of the experiment. At the end of the 8-week treatment period, body weights were recorded and rats sacrificed. Plasma was harvested for measurement of plasma ghrelin, insulin, leptin and triglyceride levels. The stomach-fundal mucosa was collected for extraction of total cellular RNA and ghrelin peptide. Total body fat was measured by quantitative nuclear magnetic resonance using an Echo MRI Whole Body Composition Analyzer (Echo Medical Systems, Houston, TX) (37). Plasma triglyceride levels were measured using a commercial kit (Sigma Chemical Co). The compositions (approximate percent of calories) of the commercial AIN-76A biscuit and HF diets are: fat 5%; protein 20%; carbohydrate 72%; and, fat 48% (beef tallow); protein 16%;
carbohydrate 34%, respectively. Two percent of the fat calories are derived from safflower oil in the beef tallow diet to supply essential fatty acids. All rats had unrestricted access to water. Food intake was monitored by giving rats a pre-weighed amount of food daily. Food intake was calculated daily by monitoring the amounts of food remaining in the food bowel plus waste below the cage. Pair-fed rats were given an additional 10% food to accommodate loss due to spillage.

**Experiment 2.** Two groups of adult male Sprague-Dawley rats (n = 10 rats/group) were fed either an AIN-76A (~12% calories-fat) or a HF diet (~40-45% calories-fat) for 5 weeks. All rats had unrestricted access to water. Plasma was collected in the ad-lib fed and fasted conditions (~24 h) from groups of AIN-76A and HF fed rats for measurement ghrelin levels by immunoassay. Food intakes were monitored for rats fed the AIN-76A and HF diets for 2 weeks as described for Experiment 1.

**Ghrelin, insulin and leptin assays**

For Experiment 1, plasma acyl-ghrelin levels were measured using a commercial ELISA kit that detects acyl-ghrelin specifically (ALPCO, Salem, NH). The sensitivity for this assay is 3.9 pg/ml. An anticoagulant (EDTA) and a protease inhibitor (p-hydroxymercuribenzoic acid, PHMB, 1 mM in the final sample volume) are added at the time of blood collection. After separation of plasma, 100 µl of 1N HCl is immediately added per ml of collected plasma and centrifuged briefly. Supernatants are saved for assay. Blood and plasma samples are kept on ice until frozen. A double antibody radioimmunoassay procedure was used to measure plasma ghrelin levels in Experiment 2 and stomach tissue ghrelin levels in Experiment 1 as described in detail previously by our laboratory (33). The ghrelin antiserum was produced in rabbits by immunization with a synthetic carboxy-terminal fragment of rat ghrelin. This ghrelin antibody
recognizes acyl and desacyl-ghrelin equally hence ghrelin levels reflect total ghrelin levels (acyl + desacyl-ghrelin). The sensitivity and ID$_{50}$ (50% inhibition of bound radio-iodinated ligand) are 0.01 and 0.2 ng/tube.

Stomach ghrelin peptide was extracted from stomach tissue specimens as reported earlier (33). For Experiment 2, plasma ghrelin was extracted from rat plasma (1.2 ml/sample) by use of C$_{18}$ Sep Paks (Waters, Milford, MA). Samples were assayed in duplicate. Plasma insulin levels were measured as described previously (24). The sensitivity and ID$_{50}$ for this insulin assay are 0.04 and 4.0 ng/ml. A double antibody radioimmunoassay procedure was used to measure plasma leptin levels. The sensitivity and ID$_{50}$ for this leptin assay are 1.0 and 6.0 ng/tube. All assays use specific antibodies that do not recognize structurally unrelated pancreatic or gastrointestinal peptides. For all assays, the intra- and inter-assay CVs are ~3-5.0 and ~8-12%, respectively.

**RNA purification and Northern blotting analysis**

Fresh tissues were homogenized immediately in 4 M guanidinium isothiocyanate containing 25 mM sodium citrate, pH 7.0, 0.5% sodium N-lauroylsarcosine, and 0.1 M β-mercaptoethanol (33). Extracts were frozen at -80°C until purification by ultracentrifugation over a cesium chloride cushion (2 ml, 5.7 M). RNA samples were separated on a 1% agarose gel (30 μg/lane) in a 20 mM 3-[N-morpholino]propanesulfonic acid running buffer system (33) and then transferred to a nylon membrane and subjected to Northern hybridization. $^{32}$P-labeled riboprobes prepared from Strip-EZ RNA kits (catalog no. 1366, Ambion, Inc., Austin, TX) were used for Northern hybridizations. Ribosomal 18S was used to normalize for variations in RNA loading and transfer. Expression levels of ghrelin or the 18S genes were quantitated by phosphoimaging.
Statistics

Values are given as means with SEMs. Data were evaluated using an ANOVA followed by the Newman-Keul's test where appropriate by means of Statview Software (Cary, NC).

RESULTS

Experiment 1. The purpose of this experiment was to examine the role of body adiposity in the reduction of stomach ghrelin production and plasma ghrelin levels that occurs in response to a long-term HF diet, and to determine the extent to which a higher percentage of ingested dietary fat is a signal to reducing stomach ghrelin production and blood ghrelin levels. A HF diet, i.e., diet-induced obesity will suppress ghrelin synthesis and secretion in rodents, and obesity will lower circulating ghrelin levels in humans (33, 50). In order to isolate the influence of increased body fat on stomach ghrelin production and secretion, T3 treatment was given to rats fed a HF diet in order to prevent body fat accumulation. Because T₃ administration has been reported to stimulate food intake (32, 36) and food intake influences stomach ghrelin homeostasis, a group of rats (HFₚHFₚHF+VEH + T3) were T3 treated and pair fed a HF diet according to the daily caloric of rats fed the HF diet but not given T3. Another group of rats (HFₚAIN+VEH + VEH) were pair-fed the HF fat diet according to the daily caloric intake of rats fed ad lib the AIN-76A diet in order to determine the extent to which a higher percentage of ingested dietary fat is a signal to reducing stomach ghrelin production and blood ghrelin levels.

The body weights of rats given the HF diet plus vehicle injections (HF + VEH) but not the body weights of HF + T3 rats were significantly higher (p<0.05) when compared to rats given the AIN-76A diet plus vehicle injections (AIN + VEH) (Figure 1). The body weights of AIN + VEH and AIN + T3 rats did not differ significantly (p>0.05). The body weights of rats
(HF_{PFAIN+VEH} + VEH) pair-fed the HF diet according to the daily caloric intake of rats fed ad lib the AIN-76A diet were significantly higher when compared to rats given the AIN-76A diet (AIN + VEH). The body weights of rats (HF_{PFHF+VEH} + T3) given T3 and pair fed a HF diet according to the daily caloric of HF rats but not given T3 (HF + VEH) did not differ significantly when compared to rats given the AIN-76A diet.

Body composition analyses indicated that the body fat contents of HF + VEH but not HF + T3 rats were significantly greater than the body fat contents of AIN + VEH rats (Figure 1). The body fat contents of HF + T3 rats were reduced significantly when compared to HF + VEH rats. The body fat contents of AIN + T3 and AIN + VEH rats did not differ significantly. The body fat contents of HF_{PFAIN+VEH} + VEH rats were increased significantly when compared to AIN rats and did not differ significantly when compared to HF + VEH rats. The body fat contents of HF_{PFHF+VEH} + T3 rats were similar to those of AIN + VEH rats and significantly lower when compared to HF + VEH rats.

With one exception, plasma triglyceride levels were significantly higher in rats fed the HF diet (HF + VEH; HF + T3; HF_{PFHF+VEH} + T3) when compared to rats fed the AIN-76A diet. In rats given HF_{PFAIN+VEH} + VEH, plasma triglyceride levels did not differ when compared to AIN + VEH rats.

Plasma ghrelin levels were reduced significantly in rats given HF + VEH or HF + T3 when compared to rats given AIN + VEH or AIN + T3 (Figure 2). When compared to AIN + VEH rats, plasma ghrelin levels were reduced significantly in rats (HF_{PFAIN+VEH}) pair-fed the HF diet according to the daily caloric intake of rats fed ad lib the AIN-76A diet (AIN + VEH). When compared to AIN + VEH rats, plasma ghrelin levels were reduced significantly in rats
(HF_{PHHF + VEH} + T3) that were T3 treated and pair fed a HF diet according to the daily caloric of 
HF + VEH rats.

In addition, stomach ghrelin mRNA levels were decreased significantly in rats given HF + VEH or HF + T3 when compared to rats given AIN + VEH or AIN + T3. In rats given 
HF_{PHFAIN + VEH} + VEH or HF_{PFHF + VEH} + T3, stomach ghrelin mRNA levels were decreased 
significantly when compared to rats given AIN + VEH or AIN + T3.

Stomach ghrelin peptide levels in HF + VEH and HF + T3 rats were reduced significantly 
when compared to stomach ghrelin peptide levels in AIN + VEH or AIN + T3 rats. Stomach 
ghrelin peptide levels of rats (HF_{PHFAIN + VEH} + VEH) pair-fed the HF diet according to the daily 
caloric intake of rats fed ad lib the AIN-76A diet were not altered significantly when compared 
to AIN fed rats. Stomach ghrelin peptide levels in HF_{PFHF + VEH} + T3 rats were reduced 
significantly when compared to AIN + VEH rats.

Plasma leptin levels in HF + VEH rats were significantly higher when compared to 
plasma leptin levels in AIN + VEH, AIN + T3, HF + T3 or HF_{PFHF + VEH} + T3 rats. In HF + T3 
and HF_{PFHF + VEH} + T3 rats, plasma leptin levels were reduced significantly when compared to HF + VEH rats. Plasma leptin levels in HF_{PFFAIN + VEH} + VEH rats were increased significantly when 
compared to AIN-76A fed rats.

Plasma insulin levels in AIN + T3 rats were significantly lower when compared to all 
other groups. Plasma insulin levels in AIN + VEH, HF + VEH, HF + T3, HF_{PFAIN + VEH} + VEH 
and HF_{PFHF + VEH} + T3 rats did not differ significantly.

Table 1 shows that daily food intakes of AIN + VEH and HF + VEH rats did not differ 
significantly. Daily food intakes of rats given AIN + T3 were significantly higher when 
compared to AIN + VEH rats.
Experiment 2. The aim of this experiment was to determine the extent to which plasma ghrelin levels increase in response to acute fasting in rats fed a long-term HF diet. Control rats were fed the AIN-76A diet (12% fat). Interestingly, in rats fed a HF diet, plasma ghrelin levels increased significantly in response to fasting (Figure 3). The plasma ghrelin increments in response to fasting were similar in rats fed HF (~1300 pg) or AIN-76A (~1400 pg) diets. Food intake was measured for a 2-week period in rats fed AIN76A and HF diets. Daily food intake, in terms of grams/day/rat in rats fed the AIN-76A diet was significantly greater when compared to rats fed the HF diet (Table 2). In terms kcal/day/rat, the daily food intakes did not differ significantly.

DISCUSSION

The primary aim of these experiments was to investigate the hypotheses that 1) the increased body adiposity in rats fed a HF diet will exert an inhibitory influence over gastric ghrelin production and systemic ghrelin levels, that 2) a higher percentage of dietary fat is a signal to reduce stomach ghrelin production and blood ghrelin levels, and that 3) an increased body adiposity will suppress the rise in circulating ghrelin levels in response to acute fasting. In the present studies, T3 treatment was used to avert body fat accumulation in rats fed a HF diet. It is well known that thyroid hormones, thyroxine (T4) and triiodothyronine (T3) are the predominant regulators of basal metabolic rate and that circulating levels of thyroid hormones have a direct correlation with energy expenditure and caloric loss (35). T3 administration may stimulate food intake (32, 36), it is possible, therefore, that stomach ghrelin homeostasis and plasma ghrelin levels are influenced partly by this T3-induced increase in food intake. Therefore, stomach ghrelin homeostasis and secretion were measured in a group of rats given T3
and pair fed a HF diet according to the daily caloric of rats fed the HF diet but not given T3. Another group of rats were pair-fed the HF diet according to the daily caloric intake of rats fed ad lib the AIN-76A diet in order to determine the extent to which a higher percentage of ingested dietary fat plays a role in the reductions of stomach ghrelin production and blood ghrelin levels during a HF diet.

It is well documented that chronic HF diets and the ensuing obesity will reduce stomach ghrelin production and plasma ghrelin levels in rodents, plasma ghrelin levels are also reduced in obese humans (33, 50). The source and identity of the inhibitory signal on ghrelin homeostasis during increased adiposity, however, are not known. One potential source of inhibitory signals is the increased body fat mass that accrues from regular ingestion of excess calories. Possible inhibitory signals secreted by adipose cells include several adipokines, such as leptin, apelin, adiponectin, resistin and others (48). Studies in humans and rodents show that circulating levels of leptin and ghrelin can be inversely correlated (12, 50); and in the isolated perfused rat stomach, leptin infusion will inhibit ghrelin secretion (28). Together, these earlier findings suggest that leptin may participate in the lowering of ghrelin homeostasis during a HF diet and obesity. In the present study, plasma ghrelin levels are reduced in two groups of rats (HF + VEH, HFADAIN+VEH + VEH) that have elevated plasma leptin levels; these rats are fed a HF diet. However, plasma ghrelin levels are also reduced in rats without elevated plasma leptin levels (HF + T3, HFADPFHF+VEH + T3). These latter findings indicate that the reduced plasma ghrelin levels in rats fed a HF diet are not dependent upon an elevation in circulating leptin levels and suggest further that the inhibitory signal during a HF diet is most likely not leptin. Alternatively, if the inhibitory signal includes leptin, leptin is a partial component. The lower plasma leptin levels in rats given HF + T3 and HFADPFHF+VEH + T3 agree with their reduced body
fat contents. In non-obese rats fed the HF diet (HF + T3, HF_{PFHF+VEH} + T3) the amount of body adiposity is not significantly different from the amount measured in lean AIN + VEH rats. The influence of other adipokines such as apelin, adiponectin and resistin, on gastric ghrelin production and secretion have not been investigated.

Another potential inhibitor of plasma ghrelin levels during a HF diet is insulin since circulating insulin levels can be elevated during chronic HF diets and obesity (15, 34, 39), and in the isolated perfused rat stomach, insulin infusion will inhibit ghrelin secretion (28). Additionally, in humans, plasma ghrelin levels decrease in response either to an insulin infusion or to a meal suggesting that insulin is a physiological inhibitor of ghrelin secretion (13, 18). It should be pointed out that other studies indicate that insulin does not affect ghrelin secretion (3). In the present report, plasma insulin levels are unaltered by the various treatment combinations, indicating that alterations in plasma ghrelin levels during HF diets are not influenced by plasma insulin.

An elevated fat mass will also result in elevated systemic levels of several cytokines, including TNF-α and IL-6 (20, 27). The effects of exogenous TNF-α and IL-6 on gastric ghrelin production and secretion have not been investigated although LPS administration, an activator of IL-6 and TNF-α secretion (29, 30, 43, 44) will lower ghrelin secretion in rats (52).

Our results indicate that the reduction in gastric ghrelin homeostasis during a HF diet is not associated primarily with an inhibitory signal from adipose tissue. Reductions in stomach ghrelin homeostasis and plasma ghrelin levels appear to be related to the ingestion of the HF - it’s an enteric signal. In non-obese rats fed the HF diet (HF + T3, HF_{PFHF+VEH} + T3) the amount of body adiposity is equal to the body adiposity measured in lean AIN + VEH rats. In spite of normal body adiposity, gastric ghrelin production and secretion remain reduced.
In our studies, a group of rats (HF_{PAIN} + VEH + VEH) were pair-fed the HF diet according to the daily caloric intake of rats fed ad lib the AIN-76A diet in order to determine the extent to which a higher percentage of ingested dietary fat is a signal to reducing stomach ghrelin production and blood ghrelin levels. Interestingly, plasma ghrelin and stomach ghrelin mRNA levels are reduced in these rats, however these rats also have elevated body fat contents making it impossible to discern the influence of a fat calorie dense diet on stomach ghrelin homeostasis and plasma ghrelin levels apart from other nutrition associated effectors. It should be pointed out however, that the reduced plasma ghrelin and stomach ghrelin mRNA levels in rats given HF_{PAIN} + VEH + VEH indicate that the inhibitory mechanism does not involve intake of excess calories since calories were restricted. We did not expect to find that rats fed a calorie restricted, HF diet, to have in increased body weights and body fat contents. These findings, however, agree with and extend earlier data showing that HF diets produce obesity partly since they produce a metabolically efficient state (41, 45, 54).

As indicated earlier, T3 treatment has been reported to stimulate food intake in rats (32, 36). Interestingly, our findings show that T3 administration to rats fed AIN-76A diet (AIN + T3) increases food intake when compared to AIN + VEH rats whereas T3 treatment of rats fed the HF diet (HF + T3) fails to increase food intake significantly when compared to HF + VEH rats. In order to determine the extent to which this possible T3 enhanced food intake plays a role in lowering stomach ghrelin production and secretion, a group of rats (HF_{PFHF} + VEH + T3) were fed the HF diet and given T3 however the daily amount of ingested calories was restricted to an amount consumed by rats fed a HF diet but not given T3. In HF_{PFHF} + T3 rats, stomach ghrelin production and secretion were reduced. Furthermore, T3 treatment of HF fed rats does not enhance food intake. Together, these findings indicate that the reduced stomach ghrelin
production and secretion in HF + T3 rats is not due to an enhanced food intake. Additionally, the persistent reductions in stomach ghrelin production and secretion in HFPFHF+VEH +T3 rats along with a normal body weight and total body fat contents further supports the idea that increased body fat does not have a role in the lowering of stomach ghrelin production and secretion in rats fed a HF diet. Although speculative, the marginal but insignificant reduction in plasma ghrelin levels of AIN + T3 rats may be attributed to the increased food intake.

Our results imply that the inhibitory signal on gastric ghrelin production and secretion during HF diet consumption either is an enteric or pancreatic hormone, ingested nutrients, or both. As expected (11), the present study shows that hyperlipidemia (accumulation of triglycerides in plasma) results during HF diets. Because blood-borne nutrients can depress ghrelin secretion, the increased levels of blood lipids may act on the systemic side of ghrelin cells to reduce ghrelin secretion. The identity of a possible inhibitory enteric hormone during HF diets or obesity is not known but it is expected to act systemically on ghrelin cells. Interestingly, our laboratory has reported that gastric lavage of soybean trypsin inhibitor (SBTI), a secretagogue for release of intestinal cholecystokinin (CCK) (40) will lower ghrelin secretion in fasted rats (22) implying that CCK might act to inhibit ghrelin secretion. Because physiologic changes occur in muscle and liver tissue during HF diets and obesity (2, 16, 19), a factor(s) from these tissues might conceivably affect ghrelin homeostasis.

Ghrelin is a potent activator of food intake and its secretion from the stomach is sensitive to caloric intake (22, 33). Our findings show that the acute elevation in secretion of gastric ghrelin in response to fasting in HF rats is unaffected by an excess of stored calories, i.e., obesity. Furthermore, the plasma ghrelin increment in rats fed the HF diet was comparable to that measured in rats fed the AIN-76A diet (AIN-76A: ~1400 vs HF: ~1300 pg/ml).
Stomach ghrelin is synthesized and circulates as acylated and desacylated variants (1, 26). Levels of desacyl-ghrelin predominate over those of acyl-ghrelin in the rat stomach and plasma desacyl-ghrelin levels are approximately 4-10-fold higher than acyl-ghrelin levels (26, 56) (Greeley, unreported findings). Although both ghrelin variants have multiple biological activities, only acyl-ghrelin stimulates food intake (31).

The enzyme that acylates ghrelin has been described recently (25, 55). Substrate for this enzyme is medium chain fatty acids, primarily C8- and C10- fatty acids. In our experiments, the HF diet consists of long chain fatty acids. In Experiment 1, plasma levels of acyl-ghrelin (C8) are measured. Although speculative, the decreased plasma acyl-ghrelin levels measured in rats fed a HF diet may reflect, in part, the composition of the diet and an inadequate level of enzyme substrate. In contrast, rats fed AIN-76A, a diet with little fat and a reduced caloric density, will have a greater likelihood of beta-oxidation and generation of medium chain fatty acids for ghrelin acylation.

**Perspectives and significance**

Ghrelin is an exceptionally intriguing stomach hormone since data indicate that luminal and systemic nutrient signals influence ghrelin secretion. Exactly how excess caloric intake and obesity exert a negative regulation over stomach ghrelin production and secretion is enigmatic. One very likely source of inhibitory signals is the elevated body fat mass. However, our results clearly indicate that adipose mass does not feed back to inhibit gastric ghrelin homeostasis. This is a primary finding of this study. Another important finding is that the inhibitory signal is enteric in nature, it may be either is a gastrointestinal or pancreatic hormone or specific ingested nutrients. It is most likely a combination of multiple inhibitory signals. Future studies should address and elucidate identities of inhibitory signals.
ACKNOWLEDGMENTS

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GRANTS

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DISCLOSURES

Authors have no disclosures to declare.
REFERENCES


inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. 


30. **Kim JH, Kim DH, Baek SH, Lee HJ, Kim MR, Kwon HJ, and Lee CH.** Rengyolone inhibits inducible nitric oxide synthase expression and nitric oxide production by down-
regulation of NF-kappaB and p38 MAP kinase activity in LPS-stimulated RAW 264.7 cells.


Table 1. Average Daily Food Intake of Rats Given AIN-76A or HF Diets with Vehicle or T3 Treatments

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (g)</th>
<th>kCal</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN + VEH</td>
<td>20.2 ± 1.2</td>
<td>69.8 ± 4.2</td>
</tr>
<tr>
<td>AIN + T3</td>
<td>26.2 ± 1.7*</td>
<td>90.6 ± 5.7*</td>
</tr>
<tr>
<td>HF + VEH</td>
<td>18.2 ± 1.6</td>
<td>82.1 ± 7.1</td>
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<tr>
<td>HF + T3</td>
<td>19.4 ± 1.2</td>
<td>87.7 ± 5.4</td>
</tr>
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Daily food intakes for the last 2 weeks of Experiment 1. * p<0.05 vs. AIN + VEH.
Table 2. Average Daily Food Intake of Rats Given AIN-76A or HF Diets

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (g)</th>
<th>kCal</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN-76A</td>
<td>21.7 ± 0.4</td>
<td>72.6 ± 1.3</td>
</tr>
<tr>
<td>HF</td>
<td>15.8 ± 0.4*</td>
<td>71.6 ± 1.7</td>
</tr>
</tbody>
</table>

Daily food intakes were monitored for a 2-week period in Experiment 2. * p<0.05 vs. AIN-76A.
**FIGURE LEGENDS**

Figure 1. Influence of a high-fat diet (HF) in combination either with vehicle (VEH) or T3 (T3) treatments on body weight, total body fat and plasma triglyceride levels in rats. Groups of rats pair-fed according to the daily caloric intakes of AIN + VEH and HF + VEH are included (HF<sub>PFAIN + VEH</sub> + VEH; HF<sub>PFHF + VEH</sub> + T3); the latter group was given T3. Control rats were fed an AIN-76A diet (AIN). Rats were fed AIN-76A (5% fat) or HF (40-45% fat) diets for 8 weeks. Body weights, total body fat and plasma triglyceride levels were measured as described in the text. * = P < 0.05 vs AIN + VEH; + = P < 0.05 vs HF + VEH. n=8-10 rats/group.

Figure 2. Role of adipose tissue in suppression of stomach ghrelin production and secretion in obese rats. As described in the text and legend for Figure 1, rats were fed an AIN-76A or HF diets for 8 weeks. Groups of AIN and HF fed rats were given vehicle or T3. Groups of rats pair-fed according to the daily caloric intakes of AIN + VEH and HF + VEH are included (HF<sub>PFAIN + VEH</sub> + VEH; HF<sub>PFHF + VEH</sub> + T3); the latter group was given T3. At the end of the treatment period, plasma was collected for measurements of plasma ghrelin, leptin and insulin levels and stomachs were harvested and extracted for measurements of stomach ghrelin mRNA and peptide levels. * = P < 0.05 vs AIN + VEH; Δ = P < 0.05 vs AIN + T3. † = P < 0.05 vs HF + VEH; ** = P < 0.05 vs all other groups. n=8-10 rats/group.

Figure 3. A HF diet does not inhibit the stomach ghrelin secretory response to acute fasting. Rats were fed either an AIN (12% fat) or HF (40-45%) diets for 5 weeks. Plasma was collected from rats in the ad lib fed and fasted (24 h) conditions for
measurement of plasma ghrelin levels. * = P < 0.05 vs ad lib fed rats; ** = P < 0.05 vs fed AIN-76A rats.
Figure 1

Bar graphs showing the effects of different treatments on body weight, total body fat, and plasma triglycerides.

- **Body Weight (g)**
  - AIN + VEH
  - HF + VEH
  - AIN + T3
  - HF + T3
  - HFPAIN + VEH
  - HFPPHF + VEH

- **Total Body Fat (g)**
  - AIN + VEH
  - HF + VEH
  - AIN + T3
  - HF + T3
  - HFPAIN + VEH
  - HFPPHF + VEH

- **Plasma Triglycerides (mg/ml)**
  - AIN + VEH
  - HF + VEH
  - AIN + T3
  - HF + T3
  - HFPAIN + VEH
  - HFPPHF + VEH

Significant differences indicated by asterisks (*) and daggers (†).
Figure 2
Figure 3: Plasma Ghrelin levels in response to fasting with different diets. AIN-76A Diet: Fed > Fasted; High-Fat Diet: Fed > Fasted. * indicates p < 0.05, ** indicates p < 0.01.