Basic nutritional investigation

High-fat diet increases ghrelin-expressing cells in stomach, contributing to obesity

Marie François Ph.D. a,b, Swapnali Barde M.Sc. c, Romain Legrand M.Sc. a,b, Nicolas Lucas M.Sc. a,b, Saida Azhar B.Sc. a,b, Mohammed el Dhaybi M.Sc. a,b, Charlène Guerin B.Sc. a,b, Tomas Hökfelt M.D. c, Pierre Déchelotte M.D., Ph.D. a,b,d, Moïse Coëffier Ph.D. a,b,d, Serguei O. Fetissov M.D., Ph.D. a,b,*

a Inserm UMR1073, Nutrition, Gut and Brain Laboratory, Rouen, France
b Institute for Research and Innovation in Biomedicine (IRIB), Rouen University, Normandy University, Rouen, France
c Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden
d Rouen University Hospital, CHU Charles Nicolle, Rouen, France

Abstract

Objectives: Mechanisms of high-fat diet (HFD)–induced obesity may involve ghrelin, an orexigenic and adipogenic hormone secreted by the stomach. Previous studies showed that obese subjects may display higher numbers of ghrelin-producing cells and increased affinity of plasma immunoglobulins (Ig) for ghrelin, protecting it from degradation. The aim of this study was to determine if a HFD in mice would increase the number of ghrelin-expressing cells and affinity of ghrelin-reactive IgG.

Methods: Obesity in mice was induced by consumption of a 13-wk HFD. The number of preproghrelin mRNA-producing cells in the stomach was analyzed by in situ hybridization and compared with chow-fed, nonobese controls and with genetically obese ob/ob mice. Affinity of ghrelin-reactive IgG was analyzed using surface plasmon resonance. Plasma levels of ghrelin and des-acylated ghrelin were measured.

Results: HFD resulted in 30% of body fat content versus only 8% in controls (P < 0.001). The number of preproghrelin mRNA-producing cells was 15% (P < 0.05) higher in HFD-fed mice than in controls, contrasting with ob/ob mice, having a 41% (P < 0.001) decrease. Both models of obesity had normal plasma levels of ghrelin but a decrease of its des-acylated form. Ghrelin-reactive IgG affinity was found in the micromolar range with mean values of the dissociation equilibrium constant 1.5-fold (P < 0.05) lower in HFD-fed versus control mice.

Conclusion: Results from the present study showed that HFD in mice induces obesogenic changes, including increased numbers of ghrelin precursor-expressing cells and increased affinity of ghrelin-reactive IgG. Such changes may contribute to the mechanisms of HFD-induced obesity.

© 2016 Elsevier Inc. All rights reserved.

Introduction

Obesity remains a major global health problem with increasing prevalence [1], emphasizing the need for understanding the underlying molecular mechanisms, including those linking nutritional factors with hormonal signals regulating food intake [2]. A broader view on how nutrients may affect hormonal regulation of feeding behavior and metabolism has been reviewed elsewhere [3,4]. The present study focused on the possible role of ghrelin in the obesogenic effects of a high-fat diet (HFD). Since its discovery in 1999 [5], ghrelin, a 28 amino acid-acylated peptide hormone, has been extensively studied for its possible role in obesity development [6,7]. Ghrelin is synthesized predominantly in the gastric mucosa [8,9] from a polypeptide precursor, the preproghrelin, which is acylated by ghrelin-O-acyltransferase [10,11]. Ghrelin is unstable and will lose its biological activity after deacylation and proteolysis by plasma enzymes [12–14]. Accordingly, des-acyl ghrelin (DAG) represents the main form of the circulating hormone [15]. Ghrelin stimulates food intake in both humans [16] and rodents [17–19], while its
chronic administrations in mice increases adiposity, supporting ghrelin’s role in the development of obese phenotypes [20].

Obese subjects usually display lower plasma concentrations of total ghrelin (acyl ghrelin [AG] and DAG) than age-matched lean controls [21,22], whereas AG alone has been found at comparable levels [23,24]. These changes contrast with a report on the increased number of ghrelin-producing cells in the stomach of obese patients [25] and with increased preproghrelin mRNA-expression levels in HFD-fed obese mice [26]. It suggests that in obesity, DAG may undergo faster degradation, possibly via increased peptidase activity [27], whereas AG is relatively well preserved. Thus, analyzing separately plasma ghrelin and DAG should be more informative for understanding the ghrelin’s role in obesity [28]. It has been shown that protection of ghrelin from degradation is possible due to its binding to ubiquitously present ghrelin-reactive immunoglobulins (Igs), which transport ghrelin without antagonizing its functionality [19]. Furthermore, such IgG in obese individuals and in genetically obese ob/ob mice were characterized by increased affinity and enhanced ghrelin’s orexigenic effects [19]. Thus, increased numbers of ghrelin precursor-expressing cells in the stomach coupled with increased affinity of plasmatic IgG, protecting ghrelin from degradation, may contribute to the ghrelin-mediated obesogenic mechanisms. However, it is not known whether such changes of the ghrelin system can be triggered by nutritional factors contributing to obesity development such as a HFD.

To answer this question, we induced obesity in mice by feeding them a standard HFD containing 60% of fat from Research Diets for 13 wk. The diet is known to induce obesity in rodents [29]. In fact, an excess of fat in nutrients provides permissive conditions for metabolic obesogenic adaptation [30, 31]. Control age-matched mice were fed with a standard rodent chow. Development of obesity was confirmed by in vivo body composition analysis. Then, the number of preproghrelin mRNA-expressing cells in the stomach was analyzed by in situ hybridization. To verify whether preproghrelin mRNA-expressing cells may change secondary to obesity, we also analyzed their number in obese ob/ob mice. Plasma IgG were extracted in HFD obese and control mice and their affinity kinetics for ghrelin was determined using surface plasmon resonance. Plasma levels of ghrelin and DAG also were measured.

Materials and methods

Animals

Animal care and experimentation complied with both French and European community regulations, and the Regional Ethical Committee (N04–11–12/2711–15) approved the experiment. Six-wk-old C57 Bi/6 male mice were purchased from Janvier Labs (Genest-St-Isle, France) and acclimated to the animal facility for 1 wk. They were maintained under controlled temperature (20°C–2°C) and with 12-h light/dark cycle, lights on at 0700. Standard pelleted rodent chow (RM1 diet, Special Diets Services, Essex, UK) and drinking water were available ad libitum. After acclimatization, mice were distributed into two groups: control (RM1 diet, Special Diets Services, Essex, UK) and drinking water were available ad libitum. After acclimatization, mice were distributed into two groups: control (RM1 diet, Special Diets Services, Essex, UK) and drinking water were available ad libitum. After acclimatization, mice were distributed into two groups: control (RM1 diet, Special Diets Services, Essex, UK) and drinking water were available ad libitum.

Tissue sampling

Mice were sacrificed by decapitation; trunk blood was collected in K3 E (15% aprotinin (250 KIU) tube (BD Vacutainer). After centrifugation (20 min at 3000g, 4°C) plasma were extracted, acidified with 1 N HCl to protect ghrelin from degradation (10% of total plasma volume), and stored at –80°C until assayed. Entire stomach, including its corpus and fundus, were dissected, whereby the latter was cut in halves between lesser and greater curvatures, frozen in liquid nitrogen and stored at –80°C before assay. The stomach tissue was cut with a cryostat (Lecia Microsystems, Nanterre, France), and 14-μm-thick transversal sections were collected on Superfrost glass slides (Thermo Scientific, Braunschweig, Germany) for in situ hybridization.

In situ hybridization

All solutions were made using diethylpyrocarbonate– (DEPC, Sigma, St. Louis, MO, USA) treated water. Tissue sections were fixed with 4% paraformaldehyde in 10 × phosphate-buffered saline (PBS), pH 7.5. After washing with PBS for 5 min, the sections were incubated with 0.5 M HCl in DEPC water for 5 min and then washed in PBS twice for 3 min. The sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 20 min. The sections were washed in PBS twice for 3 min, and immersed in a graded ethanol series (70%, 80%, and 99.5%) for 2 min each. Finally, sections were dried for 30 min and then stored at ~20°C. RNA probes specific to preproghrelin mRNA (Accession number: NM_021669.2, National Center for Biotechnology Information, Bethesda, MD) were prepared from rat stomach mRNA. The rat stomach extracted mRNA was reverse-transcribed to generate cDNA using the iScript select cDNA synthesis kit (BioRad, Hercules, CA, USA). The cDNA was amplified using the following specific primers: ghrelin-F: 5′-ACGACGACAGCCACAGCAGA3′- and ghrelin-R: 5′-TTGCAAGACGAGGAACAAGCTGGAT-3′ (product size of 335 bp from position 121 to 455) (Invitrogen, Carlsbad, CA, USA). The polymerase chain reaction (PCR) fragment was gel purified using QIAquick Gel extraction kit (Qiagen, Venlo, The Netherlands) and subcloned into PCR1 II-TOPO vector (Invitrogen). The sequence of cDNA probe was confirmed by nucleotide sequencing (KIGene, Stockholm, Sweden). The plasmids were linearized using restriction enzyme BamHI and Xba I (Promega, Madison, WI, USA) and transcribed to generate sense and antisense RNA probes. In vitro transcription and labeling were carried out using SP6/T7 RNA polymerases (Ambion, Austin, TX, USA) and digoxigenin (DIG) RNA labeling mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. The transcripts were purified using NucAway Spin Columns (Ambion). Sense probes were used as negative controls. Sections were prehybridized in a humidified chamber using 50% (vol/vol) deionized formamide, 0.1 M Tris-HCl (pH 7.6), 50 mM EDTA (pH 8.0), 40 mM NaCl, 1.25 mg/ml yeast tRNA (Roche, Basel, Switzerland), and 5 X Denhardt’s solution for 4 to 6 h at 55°C followed by hybridization overnight (14–16 h) at 55°C. The labeled probes were diluted to a final concentration 0.8 ng/μl in solution containing 50% (vol/vol) deionized formamide, 0.3 M NaCl, 0.1 M DTT, 10% (vol/vol) dextran sulfate, and 1 Grundmix solution. Grundmix solution is made of 0.2 M Tris-HCl (pH 7.6), 5 mg/ml yeast tRNA (Roche), 1 mg/ml poly-A-RNA (Roche), 10 × Denhardt’s solution and 10 mM EDTA (pH 8.0). After hybridization, sections were washed with constant stirring as follows: twice for 30 min in 1 × SSC with 0.1% SDS at 55°C, 1 h in 50% (vol/vol) formamide/0.5 × SSC at 55°C, 5 min in 1 × SSC with 0.1% SDS at 55°C, 30 min in 30 ng/μl RNase A diluted in RNase A buffer at 37°C, and twice for 10 min in 1 × SSC with 0.1% SDS at 55°C. RNase A buffer contains 0.5 M NaCl, 10 mM Tris, 0.1% SDS, and 0.5 mM EDTA. The sections were then incubated three times for 5 min in buffer 1 (100 mM Maleic acid, pH 7.5, 150 mM NaCl, 0.02% Tween 20), immersed in 1% blocking reagent (Roche) diluted in buffer 1 for 20 min, and incubated with the alkaline phosphatase-conjugated anti-DIG antibody (Roche) diluted at 1:2000 in buffer 1 with 1% blocking reagent at 4°C overnight. The sections were then washed in buffer 1 for 5 min three times, in buffer 2 (100 mM Tris, pH 9.5, 100 mM NaCl, 0.05% tween 20) for 5 min and in buffer 2 with 5 mM of tetramisole hydrochloride (Sigma) for 5 min. A chromagen solution made of 337 g/ml nitro-blue tetrazolium chloride (NBT) and 175 g/ml 5-bromo-4-chloro-3-indolylphosphate P-toluidine salt (BCIP) (Roche) in buffer 2 with 5 mM of tetramisole hydrochloride (Sigma) was made, and the sections were incubated for 1 h. The reaction was stopped with PBS. The sections were then washed with distilled water, mounted in a solution of PBS and glycerol (85%) and viewed under a light microscope (Axioskop, Zeiss, Germany). Pictures from the in situ hybridization were taken with an objective × 20. Total number of preproghrelin mRNA-positive cells was counted using Image J software (National Institutes of Health, Bethesda, MD, USA) from six representative sections of each mouse. The square area including the gastric mucosa and submucosa were delimited in Image J in each picture and the number of positive cells in the measured surface area was calculated.

Ghrelin assay

Plasma levels of ghrelin and DAG were measured using enzyme immunoassay kits from Mitsubishi Chemical Med Corp (Tokyo, Japan), according to manufacturer instructions.
ChloraminT (Thermo Scientific) in PBS with 0.05% Tween 20 (pH 7.4) and then incubated overnight at 4°C with 100 μL of mouse plasma diluted 1:200 in sample buffer (PBS, 0.02% sodium azide, pH 7.4). The plates were washed (three times) and incubated with 100 μL alkaline phosphatase-conjugated anti-mouse IgG (1:1,000, Jackson ImmunoResearch, West Grove, PA, USA) for 3 h at room temperature. Following washing, 100 μL p-nitrophenyl phosphate solution (Sigma) was added as alkaline phosphatase substrate. After 40 min of incubation at room temperature, the reaction was stopped by adding 3 N NaOH. The optical density (OD) was determined at 405 nm by subtracting the blank values resulting from the injection of HBS-EP buffer.

Statistical analysis

Data were analyzed and graphs were plotted using the GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA, USA) software, and P < 0.05 was considered statistically significant. Differences in body weight gain were analyzed by two-way repeated measurements analysis of variance (ANOVA) with the Bonferroni post-tests. Group differences were compared using the Student’s t test or the Mann–Whitney test depending on the Kolmogorov–Smirnov normality test. Pearson’s correlations were also analyzed. Data are shown as mean ± SEM.

Results

HFD effects on body weight and body composition

HFD-fed mice showed increased body weight gain starting from week 5 of the diet compared with mice fed with the regular chow (Fig. 1A). Body composition analysis showed that after 12 wk of the diet, HFD mice had a similar lean body mass as controls but that their fat body mass was significantly increased (Fig. 1B). The amount of fat mass in HFD mice corresponded to 30.1% of body weight, compared with 8% in lean mice, confirming development of obesity in HFD-fed mice and showing that their increase of body weight was exclusively due to an increase in body fat content. At the day of taking plasma and stomach tissues, ob/ob mice weighed 51.4 ± 1.8 g. Analysis of their body composition showed a slightly lower amount of lean tissue mass than in controls, but fat tissue mass was higher than in either control or HFD-fed mice (Fig. 1B). The amount of fat mass in ob/ob mice corresponded to 54.4% of their body weight.

Preproghrelin mRNA expression by in situ hybridization

In situ hybridization in the stomach of all obese and lean mice revealed typical distribution of preproghrelin mRNA-containing cells in the mucosal and submucosal layers of the gastric wall (Fig. 2A–C). In situ hybridization using the sense probe, as a negative control, showed no detectable signal, confirming the signal specificity. The mean number of gastric preproghrelin mRNA-positive cells was 15% higher in HFD-fed compared with chow-fed control mice (Fig. 2D). In ob/ob mice the number of preproghrelin mRNA expressing cells was lower than in control and HFD mice with a decrease of 41% versus controls (Fig. 2D).

Plasma ghrelin

Plasma concentrations of ghrelin were similar in control, HFD and ob/ob mice (Fig. 3A). Plasma concentrations of DAG were lower in both HFD-fed and ob/ob mice than in chow-fed control lean mice, without significant differences between the two obese models (Fig. 3B). The ratios between ghrelin and DAG did not significantly differ in HFD mice, but were elevated in the ob/ob group (Fig. 3C). Plasma levels of ghrelin and DAG did not correlate with the number of gastric preproghrelin mRNA-expressing cells (Pearson’s r = 0.05, P = 0.8 and r = 0.04, P = 0.9, for ghrelin and DAG, respectively).

ChloraminT-reactive IgG

Plasma levels of ghrelin-reactive IgG did not significantly differ between HFD and control mice (Student’s t test, P = 0.34; Fig. 4A). Analysis of IgG affinity kinetics for ghrelin showed that...
the association and dissociation rates (Fig. 4B, C) in HFD mice were not significantly affected versus controls (Mann–Whitney test, P = 0.7 and Student’s t test, P = 0.32, respectively). However, mean value of the dissociation equilibrium constant was found at a lower level in HFD mice (Fig. 4D), reflecting a 1.5-fold increased affinity of ghrelin-reactive IgG in HFD mice as compared to chow-fed lean controls.

**Discussion**

Our study revealed that a long-term HFD leading to obesity induces changes in the ghrelin system favorable for obesity development. As such, in HFD-fed mice, we found increased number of ghrelin precursor-expressing cells in the stomach and increased protective properties of plasmatic IgG for ghrelin. Because the ghrelin signaling is required for development of a full obese and diabetic phenotype [35–38], our data support the role of ghrelin in the mechanism of HFD-induced obesity. The validity of our HFD-induced obesity model of the human obesity was confirmed by a significantly increased fat content, reaching 30% of mouse body weight. Furthermore, HFD-fed mice had normal levels of active ghrelin and decreased plasma levels of DAG, a characteristic feature of the ghrelin system in obese individuals.

A decrease of DAG in obesity is often interpreted as a decrease of ghrelin production in general, in particular when measurements of total ghrelin levels are preformed, which do not discriminate between the AG and DAG forms of the hormone. Such interpretations may lead to a conclusion of a decreased ghrelin’s obesogenic effects in obesity as a possible result of a homeostatic adaptation to the positive energy balance [21, 22]. However, a separate analysis of plasma ghrelin/DAG levels [19,
does not support a reduction of ghrelin production in obesity, but instead, its increased protection from degradation. Elevated ghrelin-to-DAG ratios also were associated with less efficient bariatric surgery [39]. An increased number of preproghrelin mRNA-expressing cells found in HFD-fed mice of this study further supports an increased production of ghrelin in an obesogenic nutritional environment [40,41].

It should be noted that by showing different number of such cells, we do not imply an effect of HFD on cell proliferation, but on the number of detectable cells expressing preproghrelin mRNA and revealed by the in situ hybridization technique. Accordingly, a 15% increase of ghrelin precursor-expressing cells in HFD-fed mice may signify that in control mice such extra cells may express ghrelin mRNA below the detection level. These data reflect the plasticity of the ghrelin synthesis following nutritional modulations. The number of cells in the stomach expressing ghrelin precursor are also greatly affected by the negative energy modulation in HFD-fed mice might be a feedback regulation secondary to leptin resistance in the brain in the absence of ghrelin signaling.

In addition to the ghrelin peptide, the preproghrelin precursor gives rise also to obestatin, a 23-amino acid amidated peptide [48]. Although obestatin production was not analyzed in this study, one can expect its increased release following increased number of preproghrelin mRNA-expressing cells. Obestatin was shown to stimulate adipogenesis [49] and to inhibit lipolysis [43] suggesting that it may potentially contribute to the mechanisms of HFD-induced obesity. A role of HFD in preferentially inhibiting lipolysis was recently reported [50].

The exact reason of a decreased number of preproghrelin mRNA-expressing cells in ob/ob mice is not known. One possibility can be related to the missing developmental role of leptin in the gastrointestinal tract [51], which can be verified by the comparative analysis of the ghrelin cell number in ontogenesis. Another possibility is that absence of ghrelin signaling may turn down ghrelin precursor expression in only leptin sensitive ghrelin-producing cells; leptin was shown to increase ghrelin mRNA [52]. Moreover, although ob/ob mice were characterized by a lower number of ghrelin precursor-expressing cells, they often appeared as more intensely stained, suggesting that they may have increased content in preproghrelin mRNA compensating for a decreased cell number. Such differences in ghrelin-producing cell number and ghrelin precursor transcript expression may contribute to discrepancies in reported gastric tissue levels of preproghrelin mRNA in different models of obese mice [44,53].

Almost identical plasma levels of active ghrelin found in controls and in both models of obese mice were associated with different number of preproghrelin mRNA-expressing cells, suggesting that plasma ghrelin levels are not primarily dependent on this number. Indeed, no significant correlations between either ghrelin or DAG and the number of ghrelin precursor-expressing cells were found. It suggests that other factors modulating stability of plasma ghrelin and DAG can be more critically involved in maintaining plasma levels of these peptides.

We have previously shown that circulating IgG protect ghrelin from degradation [19]. Furthermore, plasmatic IgG were shown to bind independently ghrelin and DAG [54], suggesting that such IgG may selectively protect active ghrelin from desacylation and proteolysis. Increased affinity of ghrelin-reactive IgG in obese individuals also was associated with their enhanced protective role and increased orexigenic effects [19].

Detection of increased affinity of ghrelin-reactive IgG in HFD-fed mice, found in this study (i.e., increased ability to bind ghrelin) a priori signifies a gain of function for ghrelin. In fact, IgG with the micromolar range of affinity will not be able to antagonize ghrelin binding on its receptor, but will compete with the plasma enzymes having low binding specificity. A better preservation of ghrelin in HFD-fed mice may thus be achieved by their higher binding affinity for ghrelin. The reason for divergent effects of HFD on food intake and fat accumulation in mice is not clear; it may involve an inflammatory response by the brain to HFD [59] or hitherto nonidentified mechanisms. Although absence of hyperphagia in HFD-induced obesity in mice may be a disadvantage as a model of human hyperphagic obesity, using genetic hyperphagic mouse models of obesity (e.g., leptin deficiency) presents other limitations. Alterations that may exist in both models may possibly reflect most relevant to human obesity mechanisms. For instance, increased affinity of IgG for ghrelin was found in both ob/ob mice [19] and in HFD-fed mice in the present study, and was also detected in

![Fig. 4. Plasma levels (A) in OD and affinity kinetics (B–D) of ghrelin-reactive IgG in chow-fed controls (Ctr) and HFD-fed mice. Association (Small Kd, C) and dissociation (Small Kc, B) rates and the dissociation equilibrium constant (KD, C). Student’s t test *P < 0.05. Ctr, control; HFD, high-fat diet; Ig, immunoglobulin; OD, optical density.](image-url)
hyperphagic human obesity [19]. A mechanism underlying such changes of IgG properties may potentially involve increased stimulation by ghrelin-like microbial antigens present in obese- surgical environment. Such antigens can be present in gut microbiota [60–62] and in environmental microorganisms including viruses, due to their protein sequence homology with ghrelin [63]. For instance, such mimicry is present in a protein of influenza A virus, whereas plasma levels of antiviral antibodies in healthy individuals correlate with ghrelin-reactive IgG [64]. HFD has been shown to alter gut microbiota [65,66], suggesting that HFD-induced modifications of gut bacterial antigens may stimulate antibodies cross-reactive with host hormones. For instance, chronic supplementation of commensal *Escherichia coli* to rats and mice changed affinities of their IgG for melanocortin peptides [67,68]. Future studies should determine the origin of antigens that may cause affinity maturation of ghrelin-reactive IgG.

**Conclusion**

We found that HFD in mice induced obesogenic changes of the ghrelin system, including increased number of preproghrelin mRNA-expressing cells in the stomach and increased affinity of plasmatic IgG for ghrelin, which can underlie its increased protection from degradation. Such changes in the ghrelin system may contribute to the mechanisms underlying HFD-induced obesity.

**References**


