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Basic nutritional investigation

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

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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 pre-proghrelin mRNA-expressing 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-acyl 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.

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

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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 (N04-11-12/27/11-15) approved the experiment. Six-wk-old C57 Bl/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 (n = 10) and HFD (n = 22), and were fed a standard pelleted rodent chow (RM1) or D12492 HFD (Research Diets, New Brunswick, NJ, USA), respectively, for 13 wk. The HFD supplied 60% of calories as fat mainly by lard, 20% as carbohydrates, and 20% as protein. Body weight was measured weekly. Eleven 2-mo-old C57 Bl/6 male obese *ob/ob* were purchased from Janvier Labs (Genest-St.-Isle, France) and were housed for 3 wk at the same animal facility as previously described. Standard pelleted rodent chow and drinking water were available ad libitum. Body composition was measured by EchoMRI (EchoMRI, Houston, TX, USA) at week 12 of the HFD and in control and *ob/ob* mice [32].

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 (Leica 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. Stomach 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′-AGCACCAGAAAGCCCAGCAGAGAA-3′ and ghrelin-R: 5′-TTGCAGAGGAGGCAGAAGCTGGAT-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 36 μg/mL 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 (pH 8.0) 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 1:200 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'-indolyphosphate *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 immunosorbent assay kits from Mitsubishi Chemical Med Corp (Tokyo, Japan), according to manufacturer instructions.