Ghrelin and Body Weight Regulation in the Obese Zucker Rat in Relation to Feeding State and Dark/Light Cycle

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Ghrelin is a new orexigenic peptide primarily produced by the stomach but also present in the hypothalamus. It has adipogenic effects when it is chronically injected in rodents but in obese humans, its plasma concentration is decreased. It can reverse the anorectic effects of leptin when it is co-injected with this peptide in the brain ventricles. The Zucker fa/fa rat is a genetic model of obesity related to a default in the leptin receptor. It is characterized by a large dysregulation of numerous hypothalamic peptides but the ghrelin status of this rat has not yet been determined. Through several experiments, we determine in lean and obese Zucker rats its circulating form in the plasma, its tissue levels and/or expression, and studied the influence of different feeding conditions and its light/dark variations. Ghrelin expression was higher in the obese stomach and hypothalamus ($P < 0.05$ and $P < 0.02$, respectively). The ratio of [Octanoyl-Ser^3]-ghrelin (active form) to [Des-Octanoyl-Ser^3]-ghrelin (inactive form) was approximately 1:1 in the stomach and 2:1 in the plasma in lean and obese rats (no differences). After fasting, plasma ghrelin concentrations increased significantly in lean (+ 64%; $P < 0.001$) and obese (+ 60%; $P < 0.02$) rats. After 24 hours of refeeding, they returned to their initial *ad lib* levels. Ghrelin concentrations were higher in obese rats by 69% ($P < 0.005$), 65% ($P < 0.02$), and 73% ($P < 0.005$) in the *ad libitum*, fast, and refed states respectively. These results indicate that the obese Zucker rat is characterized by increases in the stomach mRNA expression and in peptide release in the circulation. They clearly support a role for ghrelin in the development of obesity in the absence of leptin signaling. Exp Biol Med 228:1124–1131, 2003

Key words: hyperphagia; stomach; hypothalamus; leptin; mRNA expression

Ghrelin is a new gut–brain peptide that is a natural ligand of the growth-hormone secretagogue receptor (GHS-R) (1). It consists of 28 amino acids and has a unique structure with an n-octanoyl ester at its third serine residue (1). It has been isolated from both the rat and human stomach where it is particularly abundant (1–3) but also from other organs including the hypothalamus where numerous GHS-Rs are present (4). It is released in the circulation where it exists under two molecular forms (5). The n-octanoylation of the serine residue is necessary for its biological activity (1, 5–7).

Through its binding to the GHS-R, ghrelin stimulates the growth hormone secretion (8, 9). However, like many other peptides, it is involved in other behaviors. One of its main side effects is the stimulation of food ingestion when it is injected in rodents and humans (10–12). This orexigenic effect is independent of growth hormone because it is also observed in dwarf rats that are deficient in growth hormone (10). The stimulatory effects of ghrelin are strongly assessed by its variations in different feeding conditions. First, ghrelin is released just before the beginning of a meal (13) concomitantly with a decrease of blood glucose (14). These signals could trigger feeding behavior. After meal ingestion, it progressively decreases (15). Second, its circulating levels are dependent on feeding status (16, 17). It is up-regulated by fasting and decreases after refeeding. After chronic administration, its orexigenic effects led to excess weight gain and adiposity (18, 19). All these effects are likely due to an interaction with the hypothalamic neuromodulatory pathways. Recent studies have indeed shown that one of its brain targets is the hypothalamus (20) and more precisely the arcuate nucleus where it induces Fos and Egr 1 expression (10, 21). Ghrelin acts on arcuate neurons by increasing the expression of some important orexigenic neuropeptides such as neuropeptide Y (NPY) and Agouti-related protein (AgRP) (22, 23). Its orexigenic effect is abolished by co-injection of NPY Y1 and Y5 receptor antagonists (10, 24). Ghrelin also strongly interacts with the leptin regulatory pathway at the level of the arcuate nucleus where leptin has receptors located on the NPY neurons (25).

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Its changes with feeding status are opposite to those of leptin (26). Its mRNA expression in the stomach is increased after administration of leptin (16). When it is co-injected with leptin, it abolishes the leptin- induced inhibition of food intake in a dose of dependent manner (10, 24).

The absence of leptin signaling is the cause of the obesity of several animal models. The obese Zucker rat is one of these models. Its obesity and hyperphagia are related to a default at the leptin receptor level (27). This deficiency is at the origin of a large modification of the neuropeptidergic status in the brain areas involved in the regulation of feeding and body weight (28). Old classical orexigenic peptides such as NPY and galanin are up-regulated (29, 30). This dysregulation has been extended to other orexigenic peptides such as orexins and melanin-concentrating hormone (31, 32). In addition, it is well demonstrated that chronic treatment with ghrelin induces adiposity in rodents whatever the route of injection (IP or ICV) used (18, 19). We have also shown that ghrelin is down-regulated in rats with increased adiposity due to the ingestion of high-fat diets (33). These data might indicate that ghrelin is not involved in the development of obesity. However, a recent paper has shown that obesity in some humans has been associated with mutations of the preproghrelin gene inside and outside the coding region for the mature peptide (34). Contrary to what was expected, some recent data have shown that circulating ghrelin levels are diminished in obese humans (35). Given the interaction of ghrelin with the hypothalamic neuropeptide systems and to clarify the role of ghrelin in obesity, we realized several experiences in lean and obese Zucker rats to explore its entire system. We determine its tissue and plasma levels and/or expression as well as its circulating form in the plasma. We studied the influence of different feeding conditions and its light/dark variations.

Material and Methods

Quantification of Ghrelin in Lean and Obese Rats. *Characterization of immunoreactive-ghrelin.* ***Animals.*** Adult male obese (fa/fa, mean body weight 322.2 ± 9.5 g; $n = 4$) and lean (FA/FA, mean body weight 217.4 ± 3.6 g; $n = 4$) Zucker rats (Genetics Models Inc., IN) were anaesthetized at the middle of the light cycle by carbon dioxide inhalation and sacrificed by decapitation. Trunk blood was collected in tubes containing EDTA (1.2 mg/ml) and centrifuged at 1500 g for 20 min at 4°C, distributed in aliquots and stored frozen at −25°C until assays. Stomachs were resected and stored at −80°C until used.

Tissue and plasma extractions. Tissue. Pooled stomach were homogenized in acidic solution [0.2 N HCl, EDTA (0.5 *M*) in water] (10 ml/g wet tissue) and sonicated at high setting for 45 sec. The supernatant was collected after centrifugation at 15,000 rpm for 30 min at 4°C. The samples were loaded onto a Sep-Pack C18 cartridge pre-equilibrated with 5% acetonitrile/0.1% TFA in water. The cartridge was washed with 5% acetonitrile/0.1% TFA in water and then eluted with 50% acetonitrile/0.1% TFA. The eluates were lyophilized and reconstituted in 1 *M* acetic acid/water before RP-HPLC.

Plasma. Plasma samples were pooled and acidified with an equal volume of 0.1% TFA in water. After centrifugation at 10,000 rpm for 30 min at 4°C the supernatant was collected. The samples were loaded onto a Sep-Pack C18 cartridge pre-equilibrated with 60% acetonitrile/0.1% TFA in water. The cartridge was washed with 0.1% TFA in water and then eluted with 60% acetonitrile/0.1% TFA. The eluates were lyophilized and reconstituted in 1 *M* acetic acid/water before RP-HPLC.

Reverse Phase High Performance Liquid Chromatography. Reverse phase high performance liquid chromatography (RP-HPLC) of the different extracts was performed on a C18 column (Pharmacia) with a linear gradient of acetonitrile from 10% to 60% in 0.1% TFA/water for 40 min at a 1 ml/min flow rate (1 ml fraction). Each fraction was lyophilized and reconstituted in assay buffer. Ghrelin was measured by competitive enzyme immunoassay (EIA) in the eluted fractions to determine the different immuno-reactive forms of the peptide present in the extracts. Position of the immunoreactive peaks were compared with the elution profile of rat ghrelin analogues and fragments ([Des-Octanoyl-Ser^3]-Ghrelin, [Octanoyl-Ser^3]-Ghrelin, [Des-Gln^{14}]-Ghrelin, Ghrelin 1-5, Ghrelin 3-28, Ghrelin 1-14, and Ghrelin 17-28).

Immunoreactivities. Cross-reactivity of the antibody (RAB-031-31, Phoenix Pharmaceuticals, CA) used in the immunoassays was investigated using standard curves of each of the following rat peptides: [Des-Octanoyl-Ser^3]-Ghrelin, [Octanoyl-Ser^3]-Ghrelin, Ghrelin 1-5, Ghrelin 3-28, Ghrelin 1-14, Ghrelin 17-28, and Ghrelin 23-28.

Quantification of ghrelin in the stomach and the plasma. Adult male lean homozygous FA/FA (mean body weight 395.7 ± 7.3 g; $n = 10$) and obese fa/fa (mean body weight 592.5 ± 13.3 g; $n = 14$) Zucker rats bred in our laboratory were used. Their characteristics are given in Table I. They were housed in individual wire cages in a temperature-regulated room with an automatic 12 hr/12 hr light-dark (L/D) cycle with lights on at 9 AM. The rats were killed by decapitation 4 hrs after the beginning of the light period. Food was withdrawn during this 4-hr period to have all animals in the same nutritional state. Trunk blood was sampled on aprotinin/EDTA. The brain was quickly removed and placed in a brain matrix and the hypothalamus was sampled and stored at −80°C until used for the measurement of ghrelin expression. The stomach was resected and dissected into three segments (gastric fundus, gastric body, and antrum) and stored at −80°C until used. Stomach sub-segments were homogenized in acidic solution [0.2 N HCl, EDTA (0.5 *M*) in water] (10 ml/g wet tissue) and sonicated at high setting for 45 sec. The supernatant was collected after centrifugation at 15,000 rpm for 30 min at 4°C and lyophilized. Lyophilizates were reconstituted in assay buffer and assayed for ghrelin and leptin through enzymatic immunoassays.

Table I. Characteristics of the Lean and Obese Zucker Rats Used in the Different Experiments

	Food intake (g/d)	BG (m*M*)	IRI (ng/ml)	Adiposity (g)
Quantification experiment				
lean	18.3 ± 1.2	7.8 ± 1.2	1.14 ± 0.53	6.2 ± 1.5
obese	22.8 ± 2.7[a]	7.9 ± 1.1	9.72 ± 2.38[a]	27.1 ± 3.8[a]
Fast/refed experiment				
lean	19.4 ± 0.9	6.9 ± 1.1	2.27 ± 0.64	5.7 ± 1.2
obese	25.8 ± 2.8[a]	8.1 ± 0.7[b]	6.03 ± 2.58[a]	36.6 ± 6.3[a]

Note. Adiposity was estimated by the sum of the weight of two well-differentiated fat pads (epididymal and perirenal). [a] $P < 0.001$ between lean and obese rats; [b] $P < 0.02$.

Fasting/refeeding experiment. Lean homozygous FA/FA (mean body weight 378.0 ± 9.7 g; $n = 10$) and obese fa/fa (mean body weight 669.5 ± 18.2 g; $n = 10$) Zucker rats bred in our laboratory were used for this experiment. They were 12 months old. Their characteristics are given in Table I. They were housed in individual wire cages with a 12 hr dark /12 hr light cycle (lights on at 8:30 AM). They were fed on standard laboratory rat chow (UAR A04, Villemoisson-sur-Orge, France) *ad libitum* and had tap water to drink. They were maintained in these stable conditions during several weeks before the start of the experiment. They were weighed weekly and their food intake was measured twice a week as well as the day before the experiment.

On the test day, the *ad libitum*-fed rats were lightly anesthetized with ether and blood was withdrawn from the tail vein in tubes containing aprotinin and EDTA. Food was then withdrawn but water remained available. Twenty-four hours later, a second blood sample was taken under ether anesthesia in the tail vein and a preweighed food quantity was given to the rats. A third blood sample was taken in the same conditions 24 hrs after refeeding. Food consumption corrected for spillage was measured during this period. The different blood samples were centrifuged for 30 min at 1500 g at 4°C. Plasma was aliquoted and stored at −20°C until assayed.

Diurnal rhythm of ghrelin. The same rats as for the fasting/refeeding test were used. Three blood samples were taken from the tail vein under light ether anesthesia: the first one at the lights off, the second one at the lights on, and the third in the middle of the light period.

*Quantification of Ghrelin Expression in Lean and Obese Rats. **RNA extraction.*** Hypothalamus (50 mg) or gastric fundus samples from the same rats as those used for the quantification of ghrelin in the stomach and the plasma (see above). The homogenized tissue was applied on QIA shredder (Qiagen) column and spun on micro-centrifuge at maximum speed for 2 min. After precipitation with equal volume of 70% EtOH the RNA was extracted with the RN-easy Mini Kit (Qiagen) according to the manufacturer instructions. The total cellular RNA was finally eluted in 40 µl DEPC treated water; 10 µl was used to determine the RNA concentration and the remainder was stored frozen for further use.

DNase I digestion and cDNA synthesis. One µl of 10× DNase I reaction buffer and 1 µl DNase I (GibcoBRL, Rockville, MD) at concentration 1 U/µl was added to 8 µl RNA. The reaction mixture was incubated at room temperature for 15 min and the enzyme action was terminated by addition of 1 µl of 25 mM EDTA, pH 8.0 and heated at 65°C for 10 min; 1 µl Oligo (dT) 12-18 (0.5 µg/µl) from SuperScript First-Strand Synthesis System for RT-PCR (GibcoBRL) was added to the reaction and was incubated on 70°C for 15 min, followed by 2 min on ice; 7 µl mixture (2 µl buffer containing 200 µM Tris HCl pH 7.4 and 500 mM KCl, 2 µl 25 mM MgCl2, 1 µl 10 mM dNTP mix, and 2 µl 0.1M DTT) was then added with a gentle pipetting several times and incubated at 45°C for 5 min. SuperScript Reverse Transcriptase (50 U) (GibcoBRL) was added and the reaction was carried on at 42°C for 50 min and terminated at 70°C for 15 min followed by chilling in ice. Samples were than diluted with nuclease-free water to a concentration of 2.5 µg/ml and either used directly in TaqMan reaction or kept frozen at −20C.

Real time polymerase chain-reaction. In a typical reaction, 10 µl cDNA (25 ng) was mixed with 25 µl TaqMan Universal PCR Master Mix (PE Biosystems, Roche, Branchburg, NJ), 600 nM from each primer, 200 nM probe complementary to the gene of interest, labeled with FAM dye, 200 nM from each primer, and 100 nM probe complementary to 18-S (rat housekeeping control gene) in 50 µl total volume. The reaction was finally transferred into a 96-Well Optical Reaction Plate (PE Biosystems), sealed with appropriate optical caps and ran on the TaqMan (PE Biosystems) apparatus under standard conditions recommended by the manufacturer. All expression data were normalized to GAPDH expression level from the same individual samples. The result were transferred and analyzed in Excel.

The following probes and primers were used in the real-time polymerase chain reaction (PCR) Prepro-ghrelin probe: AAAGCCCAGCAGAGAAAGGAATCCAAGA; primers: forward: GCTTCTTGAGCCCAGAGCAC and reverse: GTGGCTGCAGTTTAGCTGGTG.

*Bioassays. **Plasma assays.*** Ghrelin was measured in diluted plasma with a specific radioimmunoassay using commercially available kits (Phoenix Pharmaceuticals, Belmont, CA). According to the manufacturer, no cross- reaction is noted with galanin, NPY, orexins, MCH, and secre-

tin. Blood glucose and triacyl-glycerols were measured through enzymatic methods using kits from Biomérieux (Marcy l'Etoile, France).

Immunoreactive insulin (IRI) was measured with kits using insulin antibody coated tubes (CIS International, Gif-sur-Yvette, France). Rat insulin (Novo, Copenhagen) was used as standard.

Immunoreactive leptin was measured using a rat sandwich ELISA (Crystal Chem Inc, IL). Results are expressed as ng/ml. Detection limit was 100 pg/ml and coefficient of variation was less than 5%.

Tissue Assays. Ghrelin concentrations in stomach extracts were measured with a competitive enzyme immunoassay (EIA) using a secondary antibody pre-coated plate (Phoenix Pharmaceuticals, Belmont, CA); 50 µl of standard (ghrelin: 0.01, 0.1, 1, 10, 100 ng/ml) or samples, 25 µl of primary antiserum, and 24 µl of biotinylated ghrelin in each well were incubated at room temperature for 2 hr. After washing the plate 5 times with 300 µl/well of assay buffer, 100 µl of streptavidin-horseradish peroxidase solution was added, and incubated at room temperature for 1 hr. After six more washes, the substrate solution (3, 3', 5, 5'-tetramethylbenzidine and hydrogen peroxide) was added (100 µl/well) and incubated for 1 more hr. The reaction was terminated with 100 µl/well 2N HCl and absorbance was read at 450 nm wave-length in a microplate reader. Results are expressed as ng/ml. A good linearity was observed between 0.1 and 20 ng/ml. A 50% displacement was observed at 5 ng/ml. Detection limit was 0.1 ng/ml and intraassay coefficient of variation was less than 7%.

Leptin immunoreactivity was measured in stomach extracts by the same assay as for plasma.

Peptides. [Des-Octanoyl-Ser^3]-Ghrelin, [Octanoyl-Ser^3]-Ghrelin, [Des-Gln^{14}]-Ghrelin, Ghrelin 1-5, Ghrelin 3-28, Ghrelin 1-14, and Ghrelin 17-28 were synthesized by Phoenix Pharmaceuticals (Belmont, CA).

Statistical Analyses. All results are given as mean ± SEM. Comparisons between groups were made using multiple way analysis of variance and PLSD Fisher's t tests. Statistical significance was taken as $P < 0.05$.

Results

Quantification of Ghrelin in Lean and Obese Rats. *Characterization of Immunoreactive-Ghrelin.* Half-maximal binding of labeled rat ghrelin to the anti-rat [Octanoyl-Ser^3]-ghrelin antibody (RAB-031-31) was inhibited by rat [Octanoyl-Ser^3]-ghrelin at 5 ng/ml. This antibody exhibited 100% cross-reactivity with rat [Des-Octanoyl-Ser^3]-ghrelin. No cross-reactivities with N-terminal fragments of ghrelin, ghrelin 1-5, and ghrelin 1-14 were observed. Cross-reactivities with C-terminal fragments of ghrelin, ghrelin 3-28, ghrelin 17-28, and ghrelin 23-28 were 100%, 37%, and 28%, respectively.

Figure 1 shows the HPLC elution profile of the different molecular forms of ghrelin in stomach extracts and plasma. Two major peaks (1 and 2) of IR-ghrelin were observed in stomach and plasma extracts. Peak 2 eluted at the same retention time as that of synthetic rat [Octanoyl-Ser^3]-ghrelin, the active form of ghrelin. In contrast, peak 1 eluted at the same retention time as synthetic [Des-Octanoyl-Ser^3]-ghrelin, the inactive non-modified form of ghrelin. The ratio of [Octanoyl-Ser^3]-ghrelin to [Des-Octanoyl-Ser^3]-ghrelin was approximately 1:1 in the stomach and 2:1 in the plasma. In obese rats, the two major peaks corresponding to [Octanoyl-Ser^3]-ghrelin and [Des-Octanoyl-Ser^3]-ghrelin were also observed in extracts and their ratio did not differ significantly from the lean animals.

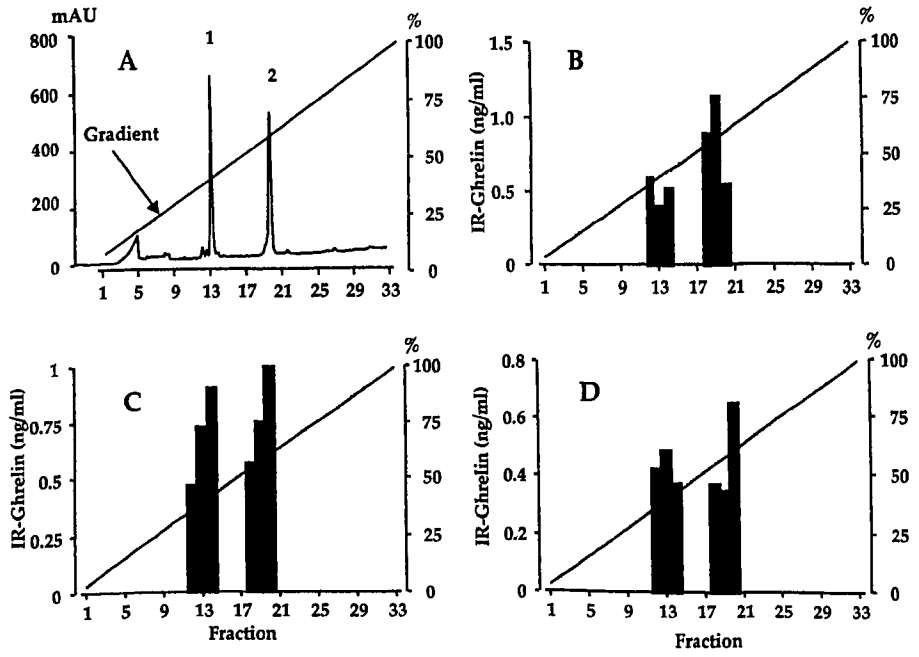

Figure 1. Elution profile on RP-HPLC of control peptides and of stomach and plasma extracts. (A) Separation of [Des-Octanoyl-Ser^3]-ghrelin (peak 1) from [Octanoyl-Ser^3]-ghrelin (peak 2) (B) plasma extract. (C and D) stomach extracts (lean: C; obese: D).

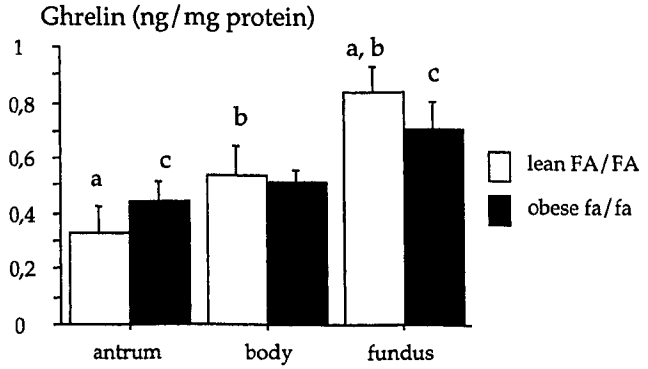

Figure 2. Ghrelin concentrations (mean ± SEM) in the different parts of the stomach of lean and obese Zucker rats. Groups sharing the same letter are significantly different. a: $P < 0.001$; b: $P < 0.035$; c: $P < 0.02$.

Quantification of Ghrelin and Leptin in the Stomach. Ghrelin concentrations in the different parts of the stomach of lean and obese rats are shown in Figure 2. There was a significant difference between the different parts of the stomach ($P < 0.0001$) but not between the two genotypes. The highest concentrations were observed in the fundus. There was no difference between antrum and body. In the fundus, it was 1.5-fold and 60% higher than in antrum in the lean rats ($P < 0.001$) and obese rats ($P < 0.02$) respectively.

Leptin concentrations in the different parts of the stomach and in the plasma are shown in Figure 3. There was a significant effect of genotype ($P < 0.025$) and of parts of the stomach ($P < 0.0001$).

As for ghrelin, the highest concentrations were observed in the fundus. In lean rats, they were 3- to 7-fold higher than in the antrum ($P < 0.0001$) and body ($P < 0.0001$), respectively.

In the obese rats, they were multiplied by a factor of five when compared with the other parts of the stomach ($P < 0.0001$). Leptin concentrations in the body were higher in the obese rats than in the lean rats ($P < 0.001$). In the fundus, the difference between lean and obese rats did not reach statistical significance ($P = 0.08$). Plasma leptin levels were markedly elevated in obese rats (×9 ; $P < 0.0001$).

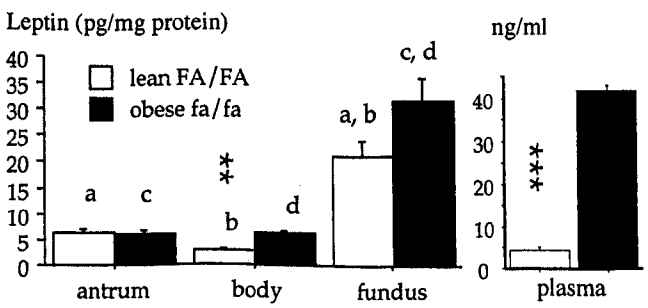

Figure 3. Leptin concentrations (mean ± SEM) in the different parts of the stomach and in the plasma of lean and obese Zucker rats. Groups sharing the same letter are significantly different with a probability of less than 0.0001. Asterisks indicate a significant difference between lean and obese rat. **: $P < 0.001$; ***: $P < 0.0001$.

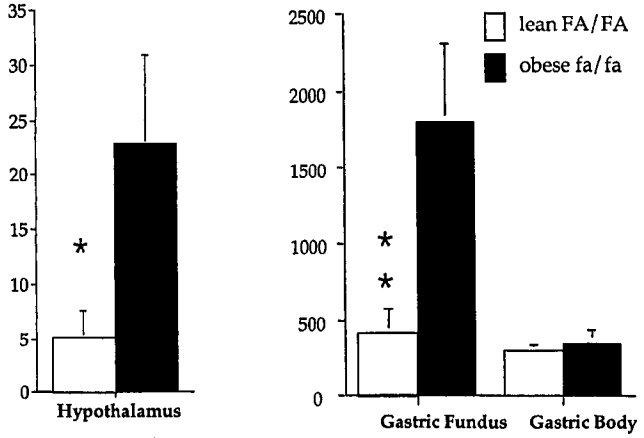

Figure 4. Ghrelin mRNA expression (mean ± SEM) in the hypothalamus (left part) and stomach (right part) in lean and obese Zucker rats. *: $P < 0.05$; **: $P < 0.02$ lean vs obese.

Quantification of Ghrelin Expression in the Hypothalamus and Stomach of Lean and Obese Rats. Ghrelin mRNA expression in the hypothalamus and in the gastric fundus and body are shown in Figure 4. There was a marked difference in the expression of ghrelin mRNA between brain and stomach. Ghrelin mRNA was about 100-fold more expressed in the stomach ($P < 0.0001$). There was also a genotype effect in both the hypothalamus and in the fundus. Ghrelin mRNA expression was higher in the obese rats than in the lean rats in both organs ($P < 0.05$ and $P < 0.02$ respectively).

*Plasma Variations of Ghrelin in Different Physiologic Situations. **Fasting/refeeding experiment.*** The effect of fasting and refeeding both for 24 hrs in lean and obese rats are shown in Figure 5. There was a very significant effect of the nutritional status ($P < 0.0001$) as well as of genotype ($P < 0.0001$) without any interaction.

After fasting, plasma ghrelin concentrations increased

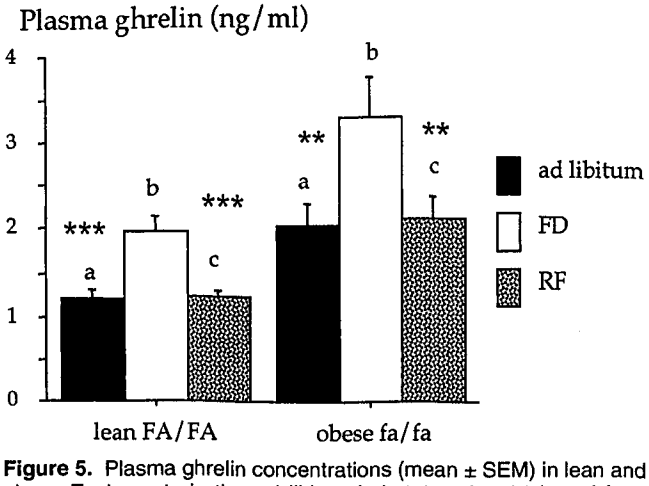

Figure 5. Plasma ghrelin concentrations (mean ± SEM) in lean and obese Zucker rats in the *ad libitum* fed state, after 24 hrs of food deprivation (FD) and after 24 hrs of refeeding (RF). Groups sharing the same letter were significantly different with a probability of at least 0.02. Stars indicate a significant difference with the fasting state in the same genotype. ***: $P < 0.001$; **: $P < 0.02$.

significantly in lean (+64%; $P < 0.001$) and obese (+60%; $P < 0.02$) rats. After 24 hrs of refeeding, they returned to their initial ad lib levels.

Whatever the nutritional situation, ghrelin concentrations in obese rats were significantly higher than in lean rats. They were higher by 69% ($P < 0.005$), 65% ($P < 0.02$), and 73% ($P < 0.005$) in the *ad libitum*, fast, and refed states respectively.

Diurnal Rhythm of Ghrelin. Plasma ghrelin concentrations at the light transition times and in the middle of the light period are shown in Figure 6. There was a significant effect of genotype ($P < 0.0001$) but no effect of time ($P = 0.15$). At each time, plasma ghrelin concentrations in obese rats were higher than in lean rats varying from 68% more at dark–light transition ($P < 0.01$) to 89% more at light–dark transition ($P < 0.005$).

Discussion

Ghrelin is new gut–brain peptide with independent stimulatory effects on both food intake and growth hormone secretion (10, 11). Besides its orexigenic effects, it has adipogenic effects as its chronic infusion in rats induces an increase in body weight (18, 19). The Zucker fa/fa rat is a widely used model of obesity characterized by massive obesity, overeating, and growth hormone metabolism alterations (36). In the present study, we explored the ghrelin system in lean (FA/FA) and obese (fa/fa) Zucker rat to better understand the role of ghrelin in the obesity syndrome induced by the fa mutation.

Our first approach was the *ex vivo* characterization of the molecular forms of ghrelin. Previous works have indeed shown that ghrelin exists under two main forms: an active form with an octanoylated serine in position 3 and an inactive form, the [Des-Octanoyl-Ser^3] ghrelin. The antibody we used recognizes the C-terminal portion of ghrelin and fully cross-reacts with the two forms of ghrelin. We confirmed that these two forms are present in the stomach and plasma and that the active form is the major form in the plasma. The molar ratio of the two forms was not different

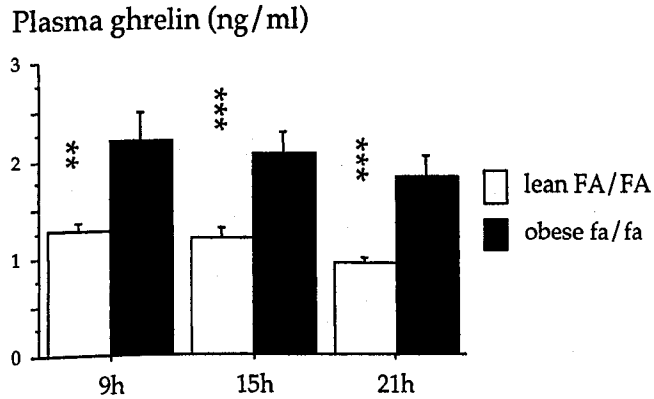

Figure 6. Plasma ghrelin concentrations (mean ± SEM) in lean and obese Zucker at the dark-light transition (9 h), in the middle of the light period (15 h) and at the light-dark transition (21 h). **: $P < 0.01$ and ***: $P < 0.005$ between lean and obese rats.

between lean and obese rats. This might be important for the biological action of the peptide in obese rats because a previous study by Toshinai *et al.* (16) has shown a selective diminution of the active form in the stomach, when the ghrelin system is activated by fast inducing, therefore a marked change in the ratio.

In a second approach, we tried to determine if the ghrelin system was modified in obesity. We checked both the peripheral and central sites of ghrelin production (e.g., the stomach and the hypothalamus). We found that the expression of ghrelin mRNA was significantly augmented in both sites in the obese Zucker rats by a factor of 4 to 5. In the stomach, this augmentation was observed only in the fundus where the highest concentrations of the peptide are measured. As there was no difference of peptide concentration in this subpart of the stomach between lean and obese rats, we could conclude an increased release of the peptide in the circulation. This was confirmed by the measurement of blood levels. The obese rats had indeed higher plasma concentrations of ghrelin than the lean rats. These results contrast with those previously published in obese humans where lower levels have been detected (35). This might indicate that the over-activation of the ghrelin system in the obese Zucker rat could contribute to the development of its overweight and adiposity.

The reasons for this activation might be linked to some important metabolic hormones such as insulin and leptin. We and others have indeed shown that ghrelin is negatively correlated with leptin and insulin (33, 35). The absence of leptin signaling in the obese rat through a defective leptin receptor could contribute to the over-expression of ghrelin mRNA as leptin receptors have been detected in the stomach and more precisely in the antrum and fundus (37, 38). Moreover, a paracrine effect of leptin in the stomach can also not be excluded. The stomach is indeed a site for leptin production (39) and we measured the highest levels of the protein in the same area (fundus) where ghrelin expression is highly expressed. A fine detection of the presence of leptin receptors on ghrelin-producing cells in this area would probably confirm this. The possible role of leptin in the regulation of ghrelin is supported by recent data obtained in another model of obesity with leptin signaling deficiency, the ob/ob mouse (17). This animal that has an obesity due to a modified leptin peptide with no biological activity is also characterized by an increase of ghrelin mRNA expression in the stomach but this augmentation (+19%) is smaller than what we measured in Zucker rats.

It should be emphasized that in the Zucker rat, the marked augmentation of ghrelin expression was not restricted to the stomach but was also observed in the hypothalamus. However, the level of expression in the brain was 100-fold lower than in the stomach. In the hypothalamus, the arcuate nucleus (ARC) is an important site of ghrelin production (1). The local release of ghrelin as well as the existence of high ghrelin levels in the blood might activate neuropeptide Y neurons in the ARC through the GHS-R

present in this area and induce an appetitive drive through the release of NPY. Several papers have recently supported this possibility (1, 22–24) through different approaches (measurement of NPY mRNA expression after ghrelin injection or use of NPY antagonists). It can also act through AGRP, which is co-localized with NPY in the arcuate nucleus (40). AGRP mRNA levels indeed increase either after central ghrelin administration in normal rats (22) or after treatment with a ghrelin agonist in NPY-deficient rats (41).

Other factors either downstream or independent of leptin can also play a role in the hyper expression of ghrelin in Zucker rats. Insulin is one of these factors. We have shown that ghrelin is inversely correlated to insulin in different feeding conditions (33). It also decreases after a meal when insulin is secreted (15). Due to its insulin resistance, the Zucker rat is likely not able to well regulate its ghrelin system through this hormone. Blood glucose, a factor in direct relation with insulin, can also play a role in this regulation as a fall in its blood concentration triggers the beginning of a meal (14) and is concomitant to a rise in ghrelin levels (13). Furthermore, ghrelin expression is upregulated by insulin-induced hypoglycemia (16). The decline in blood glucose levels induced by fasting could partly explain the large augmentation of plasma ghrelin that we measured in our lean rats after 24 h of food deprivation. Such a ghrelin increase after fast was also observed in the obese rats. This indicates that the ghrelin system is fully operational in these obese rats. In fasted obese rats, ghrelin once again might stimulate the NPY synthesis and the peptide release as indicated by increased NPY mRNA expression (42) and unchanged peptide levels in the arcuate nucleus (43) of these rats. It might not act on the AGRP system as AGRP expression is not altered by fast in obese rats (42). A difference in sensitivity of the two systems to ghrelin in obese rats might explain these results as it has been recently shown that AGRP mRNA levels augment in fasted lean rats (42). The nonactivation of the AGRP system appears to be beneficial in the obese rat for avoiding a further augmentation of its pre-existing overeating.

We did not measure any significant variations of ghrelin at the light/dark transitions that constitute crucial periods for food ingestion in the rat. Ghrelin concentrations at those times were also not significantly different from those found in the middle of the light period, which could reasonably considered as a period of relative inactivity in terms of feeding. These data could be explained by the pulsatile nature of the ghrelin release as indicated in recent papers (44, 45). In our case, the measurement of ghrelin was done during the hour around the light/dark transitions and was perhaps not sufficiently close to the beginning of meal ingestion to allow the detection of a significant change.

In conclusion, we have shown in this paper that the obese Zucker rat is characterized by an increased expression of ghrelin both at the peripheral and central levels. This increase is linked to an undisturbed regulation of its circulating levels by the feeding state. This indicates that the overactivation of a fully functional system for ghrelin might participate in the development of extra weight in the obese rat. This is reinforced by the observation of adipogenic effects of ghrelin and/or ghrelin agonist in normal rats as well as in NPY-deficient rats (18, 41). Furthermore, ghrelin levels are higher in bulimia nervosa patients (46) and transgenic rats expressing an antisense GHS-R mRNA, thus selectively attenuating GHS-R protein expression in the arcuate nucleus that have lower body weight and less adipose tissue than control rats (47). All these recent data in addition to our present results support a role of ghrelin in hyperphagia and obesity.

The Zucker fa/fa rat could constitute a good model for testing anti-obesity drugs that might act through the ghrelin pathway. As targets for these type of drugs are present in the periphery (fundus), it would be likely easier to reach these targets with the *per os* absorption of the drugs. However, the role and importance of the central ghrelin present in the arcuate nucleus are not fully understood and must be more finely determined. Experiments are presently underway in our lab to investigate this problem.

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