Age, sex, and lactating status regulate ghrelin secretion and GOAT mRNA levels from isolated rat stomach

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The 30% of ghrelin circulating levels presents Ser3 residue acylated by an n-octanoic acid; the remainder circulates as unacylated ghrelin (19). The acyl modification of ghrelin is necessary for ghrelin to bind the ghrelin receptor GHSR1a (22). The acyltransferase that catalyzes ghrelin octanoylation has recently been identified as ghrelin O-acyltransferase (GOAT) (16, 35). This enzyme is expressed in stomach and concretely colocalizes with ghrelin in mouse oxyntic mucosa (29). GOAT is essential for ghrelin octanoylation because GOAT knockout mice do not have the octanoylated ghrelin form and show an increase in des-acyl ghrelin levels compared with wild-type mice. It was found that GOAT is regulated by nutritional status (34), and their levels are highly influenced by the nature of the lipids in the diet (21).

It is widely shown that neuroendocrine peptide regulation changes with age in different locations, such as the gastrointestinal tract (10). Ghrelin is the only stomach-derived peptide with orexigenic action that communicates the gastrointestinal tract and the brain, contributing to energy homeostasis regulation (4). It might also be possible that gastric ghrelin changes with age to adapt the organism to metabolic requirements demanded through different stages of life.

Several works have studied the variations of the stomach ghrelin expression with age, and histological studies have been published about this topic. However, the controversial data is included in the references (33, 27, 15), and no studies of direct gastric secretion have been published.

From a metabolic point of view, one of the most relevant processes in rat development is the weaning period, which is associated in the offspring with drastic dietary and environmental changes. In addition, this period is characterized by alterations in the gastrointestinal tract morphology, and these weaning-related effects might possibly be affecting ghrelin production (3, 30). Circulating ghrelin levels in suckling animals are regulated in a different manner than in adults (18).

In addition, puberty is a life stage characterized by noticeably high energy requirements and alterations in circulating hormone levels. Available studies have shown that most relevant changes in ghrelin levels are produced in the pubertal stage in females under different interventions associated with estrogen level variations (e.g., ovariectomy and estradiol treatment). These approaches affect gastric ghrelin mRNA, the number of positive ghrelin cells, and plasma ghrelin.

Although several articles about gastric mRNA levels and protein content have been published (15, 18), the mechanism of gastric ghrelin regulation is still unknown, and the factor(s) regulating the secretion has yet to be identified. The recently

Ghrelin is a 28-amino acid peptide that has been isolated from the stomach as the endogenous ligand of the growth hormone secretagogue receptor-1α (GHSR1α) (22). It was found that circulating ghrelin levels were decreased by 65% after gastrectomy in humans and rodents, suggesting that the main source of ghrelin in the organism is the stomach, especially X/A-like cells (1, 9, 26).

In addition, ghrelin expression has been found along the gastrointestinal tract (19) and has also been detected in other tissues such as the hypothalamus (8), testis (2), pituitary (5), ovary (6, 12), heart (20), and placenta (14), although the contribution, if any, of these tissues to circulating ghrelin levels is ancillary.

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identified enzyme for ghrelin acylation (GOAT) has been studied in relation to nutritional status but not during development. Due to the lack of study, there are currently no data available on the regulation of ghrelin in these important stages of life. The deep study of these topics might be of great relevance for the discovery of the possible role of gastric ghrelin in neonatal and pubertal development.

The three objectives of this paper are to gain insight into 1) the control of ghrelin secretion directly from the stomach in response to different stages of life and the role of lactation, 2) the role of gonadal hormones on ghrelin secretion, and 3) the regulation of GOAT levels by age, sex, and lactation.

MATERIALS AND METHODS

Animals and Experimental Design

For all experiments, Sprague-Dawley rats were used. The animals were maintained according to protocols approved by the Animal Care Committee of Santiago de Compostela University in accordance with our institutional guidelines and the European Union standards for the care and use of experimental animals. Rats were housed in air-conditioned rooms (22–24°C) under controlled (12:12-h) light-dark cycle with free access to food and water (n = 10). Surgical procedures were performed under anesthesia by intraperitoneal injection of a mixture of ketamine-xylazine (100 mg/kg body wt ketamine + 15 mg/kg body wt xylazine). Animals were euthanized by decapitation. Trunk blood was collected and immediately centrifuged and plasma stored at −20°C for the biochemical measurements.

Experiment 1: age-related variations in plasma ghrelin and gastric ghrelin secretion. To investigate whether differential secretion of gastric ghrelin takes place during development, male and female rats (1–9 wk of age) were used to measure gastric ghrelin secretion and levels of ghrelin and testosterone in plasma (n = 10). Upon decapitation, trunk blood was collected, and the uterus was dissected out of the surrounding fat and its weight recorded after a brief drying with cellulose paper. Due to the difficulty of measuring basal estrogen levels directly by RIA, the measurements of the uterus weight were used as an index of pubertal maturation and activation of the reproductive axis. It was shown that this value is strongly associated with the estrus cycle with free access to food and water (10). Surgical procedures were performed under anesthesia by intraperitoneal injection of a mixture of ketamine-xylazine (100 mg/kg body wt ketamine + 15 mg/kg body wt xylazine). Animals were euthanized by decapitation. Trunk blood was collected and immediately centrifuged and plasma stored at −20°C for the biochemical measurements.

Experiment 2: effect of estrogen and testosterone in vitro on gastric ghrelin secretion. Explants of gastric tissue obtained from adult female rats with the method described below were incubated for 3 h with 17β-estradiol at a dose of 1 μM (β-estradiol 3-benzoate, Sigma Aldrich, Steinheim, Germany). On the other hand, the gastric explants obtained from male animals were incubated with testosterone propionate (Fluka Biochemika, Buchs, Switzerland) at a dose of 1 μM for 3 h. The medium was collected, and ghrelin levels in the culture medium were measured by RIA. The variations in ghrelin mRNA levels were tested by real-time PCR, and the ghrelin peptide content was tested by Western blot in female rat gastric tissue. Experiments 3 and 4: effects of the estrogen and testosterone ex vivo on gastric ghrelin secretion. To study the role of sexual hormones on gastric ghrelin secretion, young animals (4 wk old), females and males, were bilaterally ovariecotomized, orchiectomized, or sham operated under ketamine-xylazine anesthesia. After 3 days, rats were euthanized. One group of ovariecotomized animals was implanted subcutaneously with a Silastic tubing (1.0 mm id, 1.5 mm od, 20 mm in length) cannula containing 17β-estradiol (Sigma Aldrich). One group of male orchiectomized rats was implanted with a cannula similar to those described above but containing testosterone propionate. The variations in ghrelin mRNA levels were tested by real-time PCR, and ghrelin peptide content was tested by Western blot in female rat gastric tissue.

Experiment 5: breastfeeding effect on gastric ghrelin secretion. With the aim of testing the weaning effect on gastric ghrelin secretion, the model of delay weaning (DW) was developed by preventing pups from eating solid food from days 21 (3 wk of age) to 28 (4 wk of age), leaving the pups with the mum for this period. The corresponding controls (4 wk) that were previously weaned at 3 wk of age were used. The pup body weight was monitored. Testosterone levels were measured by RIA, and uterus weight was recorded.

Experiment 6: regulation of gastric GOAT mRNA expression by age and breastfeeding. To test the possible changes in the GOAT with age, the mRNA levels of this enzyme were studied by real-time quantitative PCR in the same experimental groups where mRNA ghrelin levels were tested (2, 4, 6, and 8 wk of age). To test the effect of the weaning in gastric GOAT expression, real-time RT-PCR for GOAT was performed in stomach mucosa from the animal groups in experiment 5.

RNA Isolation and Real-Time Quantitative RT-PCR

Total RNA was isolated from rat stomach mucosa using Trizol (Invitrogen) according to the manufacturer’s recommendations. Extracted total RNA was purified with DNase treatment by means of DNA-free kit as a template (Ambion) to generate first-strand cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR was performed using an ABI PRISM 7300 HT Sequence Detection System (Applied Biosystems) with specific Taqman quantitative RT-PCR primers and probes (Table 1). For the analysis, gene expression levels of ghrelin and GOAT were normalized using 18S rRNA as a housekeeping gene (TaqMan; Applied Biosystems) and hypoxanthine phosphoribosyltransferase 1, respectively, and expressed in respect to the average value for the control group (13).

Western Blot

Immunodetection. Whole tissue samples were prepared by homogenization and mild sonication in cold RIPA buffer [containing 200 mM Tris-HCl (pH 7.4), 130 mM NaCl, 1% (vol/vol) glycerol, 0.1% (vol/vol) SDS, 1% (vol/vol) Triton X-100, and 10 mM MgCl2] with antiproteases and antiphosphatases (Sigma-Aldrich, St. Louis, MO). Tissue lysates were centrifuged for 10 min at 18,000 g in a microfuge at 4°C. Then, equal amounts of protein (10 μg/well) were run on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and electrophoresed onto nitrocellulose membranes. The membranes were probed successively with primary antibodies and alkaline phosphatase-labeled secondary antibodies (GE Healthcare, Fairfield, CT). Specific antigen-antibody binding was visualized using a chemiluminescence method according to the manufacturer’s instructions (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific; Pierce). Primary anti-ghrelin (SC-10368), diluted 1:2,000, was purchased from Santa Cruz Biotechnology.

Western blots were done by pooling tissue extracts from three different rats belonging to each group and repeated in three independent experiments; β-actin (A-5316; Sigma Chemical) detection was performed in all tissue Western blots as loading control.

Tissue Explants Culture

A model of tissue explants culture was developed as described. In brief, to obtain ex vivo tissue, the stomachs were immediately excised...
and transported to the incubator in sterile Krebs-Ringer-HEPES buffer (125 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l MgSO₄, 1.3 mmol/l KH₂PO₄, 2 mmol/l CaCl₂, 6 mmol/l glucose, 25 mmol/l HEPES, pH = 7.4). After blood vessel and connective tissue elimination, stomach tissue was washed with sterile Krebs-Ringer-HEPES. Tissue explants, mostly gastric fundus, were placed in six-well dishes containing 2.5 ml of Dulbecco’s modified Eagle’s medium supplemented with penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml) and incubated at 37°C under a humidified atmosphere of 95% air-5% CO₂. After a preincubation period of 1 h the medium was discarded, and 2.5 ml of fresh medium was dispensed into each well. Culture medium was then collected at 2 h, and tissue was weighed with a precision scale. Medium was stored at −20°C until ghrelin assay.

Biochemical Analysis

Total and active ghrelin levels were determined by means of a double-antibody RIA using reagent kits and methods provided by Linco Research (St. Charles, MO). Samples for measuring the secretions from the tissue explants were obtained directly by collecting the culture medium. Samples were analyzed by RIA, as described previously (32). The limit of assay sensitivity was 93 pg/ml for total and 7 pg/ml for active ghrelin. Results were expressed as nanograms per milliliter of total ghrelin and picograms per milliliter of active ghrelin per gram of tissue in the culture medium. Testosterone concentrations in serum samples were measured using a commercial kit purchased from ICN Biomedical (Costa Mesa, CA), following the manufacturer’s instructions. The sensitivity of the assay was 100 pg/ml.

Statistical Analysis

Data were expressed as means ± SE. The statistical analysis comparing the different groups was assessed using the nonparametric Mann-Whitney test. P ≤ 0.05 was considered significant.

RESULTS

Age-Related Variations in Gastric Ghrelin Secretion Compared with Plasma Ghrelin Levels and mRNA

Plasmatic ghrelin levels did not change with age in either males or females (Fig. 1, A and E). The total gastric ghrelin secretion corrected per gram of gastric tissue increased with the first 3 wk of age, and at 4 wk of age it was dramatically decreased in both male [30.8 ± 2.1 ng/ml at 3 wk vs. 11.2 ± 0.5 ng/ml at 4 wk and 19.1 ± 1.2 ng/ml at 5 wk (P ≤ 0.05, P ≤ 0.01); Fig. 1B] and female animals [20.4 ± 1.5 ng/ml at 3 wk of age, 7.9 ± 0.5 ng/ml at 4 wk of age, 20.3 ± 12.9 ng/ml at 5 wk of age (P ≤ 0.05, P ≤ 0.01 vs. 3 wk of age); Fig. 1F].

The other outstanding finding was a rebound peak in gastric ghrelin secretion found at −5−6 wk of age in both sexes (19.1 ± 1.2 ng/ml at 5 wk, 27.6 ± 2.5 ng/ml at 6 wk, 19.6 ± 2.4 ng/ml at 7 wk; P ≤ 0.05, P ≤ 0.01 vs. 6 wk of age; Fig. 1B). However, in the 6-wk-old female group, gastric ghrelin secretion was increased to values characteristic of the adult stage, remaining constant throughout adulthood (Fig. 1F). The age of 4 wk was coincident with the initial pubertal stage in females, as shown by the uterus weight data. Between the 1st and the 3rd wk of life, the uterus remained small in size (0.05−0.02 g); however, in the 4th wk it experienced a considerable increase in volume (0.2 g at 4 and 5 wk), and after the 6th wk the uterus remained constant throughout adult stage (Fig. 1H). This parameter can be considered as an index of pubertal maturation, and it is directly related to circulating levels of estrogen (24). The latter indicates that at 4 wk of age the animals began the pubertal stage with an increase in estrogen levels. These data were corroborated after the evaluation of the vaginal opening. The vaginal opening is an external index of puberty onset in female rats that allows recapitulation of proper functional activation of all of the levels of reproductive axis and sexual maturation. Females showed complete vaginal opening at a mean of 35.8 ± 0.35 days postpartum. Alternatively, the measurements of testosterone levels in males showed a peak at just 6 wk of age, coinciding with the pubertal stage in males. The levels of testosterone measured by RIA were of 0.02 ± 0.002 ng/ml between the 1st and the 5th wk of age and exhibited a noticeable increase at 6 wk of age (0.12 ± 0.05 ng/ml) through the adult age (Fig. 1D). The age of occurrence of BPS in control rats was 41.3 ± 0.15 days, and 100% of animals (10 of 10) presented complete preputial separation at 42 days postpartum, which corroborated the testosterone level measurements.

The mRNA levels for gastric ghrelin determined by real-time PCR showed that gastric mRNA ghrelin peaked in 4-wk-old females (2-wk-old females: 100 ± 17 arbitrary units vs. 4-wk-old females: 217 ± 45 arbitrary units, P ≤ 0.05; Fig. 1G), whereas in males there were no mRNA ghrelin variations found with age (Fig. 1C).

In Vitro Estrogen and Testosterone Exposure on Gastric Ghrelin Secretion

The addition of testosterone propionate (1 μM) directly onto gastric tissue explant medium did not affect gastric ghrelin

Table 1. Primers and probes for real-time qPCR analysis

<table>
<thead>
<tr>
<th>mRNA (GenBank Accession No.)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghrelin (AB029433.1)</td>
<td>5′GACGCGAGCCAGCAGGAGAA-3′</td>
</tr>
<tr>
<td>GOAT (NM_001107317)</td>
<td>5′GGCCGAGCTTTTCTCTCT-3′</td>
</tr>
<tr>
<td>HPRT1 (NM_012583)</td>
<td>5′AGGGATAGCTACGGAGGAA-3′</td>
</tr>
</tbody>
</table>

GOAT, ghrelin O-acyltransferase; HPRT1, hypoxanthine phosphoribosyltransferase 1.
secretion (Fig. 2A). On the contrary, estradiol (1 μM) induced a significant decrease in gastric ghrelin secretion (11.0 ± 0.7 vs. 14.5 ± 0.5 ng/ml in control animals, *P ≤ 0.05; Fig. 2B). However, this treatment did not affect mRNA ghrelin levels in gastric tissue, peptide ghrelin content, or GOAT mRNA levels (Fig. 2, C, D, and E).

**Effect of Ex Vivo Testosterone on Gastric Ghrelin Secretion**

Ghrelin measurements in the medium secreted from gastric tissue explants extracted from young male rats of several experimental groups were performed: 3-day orquidectomized rats, testosterone propionate-treated rats after 3 days of being orquidectomized, and the corresponding sham-operated controls.

Young animals presented low levels of gastric ghrelin secretion as well as low levels of testosterone. Orquidectomy did not affect gastric ghrelin secretion in the same way that testosterone treatment had no effect in orquidectomized males (Fig. 3C). These effects at gastric level were reflected in the circulating levels as well as mRNA levels of the hormone (Fig.
and E). Alternatively, the transcription of GOAT showed a significant increase in orquidectomized testosterone-treated males with respect to operated animals (119.1 ± 10.1 vs. 90.8 ± 11.1 arbitrary units, P ≤ 0.05; Fig. 3G).

Effect of Estrogens Ex Vivo on Gastric Ghrelin Secretion

Gastric ghrelin secretion was studied in young female rats under several experimental approaches: animals 3 days after being subjected to surgical ovariectomy, animals with estrogen replacement after 3 days of ovariectomy, and the sham-operated control animals (25). It was found that estrogen treatment in young ovariectomized females reversed the stimulatory effect on gastric ghrelin secretion exerted by the ovariectomy (control animals: 23.3 ± 1.1 ng/ml; ovariectomized animals: 38.1 ± 2.6 ng/ml; ovariectomy + estradiol treatment: 26.8 ± 2.1 ng/ml; P ≤ 0.01 vs. control, P ≤ 0.01 vs. ovariectomy; Fig. 3D). But this effect was not reproduced over mRNA ghrelin levels and GOAT expression (Fig. 3, F and H). The peptide content of ghrelin in gastric tissue of ovariectomized animals was significantly decreased (64.7 ± 5.9% over control, P ≤ 0.05 vs. control), and this effect was not reverted after estradiol administration (69.2 ± 7.3% over control, P ≤ 0.05 vs control) (Fig. 3I).

Breastfeeding Effect on Gastric Ghrelin Secretion

Two experimental models were performed to determine the implication of maternal food intake on gastric ghrelin secretion. The first of those included young animals of 4 wk of age that had been weaned at the end of the 3rd wk of life (21 days old). The second group was comprised of young animals that were still not weaned, named DW models. In the female group, DW altered neither the gastric secretion nor the plasmatic ghrelin levels (Fig. 4, A and B). On the other hand, in the male group the delay of the weaning produced an increase in gastric ghrelin secretion (control animals: 51.6 ± 2.9 vs. DW: 61.3 ± 3.3 ng/ml, P ≤ 0.05; Fig. 4B). In circulating ghrelin levels, the variation was not statistically significant (Fig. 4A).

Gastric mRNA levels of ghrelin were measured by quantitative real-time RT-PCR. A decrease in gastric mRNA levels was found in females subjected to DW (control: 100 ± 12.8 arbitrary units vs. DW: 44.9 ± 8.9 arbitrary units, P ≤ 0.01). Contrarily, gastric mRNA in males was not affected by DW (Fig. 4C).

Body weight was measured in the different groups, and surprisingly, it was found that DW was associated with a decrease in body weight in both males and females (control male: 74.5 ± 2.6 g, DW male: 62.5 ± 2.8 g, P ≤ 0.05; control female: 67.9 ± 2.5 g, DW female: 54.6 ± 1.5 g, P ≤ 0.05; Fig. 5C).
Estrogen and testosterone levels were calculated in each one of these experimental groups, and it was discovered that DW caused a diminution of the uterus weight in females (control: 0.058 ± 0.007 g vs. DW: 0.035 ± 0.003 g, \( P \leq 0.05 \); Fig. 5B). In addition, the DW decreased the testosterone circulating levels in male animals (control: 0.012 ± 0.002 ng/ml vs. DW: 0.007 ± 0.001 ng/ml, \( P \leq 0.05 \); Fig. 5A).
GASTRIC GHRELIN, GOAT, AND AGE

Fig. 4. A: plasmatic ghrelin concentration in 4-wk-old male and female rats. B: gastric ghrelin secretion from tissue explants from 4-wk-old male and female rats to the incubation medium. C: ghrelin mRNA expression in gastric mucosa by quantitative real-time RT-PCR standardized by 18S mRNA levels from male and female rats to the incubation medium. Values are means ± SE. *P < 0.05, **P < 0.01 vs. control; DW, delay weaning.

Age and Breastfeeding Effect on GOAT mRNA in Stomach Mucosa

In the male groups, an increase in GOAT mRNA levels was found with age (2 wk old: 100 ± 12 arbitrary units, 4 wk-old males 366 ± 66 arbitrary units, 6 wk-old males 740 ± 91.5 arbitrary units, P < 0.01 vs. 2 wk-old males; 8 wk-old males 814 ± 155 arbitrary units, P < 0.01 vs. 2 wk-old males, P < 0.05; Fig. 6B). Contrarily, the female group showed a peak in GOAT mRNA levels at 6 (519 ± 68 arbitrary units) vs. 2 wk of age (160 ± 43.6 arbitrary units) (P < 0.001; Fig. 6B). Acyl ghrelin secretion by the stomach was measured by RIA. An increase was found in the amount of active ghrelin secreting from the stomach with age in both males (2 wk old: 70.8 ± 5.2 pg/ml; 4 wk old: 72.9 ± 8.9 pg/ml; 6 wk old: 146 ± 23 pg/ml; 8 wk old: 240 ± 28 pg/ml) and females (2 wk old: 75.5 ± 8.8 pg/ml; 4 wk old: 102.05 ± 18.7 pg/ml; 6 wk old: 140 ± 15 pg/ml; 8 wk old: 238.8 ± 45.6 pg/ml) (Fig. 6A).

Conversely, breastfeeding in the male group induced a decrease in gastric GOAT mRNA levels as probed by the DW group values (control: 100 ± 14 arbitrary units vs. DW: 66 ± 11 arbitrary units, P < 0.05). In contrast, the delay of weaning did not affect GOAT mRNA levels (Fig. 6C) in 4-wk-old females.

DISCUSSION

An organ culture model of gastric tissue developed and validated by our group, (31, 32) has been used in the present study to assess the direct regulation of ghrelin secretion by the stomach during postnatal life. With this model, it has been shown that age, sexual steroids, and dietary modifications regulate the stomach ghrelin secretor function independently of the mRNAs and circulating levels of this hormone. The most relevant findings in this paper were the following. First, the hormonal modifications associated with the pubertal period, i.e., variations of estrogen and testosterone circulating levels, regulate gastric ghrelin secretion. Second, weaning is strongly implicated in gastric ghrelin regulation. The weaning delay affects sexual hormone levels, body weight, and ghrelin secretion. Third, all of the above-mentioned factors are closely implicated in gastric GOAT mRNA regulation.

In the present study, it was shown that plasmatic ghrelin levels were not affected in a significant way by age (Fig. 1, A and E). According to the published results (2), our data indicate an increase in mRNA ghrelin expression in young (4 wk old) female rats (Fig. 1G). However, the most novel results showed that ghrelin secretion from the stomach was altered in female animals at the beginning of the pubertal period (4 wk old) (Fig. 1F), coinciding with an increase in the uterus weight (Fig. 1H). In males, puberty starts at 6 wk of age, as shown by the increase in testosterone levels (Fig. 1D), coinciding with the highest levels of gastric ghrelin secretion (Fig. 1B). Taking into account that puberty is a period characterized by significant variations in estrogen and testosterone circulating levels, it suggests that both hormones may act on the stomach to regulate ghrelin production. To confirm this affirmation, a culture system of gastric tissue explants (32) was used in both ex vivo and in vitro studies. Using this approach, it was found that a direct in vitro effect of estrogen treatment on this tissue induces a significant decrease in ghrelin secretion (Fig. 2B), with no effect on mRNA and protein levels (Fig. 2, C and D). On the contrary, direct treatment with testosterone on the...
The stomach did not affect gastric ghrelin secretion (Fig. 2A). These data are supported by the fact that estrogen receptor-α expression has been reported in ghrelin-producing cells of rat stomach (7). A previous study, using isolated stomach cells, found that estrogen treatment significantly stimulates ghrelin mRNA expression and the number of immunopositive cells for ghrelin (28). These results are in agreement with our findings, as proven by an increase in gastric ghrelin mRNA in 4-wk-old females (Fig. 1G) that present high levels of estrogen during this stage of life (Fig. 1H).

In this article, the effect of estrogen and testosterone modulation on gastric ghrelin secretion was tested ex vivo. This physiological regulation of gastric ghrelin secretion by the estrogens in the puberty stage was reproduced in the laboratory through different approaches of estrogen manipulation. The increase in estrogen levels produced in early-stage pubertal animals (4 wk old) was blocked by surgical ovariectomy, and because of its manipulation, the drop in gastric ghrelin secretion was prevented as well, thus keeping the secretion within the range of basal values found between the 1st and the 3rd wk of life (Fig. 3D). Moreover, when the estrogen values were replaced by exogenous treatment, the ovariectomy effect was reverted. The increase in gastric ghrelin secretion with ovariectomy produces a decrease in the ghrelin levels inside the tissue (Fig. 3I). However, the mRNA levels were not significantly affected, although a slight tendency to increase was
observed (Fig. 3E). Together, these results suggested a new mechanism for gastric ghrelin production regulation, proposing that the decrease in ghrelin secretion from the stomach may be reflected in a greater amount of protein storage in ghrelin-positive cells, as consequence of the increase in the mRNA expression found after estrogen treatment.

The decreased gastric ghrelin secretion found in 4-wk-old males (Fig. 1B) cannot be explained as a consequence of variations in testosterone (Fig. 1D). A modification of diet, i.e., the weaning, takes place immediately prior to the 4th wk of life. This process is characterized by the step from breastfeeding to solid food ingestion. Possibly gastric ghrelin secretion modifications found at 4 wk might be produced as a consequence of the weaning since it has several effects on the maturation of ghrelin-producing cells and morphology in the gastrointestinal tract (3). The direct ghrelin secretion by the stomach was measured in animals subjected to a delay in weaning. In the female pups, the delay of weaning did not affect gastric ghrelin secretion (Fig. 4B), probably due to the powerful effect of estrogen variations produced at this age, which may be masking a possible effect. However, the mRNA ghrelin levels were strongly inhibited by the delay in weaning (Fig. 4C) that coincides with the increase in ghrelin mRNA levels found in weaned females (Fig. 1G).

Opposite of females, the delay weaning in male pups prevented the drop in gastric ghrelin secretion evoked in 4-wk-old weaned animals (Fig. 4B). However, ghrelin mRNA was not affected by the weaning in males (Fig. 4C), and this is supported by the lack of effect of age in ghrelin mRNA (Fig. 1C). Our results are in contrast with previous data from another group that found a decrease in plasma and mRNA ghrelin levels and ghrelin cell density in delayed weaning rats (11). The discrepancy could be explained due to the fact that they did not test the direct ghrelin secretion from the stomach as well as several methodological differences (e.g., the age of weaning). The possibility that maternal ghrelin could be crossing to the pup through ingestion of milk should be considered since it is suggested by the data of plasmatic ghrelin levels (Fig. 4A). However, with this explants model, direct secretion from the pup stomach was measured (Fig. 4B), and together with the data of mRNA (Fig. 4C) there is evidence that the stomach has its own mechanism of ghrelin regulation under diet modifications.

Body weight was also affected by delayed weaning (Fig. 5C). This last finding was described previously by another group and is in agreement with the fact that a prolonged period of breastfeeding has been shown to reduce the risk of developing obesity, although the mechanism behind this is still unknown (17).

In the present work, a diminution in testosterone levels in males as well as in uterus weight in female pups with the delay of weaning was shown for the first time (Fig. 5, A and B). These data might indicate a delay in the onset of puberty in rats subjected to delay weaning, but this topic deserves future investigation.

In 2008, two different groups characterized the enzyme in charge of ghrelin acylation (GOAT) (16, 35). Their findings suggest a role of GOAT producing an adaptive response to prevent alterations in energy balance and body weight homeostasis (13, 21, 34). Under this context, it is possible that GOAT mRNA levels change through the different periods of life to adjust the organism to energetic requirement variations with age. To the best of our knowledge, the data presented in this work showed for the first time an age-related change in the GOAT mRNA pattern at the gastric level in both males and females. In males (Fig. 6B), GOAT mRNA increases linearly with age parallel to body weight gain (data not shown). However, in the females, the maximum value for GOAT mRNA was found in 6-wk-old females (Fig. 6B), which is the initial adult age as well as the moment when gastric ghrelin secretion is stabilized and maintains values characteristic of the adult stage thereafter (Fig. 1F). The fact that the growing period in females finishes at a younger age than in males (data not shown) might be linked to the differences in GOAT mRNA pattern between males and females. During the growing period and especially during the puberty, the energetic requirements are higher than in other periods of life, and it is just at this time when the GOAT production is more elevated in both males and females. The main function of GOAT consists of the acylation of ghrelin to produce the active form of this peptide with orexigenic and adiposity inductor actions by its binding to the GHSR1a. In this context it is possible that, in periods of life characterized by a negative energy balance as a consequence of the elevated energetic requirements, GOAT increases to save energy to counteract this negative energy balance. In this model GOAT is proposed as a mechanism of body weight defense, allowing the organism to adapt to the different necessities of each period of life by switching the rate of acyl/desacyl ghrelin. Reinforcing this hypothesis are the data showing an increase in gastric acyl ghrelin (Fig. 6A) secretion with age in parallel with the pattern of GOAT mRNA levels in the stomach.

On the other hand, GOAT mRNA levels were tested as well in 4-wk-old male and female rats subjected to a delay weaning. Likewise with gastric ghrelin secretion (Fig. 4B) in the female group, the delay of weaning did not affect GOAT mRNA levels, probably because of puberty at this age masking the effects that take place. In the same manner as gastric ghrelin secretion (Fig. 4B) in the male group, GOAT mRNA levels are highly affected by delay weaning (Fig. 6C). The GOAT decrease in delay weaning coincides with decreased testosterone levels, suggesting a regulation of GOAT levels by testosterone. Moreover, this regulation was probed for the first time in the present work, since the treatment with testosterone to orquidectomized males induces an elevation of GOAT mRNA levels in gastric tissue (Fig. 3G).

In summary, the present study demonstrates for the first time that gastric ghrelin secretion is regulated through postnatal life independently of gastric expression and circulating levels of this hormone. All together, the present findings indicate a strong regulation of gastric ghrelin secretion by estrogens. The weaning strongly regulates gastric ghrelin secretion. Furthermore, animals subjected to delayed weaning present a lower body weight than the corresponding controls. For the first time, it is shown that a noticeable decrease in testosterone and estrogen circulating levels is associated with delay of weaning. GOAT mRNA levels in the stomach are strongly regulated by age, breastfeeding, and testosterone.

In conclusion, the data presented in this article might be indicating that the stomach itself could be regulating its own ghrelin production throughout life independently of other organs to adapt the organism to the metabolic requirements demanded through each stage of life.

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REFERENCES


