Gastric ghrelin cell development is hampered and plasma ghrelin is reduced by delayed weaning in rats

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Abstract

The duration of breastfeeding has attracted much interest, as a prolonged period of breastfeeding has been shown to reduce the risk of developing obesity. The mechanism behind the reduced risk is, however, poorly understood. The novel hormone ghrelin augments appetite, promotes body weight increase and increases adiposity. The majority of circulating ghrelin emanates from endocrine cells in the oxyntic mucosa of the stomach. In newborn humans and rodents, the number of ghrelin cells is low after birth until weaning, when the cell population is greatly expanded. To date, information about the influence of weaning perturbations on ghrelin cell development is scarce. Therefore, we studied the effect of delayed weaning on gastric ghrelin expression and plasma ghrelin concentration. To this end, special food separator cages were used to prevent the pups from eating solid food, forcing them to drink milk up to 21 days of age. Gastric

ghrelin expression was examined by immunocytochemistry and *in situ* hybridisation, and plasma concentrations were assessed by RIA. Our data showed that gastric ghrelin expression and plasma ghrelin concentration are maintained at a lower level by delayed weaning. We also found that the relation between gastric ghrelin expression and body weight was altered by delayed weaning. Thus, control rats displayed a positive correlation between ghrelin expression and body weight, while no such correlation was evident in animals with delayed weaning. We conclude that delayed weaning exerts a negative influence on ghrelin expression, and that the onset of solid food intake may trigger normal ghrelin expression. Therefore, we suggest that ghrelin may constitute a hormonal link between the duration of breastfeeding and body weight development.

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Introduction

Ghrelin is a 28 amino acid peptide originally isolated from rat stomach as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R; Kojima *et al.* 1999). Ghrelin is mainly produced by endocrine cells in the oxyntic mucosa of the stomach, but ghrelin expression has been reported in a wide range of tissues including gastric antrum, intestine (Date *et al.* 2000, Dornonville de la Cour *et al.* 2001) and pancreas (Wierup *et al.* 2002, 2004); for a review see Korbonits *et al.* (2004). In addition to the somatotrophic effects of ghrelin, the peptide stimulates food intake and increases adiposity (for review see Korbonits *et al.* (2004) and van der Lely *et al.* (2004)).

The regulation of appetite and food intake is highly complex and many hormonal as well as neuronal pathways participate (Druce & Bloom 2006). Recent data suggest that important programming of satiety and hunger signalling occurs during the neonatal period, and that these settings may influence appetite and food intake later in life (Cripps *et al.* 2005). Interestingly, a role for ghrelin in these early settings

has recently been argued (Grove & Cowley 2005). The duration of breastfeeding has attracted much interest, as a prolonged period of breastfeeding has been shown to reduce the risk of developing obesity. The mechanism behind the reduced risk is, however, poorly understood (Kramer 1981, Harder *et al.* 2005, Gillman *et al.* 2006), for review see Arenz *et al.* (2004).

In the developing rat, gastric density of ghrelin cells is low until the weaning period when the ghrelin cell population is greatly expanded (Björkqvist *et al.* 2002, Hayashida *et al.* 2002, Wierup *et al.* 2004). Similar observations have been made in humans (Wierup *et al.* 2002). During the neonatal and pre-weaning period, the pancreas has been suggested to be an important source of circulating ghrelin, since islet ghrelin cells are numerous neonatally both in rodents and in humans (Wierup & Sundler 2004, Wierup *et al.* 2004). Studies of the regulation of gastric ghrelin cell development have suggested that the ghrelin cell population is not affected by gastrin in the rat (Dornonville de la Cour *et al.* 2001), although gastrin null mutant mice as well as mice lacking both gastrin and cholecystokinin have affected ghrelin cell

intramucosal topography, but normal density of ghrelin cells (Friis-Hansen *et al.* 2005). Even though it is clear that the gastric ghrelin cell population expands during the weaning period, there is, to our knowledge, no information on how ghrelin cell development is affected by weaning perturbations. Therefore, we studied the effect of delayed weaning on ghrelin cell development, ghrelin mRNA expression and plasma ghrelin concentrations.

Materials and Methods

Animals

Sprague–Dawley rats (Møllegaard Breeding and Research Ltd, Skensved, Denmark) were used. The rats were kept under standardised light conditions (a light period of 12 h, from 0800 to 2000 h) and at a constant temperature of 20 ± 1 °C with a relative humidity of $50 \pm 10\%$. Each dam was housed separately with its litter. The day of birth was assigned as day 0, and within 3 days the litters were restricted to nine to thirteen pups per litter. The pups were kept with their mother and were fed with tap water and a pelleted rat chow *ad libitum* (Altromin, Breeding diet, B&K Universal AB, Sweden, containing 22.5% protein, 5% fat, vitamins and minerals and the rest carbohydrates). The experiments were approved by the Animal Ethics Committees in Lund and Malmö.

Design of delayed weaning experiments

To study the effects of delayed weaning, three litters ($n=20$ in total) were prevented from eating solid food, from day 14 to day 21, by placing the animals in specially designed cages with elevated cage lid, food pellets at a height of 17 cm above the cage floor (as compared with 8 cm in the control group). Thereby, the food was only available for the dam, while the pups were offered the dam's milk only. This group is hereinafter referred to as the delayed weaning (DW) group. Four control litters ($n=32$ in total), kept with their mothers in standard cages, were given extra access to solid food by placing food pellets on the cage floor once daily from day 14 to day 21. On day 21, the pups were separated from their mothers for 3 h prior to killing. The pups were lightly sedated with CO₂ and thereafter anaesthetised with a mixture of Ketamin (Ketalar, Park–Davis, Solna, Sweden, 0.5 mg/g body weight) and Azaperon (Stresnil, Janssen–Cilag Pharma, Vienna, Austria, 0.4 mg/g body weight) in 0.15 M NaCl. On killing, the stomach content was checked and the food separator group had mainly clotted milk, while the control group had a mixture of milk and chow fragments.

Tissue preparation

For histochemical analysis, the stomach and pancreas were dissected, fixed overnight in Stefanini's solution (2% paraformaldehyde and 0.2% picric acid in 0.1 M PBS, pH

7.2), rinsed thoroughly in Tyrode solution containing 10% sucrose and frozen on dry ice. Sections (10 µm thickness) were cut and thaw-mounted on slides.

Immunocytochemistry

Antibodies were diluted in PBS (pH 7.2) containing 0.25% BSA and 0.25% Triton X-100. Sections were incubated with primary antibodies (rabbit anti-ghrelin: code H-031-31, diluted at a ratio of 1:3200, Phoenix, Belmont, CA, USA; guinea pig anti-gastrin: code 8818, dilution of 1:1280, EuroDiagnostica, Malmö, Sweden; guinea pig anti-proinsulin: code 9003, diluted at a ratio of 1:2560, EuroDiagnostica) overnight at 4 °C in moisturising chambers. The sections were rinsed in PBS with Triton X-100 for 2×10 min. Thereafter, secondary antibodies with specificity for rabbit- or guinea pig-IgG, and coupled to either fluorescein isothiocyanate or Texas-Red (Jackson, West Grove, PA, USA), were applied on the sections. Incubation was for 1 h at room temperature in moisturising chambers. The sections were again rinsed in PBS with Triton X-100 for 2×10 min and then mounted in PBS:glycerol, 1:1. The specificity of immunostaining was tested using primary antisera pre-absorbed with homologous antigen (100 µg peptide/ml antiserum at working dilution). Double immunofluorescence was also used, with combinations of primary antibodies (rabbit and guinea pig antibodies). The two primary antibodies were incubated simultaneously overnight at 4 °C, followed by rinsing in PBS with Triton X-100 for 2×10 min. Thereafter, the two secondary antibodies were incubated simultaneously for 1 h at room temperature. In these studies, the controls included tests for inappropriate binding of the secondary antibodies.

In situ hybridisation

The ghrelin probe (Wierup *et al.* 2004) was complementary to the sequence 172–201 of rat ghrelin cDNA (GenBank accession number NM_021669; Kojima *et al.* 1999). BLAST search in the GeneBank database demonstrated lack of significant sequence similarity with any other mammalian cDNA. The probe was synthesised by DNA-technology A/S (Aarhus, Denmark) and was 3'-endtailed by [³⁵S]dATP (Perkin–Elmer, Stockholm, Sweden; Mulder *et al.* 1993). The *in situ* hybridisation protocol has been described previously (Mulder *et al.* 1993). In brief, sections were rapidly air-dried, fixed in 4% paraformaldehyde for 15 min, washed 2×5 min in PBS and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Thereafter, sections were dehydrated in graded ethanols, treated with chloroform for 5 min, ethanol (95.5%) for 5 min and again air-dried. Hybridisation was carried out in sealed moisturising chambers at 37 °C overnight, using a probe concentration of approximately 1 pmol/ml, followed by stringent post-hybridisation washing ($1 \times$ SSC; 0.15 M NaCl, 0.015 M sodium citrate). The slides were dipped in NTB 2 emulsion (Eastman Kodak Company, Rochester, NY, USA) and stored

in light sealed boxes at 4 °C for 10–18 days. They were then developed in Kodak D-19, fixed in Kodak polymax and mounted on Kaiser's glycerol gelatine. As controls, sense probe or excess unlabelled probe was used, as described previously (Wierup *et al.* 2002).

Imaging and cell quantification

Immunofluorescence was examined in an epifluorescence microscope (Olympus, BX60, Tokyo, Japan). By changing filters, the location of the different secondary antibodies in double staining was determined. *In situ* hybridisation radiolabelling was examined in bright- or dark-field. Images were captured with a digital camera (Olympus, DP50). Ghrelin immunoreactive (IR) cells were counted in immunostained sections of gastric fundus, according to previously published protocols (Sundler *et al.* 1995), using a fluorescence microscope (250× magnification; objective 25×, eyepiece 10× and visual field 0.9 mm). All ghrelin IR cells were counted in transverse sections (perpendicular to the mucosa surface) within 10–15 visual fields, covering the entire height and length of the mucosa (from rumen to antrum). This was repeated on three consecutive sections from each animal ($n=17$ for each group). The oxyntic identity of the mucosa was always verified by the lack of gastrin immunoreactivity. Pancreatic ghrelin cell density was counted as reported previously (Wierup *et al.* 2004). To quantify labelling for ghrelin mRNA in the fundus, images were taken as above ($n=8$ for each group). The density of *in situ* hybridisation labelling for ghrelin mRNA was calculated using Image Pro Plus software. Cells with labelling were delineated and the total cell area was calculated. Thereafter, the area of the silver grains within the delineated area was calculated. The density of labelling was expressed as labelled area per total cell area. At least 100 cells per animal were analysed. Oxyntic mucosa thickness was measured on transversely sectioned parts of the mucosa using Image Pro Plus software; at least 50 measurements were performed for each animal ($n=10$ for each group).

RIA for plasma ghrelin

Blood samples were taken from the heart in anaesthetised animals and collected into ice-chilled tubes containing 1.5 mg EDTA and 20 000 IU aprotinin (Trasylol; Bayer, Leverkusen, Germany). Thereafter, the samples were centrifuged and plasma was collected and stored at –80 °C before further handling. The concentration of total ghrelin (acylated and non-acylated) was measured using a commercially available rat ghrelin RIA kit (RK-031–31, Phoenix, Belmont, CA, USA).

Statistical analysis

Results are shown as mean $\pm$ S.E.M. Morphometric and plasma data were analysed using Student's unpaired *t*-test. Correlation

analysis was performed using Spearman's test. Differences with a value of $P<0.05$ were considered significant.

Results

Stomach growth and immunocytochemistry for ghrelin

In control rats, numerous ghrelin cells were seen from the base to the neck region of the glands in the oxyntic mucosa, as expected (Fig. 1A). However, in the DW group, the number of ghrelin cells in the oxyntic mucosa was markedly reduced

Figure 1 Fluorescence photomicrographs covering the entire height of the glands of rat oxyntic mucosa immunostained for ghrelin. At 21 days of age, rats with delayed weaning (B) kept in food separator cages display fewer ghrelin immunoreactive cells when compared with normally weaned control rats (A). Scale bar = 50 μ m.

(Fig. 1B). Morphometrical analysis of ghrelin cells per unit length of mucosa revealed that the DW rats had 30% lower ghrelin cell density than the control rats (DW group: 24.3 ± 0.7 cells/mm versus control group: 34.0 ± 1.4 cells/mm; $P < 0.0001$; Fig. 2A). There was no difference in stomach weight between the groups (DW group: 7.5 ± 0.5 g versus control group: 7.6 ± 0.4 g; NS; Fig. 2B), indicating that there was no general negative influence on stomach growth that could explain the observed difference in ghrelin cell density. Further, there was no difference in the thickness of the oxyntic mucosa between the groups (DW group: 430 ± 17 μ m versus control group: 454 ± 23 μ m; NS; Fig. 2C). On the other hand, the DW group had significantly lower body weight than the controls (DW group: 46.3 ± 1.3 g versus control group: 56.1 ± 1.1 g; $P < 0.0001$; Fig. 2D). To test if the difference in ghrelin cell density between the two groups could be explained by the difference in body weight, ghrelin cell density was normalised to body weight. Although less pronounced, the DW group still displayed significantly lower ghrelin cell density than the control group (DW group: 0.53 ± 0.036 cells/mm per g vs control group: 0.60 ± 0.021 cells/mm per g; $P = 0.031$; Fig. 2E). The reduced difference between the two groups that was seen after normalisation for body weight prompted analysis of the relationship between ghrelin cell density and body weight. Correlation analysis revealed that in the control group, the ghrelin cell density was positively correlated with the body weight ($r = 0.61$; $P = 0.0089$; Fig. 3A). Interestingly, no such correlation was evident in the DW group ($r = -0.14$; $P = 0.61$; Fig. 3B). Quantification of pancreatic ghrelin cell density revealed that there was no difference in the islet ghrelin cell density between the two groups (DW group: 0.26 ± 0.03 cells/visual field versus control group 0.22 ± 0.08 cells/visual field; NS; Fig. 4).

In situ hybridisation for ghrelin mRNA

Cells with labelling for ghrelin were numerous in the oxyntic mucosa of control animals, as expected (Fig. 5A). In the DW group such cells were fewer (Fig. 5B) and morphometrical analysis revealed that ghrelin mRNA expression, measured as the labelling density per cell, was 60% lower in the DW group than in the control group ($P < 0.01$; Fig. 6A). No or only very weak background labelling was detected in control sections when using sense probe or excess of unlabelled probe (not shown).

Plasma ghrelin

The DW group displayed 20% lower circulating concentrations of total ghrelin (des-acyl ghrelin and acylated ghrelin) than control animals (DW group: 140 ± 9 pmol/l versus control group: 173 ± 9 pmol/l; $P = 0.029$; Fig. 6B).

Discussion

The ghrelin cell density in both neonatal human and rat oxyntic mucosa is low when compared with the density in adults (Hayashida *et al.* 2002, Wierup *et al.* 2002, 2004). In rats, the ghrelin cell density remains low until the weaning period when it is greatly expanded, so that the density has reached adult levels at 20 days of age (Björkqvist *et al.* 2002, Hayashida *et al.* 2002, Wierup *et al.* 2004). Here, we show that delayed weaning, caused by preventing pups from eating solid food from day 14 to day 21, prevents the normal rise in both gastric ghrelin expression and plasma ghrelin concentration. Thus, fundic ghrelin cell density and ghrelin mRNA expression as well as circulating ghrelin concentration were

Figure 2 Morphometrical analysis revealed that the delayed weaning (DW) group has fewer ghrelin immunoreactive cells/mm mucosa than control (C) rats (A). The stomachs of DW rats and controls have similar weight (B) and comparable thickness of the oxyntic mucosa (C). DW rats have lower body weight than control rats (D); this does, however, not explain the difference in ghrelin cell density (in A), since a difference remains when compensating for body weight (E). * $P < 0.0001$, † $P < 0.05$ DW versus C.

Figure 3 Normal control rats have a positive correlation between oxyntic mucosa ghrelin cell density and body weight (A) ($r=0.61$; $P=0.0089$); no such correlation is seen in the delayed weaning group ($r=-0.14$; $P=0.61$) (B).

Figure 4 Fluorescence photomicrographs of rat pancreas double-immunostained for ghrelin (A and B) and insulin (C and D). A, C: delayed weaning. B, D: control. Delayed weaning does not affect the density of ghrelin cells in the pancreas. Location of ghrelin cells indicated by arrow heads. Scale bar = 50 μ m.

Figure 5 Rat oxyntic mucosa with *in situ* hybridisation labelling for ghrelin mRNA. Delayed weaning (DW) rats (B) display fewer cells with labelling for ghrelin than control rats (A). Note that the density of labelling per cell is weaker in the DW rats compared with the normal control rats. Scale bar=50 μ m.

significantly lower in the DW group when compared with the control group. The mechanism underlying our findings is not known, but could be related to the different nutrient composition of milk versus solid food. Rat milk contains high fat (60%) and low carbohydrates (<10%; Girard *et al.* 1992) when compared with the solid food used here. Intake of high-fat diet has been associated with low plasma levels of a ghrelin (Beck *et al.* 2002). One possibility is therefore that the high fat content of the milk suppresses ghrelin. Another possibility is that ghrelin is present in rat milk, as reported for human milk (Aydin *et al.* 2006, Kierson *et al.* 2006), and acts to suppress the endogenous ghrelin expression. Further studies are warranted to elucidate these mechanisms. While this study was in progress, it was demonstrated that early weaned rats have higher concentrations of total ghrelin in the stomach (Nishi *et al.* 2005). Thus, weaning appears to be important for regulating gastric ghrelin expression, and ghrelin follows the pattern of other gut hormones (Björkqvist *et al.* 2002). In contrast, other processes involved in gastric maturation, for example proliferation of the gastric

epithelium, are less dependent on nutrition, but instead more dependent on time (Gama & Alvares 2000). In the present study, we also found that the ghrelin cell density in the oxyntic mucosa was positively correlated to body weight in the control group. The observed correlation indicates a relationship between ghrelin cell density and body weight development. In contrast, no such correlation could be detected in the DW group. The observed difference indicates that the onset of solid food intake may trigger ghrelin cell development and could be required for establishing the normal relationship between ghrelin and body weight. We also found that the DW rats had a lower body weight than the control group; these findings are in agreement with previous observations made in rats (Lee *et al.* 1982, Gama & Alvares 2000) and humans (Kramer *et al.* 2004); for review see Rogers *et al.* (1997). The present finding of lower plasma concentration of ghrelin in the DW group is important and illustrates that the reductions in ghrelin cell density and ghrelin mRNA expression impact on systemic levels of ghrelin.

Figure 6 (A) Quantification of labelling density for ghrelin mRNA per cell. Delayed weaning (DW) rats display lower ghrelin mRNA expression per cell than control rats. DW rats have lower plasma concentration of total ghrelin when compared with control rats (B). * $P < 0.05$, [†] $P < 0.01$ DW versus C.

We previously reported that during the weaning period, when gastric ghrelin cell density was increasing, the density of ghrelin cells in the pancreatic islets decreased (Wierup *et al.* 2004). Since, in the present study, we observed a lower gastric ghrelin cell density in the DW rats, one could suspect that the normal decline in islet ghrelin cell density would be delayed. However, our present data showed that the islet ghrelin cell population was not affected by delayed weaning.

It is well documented that breastfeeding reduces the risk of the baby developing obesity, and it has also been argued that the duration of breastfeeding in humans may affect body weight later in life (Arenz *et al.* 2004). However, the mechanism behind this potentially protective effect is poorly understood. It was recently demonstrated that mice lacking ghrelin or GHS-R are resistant to diet-induced obesity when fed a high-fat diet during the early post-weaning period (Wortley *et al.* 2005, Zigman *et al.* 2005). In view of these findings, our present data showing decreased ghrelin expression and plasma ghrelin as a result of delayed weaning are intriguing. Although we have not examined body weight later than day 21, we suggest that ghrelin may represent a hormonal link between the duration of breastfeeding and body weight development later in life. Interestingly, Pinto *et al.* (2004) demonstrated that ghrelin is able to alter the ratio of excitatory to inhibitory synapses on hypothalamic pro-opiomelanocortin neurons. These neurons are indeed known to regulate satiety (for review see Harrold & Williams 2006), and it is not inconceivable that synaptic alterations during their development may have a long-term impact on their excitability. Thus, it can be hypothesised that lower ghrelin levels, caused by delayed weaning, lead to alterations in synapses in appetite regulating neurons, which in turn may lead to altered long-term appetite settings.

In conclusion, this study demonstrates for the first time that delayed weaning maintains low gastric ghrelin expression and low plasma levels of ghrelin. In addition, the increase in plasma ghrelin and gastric ghrelin expression seen at weaning appear to be related to the onset of solid food intake and not merely to age.

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