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Changes of Plasma Obestatin, Ghrelin and NPY in Anorexia and Bulimia Nervosa Patients Before and After a High-Carbohydrate Breakfast

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Summary

Peptides ghrelin, obestatin and neuropeptide Y (NPY) play an important role in regulation of energy homeostasis, the imbalance of which is associated with eating disorders anorexia (AN) and bulimia nervosa (BN). The changes in ghrelin, obestatin and NPY plasma levels were investigated in AN and BN patients after administration of a high-carbohydrate breakfast (1604 kJ). Eight AN women (aged 25.4±1.9; BMI: 15.8±0.5), thirteen BN women (aged 22.0±1.05; BMI: 20.1±0.41) and eleven healthy women (aged 25.1±1.16; BMI: 20.9±0.40) were recruited for the study. We demonstrated increased fasting ghrelin in AN, but not in BN patients, while fasting obestatin and NPY were increased in both AN and BN patients compared to the controls. Administration of high-carbohydrate breakfast induced a similar relative decrease in ghrelin and obestatin plasma levels in all groups, while NPY remained increased in postprandial period in both patient groups. Ghrelin/obestatin ratio was lower in AN and BN compared to the controls. In conclusions, increased plasma levels of fasting NPY and its unchanged levels after breakfast indicate that NPY is an important marker of eating disorders AN and BN. Different fasting ghrelin and obestatin levels in AN and BN could demonstrate their diverse functions in appetite and eating suppression.

Key words

Anorexia and bulimia nervosa • Ghrelin • Obestatin • NPY • High-carbohydrate breakfast

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Introduction

Anorexia nervosa (AN) and bulimia nervosa (BN) are psychiatric and somatic diseases, occurring mainly in young women, characterized by abnormal eating behaviour and an imbalance in energy homeostasis. Peptides of gut-brain axis play a pivotal role in the regulation of energy homeostasis. Neuropeptide Y (NPY), belonging to family of peptides synthesized in neural tissue of central (hypothalamus and steam brain) and peripheral nervous system, exerts diverse biological and pathological actions that bear on all major vital systems (Hillebrand *et al.* 2002). Recent studies have suggested that NPY is not merely an "orexigen", but acts to stimulate behaviour which precedes the food intake and actually inhibits intake per se (Sederholm *et al.* 2002, Ammar *et al.* 2005). The treatment with NPY increased physical activity and decreased food intake and caused a loss of body weight in rats (Nergardh *et al.* 2007). This finding can be in line with clinical observation in AN patients who are physically hyperactive and eat only a little food in spite of having depleted body fat and pathologically up-regulated hypothalamic orexigenic peptides (Holtkamp *et al.* 2003, Nergardh *et al.* 2007).

One of the main orexigenic peptides ghrelin, discovered by Kojima *et al.* (1999), is synthesized mainly in the stomach and transmits changes in food intake to the central nervous system through ghrelin receptors, which are localized on NPY neurons in the brain and the most of peripheral tissues (Papotti *et al.* 2000). Ghrelin has been shown to stimulate appetite and is involved in the regulation of energy balance. Increased fasting plasma ghrelin levels have been reported in underweight AN patients in several studies (Nedvidkova *et al.* 2003, Troisi *et al.* 2005, Janas-Kozik *et al.* 2007, Dostalova and Haluzik 2009), while only small group of researchers found increased fasting plasma ghrelin levels also in BN patients with a normal body mass index (BMI) (Tanaka *et al.* 2002, Kojima *et al.* 2005).

The most of authors have reported normal baseline ghrelin levels in symptomatic bulimic subjects (Monteleone *et al.* 2005, Rouach *et al.* 2007, Monteleone *et al.* 2008). Recently, gut peptide obestatin identified by Zhang *et al.* (Zhang *et al.* 2005) was found to originate from post-translational processing of the preproghrelin peptide in a similar manner as ghrelin. It was observed that obestatin had a suppressive effect on food intake in mice when injected peripherally or into the brain ventricles (Zhang *et al.* 2005, Lagaud *et al.* 2007). The majority of subsequent studies, however, did not reproduce the anorexigenic property of obestatin that was initially reported (Gourcerol *et al.* 2007, Monteleone *et al.* 2008, Nakahara *et al.* 2008, Germain *et al.* 2009). In our previous study, we observed a decrease in obestatin and ghrelin in the circulation of healthy subjects following a high-carbohydrate breakfast (Sedlackova *et al.* 2008), an increase in fasting plasma ghrelin and obestatin levels in AN patients, and a decrease in fasting plasma ghrelin and obestatin levels in obese patients (Zamrazilova *et al.* 2008). Since it is generally believed that ghrelin exerts its orexigenic effect mainly by activation NPY neurons (Goto *et al.* 2006), and because changes in basal plasma ghrelin and obestatin have been found in patients with AN and BN, we believe that these hormones may be involved in the pathophysiology of these disorders. Thus, in the present study, we measured the fasting and postprandial responses of ghrelin, obestatin and NPY to a high-carbohydrate breakfast. We assessed the changes of plasma levels of these hormones during the postprandial period to find out more about their relationship to food intake in AN and BN patients. The control group used for comparison consisted of healthy women.

Material and Methods

Study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Institute of Endocrinology in Prague. Prior to the study each participant signed an informed consent form.

Study subjects

Eight AN women patients with restrictive and purgative type of anorexia nervosa (age: 25.4±1.9 years; BMI: 15.8±0.5 kg/m^2), thirteen BN women patients (age: 22.0±1.05 years; BMI: 20.1±0.41 kg/m^2) and eleven healthy women (age: 25.1±1.16 years; BMI: 20.9±0.40 kg/m^2) were recruited for this study. All subjects included in the study were nonsmokers, had no allergies and had been free of medications for at least two weeks prior the study. AN and BN patients were diagnosed according to the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders, American Psychiatric Association, 1994. All AN and BN patients were clinically stable and in relatively good health, except for their eating disorder and amenorrhea in AN patients and/or irregular cycle in BN patients. In AN patients group were included 5 patients with restrictive type and 3 patients with purgative type of AN. In BN patients group the average frequency of binge-purging episodes was 2.8x per day. Average duration of eating disorder was counted for all patients. In AN patients, the average duration of disorder was 6 years and 10 months, in BN patients 5 years and 6 months. All patients were investigated after 1 week of hospitalization at the Department of Psychiatry at Charles University, Prague. Healthy volunteers (C) had no history of cardiovascular disease, eating disorders or other psychiatric disease, had normal electrocardiogram (ECG), blood count, liver and renal function. All healthy women had regular menstrual cycle and were in follicular phase of the cycle.

Blood tests conducted before initiation of the study confirmed normal values for blood count, fasting blood glucose, liver and renal function. Participants were recommended to avoid vigorous physical activity during the 14-hour period before blood samplings. All subjects consumed a standardized dinner at 6.00 PM and were then asked to fast overnight. Reported duration of sleep in the night preceding blood sampling was comparable in all studied subjects. All participants were admitted to the Institute of Endocrinology at 7.30 AM. Overall, the study lasted about 3.5 hours and the protocol consisted of high-

carbohydrate breakfast consumption and blood withdrawals. Body composition was measured using method of bioimpedance (Tanita, Japan) together with other physical examination before the beginning of the test.

Study design

Each subject received a high-carbohydrate breakfast with a total energy content of 1604 kJ, consisting of 81.9 g carbohydrates, 8.8 g proteins and 3.4 g fats in the form of a white bread roll (90 g) and strawberry jam (50 g). In addition, the subjects consumed 250 ml of fruit tea without sugar or other sweetener with the meal. Participants were given 15 minutes to consume their meal. Blood samples were drawn from the cubital vein using an intravenous cannula, the first blood drawn was collected before meal, and then 30, 60, 90, 120 and 150 min after breakfast consumption. Blood samples were collected into chilled polypropylene tubes containing Na_2EDTA and antilysin. Plasma was immediately separated by 15-min centrifugation at 5 °C and stored at –70 °C until being assayed.

Analytical measurements

Plasma obestatin immunoreactivity was measured by a commercial RIA kit (Phoenix Pharmaceuticals Inc., Belmont, CA, U.S.A.), the intra- and interassay variability was 5.0 % and 14.2 %, respectively, sensitivity was 50 pg/ml. Total plasma ghrelin and NPY were determined by commercially available RIA kits (Linco Research, Inc., St. Charles, Missouri, U.S.A.). The intra- and interassay variability for total ghrelin was 6.4 % and 16.3 %, sensitivity was 93 pg/ml, for NPY the intra- and interassay variability was 5.0 % and 8.4 % respectively, sensitivity was 3 pmol/l.

Statistical analysis

The dependence of hormone levels on patient's status and stage of the meal test was evaluated using repeated measures ANOVA model consisting of subject factor, between-subject factor "patient's status", within-subject factor "stage of the meal test" and interaction "patient's status" × "stage of the meal test". Least significant difference multiple comparisons followed the ANOVA testing. The level of statistical significance $P<0.05$ was chosen for both ANOVA and multiple comparisons. Due to non-Gaussian data distribution in all dependent variables these underwent power transformations to attain distributional symmetry and a constant variance both in the data and residuals.

The relationships between NPY, and patient's status, ghrelin and obestatin levels were evaluated using multivariate regression (the method of orthogonal projections to latent structures), which was robust to multicollinearity. Gaussian distribution and occurrence of non-homogeneities persisting in the data after power transformation were checked using normal probability plot and Hotteling's statistic, respectively.

The statistical software Statgraphics Centurion v. XV from Statpoint, Inc. (Herndon, Virginia, USA) and SIMCA v. 12.0 from Umetrics (Umeå, Sweden) were used for data analysis.

Results

The fasting plasma ghrelin, obestatin and NPY levels, and their postprandial responses to high-carbohydrate breakfast in C, AN and BN patients, as well as the ghrelin/obestatin ratio are summarized in Figure 1. Basic anthropometric and biochemical parameters for all groups are summarized in Table 1.

Table 1. Anthropometric and biochemical parameters (AN, BN, C).

Variable	AN (n=8)	BN (n=13)	C (n=11)
Age (years)	25.4±1.9	22.0±1.05	25.1±1.16
BMI (kg/m^2)	15.8±0.5*	20.1±0.41	20.9±0.40
Lean body mass (%)	87.5±1.2*	74.2±2.3	69.9±2.7
Fat mass (%)	11.9±1.4*	25.3±1.3	29.2±1.6
IGF-I (ng/ml)	253.42±33*	395.77±54	455.76± 41
IGF BP-III (ng/ml)	2832.54±138*	3286.17±241	3478.56±112

Data are expressed as mean ± SEM. Index * means significant differences between AN, BN and C ($p<0.05$).

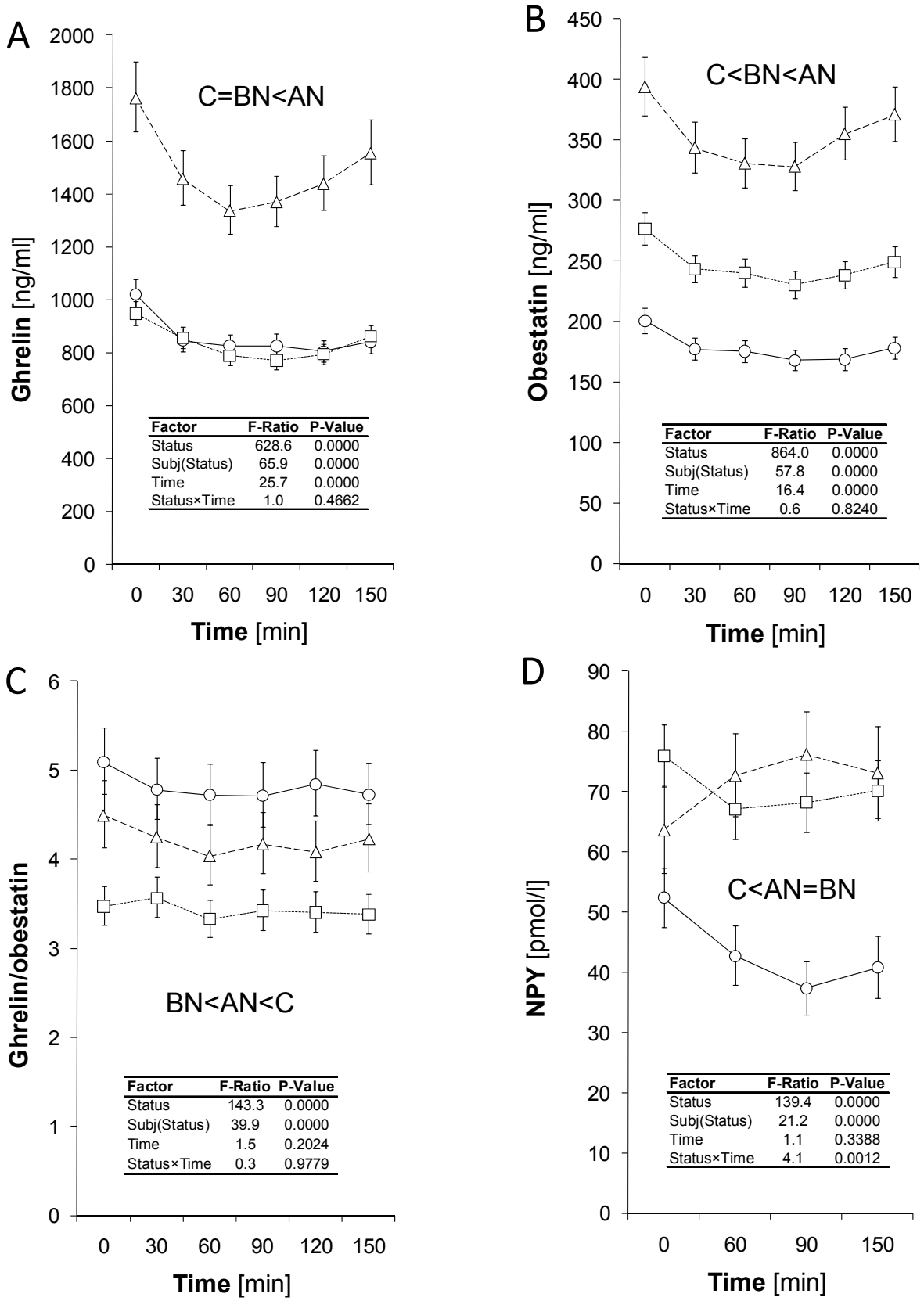

Fig. 1. Serum levels of NPY, ghrelin, obestatin and ghrelin/obestatin ratio during the meal test. The circles, triangles and squares with error bars represent the retransformed means with their 95 % confidence intervals for the controls (C), patients suffering with anorexia nervosa (AN), and patients suffering with bulimia nervosa (BN), respectively as evaluated by repeated measures ANOVA followed by least significant difference multiple comparisons. For the details see Statistical data analysis. The character "<" in the smaller embedded table means "significantly lower than" as found using least significant difference multiple comparisons for the levels of the factor "Status".

Table 2. Dependence of NPY on ghrelin, obestatin and on the anorexia and bulimia status as evaluated using multivariate regression (ortogonal projection to latent stuctures).

Variables		Parameter	95 % Confidence interval of the parameter	Parameter/ 95 % CI	Significance of the parameters	Correlation with the common predictive component
NPY	*Dependent variable*	1.000	0.289	3.47	p<0.01	0.506
Anorexia	*Predictors*	0.391	0.037	10.66	p<0.01	0.574
Bulimia		0.272	0.099	2.76	p<0.01	0.400
Controls		–0.626	0.085	–7.34	p<0.01	–0.919
Ghrelin		0.318	0.089	3.56	p<0.01	0.467
Obestatin		0.553	0.089	6.22	p<0.01	0.812
Time		–0.065	0.120	–0.54	NS	–0.095
$R^{2\,a)}$				25.6 %		
$Q^{2\,b)}$				23.9 %		

[a)] R^2 is the percent of variation of dependent variable explained by the model. [b)] Q^2 is the percent of variation of dependent variable predicted by the model according to cross validation.

The fasting plasma ghrelin levels were significantly increased in AN patients as documented by significance factor "patient's status" and significant multiple comparisons AN vs. C and AN vs. BN (Fig. 1A). The effect of breakfast was also significant showing U-shaped dependence regardless of the patient's status, reaching lowest level in 60th minute as clearly documented by significant factor "stage of the meal test", by insignificant interaction "patient's status" × "stage of the meal test" and by multiple comparisons for individual stages of the test.

As documented by insignificant interaction "patient's status" × "stage of the meal test" and by multiple comparisons for individual stages of the test, plasma obestatin levels postprandially showed a similar trend to ghrelin, with the minimum in the 90th minute. Obestatin levels were significantly higher in AN as compared to BN, and levels in BN were significantly higher than in C, as shown from significant factor "patient's status" and from multiple comparisons for individual groups (Fig. 1B).

The fasting ghrelin/obestatin ratio was lower in AN patients than in C and the ratio in BN was significantly lower than that for AN. These results are obvious from Figure 1C showing significant factor "patient's status" and significant differences when using multiple comparisons for individual groups. The effect of the meal test was insignificant for the ghrelin/obestatin ratio.

NPY exhibited no differences between AN and BN but both groups showed higher levels when compared with C. As documented by significant interaction "patient's status" × "stage of the meal test" as well as by multiple comparisons the effect of the meal test differed from group to group. While AN showed insignificant decrease and BN insignificantly increasing trend, C expectedly significantly decreased up to the 60th min.

The relationships between NPY, ghrelin and obestatin were analyzed using multivariate regression. As demonstrated in Table 2, NPY was positively associated with BN and AN, and negatively with C. This means that NPY levels are higher in AN and BN than in C. NPY was strongly positively correlated with obestatin and less strongly but still significantly with ghrelin. Using the regression model, NPY levels were independent of the stage of the meal test. The last finding is in contradiction with the results of ANOVA test, evidently due to absence of interaction "patient's status" × "stage of the meal test" in the regression model.

Patients with AN had significantly lower fat mass (%) and significantly higher lean body mass (LBM,

%) comparing to other two groups. The BMI was counted for every group, AN patients had significantly lower BMI comparing to BN patients and the controls. BN patients had significantly higher BMI compared with AN patients and similar to the controls.

Discussion

The most important finding of the present study is that fasting NPY was increased in AN and BN patients and did not change after a high-carbohydrate breakfast, in spite of the decreased ghrelin and obestatin levels, in underweight AN and in BN patients compared with healthy women. Further, in contrast to AN patients, BN patients had increased fasting obestatin, but not fasting ghrelin levels. The ghrelin to obestatin ratio was lower in both patient groups compared with the control group.

In our previous study, we documented that the standard breakfast-induced suppression of increased plasma ghrelin in AN patients was almost completely absent (Nedvidkova *et al.* 2003). The type of nutrients and total calories provided in breakfast may influence the ghrelin response to food intake as we observed in the study with a high-fat meal (Nedvidkova *et al.* 2003), similarly as with other authors (Erdmann *et al.* 2004, Nakai *et al.* 2004, Otto *et al.* 2005). The duration of total anorectic status and the variation in the clinical characteristics of patient samples, has been suggested to explain these discrepancies. For example, Tanaka *et al.* (2003) observed a decrease of increased fasting plasma ghrelin levels to oral glucose administration in dependence on AN type, with the decrease evident in the restrictive type of AN and a normal but delayed response in AN patients with habitual binge-purge behaviour. The initially claimed anorexigenic role of obestatin in mice (Zhang *et al.* 2005, Guo *et al.* 2008, Monteleone *et al.* 2008) was highly negated by a number of authors who recently reported that they have been unable to reproduce these initial findings (Samson *et al.* 2007, Monteleone *et al.* 2008, Germain *et al.* 2009). In previous study we observed a similar decrease of plasma ghrelin and obestatin in healthy subjects following a high-carbohydrate breakfast (Sedlackova *et al.* 2008). The present and other studies indicate that both fasting plasma ghrelin and obestatin concentrations are increased in AN patients compared with the controls (Harada *et al.* 2008, Monteleone *et al.* 2008, Nakahara *et al.* 2008, Germain *et al.* 2009), but we observed the ghrelin/obestatin ratio to be decreased in the postprandial period in these patients. These results indicate that obestatin secretion is higher than ghrelin secretion in AN individuals compared with the controls. This could have different effects on food intake.

In comparison with underweight AN patients we observed normal fasting ghrelin levels in BN patients, also documented by other researchers (Nakazato *et al.* 2004, Monteleone *et al.* 2005, Troisi *et al.* 2005). These authors suppose that it is unlikely that ghrelin plays a role in the pathophysiology of BN (Monteleone *et al.* 2005, Troisi *et al.* 2005). On the other hand, Tanaka *et al.* (Tanaka *et al.* 2002) demonstrated that mean plasma ghrelin levels in BN patients were significantly higher than in the controls, though mean BMI between the groups were not significantly different. These findings may lead to presumption that an abnormal eating behaviour with habitual binge eating and purging of different intensity may have a different influence on gastric secretion and circulating ghrelin levels in BN. The increased fasting plasma obestatin but not ghrelin levels in BN patients could be explained by action of these two peptides on different levels in eating behaviour regulations. Further research in physiology and pathophysiology of obestatin is needed for clarifying these findings. It may be supposed that regulation of energy homeostasis could depend upon the ratio between ghrelin and obestatin peptides. We documented a decrease of the ghrelin to obestatin ratio in both AN and BN patients. However, we did not confirm the higher ghrelin to obestatin ratio in AN patients observed by Monteleone *et al.* (2008). Monteleone *et al.* believe that a higher prevalence of binge-purging in the AN patients in their group may have been the cause of this discrepancy (Monteleone *et al.* 2008). Our results support previous observations of Germain *et al.* (2009) who described decreased acylated ghrelin to obestatin and total ghrelin to obestatin ratios in AN patients as compared with constitutionally thin and the control subjects. We may speculate that increased expression of the preproghrelin gene likely does not occur in a one to one ratio for ghrelin and obestatin and may be one of causes of the lower ghrelin to obestatin ratio in our AN and BN patients.

To date no study has reported the increase of fasting NPY and mostly absent response to a high-carbohydrate breakfast in both AN and BN patients compared with healthy women. Currently we cannot confidently explain these findings. Recently, a novel peripheral site for NPY biosynthesis was found in adipocytes where NPY stimulates proliferation of primary preadipocytes (Kos *et al.* 2007, Yang *et al.* 2008) and in this manner participates on adipogenesis. We may

speculate that by this manner NPY leads to increased functioning of the defensive system of the organism before exhaustion of energy reserves. However, the possibility that higher NPY biosynthesis in the adipose tissue may occur or rather its higher brain secretion leads to its increased plasma levels in AN and BN patients is difficult to interpret and needs to be clarified by further studies.

The increased plasma ghrelin stimulates NPY synthesis in the brain, which in turn stimulates food intake (Gil-Campos *et al.* 2006). However, we found increased plasma NPY levels and decreased plasma ghrelin levels during the postprandial period in AN patients. Thus, we did not demonstrate that feedback between ghrelin and NPY in AN patients exists. In BN patients with normal BMI levels, we found increased basal NPY. Increased response of NPY to exercise compared with the controls was also observed in our previous study (unpublished results). This may indicate that basal plasma NPY levels are not directly related to lower BMI, but rather to eating disorder itself.

On the basis of the strongly positive correlation between NPY and obestatin and the weaker, but still significant, correlation with ghrelin, we suppose that obestatin might influence the appetite differently from the ghrelin molecule. The increase of both fasting ghrelin and obestatin levels in AN but only plasma obestatin in BN demonstrates that obestatin might use other pathways to modulate appetite than ghrelin does. Higher fasting NPY levels with antilipolytic properties and almost absent response to high-carbohydrate breakfast in both patient groups might indicate protection of the organism before energetic exhaustion.

In conclusion, we assume that increased fasting NPY levels unchanged after a high-carbohydrate breakfast indicates that NPY may be an important marker for disturbed eating behaviour in AN and BN. The distinct fasting ghrelin and obestatin levels together with simultaneously increased NPY levels in AN and BN patients may document different pathways or efficiency of obestatin and ghrelin to modulate the appetite and eating behaviour. Further studies are needed to elucidate, if changes in plasma hormone levels in patiens with AN and BN are the cause or rather the consequence of eating disorders.

Conflict of Interest

There is no conflict of interest.

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