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Plasma Obestatin and Ghrelin Levels in Subjects With Prader–Willi Syndrome

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Abstract

Prader–Willi syndrome (PWS) is an obesity syndrome characterized by rapid weight gain and excessive food intake. Food intake is regulated by the hypothalamus but directly influenced by gastrointestinal peptides responding to the nutritional status and body composition of an individual. Ghrelin, derived from preproghrelin, is secreted by the stomach and increases appetite while obestatin, a recently identified peptide derived post-translationally from preproghrelin, works in opposition to ghrelin by decreasing appetite. The objective of this study was to measure fasting obestatin and ghrelin levels in peripheral blood of subjects with PWS and compare to age and gender matched control subjects. Plasma obestatin and ghrelin levels were measured in subjects with PWS ($n = 16$, mean age 16.0 ± 13.3 years; age range 1–44 years) and age gender matched control subjects ($n = 16$). Significantly higher obestatin levels were seen in the 16 PWS subjects (398 ± 102 pg/ml) compared with 16 controls (325 ± 109 pg/ml; matched t -test, $P = 0.04$), particularly in 5 young (< 3 years old) PWS subjects (460 ± 49 pg/ml) compared with 5 young controls (369 ± 96 pg/ml; matched t -test, $P = 0.03$). No significant difference in ghrelin levels was seen between the PWS and comparison groups. No significant correlation was observed for either peptide when compared with body mass index but a significant negative correlation was seen for ghrelin and age in PWS subjects. Our observations suggest that obestatin may be higher in infants with PWS compared to comparison infants. The possibility that obestatin may contribute to the failure to thrive which is common in infants with PWS warrants further investigation.

Keywords

Prader–Willi syndrome (PWS); obestatin; ghrelin; obesity; appetite/satiety hormones

INTRODUCTION

Prader–Willi syndrome (PWS) is a complex neurodevelopmental disorder characterized by infantile hypotonia, hypogonadism, small hands and feet, short stature, growth hormone deficiency, hyperphagia, early childhood obesity, mental deficiency, and a typical facial appearance [Butler, 1990; Cassidy, 1997; Bittel and Butler, 2005]. A de novo paternally derived chromosome 15q11–q13 deletion is seen in about 70% of subjects while maternal

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disomy 15 (both chromosome 15s from the mother) is seen in about 25% of cases and an imprinting center defect in the remaining subjects [Butler, 1990; Cassidy, 1997; Nicholls and Knepper, 2001; Bittel and Butler, 2005].

The course and natural history of PWS has been divided into two distinct stages [Butler, 1990; Cassidy, 1997; Butler and Thompson, 2000; Butler et al., 2006]. The first stage occurs during infancy and is characterized by central hypotonia, feeding difficulties and failure to thrive. The second stage is characterized by rapid weight gain due to hyperphagia and food foraging leading to obesity. The severe hyperphagia mediated obesity appears to result from a faulty satiety mechanism [Shapira et al., 2005; Butler et al., 2006; Holsen et al., 2006].

The regulation of food intake is complex and controlled through the hypothalamus including the melanocortin and neuropeptide Y (NPY) systems in the arcuate nucleus [Schwartz et al., 2000]. The NPY Y2 receptor (Y2R) is thought to be an inhibitory presynaptic receptor and is accessible to peripheral hormones [Broberger et al., 1997]. Peptide YY (PYY) is a Y2R agonist and released by the gastrointestinal tract [Adrian et al., 1985; Pedersen-Bjergaard et al., 1996].

Ghrelin, a novel 28-amino acid peptide, is derived by post-translational processes from preproghrelin consisting of 117 residues, secreted by the stomach with specific receptors in the brain. There are two forms of ghrelin, an inactive form and an active (acylated) form which conveys information to the brain thereby increasing appetite, food intake and body weight and influences the release of growth hormone [Tschop et al., 2000; Tschop et al., 2001; Cummings et al., 2002b]. Ghrelin levels are inversely correlated with body weight and are higher during weight loss [Tschop et al., 2000, 2001; Shiiya et al., 2002]. Leptin which is secreted by adipose tissue in direct relationship to the total fat mass of an individual plays a role in eating behavior promoting satiety and body mass regulation. The JAK/STAT signal transduction pathway is involved in this process [Dagogo-Jack et al., 1996; Lahlou et al., 1997; Fruhbeck, 2006]. Leptin and ghrelin both induce central effects through the neuropeptide Y receptor pathway in the hypothalamus [Chanoine et al., 2002].

Recently, another peptide derived post-translationally from preproghrelin was isolated from rat stomach and named *obestatin*. Obestatin is a 23-amino acid peptide flanked by a glycine residue at the C-terminus. There appears to be two forms of obestatin, an inactive form and an active (amidated) form. Both ghrelin and obestatin are post-translationally modified and act through distinct receptors [Zhang et al., 2005]. Contrary to the appetite stimulating effects of ghrelin, treatment of rats with obestatin suppresses food intake, inhibits jejunal contraction and decreases body weight gain [Zhang et al., 2005]. Thus, these two peptides appear to have opposing action on appetite and weight regulation but are derived from the same gene and polypeptide.

Obestatin reportedly binds to an orphan G protein-coupled receptor (GPR39) but does not readily cross the blood brain barrier and is rapidly degraded [Pan et al., 2006]. It is highly conserved across species with sequence homologies of 87% between rodent and human obestatin [Zhang et al., 2005]. Serum obestatin concentrations in rodents are more than 300 pg/ ml and not affected by fasting or refeeding [Pan et al., 2006] but limited data exists in

humans specifically in PWS [Park et al., 2006]. Park et al. [2006] studied 15 children (ages 10–12 years) with PWS and found that plasma obestatin was not elevated in PWS relative to control subjects and is not regulated by insulin in either PWS children or obese control subjects.

PYY, ghrelin, leptin and now obestatin, are primary candidates to investigate in PWS, particularly during infancy and early childhood when eating behavior is markedly different during stage one compared with stage two. Leptin levels generally reflect the higher body weight or fat mass in PWS subjects [Butler et al., 1998; Pietrobelli et al., 1998; Myers et al., 2000]. Fasting plasma ghrelin levels are generally elevated in subjects (infants, children and adults) with PWS compared with lean and obese control subjects [DeParigi et al., 2002; Cummings et al., 2002a; Haqq et al., 2003; Butler et al., 2004]. Expression of the ghrelin gene has also been reported in different brain regions in both PWS and control subjects [Talebizadeh et al., 2005]. Analysis of ghrelin gene expression by RT-PCR was examined in six different regions of the brain and detected in all subjects (PWS, Angelman syndrome and control subjects) but no obvious differences were seen in the pattern of expression between the subjects [Talebizadeh et al., 2005]. Our current study of fasting plasma obestatin levels including individuals of all ages (infants to adults) with PWS along with age and gender matched controls is the first to compare plasma obestatin levels with ghrelin, clinical and genetic subtype data.

SUBJECTS AND METHODS

Subjects

Sixteen subjects with PWS (8 females, 8 males) were studied with an average age $\pm$ SD of 16.0 ± 13.3 years and age range of 12 months to 44.3 years (Table I). PWS was confirmed by genetic testing (chromosome analysis with FISH and methylation PCR). Seven subjects had the typical 15q11-q13 deletion seen in PWS and nine had maternal disomy 15. Five subjects with PWS and five matched control subjects were less than 3 years of age. Informed consent was approved through the local institutional review board and obtained from the patient, parent or by assent from the adolescent. Height and weight were obtained for each subject and body mass index (BMI) calculated (kg/m^2). The average $\pm$ SD for BMI for the PWS subjects was 30.3 ± 14.1 and 24.3 ± 8.5 for the control subjects. Similarly, aged matched control subjects (8 females, 8 males) were selected from a larger pool of control subjects recruited from a research program on PWS including clinical description and genotype/phenotype correlations. The average age $\pm$ SD for the control subjects was 15.9 ± 13.8 years and age range of 12 months to 44 years (Table I). Three of our children with PWS (one female age 1 year 8 months; two males, ages 1 year and 2 years 7 months) were treated with growth hormone for a few weeks to 24 months duration.

Obestatin and Ghrelin Assay

Peripheral fasting blood was collected in a refrigerated EDTA vacutainer tube containing aprotinin, a specific preservative for gastrointestinal protein. The plasma was immediately separated after centrifugation and stored at -70°C until used. Obestatin and ghrelin levels expressed in picograms per milliliter were measured commercially by Inter Science Institute

(Inglewood, CA) using radioimmunoassay (RIA) following established protocols published elsewhere [Adrian et al., 1985; Shiiya et al., 2002; Butler et al., 2004]. Specifically, the obestatin assay is based upon the competition between labeled ^{125}I -obestatin (human, monkey) and obestatin (either standard or unknown) bound to a limited quantity of antibodies specific for obestatin in each reaction mixture. It uses polyclonal antibodies raised in rabbit against a synthetic obestatin peptide [Adrian et al., 1985; Shiiya et al., 2002; Butler et al., 2004]. A similar process was required for development of the ghrelin assay and standardized in the laboratory. Plasma samples were tested for the active (amidated) form of obestatin by RIA.

Laboratory specificity was determined for both obestatin and ghrelin at 50% inhibition of binding level for these peptides compared with binding to other gastrointestinal peptides. Cross reactivity for both obestatin and ghrelin was 100% for their respective assay and $<0.01\%$ for other measured gastrointestinal and related peptides, such as ghrelin, motilin, PYY, alpha-MSH, CART and AGRP for the obestatin assay. Sensitivity determined as the least amount of ghrelin and obestatin that can be distinguished from zero was 50 pg/ml. Based on three controls assayed in different runs in the laboratory setting, the intra-assay and inter-assay coefficients of variation were 8.0% and 4.1% for obestatin and 5.5% and 2.0% for ghrelin, respectively. The expected range for fasting ghrelin was 520–700 pg/ml (for normal weight adults) and up to 120 pg/ml for obese subjects following post-gastric bypass surgery (Inter Science Institute) [Butler et al., 2004]. Reference laboratory standards in humans have not been established at this time for fasting plasma obestatin levels.

We used the statistical package, SPSS v 12.0, for evaluating data. Statistical analyses for our study included comparison of group means by parametric paired samples and independent samples *t* tests. Pearson correlation coefficients were calculated to examine covariation between ghrelin and obestatin levels, ghrelin and age, ghrelin and BMI, obestatin and age, and obestatin and BMI for subjects with PWS and matched controls. Significance for all tests was set at a $P = 0.05$.

RESULTS

Sixteen subjects with PWS (8 females and 8 males) were analyzed with a mean age $\pm$ SD of 16.0 ± 13.3 years and mean BMI $\pm$ SD of 30.3 ± 14.1 kg/m² and 24.3 ± 8.5 for matched controls (Table I). Eight of the 16 subjects with PWS and four of the controls had BMI scores greater than the 85th centile which supported an overweight to obese status. The 85th centile for BMI is used to demarcate an overweight status while 95th centile is used to demarcate obesity in children (see the American Obesity Association website at http://www.obesity.org/subs/fastfacts/obesity_youth.shtml). The mean ghrelin $\pm$ SD concentration was 626 ± 457 pg/ml and 398 ± 102 pg/ml for obestatin in subjects with PWS. The mean $\pm$ SD ghrelin and obestatin levels were 521 ± 269 pg/ml, and 325 ± 109 pg/ml, respectively in our controls (Table II). There was a significantly higher plasma obestatin level in the 16 PWS subjects when age and gender matched with 16 controls ($P = 0.04$). In addition, young PWS subjects (< 3 years of age) were found to have higher obestatin levels when compared with matched control subjects ($P = 0.03$) although the number of subjects in each group was small ($N = 5$). PWS males also had higher plasma obestatin levels compared with matched

control males ($P=0.04$). Although there was a trend toward higher ghrelin levels in the PWS group compared to controls, it did not meet statistical significance in our study (Table II). No significant difference was found in ghrelin or obestatin levels in our PWS or control subjects older than 3 years of age. Generally, no difference was found in average obestatin or ghrelin levels between males or females with PWS or for controls but higher ghrelin levels were found in male controls compared with female controls ($P=0.002$) in our study. When comparing non-obese subjects with or without PWS, higher obestatin levels were seen for the non-obese PWS subjects only ($P=0.005$).

Three subjects with PWS were receiving growth hormone for a few weeks to 24 months duration (one female age 1 year 8 months; two males, ages 1 year and 2 years 7 months). No difference was found in ghrelin or obestatin levels in those PWS subjects treated with growth hormone compared to other PWS subjects. There was also no significant difference in ghrelin or obestatin levels in the PWS subjects with the 15q deletion compared to those with maternal disomy 15 (Table II).

Pearsonian correlation analysis was also performed to compare ghrelin with obestatin levels, ghrelin with age, ghrelin with BMI, obestatin with age, and obestatin with BMI for subjects with PWS and matched controls. Generally, no significant correlations were found for the parameters studied; however, there was a significant negative correlation ($r=-0.52$; $P=0.04$) for ghrelin level and age in the subjects with PWS (see Fig. 1).

DISCUSSION

PWS is considered the most common known genetic cause of morbid obesity in humans, hence it is an ideal syndrome to investigate the relationship of the two peptide hormones (obestatin and ghrelin) produced from the same gene with apparently opposing action in regulating appetite and energy metabolism. We analyzed the relationship between fasting plasma obestatin and ghrelin levels in both PWS and age and gender matched controls for obesity status and genetic subtype effects to determine if obestatin levels are altered in PWS subjects. We found no differences in fasting obestatin or ghrelin levels when comparing PWS genetic subtypes (deletion or UPD); ghrelin or obestatin levels in males or females with PWS; or PWS subjects receiving growth hormone. Because only three subjects were on growth hormone, it is not possible to form firm conclusions in our study regarding the impact of growth hormone on obestatin regulation. However, the lack of impact of growth hormone therapy on plasma ghrelin or obestatin levels in our PWS children is supportive of a previous report; [Janssen et al., 2001]. Nevertheless, the effect of obestatin on growth hormone secretion is unclear in rats and humans [Bresciani et al., 2006; Holst et al., 2006; Lauwers et al., 2006; Nogueiras et al., 2006; Yamamoto et al., 2006].

Our PWS children were divided into two groups by age, for example, > 3 years old, and no significant difference was found in obestatin or ghrelin levels in the older age group. However, the young PWS subjects < 3 years old had higher obestatin levels when compared with age and gender matched controls (Table II). Because infants with PWS commonly have failure to thrive, it is tempting to speculate that obestatin, an anorexigenic peptide, may be a contributing factor. Although our sample sizes were small, significantly higher obestatin

levels were seen in PWS males compared with control males. PWS males have hypogonadotropic hypogonadism with low testosterone levels and body composition similar to females [Butler et al., 2006]. These observations may also impact on obestatin regulation in humans. However, no difference was observed when comparing obese versus non-obese PWS or obese versus non-obese control subjects. Furthermore, higher obestatin levels were seen in the non-obese PWS subjects compared with non-obese controls.

The genes for ghrelin, and therefore obestatin and their receptors are not localized on chromosome 15. Therefore, higher obestatin levels in the young PWS subjects compared with matched controls cannot be attributed to chromosome 15 abnormalities in PWS. However, there is limited information on processing and activation (amidation) of obestatin in humans, particularly in PWS. There is supportive evidence for altered or defective processing of precursor proteins, particularly vasopressin and polypeptide 7B2 in the hypothalamus in some PWS subjects, [Gabreels et al., 1998]. It is possible that defective post-translational processing in PWS may include preproghrelin processing by prohormone convertase 2 with implications for appetite and energy metabolism by perturbing the precise regulation of active and inactive forms of obestatin and ghrelin. If the measurable ghrelin in PWS is mostly in an inactive form, this would explain, at least in part, why higher levels of growth hormone are not observed in PWS children. Recent reports indicate that insulin has an inhibitory effect on ghrelin secretion independent of plasma glucose levels [Mohlig et al., 2002; Saad et al., 2002]. Furthermore, the active (acylated) form of ghrelin is suppressed by insulin and this suppression is correlated with insulin sensitivity in PWS children [Paik et al., 2006]. Future studies are warranted to address the interaction of ghrelin, PYY, leptin and other appetite related hormones including obestatin in humans, and their distribution, function and receptor status peripherally and centrally. The regulatory processes which control the transcription, translation and production of active and inactive forms of obestatin and ghrelin determine how these two peptide hormones interact to affect appetite and energy metabolism. A clear understanding of the biology of obestatin/ghrelin is important for defining the molecular abnormalities which produce the phenotype associated with PWS and ultimately lead to better therapeutic interventions.

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FIG. 1.

Correlation analyses for plasma ghrelin with obestatin; obestatin with BMI; ghrelin with BMI; obestatin with age; and ghrelin with age for subjects with Prader-Willi syndrome and age and gender matched controls. A significant negative correlation ($P = 0.04$) was seen only for ghrelin and age for subjects with Prader-Willi syndrome. Arrows denote individuals with a BMI greater than the 85th centile indicating an overweight to obese status. Numbers by each symbol indicate age and gender matched Prader-Willi syndrome and control subjects. Filled symbols represent individuals ≤ 3 years of age and open symbols represent individuals > 3 years of age.

TABLE I

Descriptive Statistics for Control and Prader-Willi Syndrome Groups

Variables	Control (N = 16)			Prader-Willi syndrome (N = 16)			P-value*
	Mean	SD	Range	Mean	SD	Range	
Age (years)	15.9	13.8	1.0–44.0	16.0	13.3	1.0–44.3	0.82
Body mass index (Kg/m ²)	24.3	8.5	15.9–48.6	30.3	14.1	15.2–67.1	0.10
Ghrelin (pg/ml)	521	269	165–918	626	457	233–1750	0.47
Obestatin (pg/ml)	325	109	116–487	398	102	123–543	0.04

* P-values calculated using paired samples t-test.

TABLE II

Number of Subjects, Means, Standard Deviations, and *P* Values for Plasma Ghrelin and Obestatin Levels in Prader-Willi Syndrome and Control Subjects

	N	Ghrelin (pg/ml)			Obestatin (pg/ml)		
		Mean	SD	P-value	Mean	SD	P-value
Matched variables							
Control	16	521	269	0.47*	325	109	0.04*
PWS	16	626	457		398	102	
Control >3 years old	11	480	277	0.93*	305	113	0.19*
PWS>3 years old	11	492	256		370	109	
Control 3 years old	5	610	255	0.43*	369	96	0.03*
PWS 3 years old	5	921	678		460	49	
Male control	8	704	191	0.48*	351	86	0.04*
Male PWS	8	576	150		432	72	
Female control	8	337	203	0.14*	299	129	0.31*
Female PWS	8	676	510		364	120	
Independent variables							
Deletion	7	583	462	0.75**	436	73	0.20**
UPD	9	660	478		369	115	
Control > 3 years old	11	480	277	0.39**	305	113	0.29**
Control 3 years old	5	610	255		369	96	
PWS > 3 years old	11	492	256	0.08**	370	109	0.10**
PWS 3 years old	5	921	678		460	49	
Male control	8	704	191	0.002**	351	86	0.36**
Female control	8	337	203		299	129	
Male PWS	8	576	150	0.68**	432	72	0.19**
Female PWS	8	676	510		364	120	
Obese control	4	527	382	0.96**	386	105	0.21**
Non-obese control	12	518	242		305	107	
Obese PWS	8	521	285	0.38**	359	123	0.12**

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	N	Ghrelin (pg/ml)			Obestatin (pg/ml)		
		Mean	SD	P-value	Mean	SD	P-value
Non-obese PWS	8	731	584		438	59	
Obese control	4	527	382	0.98**	386	105	0.72**
Obese PWS	8	521	285		359	123	
Non-obese control	12	518	242	0.27**	305	107	0.005**
Non-obese PWS	8	731	584		438	59	

PWS, Prader–Willi syndrome; UPD, uniparental disomy 15.

* P-values calculated using paired samples *t*-test.

** P-values calculated using independent samples *t*-test.