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INFLUENCE OF PRETERM DELIVERY ON GHRELIN AND OBESTATIN CONCENTRATIONS IN MATERNAL PLASMA, MILK AND THEIR EXPRESSION IN MAMMARY EPITHELIAL CELLS

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Ghrelin and obestatin are gastrointestinal peptides with a potential role in the programming of metabolism in newborns. The present study aimed to investigate the influence of preterm delivery on ghrelin and obestatin concentrations in the maternal blood plasma and breast milk as well as their gene expressions in the mammary epithelial cells (MECs). On the 3rd day after delivery, milk and plasma samples were collected from mothers that carried to term or gave birth prematurely (< 36 weeks of gestation) and analyzed for ghrelin and obestatin concentrations. MECs isolated from the milk were analyzed for the relative expression of GHRL splice variants. In both groups ghrelin concentrations were significantly lower in milk than in blood plasma. In the preterm group obestatin concentrations were significantly higher in milk than in blood plasma but significantly lower in comparison to that of the control mothers. The expression of GHRL mRNAs was higher ($P < 0.05$) in MECs isolated from the preterm group as compared to those isolated from control mothers. The concentration of obestatin (but not ghrelin) in the breast milk is dependent on the term of pregnancy. Moreover, the lactating mammary gland is one of the sources of ghrelin and obestatin.

Key words: *ghrelin, obestatin, milk, plasma, mammary epithelial cells, preterm, neonate, maternal plasma*

INTRODUCTION

Nutritional programming is a concept, which is based on the hypothesis that certain factors which influence organisms during the critical developmental period determine the risk for development of diseases in adulthood. The role of human breast milk in nutritional programming is pivotal. In the earlier studies it has been shown that infants who are breastfed have better cognitive functions and possibly reduced incidence of autoimmune diseases (type I diabetes, inflammatory bowel disease) or metabolic disorders (obesity, hypertension) in childhood and adolescence (1). Biochemical substances responsible for the programming effects of human breast milk have not yet been identified. However, certain peptides involved in glucose and lipid metabolism have been found in human breast milk, which may suggest a role for these peptides in the development of metabolic syndrome later in adulthood.

Ghrelin and obestatin are gastrointestinal peptides derived from the common pre-prohormone precursor and are involved in the regulation of metabolic functions. The human preproghrelin polypeptide precursor is encoded by a single-copy gene (GHRL) that is located on the short arm of chromosome 3. In humans, exons 1 to 4 encode the 117-amino acid preproghrelin, with exons 1 and 2 coding for the 28-amino acid peptide hormone ghrelin. After cleavage of a 23-amino acid signal peptide, proghrelin is processed to form ghrelin, and the third ghrelin residue (serine) is post-translationally octanoylated (acylated) by the enzyme ghrelin O-acyl transferase (GOAT, encoded by MBOAT4). The C-terminus of proghrelin (encoded by exons 3 and 4) is further processed to give rise to the 23-amino acid peptide hormone obestatin, which effects are independent from those of ghrelin (2). It is postulated that the exon 3-deleted transcript lacks the exon region coding for obestatin, suggesting that the physiological ratio of ghrelin to obestatin may be regulated by means of alternative splicing (3).

Although these both peptides are derived from a common pre-prohormone, different post-translational processing determines their functions, which are reported to be opposite in several physiological processes. Several studies have shown that ghrelin, besides affecting energy and glucose homeostasis, has also an effect on cardiovascular (4), gastrointestinal, pulmonary and immune functions, cell proliferation and differentiation, bone physiology (5). Moreover, previous studies have shown the relation between ghrelin concentration in blood serum and central type of adipose tissue distribution (6). Although obestatin's role in the control of metabolism is still unclear,

previous studies have shown that the ghrelin/obestatin balance is essential for modulating energy homeostasis and adaptation of the organism to nutritional challenges. For example, dysregulation in the ghrelin/obestatin ratio was observed in anorexia nervosa and obesity (7) both in childhood (8) and adulthood (9). Substantial amounts of ghrelin and obestatin were previously reported in colostrum and human breast milk, suggesting a role of hormones in the metabolism programming in newborns (10). Many authors have demonstrated that the nutritional composition of human breast milk from a mother who delivers prematurely is different from that from a mother who delivers at term (11, 12). However, studies on metabolic hormone concentrations in breast milk depending on the time of delivery are very limited. The present study aimed to investigate whether preterm delivery has an effect on the process of metabolic programming. For these purposes we assessed the concentrations of ghrelin and obestatin in the breast milk of mothers who gave birth at full-term in comparison to those who gave birth prematurely. Moreover, due to the fact that the origins of ghrelin and obestatin in breast milk are not fully investigated, their concentrations in blood plasma, as well as their gene expression in the mammary gland were also evaluated.

MATERIALS AND METHODS

Study subjects

The study protocol was approved by the ethics committee at Warsaw Medical University (resolution no. KB/135/2012). The present study was conducted at the Princess Anna Mazowiecka Clinical Hospital in Warsaw.

The study was performed on 40 volunteer women that gave their consent for being involved in the experiment. The study was conducted on lean mothers (pregnancy BMI < 25) between the ages of 25 – 35 years old who had undergone vaginal delivery. Exclusion criteria included smoking nicotine, prescribed medications, multiple gestation and diabetes. Mothers were divided into two groups on the basis of gestation period: control group (C): > 36 weeks gestation (n = 20) and preterm group (P): < 36 weeks gestation (n = 20), (*Table 1*). On the 3rd day after delivery blood plasma and breast milk samples were collected.

Sampling blood and breast milk

Blood and breast milk sampling was done once, on the 3rd day after delivery, 2 h after consumption of the morning meal (between 10.00 – 11.00 a.m.) when the concentration of ghrelin was claimed to be the lowest. Approximately 2 ml of breast milk and 5 ml of blood were collected into sterile tubes containing EDTA and aprotinin (0.6 TIU/ml of milk/blood). Samples were centrifuged at 1.500 × g for 15 min at 4°C. Blood plasma was harvested, distributed into Eppendorff tubes, deep frozen (–80°C) and stored until analysis. The fat layer and the skimmed milk from the breast milk samples were discarded. The tubes containing the cell pellet were filled up with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.2 mM EDTA and then centrifuged. Centrifuging (1500 g; 15 min; 4°C) and washing with cell buffer was repeated 3 times. After the 3rd wash the cell pellet was resuspended in 1 ml of cell buffer. The cell suspension (20 µl) was then examined under a light microscope for cell viability and cell count.

Isolation of mammary epithelial cells (MECs)

Twenty five microliters of magnetic beads coated with monoclonal antibodies for epithelial marker (HEA125, Dynabeads® Epithelial Enrich, Invitrogen Dynal, Norway) were added into the cell pellet suspension in cell buffer and incubated for 30 min at 4°C with gentle orbital shaking. The volume of beads used per sample was chosen on the basis of the manufacturer's recommendation and the analysis of MECs number using fluorescence-activated cell sorting (FACS) flow cytometry. FACS samples containing beads were incubated with goat anti-mouse Ig1 antibody conjugated with FITC (Abcam, Great Britain). After incubation with magnetic beads, tubes were placed in the magnet (DynaMag™, Invitrogen, Norway) for 3 min. The supernatant was then removed and fresh cold cell buffer was added and the tubes were placed on the magnet. This was repeated 3 times. After 3 repetitions the bead suspension was transferred to a new tube, immerse in liquid nitrogen and then stored at –80°C until RNA isolation.

Biochemistry

Glucose, triglyceride and cholesterol (total and HDL) concentrations in blood plasma were determined spectrophotometrically (MAXMAT PL multidisciplinary diagnostic platform, Erba Diagnostics France SARL, France) using ELITech ready-to-use reagents (ELITech Group, France) according to method described previously (13).

Obestatin and ghrelin radioimmunoassay

According to the kits manufacturer's instruction breast milk and plasma samples underwent acidification before peptides

Table 1. Characteristics of mothers and concentration of triglycerides, cholesterol and glucose in maternal blood plasma (mmol/L). Results are presented as means ± S.E.M. Asterisks indicate differences between groups; *P < 0.05; ***P < 0.001, ****P < 0.00001. Unpaired t-test or Mann-Whitney test, P < 0.05 was considered as significant.

	C	P
Age of mother (years)	30 ± 2.8	30 ± 3.8
Pregnancy BMI (kg/m^2)	21 ± 2	22 ± 1.6
Pregnancy weight gain (kg)	14 ± 4.6	12.6 ± 3.7
Gestation week at delivery	39 ± 1	33.8 ± 2.3 ****
Triglycerides	2.58 ± 0.47	2.58 ± 0.62
Total cholesterol	6.41 ± 0.93	5.54 ± 0.57***
HDL	1.60 ± 0.22	1.31 ± 0.16****
LDL	4.45 ± 0.24	3.76 ± 0.16*
Glucose	4.27 ± 0.56	4.60 ± 0.38****

extraction. Breast milk and plasma samples were assayed for obestatin and ghrelin concentrations using commercially available radioimmunoassay (RIA) kits: Ghrelin (Human, Monkey) RIA Kit (sensitivity 84.7 pg/ml; Phoenix Pharmaceuticals, Inc., USA) and Obestatin (Human, Monkey) Ultra-Sensitive RIA kit, (sensitivity 77.59 pg/ml; Phoenix Pharmaceuticals, Inc., USA) according to the manufacturer's instructions. Spike and recovery for 200 pg/ml of obestatin and total ghrelin were measured in 3 different plasma or breast milk samples. The assays were performed in one run. The mean percentage recovery for obestatin and ghrelin in plasma was 95% and 96%, respectively. The intra-assay coefficient of variation (CV) for obestatin and ghrelin in plasma was 6.0% and 7.0%, respectively. Prior to performing the assays, milk samples were sonified for 15 min. Mean percentage recovery was 90% for both obestatin and ghrelin in breast milk. The intra-assay CV for obestatin and ghrelin in breast milk was 6.6% and 6.0%, respectively.

Determining the relative gene expression

Total RNA from the mammary epithelial cells was isolated using a NucleoSpin® RNA XS Kit (MACHEREY-NAGEL Gmbh & Co; Duren, Germany) according to the manufacturer's instructions. The purity and concentration of isolated RNA were spectrophotometrically quantified by measuring the optical density at 230, 260 and 280 nm in a NanoDrop 1000 instrument (Thermo Fisher Scientific Inc., Waltham, USA). The RNA integrity was verified by electrophoresis using 1% agarose gel stained with ethidium bromide. Maxima™ First StrandcDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific Inc., Waltham, USA) was used to prepare cDNA synthesis. As a starting material for this PCR synthesis, 10 ng of total RNA was used.

Real-time RT-PCR was carried out using HOT FIREPol EvaGreen®qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) components and HPLC-grade oligonucleotide primers synthesised by Genomed (Poland). Specific primers for determining the expression of mRNAs for housekeeping genes and GHRL mRNAs variants containing 1st and 2nd exons (GHRL E1-2; including sequence encoding ghrelin) or 3rd and 4th exons (GHRL E3-4; including sequence encoding obestatin) of ghrelin/obestatin prepropeptide were designed using Primer 3 software (14, 15) (*Table 2*). One tube contained: 4 µl PCR Master Mix (5 x), 14 µl RNase-free water, 1 µl primers (0.5 µl each, working concentration was 0.25 µM) and 1 µl cDNAtemplate. The tubes were run on the Rotor-Gene 6000 (Qiagen, Duesseldorf, Germany). The following protocol was used: 95°C for 15 min (activation of the Hot Star DNA polymerase) and then 35 PCR cycles including 95°C for 5 s (denaturation), 60°C for 20 s (annealing) and 72°C for 10 s (extension). After the cycles, a final melting curve analysis under continuous fluorescence measurements was performed to confirm the specificity of the amplification. Additionally, the size of the real-time PCR products and the specificity of the reaction were also confirmed by electrophoresis in 4% agarose gel, stained with ethidium bromide in the presence of molecular weight marker (HyperLadder™ 25bp, Bioline, United Kingdom) (*Fig. 1*). PCR amplicons were also sequenced (Genomed, Warsaw, Poland) to confirm their specificity.

Relative mRNA expression was calculated using the comparative quantification option of the Rotor Gene 6000 software version 1.7. (Qiagen, Duesseldorf, Germany). Initially, 3 housekeeping genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB) and cyclophilin C (PPIC) were tested. Based on the results of analysis performed with BestKeeper software (16), ACTB was chosen as the best endogenous reference gene. The results are presented as a relative expression of the target GHRL mRNA variant versus ACTB, relative expression value and mean ± S.E.M. The average relative quantity of GHRL E1-2 mRNA expression in control group was set to 1.0.

Statistical analysis

The data are expressed as means ± S.E.M. The significance of differences between the experimental groups was assessed using the Unpaired t-test or Mann-Whitney test. The Pearson test was used for correlation analysis. In all statistical analyses, $P < 0.05$ was considered significant (Prism 6 for Mac OS X, Version 6.0h, Graph Pad Software, San Diego, CA, USA).

Table 2. Summary of genes analyzed by real-time PCR with their full name and abbreviation.

GenBank Acc. No.	Gene	Amplicon size [bp]	Forward/ reverse	Sequence 5'→ 3'
NM_001256799.1	***GAPDH*** glyceraldehyde-3-phosphate dehydrogenase	102	forward	GATTCCACCCATGGCAAAT
			reverse	GGAGGGATCTCGCTCCTG
NM_001101.3	***ACTB*** B-actin	116	forward	CTCTTCCAGCCTTCCTTCCT
			reverse	AGCACTGTGTTGGCGTACAG
NM_000943.4	***PPIC*** cyclophilin C	140	forward	AAGTTGTGCCCAAGACAGTG
			reverse	AGTGCCATCTCCAGTGGTG
NM_016362	***GHRL*** ghrelin/obestatin prepropeptide **exon 1 and 2**	101	forward	ACACCAGAGAGTCCAGCAGA
			reverse	CTTGACCTCCATCTTCCGGG
NM_016362	***GHRL*** ghrelin/obestatin prepropeptide **exon 3 and 4**	124	forward	CTGGGAAGAGGCCAAAGAG
			reverse	ACAGTCGTGGGAGTTGCTG

RESULTS

Metabolic profile

No differences in plasma triglyceride concentrations were observed between the two examined groups. Plasma total cholesterol, HDL and LDL cholesterol concentrations were significantly lower in the preterm group as compared to the control group. Plasma glucose concentration was significantly higher in the preterm mothers than in the control mothers (*Table 1*).

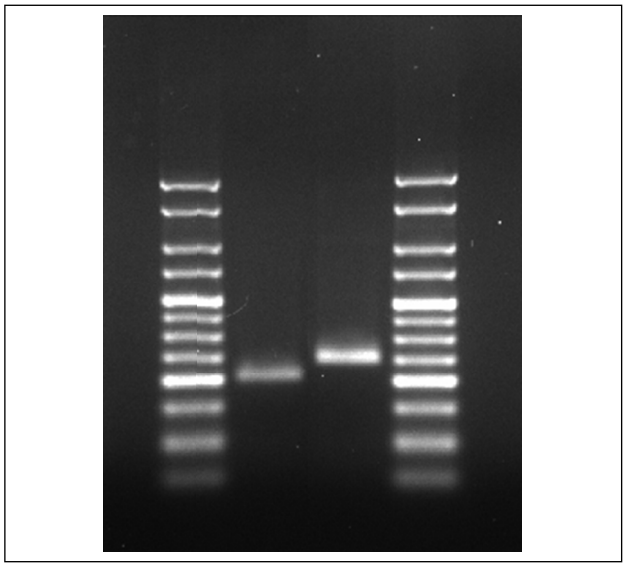

Fig. 1. Representative electrophoresis of PCR products loaded on 4% native agarose gel obtained with primers GHRL (exon 1 – 2) (line 2) and GHRL (exon 3 – 4) (line 3) for the RNA samples extracted from the mammary epithelial cells. Lines 1 and 4: DNA molecular weight marker HyperLadder™ 25b. The size of the band (bp) was as follows (staring from the top): 500, 400, 300, 250, 200, 175, 150, 125, 100, 75, 50, 25.

Ghrelin and obestatin concentrations in blood plasma and breast milk

Ghrelin concentrations in blood plasma and breast milk were not significantly different between the two groups assessed. Moreover, in both groups of mothers ghrelin concentrations in breast milk were significantly lower than that in blood plasma (*Fig. 2a*).

Obestatin concentrations in both blood plasma and breast milk were significantly lower in the preterm group in comparison to that observed in the control group. Moreover, in the preterm group obestatin concentrations in breast milk were significantly higher than that in blood plasma (*Fig. 2b*).

No relationship between the obestatin concentration in breast milk and that in maternal blood plasma was observed in both groups of mothers. In the preterm group, positive correlations between plasma obestatin and plasma and breast milk ghrelin concentrations ($r = 0.6934$, $P = 0.0062$ and $r = 0.4771$, $P = 0.0494$, respectively) were observed. A significant negative correlation was observed between maternal blood plasma ghrelin and breast milk obestatin concentrations ($r = –0.3716$, $P = 0.0486$) in the full term mothers (*Table 3*).

Ghrelin/obestatin ratio in blood plasma and milk

No significant differences in the ghrelin/obestatin ratio were observed between the two groups of mothers, both in the blood plasma and in the breast milk. In both the control and preterm groups the ghrelin/obestatin ratio in breast milk was significantly lower than that in blood plasma (*Fig. 2c*).

Expression of GHRL mRNA variants in mammary epithelial cells

The expression of GHRL E3-4 mRNA was significantly higher ($P < 0.001$) than that of GHRL E1-2 mRNA in the epithelial cells isolated from breast milk from the control and preterm groups. Moreover, in the cells derived from the mothers who gave birth prematurely, the expression of GHRL mRNAs

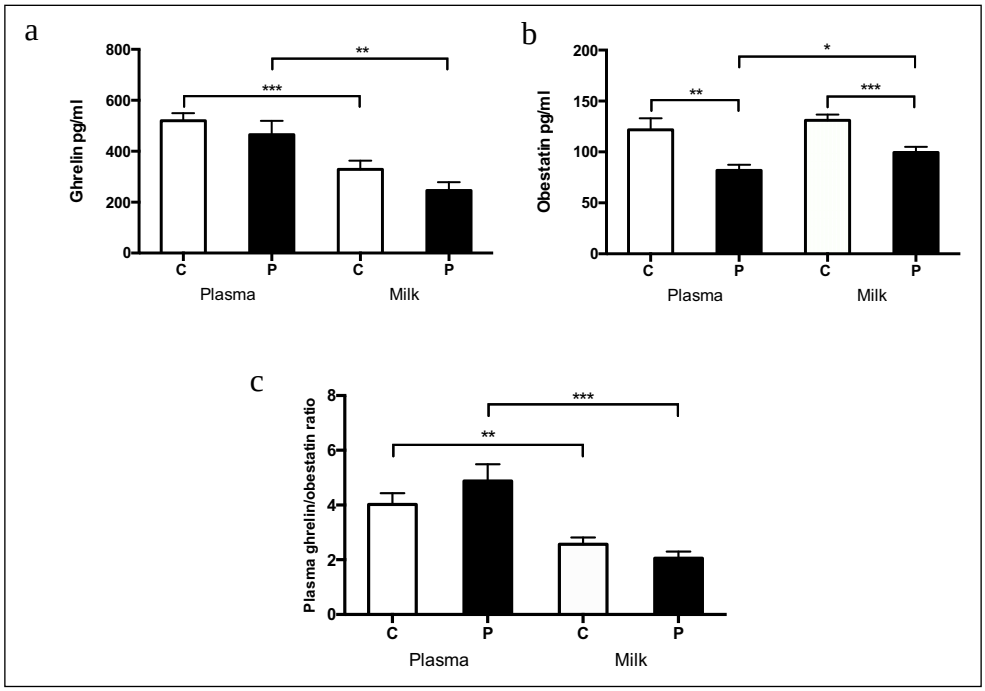

Fig. 2. Concentration (pg/mL) of ghrelin (a), obestatin (b) and ghrelin/obestatin ratio (c) in blood plasma and breast milk of women who gave birth at full term (C) or prematurely (P). Results are presented as means ± S.E.M. Asterisks indicate differences between groups; *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$. Unpaired t-test or Mann-Whitney test.

Table 3. Correlations between obestatin and ghrelin in blood plasma and breast milk. Results are presented as means ± S.E.M. Asterisks indicate differences between groups and between breast milk and plasma within the treatment; *P < 0.05; **P < 0.01. Pearson test was used for correlation analysis.

	C	P
Milk obestatin vs. milk ghrelin	r = –0.1285 P = 0.2946	r = –0.1045 P = 0.3799
Milk obestatin vs. plasma ghrelin	r = –0.3716 P = 0.0486*	r = –0.2138 P = 0.2315
Plasma obestatin vs. milk ghrelin	r = 0.5152 P = 0.0433*	r = 0.4777 P = 0.0494*
Plasma obestatin vs. milk obestatin	r = 0.0025 P = 0.4957	r = –0.2385 P = 0.1627
Plasma obestatin vs. plasma ghrelin	r = –0.1815 P = 0.2673	r = 0.6934 P = 0.0062**
Plasma ghrelin vs. milk ghrelin	r = 0.2305 P = 0.1641	r = –0.0680 P = 0.4212

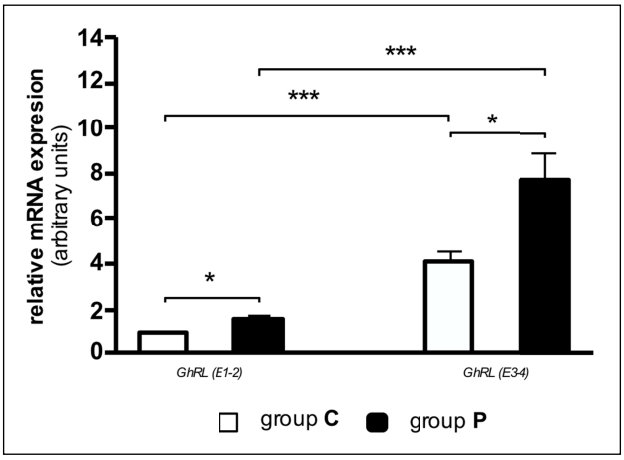

Fig. 3. Relative GHRL mRNA expression in MECs of women who gave birth at full term (C) or prematurely (P). (GHRL E1-2) exons no 1 and 2 including sequence encoding ghrelin or exons no 3 and 4 (GHRL E3-4) including sequence encoding obestatin. Results are presented as mean ± S.E.M. Asterisks indicates values that differ significantly *P < 0.05; ***P < 0.001. Unpaired t-test or Mann-Whitney test.

was significantly higher (P < 0.05) than that observed in the cells isolated from the mothers who gave birth at full term (*Fig. 3*).

DISCUSSION

The obestatin concentrations observed in the blood plasma and breast milk in the present study were significantly lower than those reported by Aydin *et al*. (10) and by Savino *et al*. (17). Several factors could be responsible for such conflicting results. First of all, in the present study the blood samples were collected 2 h after the morning meal when the plasma level of ghrelin was reported to be the lowest. In the study by Aydin *et al*. (10), the blood samples were collected just before breakfast. Additionally, the subject inclusion criteria in the present study were more specific than those used by Aydin *et al*. (10). Both BMI of the mothers, as well as the week of pregnancy during which they gave birth are crucial factors, which could influence obestatin and ghrelin concentrations. In the present study, significantly lower concentrations of total and LDL cholesterol were observed in the preterm mothers together with the negative correlation between plasma obestatin and LDL cholesterol concentrations. Oluwole *et al*. (18) reported that decreased concentrations of total cholesterol are associated with preterm birth. This could be explained by the fact that circulating low-density lipoprotein cholesterol is the chief substrate for placental progesterone biosynthesis (19). On the other hand, in *in vitro* studies on human luteal cells a significant reduction in the release of progesterone after incubation with obestatin was observed (20). To the best of our knowledge, our study is the first, which displayed obestatin and ghrelin expressions in the mammary epithelial cells during lactation. In the previous studies it has been shown that mammary epithelial cells isolated from milk are a high quality source of mammary mRNA obtained in non-invasive manner.

The radioimmunological assessment of obestatin and ghrelin concentrations in blood plasma and breast milk, and analysis of the GHRL splice variant expression show that obestatin and ghrelin are controlled independently and that preterm birth is associated with maternal changes in obestatin synthesis in the mammary glands. Basing on the currently available knowledge, it is difficult to clearly explain the mechanism of this process. However, the results of our other studies on the effect of obestatin on intestinal contractility in neonatal rats may shed some light on the biological meaning of these findings (21). In the above-mentioned studies it was observed that obestatin, when administered enterally for 7 consecutive days to rat pups (starting from 14th day of life), significantly decreased intestinal contractility. With regard to the concept that mother's milk is the best food for the baby, it is reasonable that milk for premature infants whose gastrointestinal tract is immature and very susceptible to functional disorders, should have lower concentration of peptide, which strongly inhibits intestinal contractility.

Ghrelin concentrations in colostrum and breast milk have been reported previously (22, 23). Our results are in

agreement with those of Kiresen *et al*. (22) who observed no differences in ghrelin concentrations between full term and preterm mothers' breast milk, as well as a significantly higher plasma concentration of this peptide in blood plasma than in milk. These results may indicate that plasma and milk concentrations of ghrelin are not associated with the week of giving birth but rather with the regulation of maternal energy balance. Expression of GHRL splice variants containing exon 1 and 2, which are translated to ghrelin (3), confirmed that lactating mammary glands synthesize ghrelin. Moreover, expression for both variants- ghrelin and obestatin (exon 3 and 4) were significantly higher in preterm mothers. These observations are in contrast to results obtained from the radioimmunological assessment of the peptide concentrations. Several aspects must be taken into consideration in order to understand these results. First of all, the concentration of obestatin and ghrelin in breast milk is a result of the synthesis of these peptides in the mammary glands and their transportation from the maternal circulation. Moreover, due to complex regulation control, the transcript level of the gene in many cases does not correspond to the peptide level. We also cannot exclude that some amounts of ghrelin and obestatin synthesized in the mammary glands have a paracrine or autocrine role within the mammary tissue.

In conclusion, it was shown that the concentration of obestatin (but not ghrelin) in breast milk is dependent on the term of giving birth. Moreover, lactating mammary glands are one of the sources of ghrelin and obestatin. It is clear that more studies are required to elucidate the mechanism by which the term of the labor determines the obestatin concentration in the breast milk and the role of milk-born obestatin in the development of infants.

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