Gherlin expression in women with polycystic ovary syndrome - a preliminary study

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Summary

The etiology and pathogenesis of polycystic ovary syndrome (PCOS) is still unknown. Using real-time PCR, we detected that polycystic ovaries showed almost ten times lower expression of ghrelin mRNA than normal ovaries, whereas the mRNA levels in blood cells were similar in both study groups. This suggests that the presence of ghrelin in PCOS and normal ovaries may have an autocrine/paracrine modulatory effect on ovary functions and local significance in the etiology of PCOS.

Key words: Ghrelin expression, PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies in women of reproductive age. The disorder is characterized by menstrual disturbances (amenorrhea, oligomenorrhea, dysfunctional uterine bleeding), signs of hyperandrogenism (hirsutism, alopecia, acne, elevated serum concentrations of androgens) and polycystic ovaries at ultrasound [1]. Polycystic ovary syndrome is defined on the basis of clinical and biochemical criteria, with the exclusion of other hyperandrogenic disorders such as nonclassic adrenal 21-hydroxylase deficiency, hyperprolactynemia, or androgen-secreting neoplasms [2]. The syndrome is associated with greatly increased risk of type 2 diabetes mellitus, cardiovascular disease, endometrium and breast cancer. Many environmental factors seem to participate in the pathogenesis of PCOS, but their role and mechanism of action is actually unknown.

The role of genes responsible for steroid hormone synthesis and action, genes involved in carbohydrate metabolism and genes involved in gonadotropin action in the pathophysiology of PCOS has also been considered [3, 6]. In our study we try to determine the role of ghrelin in PCOS etiology.

Ghrelin is an endogenous ligand for GH secretagogue receptor (GHS-R) type 1a, a G-protein coupled receptor [7]. It is a 28-amino-acid peptide with an octanoyl group on the third N-terminal amino acid serine, that appears to be essential for its biological activity. The ghrelin gene is located on human chromosome 3p26-p25.

Ghrelin is mainly expressed in the enteroendocrine cells of the stomach but its expression is also detected in a number of other tissues, including the hypothalamus, pituitary and intestine [6-11].

It is regarded as a very important regulator of energy homeostasis and GH secretion.

Through activation of NPY/AGRP neurons, ghrelin modulates hormonal and neural pathways that regulate food intake and body fat mass and can contribute to the pathophysiology of obesity [12, 13]. The aim of the present study was to evaluate ghrelin expression in blood cells and ovary tissue of PCOS women.

Materials and Methods

Ovarian tissue and blood samples were obtained from four patients aged 25.5 ± 5.0 years who had been treated for infertility with bilateral wedge resection therapy at the Department of Gynaecological Endocrinology, University of Medical Sciences in Poznań, Poland. The clinical characteristics of patients were typical for PCOS. The diagnosis of PCOS was made according to established guidelines, including hyperandrogenemia, oligoovulation, and the exclusion of 21-hydroxylase deficiency, Cushing syndrome and hyperprolactinemia [14]. The serum levels of LH, FSH prolactin, oestradiol, dehydroepiandrosterone (DHEA), testosterone and sex hormone binding globulin (SHBG) were determined by radioimmunoassay and the concentration of urinary 17-keto steroids (17KS) was estimated as described in a previous study [15].

The size of the ovaries was determined by ultrasonography before the surgery and reexamined during the procedure of wedge resection.

Each specimen was examined microscopically and all the ovaries were diagnosed as being polycystic. The control group consisted of blood samples and ovaries tissue from women after surgeries for nonovarian reasons (age 29.42 ± 2.8). They did not show any endocrinological disorders. None were receiving exogenous hormones.

The investigation was approved by the Ethical Committee of the University of Medical Sciences in Poznań.

Tissue samples were quickly cut into 40 mg samples, placed in 1.5-ml tubes and stored in RNAlater RNA Stabilization Reagent (Qiagen) at –80°C until further processing. Disruption

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and homogenization of tissue samples was carried out with mortar and pestle.

Blood samples were obtained just before the RNA isolation procedure, as frozen samples cannot be used.

Total RNA was prepared using Rneasy Mini Kit *(Quiagen)* and QIAamp RNA Blood Mini *(Qiagen)* according to the manufacturer's protocol; 20 mg of each tissue sample and 1 ml of whole blood samples were used for isolation of total cellular RNA. After addition of Rnazin, RNA was stored at –80°C.

Reverse transcription and real-time PCR were performed with published primers [14]. Primers used in this study were designed for human ghrelin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and both sets cross exon-intron boundaries. The possibility of DNA co-amplification was excluded by failure of product detection in real-time PCR with DNA template.

RT and PCR reactions were performed separately ('two-enzyme/two-tube' reaction). First strand cDNA synthesis was performed with the Super Script™ Rnase H- RT kit according to the manufacturer's protocol. In short, the RT reaction conditions were the following: 20 µl reaction mixture including aproximmately 150 g of total RNA (9 µl) and 2 pM of gene specific primers, 1 µl 10mM dNTP mix, 4 µl 5x first strand buffer, 2 µl 0,1M DTT, 200 units of Super Script™ II and sterile, distilled water.

The use of mRNA-specific primers was selected on the basis of decreasing background priming. In order to minimize primer dimer formation in following real-time PCR assay, we performed two-tube reverse transcription for each RNA sample: one with reverse primer for ghrelin and one with reverse primer for GAPDH. First strand cDNA was stored at –20°C.

Detection of the amplified product was performed with SYBR Green I, a fluorescent intercalating dye [17, 18].

Temperature profiles of real-time PCR were as follows: the reaction mixture was denatured at 95°C for 10 min followed by 40 cycles of PCR at 95°C for 15 sec and 60°C for 30 sec (two-step PCR) or 95°C for 15 sec, 52°C for 15 sec and 60°C for 30 sec (three-step PCR). Annealing temperatures were determined empirically to minimalize primer-dimer formation. Quantification of mRNA samples was carried out by relating the PCR threshold cycle obtained from tissue samples to specific standard curves. The log copy numbers of target and GAPDH in each sample were calculated from the regression line according to the formula: *relative abundance* = $10^{(C_t-b)/m}$, where C_t = *threshold cycle, b=y – intercept* and *m=slope.*

The relative abundance of the target was divided by the relative abundance of GAPDH in each sample to generate a normalized abundance.

Quality standards - as RNA isolation from tissue may result in a different quality and quantity of nucleic acids, amplification of GAPDH mRNA was used as a standard. Samples negative for GAPDH should be excluded from quantification. One no-template control, prepared at the end of the experiment, was included with every amplification run. This allowed us to monitor any contamination arising during the handling of the reagents.

Results

Clinical and biochemical characteristics of the study

The patients had undergone menarche between 11 and 13 years, and their past and family histories were non-contributory. All of the patients were infertile and oligomenorrheic. The characteristics of the women diagnosed with PCOS are given in Table 1.

Table 1. — *Characteristics of women diagnosed with PCOS.*

BMI (kg/m²)	22.0 ± 0.50
SHBG (nmol/l)	101.18 ± 43.82
LH (mlU/ml)	12.17 ± 11.23
FSH (mlU/ml)	4.60 ± 3.25
LH/FSH	2.41 ± 0.59
PRL (ng/ml)	12.48 ± 4.47
Estradiol (pg/ml)	131.25 ± 90.86
Glucose (mg/dl)	90.75 ± 11.17
Insulin (ug/ml)	25.25 ± 7.90
Hirsutism F-G score (points)	19.30 ± 1.80
Testosterone (ng/ml)	1.03 ± 0.41
DHEA (mg/day) < 24 hours urine collection >	2.47 ± 1.19
17KS (mg/day) < 24 hours urine collection >	9.60 ± 3.01

BMI: body mass index.

Ultrasound examination showed that the ovaries were enlarged bilaterally and the diameters along the major axes were significantly greater than in the control ovaries. In all ovaries of the PCOS patients examined by means of ultrasonography, the number of immature follicles, ranging from 5 to 9 mm, were greatly increased as compared to the control ovaries. This observation was confirmed during wedge resection and the results obtained were not significantly different from those obtained by ultrasound examination.

All the polycystic ovaries on microscopic examination showed fibrotic tissue degeneration of follicles with signs of atrophy of granulosa and hypertrophy of theca folliculi. In the majority of polycystic ovaries atretic follicles less than 6 mm in diameter were dominant. The ovaries did not contain mature follicles or corpora lutea. All the control ovaries, on microscopic examination showed normal structure with no signs of follicular or thecal hypertrophy and some mature follicles and corpora lutea were observed.

Ovary and blood ghrelin expression

The expression of ghrelin was investigated in a normal and polycystic ovary, as well as in blood cells of both study groups of women using real-time PCR. Ghrelin mRNA concentrations in blood cells were similar in the PCOS and the control group of women (Table 2). Ghrelin mRNA was also expressed in polycystic ovary tissue and the normal ovary. However, we detected a ten times lower level of ghrelin mRNA expression in polycystic ovaries compared with normal ovaries (Table 2).

Table 2. — *Real-time PCR data (GHR/GAPDH ratio) as a percent change from controls;* [b] *– blood sample.*

Diagnosis number of subjects	BMI (kg/m²)	Real-time PCR ghrelin/GAPDH ratio
PCOS (4)	22.0 ± 0.5	5.417[b]
PCOS (4)	22.0 ± 0.5	0.00010
Control group (4)	20,5 ± 0.5	5.930[b]
Control group (4)	20.5 ± 0.5	0.00162

BMI: body mass index.

Discussion

Ghrelin is involved in the regulation of GH release, but it has recently been suggested, that ghrelin can also be regarded as one of the peptides, which can play an important role in food intake regulation and some reproductive processes. It is also involved in a large number of endocrine and non-endocrine pathways [20-25]. The distribution of ghrelin and its receptor was investigated in various human tissues. Ghrelin was found in the stomach, other parts of the gut, and in the adrenal gland, atrium breast, buccal mucosa, esophagus, fat tissue, gall bladder, human lymphocytes, kidney, colon, liver, lung, lymph nodes, muscle, testis and ovary [8-11].

Using the real-time PCR method we also detected that ghrelin mRNA may be expressed in the polycystic ovary. However, polycystic ovaries showed significantly lower expression of ghrelin mRNA, than normal ovaries, whereas the mRNA level in blood cells was similar in patients with PCOS and in normal subjects. The results of our preliminary study suggest that the presence of ghrelin mRNA in polycystic and normal ovaries implies that the locally synthesized hormone may have clinical relevance (autocrine/paracrine modulatory effect on ovary functions). Additionally the lower mRNA expression of ghrelin in the polycystic ovary can have local significance in the pathophysiology of polycystic ovary syndrome. Literature on the possible role of ghrelin in the ovary and particularly in polycystic ovary is very scarce and contradictory [19-21]. Pagotto *et al.* [19] examined ten obese women with PCOS and compared them with ten age- and body mass index (BMI)-matched obese subjects without the syndrome and indicated a marked negative correlation between ghrelin and androstendione level, suggestive of an interaction between ghrelin and steroid synthesis or action.

Gaytan *et al.* [24] found, that strong ghrelin immunostaining was demonstrated in ovarian hillus-interstial cells of the rat ovary and was not detected in an ovarian follicle at any of the developmental stages. The observations of Caminos *et al.* [25] suggested that ghrelin expression was predominantly located in the luteal compartment of the ovary.

Apart from ghrelin expression in the ovaries, the serum ghrelin levels in PCOS women and controls were also studied. Orio *et al.* [20] demonstrated, that in women with PCOS plasma ghrelin concentrations are not different from those in controls and are not influenced by an abnormal pattern of the disorder. In both groups there was a significant inverse correlation between ghrelin concentrations and BMI. Another study indicated, that obese women with polycystic ovary syndrome have lower plasma ghrelin levels negatively correlated with insulin sensitivity [26]. A significant difference in ghrelin serum plasma levels between PCOS and a control group was described by Waśko *et al.* [15], whereas there was no significant difference in BMI between groups. All PCOS women have a higher ghrelin level than controls. Similarly in another study ghrelin levels were decreased in PCOS women and highly correlated to the degree of insulin resistance in PCOS women. However, this study did not explain whether a low ghrelin level in PCOS was a cause or a consequence of insulin resistance [27]. It is also interesting that treatment with drugs which lower serum androgen levels can affect serum ghrelin levels. The contradictory results on the influence of ghrelin on PCOS suggest that ghrelin could be a possible factor in the etiology of this syndrome. However, the molecular mechanism, its actions and pathways remain unknown and need further study.

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