Follistatin and activin A serum concentrations in obese and non-obese patients with polycystic ovary syndrome

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BACKGROUND: Activin promotes ovarian follicular development, inhibits androgen production and increases FSH and insulin secretion. Follistatin, an activin-binding protein, neutralizes activin bioactivity. Therefore, a decrease in the ratio of activin/follistatin might encourage characteristic features of polycystic ovary syndrome (PCOS). We investigated whether women with PCOS showed disordered follistatin and/or activin serum concentrations.

METHODS: The study group included 24 obese and 20 non-obese (body mass index ≥ and <27 kg/m² respectively) clomiphene-failure PCOS patients. The control group included 16 obese and 46 non-obese patients with normal ovulatory cycles. Blood samples were obtained from the patients on day 3–5 of a progesterone-induced or spontaneous cycle and were assayed for LH, FSH, testosterone, 17-hydroxy-progesterone, androstenedione, follistatin, activin A, fasting glucose and insulin.

RESULTS: Follistatin concentrations were comparable between obese and non-obese PCOS and controls respectively (35, 513 ± 74, 661 ± 87 and 595 ± 43 pg/ml in obese and non-obese PCOS and controls respectively). Stepwise regression analyses for relationships between follistatin or activin A levels and all other variables indicated that follistatin was significantly and independently positively affected by PCOS (P < 0.0001), age (P < 0.02), androstenedione (P < 0.03) and weight (P < 0.05). Activin A was significantly and independently negatively affected by PCOS (P < 0.003) and FSH (P < 0.03), and positively affected by weight (P < 0.009) and androstenedione (P < 0.02).

CONCLUSIONS: Serum follistatin is increased in PCOS patients, regardless of obesity. PCOS is the most significant variable that relates to high follistatin and low activin A serum concentration. A high follistatin/activin ratio could well contribute to the pathophysiology of PCOS.

Key words: activin A/follistatin/obesity/PCOS

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder, characterized by typical ovarian morphology, follicular arrest, anovulation, hyperandrogenism and insulin resistance, which are not always fully associated (Yen, 1999). The aetiology of PCOS has not yet been fully elucidated, although the observation of familial aggregation (Legro et al., 1998; Urbanek et al., 1999) is consistent with a genetic basis for this disorder. Growth factors are heavily involved in the pathophysiology, either contributing to or as a consequence of the arrested development of follicles, abnormal steroidogenesis, and hyperinsulinaemia (Homburg, 1998).

Follistatin is a monomeric glycosylated polypeptide chain, which was initially identified in and isolated from follicular fluid on the basis of its inhibition of pituitary FSH secretion (Robertson et al., 1987; Ueno et al., 1987). Later it was well documented that follistatin exerts this and other activities by neutralizing activin bioactivity, being an activin-binding protein (Mather et al., 1997). Both follistatin and activin are expressed in numerous tissues, including the gonads, pituitary, adrenal cortex, liver and pancreas. Activin, a member of the transforming growth factor-β superfamily, promotes ovarian follicular development, inhibits thecal cell androgen production, increases pituitary FSH secretion and modulates pancreatic β-cell insulin secretion (Shibata et al., 1996; Mather et al., 1997; Florio et al., 2000). A decrease in concentration or functional activity of activin, as well as an increase in follistatin, might therefore encourage characteristic features of PCOS.

Some studies support the assumption that high follistatin gene expression might be linked to PCOS. In transgenic mice, overexpression of follistatin resulted in arrested ovarian folliculogenesis, with or without suppression of serum concentrations of FSH (Guo et al., 1998). Urbanek et al. tested 37
candidate genes for linkage and association with PCOS or hyperandrogenism in data from 150 families (Urbanek et al., 1999). The strongest evidence for linkage was with the follistatin gene. Although later studies failed to identify any mutation in the follistatin gene in PCOS (Liao et al., 2000; Urbanek et al., 2000), these observations led us to investigate whether women with PCOS showed disordered follistatin and/or activin serum concentrations.

Recently, Norman et al. published their findings that circulating follistatin concentrations are higher and activin concentrations are lower in PCOS (Norman et al., 2001). However, the question of a correlation of these findings with body mass index (BMI) has been left open. Therefore, we compared follistatin and activin A serum concentrations in obese and non-obese patients with PCOS or normal ovarian function.

Materials and methods

Subjects
One hundred and six patients aged 21–40 years and undergoing treatment in the infertility clinic in Shaare-Zedek Medical Center between March and December 2000 were included in the study. The study group (with PCOS) included 44 patients with typical ultrasonic ovarian morphology (≥10 follicles with a diameter of <9 mm) (Adams et al., 1985) and chronic menstrual irregularity (oligo- or amenorrhea). They had all failed to ovulate in response to a spontaneous or progesterone-induced bleed (clomiphene-resistant PCOS). The control group included 62 patients with normal ovulatory cycles with a mean length of 27–32 days and no endocrine abnormalities, such as hyperprolactinaemia or abnormal thyroid function. Each group was further divided into obese and non-obese (BMI ≥ and <27 kg/m² respectively). Patients being treated with steroids, oral hypoglycaemic agents or insulin were not included. All patients were in good physical and mental health. The Shaare-Zedek Medical Center Research and Ethics Committee, Jerusalem, approved the study protocol.

Protocol
Blood samples were obtained from PCOS patients after 35–90 days of amenorrhoea with no hormonal treatment. After ruling out the possibility of pregnancy (β-human chorionic gonadotrophin <10 IU/l) or spontaneous ovulation (progesterone <15 nmol/l), medroxyprogesterone acetate (Aragest, Dexon, Hadera, Israel) 10 mg/day was administered for 90 days. Second blood samples were obtained on day 3–5 of the cycle. Blood samples were obtained from the control group patients on day 3–5 (early follicular) and 20–22 (mid-luteal, confirmed by progesterone >25 nmol/l) of the cycle.

The samples were centrifuged and sera were stored at -20°C. Samples from day 3–5 were assayed for LH, FSH, testosterone, oestradiol, 17-hydroxy-progesterone (17-OH-P) and androstenedione (Δ4A). All samples were assayed for total follistatin and total activin A.

Overnight fasting glucose and insulin concentrations were determined without regard to cycle day. Insulin resistance (IR) was considered to be present if fasting glucose/insulin ratio was <4.5 (Legro et al., 1998).

Methods
Serum concentrations of testosterone, oestradiol, Δ4A and 17-OH-P were determined using the Coat-a-Count radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA). FSH and LH levels were measured using the Abbott IMX system (Abbott Diagnostics, Abbott Park, IL, USA). Follistatin was measured using two site-specific enzyme-linked immunosorbent assays (ELISA) for follistatin 288 as reported previously (Evans et al., 1998). The assay sensitivity was 19 pg/ml and the between assay variation was 15%. The cross reactivity with follistatin 315 was 9.9%. Activin A was measured using highly sensitive two-site ELISA (Serotech, Oxford, UK). Glucose was measured by the glucose oxidase technique with a Vitros 950 instrument (Johnson and Johnson, USA) and insulin was measured with an Assim instrument (Abbott Diagnostics).

Statistics
Results are presented as mean ± SE, unless otherwise indicated. Student’s t-test was used to test for differences between groups. Normality of distribution was found for all variables. Stepwise regression analyses were performed with follistatin and activin A concentrations as dependent variables and diagnosis (PCOS or control), age, weight, BMI, LH, FSH, testosterone, Δ4A and insulin sensitivity (normal or insulin-resistant) as independent parameters. Statistics Package for Social Sciences was used for all statistical analyses. Statistical significance was defined as P < 0.05.

Results
Serum follistatin concentration on cycle day 3–5 in PCOS patients was significantly higher than in the controls (mean ± SE; 1100 ± 91 and 601 ± 39 pg/ml respectively, P < 0.0001). Weight (73.3 ± 2.7 and 66.3 ± 2.1 kg respectively) and BMI (27.3 ± 0.9 and 24.8 ± 0.7 kg/m²) of the PCOS patients were also significantly higher (P < 0.05). Therefore, each group was further divided into obese and non-obese (BMI ≥ and <27 kg/m² respectively) and the four groups were compared.

Serum follistatin and activin A on cycle day 3–5 in obese and non-obese PCOS and control groups are shown in Figure 1. Follistatin levels were comparable between obese and non-obese PCOS patient groups (mean ± SE; 1171 ± 103 and 1045 ± 159 pg/ml respectively) and significantly higher than their respective controls (628 ± 61 and 592 ± 49 pg/ml, P < 0.001 and P < 0.05 respectively). Activin A concentrations were comparable between the four groups (590 ± 35, 513 ± 74, 661 ± 87 and 595 ± 43 pg/ml in obese and non-obese PCOS groups and their corresponding controls respectively). Follistatin to activin ratios were 2.4 fold higher in PCOS patients compared with the controls (2.7 ± 0.21 and 0.92 ± 0.10 respectively, P < 0.0001) (Figure 2).

Clinical data and cycle day 3–5 serum hormone concentrations (mean ± SE) in obese and non-obese PCOS and control patients are shown in Table I. LH concentrations were 3-fold higher (P < 0.001) in both obese and non-obese PCOS patients compared with their respective controls, in the face of comparable FSH concentrations. LH concentrations were 50–60% higher (P < 0.05) in non-obese than obese groups. Testosterone concentrations were 2.5 and 1.5 fold higher in non-obese (P < 0.05) and obese (P = 0.05) PCOS patients than in their respective controls. Δ4A concentrations were double in obese PCOS patients compared with their respective controls (P < 0.001) and 1.5 fold higher in non-obese patients compared with obese controls (P < 0.05). Serum oestradiol concentrations were comparable in all groups. Concentrations
levels were also similar before and after progesterone-induced bleeding (1034 ± 51 and 1100 ± 91 pg/ml respectively). In the control group, serum follistatin concentrations were similar on cycle day 3–5 and 20–22 (605 ± 41 and 629 ± 46 pg/ml respectively). Serum activin A levels were also similar before and after progesterone-induced bleeding in the PCOS group (649 ± 80 and 593 ± 70 pg/ml respectively) and on cycle day 3–5 and 20–22 in the control group (599 ± 35 and 666 ± 69 pg/ml respectively).

To determine which independent variables affect serum follistatin and activin A concentrations, stepwise linear regression analyses with follistatin and activin A as the dependent variables and the presence of PCOS and IR, age, weight, BMI, serum LH, FSH, T, Δ4A and 17-OH-P concentrations were performed. Eighty-eight patients, who had no missing data, were included in the regression analyses. Follistatin concentrations were significantly and independently positively affected by PCOS (P < 0.0001), age (P < 0.02), Δ4A levels (P < 0.03) and weight (P < 0.05). Activin A concentrations were significantly and independently negatively affected by PCOS (P < 0.003) and day 3–5 FSH concentrations (P < 0.03), and positively affected by weight (P < 0.009) and Δ4A levels (P < 0.02). IR correlated with follistatin concentrations in both PCOS and control groups (r = 0.30 and r = 0.28 respectively, P < 0.05). However, stepwise regression analysis showed that IR was not a statistically significant independent factor affecting follistatin concentrations; it also positively correlated with weight and BMI (r = 0.34 and r = 0.31 respectively, P < 0.05), and negatively correlated with LH (r = -0.41, P < 0.01) in the PCOS patients.

Discussion

Our study was instigated by recent reports that follistatin can encourage and activin can oppose many features of PCOS, such as increased ovarian androgen synthesis, decreased pituitary FSH, disturbed pancreatic insulin secretion and inhibition of follicular development. Our data indicated that the serum follistatin/activin ratio was 2.4 fold higher in PCOS patients compared with controls. Follistatin was increased by 80–90% in PCOS patients, independent of obesity. A negative influence of PCOS on activin A levels was found by stepwise regression analysis. Moreover, PCOS was the most significant variable that independently increased follistatin and decreased activin A serum levels when weighed against age, weight, BMI and hormonal status. All these results achieved high statistical significance (P < 0.003 to P < 0.0001).

Following the completion of our study, Norman et al. published their findings and quite independently discovered follistatin and activin concentrations in PCOS, very similar to our findings (Norman et al., 2001). We have, in addition, answered the question which Norman et al. left open, i.e. a correlation of these findings with BMI. We have demonstrated that weight is not the explanatory factor.

It has been shown that virtually all circulating follistatin in women (Muttukrishna et al., 1996; McConnell et al., 1998) and girls (Foster et al., 2000; Phillips et al., 2000b) is activin-bound. In contrast, a substantial amount of free follistatin has been detected in the follicular fluid and pituitary. These observations are compatible with the hypothesis that the main biological role of circulating follistatin is to restrict activin bioavailability. Currently, there is no reliable method to measure free activin levels due to disruption of activin/follistatin complexes during the assay procedures. Using the same immunoassay described above, changes in the ratio between total follistatin and activin A were found in pregnant women with
hypertensive disorders (D’Antona et al., 2000), suggesting that unbound, biologically active activin A is increased in these disorders. Our results support the assumption that high follistatin levels accompanied by a low biologically active activin A level contribute to the pathophysiology of PCOS.

The source of serum follistatin and activin in women is unascertained. Both peptides are secreted from ovarian granulosa cells in response to FSH stimulation (Vale et al., 1990). The concentrations of follistatin and activin A in follicular fluid exceed serum concentrations by as much as 100–200 and 30-fold respectively (Ericsson et al., 1995; Khoury et al., 1995; Muttukrishna et al., 1996; Evans et al., 1998). However, follistatin concentrations remain stable with no consistent trend across the menstrual cycle (Demura et al., 1993; Khoury et al., 1995; Kettel et al., 1996; Evans et al., 1998). Furthermore, serum follistatin concentrations have been found to be similar in eugonadal women, women with hypothalamic amenorrhoea and women after oophorectomy (Khoury et al., 1995), and did not change during puberty or after ovarian suppression with gonadotrophin-releasing hormone analogue administration (Kettel et al., 1996). Other investigators found that serum total activin A concentration did not vary significantly across the peri-menopause in women (Muttukrishna et al., 2000) or pubertal stages in girls (Foster et al., 2000). However, follistatin concentrations in late puberty were less than those in early puberty (Phillips et al., 2000b). Post-menopausal women had higher (Kettel et al., 1996) or similar (Khoury et al., 1995) follistatin levels than young cycling women. Variation in serum activin A during the normal menstrual cycle is much less than that observed for inhibins (Harada et al., 1996; Knight et al., 1996; Muttukrishna et al., 1996). No changes in serum activin A were found in IVF patients after pituitary down-regulation and during FSH ovarian stimulation (Lockwood et al., 1996). In the present study, we also found no differences in either follistatin or activin A levels between early follicular and mid-luteal phases in normal cycling women, or before and after progesterone administration in PCOS women. These data argue against the ovary as a significant source of circulating follistatin or activin A.

Interestingly, no differences in the levels of follistatin were found in follicular fluids from healthy, atretic or polycystic ovaries (Erickson et al., 1995). There was no difference in follicular fluid activin A concentration between PCOS and size-matched follicles from normally cycling women (Magoffin and Jakimik, 1998). Lambert-Messerlian et al. found increased levels of follicular fluid follistatin concentrations with follicular volume in normal cycling IVF patients, but not in PCOS patients (Lambert-Messerlian et al., 1997). It is difficult to explain why follistatin mRNA was entirely absent from sections of polycystic ovaries examined by in-situ hybridization compared with normal ovaries (Roberts et al., 1994). Together, our finding and the above reports implicate an extra-gonadal origin of the high circulating follistatin in PCOS.

Nevertheless, both follistatin and activin have multiple sites of production and multiple inducers. For example, changes in activin/follistatin have been found in atherosclerosis (Kozaki and Ouchi, 1998), inflammatory response (Jones et al., 2000), septicaemia (Michel et al., 1998) and neoplasia (Danila et al., 2000). The exclusion of patients treated with anticoagulants such as heparin is also important, since heparin administration can induce an increase in circulating levels of both activin A and follistatin (Phillips et al., 2000a). An increased synthesis or secretion of follistatin from the liver, adrenal glands, pancreas, endothelial cells or another organ or tissue in PCOS patients could be speculated as a source of the high serum follistatin concentrations in this syndrome. In fact, follistatin mRNA levels in cultured fibroblasts from PCOS and control women hardly differ (Urbanek et al., 2000). However, as with insulin resistance in PCOS, there might be tissue specificity and fibroblasts are not the main physiological source of serum follistatin.

In 1999 a large group of investigators reported strong evidence for a link between the follistatin gene and PCOS (Urbanek et al., 1999). However, in a later expanded study of 249 families, the evidence for linkage between PCOS and the single rare mutation found was weak (Urbanek et al., 2000). Furthermore, no mutations could be found in the entire coding region of the follistatin gene in 64 Chinese patients with PCOS (Liao et al., 2000). However, the whole process of follistatin mRNA and protein synthesis, processing, binding and secretion is very complex. The existence of multiple follistatin isoforms with different bioactivities may provide another mechanism.

### Table 1. Clinical data and day 3–5 serum hormone concentrations in obese and non-obese polycystic ovary syndrome (PCOS) and control patients (values are mean ± SE)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Obese PCOS (n = 24)</th>
<th>Non-obese PCOS (n = 20)</th>
<th>Obese controls (n = 16)</th>
<th>Non-obese controls (n = 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29.4 ± 1.0</td>
<td>27.3 ± 0.8a</td>
<td>30.5 ± 1.2</td>
<td>29.9 ± 0.6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>86.4 ± 2.4b</td>
<td>57.5 ± 2.1</td>
<td>87.4 ± 3.9b</td>
<td>58.9 ± 1.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.0 ± 0.8b</td>
<td>21.7 ± 0.7</td>
<td>32.1 ± 1.2b</td>
<td>22.3 ± 0.4</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>10.2 ± 1.5c,d</td>
<td>16.4 ± 2.1c</td>
<td>3.4 ± 0.3d</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>5.1 ± 0.4</td>
<td>5.6 ± 0.4</td>
<td>5.3 ± 0.4</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>2.4 ± 0.2</td>
<td>4.3 ± 1.0d</td>
<td>1.6 ± 0.3</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Δ4A (nmol/l)</td>
<td>7.8 ± 0.8b</td>
<td>7.5 ± 1.5</td>
<td>3.9 ± 0.3d</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>Oestradiol (pmol/l)</td>
<td>190 ± 25</td>
<td>237 ± 45</td>
<td>137 ± 19</td>
<td>168 ± 13</td>
</tr>
<tr>
<td>17-OH-P (nmol/l)</td>
<td>4.5 ± 1.0b</td>
<td>2.9 ± 0.3d</td>
<td>2.0 ± 0.3</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>

*a* *P < 0.05, PCOS versus corresponding controls; b* *P < 0.0001, obese versus corresponding non-obese; c* *P < 0.001, PCOS versus corresponding control; d* *P < 0.05, obese versus corresponding non-obese.*

**BMI** = body mass index; Δ4A = androstenedione; 17-OH-P = 17-hydroxy-progesterone.
for the control of follistatin activity through alterations in post-transcriptional processing (Esch et al., 1987).

In summary, we found that the serum follistatin/activin ratio was increased in PCOS patients compared with controls. Serum follistatin was increased in PCOS patients regardless of obesity. Using stepwise regression, we showed that PCOS was the most significant variable that increased follistatin and decreased activin A serum levels when weighed against age, weight, BMI and hormonal status. We postulate that by neutralizing the bioactivity of activin, high serum follistatin levels could well contribute to the pathophysiology of PCOS. While the measurement of follistatin and activin will probably not be relevant for the diagnosis of PCOS, their role in the pathophysiology of this condition and the possibility of developing therapeutic alternatives make further investigation imperative.

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References


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