Abstract

Objective: Polycystic ovary syndrome (PCOS) is associated with insulin resistance and a high incidence of obesity. Leptin, the product of the ob gene, is involved in the regulation of energy balance and obesity and circulates in both free and bound forms. The soluble leptin receptor (sOB-R) is the most important leptin-binding protein, thus influencing the biologically active free leptin level.

Design: We assessed the correlation of metabolic and endocrine parameters with leptin and sOB-R levels in 122 PCOS women (aged 27 ± 5.7 years) and 81 healthy controls (aged 25 ± 4.0 years).

Methods: Leptin and sOB-R levels were measured using ELISA kits. In addition, anthropometric variables, body fat and endocrine parameters were evaluated and a glucose tolerance test performed to assess indices of insulin resistance and glucose metabolism.

Results: In PCOS patients, no correlation was found between leptin or sOB-R and parameters of hyperandrogenism. However, as expected, body mass index (BMI), body fat, waist circumference and indices of insulin resistance were significantly correlated with leptin in PCOS subjects and controls. In a subgroup analysis of lean, overweight and obese PCOS patients, significant differences were found in leptin (29.7 ± 20.7 vs 45.4 ± 25.0 vs 67.7 ± 28.8 ng/ml, \( P = 0.0001 \)) and sOB-R (8.0 ± 3.4 vs 6.4 ± 2.5 vs 5.7 ± 2.3 ng/ml, \( P < 0.05 \)). Compared with BMI-matched controls, lean PCOS patients had lower sOB-R levels (8.0 ± 3.4 vs 12.7 ± 4.7 ng/ml, \( P = 0.0001 \)) and higher free leptin indices (4.5 ± 3.9 vs 2.8 ± 2.2, \( P = 0.0285 \)).

Conclusion: Taking into account that low sOB-R levels supposedly compensate diminished leptin action, PCOS per se might cause leptin resistance.

Introduction

Polycystic ovary syndrome (PCOS) is among the most common endocrine disorders, affecting more than 5% of women of reproductive age (1, 2). An association of PCOS with peripheral insulin resistance, compensatory hyperinsulinemia and alterations in β-cell function as the cause of its predisposition to develop a metabolic syndrome (type 2 diabetes mellitus, hypertension, lipid disorders, obesity) has been established (3). Most relevant risk factors of cardiovascular disease are elevated in PCOS women (4). However, it is unclear whether this risk results in an increase in cardiovascular morbidity and mortality later in life. On the other hand, there is clear evidence that insulin resistance and hyperinsulinemia aggravate ovarian androgen overproduction and enhance skin manifestations. According to the majority of studies, most PCOS women are insulin resistant and overweight or obese. Adipose tissue has been established as a major endocrine organ. Through the release of peptides, such as adiponectin, resistin and leptin (5), it is involved in the pathogenesis of several metabolic disorders. Leptin, the gene product of the ob gene, is thought to provide the central nervous system with feed-back information about fat storage of the body. Thus, leptin is thought to be a part of the regulation of appetite, food intake and the metabolic rate (6). Obese humans present with hyperleptinemia as an indicator of leptin resistance which, in turn, has been suggested to play a major role in the pathogenesis of obesity (7).

The consensus of published studies did not find a PCOS-specific influence on leptin, as no significant differences were found between body mass index (BMI)-matched PCOS women and healthy controls (8–11). The main determinants of leptin concentration in these studies were fat mass and the degree of obesity or insulin resistance.
In mice, a genetic defect in the ob gene results in severe obesity and type 2 diabetes mellitus, as well as in infertility (6). In this mouse model, leptin injections restore fertility. Leptin treatment in normal female mice accelerates puberty (12). In humans, granulosa cells have been shown to secrete leptin (13) and leptin seems to influence adrenal androgen formation (14). However, the role of leptin in ovarian function or on the reproductive system remains unclear. On the one hand, leptin levels did not influence follicular maturation and ovulation in PCOS patients (10) but, on the other hand, Carmina and co-workers (15) demonstrated a negative correlation between leptin and dehydroepiandrosterone sulphate (DHEAS) levels in 21 lean PCOS women, suggesting that androgens may play a role in suppressing serum leptin. Another study showed that leptin concentrations correlated inversely with luteinizing hormone (LH) levels, independent of body weight and insulin resistance (16). In contrast again, Laughlin et al. (17) proposed that leptin is neither involved in the hypersecretion of LH nor in the regulation of LH pulsatility. During luteal phase, Sinha et al. (18) also showed that leptin secretion is not related to LH secretion.

Both metabolic and endocrine parameters might correlate better with free leptin rather than with total leptin levels, as the former represents the biologically active fraction that is available for direct interaction with its receptor. The levels of free leptin can either be measured directly or assessed by determination of the soluble leptin receptor (sOB-R), which accounts for the majority of serum leptin-binding activity (19). The sOB-R has been shown to correlate inversely with total serum leptin levels and BMI in women of reproductive age (20). Kado et al. (20) hypothesized that obese women are able to conversely increase their leptin activity by minimizing the bound leptin fraction, thus influencing the biological activity of leptin. In line with this, Sinha et al. (21) reported that in lean subjects leptin circulates predominantly in the bound form, whereas in obese patients, due to low sOB-R levels, leptin circulates mostly in the free form. Similar results were found for Japanese men and women, showing a negative correlation between sOB-R levels and BMI, parameters of insulin resistance, cholesterol and leptin levels (22). Weight loss, following gastric restrictive surgery, leads to a reduction in circulating leptin levels and to an increase in sOB-R (23). In 114 Greek students, determination of total energy intake and macronutrient composition by food records was undertaken in order to assess an association between sOB-R and dietary variables. The sOB-R was mostly correlated with the total amount of energy intake and not so much with food composition (24). In adolescent girls with anorexia nervosa, higher sOB-R levels and a lower free leptin index (FLI) were found compared with healthy control women (25). In this cohort, sOB-R levels were also correlated with cortisol levels. Additionally, sOB-R and FLI but not total leptin levels were closely related to parameters of sexual maturation and the onset of puberty (26).

We here present an analysis of sOB-R and its correlation with obesity, leptin resistance, insulin resistance and other endocrine parameters in PCOS women.

Subjects and methods

Study population

PCOS patients (n = 122) were recruited from the outpatient clinics of the Division of Endocrinology, Department of Medicine and the Department of Gynecology at the University of Duisburg-Essen. Some patients were also attracted by the clinic’s PCOS homepage (www.pco-syndrom.de). Based on the criteria derived from the 1990 National Institutes of Health (NIH) conference, diagnosis of PCOS was established when either oligomenorrhea (cycles lasting longer than 35 days) or amenorrhea (less than two menstrual cycles in the past 6 months) and either clinical signs of hyperandrogenism (hirsutism or obvious acne or alopecia and/or an elevated total testosterone in combination with an elevated free androgen index (FAI) (normal range: testosterone <2.0 nmol/l, FAI < 3.8) were found, and other pituitary, adrenal or ovarian diseases could be excluded. In addition, all women an adrenocorticotropic test with measurement of 17-hydroxyprogesterone was performed. When the difference of both values (baseline and after 60 min) was greater than 2 ng/ml or the stimulated value was greater than 10 ng/ml, a genetic analysis (21-hydroxylase deficiency) was added. Hirsutism was routinely graded by two physicians independently using the common modified Ferriman–Gallway (FG) score. FG scores never differed by more than 2 and when not identical were re-evaluated by a third physician and the median value used. This method to assess hirsutism requires the visual scoring of the extent of terminal hairs in nine body areas, namely (a) upper lip, (b) chin, (c) chest, (d) upper abdomen, (e) lower abdomen, (f) upper back, (g) lower back, (h) thighs and (i) upper arms. The lower arms or lower legs are not included in the hair assessment. Each area is scored from 0 to 4, resulting in a possible maximum score of 36. Hirsutism was diagnosed when a score above 5 was evaluated. All PCOS women also fulfilled the 2003 Rotterdam criteria. All recruited women were required to be otherwise healthy. Healthy controls (n = 81) were matched according to BMI (<25 kg/m²) with a subgroup of lean PCOS women (n = 44). Controls were recruited from a screening program for employees instituted at the University of Duisburg-Essen. In control women, all NIH criteria of PCOS were excluded. PCOS as well as control subjects had not taken any medication known to affect carbohydrate metabolism or endocrine parameters for at least
Data collection
In PCOS subjects and control women, clinical parameters were assessed by physical examination, including the degree of hirsutism by evaluating the FG score and anthropometric measurements including body weight in kg (BW) and waist (W) and hip (H) circumference in cm. Body fat (F) was measured using the Body FAT Watcher (NAIS Wellnesslife GmbH, Duesseldorf, Germany) and BMI was calculated as weight/(height)² (kg/m²). Parameters of insulin resistance and β-cell function were evaluated using a 3-h oral glucose tolerance test (OGTT). After an overnight fast of 12 h, patients ingested 75 g glucose and had their glucose and insulin levels determined at baseline and at 30, 60, 90, 120 and 180 min. Insulin resistance and β-cell function were determined by the homeostasis model assessment (HOMA) (27) and hyperinsulinemia by calculating the area under the insulin response curve (AUC-I). In addition, whole-body insulin sensitivity (ISI_Matsuda) (28), which combines hepatic and peripheral insulin sensitivity, was measured by the formula: 10,000/square root of (fasting glucose × fasting insulin) × (mean glucose × mean insulin during OGTT times 0, 30, 60, 90, 120 min). Prior to OGTT (between 0800 and 0900 h), blood samples were drawn for serum leptin, sOB-R and all other hormonal and metabolic parameters. The FAI was estimated as testosterone (nmol/l)/sex hormone-binding globulin (SHBG) (nmol/l) × 100. The ratio of leptin/sOB-R was used as the FLI. Except for amenorrhoic women, all laboratory determinations were performed in the early follicular phase of the cycle.

Biochemical assays
Automated chemiluminescence immunoassay systems were used to determine levels of LH, estradiol, testosterone, cortisol, thyrotropin (TSH), cholesterol (CHOL), triglycerides (TG) and blood glucose (G) (ADVIA Centaur; Bayer Vital, Fernwald, Germany), androstenedione, DHEAS, insulin and SHBG (IMMULITE 2000: DPC Biermann, Bad Nauheim, Germany) and insulin-like growth factor (IGF-I) (Nichols Advantage, Nichols Institute Diagnostics, Bad Vilbel, Germany). The glycosylated fraction of hemoglobin-A1 (HbA1c) was determined by an automated HPLC method on an A1C 2.2 glycohemoglobin analyser (TOSOH-Eurogenetics, Cologne, Germany). Leptin and sOB-R were measured using ELISA kits (Diagnostic Systems Laboratories (DSL), Webster, TX, USA). The DSL ACTIVE Human Leptin ELISA is an enzymatically amplified ‘two-step’ sandwich-type immunoassay. The DSL ACTIVE Leptin Soluble Receptor ELISA is a ‘one-step’ sandwich-type immunoassay. The theoretical sensitivity or minimum detection limit as calculated by interpolation of the mean plus two standard deviations of 12 replicants of the zero standard was 0.05 ng/ml for leptin and 0.14 ng/ml for sOB-R. Intra-assay variation was < 5% and interassay variation was < 8% for all measured parameters.

Ethical approval
The study protocol was approved by the Ethics Committee of the University of Duisburg-Essen. All participants gave written informed consent before entering the study.

Statistical analysis
Data are presented as median plus range for non-parametric data. For better comparison, means±S.D. is also shown. Correlations between variables were examined by Spearman’s correlation coefficient (rs) because analysed data were not normally distributed. Differences between the groups were evaluated with the Mann–Whitney U test. P values < 0.05 were considered significant. The box-plot graph was computed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA, USA).

Results
In PCOS women, leptin levels correlated significantly with BMI (Fig. 1). In addition, leptin was negatively correlated with sOB-R levels both in PCOS patients and in controls, but sOB-R levels were lower in PCOS women than in healthy women (Fig. 2). In PCOS patients, leptin was correlated with metabolic parameters such as insulin resistance, glucose and lipid metabolism (Table 1). A significant correlation was also observed between sOB-R and BMI (rs = −0.21, P = 0.0285), body fat, waist and insulin resistance (IR) but not with serum lipids or parameters of glucose metabolism (Table 1). Analysis of endocrine parameters revealed a significant correlation between leptin and FAI (rs = 0.29, P = 0.0022) as well as SHBG (rs = −0.43, P < 0.0001), but not with total testosterone (rs = −0.15), androstenedione (rs = −0.10), DHEAS (rs = −0.04), estradiol (rs = −0.01), LH (rs = 0.09), cortisol (rs = −0.08) or IGF-I (rs = 0.00) (all P > 0.05). No correlation between hirsutism scores and leptin or sOB-R was found. Interestingly, both leptin (rs = 0.25, P = 0.0060) and sOB-R (rs = −0.22, P = 0.0173) correlated with TSH. No correlations were found between sOB-R and sex steroids, cortisol or lipids.

Lean PCOS patients (n = 44) and BMI-matched controls (n = 81) did not differ in anthropometric parameters or leptin levels (Table 2). Although ten PCOS patients had a HOMA-IR > 2 and five of these had a
HOMA-IR > 2.6 (the limit for insulin resistance for this age group in our reference population), mean HOMA-IR of the PCOS patients was not significantly different from that of the control group (Table 2). However, sOB-R levels were significantly lower in lean PCOS women compared with these controls (Fig. 3). As expected from the lower sOB-R, PCOS patients had a higher FLI than controls (PCOS 4.5±3.9 vs controls 2.8±2.2, P = 0.0285). Lean PCOS women had significantly lower triglyceride levels (0.82±0.36 vs 1.09±0.48 mmol/l, P = 0.0021) and total cholesterol levels (4.7±0.7 vs 5.2±1.1 mmol/l, P = 0.0164), not explained by the differences in TSH, as thyroid function was not correlated with lipid metabolism in women with normal body weight.

In the lean PCOS subgroup, correlation of leptin with BMI remained significant (r = 0.5041, P = 0.0007) but not the correlation between leptin and sOB-R (r = −0.0689, P = 0.664). PCOS subjects were found to have higher androgen levels and hirsutism scores (7±4 vs 2±2) than controls but did not differ in cortisol or IGF-I levels (data not shown). PCOS patients also had significantly higher TSH levels than controls (2.0±1.0 vs 1.5±0.6 mU/l, P = 0.0038), in part due to four PCOS patients with elevated TSH levels (3.6–5.0 mU/l). However, excluding these from the evaluation (n = 40; TSH 1.8±0.8 mU/l), differences in TSH levels and sOB-R levels (PCOS 7.95±3.4 vs controls 12.6±4.7 ng/ml) still remained significant (P = 0.0159 and P < 0.0001 respectively). Thus, while TSH correlated with sOB-R in the entire PCOS cohort, no such correlation was found in lean PCOS patients and healthy controls.

To evaluate the impact of BMI on leptin and sOB-R levels, PCOS patients were stratified into three groups according to BMI: <25 kg/m², PCOS lean, n = 44; 25–29.9 kg/m², the overweight group, n = 20 and ≥ 30 kg/m², PCOS obese, n = 58. As expected, metabolic variables correlated with BMI in PCOS patients (Table 2). Leptin increased and sOB-R decreased with higher BMI values. SHBG levels were

Figure 1 Correlation of BMI and leptin levels in PCOS women. Total leptin was determined in 122 PCOS patients as described in Subjects and methods. Spearman’s evaluation of correlation gave an r = 0.56, P < 0.0001.

Figure 2 Correlation of leptin with sOB-R levels in PCOS patients and healthy controls. Leptin and sOB-R levels were determined in 122 PCOS patients (●) and 81 healthy controls (□) by ELISA as described in Subjects and methods. In both PCOS and controls, leptin was significantly correlated with sOB-R (r = −0.38, P < 0.0001 and r = −0.27, P = 0.0142 respectively). However, the sOB-R levels of the PCOS patients were shifted to lower values by about 4 ng/ml.
also different between the groups, but all other endocrine parameters were similar (Table 2).

The PCOS cohort was also divided into a hyperandrogenemic group (HA) (n = 103) and a group with normal testosterone levels (N) (n = 19) to analyse the impact of androgens on leptin and sOB-R levels. Neither group differed in hirsutism scores (HA 10.4 ± 7.3 vs N 10.1 ± 6.0, P = 0.8796). Leptin and sOB-R values were similar in the HA and the N subgroup (leptin P = 0.75, sOB-R P = 0.645). Furthermore, oligo- and amenorrheic (n = 78) and ammenorrheic (n = 44) PCOS women had similar leptin and sOB-R levels (data not shown).

**Discussion**

Leptin levels are increased in obesity and may play a role in the development of insulin resistance and type 2 diabetes mellitus (7). In our study, leptin levels correlated significantly with BMI and body weight in both PCOS women and controls. The difference in mean leptin levels in lean, overweight and obese patients was highly significant. In agreement with previous studies (8), leptin correlated with other metabolic parameters including lipid metabolism and insulin resistance in this German PCOS cohort. However, in conformity with other studies (9), our lean PCOS women and healthy controls, with similar anthropometric variables and parameters of insulin resistance, did not differ in total leptin levels. Similar results were found in PCOS patients from Australia (29), Brazil (16), Canada (10), Finland (30), Italy (15), Sweden (8), Turkey (11) and the USA (17, 31).

In the entire PCOS cohort, as well as in the lean subgroup, no correlation was found between leptin concentrations and androgens or gonadotropins. The correlation of leptin with FAI resulted from its influence on SHBG. The inverse correlation between leptin and SHBG has previously been described by Laughlin et al. (17). Another study on leptin also reported a
correlation of leptin with FAI (8). Our results are also in agreement with data from Pirwany and colleagues (10) who did not find any correlation between leptin and testosterone as well as LH but a significant correlation between leptin and FAI. Furthermore, they did not find any correlation of leptin levels and follicular development or ovulation. We also did not find differences in leptin levels in either oligomenorrhoic or amenorrhoic women.

Glucocorticoids are potent regulators of leptin expression. In rats, the administration of glucocorticoids leads to an up-regulation of leptin expression (32). In contrast, Cushing’s syndrome does not elevate leptin levels in humans (33). On the other hand, leptin reduced cortisol secretion by 52% in adrenocortical cells isolated from obese patients (34). We did not find a correlation between leptin and cortisol levels or IGF-I, consistent with other studies (35, 36). A positive correlation of leptin with estradiol levels was detected in 28 PCOS women published by Mendonca et al. in 2004 (37). No such correlation was found in a study from Sweden (8) and in our study population.

In PCOS women, the present study confirms that circulating leptin levels correlate with parameters of insulin resistance and the metabolic syndrome but has no detectable effect on the reproductive features of PCOS. Thus, leptin appears to impair ovulation and reproduction only in extreme leptin deficiency, such as in the ob/ob mouse. No such extreme leptin deficiency has been described in PCOS patients.

Some studies have focused on the relationship between leptin and thyroid function. Thyroid hormones regulate the expression of leptin mRNA and secretion of leptin by adipocytes in vitro (38). In PCOS women, free thyroxine (fT4) was found to be lower than in controls (39) in some studies but not in all (40). However, even in the former study, only a weak and non-significant correlation between fT4 and leptin was found. We have recently described a higher incidence of elevated TSH levels and autoimmune thyroiditis in PCOS women (40), which was confirmed in this study. Pinkney et al. (41) found no differences in TSH levels of lean or obese euthyroid patients, with a significant correlation of leptin and TSH only in the obese subgroup. In agreement with these data, TSH correlated with leptin only in overweight and obese, but not in lean PCOS women in our German sample. In another study from Italy, subclinical hypothyroidism did not influence leptin levels in obese patients (42). Thus, the impact of thyroid function on leptin appears to be quite limited.

In obesity, high leptin levels are found to be indicative of leptin resistance (7). Potential mechanisms that may mediate leptin resistance include impairment of brain leptin transport or leptin receptor internalization, receptor mutations and post-receptor signaling defects. Furthermore, the active hormone may be reduced by binding proteins or soluble receptors (43). As only free leptin is found in cerebrospinal fluid, its free fraction rather than total leptin appears to be biologically active (44). sOB-R represents the main leptin-binding compound in plasma, thus regulating its free fraction in the circulation. Several studies have shown that sOB-R levels are inversely correlated with BMI (45), confirmed in our study by comparison of PCOS women divided into different BMI subgroups. sOB-R levels also decreased with higher body fat content and insulin resistance. Lower sOB-R levels increase the free leptin index, a mechanism likely to compensate, at least in part, for leptin resistance. All PCOS women, regardless of their BMI, presented with lower sOB-R levels and higher free leptin indices than healthy controls. The reduction of sOB-R could not be explained by differences in body fat content or other metabolic parameters.

The interplay of sOB-R and the reproductive system is quite unclear. While some studies have found an association of sOB-R with estradiol and free testosterone (46), sex hormones did not affect sOB-R levels in a Japanese population (45). In analogy to leptin, sOB-R did not correlate with androgens, estradiol or gonadotropins in our German PCOS cohort. The inverse relationship of sOB-R with IGF-I described in adolescents (47) could not be confirmed in our study, most likely because of the prevalence of adult women in our study population.

Yannakoulia and co-workers (24) demonstrated that sOB-R is positively correlated with total energy intake and inversely correlated with energy intake from...
dietary fat. They speculated that the macronutrient composition of the diet influences serum concentrations of free leptin. Another recent study found a positive correlation between free leptin and dietary carbohydrate content but not with lipid or protein content (48). The authors hypothesized that the reduction of SOβ-R levels is tantamount to or represents a compensatory mechanism to overcome leptin resistance. Except for slightly lower triglycerides and cholesterol levels in lean PCOS subjects compared with their BMI-matched controls, we cannot exclude differences in dietary composition in our PCOS and control groups, as no food records from our study population were available.

As neither androgens nor estrogens seem to explain the higher leptin (49) and lower SOβ-R levels in women (46), body composition has been speculated to account for these differences. Consistent with this hypothesis, reduction of trunk fat in anorectic women results in higher SOβ-R levels and, consequently, lower FLI (50). Waist-to-hip ratio, as an approximate measure of visceral obesity, did not differ in PCOS women and controls in our study (data not shown) but an exact evaluation of trunk, subcutaneous and extremity fat was not performed.

A recently published twin study of pubertal females studying the genetic and environmental influences on the variations of leptin and SOβ-R levels showed that leptin is mostly influenced by body composition but SOβ-R by genetic background (26). The lower SOβ-R levels in PCOS women, unexplained by body weight, body fat content or hormonal parameters, may indicate that genetic determinants are more important than environmental factors in its contribution to free leptin regulation. In conclusion, we are the first to demonstrate low SOβ-R levels in PCOS women as a possible mechanism to compensate for leptin resistance. Neither leptin nor SOβ-R levels were influenced by hyperandrogenism or the severity of menstrual disturbances. Rather than environmental factors, food or body composition, genetic differences might account for lower SOβ-R levels in PCOS women.

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