Omental adipose tissue overexpression of fatty acid transporter CD36 and decreased expression of hormone-sensitive lipase in insulin-resistant women with polycystic ovary syndrome

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BACKGROUND: Elevated free fatty acids (FFAs) are involved in insulin resistance in polycystic ovary syndrome (PCOS). We investigated the role of fatty acid transporter CD36, hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) in regulation of lipolysis in insulin-resistant women with PCOS.

METHODS: CD36, HSL and ATGL proteins were analyzed in omental adipose tissue from 10 women with PCOS and 10 healthy, BMI- and age-matched controls by western blotting.

RESULTS: Women with PCOS had higher fasting and 2 h insulin levels ($P < 0.002$, $P < 0.029$, respectively) and a higher homeostasis model insulin resistance index ($P < \text{HOMA}_{IR}$, 0.005) and a lower fasting glucose-to-insulin ratio ($G_0/I_0$) ($P < 0.001$) than controls. CD36 protein levels in the PCOS women were higher (268% of control levels, $P < 0.05$) and HSL protein levels were lower (43% of control levels, $P < 0.05$). However, ATGL protein levels were not different in the two groups. Fasting serum insulin levels showed a positive correlation with CD36 levels ($P = 0.01$, $r = 0.875$) and a negative correlation with HSL levels ($P = 0.045$, $r = -0.73$). Furthermore, a positive correlation was found between serum testosterone levels and CD 36 protein levels ($P = 0.025$, $r = 0.817$) but the correlation did not reach significance after controlling for HOMA$_{IR}$. After adjusting insulin resistance index of HOMA$_{IR}$ by analysis of covariance, only CD36 differed between PCOS and control ($P = 0.026$).

CONCLUSIONS: Our results suggest that, in insulin-resistant women with PCOS, changes in CD36 and HSL expression may result in altered FFA uptake.

Key words: polycystic ovary syndrome / free fatty acids / CD36 / hormone-sensitive lipase

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrinopathy characterized by chronic anovulation, hyperandrogenism and polycystic ovaries on ultrasonography (Rotterdam, 2004) which affects 5–10% of women of reproductive age (Dunaif, 1997; Diamanti-Kandarakis et al., 1999). Recent studies have shown that 50% of women with PCOS fulfill the criteria of metabolic syndrome (Ehrmann et al., 1999; Glintborg et al., 2004). PCOS is frequently associated with insulin resistance accompanied by compensatory hyperinsulinemia, creating a 5- to 8-fold increased risk of the development of type 2 diabetes mellitus compared with weight-matched female controls (Glintborg et al., 2004). Furthermore, significantly increased risks of hypertension, coronary heart disease and obesity...
Markers of lipolysis in polycystic ovary syndrome

Fatty acids are deposited as triacylglycerol (TG) in white adipose tissue and are mobilized (lipolysis) in the form of free fatty acids (FFAs) into the circulation to meet the energy demands of the organism. Elevated circulating levels of FFAs, released from adipose tissue stores, are a key factor in the development of insulin resistance and metabolic syndrome (Boden, 1997). Hormone-sensitive lipase (HSL) was initially regarded as the only rate-limiting lipolytic activity in mammalian adipocytes (Saltiel, 2000). However, HSL-null mice are not obese (Harada et al., 2003; Fortier et al., 2004) and, although their catecholamine-induced lipolysis is disturbed, their adipocytes show residual basal lipolysis (Oagua et al., 2000). These data suggest that HSL is the rate-limiting enzyme in the cellular catabolism of triglycerides in adipose tissue and muscle, but that other non-HSL lipases are responsible for the hydrolysis of triglycerides to diglycerides in adipose tissue. A novel triglyceride lipase, termed adipose tissue triglyceride lipase (ATGL), desnutrin, or iPLA2γ, has been isolated (Hinkle et al., 2004; Zimmermann et al., 2004). Recent studies have shown that HSL is the major lipase for catecholamine- and natriuretic peptide-stimulated lipolysis, whereas ATGL mediates the hydrolysis of triglycerides during basal lipolysis (Langin et al., 2005; Rydén et al., 2007). ATGL and HSL mRNA and protein levels are reduced in obese insulin-resistant humans compared with insulin-sensitive subjects (Jocken et al., 2007). In PCOS women, HSL protein expression and norepinephrine-induced lipolysis are significantly decreased, with no change in ATGL protein expression (Rydén et al., 2007); however, these PCOS women were not insulin-resistant compared with the BMI- and age-matched normal subjects.

Fatty acid transporter CD36, a class B scavenger receptor, is an 88 kDa heavily N-linked glycosylated membrane protein (Platt and Gordon, 1998) expressed on the surface of monocytes and macrophages (Febbraio et al., 2001) and an extensive range of cells and tissues, such as microvascular endothelial cells, intestinal cells, smooth muscle cells, adipose tissue, skeletal muscle and cardiomyocytes (Swerlick et al., 1992; Abumrad et al., 1993). CD36 is presumed to be an endocytic receptor for long-chain fatty acids and to be associated with their uptake. In ob/ob mice, CD36 mRNA levels in the liver and adipose tissue are increased (Memon et al., 1999). Furthermore, thiazolidinediones (insulin sensitizers) or agonists of peroxisome proliferator-activated receptor-γ, such as rosiglitazone, increase CD36 expression in monocytes/macrophages, adipose tissue and muscle (Tontonoz and Nagy, 1999; Wilmens et al., 2003). Moreover, CD36 protein levels are sensitive to insulin, and a recent study in skeletal muscle showed that insulin can acutely (within 3 h) increase the CD36 protein content by 1.5-fold (Corpeleijn et al., 2008). Since CD36 is expressed in tissues important in fatty acid metabolism, this may indicate that it plays an important role in insulin resistance. Glintborg et al. (2008) reported that levels of soluble CD36 (sCD36) are significantly increased in PCOS patients compared with control subjects and that its expression correlates with measures of insulin sensitivity independent of central fat mass. However, adipose tissue, especially that from the intra-abdominal cavity, is the main target of insulin action and glucose homeostasis in humans (Ciaraldi et al., 1997). In addition, PCOS is frequently associated with visceral obesity, suggesting that omental adipose tissue might play an important role in the pathogenesis of the syndrome (Corton et al., 2007). There are no data on CD36 protein expression in visceral adipose tissue in women with PCOS.

The aim of this study was to investigate the role of CD36 in fatty acid transport and the role of HSL and ATGL in the regulation of lipolysis in insulin-resistant women with PCOS.

Materials and Methods

Subjects
Ten women who fulfilled the inclusion criteria for PCOS detailed below were enrolled in this study. All were in good health and had not taken oral contraceptives within the last 3 months. The protocol was reviewed and approved by the Institutional Review Boards of the Shin Kong Wu Ho-Su Memorial Hospital. The patients entered this study only after giving their written informed consent.

PCOS was defined by clinical, laboratory and ultrasound criteria according to the consensus criteria reported by the Rotterdam group (2004). The clinical criteria included chronic anovulation or oligomenorrhea (menstrual interval > 6 weeks) or amenorrhea (no menstrual loss for > 3 months) dating from menarche, the biochemical criterion was elevated total serum testosterone levels (>0.8 ng/ml, normal range 0.06–0.80 ng/ml), although the ultrasound criteria were enlarged ovaries with an increased stroma and the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter and/or an increased ovarian volume (>10 ml) on transvaginal ultrasonographic examination (Balan et al., 2003). Serum prolactin and thyroid hormone levels were checked in all patients and were within normal limits. Cushing’s syndrome, congenital adrenal hyperplasia and androgenic tumors were excluded by appropriate testing.

Ten healthy, BMI- and age-matched women served as controls. None were hirsute, and all had a normal regular cycling menstrual period. None were taking oral contraceptives. All had a normal appearance of the ovaries on ultrasound and normal LH and FSH levels and none had elevated androgen levels.

Reagents
Polyclonal chicken antibodies against human HSL were purchased from ProSci Incorporated (Poway, CA, USA). Polyclonal rabbit anti-ATGL antibodies were purchased from Cayman Chemical (Ann Arbor, MI, USA). Polyclonal rabbit antibodies against human CD36 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Human anti-β-actin antibodies were purchased from Chemicon Inc. (Temecula, CA).

Oral glucose tolerance test, fasting glucose-to-insulin ratio and homeostasis model insulin resistance index
A 2 h oral glucose tolerance test (OGTT) with 75 g of glucose load was performed during the early follicular phase (days 3–7) on all women after an overnight fast. In the case of amenorrheic women, progesterone was given to induce withdrawal bleeding. Four blood samples were collected from the antecubital vein at 0, 30, 60 and 120 min after an overnight fast. In the case of amenorrhoeic women, progesterone was given to induce withdrawal bleeding. Four blood samples were collected from the antecubital vein at 0, 30, 60 and 120 min and serum stored at −20°C until assayed for glucose and insulin. The fasting glucose to insulin ratio (G0/I0) was measured as described previously (Legro et al., 1998). The homeostasis model insulin resistance index (HOMA2) was calculated using the formula: fasting glucose (mg/dl) x fasting insulin (μU/ml)/405 (Matthews et al., 1985). A HOMA2 value of >3.8 or a G0/I0 ratio ≤4.5 indicates insulin resistance in PCOS (Legro et al., 1999; Kauffmann et al., 2002).
Hormonal profile
Blood was withdrawn from the antecubital vein for the measurement of serum estradiol (E2), FSH, LH and testosterone levels on the day of the OGTT before and after laparoscopic ovarian electrocautery (LOE). For women with amenorrhea, 75 mg of progesterone was given i.m. to induce withdrawal bleeding and the blood sample was collected on cycle day 3 or 4. Serum levels of FSH, E2, testosterone and LH were measured by immunoassay using Immulite® kits (Diagnostic Products Corporation, Los Angeles, CA, USA). For FSH, the sensitivity was 0.1 mIU/ml and the intra-assay and inter-assay coefficients of variance 7.7 and 7.9%, respectively, although the corresponding values were 0.1 mIU/ml, 6.5%, and 7.1% for LH; 15 pg/ml (55 pmol/l), 6.3%, and 6.4%, for E2; and, 0.1 ng/ml, 4.0%, and 5.6% for testosterone.

Adipose tissue sampling
Adipose tissue weighing about 5–6 g was obtained from the omental fat tissue of all PCOS women by laparoscopy on the day of laparoscopic ovarian drilling. For control subjects, adipose tissue was obtained during laparoscopic examination for tubal infertility or sterilization. The adipose tissue of the PCOS and control subjects was extracted via a 5 mm trocar inserted in the umbilical area and immediately stored at −80°C until tested by western blotting.

Western blotting
Whole cell lysates of PCOS and control adipose tissue were prepared by sonication at 4°C in lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM HEPES, pH 7.8, 10 μg/ml of leupeptin, 20 μg/ml of aprotinin, 125 μM diethiothreitol and 1 mM phenylmethylsulfonyl fluoride) and analyzed on the same western blot. Samples (50 μg of total protein) were mixed with 50 μl of 2× sodium dodecyl sulfate (SDS)-mercaptoethanol sample buffer and boiled for 10 min, then the proteins were separated on 7.5% SDS gels and transferred to a polyvinylidene fluoride membrane. The membrane was then blocked for 1 h at room temperature using 5% skimmed milk in phosphate-buffered saline (PBS) containing 0.5% Tween-20, immunoblotted with antibodies against human CD36, HSL or ATGL diluted in PBS and horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) diluted in PBS, followed by detection with Chemiluminescence Reagent (Amersham Bioscience, Buckinghamshire, England). The band density was measured by densitometry, using Image Master VDS and Image Quant Analysis Software (Amersham Pharmacia Biotech, Hong Kong). The relative protein levels of CD36, HSL, ATGL and β-actin in the original total protein lysate from the adipose preparations were obtained. CD36, HSL and ATGL protein expression was normalized to β-actin protein expression. All antibodies were produced by immunizing host with a synthetic peptide derived from the sequence of human CD36, HSL or ATGL, purified by peptide affinity chromatography and confirmed using control peptides.

Statistical analysis
Data are presented as the mean ± SD. Statistical analysis was carried out using Student’s t test. Correlations between variables were evaluated by Pearson’s correlation coefficient or partial correlation analysis when appropriate. A General Linear Model Analysis of Covariance (ANCOVA) was used and introduces insulin resistance (HOMAIR) as a covariate, in order to characterize the impact of insulin resistance on the differences in HSL, ATGL and CD36 between PCOS and controls. Computations were performed using SPSS (Statistical Packages of Social Sciences, SPSS for Windows, Version 13.0, Inc., Chicago, IL, USA) software. In all cases, the threshold for significance was taken as P < 0.05.

Results
Clinical and endocrine metabolic characteristics
The clinical features and baseline hormonal and metabolic parameters for the control and PCOS women are shown in Table I. The mean age of the PCOS women was 29.5 ± 3.3 years and that of the controls 28.3 ± 3.3 years. Women with PCOS had significantly higher serum LH/FSH ratios and serum testosterone levels than controls. The fasting insulin, 2 h insulin post-75-g glucose load values and the HOMAIR were significantly higher in PCOS women than in controls (P < 0.05), consistent with the presence of insulin resistance. In addition, the G0/I0 ratio was significantly lower in the PCOS women than in the controls (P = 0.001). ANCOVA revealed a significant main effect of insulin resistance index of HOMAIR (P < 0.05) for HSL, ATGL and CD36. After adjusting HOMAIR by ANCOVA, HSL and ATGL did not differ significantly between PCOS and control. However, CD36 was significantly different (P = 0.026) between PCOS and control after adjusting HOMAIR by ANCOVA.
CD36 protein expression in omental adipose tissue
As shown in Fig. 1, CD36 levels in adipose tissue were significantly higher in the 10 PCOS women than in the controls (268% of control levels).

HSL protein expression in omental adipose tissue
As shown in Fig. 2, HSL levels in the 10 PCOS women were significantly lower than in the controls (43% of control levels). The HSL antibody may detect phosphorylated (activated) HSL and non-phosphorylated forms of HSL, hence the diffuse nature of the band.

ATGL protein expression in omental adipose tissue
As shown in Fig. 3, there was no significant difference in ATGL levels in adipose tissue from PCOS women and controls.

Correlations
We studied the relationships between levels of CD36 and HSL and ATGL in human omental adipose tissue. CD36 levels showed a significant negative correlation with HSL levels ($P = 0.027, r = -0.71$). No significant correlation was found between CD36 and ATGL levels or between HSL and ATGL levels. There was a significant negative correlation between HSL levels and either fasting insulin levels ($P = 0.045, r = -0.73$) or 2 h insulin ($P = 0.034, r = -0.55$). Furthermore, CD36 levels showed a significant positive correlation with fasting insulin ($P = 0.01, r = 0.875$) and were positively correlated with 2 h insulin, although the result did not reach statistical significance ($P = 0.068, r = 0.582$). Insulin levels did not correlate with ATGL levels. CD36 levels showed a significant positive correlation with testosterone levels ($P = 0.025, r = 0.817$). However, after controlling for HOMAIR by partial correlation analysis, CD36 levels did not correlate with testosterone levels ($P = 0.214, r = 0.450$).

Discussion
The role of HSL and ATGL in human fat cell lipolysis is not clear. In this study, we found that HSL expression was significantly lower (43% of control levels) in women with PCOS, whereas there was no significant
difference in ATGL levels, suggesting that HSL is more important than ATGL in the regulation of lipolysis in women with PCOS. In contrast, CD36 expression was increased in women with PCOS (268% of control levels). Our data suggest that HSL and CD36 are equally important in the regulation of TG mobilization and lipolysis in insulin-resistant women with PCOS.

The studied patients are lean (mean BMI 24.3 ± 6.3) but they present a severe hyperinsulinemia both in fasting condition and after a 2 h glucose tolerance test. Chinese women with POCIS are at increased risk for insulin resistance and glucose intolerance compared with normal control even though the mean BMI was only 22.4 (Wei et al., 2008). The prevalence of impaired glucose tolerance and diabetes mellitus in lean Chinese Taiwan women was 7.6 and 3.1%, respectively (Wei et al., 2008). We selected PCOS women with hyperinsulinemia and insulin resistance in this study.

In the present study, we found that ATGL protein levels were not altered in PCOS with insulin resistance, although HSL protein expression was significantly decreased. HSL is mainly required for stimulated catecholamine-induced lipolysis in adipocytes (Langin et al., 2005; Rydén et al., 2007). Our result is consistent with the findings of Rydén et al. (2007) that ATGL is less important than HSL in regulating catecholamine-induced lipolysis and that ATGL expression is not influenced by obesity or PCOS. These two sets of results also suggest that the reduced expression of HSL may be secondary to insulin resistance in women with PCOS (Jocken et al., 2007). Insulin resistance in PCOS is caused by an increase of FFA flow from visceral adipose tissue to the liver (Holte et al., 1994; Mai et al., 2008). Therefore, the observed reduction of HSL in omental tissue is a secondary effect of insulin, trying to limit the flow of FFAs to the liver. Furthermore, the decreased HSL protein expression could promote the development of obesity seen in PCOS via a decrease in lipolytic activity (Large and Arner, 1998). Lipolytic catecholamine resistance is seen in women with PCOS (Large and Arner, 1998) and is probably attributable to a combination of a decrease in β-adrenergic receptors, the regulatory II domain component of protein kinase-A, and HSL (Faulds et al., 2003). Nevertheless, HSL-null mice are not obese (Harada et al., 2003; Fortier et al., 2004).

Fatty acids are important for many biological functions and fatty acid transporter CD36 is a key protein in the regulation of FFA uptake across the plasma membrane in adipose tissue and skeletal tissue (Luiken et al., 2002a, b). Fatty acid transport and/or transporter expression is altered in animal models of insulin resistance (Memon et al., 1999; Luiken et al., 2002a). Levels of fatty acid transporter FAT/CD36 are increased in ob/ob mice, (Memon et al., 1999), but are unchanged in adipose tissue in streptozotocin-induced diabetes mice (Luiken et al., 2002b). We found that CD36 protein expression was significantly increased in women with PCOS. This is the first report that, in adipose tissue in insulin-resistant women with PCOS, CD36 may play a role in the insulin resistance, although the mechanism remains undefined. Corpeleijn et al. (2008) showed that the increase in FAT/CD36 protein content in obese humans is positively correlated with insulin resistance, as measured by hyperinsulinemic euglycemic clamp.

Several studies have shown that insulin can down-regulate ATGL and HSL mRNA levels in 3T3-L1 adipocytes in a dose-dependent manner (Kralisch et al., 2005; Kim et al., 2006; Kershaw et al., 2006). However, our data did not show any change in ATGL protein expression. In contrast, HSL protein expression showed a significant negative correlation with fasting and 2 h insulin in insulin-resistant women with PCOS. The CD36 protein content of cardiomyocytes and skeletal muscle is up-regulated by insulin and this increase contributes to increased FFA uptake capacity (Corpeleijn et al., 2008). Our present study also showed that CD36 levels showed a significant positive correlation with serum insulin levels. Furthermore, ANCOVA revealed a significant main effect of insulin resistance index of HOMAIR (P < 0.05) for HSL, ATGL and CD36 in women with PCOS. These results indicate that the CD36 and HSL protein content of adipose tissue is regulated by insulin in vivo.

The roles of CD36 and HSL in the regulation of insulin resistance in women with PCOS are still unclear. Bonen et al. (2004) reported that CD36 protein translocation is impaired in insulin resistance, resulting in an increased amount of CD36 at the sarcolemma and reduced translocation after insulin stimulation in vitro (Bonen et al., 2004). The increase in CD36 protein may be a mechanism to compensate for the reduced translocation. However, the impaired CD36 translocation cannot explain the decreased expression of HSL protein in women with PCOS. Several studies confirmed that insulin resistance and hyperinsulinemia in PCOS are caused by a chronic increase in FFA secretion (Holte et al., 1994; Mai et al., 2008). The adverse effects of hyperinsulinemia may stimulate secretion of CD36 protein in PCOS and thus increase FFA uptake and the increased cellular FFA concentrations may induce a negative feedback, and thus suppress HSL expression and reduce lipolysis of TGs. In addition, the high levels of insulin in insulin-resistant women with PCOS may further down-regulate HSL expression, establishing a vicious circle, and this could explain why CD36 expression is increased and HSL expression decreased in insulin-resistant women with PCOS.

In the present study, CD36 levels showed a significant positive correlation with serum testosterone levels. This is an interesting finding and has not been reported previously. High testosterone levels increase scavenger receptor B1 mRNA and protein levels in cultured HepG2 hepatocytes and primary human monocyte-derived macrophages (Langer et al., 2002). Since CD36 is classified as a class B scavenger receptor (Platt and Gordon, 1998), it would not be too surprising that testosterone may also up-regulate CD36 protein expression in women with PCOS. However, this result differs from Glintborg et al.’s study (2008), which showed no significant correlations between sCD36 and testosterone levels. Furthermore, Yao et al. (2004) reported that testosterone levels in follicular fluid in polycystic ovaries were negatively related to CD36 mRNA expression in follicle theca interna. Although our results from adipose tissue are interesting, further studies are needed for verification, owing to the limited number of samples available for the study. In addition, it is difficult to draw a conclusion from correlations between omental tissue expression and blood levels of testosterone. Moreover, the significant positive correlation between CD36 and testosterone is most likely the effect of insulin resistance in women with PCOS, since after controlling for HOMAIR by partial correlation analysis the correlation was not significant.

The limitation of this study is all the control and women with PCOS were not matched for insulin resistance although they were matched for BMI and age. Only women with PCOS were insulin resistant in this study. CD36 and HSL are expressed on tissues important in fatty acid metabolism, such as adipose tissue, skeletal muscle, liver...
etc; this may imply an important role for CD36 in insulin resistance in PCOS. The observed protein levels could be due to the different insulin sensitivity between control and PCOS women. However, because of difficulties in recruiting lean control subjects with insulin resistance but no other associated endocrine disorder (such as Cushing’s syndrome, congenital adrenal hyperplasia), we were only able to recruit healthy lean subjects without insulin resistance as the normal controls in this study. Furthermore, the number of studied patients is small and only larger studies may indicate the real role of insulin in regulating HSL expression in adipose tissue.

In conclusion, our results clearly demonstrate that an overexpression of CD36 and decreased expression of HSL may play a pivotal role in the pathogenesis of insulin resistance in women with PCOS.

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