Expression levels of haem oxygenase-1 in the omental adipose tissue and peripheral blood mononuclear cells of women with polycystic ovary syndrome

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BACKGROUND: Haem oxygenase (HO)-1, an enzyme that degrades haem, plays a key role in the regulation of the inflammatory response and insulin resistance. The aim of this study was to evaluate the role of HO-1 in the regulation of insulin resistance and glucose tolerance in women with polycystic ovarian syndrome (PCOS).

METHODS: Omental adipose tissue and human peripheral blood mononuclear cells (PBMCs) from seven women with PCOS and five healthy controls, matched for BMI and age, were analysed using western blotting and the real-time PCR.

RESULTS: Women with PCOS were found to have significantly higher fasting and 2-h insulin levels, a significantly higher homeostasis model assessment insulin resistance index and a lower fasting glucose-to-insulin ratio (G0/I0) than the controls. The level of HO-1 protein in omental fat (P = 0.002), and the expression of HO-1 mRNA in omental fat and PBMCs from the women with PCOS were significantly lower (P = 0.002 and 0.05, respectively) than those of the controls. The expression of adiponectin mRNA in omental fat was also significantly lower (P = 0.02) in the women with PCOS than in the controls. However, there were no significant differences in the expression of tumour necrosis factor-α or interleukin-6 between the two groups. The level of HO-1 protein showed a significant positive correlation with the expression of HO-1 mRNA (r² = 0.786, P = 0.037) and adiponectin mRNA (r² = 0.7276, P < 0.05). Serum insulin and glucose levels and BMI showed a significant negative correlation with the level of HO-1 (P < 0.05).

CONCLUSIONS: Our results suggest that the HO-1–adiponectin axis may be associated with the regulation of insulin resistance and glucose intolerance in women with PCOS.

Key words: polycystic ovary syndrome / haem oxygenase-1 / adiponectin / insulin resistance

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrinopathy that is characterized by chronic anovulation, hyperandrogenism and polycystic ovaries on ultrasonography (2004). It affects 5–10% of women of reproductive age (Dunaif, 1997; Diamanti-Kandarakis et al., 1999), and 60% of the affected women are obese. PCOS is associated frequently with insulin resistance accompanied by compensatory hyperinsulinaemia, which increases the risk of developing type 2 diabetes mellitus for patients with PCOS by 5- to 8-fold when compared with weight-matched female controls (Glintborg et al., 2004). Furthermore, significantly increased risks of hypertension, coronary heart disease and obesity in women with PCOS have been documented in several studies (Carmina et al., 2006; Cussons et al., 2006;
Shroff et al., 2007; Dokras, 2008). Recent studies have also demonstrated that women with PCOS have increased serum levels of C-reactive protein (Boulman et al., 2004; Talbott et al., 2004) and interleukin-8 (IL-8) (Escobar-Morreale et al., 2003). The presence of chronic inflammation in women with PCOS is found to be associated with insulin resistance and cardiovascular disease.

Haem oxygenase (HO), which is the rate-limiting enzyme in haem catabolism, catalyses the conversion of haem into biliverdin, carbon monoxide (CO) and free iron (Abraham and Kappas, 2008). HO provides both antioxidant and ant apoptotic activities, which can be attributed to its products, bilirubin/biliverdin and CO (Ollinger et al., 2007). HO-1 is an inducible molecule, which is produced by oxidant stress, and it plays a crucial role in the protection against oxidative insult in diabetes (Abraham and Kappas, 2008) and cardiovascular disease (Yet et al., 2001). Recent studies have demonstrated that HO-1 is down-regulated in abnormal metabolic states, and overexpression of HO-1 might inhibit the inflammatory response and ameliorate metabolic disorders. For example, an in vitro study showed that the expression of HO-1 in the lungs of humans with cystic fibrosis resulted in protection against injury/apoptosis induced by bacteria (Zhou et al., 2004). Furthermore, HO activity and levels of HO-1 protein were reduced, whereas those of tumour necrosis factor (TNF)-α and IL-6 were increased, in Zucker obese (ZF) rats, when compared with Zucker lean rats (Kim et al., 2008). Recently, overexpression of HO-1 was found to improve insulin sensitivity and glucose tolerance and to decrease insulin levels and reduce adiposity in obese rats and obese diabetic mice (Li et al., 2008; Nicolai et al., 2009). Moreover, up-regulation of the HO system by haemin ameliorated insulin resistance in animal models of diabetes, and the antidiabetic effect was sustained for 3 months after therapy (Ndisang et al., 2009). These results suggest that HO-1 might be an important link between increased inflammation and insulin resistance in human diseases. However, the role of HO-1 in metabolic disorders in women with PCOS needs to be clarified.

Adipose tissue plays an important role in insulin resistance, and a variety of cytokines that are related to insulin resistance are secreted by adipose tissue, such as leptin, adiponectin, TNF-α and IL-6, as well as HO-1 (Berg and Scherer, 2005; Li et al., 2008). Adiponectin, a protein of 244 amino acids, is an insulin-sensitizing hormone that is expressed exclusively in white adipose tissue (Tsao et al., 2002). Adiponectin belongs to the complement C1q family and contains an NH2-terminal collagen-repeat domain and a COOH-terminal globular head domain (Shapiro and Scheree, 1998). The level of adiponectin has been reported to be reduced in obese women (Orio et al., 2003) and in women with insulin resistance and diabetes (Sieminska et al., 2004; Spranger et al., 2004). Recently, there have been several reports that up-regulation of HO-1 increases the level of adiponectin in the culture medium of human bone marrow-derived adipocytes (L’Abbate et al., 2007; Kim et al., 2008; Li et al., 2008).

The aim of this study was to assess, by western blotting, the expression of HO-1 protein in adipose tissues from women with PCOS. We also examined the expression of HO-1 mRNA in omental fat and human peripheral blood mononuclear cells (PBMCs) from women with PCOS using the real-time PCR. PBMCs from venous blood samples are the most accessible tissue for the analysis of gene expression. Analysis of the expression of HO-1 in PBMCs is more accurate and might provide more valuable information than the measurement of plasma levels. We also evaluated the expression of adiponectin, TNF-α and IL-6 mRNA and investigated the relationships between these cytokines and HO-1 in women with PCOS. This is the first reported study to evaluate the role of HO-1 in insulin resistance in women with PCOS.

Materials and Methods

Participants

Seven non-obese Chinese women (BMI <27 kg/m²) 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 previous 3 months. The protocol was reviewed and approved by the Institutional Review Board of the Shin Kong Wu Ho-Su Memorial Hospital. Patients and controls were only entered into the study after informed written consent had been obtained.

PCOS was defined by clinical, laboratory and ultrasound criteria in accordance with the consensus criteria reported by the Rotterdam group (2004). All the women with PCOS had menstrual disturbances and hyperandrogenism and/or polycystic ovaries. The clinical criteria included oligomenorrhea (menstrual interval >6 weeks) or amenorrhea (no menstrual loss for ≥3 months), dating from menarche. None of the subjects had acanthosis nigricans. The biochemical criteria were an increased serum concentration of LH (>6 mIU/ml), a normal serum concentration of FSH and an elevated serum concentration of total testosterone. The ultrasound criteria were enlarged ovaries with an increased amount of stromal tissue, the presence in each ovary of 12 or more follicles that measured 2–9 mm in diameter and/or an increased ovarian volume (>10 ml) when viewed on transvaginal ultrasonographic examination. Serum levels of proactin and thyroid hormone were checked in all patients and were within the normal limits. Cushing’s syndrome and androgenic tumours were excluded by appropriate testing. Congenital adrenal hyperplasia was excluded by documenting a serum level of 17-hydroxyprogesterone in the morning of <2 ng/ml.

Five healthy women, who were matched for BMI and age, served as controls. None were hirsute, and all had a normal regular menstrual cycle. None were taking an oral contraceptive drug. All had a normal appearance of the ovaries on ultrasound and normal levels of LH and FSH, and none had elevated levels of androgen.

Oral glucose tolerance test, fasting glucose-to-insulin ratio and the homeostasis model assessment insulin resistance index

A 2-h oral glucose tolerance test with a glucose load of 75 g was performed during the early follicular phase (Days 3–7) of all the participants 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 the serum was 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., 1999). The homeostasis model assessment-insulin resistance (HOMAIR) index was calculated using the formula: fasting glucose (mg/dl) × fasting insulin (mIU/ml)/405 (Matthews et al., 1985). A HOMAIR value of ≥3.8 or a G0/I0 ratio ≤4.5 indicates insulin resistance in PCOS (Legro et al., 1998; Kauffman et al., 2002).

Hormonal profile

Blood was drawn from the antecubital vein of all the participants during the early follicular phase to measure serum levels of E2, FSH, LH and
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Western blotting
Whole-cell lysates were prepared from the PCOS and control adipose tissue by sonication at 4°C in lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM HEPES, pH 7.8, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 125 μM dithiotreitol and 1 mM phenylmethylsulfonyl fluoride) and analysed on the same western blot. Samples (50 μg of total protein) were mixed with 50 μl of 2× 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 blocked for 1 h at room temperature with 5% skimmed milk in phosphate-buffered saline (PBS) that contained 0.5% Tween 20 and then immunoblotted with antibodies against human HO-1 (Stressgen Biotechnologies, Victoria, BC, Canada) diluted in PBS followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted in PBS. Antibody binding was detected using Chemiluminescence Reagent (Amersham Bioscience, Little Chalfont, UK).

Quantitative analysis of inflammatory marker transcripts by real-time PCR
Total RNA was extracted from the adipose tissue and PBMCs isolated from each participant using Tr Reagent (Sigma-Aldrich, St Louis, MO, USA). The integrity of the total RNA was assessed by measuring UV absorbance at 260 nm (Genequant RNA/DNA Calculator; Pharmacia, LKB Biochrom, UK). Aliquots of RNA (1 μg) were reverse transcribed using a TaqMan High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and 100 ng of cDNA were used to detect and quantify levels of the marker transcripts by real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) diluted in PBS and transferred to a polyvinylidene fluoride membrane. The membrane was blocked for 1 h at room temperature with 5% skimmed milk in phosphate-buffered saline (PBS) that contained 0.5% Tween 20 and then immunoblotted with antibodies against human HO-1 (Stressgen Biotechnologies, Victoria, BC, Canada) diluted in PBS followed by horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted in PBS. Antibody binding was detected using Chemiluminescence Reagent (Amersham Bioscience, Little Chalfont, UK).

Statistical analysis
The data are presented as the median (range). Statistical analysis was carried out by non-parametric testing using the Mann–Whitney U-test to compare the two groups. Correlations between variables were evaluated by Spearman’s correlation coefficient. Computations were performed using SPSS software (Statistical Package for the Social Sciences, SPSS for Windows, Inc., Version 13.0, Chicago, IL, USA). 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 participants and the women with PCOS are shown in Table I. The median age of the women with PCOS was 30 (24–38) years and that of the controls was 27.5 (24–33) years. The women with PCOS had significantly higher ratios of serum LH/FSH and serum levels of testosterone than controls. The fasting insulin levels, insulin levels 2 h after 75-g glucose loading and the HOMAIR were significantly higher in the women with PCOS than in the controls (P <

### Table I Clinical and metabolic characteristics of women with PCOS and control.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 5)</th>
<th>PCOS (n = 7)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>27.5 (24–33)</td>
<td>30 (24–38)</td>
<td>NS</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>160 (148–170)</td>
<td>161 (143–178)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>53.5 (45–75)</td>
<td>60 (49–79)</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21 (19–27)</td>
<td>24 (18–27)</td>
<td>NS</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>4.8 (3.3–9.4)</td>
<td>5.9 (2.3–8.9)</td>
<td>NS</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>5.3 (2.1–13)</td>
<td>12 (5.8–19.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1.1 (0.3–2.5)</td>
<td>2.4 (0.8–5.6)</td>
<td>0.019</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>44.5 (25–93)</td>
<td>51 (21–103)</td>
<td>NS</td>
</tr>
<tr>
<td>T (ng/ml)</td>
<td>0.3 (0.1–0.6)</td>
<td>0.8 (0.4–1.26)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>86 (80–93)</td>
<td>90 (78–107)</td>
<td>NS</td>
</tr>
<tr>
<td>2-h glucose (mg/dl)</td>
<td>85 (56–105)</td>
<td>113 (83–206)</td>
<td>0.004</td>
</tr>
<tr>
<td>Fasting insulin (mIU/ml)</td>
<td>5.5 (2–18)</td>
<td>16 (9.3–56)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2-h insulin (mIU/ml)</td>
<td>13 (2–28)</td>
<td>56 (25–380)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HOMAIR</td>
<td>1.2 (0–2.1)</td>
<td>3.6 (2.1–15.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G0/I0</td>
<td>16 (8.9–40)</td>
<td>9.0 (2.7–18)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

All values are the median (range). BMI, body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, estradiol; T, testosterone; HOMAIR, homeostasis model assessment of insulin index; G0/I0, fasting glucose-to-insulin ratio.
*Mann–Whitney U-test, PCOS versus control.
In addition, the G₀/I₀ ratio was significantly lower in the women with PCOS than in the controls \( (P < 0.0001) \).

**Expression of HO-1 protein in omental adipose tissue by western blot analysis**
As shown in Fig. 1, the level of HO-1 protein in adipose tissue was significantly lower in the women with PCOS than in the controls \( (P = 0.002) \).

**Expression of HO-1, adiponectin, TNF-α and IL-6 mRNA in adipose tissue and HO-1 mRNA in PBMCs by real-time PCR**
We compared the expression of HO-1, adiponectin, TNF-α and IL-6 mRNA in adipose tissue and HO-1 mRNA in PBMCs between the women with PCOS and the controls using real-time PCR. As shown in Fig. 2, we found that the expression of HO-1 mRNA in adipose tissue was significantly lower in the women with PCOS than in the controls \( (P = 0.002) \). The expression of HO-1 mRNA in PBMCs (Fig. 3) was also significantly lower in the women with PCOS than in the controls \( (P = 0.05) \). The expression of adiponectin mRNA in adipose tissue was also significantly lower in the women with PCOS than in the controls \( (P = 0.02) \) (Fig. 4a). However, there were no significant differences with respect to TNF-α (Fig. 4b) and IL-6 (Fig. 4c) expression between the women with PCOS and the controls \( (P > 0.05) \).

**Correlations**
We analysed the relationships between the levels of HO-1 in human omental adipose tissue or PBMCs and metabolic parameters or cytokines. The level of HO-1 protein in adipose tissue showed a significant positive correlation with HO-1 mRNA expression in adipose tissue and PBMCs \( (r^2 = 0.866, P = 0.003 \text{ and } r^2 = 0.786, P = 0.037, \text{ respectively}) \) in women with PCOS. The level of HO-1 protein showed a significant negative correlation with fasting insulin levels \( (P = 0.045, r^2 = 0.63) \), insulin levels 2 h after glucose loading \( (P = 0.034, r^2 = 0.55) \) and HOMA-IR \( (P = 0.026, r^2 = 0.48) \). Furthermore, HO-1 protein levels showed a significant negative correlation with BMI \( (P = 0.01, r^2 = 0.575) \) and with 2-h glucose levels \( (P = 0.023, r^2 = 0.520) \). The level of HO-1 protein showed a significant positive correlation with the expression of adiponectin mRNA \( (P < 0.05, r^2 = 0.727) \). However, HO-1 protein levels did not correlate with the expression of TNF-α or IL-6 mRNA or with the level of testosterone \( (P > 0.05) \).
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Discussion

The study reported herein was performed to investigate the role of HO-1 expression in the insulin resistance seen in women with PCOS. The main finding was that levels of HO-1 mRNA and protein were reduced significantly, in both omental fat and PBMCs, in women with PCOS when compared with the controls. This result indicated that the expression of HO-1 is related closely to insulin resistance in women with PCOS. The present study is the first to report the expression of HO-1 in women with PCOS. Furthermore, the expression of HO-1 showed a significant positive correlation with the expression of adiponectin, which suggests the existence of an HO-1—adiponectin axis in the regulation of insulin resistance in women with PCOS.

A recent study demonstrated that the induction of HO-1 reduced adiposity, improved insulin sensitivity and glucose tolerance and decreased insulin levels in obese diabetic mice (Nicolai et al., 2009). A further study also reported that up-regulation of the HO system might ameliorate postprandial and fasting hyperglycaemia in Goto–Kakizaki rats, a non-obese insulin-resistant model with type 2 diabetes (Ndisang et al., 2009). These findings indicate that hyperinsulinaemia and hyperglycaemia might down-regulate the activity of HO-1. PCOS is a common metabolic disorder that is associated with insulin resistance accompanied by compensatory hyperinsulinaemia and hyperglycaemia, and thus with an increased risk for the development of type 2 diabetes. These findings could explain why the level of HO-1 was lower in women with PCOS than in controls matched for age and BMI. However, Johnson et al. (2006) presented contradictory findings that HO-derived production of CO was increased in obese Zucker rats with metabolic syndrome, but that the administration of an inhibitor of HO-1 did not improve the metabolic disorder.

In the present study, we found that HO-1 protein was correlated with the level of insulin and with BMI in women with PCOS. Hyperinsulinaemia was negatively correlated with HO-1 expression in women with PCOS in the present study, which indicates that HO-1 might regulate insulin sensitivity in PCOS. Our results are consistent with the findings of Shakeri-Manesch et al. (2009), who showed that HO-1 is negatively correlated with the waist-to-hip ratio and the HOMA\textsubscript{IR} value. All these results demonstrate that HO-1 might be an important link between increased fat mass and insulin resistance in humans.

Li et al. (2008) reported that the inhibition of HO activity decreased adiponectin levels but increased the secretion of TNF-\(\alpha\), IL-6 and IL-1\(\beta\) in ob mice. They also found that the activation of HO-1 prevented weight gain, decreased fat content, increased adiponectin and decreased TNF-\(\alpha\), IL-6 and IL-1\(\beta\) levels (Li et al., 2008). Ndisang et al. (2009) demonstrated similar results and showed that haemin therapy might improve insulin sensitivity and glucose metabolism via the up-regulation of HO-1 activity and might increase adiponectin levels by 2.1-fold. Haemin also increased the expression of glucose transporter 4 (GLUT4) in adult spontaneously hypertensive rats (Ndisang et al., 2009). The synergistic interactions among HO-1, adiponectin and GLUT4 increased insulin sensitivity and improved insulin resistance. In the present study and in our previous studies (Seow et al., 2004, 2009), we found that the expression of GLUT4 and adiponectin was significantly lower in women with PCOS than in the controls and that the level of HO-1 protein was positively correlated with the expression of adiponectin. In fact, adiponectin, GLUT4 and HO-1 are related closely to the regulation of insulin sensitivity and glucose tolerance in women with PCOS.

In the present study, we found that HO-1 was not correlated significantly with the level of testosterone in women with PCOS. Hyperandrogenism is an important clinical feature of PCOS (Dunaif, 1997). It is thought to be an important aetiological factor in the development of PCOS and insulin resistance. However, the exact pathophysiology of PCOS is complex and remains largely unclear. Therefore, although HO-1 protein did not correlate with the level of testosterone, this does not mean that HO-1 is not associated with the regulation of insulin resistance and glucose intolerance in PCOS.

A limitation of the present study was the small sample size. However, we investigated the expression of HO-1 in tissue from two different sources, i.e. omental adipose tissue and PBMCs, in
women with PCOS and in controls. Adipose tissue is important in the regulation of insulin resistance in women with PCOS. PBMCs from venous blood samples are the most accessible tissue for the analysis of gene expression and it is more accurate to measure gene expression and insulin resistance in these cells than to determine plasma levels. It is noteworthy that both the level of HO-1 protein in omental adipose tissue \( (P = 0.002) \) and the expression of HO-1 mRNA in adipose tissue and PBMCs from the women with PCOS were significantly lower \( (P = 0.002 \text{ and } 0.05) \) respectively than those of the controls. Nevertheless, a larger study might be indicated to investigate the true role of HO-1 in the regulation of insulin action in women with PCOS. The other limitation of the present study is that we evaluated only the levels of protein and mRNA expression for HO-1, and we did not evaluate the activity of HO-1. However, given that the results for both HO-1 protein and gene expression were similar, we believe that HO-1 is associated with the regulation of insulin resistance in PCOS.

In conclusion, the expression of HO-1 in both adipose fat and PBMCs from women with PCOS was significantly lower than that of controls. Furthermore, HO-1 was positively correlated with the level of adiponectin. Therefore, an HO-1–adiponectin axis may be involved in the regulation of insulin resistance and glucose intolerance in women with PCOS.

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