Expression of visfatin mRNA in peripheral blood mononuclear cells is not correlated with visfatin mRNA in omental adipose tissue in women with polycystic ovary syndrome

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BACKGROUND: Visfatin, which is secreted predominantly from visceral adipose tissue, has an insulin-mimetic action and may play a role in the regulation of insulin sensitivity in humans. Peripheral blood mononuclear cells (PBMCs) from venous blood samples are the most accessible tissue for the analysis of gene expression. The aims of the study were to compare the expression of visfatin in PBMCs with that in omental adipose tissue in women with polycystic ovary syndrome (PCOS).

METHODS: Visfatin mRNA was measured in omental adipose tissue and PBMCs from 10 women with PCOS and 10 healthy controls, matched for BMI and age, using the real-time polymerase chain reaction (PCR).

RESULTS: The expression of visfatin mRNA in both omental adipose tissue and PBMCs from the women with PCOS was significantly higher ($P = 0.01$ and $P = 0.05$, respectively) than that in the controls. This finding indicated that mononuclear cells are a potential source of visfatin in women with PCOS. However, only the expression of visfatin mRNA in adipose tissue, not that in PBMCs, showed a significant positive correlation with insulin levels 2 h after glucose loading ($P = 0.044$, $r^2 = 0.45$), and with homeostasis model assessment--insulin resistance (HOMA$_{IR}$; $P = 0.035$, $r^2 = 0.47$). In addition, the expression of visfatin mRNA in PBMCs did not correlate with the expression of visfatin mRNA in omental adipose tissue.

CONCLUSIONS: PCOS is associated with increased visfatin mRNA concentrations in PBMCs and in omental adipose tissue. However, only visfatin mRNA concentration in omental adipose tissue is closely correlated with BMI and insulin resistance.

Key words: polycystic ovary syndrome / visfatin / adiponectin / adiponectin receptor / insulin resistance

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrinopathy that is characterized by chronic anovulation, hyperandrogenism and polycystic ovaries on ultrasonography (Rotterdam, 2004). PCOS is associated frequently with insulin resistance accompanied by compensatory hyperinsulinaemia, which increases the risk of developing type 2 diabetes mellitus, hypertension, coronary heart disease and obesity when patients are compared with weight-matched female controls (Glintborg et al., 2004; Carmina et al., 2006; Cussons et al., 2006; Shroff et al., 2007; Dokras, 2008).

Recently, several novel adipokines that are secreted only by fat cells, such as visfatin, adiponectin and resistin, have been suggested to play a role in insulin resistance and diabetes. Visfatin, known previously as pre-B cell colony-enhancing factor, is a recently identified adipocytokine that is expressed predominantly in, and secreted from, visceral fat.
deposits in humans and mice (Fukuhara et al., 2005). Visfatin binds to, and activates, the insulin receptors in different insulin-sensitive cells in vitro, and plasma glucose is lowered acutely by treatment with visfatin (Fukuhara et al., 2005). Furthermore, heterozygous mice in which the visfatin gene has been knocked out have higher plasma glucose levels than wild-type littermates (Fukuhara et al., 2005). These findings suggest that visfatin has insulin-mimetic action and could play a role in the regulation of insulin sensitivity in humans. Berndt et al. (2005) found that the plasma concentration of visfatin correlates positively with the visceral expression of visfatin mRNA, and negatively with the subcutaneous expression of visfatin mRNA. However, no difference was found in the expression of visfatin between visceral and subcutaneous adipose tissues, and there was no correlation between plasma visfatin and visceral fat mass (Berndt et al., 2005). Furthermore, the serum level of visfatin was found to be increased only in lean women with PCOS (Kowalska et al., 2007).

A relationship between the plasma concentration of visfatin or the expression of visfatin in visceral adipose tissue and insulin resistance in women with PCOS has been reported in several studies (Tan et al., 2006; Chan et al., 2007; Kowalska et al., 2007; Panidis et al., 2008; Gen et al., 2009; Jongwutiwes et al., 2009; Ozkaya et al., 2010; Yildiz et al., 2010). Most studies have demonstrated that women with PCOS have significantly higher serum visfatin levels than control subjects of similar age and BMI (Kowalska et al., 2007; Panidis et al., 2008; Jongwutiwes et al., 2009; Plati et al., 2010). Tan et al. (2006) reported, further, that visfatin mRNA was up-regulated significantly in both the abdominal and omental adipose tissue of women with PCOS when compared with normal controls. Moreover, visfatin levels were correlated negatively with insulin sensitivity in PCOS (Kowalska et al., 2007). All these studies indicate that visfatin is associated with insulin resistance and that it might play a role in the pathophysiology of PCOS.

Peripheral blood mononuclear cells (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 plasma levels. Macrophages in human visceral adipose tissue have been reported to be a source of visfatin and resistin (Curat et al., 2006). However, there are no data on the expression of visfatin in PBMCs, or on the relationship between visfatin expression in PBMCs and visceral adipose tissue in women with PCOS. In light of this, we studied the expression of visfatin mRNA using the real-time polymerase chain reaction (PCR) in paired samples of PBMCs and omental adipose tissue from women with PCOS and from controls matched for BMI and age. If the level of visfatin mRNA in PBMCs were to be higher and related more closely to insulin resistance than that in adipose tissue in women with PCOS, it would facilitate future investigations into the role of visfatin since these could use PBMCs.

Materials and Methods

Participants

Ten non-obese Chinese women (BMI < 27 kg/m²) who fulfilled the inclusion criteria for PCOS detailed later 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 were only entered into the study after informed written consent had been obtained.

The presence of PCOS was defined by clinical, laboratory and ultrasonographic criteria in accordance with the consensus criteria reported by the Rotterdam Group (2004). All the women with PCOS had menstrual disturbances, hyperandrogenism and/or polycystic ovaries. The clinical criteria included oligomenorrhea (menstrual interval > 6 weeks) or amenorrhea (no menstruation 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 (≥ 0.8 ng/ml, normal range 0.06–0.80 ng/ml). The ultrasonographic criteria were: enlarged ovaries with an increased amount of stromal tissue, the presence of 12 or more follicles in each ovary that measured 2–9 mm in diameter and/or an increased ovarian volume (> 10 ml) when viewed by transvaginal ultrasonography. Serum levels of prolactin 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 of < 2 ng/ml in the morning.

Ten healthy women, who were matched with the study group for BMI (± 2 kg/m²) and age (± 3-year-old), served as controls. None was hirsute, and all had a normal regular menstrual cycle. None was taking an oral contraceptive drug. All had a normal appearance of the ovaries on ultrasound examination 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 the menstrual cycle of all the participants after an overnight fast. In the case of amenorrhoic 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 the ingestion of glucose 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 (HOMA IR) index was calculated using the formula: fasting glucose (mg/dl) × fasting insulin (µIU/ml)/405 (Matthews et al., 1985). A HOMA IR value of ≥ 3.8 or a G₀/I₀ ratio ≤ 4.5 indicates insulin resistance in women with 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 testosterone. For women with amenorrhea, 75 mg of progesterone was given intramuscularly to induce withdrawal bleeding, and the blood samples were 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 were 7.7 and 7.9%, respectively. 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.

Isolation of human PBMCs

Blood samples (5 ml) were collected in EDTA tubes, transferred to a centrifuge tube and centrifuged at 3000g for 30 min at 4 °C. After centrifugation, the plasma in the upper layer was discarded and the middle layer,
which contained the mononuclear cells, was aspirated carefully into a new centrifuge tube. The mononuclear layer was diluted with three volumes of phosphate-buffered saline (PBS), and the diluted mononuclear suspension was overlain on 3.0 ml of Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) in a new centrifuge tube, and centrifuged at 3000g for 30 min at 4°C. The mononuclear cells, which were at the interface, were aspirated into a new centrifuge tube and centrifuged at 14 000g at 4°C for 10 min. The supernatant was discarded, the cell pellet suspended in PBS and the suspension stored at −80°C until used for RNA extraction.

**Sampling of adipose tissue**

Adipose tissue that weighed ~5–6 g was obtained by laparoscopy from the omental fat tissue of all the women with PCOS on the day of laparoscopic ovarian drilling. For control participants, adipose tissue was obtained during laparoscopic examination for tubal infertility or sterilization. For all participants, the adipose tissue was extracted via a 5-mm trocar inserted in the umbilical area and was stored immediately at −80°C until analysed by real-time PCR.

**Quantitative analysis of adipocytokines in adipose tissue and PMBC transcripts by real-time PCR**

Total RNA was extracted from the adipose tissue and PBMCs isolated from each participant using TRI Reagent (Sigma-Aldrich). The integrity of the total RNA was assessed by 1% agarose gel electrophoresis, and the concentration of the RNA was determined 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 random primers. Real-time PCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems) using the comparative C_\text{t} quantification method. TaqMan Gene Expression Assays (Applied Biosystems) that contained specific primers (assay ID for visfatin: Hs00237184_m1; for GAPDH: Hs99999905_m1), TaqMan MGB probe (FAM dye-labelled), TaqMan Fast Universal PCR Master Mix and 100 ng of cDNA were used to detect and quantify the levels of visfatin mRNA in the adipose tissue and the PBMCs. GAPDH mRNA was used as an internal control. The C_\text{t} value for GAPDH was subtracted from that for the target genes (ΔC_\text{t}). The ΔC_\text{t} value for the PCOS group was compared with that for the controls. The following reaction conditions were used: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s/60°C for 1 min.

**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 and multiple regression analysis. The 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**

**Demographic data**

The clinical features and baseline hormonal and metabolic parameters for the control participants and the women with PCOS are shown in Table I. As expected, the women with PCOS had significantly higher ratios of serum LH/FSH and serum levels of testosterone than the controls. The 2-h glucose levels, fasting insulin levels, insulin levels 2 h after 75-g glucose loading and the HOMA_\text{IR} were significantly higher in the women with PCOS than in the controls (P < 0.05).

**Expression of visfatin mRNA in omental adipose tissue in controls and women with PCOS**

As shown in Fig. 1, the level of visfatin mRNA in adipose tissue was significantly higher in the women with PCOS than in the controls (P = 0.01).

**Expression of visfatin mRNA in PBMCs in controls and women with PCOS**

The expression of visfatin mRNA in PBMCs (Fig. 2) was also significantly higher in the women with PCOS than in the controls (P = 0.05).

**Association of visfatin with other covariates**

We analysed the relationships between the level of expression of visfatin mRNA in human omental adipose tissue or PBMCs and various metabolic parameters. There was no significant correlation between visfatin expression in PBMCs and BMI (P = 0.480) or between visfatin expression in adipose tissue and BMI (P = 0.488). Visfatin expression in PBMCs did not correlate with the expression of visfatin mRNA in omental adipose tissue. Furthermore, visfatin expression in PBMCs did not correlate with insulin or glucose levels or HOMA_\text{IR}. However, the expression of visfatin mRNA in adipose tissue showed a significant positive correlation with insulin levels 2 h after

**Table I Clinical and metabolic characteristics of women with PCOS and controls.**

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 10)</th>
<th>PCOS (n = 10)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>28.5 (22–32)</td>
<td>29.2 (25–37)</td>
<td>0.220</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>157 (150–162)</td>
<td>162 (155–165)</td>
<td>0.147</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>48.5 (45–54)</td>
<td>52 (49–67)</td>
<td>0.132</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>20 (19–21)</td>
<td>21.9 (19.7–23)</td>
<td>0.110</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>6.1 (5.2–8.3)</td>
<td>6.2 (4.3–7.0)</td>
<td>0.958</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>5.6 (3.4–8.2)</td>
<td>9.4 (7.3–13)</td>
<td>0.001</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1.0 (0.7–1.0)</td>
<td>1.55 (1.2–1.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E2 (pg/ml)</td>
<td>40 (19–453)</td>
<td>39.5 (15–46)</td>
<td>0.852</td>
</tr>
<tr>
<td>T (pg/ml)</td>
<td>0.3 (0.2–0.5)</td>
<td>0.75 (0.6–0.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2-h glucose (mg/dl)</td>
<td>93.5 (81–98)</td>
<td>121 (90–190)</td>
<td>0.04</td>
</tr>
<tr>
<td>Fasting insulin (mIU/ml)</td>
<td>7.7 (1.6–11.6)</td>
<td>18.7 (143–21)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2-h insulin (mIU/ml)</td>
<td>23 (11–36)</td>
<td>76 (55–150)</td>
<td>0.002</td>
</tr>
<tr>
<td>HOMA_\text{IR}</td>
<td>1.6 (0.3–2.8)</td>
<td>4.5 (3.1–4.74)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G_0/I</td>
<td>11.6 (8.5–15.9)</td>
<td>5.1 (4.37–6.4)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

E2, estradiol; T, testosterone, HOMA_\text{IR}, homeostasis model assessment of insulin index; G_0/I, fasting glucose-to-insulin ratio.

All values are the median (range). Bold values are considered significant, p < 0.05.

*P*: Mann–Whitney U-test, PCOS versus Control.
Visfatin has been reported to have a positive correlation with visceral fat mass and also with the BMI/waist to hip circumference ratio (WHR) (Fukuhara et al., 2005; Chen et al., 2006). However, some studies have revealed conflicting data regarding the relationship between visfatin and visceral fat (Berndt et al., 2005; Tan et al., 2006). Kowalska et al. (2007) reported that serum visfatin was increased only in lean women with PCOS, but the present study found no significant correlation between visfatin and BMI although this could be due to a lack of statistical power. The exact relationship between visfatin and BMI needs to be clarified further. In addition, our study failed to show a positive correlation between visfatin and the total levels of testosterone in omental adipose tissue and PBMCs. This is in conflict with the report of Kowalska et al. (2007) that visfatin is associated with markers of hyperandrogenism in lean women with PCOS. However, further study is needed owing to the small sample size of the current study.

Visfatin has been proposed to have an insulin-mimetic action that is mediated by a distinct binding site on the insulin receptor, and to contribute to the development of metabolic syndrome and type 2 diabetes (Fukuhara et al., 2005). 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 (Dunaif, 1997). These could explain why the expression of visfatin was higher in women with PCOS than in controls matched for age and BMI (Kowalska et al., 2007; Panidis et al., 2008; Jongwutiwes et al., 2009; Platı et al., 2010). In the present study, visfatin expression in omental adipose tissue, but not in PBMCs, was correlated significantly with insulin and HOMAIR. PBMCs from venous blood samples are the most accessible tissue for the analysis of gene expression. The increased macrophage population that occurs in obese individuals might be responsible for the enhanced production of visfatin and resistance in humans (Curat et al., 2006). This phenomenon (increased PBMC cell number) can also be seen in PBMCs in non-obese women with PCOS, and that is why the expression of visfatin in PBMCs was higher in women with PCOS than in controls.

A limitation of the present study was the small number of participants. However, we investigated the expression of visfatin in tissue from two different sources, i.e. omental adipose tissue and PBMCs, in women with PCOS and in controls. It is noteworthy that the expression of visfatin mRNA in both omental adipose tissue and PBMCs from the women with PCOS was significantly higher ($P = 0.01$ and $P = 0.05$, respectively) than that in the tissues from the controls. Furthermore, the opportunity to obtain omental adipose tissue from women with PCOS who are matched on age and BMI during laparoscopic ovarian drilling is limited, and this impeded participant recruitment. The other limitation is that we did not assess the expression of visfatin protein in these women. The expression of visfatin mRNA does not correlate with protein production. A larger study might be indicated to investigate the true role of visfatin in the regulation of insulin action in women with PCOS.

In conclusion, we present novel data that indicate that the expression of visfatin mRNA is increased significantly, in both omental fat and PBMCs, in women with PCOS when compared with controls. However, only the expression of visfatin in omental adipose tissue, not that in PBMCs, is related to the insulin resistance index in women with PCOS.

**Discussion**

In this study, we showed that the level of visfatin mRNA was increased significantly, in both omental adipose tissue and PBMCs, in women with PCOS when compared with controls. This indicated that mononuclear cells are a source of secreted visfatin in addition to adipose tissue in women with PCOS. However, only the expression of visfatin in omental adipose tissue, and not that in PBMCs, was associated significantly with insulin and HOMAIR in women with PCOS. Furthermore, the expression of visfatin mRNA in PBMCs showed no significant correlation with the testosterone level in either PBMCs or omental adipose tissue.

Visfatin may indicated that the expression of visfatin in omental adipose tissue, but not in PBMCs, is associated with insulin resistance in women with PCOS, a finding that has not been described previously.
Authors’ roles
K.-M.S. was involved in manuscript writing and study design. J.-L.H. took part in acquisition of data and discussion. P.-H.W. conducted data analysis. L.-T.H. was involved in interpretation of data. C.-C.J. played a role in design and manuscript drafting.

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