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Decreased serum paraoxonase 1 (PON1) activity: an additional risk factor for atherosclerotic heart disease in patients with PCOS?

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BACKGROUND: Patients with polycystic ovary syndrome (PCOS) may have an increased risk for the development of hypertension and atherosclerotic heart disease (AHD), the pathophysiological mechanisms of which are not clear. Paraoxonase1 (PON1) is a high-density lipoprotein-associated enzyme that prevents oxidative modification of low-density lipoprotein. The aim of this study was to measure the serum levels of PON1 activity in patients with PCOS and to compare with those of regularly cycling controls. METHODS: Serum lipid parameters, malondialdehyde (MDA) levels and PON1 activity, were measured in PCOS patients (n = 23) and regularly cycling, age-, body mass index- and smoking status-matched controls (n = 23). All patients had normal glucose tolerance test as assessed by a 75 g oral glucose tolerance test. None of the patients had clinically evident hypertension or AHD. RESULTS: Apart from the mean serum PON1 activity, all parameters in the lipid profile including serum MDA levels were comparable between the two groups. There were no significant differences in respect to fasting glucose (4.64 ± 0.5 versus 4.43 ± 0.83 mmol/l) and fasting glucose insulin ratio (11.06 ± 8.26 versus 11.49 ± 4.90) among the two groups (P > 0.05). However, HOMA insulin resistance index was significantly higher in patients with PCOS compared with the controls (2.06 ± 0.86 versus 1.51 ± 0.49; P = 0.01). Also, mean serum PON1 activity was significantly lower in the PCOS group compared with the controls (151.2 ± 90.8 versus 217.7 ± 101.6, respectively; P = 0.027). CONCLUSIONS: Reduced serum PON1 activity might contribute to the increased susceptibility for the development of AHD in women with PCOS.

Key words: atherosclerotic heart disease/cardiovascular disease/insulin resistance/paraoxonase/polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) is the most common reproductive endocrinopathy, affecting 5–10% of women of childbearing age (Knochenhauer *et al*., 1998). Patients with PCOS may have an increased risk for the development of diabetes mellitus, hypertension and atherosclerotic heart disease (AHD) (Guzick *et al*., 1996; Birdsall *et al*., 1997; Wild, 2002; ACOG Practice Bulletin, 2003). Although the exact pathophysiological mechanisms are not clear, insulin resistance, unfavourable lipid profile, endothelial dysfunction, activation of the coagulation cascade and impaired fibrinolysis may contribute to increased cardiovascular disease risk (Guzick *et al*., 1996; Birdsall *et al*., 1997; Wild, 2002; ACOG Practice Bulletin, 2003).

Serum paraoxonase 1 (PON1), synthesized in the liver, is a high-density lipoprotein (HDL)-associated enzyme that prevents oxidative modification of low-density lipoprotein (LDL). Serum PON1 is located on HDL-cholesterol and is responsible for the antioxidant activity of HDL (Mackness *et al*., 1998a; b; 2002; James *et al*., 2000; Jarvik *et al*., 2000). Because oxidative stress may impair insulin action (Rudich *et al*., 1997), reduced serum paraoxonase activity may contribute to insulin resistance. This hypothesis is supported by the finding of reduced serum paraoxonase activity in insulin-resistant disorders such as type 2 diabetes mellitus (Mackness *et al*., 1998a; b; Sakai *et al*., 1998) and AHD (Mackness *et al*., 1998a; b; 2002; James *et al*., 2000; Jarvik *et al*., 2000). Recently, San Millan *et al*. (2004), in a genomic variation study, have reported that PCOS patients were more homozygous for a variant of *PON1* gene (–108T) compared with the controls. Since homozygosity for –108T alleles might contribute to reduced PON1 expression, the authors have speculated that the resulting reduced PON1 and higher oxidative stress may contribute the increased insulin resistance in patients with PCOS. However, serum PON1 activity was not examined in this study.

The aim of the present study was to measure the serum levels of PON1 activity in patients with PCOS and to compare with those of age-, body mass index (BMI)- and smoking status-matched regularly cycling controls.

Materials and methods

Study population

The study group consisted of 23 consecutive women with PCOS. The diagnosis of PCOS was based on the revised 2003 Rotterdam ESHRE/ASRM consensus criteria and exclusion of related disorders (Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group, 2004). Oligoamenorrhea was defined as six or less menses per year. Sonographic diagnosis of PCOS was confirmed if there were 10 or more subcapsular follicular cysts, 2–8 mm in diameter, arranged around a thickened ovarian stroma (Adams *et al.*, 1986). The controls did not have any androgenic skin changes. None of the patients in the PCOS and control groups had clinically evident hypertension or AHD. Twenty-three age-, BMI- and smoking status-matched patients with regular cycles and normal transvaginal ultrasonography served as the controls. All participants underwent a 75 g oral glucose tolerance test (OGTT) after 3 days of a 300 g/day carbohydrate diet and a 12-h overnight fast. After a venous blood sample was obtained for fasting glucose and insulin, 75 g of glucose was ingested, and further blood samples were obtained 30, 60, 90 and 120 min later to measure serum glucose and insulin levels. The diagnosis of glucose intolerance was based on World Health Organization criteria: diagnoses of impaired glucose tolerance and type 2 diabetes were made when 2-h plasma glucose levels were 140–199 and 200 mg/dl, respectively. All patients in the PCOS and control groups had not taken any medication for 3 months before they were enrolled into the study.

Blood samples were obtained during days 3–7 after spontaneous or progesterone-induced menses in the control and PCOS groups. Fasting glucose, FSH, LH, testosterone, estradiol (E_2) and prolactin, and DHEAS, glucose, triglyceride, total cholesterol, HDL, LDL, ApoA1, ApoB, and MDA levels and PON1 activity were measured in serum samples obtained in early morning. Medroxyprogesterone acetate (Farlutal, Deva, Istanbul; 10 mg/day for 10 days) was prescribed to induce progesterone withdrawal bleeding, when necessary.

Laboratory methods

Plasma glucose was measured by the glucose oxidase technique (Roche Diagnostics GmbH, Mannheim, Germany). The serum concentrations of FSH, LH, testosterone, E_2, prolactin and DHEAS were measured by chemiluminescent enzyme immunoassay (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA, USA). Serum HDL, total cholesterol and triglyceride concentrations were measured by enzymatic assay (Boehringer, Mannheim, Germany) using a Hitachi 717 moduler analyser. The LDL level was calculated using the Friedewald equation [LDL cholesterol = total cholesterol – (HDL cholesterol + 0.45 × triglycerides)]. Serum concentrations of homocysteine were measured by fluorescence polarization immunoassay (Abbott, Germany). Insulin concentration was measured by immunoradiometric assay (Berthold gamma counter). The estimate of insulin resistance by the homeostasis model assessment (HOMA) was calculated by the following formula (Matthews *et al*., 1985): HOMA index = fasting insulin (U/m) × (fasting glucose mmol/l)/22.5. Malondialdehyde was measured by spectrophotometric assay (thiobarbituric acid reactivity) (Jialal and Devaraj, 1996).

Fasting venous blood was collected in tubes containing EDTA or potassium fluoride or in plain tubes. All blood samples were centrifuged at 1500 *g* for 5 min at 4°C and was stored at 4°C for lipoprotein isolation and used within 24 h. For paraoxonase activity, a serum aliquot was stored at –70°C.

PON1 activity was assessed by the rate of enzymatic hydrolysis of 1.0 mmol/l paraoxon (*O*,*O*-diethyl-*O*-*p*-nitrophenylphosphate; Sigma Chemical Co.) to *p*-nitrophenol in 1 mmol/l $CaCl_2$ and 2 mmol/l NaCl in 0.1 mol/l Tris–HCl (pH 8.0) at a final concentration of 1.2 mmol/l. The amount of *p*-nitrophenol generated was monitored with a continuously recording spectrophotometer (UV-1601 Shimadzu) by the increase in absorbance at 412 nm and 25°C. The amount of *p*-nitrophenol generated was calculated from the molar absorbtivity at pH 8.0, which was 17 000/mol/cm. One unit of PON1 activity is defined as 1 nmol of 4-nitrophenol formed per minute, under the above assay condition (Gan *et al.*, 1991; Mackness *et al.*, 1991).

The intra- and interassay coefficients of variation for insulin were 4% and 3.5%, respectively; for PON1 they were 2.9% and 5.4%; for MDA they were 3.3% and 3.1%; for total cholesterol they were 2.4% and 2.2%; for triglycerides they were 4.5% and 6.1%; for HDL they were 1.8% and 2.1%; for LDL they were 3.5% and 3.4%; for homocysteine they were 2.5% and 2.4%; for glucose they were 1% and 1.5%; for LH they were 3.6% and 6.2%; for FSH they were 5.3% and 5%; for estrogen they were 2.2% and 2.9%; for T they were 2.4% and 4%; for free T they were 3.3% and 3.7%; and for DHEAS they were 4% and 3.5%.

Statistical analyses

A power analysis was performed to estimate the number of patients needed in each group. It was assumed that 20 nmol/ml/min change in PON activity was clinically significant. Assuming a two-sided test with a probability of a type I error of 0.05, a statistical power of 95%, 20 patients were required in each group.

Data were expressed as mean ± SD. Mann–Whitney *U*-test and Student's *t*-test were used for statistical analyses. Type I error was set at 0.05. Correlation analysis between serum PON1, hormones and lipids were performed using Pearson's correlation coefficient. Data were analysed with SPSS, version 10.0 (SPSS, Inc., Chicago, IL, USA) for Windows 98 (Microsoft Corp.).

Results

The demographic features and the mean serum hormone levels of the PCOS and control groups are given in Table I. The two groups were matched in terms of age, BMI and smoking status (Table I). The mean serum LH, testosterone, free testosterone and DHEAS levels were significantly higher in the PCOS group (Table I). Three patients in both groups were light smokers.

Apart from mean serum PON1 activity, all parameters in the lipid profile and mean serum MDA levels were comparable between the two groups ($P > 0.05$). The mean serum PON1 activity was significantly lower in the PCOS group compared to the controls (151.2 ± 90.8 versus 217.7 ± 101.6, respectively; $P = 0.027$) (Table II). There was a significant negative

Table I. The demographic features and baseline hormone levels of the PCOS and control groups

	PCOS (n = 23)	Controls (n = 23)
Age (years)	24.4 ± 4.8	25.1 ± 4.5
BMI (kg/m^2)	23.0 ± 2.9	22.2 ± 3.4
Smokers (n)	3	3
FSH (mIU/ml)	4.9 ± 2.3	4.3 ± 0.7
LH (mIU/ml)[a]	12.4 ± 4.4	8.2 ± 3.6
E_2 (pg/ml)	39.1 ± 8.8	34.2 ± 12.4
Testosterone (ng/dl)[b]	81.1 ± 20	45.4 ± 16.9
Free testosterone (pg/ml)[c]	6.6 ± 2.3	4.7 ± 1.4
SHBG (nmol/l)[d]	31.9 ± 22.3	56.3 ± 12.9
DHEAS (μg/dl)[e]	250 ± 80	178.3 ± 50.1

[a]$P < 0.01$; [b]$P < 0.001$; [c]$P < 0.001$; [d]$P = 0.01$; [e]$P < 0.02$.
SHBG, sex hormone-binding globulin.

Table II. The lipid profile, serum MDA and PON1 activity of the PCOS and control groups

	PCOS (n = 23)	Controls (n = 23)
Triglycerides (mg/dl)	88.4 ± 40.6	107.9 ± 72.8
Total cholesterol (mg/dl)	185.6 ± 45.6	187.7 ± 38.7
HDL (mg/dl)	59.9 ± 11.1	60.2 ± 14.2
LDL (mg/dl)	108.1 ± 38.5	105.7 ± 35.9
Apo A_1 (mg/dl)	144.2 ± 30.4	137.8 ± 20.2
Apo B (mg/dl)	79.8 ± 23.5	90.0 ± 24.9
Homocysteine (μmol/l)	14.0 ± 4.2	9.72 ± 5.76
MDA (μmol/l)	3.60 ± 1.22	3.53 ± 1.0
Fasting glucose (mmol/l)	4.64 ± 0.5	4.43 ± 0.83
Fasting glucose/insulin ratio	11.06 ± 8.26	11.49 ± 4.90
HOMA[a]	2.06 ± 0.86	1.51 ± 0.49
PON1 activity (nmol/ml/min)[b]	151.2 ± 90.8	217.7 ± 101.6

Data are presented as mean ± SD.
[a]P = 0.01; [b]P = 0.027.

correlation between serum total testosterone levels and the serum PON1 activity among all groups (r = –0.54; P <0.001). In addition, there was a significant negative correlation between serum total testosterone levels and the serum PON1 activity in PCOS group (r = –0.54; P = 0.01) (Figure 1). There were no significant differences in respect to fasting glucose (4.64 ± 0.5 versus 4.43 ± 0.83) and fasting glucose insulin ratio (11.06 ± 8.26 versus 11.49 ± 4.90) between the groups (P > 0.05). However, HOMA index was significantly higher in patients with PCOS compared with the controls (2.06 ± 0.86 versus 1.51 ± 0.49; P = 0.01).

Discussion

PCOS is currently considered to be a part of the metabolic syndrome. Women with PCOS cluster risk factors associated with cardiovascular disease. These risk factors include insulin resistance, obesity, unfavourable lipid profile, endothelial dysfunction and activation of the coagulation cascade and impaired fibrinolysis. Whether having PCOS is an independent risk factor for AHD remains unclear (Wild, 2002). Although the risk of myocardial infarction has been reported to increase by 7.4-fold in a retrospective case–control study (Dahlgren *et al*., 1992), no prospective study has yet demonstrated an increased risk of AHD in patients with PCOS. However, a number of studies using surrogate endpoints have suggested that patients with PCOS may be at an increased risk for AHD (Guzick *et al*., 1996; Birdsall *et al*., 1997; Wild, 2002; ACOG Practice Bulletin, 2003).

Increased incidence of subclinical atherosclerosis in carotid arteries has been reported in patients with PCOS (Guzick *et al*., 1996). Patients with PCOS have been reported to have dyslipidemia, including elevated levels of cholesterol, LDL, Apo B, triglycerides and lipoprotein C III, and lower levels of total HDL, HDL_2 (Wild *et al*., 1985; 1992). On the other hand, others have failed to demonstrate any difference between PCOS and controls and have submitted that the observed changes in triglycerides and HDL cholesterol were secondary to obesity (Graf *et al*., 1990; Holte *et al*., 1994). We noted comparable lipid profile in PCOS patients and the controls. This lack of difference may be due to the inclusion of lean PCOS patients (mean BMI 23.0 ± 2.9 kg/m^2) in this study.

Serum PON1 prevents oxidative modification of the LDL and is responsible for the antioxidant activity of HDL. Experimental studies using transgenic PON1 knockout mice revealed an anti-atherosclerotic characteristic of the PON1 enzyme (Mackness *et al*., 1998b; 2002). In recent studies, reduced serum PON1 activity has been reported to be associated with increased risk of AHD (Mackness *et al*., 1998b; 2002; James *et al*., 2000; Jarvik *et al*., 2000) and insulin resistance (Mackness *et al*., 1991; Ikeda *et al*., 1998; Leviev *et al*., 2001). Significantly lower PON1 activity has been reported after myocardial infarction when compared with age- and gender-matched controls (Ayub *et al*., 1999). Decreased serum PON1 activity has been reported with other states associated with increased atherosclerosis, including diabetes, hypercholesterolemia and

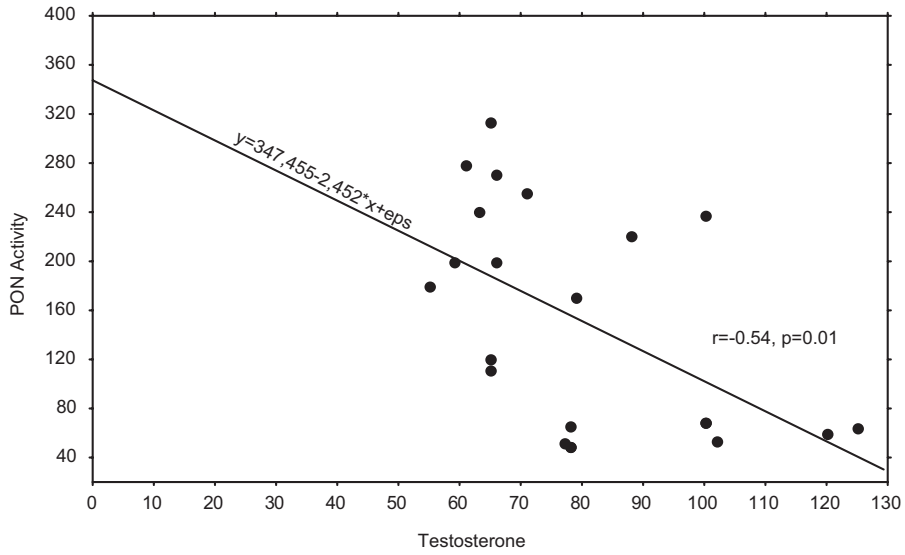

Figure 1. Scatterplots graphic for serum PON1 and testosterone levels of the PCOS group.

renal diseases (Mackness *et al*., 1991; Hasselwander *et al*., 1998; Ikeda *et al*., 1998; Ak *et al*., 2002). Therefore, PON1 activity may reflect the antioxidant and anti-atherogenic capacity (Mackness *et al*., 1998b; 2002).

Fortunato *et al*. (2003) reported that paraoxonase gene polymorphism might be an independent risk factor for increased carotid intima-media thickness in middle-aged women. To our knowledge, there is only one study in the literature evaluating the association of PON1 polymorphism and PCOS (San Millan *et al*., 2004). San Millan *et al*. genotyped the three variants in the genes encoding *PON1* in 72 PCOS patients and 42 healthy controls. They noted that patients with PCOS were more homozygous for a variant of *PON1* gene allele (–108T). The –108 C→T polymorphism in PON1 was distributed differently in PCOS patients compared with controls. In agreement with the different distribution of the *PON1* genotype, PCOS patients were more frequently homozygous for the –108T variant in *PON1* (PCOS 36.6% versus controls 9.5%; $P = 0.002$), compared with healthy controls. Furthermore, the influence of the genomic variants on clinical and biochemical markers of hyperandrogenism was studied. Compared with carriers of –108C alleles, subjects homozygous for –108T alleles of the –108 C→T polymorphism in PON1 presented with significantly increased hirsutism scores, and total testosterone, free testosterone and androstenedione concentrations. However, the serum PON1 activity was not studied in this study.

Homozygosity for –108T alleles has been studied in a non-PCOS population and has been reported to be more frequent in non-diabetic subjects showing abnormal fasting glucose concentrations, and therefore suspected to have insulin resistance, compared with subjects with normal serum glucose concentrations (Leviev *et al*., 2001). Because oxidative stress may impair insulin action (Rudich *et al*., 1997), reduced serum PON1 activity may contribute to insulin resistance. This hypothesis is supported by the finding of reduced serum paraoxonase activity in insulin-resistant disorders such as type 2 diabetes mellitus (Mackness *et al*., 1998a; Sakai *et al*., 1998).

Liver *PON1* mRNA expression is influenced by genetic and environmental factors, and both androgens and proinflammatory mediators decrease liver PON1 expression (bin Ali *et al*., 2003). Of interest, both androgen excess and proinflammatory genotypes are associated with PCOS (Peral *et al*., 2002; Villuendas *et al*., 2002; Escobar-Morreale *et al*., 2003). Serum PON1 activity has been reported to be lower in male mice (bin Ali *et al*., 2003). Following castration of male mice, hepatic *PON1* mRNA had increased by 170%. In our study, we noted a significant negative correlation between serum total testosterone levels and serum PON1 activity in the PCOS group ($r = –0.54$; $P < 0.01$).

In recent studies, reduced serum PON1 activity has been reported to be associated with insulin resistance (Mackness *et al*., 1991; Ikeda *et al*., 1998; Leviev *et al*., 2001). Also, serum PON activity significantly lower in patients with diabetes mellitus and lower serum paraoxonase activity has been associated with increased susceptibility to atherosclerosis, neuropathy, retinopathy and other complications in diabetic population compared with healthy controls (Abbott *et al.*, 1995; Ikeda *et al.*, 1995). We noted a significantly higher insulin resistance in the PCOS group as reflected by elevated HOMA index. We speculate that insulin resistance is associated with decreased PON1 activity in patients with PCOS.

There is a paucity of data on the oxidative status of patients with PCOS. Reduced antioxidant status and increased oxidative stress in women with PCOS have recently been reported (Sabuncu *et al*., 2001; Fenkçi *et al*., 2003). Fenkçi *et al*. (2003) studied the total antioxidant status of 30 patients with PCOS and found that it was significantly lower compared with regularly cycling, age- and BMI-matched controls. Similarly, Sabuncu *et al*. (2001) reported an increased oxidative and decreased antioxidative status in 27 patients with PCOS. These two studies raise the possibility that increased oxidative and/or decreased antioxidative status may contribute to the apparently increased risk of cardiovascular disease in women with PCOS, in addition to classical risk factors such as insulin resistance, hypertension, central obesity and dyslipidemia. Whether reduced antioxidative and/or increased oxidative status is an independent risk factor for increased AHD in patients with PCOS awaits further studies.

MDA is a natural product of lipid peroxidation and reflects the oxidant status of the biological systems. It has been demonstrated that high MDA levels are associated with high oxidative stress, diabetes mellitus and AHD (Slatter *et al*., 2000; Jung *et al.*, 2004). Atherosclerotic heart disease is associated with an imbalance between oxidant levels and cellular antioxidant defence mechanism. Enhanced oxidative stress may facilitate the development of AHD (Jung *et al*., 2004). MDA has been suggested to be used as a marker of extent and severity of AHD (Polidori *et al.*, 2002). In contrast to the studies by Fenkçi *et al*. (2003) and Sabuncu *et al*. (2001), we did not note a significant difference with respect to MDA levels among the groups. A lack of significant difference in our study may be attributable to younger age, normal lipid profile, lower BMI and limited sample size of our study.

To our knowledge, this is the first study reporting reduced serum PON1 activity in patients with PCOS. We suggest that decreased serum PON1 activity in PCOS patients may contribute to insulin resistance and AHD. All our patients had normal glucose tolerance as assessed by a 75 g OGTT. Further studies with more sensitive tests are warranted to establish the relationship between insulin resistance and PON1 in patients with PCOS.

Obesity *per se* may contribute to decreased serum PON1 activity. Recently, Ferretti *et al*. (2005), in a non-PCOS patient population, reported that the HDL-PON activity in obese subjects was significantly lower compared with age- and sex-matched controls. Since the mean BMI of the PCOS patients in our study was ~23 kg/m^2, further studies are warranted to evaluate the serum PON1 activity in lean and obese patient subgroups with PCOS.

In conclusion, despite comparable lipid profile and serum MDA levels, we noted significantly lower mean serum PON1 activity in patients with PCOS. We hypothesize that reduced serum PON1 activity may contribute to the increased susceptibility for the development of insulin resistance and AHD in women with PCOS. Further prospective studies with large sample sizes are warranted to elucidate the relationship

between reduced PON1 activity, testesterone and other androgens, insulin resistance and apparently increased AHD in lean and obese patients with PCOS.

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