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Research paper

Effect of hyperglycemia and hyperinsulinemia on glutathione peroxidase activity in non-obese women with polycystic ovary syndrome

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ABSTRACT

OBJECTIVE: In order to gain deeper insight into molecular mechanisms underlying oxidative stress (OS) and its relation to insulin resistance and hyperandrogenemia, plasma markers of OS and antioxidant glutathione-peroxidase (GPX) activity were studied in non-obese polycystic ovary syndrome (PCOS) women via the oral glucose tolerance test (OGTT) and hyperinsulinemic euglycemic clamp. DESIGN: In 36 PCOS women, plasma nitrotyrosine, thiol groups, uric acid (UA) and GPX activity were studied during OGTT and clamp. Insulin resistance was assessed by the homeostasis model (HOMA-IR), quantitative insulin sensitivity check index (QUICKI), Matsuda insulin sensitivity index (ISI) and M/I ratio. RESULTS: In PCOS patients, significant positive correlations were obtained for UA with testosterone (r=0.385, p=0.039) as well as indices of insulin resistance. Acute hyperglycemia during OGTT induced alteration in both OS markers and GPX. The change in nitrotyrosine and GPX during OGTT correlated with testosterone (r=0.543, p=0.036 and r=-0.457, p=0.025, respectively). The most significant association was found between OS markers and ISI. CONCLUSIONS: Our results indicate that non-obese PCOS women are prone to oxidative stress induced by hyperglycemia, but this seems not to be related to the direct effect of hyperinsulinemia during clamp. Oxidative stress markers correlated with indices of insulin resistance and circulating testosterone.

Key words: Glutathione peroxidase, Insulin resistance, Nitrotyrosine, Oxidative stress, Polycystic ovary syndrome

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INTRODUCTION

Polycystic ovary syndrome (PCOS), a frequent disorder of reproductive-aged women, is accompanied by oxidative stress in which increased production of free radicals is followed by decreased serum total

antioxidant levels.[1-5] Furthermore, it has been shown that even lean women with PCOS exhibit oxidative stress.[4,5] Nevertheless, the molecular mechanisms underlying oxidative stress and its link with insulin resistance and hyperandrogenism remain unclear.

It is well known that in PCOS, glucose ingestion induces increased production of reactive oxygen species (ROS) together with an inflammatory response that is independent of obesity.[2,6] These findings are mainly based on observed increased ROS generation and NADPH activity as well as increased activation of NFκB and TNFα mRNA content of mononuclear cells (MNCs) isolated from PCOS women after the glucose challenge.[2,6] In contrast, MNCs of normal weight ovulatory women are less sensitive to hyperglycemia and exhibit a lower increase in ROS generation in response to glucose ingestion.[6] It has been suggested that both insulin resistance and hyperandrogenemia might be responsible for this pro-oxidant response. Specifically, in the most recent findings of Gonzales et al (2011), acute oral androgen administration in normal weight ovulatory women promoted MNC activation and increased MNC sensitivity to glucose ingestion in a similar manner to PCOS.[7] This implies that hyperandrogenism, the hallmark feature of PCOS, might play the role of progenitor of diet-induced oxidative stress in the disorder. Indeed, the data on vascular smooth muscle cells from male Wistar-Kyoto rats and spontaneously hypertensive rats showed that testosterone treatment causes increased ROS generation as well as increased levels of Nox1 and Nox4 mRNA and expression of the NADPH subunit, p47phox.[8] Based on these findings, it could be speculated that transient increase in ROS generation during hyperglycemia in PCOS is associated with both insulin resistance and increased testosterone production. Consequently, the question arises whether antioxidant enzymes are involved in this phenomenon.

In order to gain deeper insight into the association of hyperglycemia-induced oxidant stress with both hyperandogenism and insulin resistance, in this study we examined the correlation between markers of oxidative damage as well as plasma GPX activity in PCOS patients and parameters of insulin resistance together with testosterone levels.

SUBJECTS AND METHODOLOGY

Subjects

Thirty-six patients with PCOS and 11 age- and BMI-matched healthy women with regular ovulatory menstrual cycles were included in the study. The diagnosis of PCOS was made on the basis of the revised 2003 Rotterdam ESHRE/ARSM consensus criteria (2004).[9] PCOS index case qualifications were applied according to the definitions of Laven et al (2002) for oligomenorrhea and amenorrhea.[10] Anovulation was defined as serum progesterone less than 10 nmol/L, or in patients with normal menses at least 2 consecutive low levels of serum progesterone (<10 nmol/L). Hirsutism was defined according to the modified Ferriman-Gallwey scoring system: a woman with a score ≥8 points was considered hirsute.[11] Hyperandrogenemia was defined by serum total testosterone >2 nmol/L, which was based on examination of 56 nonselected women presenting for routine controls who were not hirsute, had regular cycles and had received no hormonal therapy.[12] Transvaginal ultrasonography was used for the diagnosis of polycystic ovaries.[13] In all examined subjects, body mass index (BMI) was calculated and waist circumference (WC) determined.

In PCOS patients, impaired fasting glucose (fasting venous glucose >6 mmol/L), pregnancy, hypothyroidism, nonclassic 21-hydroxylase deficiency, hyperprolactinemia, Cushing's syndrome and androgen-secreting tumors were excluded by appropriate tests. No subjects had received any oral contraceptives, glucocorticoids, anti-androgens, ovulation inducing agents, anti-diabetic and anti-obesity drugs, or other hormonal drugs for at least three months before the study. The study was approved by the Institutional Ethical Committees and written consent was obtained from all subjects.

Biochemical and hormonal testing

PCOS women and controls were investigated during the follicular phase of the menstrual cycle and those PCOS women with amenorrhea were evaluated after confirmation of low estrogen and progesterone levels. Plasma glucose (mmol/L) was determined by the glucose oxidase method (Randox) using the autoanalyser (Beckman, Austria). Uric acid

(mmol/L) was determined by the colorimetric method (Randox, UK). High-sensitivity C-reactive protein (CRP, ng/mL) concentration was determined by the immunonephelometric method (DADE Behring, Germany). Fibrinogen (g/L) was calculated from the prothrombin time expressed in seconds and converted to percentages of normal by using a reference curve which was established by serial dilutions of calibration plasma (Standard human plasma, DADE Boehring, Germany). Serum insulin (mU/L) concentrations were determined by radioimmunoassay [RIA INSULIN (PEG), INEP, Belgrade, Serbia]. Insulin resistance (IR) was determined by the homeostasis model assessment [HOMA-IR=insulin (mU/L) × glucose (mmol/L)/22.5],[14] quantitative insulin sensitivity check index [QUICKI=1-(Log (insulin, mU/L) + Log (glucose, mg/dL)][15] and insulin sensitivity index-Matsuda [$ISI_{Matsuda}$=10000/[Fasting glucose, mg/dL × Fasting insulin, mU/L) × (Mean glucose in OGTT, mg/dL × Mean insulin during OGTT, mU/L)].[16] Serum testosterone (nmol/L), androstenedione (ng/mL), dehydroepiandrosterone sulfate (DHEAS, nmol/L) and sex-hormone binding globulin (SHBG, nmol/L) were measured by radioimmunoassay (Testo-CT2, R-GM-100, DHEAS-CT and SHBG-RIACT, respectively; CIS Bio International, Gifsur-Yvette, France). Free androgen index (FAI) was calculated from total testosterone and SHBG levels using the formula [(testosterone/SHBG) × 100].

The plasma level of nitrotyrosine (nmol/L) was monitored by enzyme immunoassay (Oxis International Inc). The plasma thiol groups content (μmol/L) and glutathione peroxidase activity (GPX, U/L) were determined as decribed elsewhere.[17,18]

The standard oral glucose tolerance test (OGTT) was performed after an overnight fast, with 75g of glucose. Glucose and insulin were determined at 0, 30, 60, 90 and 120 min of the test. The euglycemic hyperinsulinemic clamp (hereinafter "clamp") was performed two days after OGTT and also after the overnight fasting. In accordance with DeFronzo, the clamp was started with a primed continuous infusion of crystalline insulin (Novo Nordisk, Denmark) and was continued during 120 min at a rate of 0.1 U/kg/h.[19] Blood glucose level was calculated from three glucose measurements 5 minutes apart, obtained immediately before the start of the study and was maintained at a euglycemic level between 4.5 and 5.6mmol/L by variable infusion of 20% glucose. The whole body disposal rate (M, mg per kg per min) was determined as the mean of the glucose infusion rate during the last 30 min of the clamp (steady state rate), according to DeFronzo.[19] In order to correct for any variations in the plasma insulin level, the insulin sensitivity index (M/I) was calculated by dividing the mean M by the steady state plasma insulin level during the last 30 min of the clamp and multiplying by 100.[19]

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS, version 17.0; SPSS Inc, Chicago, IL, USA). Comparison between groups was made by using the parametric t-test and nonparametric Mann-Whitney U-test, as appropriate. Association between different variables and oxidative stress markers were determined by using the Pearson correlation coefficient. A p value <0.05 was considered statistically significant.

RESULTS

Clinical and biochemical parameters obtained from PCOS patients and healthy controls are shown in Table 1. There was no statistical difference in age, BMI, WC, fasting blood glucose, fibrinogen, CRP, SHBG, DHEAS and androstenedione between the PCOS group and the control group. Basal insulin and HOMA-IR were significantly higher and QUICKI significantly lower in PCOS patients in comparison to the controls (15.14 ± 6.96 vs. 9.74 ± 4.33 mmol/L, p=0.007, 3.18 ± 1.55 vs. 1.98 ± 1.02, p=0.007, and 0.32 ± 0.03 vs. 0.36 ± 0.06, p=0.004, respectively). The PCOS group in comparison to controls had significantly higher values of testosterone and FAI (2.55 ± 1.04 vs. 1.83 ± 0.77 nmol/L, p=0.034, and 8.66 ± 8.51 vs. 3.35± 1.36 %, p <0.01, respectively).

The baseline differences of oxidative damage parameters and plasma GPX antioxidant enzyme activity between PCOS and normal subjects are displayed in Table 1. Nitrotyrosine and uric acid (UA) were significantly higher (322.2 ± 104.4 nmol/L vs. 266.5 ± 196.9 nmol/L, p=0.048, and 263.4 ± 69.18mmol/L vs. 203.3 ± 31.45 mmol/L, p=0.012, respectively), while plasma thiol groups were lower in PCOS women

Table 1. Basal clinical and biochemical parameters in non-obese PCOS patients and controls

	PCOS (n=36)	Controls (n=11)	p value
Age (years)	26.31±4.25	27.36±4.11	0.460
BMI (kg/m^2)	21.99±3.17	21.19±1.97	0.474
Waist circumference (cm)	74.47±8.78	70.59±6.12	0.161
Basal glucose (mmol/L)	4.69±0.49	4.41±0.70	0.214
Basal insulin (mU/L)	15.14±6.96	9.74±4.33	0.007
HOMA-IR	3.18±1.55	1.98±1.02	0.007
QUICKI	0.32±0.03	0.36±0.06	0.004
$ISI_{Matsuda}$	4.59±2.61	7.35±5.68	0.030
Fibrinogen (g/L)	3.39±0.92	2.91±0.77	0.134
CRP (mg/L)	1.16±1.15	0.69±0.66	0.246
Testosterone (nmol/L)	2.55±1.04	1.83±0.77	0.034
SHBG (nmol/L)	43.86±22.66	60.92±29.33	0.072
FAI	8.66±8.51	3.35±1.36	0.001
DHEAS (nmol/L)	8.18±4.09	6.69±2.61	0.304
Androstenedione (ng/mL)	3.36±1.29	2.44±1.13	0.059
M (mg/kg/min)*	9.79±4.01	8.08±2.06	0.184
M/I§ (mg/kg/min/ mU/L)x100	9.93±6.15	6.89±4.56	0.084
Thiol groups (μmol/L)	7.42±1.20	8.39±1.31	0.043
Nitrotyrosine (nmol/L)	322.20±104.38	266.45±196.97	0.048
Uric acid (mmol/L)	263.42±69.18	203.25±31.45	0.012
GPX (U/L)	376.52±110.49	355.83±91.03	0.516

*M- whole body glucose disposal rate during last 30 min of euglycemic hyperinsulinemic clamp; §M/I – M adjusted for the steady state plasma insulin level.

compared to the controls (7.42 ± 1.20 μmol/L vs. 8.39 ± 1.31 μmol/L, p=0.043). No significant difference was observed in antioxidant GPX activity in PCOS patients in comparison to controls (p >0.05). In PCOS patients, the significant positive correlations were obtained for UA, as surrogate marker of xanthine oxidase activity with testosterone (r=0.385, p=0.039), as well as indices of insulin resistance, specifically basal insulin, HOMA-IR, ISI and M/I (Table 2).

Acute hyperglycemia during OGTT induced alteration in both the parameters of oxidative damage of proteins and activity of antioxidant GPX activity in PCOS women. As depicted in Figure 1, the percent of change in plasma nitrotyrosine in response to the oral glucose challenge was significantly higher in women with PCOS compared with the weight-matched controls (p<0.05), while for the thiol groups this change did not reach statistical significance (p=0.629). In contrast, OGTT induced a more pronounced fall in GPX activity in PCOS women in comparison to controls (p<0.05) (Figure 1). The alteration of oxidative stress parameters during hyperglycemia correlated with the hyperandrogenism present in PCOS women. Specifically, plasma levels of testosterone were positively correlated with the percentage of change in nitrotyrosine concentrations (r=0.543, p=0.036) as well as with percentage of change in GPX activity during OGTT (r=-0.457, p=0.025) (Figure 2). The data on the association between investigated oxidative stress markers and indices of insulin resistance are depicted in Table 2. When analyzing indices of insulin resistance, linear correlations were obtained between ISI with both HOMA-IR and QUICKI (r=

Table 2. Correlations of oxidative stress markers with insulin resistance indices in non-obese PCOS

			OGTT						IC	
	Basal insulin (mU/L)		HOMA-IR		QUICKI		$ISI_{Matsuda}$		M/I (mg/kg/min/mU/L)x100	
Percentage of change*	r	p	r	p	r	p	r	p	r	p
Nitrotyrosine	0.524	0.031	0.541	0.031	0.631	0.066	0.490	0.039	0.046	0.861
Thiol groups	0.329	0.196	0.333	0.192	0.120	0.634	0.213	0.396	0.208	0.439
GPX activity	0.356	0.161	0.325	0.203	0.383	0.159	0.607	0.021	0.115	0.696
Uric acid[a] (mmol/L)	0.378	0.021	0.374	0.034	0.151	0.408	0.380	0.038	0.404	0.029

*The percent of change in oxidative stress markers was determined when fasting samples were compared with the samples collected 2 h after OGTT or insulin clamp. [a]Basal values. OGTT: oral glucose tolerance test; IC: hyperinsulinemic clamp.

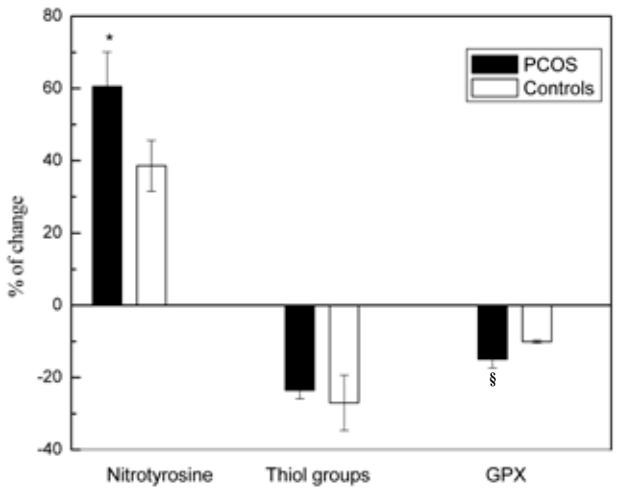

Figure 1. The percentage change in plasma nitrotyrosine, thiol groups and GPX activity when fasting samples were compared with the samples collected 2 h after glucose ingestion. *The percentage change in nitrotyrosine concentrations of PCOS women was greater compared with that of lean controls, $p<0.05$. §The percentage change in GPX activity of PCOS women showed a more pronounced decrease compared with that of lean controls, $p<0.05$.

-0.885, $p<0.001$ and $r= 0.886$, $p< 0.001$, respectively) as well as with M/I ($r=0.425$, $p=0.004$).

In order to test the effect of hyperinsulinemia on the oxidative damage markers and antioxidant plasma GPX activity, we determined the alteration in nitrotyrosine, thiol groups and GPX activity during the euglycemic hyperinsulinemic clamp. No significant change in all tested parameters was observed during the clamp (data not shown).

DISCUSSION

In this study, we showed that both plasma markers of protein oxidative damage and GPX antioxidant activity are associated with measures of insulin resistance in young, non-obese PCOS women. During the oral glucose challenge, the alteration in oxidative stress markers significantly correlated with circulating testosterone.

Under fasting conditions, women with PCOS are exposed to subtle oxidative stress on account of the increased plasma nitrotyrosine and uric acid, decreased content of plasma thiol groups, followed by no changes in antioxidant glutathione peroxidase activity. Recent evidence suggests that major causes of increased generation of ROS in PCOS might be impairment of the mitochondrial electron transport chain[20] together with increased enzymatic activities of the NADPH oxidase and xhantine oxidase.[5,21] Increased ROS production might affect the activity of redox-sensitive signaling pathways, such as the PI_3K–PDK-1–Akt signaling axis, which is involved in

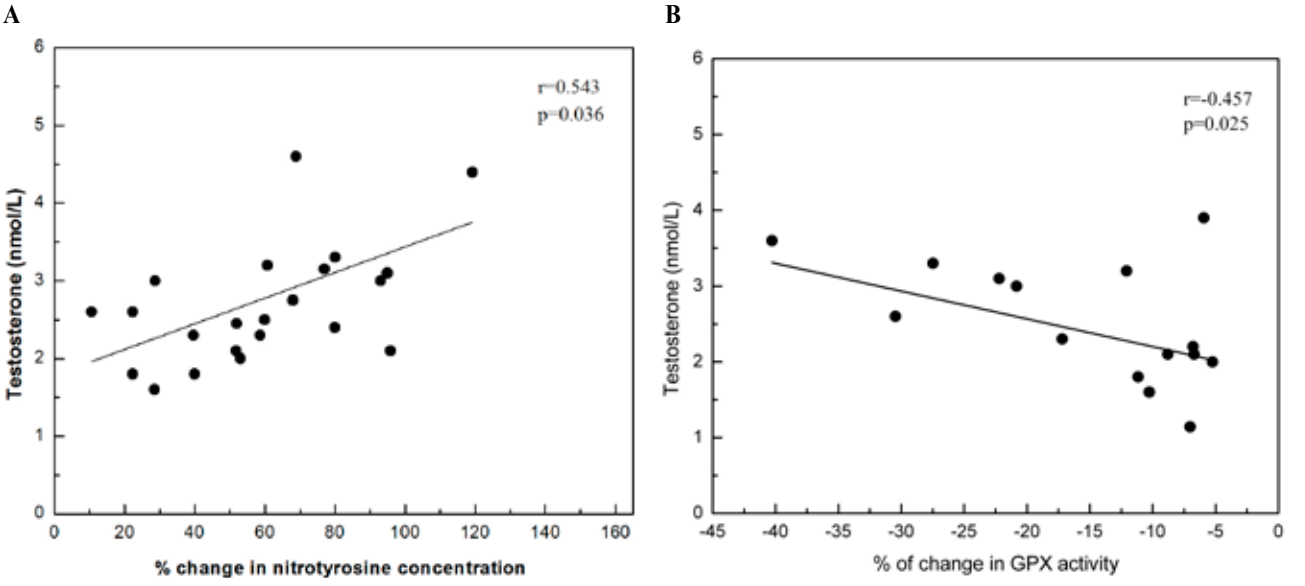

Figure 2. A. Correlation between testosterone and the percentage change in plasma nitrotyrosine. **B.** Correlation between testosterone and the percentage change in plasma GPX activity. The percentage change in plasma oxidative stress markers was determined when fasting samples were compared with the samples collected 2 h after OGTT.

pleiotropic biological actions of insulin.[22,23] As recently demonstrated *in vitro* on cultured human umbilical vein endothelial cells, a free radical peroxynitrite dose dependently inhibits Akt activity.[23]

Our results on increased concentrations of nytrotyrosine in healthy women as an indirect marker of peroxynitrite generation in response to acute hyperglycemia may be the normal *in vivo* response and are in accordance with the results of Ceriello et al (2008).[24] However, the more pronounced increase in plasma nitrotyrosine during the oral glucose challenge in PCOS women in comparison with controls clearly implies that these women are more sensitive to oxidative stress in response to glucose ingestion. In this process, increased ROS generation with consequent decreased NO bioavailability may influence both insulin signaling and endothelial dysfunction.[25] In our study, in the fasting state no changes were observed for plasma GPX activity in PCOS in comparison with healthy women, this being in accordance with recent findings.[5,26] Another demonstration of increased protein oxidation during the glucose challenge is a significant decrease in GPX activity that was observed in PCOS compared to controls. This finding is biologically plausibile, since it has been shown that peroxynitrite itself can inhibit superoxide dismutase and other antioxidant molecules, leading to positive feedback cycles of oxidant generation and exacerbation of the oxidative cellular injury.[27]

Until now, the role of the antioxidant enzyme GPX in glucose metabolism and insulin function is still unclear. In the last decade, it has been explicitly confirmed that activity of the GPX3 isoenzyme, which accounts for a major part of the plasma activity, is down-regulated in obesity.[28] Bearing in mind that GPX3 expression is greatly suppressed by prooxidative conditions present in obesity (i.e. high levels of TNFα and hypoxia), we chose normal weight PCOS women for the investigation of acute hyperglycemia on alteration in plasma GPX activity.

We did not show a direct effect of hyperinsulinemia during the euglycemic hyperinsulinemic clamp on the change in oxidative damage markers and antioxidant plasma GPX activity among our PCOS women. This could be related to the fact that we did not observe any differences in either M or M/I index between investigated subjects. These results, which are in concordance with other studies, imply that lean PCOS could be hyperinsulinemic without being truly insulin resistant.[29-31] M and M/I indexes represent predominantly insulin resistance in insulin-sensitive organs, such as skeletal muscle and adipose tissue, but not in the liver, as this method using high insulin levels almost completely suppresses hepatic glucose production, specifically in non-obese subjects,[32] as were our PCOS women. A significant correlation between M/I and UA in our PCOS women can be explained by recent identification of the importance of hyperuricemia in predicting visceral obesity and insulin resistance.[33] The most significant association that was found between oxidative stress markers, specifically GPX and $ISI_{Matsuda}$, is in line with previous speculation on the influence of insulin resistance on the diminished antioxidant capacity in PCOS women.[5] Future studies with clamp modifications are needed for more focused analyses of the possible link between the deleterious effects of hyperinsulinemia on change of the parameters of oxidative stress and consequent early vascular damage in PCOS women.

The main androgen in the circulation in women with PCOS is testosterone. Although some women with PCOS may have normal testosterone but increased levels of androstenedione and DHEAS, no such case was identified in our study. Our results regarding the association between plasma testosterone and alteration in oxidative stress parameters during acute hyperglycemia further confirm the link between oxidative stress and hyperandrogenism in this disorder. *In vitro* studies have shown that ovarian steroidogenic enzymes responsible for androgen production are stimulated by oxidative stress and inhibited by antioxidants. In fact, oxidative stress may exert a dual effect on theca cells. More pronounced oxidative stress as well as antioxidants (e.g. vitamin E succinate) inhibits proliferation of theca cells, while modest oxidative stress stimulates their proliferation *in vitro*.[34] Furthermore, oxidative stress impairs insulin signaling, resulting in a compensatory hyperinsulinemia, which in turn further stimulates thecal steroidogenesis.[35]

In conclusion, our women with PCOS exhibited high ROS generation that is independent of obesity. This indicates that oxidative stress is a feature of

PCOS per se and that it could contribute either to insulin resistance or to hyperandrogenemia. Among our non-obese women with PCOS, oxidative stress was related to hyperglycemia and circulating testosterone but seemed not to be related to the direct effect of hyperinsulinemia during the standard hyperinsulinemic euglycemic clamp.

GRANTS

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