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Amelioration of insulin resistance in women with PCOS via reduced insulin receptor substrate-1 Ser^{312} phosphorylation following laparoscopic ovarian electrocautery

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BACKGROUND: Increased Ser^{312} phosphorylation of insulin receptor substrate (IRS)-1 is one possible molecular mechanism of insulin resistance in polycystic ovary syndrome (PCOS). We investigated whether laparoscopic ovarian electrocautery (LOE) improved insulin sensitivity in women with PCOS and examined the underlying molecular mechanism of LOE. METHODS: Adipose tissue and blood samples from 12 women with PCOS before, and 3 months after, LOE were analysed. RESULTS: Before LOE, women with PCOS were found to have significantly higher 2 h glucose, fasting and 2 h insulin levels, homeostasis model insulin resistance index and lower fasting glucose-to-insulin ratio (G_0/I_0) than healthy, lean, age-matched controls. Serum levels of glucose and insulin were significantly decreased, and G_0/I_0 ratio was significantly increased 3 months after LOE. Levels of activated extracellular signal-regulated kinase 1/2 in PCOS women were higher than in controls, but were significantly decreased after LOE. Levels of insulin receptor, glucose transporter-4 and phosphatidylinositol 3-kinase were lower in PCOS patients before LOE than in controls and increased after LOE. Levels of Ser^{312}-phosphorylated IRS-1 in PCOS women before LOE were higher than in controls and decreased significantly after LOE, whereas IRS-1 tyrosine phosphorylation in PCOS women before LOE was lower than in controls and increased significantly after LOE. CONCLUSION: Over the short observation period of this study, our results demonstrated that LOE effectively ameliorated insulin resistance in women with PCOS via decreased IRS-1 Ser^{312} phosphorylation.

Key words: extracellular signal-regulated kinase/laparoscopic ovarian electrocautery/phosphatidylinositol 3-kinase/serine phosphorylation

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrinopathy characterized by chronic anovulation, hyperandrogenism and multiple small subcapsular cystic follicles in the ovary on ultrasonography (Franks, 1995), which affects 5–10% of women of reproductive age (Dunaif, 1997; Diamanti-Kandarakis *et al.*, 1999; Asuncion *et al.*, 2000). It is frequently associated with insulin resistance accompanied by compensatory hyperinsulinaemia and therefore presents an increased risk of type 2 diabetes (Ehrmann *et al.*, 1999; Legro *et al.*, 1999). *In vitro* and *in vivo* studies have shown that, in women with PCOS, the sensitivity of glucose metabolism to insulin is subnormal and that modest hyperinsulinaemia prevails (Dunaif *et al.*, 1991).

Insulin action is initiated when insulin binds to the insulin receptor. Insulin binding induces autophosphorylation of the insulin receptor on specific tyrosine residues and increases the intrinsic kinase activity of its β-subunit. The tyrosine-phosphorylated protein then activates phosphatidylinositol (PI)3-kinase through insulin receptor substrate (IRS)1/2, then PI3-kinase mediates insulin's action on glucose metabolism, antilipolysis and protein synthesis (Virkamaki *et al.*, 1999). Another signaling pathway acts through activation of the mitogen-activated protein kinase (MAPK) isoform of extracellular signal-regulated kinases (ERKs) 1 and 2 to mediate the mitogenic and other gene-regulatory effects of insulin (Virkamaki *et al.*, 1999).

The molecular mechanism of insulin resistance in PCOS is unknown. However, several studies have demonstrated that PCOS manifests a post-binding defect in insulin signaling in adipocyte and decreased activity of PI3-kinase in muscle biopsies during euglycemic hyperinsulinaemic clamps (Ciaraldi *et al.*, 1992; Dunaif *et al.*, 2001; Seow *et al.*, 2004). Dunaif et al. (1995) reported that the impaired action of insulin on glycogen synthesis in cultured skin fibroblasts from POCS women is associated with constitutively increased insulin receptor (IR)β-subunit serine phosphorylation and decreased insulin receptor tyrosine kinase activity. In addition, Corbould *et al.* (2005) showed that in cultured skeletal muscle cells from PCOS women Ser^{312} phosphorylation of IRS-1 is constitutively increased. Furthermore, MAPK activity that is constitutively increased in the skeletal muscle of women with PCOS (Corbould *et al.*, 2006) suggests that ERK1/2 or ERK-regulated kinases are responsible for the increased Ser^{312} phosphorylation of IRS-1. These observations provide strong support for the hypothesis that increased Ser^{312} phosphorylation is an important mechanism for insulin resistance in PCOS.

Laparoscopic ovarian electrocautery (LOE) is an alternative treatment for PCOS women who are clomiphene citrate-resistant (Li *et al.*, 1998). Wedge resection was the forerunner of LOE and was first introduced by Stein and Leventhal (1935) for seven anovulatory women with PCOS and resulted in resumption of menses and pregnancy. In addition, following LOE in women with PCOS, there is a marked decrease in serum androgen levels, an increase in FSH levels, a reduction in the amplitude of LH pulses, a reduction in the LH/FSH ratio and a reduction in ovarian volume (Campo *et al.*, 1993; Amer *et al.*, 2002). Furthermore, several studies have reported that LOE has a long-term effect (more than 6 years) on the normalization of endocrine abnormality in women with PCOS (Neather *et al.*, 1994; Gjonnaess, 1998; Amer *et al.*, 2002). However, the mechanism of action of LOE is unclear.

The aim of this study was to evaluate whether LOE could correct hyperinsulinaemia in women with PCOS and to assess the possible molecular mechanism involved in the normalization of serum insulin and glucose levels. We hypothesized that LOE would correct hyperinsulinaemia in women with PCOS via reduced Ser^{312} phosphorylation of IRS-1 in adipocytes. To investigate this possibility and to evaluate the mechanism of the post-binding defect in insulin receptor-mediated signal transduction, we examined insulin receptor protein tyrosine kinase activity in the adipose tissue of PCOS women obtained by laparoscopic surgery before, and three months after, ovarian electrocautery surgery.

Materials and methods

Subjects

Twelve women (four obese and eight non-obese: obesity defined as body mass index (BMI) $\geq 31\ kg\ m^{-2}$, non-obese as BMI $<31\ kg\ m^{-2}$) who fulfilled the inclusion criteria for PCOS below were enrolled in this study. All were in good health and had not taken oral contraceptives within the last 3 months. All PCOS women had anovulatory infertility of >1 year duration and had been unsuccessfully treated with clomiphene citrate at up to $150\ mg\ day^{-1}$ for 5 days prior to LOE. The protocol was reviewed and approved by the Institutional Review Boards of both the Shin Kong Wu Ho-Su Memorial Hospital and Taipei Veteran General Hospital. All patients entered this study only after informed written consent was obtained.

PCOS was defined by clinical, laboratory and ultrasound criteria according to the consensus criteria reported by the Rotterdam group (2004). All PCOS women had menstrual disturbances and hyperandrogenism and/or polycystic ovaries. The clinical criteria included oligomenorrhoea (menstrual interval >6 weeks) or amenorrhoea (no menstrual loss for ≥3 months) dating from menarche. None of the subjects had acanthosis nigricans. The biochemical criteria were an increased LH concentration ($>6\ mIU\ ml^{-1}$, normal follicular range $1–6\ mIU\ ml^{-1}$), a normal FSH concentration and an elevated total serum testosterone concentration ($>0.8\ ng\ ml^{-1}$, normal range $0.06–0.80\ ng\ ml^{-1}$). 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) under transvaginal ultrasonographic examination (Balen *et al.*, 2003). Serum prolactin and thyroid hormone levels 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 were excluded by a morning serum 17-hydroxyprogesterone level of $<2\ ng\ ml^{-1}$.

Ten healthy, lean, 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

Monoclonal mouse antibodies against the human IR β-subunit, p85 regulatory subunit of PI3-kinase, or IRS-1 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Polyclonal rabbit anti-GLUT-4 antibodies and human anti-β-actin antibodies were purchased from Chemicon Inc. (Temecula, CA). Polyclonal mouse antibodies against human ERK1/2, phospho-ERK1/2 and phospho-c-Jun N-terminal kinase (JNK) were from Cell Signaling Technology (Beverly, Mass, USA). Polyclonal rabbit antibodies against human phospho-Ser^{312}-IRS-1 and phospho-tyrosine-IRS-1, not directed at a specific site, were purchased from Sigma Chemical Co. (St Louis, Mo, USA).

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 after an overnight fast during the early follicular phase (days 3–7) on all women before, and 3 months after, LOE. For amenorrhoeic women, progesterone was given to induce withdrawal bleeding. Four blood samples were collected at 0, 30, 60, and 120 min and the plasma stored at −20°C until assayed for glucose and insulin. The fasting glucose-to-insulin ratio (G_0/I_0) was measured as described previously (Legro *et al.*, 1998). The homeostasis model insulin resistance index ($HOMA_{IR}$) was calculated using the formula: fasting glucose ($mg\ dl^{-1}$) × fasting insulin ($\mu IU\ ml^{-1}$)/405 (Matthews *et al.*, 1985). A $HOMA_{IR}$ value of ≧3.8 or G_0/I_0 ratio ≤4.5 indicates insulin resistance in PCOS (Kauffman *et al.*, 2002).

Hormonal profile

Blood was withdrawn from the antecubital vein for serum E2, FSH, LH and testosterone measurements on the day of the OGTT before

and after LOE. For women with amenorrhoea, 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^{-1} and the intra-assay and inter-assay coefficients of variance 7.7% and 7.9%, respectively. The corresponding values were 0.1 mIU ml^{-1}, 6.5% and 7.1% for LH; 15 pg ml^{-1} (55 pmol l^{-1}), 6.3% and 6.4% for E2 and 0.1 ng ml^{-1}, 4.0% and 5.6% for testosterone.

Laparoscopic ovarian electrocautery

The procedure was performed in the lithotomy position using video-monitoring equipment. All procedures were performed by the first author (K.M.S) and an assistant doctor. Before and during the operation, 500 ml of 5% dextrose in water (125 ml h^{-1}) was given intravenously to stimulate insulin secretion and thus insulin signaling. A 10 mm trocar was inserted in the umbilical position and two 5 mm trocars in the right- and left-side lower quadrant lateral to the inferior epigastric artery 6–8 cm oblique to the pubic rami. A pair of grasping forceps was introduced through one of the 5-mm trocars to grasp the utero-ovarian ligament and lift the ovary away from the bowel and ureter. A 2.5-mm hook monopolar diathermy needle electrode was introduced through the other 5-mm trocar. Cauterization of the ovaries was performed using a Force 2 Valleylab® electrosurgical generator (Valleylab Inc., Boulder, CO, USA). A monopolar coagulating current at 40-W power setting was used and a total of 10 punctures made in each ovary in all the PCOS women. The duration of each penetration was about 3 s. The bilateral ovaries were cooled by irrigation with distilled water and a check for bleeding was performed. All patients were discharged the same day without intraoperative complications.

Adipose tissue sampling

Adipose tissue weighing about 5–6 g was obtained by laparoscopy on the day of LOE from the omental fat tissue of all PCOS women before LOE. For control subjects, adipose tissue was obtained at the same time as the laparoscopic examination for tubal infertility. The adipose tissue of PCOS and control subjects was extracted via one of the two 5-mm trocars and immediately stored at −80°C until tested by western blotting. Three months after the LOE, a second-look operation was performed by laparoscopy. Peri tubal or peri ovarian adhesions were recorded and adhesiolysis performed, then 5–6 g of adipose tissue was removed and stored at −80°C until analysis.

Western blotting

Whole cell lysates from PCOS and control patients were prepared by sonication in lysis buffer (1% Triton X-100, 50 mM KCl, 25 mM HEPES, PH 7.8, 10 μg ml^{-1} of leupeptin, 20 μg ml^{-1} of aprotinin, 125 μM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and analysed on the same western blots. Samples (50 μg of total protein) were mixed with 50 μl of SDS sample buffer and boiled for 10 min, then the proteins were separated on 7.5% SDS gels (for phospho-ERK, ERK, phospho-JNK, PI3-kinase, IR-β and GLUT 4) or 5% SDS gels (for phospho-Ser^{312} IRS-1 and phospho-tyrosine IRS-1) 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 containing 0.5% Tween-20 and immunoblotted with antibodies against human phospho-ERK, ERK, phospho-JNK, PI3-kinase, IR-β, anti-GLUT 4, phospho-Ser^{312} IRS-1, or phospho-tyrosine IRS-1 followed by incubation for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch) and was revealed with Chemiluminescence Reagent (Amersham Bioscience, Buckinghamshire, England). Chemiluminescence was quantified using a Personal Densitometer (Molecular Dynamics, USA) with a range of 0.01–4.0 optical density (OD) units.

Statistical analysis

Data are presented as the mean ± SD. Statistical analysis was carried out using Student's *t*-test or paired *t*-test, as appropriate. Computations were performed using SPSS (Statistical Package for Social Science; Window version 10.0) software. In all cases, the threshold for significance was taken as $P < 0.05$.

Results

The mean age of the PCOS women was 29.5 ± 4.9 years and that of the controls 28.3 ± 3.3 years. None of the subjects had type 2 diabetes. Four of the women with PCOS had impaired glucose tolerance, whereas the other eight women with PCOS and all control subjects had normal oral glucose tolerance. Three of the PCOS women still required progesterone stimulation for withdrawal bleeding after LOE (3/12, 25%). Resumption of menses by LOE in nine of the patients (9/12, 75%).

Clinical and endocrine metabolic characteristics

The clinical features and baseline hormonal and metabolic parameters for the control and PCOS women before, and three months after, LOE are shown in Table I. There was no significant difference in BMI between women with PCOS and controls, but the PCOS women had a higher waist–hip ratio. As expected, women with PCOS had significantly higher LH/FSH ratios and serum testosterone levels than controls. Fasting glucose levels did not differ significantly, but the 2 h glucose result after a 75 g glucose load was significantly higher in PCOS women than controls ($P < 0.05$). The fasting insulin and 2 h insulin post-75-g glucose load values were significantly higher in PCOS women than controls ($P < 0.05$), consistent with the presence of insulin resistance. In addition, the $HOMA_{IR}$ was significantly higher in PCOS women than controls (4.8 ± 3.8 versus 1.03 ± 0.7, $P = 0.005$). Further, the G_0/I_0 ratio was significantly lower in PCOS before LOE, compared with control ($P = 0.001$).

The clinical features and baseline hormonal and metabolic parameters of PCOS women before, and three months after, LOE were also compared. Serum LH, the LH/FSH ratio and testosterone were significantly lower 3 months after LOE. Furthermore, the 2 h glucose, fasting insulin and 2 h insulin levels were significantly improved after LOE ($P < 0.05$). The $HOMA_{IR}$ value was reduced to 2.5 ± 1.7 after LOE, but the difference was not statistically significant. However, the G_0/I_0 ratio was significantly higher in women with PCOS after LOE compared with before (9.9 ± 3.8 versus 5.4 ± 2.0, $P = 0.003$). Five of the patients were found to have a mild peri-ovarian or peritubal adhesion (Shimanuki *et al.*, 1987) (33.3%, 5/12) in the second-look operation.

Glucose tolerance

The PCOS women had significantly increased levels of glucose (Figure 1A) and insulin (Figure 1B) during a 75-g OGTT compared to the age- and weight-matched controls.

Table I. Clinical and endocrine-metabolic characteristics of women with PCOS and control subjects

	Controls ($n = 10$)	PCOS before LOE ($n = 12$)	P^a	PCOS after LOE ($n = 12$)	P^b
Age (year)	28.3 ± 3.3	29.5 ± 4.9	NS	29.7 ± 4.8	NS
Height (cm)	159.7 ± 6	159.6 ± 10	NS	159.6 ± 10	NS
Weight (kg)	57.2 ± 14.9	65.2 ± 17.8	NS	64.8 ± 16.8	NS
BMI (kg m^{-2})	22.5 ± 14.9	25.3 ± 6.3	NS	25.1 ± 6.0	NS
Hip (cm)	92. 5± 5.3	101.1 ± 12.1	0.036	102 ± 12.8	NS
Waist (cm)	70.5 ± 7.2	84.7 ± 13.6	0.008	82.6 ± 13	NS
WHR	0.76 ± 0.04	0.83 ± 0.08	0.014	0.81 ± 0.04	NS
FSH (mIU m^{-1})	5.2 ± 1.7	5.7 ± 1.6	NS	6.6 ± 1.3	NS
LH (mIU m^{-1})	6.2 ± 3.8	13.1 ± 3.9	<0.0001	5.9 ± 1.9	<0.0001
LH/FSH	1.2 ± 0.84	2.5 ± 1.0	0.004	0.9 ± 0.3	<0.0001
E_2 (pg ml^{-1})	46.3 ± 19.9	50.5 ± 30.4	NS	45.3 ± 18.3	NS
T (ng ml^{-1})	0.38 ± 0.1	0.83 ± 0.2	<0.0001	0.45 ± 0.2	<0.0001
Fasting glucose (mg dl^{-1})	86.1 ±3.8	92 ± 8.9	NS	90 ± 6.0	NS
2 h glucose (mg dl^{-1})	95 ± 24.9	128 ± 34	0.034	100 ± 21	0.005
Fasting insulin (mIU ml^{-1})	4.8 ± 3.1	21.1 ± 14	0.002	10.1 ± 2.6	0.024
2 h insulin (mIU ml^{-1})	14.4 ± 9.75	138.7 ± 163.5	0.029	34.3 ± 15.4	0.042
$HOMA_{IR}$	1.03 ± 0.7	4.84 ± 3.8	.005	2.5 ± 1.7	NS
G_0/I_0	19.4 ± 9.6	5.4 ± 2.0	0.001	9.9 ± 3.8	0.003

Mean ± SD.
LOE, Laparoscopic ovarian electrocautery; BMI, body mass index; WHR, waist to hip ratio; FSH, follicle stimulating hormone; LH, luteinizing hormone; E_2, estradiol; T, testosterone; $HOMA_{IR}$, homeostasis model insulin resistance index assessment; G_0/I_0, fasting glucose-to-insulin ratio.
[a]Student's *t*-test, PCOS versus control.
[b]Paired *t*-test, PCOS before LOE versus after LOE.

Three months after LOE, glucose and insulin levels were significantly decreased; insulin levels in the PCOS women were significantly higher than in controls, but there was no significant difference in glucose levels between the two groups (Figure 1).

Mitogenic signaling in adipose tissue

In adipose tissue from PCOS women, levels of phospho-ERK 1/2 before LOE were significantly higher than in controls and decreased significantly after LOE to control levels (Figure 2). However, no difference was seen in the amounts of phospho-JNK protein between PCOS women and controls before LOE or between PCOS women before and after LOE (data not shown).

Phosphatidylinositol 3-kinase expression

To determine whether levels of IR β-subunit, PI3-kinase and GLUT-4 increased after LOE in women with PCOS, adipose tissue from all 12 PCOS women was studied. Levels of IR β-subunit in PCOS women before LOE were significantly lower ($P < 0.05$) than in controls and increased significantly after LOE to control levels (Figure 3A). GLUT-4 levels in PCOS women before LOE were significantly lower than in controls and increased significantly after LOE to levels much higher than in before LOE (Figure 3B). PI3-kinase levels before LOE in PCOS women were similar to these in controls and increased markedly after LOE (Figure 3C).

IRS-1 Ser^{312} phosphorylation

We examined the levels of IRS-1 Ser^{312} phosphorylation (Figure 4A) and IRS-1 tyrosine phosphorylation (Figure 4B) in adipose tissue of women with PCOS before LOE and found that they displayed constitutively increased Ser^{312} IRS-1 phosphorylation and decreased IRS-1 tyrosine phosphorylation. Levels of total IRS-1 protein did not differ

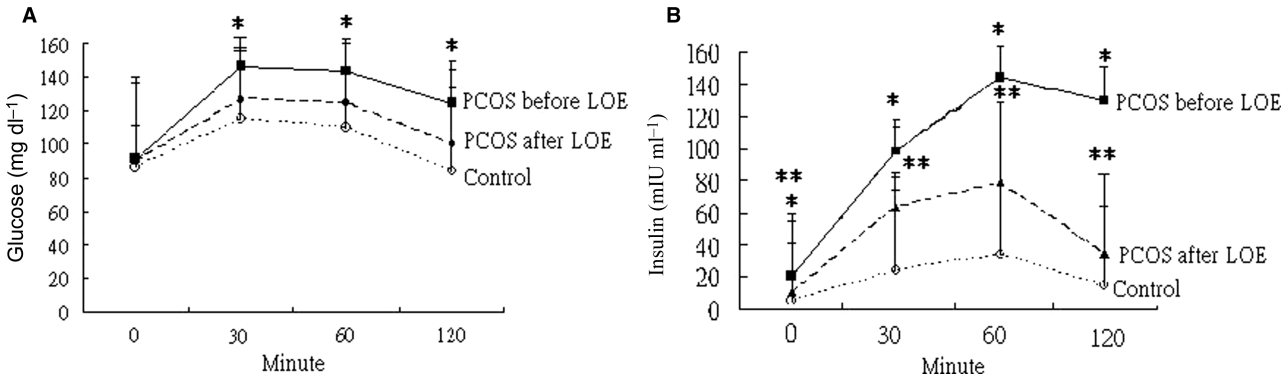

Figure 1. Basal glucose and insulin responses and responses after a 75-g oral glucose load in controls and women with polycystic ovary syndrome (PCOS) before and after LOE. (**A**) glucose; (**B**) insulin. $N = 10$ for controls, 12 for PCOS $*P < 0.05$, PCOS before LOE versus after LOE or control; $**P < 0.05$, PCOS after LOE versus control.

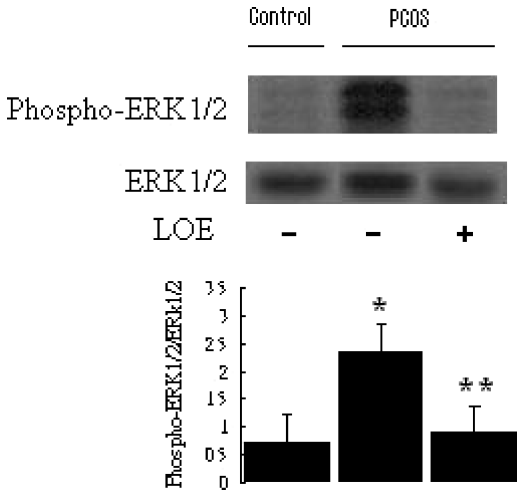

Figure 2. Mitogenic signaling in adipose tissue from controls and PCOS women before and after LOE (obese, $n = 4$; non-obese, $n = 8$) and control ($n = 10$). Cell lysates were immunoblotted with antibodies to phospho-ERK1/2 or total ERK1/2. Upper panel: typical result. Lower panel: phospho-ERK1/2 results for 10 controls and 12 PCOS women. $*P < 0.05$, PCOS versus control; $**P < 0.05$, PCOS before versus after LOE. The experiment was performed twice with similar results. The pre- and post-LOE blots refer to the same PCOS patient.

between PCOS women and controls. After LOE, Ser^{312} phospho-IRS-1 levels whereas significantly reduced to control levels, while levels of phospho-tyrosine IRS-1 were significantly increased to control levels in all PCOS women, including those who did not have regular menses post-LOE.

Discussion

In this study, we found that, in addition to correcting hyperandrogenism, LOE surprisingly improved insulin sensitivity in women with PCOS. This result differs from that of Lemieux *et al.* (1999), who found that LOE failed to correct hyperinsulinaemia, although it successfully restored the ovulatory cycle and resulted in a marked reduction of serum androgen levels. The present study is the first to report that LOE can ameliorate insulin resistance in women with PCOS.

Women with PCOS have a high incidence of insulin resistance ($HOMA_{IR} > 3.8$ or G_0/I_0 ratio ≤ 4.5) (Legro *et al.*, 1998; Kauffman *et al.*, 2002); in the present study, the incidence was 41.6% (by $HOMA_{IR}$ method) or 33.3% (by G_0/I_0 method). Although there was no significant difference in fasting glucose levels between control and PCOS women, significantly higher fasting insulin levels were found in the PCOS women, indicating that the normal fasting glucose value is due to the effect of compensatory hyperinsulinaemia, i.e. increased insulin secretion to overcome the insulin resistance (Goke, 1998). Furthermore, the significant difference in the 2 h glucose and 2 h insulin levels in women with PCOS indicates that insulin sensitivity was decreased, since higher serum insulin levels failed to normalize the 2 h glucose levels (Goke, 1998). Insulin sensitivity was significantly improved after LOE, since serum glucose and insulin levels were decreased. Nevertheless, insulin levels after a 75-g glucose load in PCOS women were still significantly higher after LOE than in controls. This may be due to the short-term (3 months) nature of the study, and we believe serum insulin levels may continue to decrease to more normal levels if LOE has a long-term effect on the normalization of endocrine abnormalities (Gjonnaess *et al.*, 1998; Amer *et al.*, 2002) and insulin levels in women with PCOS.

LOE is performed on infertile women with clomiphene citrate-resistant PCOS (Stein and Leventhal, 1935). It can induce a significant reduction in LH and androgen levels in women with PCOS (Naether *et al.*, 1994; Gjonnaess, 1998; Amer *et al.*, 2002). A significant reduction in serum insulin

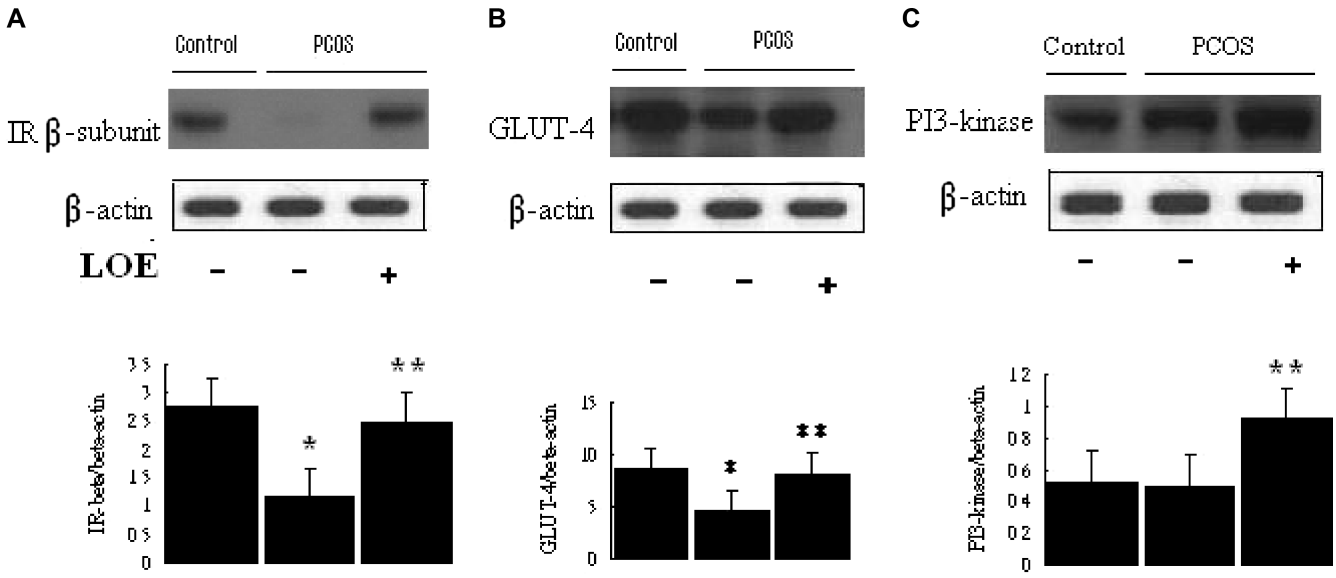

Figure 3. Levels of insulin receptor (IR) β-subunit, GLUT-4 and PI3-kinase in controls and PCOS women before and after LOE. Cell lysates were immunoblotted with antibodies to IRβ-subunit (**A**) GLUT-4 (**B**), or PI3-kinase (**C**). $*P < 0.05$, PCOS versus control; $**P < 0.05$, PCOS before versus after LOE. The experiment was performed twice with similar results. The pre- and post-LOE blots refer to the same PCOS patient.

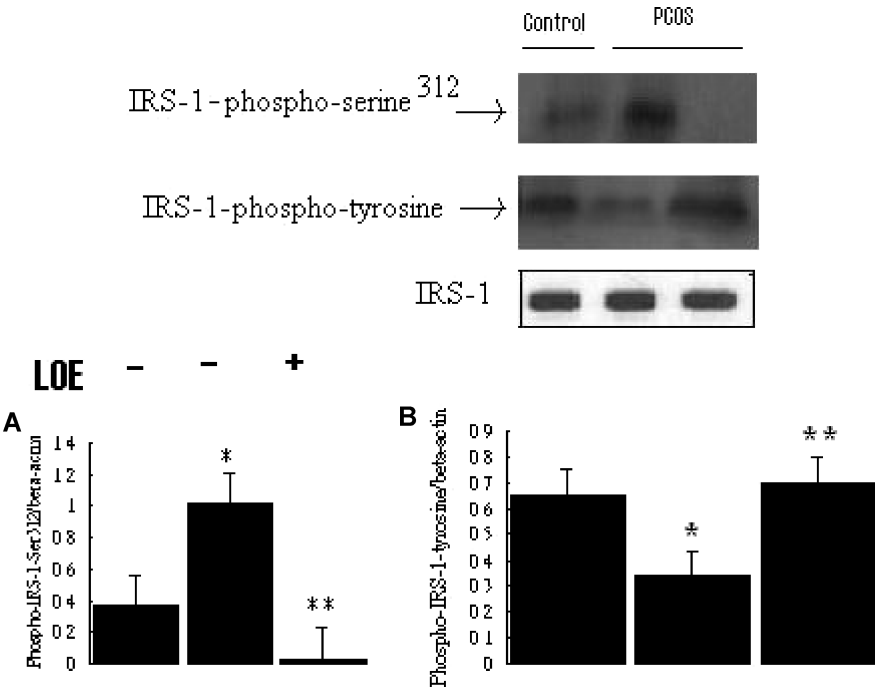

Figure 4. Insulin receptor substrate (IRS-1) serine and tyrosine phosphorylation in adipose tissue from controls and PCOS women before and after LOE. Cell lysates were immunoblotted with antibodies to phospho-Ser^{312} IRS-1, phospho-tyrosine IRS-1 or total IRS-1. *$P < 0.05$, PCOS versus control; **$P < 0.05$, PCOS before versus after LOE. The experiment was performed twice with similar results. The pre- and post-LOE blots refer to the same PCOS patient.

levels was seen in the present study, suggesting that androgens are related in a causal fashion to insulin sensitivity. Several studies have suggested that elevated circulating insulin levels impede ovulation (Lobo *et al.*, 1982; Nestler *et al.*, 1998; Vandermolen *et al.*, 2001). The increased insulin sensitivity due to LOE improves spontaneous ovulation and promotes fertility (Amer *et al.*, 2002; Campo *et al.*, 1993). This is consistent with the findings that administration of agents, such as metformin or rosiglitazone, increases insulin sensitivity, thus inducing ovulation and increasing the pregnancy rate (Nestler *et al.*, 1998; Vandermolen *et al.*, 2001).

The only adverse effect of LOE is pelvic adhesions (Naether *et al.*, 1994; Gjonnaess, 1998; Amer *et al.*, 2002). In the present study, 33.3% of the patients were found to have post-operative periovarian or peritubal adhesions; however, all were mild. Considering the excellent effect of LOE in the correction of insulin resistance, the side-effects (i.e. pelvic adhesions) should not discourage the use of this procedure in PCOS. Furthermore, studies have shown that the endocrine changes seen after LOE are stable over the long term (Gjonnaess *et al.*, 1998; Amer *et al.*, 2002). Thus, if the improvement in insulin sensitivity due to LOE is found to be a long-term effect, LOE may be effective in decreasing the risk of type 2 diabetes in women with PCOS.

The exact molecular mechanism of insulin resistance in PCOS is controversial. However, several studies have shown that insulin resistance in PCOS is due to post-binding defects in signal transduction and that there are multiple defects in insulin action in PCOS that affect metabolism (Ciaraldi *et al.*, 1992; Dunaif *et al.*, 2001; Seow *et al.*, 2004). Our results are consistent with those of these previous studies, since levels of phospho-tyrosine IRS-1, PI3-kinase and GLUT-4 transporter were decreased and those of phospho-ERK and Ser^{312} phospho-IRS-1 increased. After LOE, the observed abnormalities in the insulin-signaling pathway were reversed in women with PCOS. To the best of our knowledge, these are the first results obtained in human adipose tissue showing that multiple defects in the insulin receptor-signaling pathway are present in PCOS and are reversed by LOE. However, although we found multiple defects, we believe that the decreased IRS-1 tyrosine phosphorylation and increased IRS-1 Ser^{312} phosphorylation seen in women with PCOS (Corbould *et al.*, 2005, 2006; Dunaif *et al.*, 1995) may be the initial defect in insulin resistance in PCOS. Increased IRS-1 Ser^{312} phosphorylation would inhibit insulin receptor tyrosine kinase activity and prevent the signal propagation that underlies many biological effects of insulin, leading to decreased activation of the signaling pathway. Evidence for this hypothesis comes from the observed reversal of impaired insulin receptor signaling by serine kinase inhibitors in human fibroblasts from women with PCOS (Li *et al.*,2002). After LOE, IRS-1 Ser^{312} phosphorylation was significantly decreased and IRS-1 tyrosine phosphorylation increased, thus restoring the insulin signaling pathway to increase glucose uptake and improve hyperinsulinaemia.

The increased activation of the ERK1/2 pathway seen in PCOS women in this study suggests that ERK1/2 is involved in serine phosphorylation of IRS-1, resulting in decreased tyrosine phosphorylation and inhibition of PI3-kinase activation. This result is consistent with Corbould *et al.*'s (2006) report that ERK1/2 is responsible for constitutive phosphorylation of IRS-1 Ser^{312} in women with PCOS. However, the mechanism by which ERK1/2 regulates IRS-1 Ser^{312} phosphorylation is unknown.

Nevertheless, in the present study, we found that LOE decreased ERK1/2 activation and IRS-1 Ser^{312} phosphorylation in PCOS women, resulting in ameliorated insulin resistance.

Obese women are reported to have lower levels of insulin receptor, IRS and PI3-kinase than non-obese women (Goodyear *et al.*, 1995). We have measured levels of activated ERK1/2, Ser^{312} -phosphorylated IRS-1, tyrosine-phosphorylated IRS-1, IR β-subunit, PI3-kinase and GLUT-4 in both obese and non-obese PCOS women, and found that these two groups have no statistical differences of these proteins and that, in both groups, these levels are lower than in controls (data not shown). These results suggest that non-obese and obese PCOS women have a similar risk of developing insulin resistance or type 2 diabetes.

Insulin is known to stimulate JNK activation, resulting in activation of skeletal muscle glycogen synthase *in vivo* (Moxham *et al.*, 1996). However, in the present study, we did not find any difference in JNK activation in adipose tissue between PCOS women and controls. Other studies have shown that insulin fails to activate JNK in skeletal muscle and other cell lines (Dong *et al.*, 1996; Goodyear *et al.*, 1996). In the present study, we demonstrated that JNK was not involved in the insulin resistance of PCOS.

In conclusion, our results clearly demonstrate that, within the short observation period of this study, LOE reduces serum androgen levels and ameliorates insulin resistance in women with PCOS. LOE may decrease compensatory hyperinsulinaemia in PCOS via a reduction in IRS-1 Ser^{312} phosphorylation. LOE may therefore be an effective tool for reducing the risk of type 2 diabetes in women with PCOS. However, further studies will be needed to determine the long-term effects of LOE in the improvement of insulin sensitivity in PCOS.

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