Levels of Rabs and WAVE family proteins associated with translocation of GLUT4 to the cell surface in endometria from hyperinsulinemic PCOS women

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Submitted on March 23, 2010; resubmitted on July 21, 2010; accepted on July 29, 2010

BACKGROUND: Polycystic ovary syndrome (PCOS) is an endocrine–metabolic disorder highly associated with insulin resistance and compensatory hyperinsulinemia. It is known that the insulin signaling pathway is impaired in endometria from PCOS hyperinsulinemic women, but no information is available about molecules associated with cell surface GLUT4 translocation. We therefore evaluated the protein levels of AS160 target molecules, Rab8A and Rab10, and the WAVE family proteins involved in the cortical-actin remodeling, Neural Wiskott-Aldrich Syndrome Protein (N-WASP) and WASP, in endometria from hyperinsulinemic PCOS women and controls.

METHODS: Protein levels were assessed by western blot, immunohistochemistry and immunofluorescence in proliferative (PE) and secretory (SE) phase endometria from control women and in endometria from hyperinsulinemic PCOS women (PCOS h-INS).

RESULTS: Similar levels were detected for Rab10 in the three studied groups; however, Rab8A levels decreased in SE (P<0.05) while higher levels were obtained in PCOSE h-INS compared with PE (P<0.05). In the normal menstrual cycle, Neural Wiskott-Aldrich syndrome protein (N-WASP) and WASP levels were increased in SE versus PE (P<0.05), but in PCOSE h-INS, the levels were diminished compared with PE (P<0.05).

CONCLUSIONS: SE is characterized by protein expression changes associated with glucose uptake. In endometria from PCOS women with hyperinsulinemia, reduced levels of WAVE family proteins could compromise the cell surface GLUT4 exposure and the consequent glucose uptake in this tissue.

Key words: endometrium / Rabs / WASP / hyperinsulinemia / GLUT4

Introduction

Polycystic ovary syndrome (PCOS) is an endocrine–metabolic disorder that affects 5–10% of fertile-age women (Diamanti-Kandarakis, 2008). This pathology is characterized by clinical and/or biochemical hyperandrogenism, together with at least oligo- and/or amenorrhea or polycystic ovary assessed by ultrasonography (Azziz et al., 2004). These women also show a 2-fold recurrent and spontaneous miscarriage rate, compared with the normal population (Giudice, 2006). Moreover, between 50 and 70% of PCOS women develop an insulin-resistant condition with compensatory hyperinsulinemia (Giudice, 2006; Salley et al., 2007) that could compromise the energy availability in insulin-responsive tissues.

It is known that endometrial tissue expresses the molecules involved in the insulin signaling pathway, including the insulin-dependent glucose transporter GLUT4. In addition, PCOS endometria exhibit alterations on this signaling pathway, such as lower expression of IRS-1, IRS-1 Y612 and AS160 T642 phosphorylation and GLUT4 protein levels (Mioni et al., 2004; Mozzanega et al., 2004; Fornes et al., 2010), suggesting an endometrial insulin-resistant condition. However, it is unknown whether the endometria from PCOS women exhibit abnormalities in the expression of molecules involved in the GLUT4 traffic to the cell surface.

Insulin, fundamentally through the PI3K/Akt pathway, induces the phosphorylation of the AS160 protein (Akt substrate of 160 kDa) in the threonine 642 site (pAS160T642), reducing its GTPase activity...
upon Rabs proteins insertion in GLUT4 vesicles (Sano et al., 2003; Zeigerer et al., 2004; Larance et al., 2005; Minea et al., 2005; Kramer et al., 2006). To date, the major AS160 target candidates are the small G proteins, Rab8A and Rab10, which are present in GLUT4 vesicles of muscle and an adipose cell line (Ishikura et al., 2007; Sano et al., 2007, 2008). No knowledge is available regarding the expression level of these molecules in human endometria throughout the menstrual cycle, nor if these proteins are present in the same endometrial compartment or if the protein expression is affected by the hyperandrogenemic and/or hyperinsulinemic condition of PCOS.

On the other hand, for GLUT4 cell membrane exposition, cortical actin must be reorganized in an insulin-dependent manner, independently of Akt pathway, where proteins like Rac and WAVE family molecules, WASP and N-WASP, have been involved (jiang et al., 2002; Brozinick et al., 2004; Kanazaki, 2006; JeBailey et al., 2007; Randhawa et al., 2008). Previous studies in 3T3-L1 cells have demonstrated that the inactivation of N-WASP leads to a lower insulin-stimulated GLUT4 translocation to the plasma membrane (jiang et al., 2002). No reports have been published about WASP activity in response to insulin in any cell model, although several reports have related WASP with the cytoskeleton reorganization. In addition, WASP may interact with molecules associated with the rafts insulin pathway involved with the GLUT4 translocation to the cell surface (Tian et al., 2000; Millard et al., 2004; Samarin, 2005; Takenawa and Suetsugu, 2007).

Since several reports have shown impairment of the insulin pathway in PCOS endometria, this study aims to evaluate if the high plasma levels of insulin and androgens present in PCOS women changes the expression of molecules associated with the traffic of GLUT4 to the cell membrane, specifically, Rab8A and Rab10 proteins as well as WASP and N-WASP-actin-related molecules in this tissue. Also, we investigated if these molecules are modified in the endometrium throughout the normal menstrual cycle.

**Materials and Methods**

**Subjects**

Human endometria were obtained with a Pipelle suction curette from the corpus of the uterus of control women and of hyperandrogenic and hyperinsulinemic women with PCOS. Glucose and insulin levels were evaluated by an oral glucose tolerance test with 75 g load of glucose. In order to determine a hyperinsulinemic condition, we measured plasma glucose and insulin levels at 2 h post load of glucose. The diagnosis of hyperinsulinemia was determined when 120 min insulin levels were 2 SDs of insulin concentration over the mean of the control group, as in previous studies (Maliqueo et al., 2003; Fornes et al., 2010). Additionally, homeostasis model assessment (HOMA) index was calculated for all patients (fasting glycemia (mg/dl) and insulinemia divided by 405). The insulin values for patients in the PE phase were 49.6 ± 11.6 μU/ml, in the SE phase 51.8 ± 12.5 μU/ml and for PCOS hyperinsulinemic patients 150.5 ± 46.1 μU/ml. All women had normal glycemia values in the oral tolerance glucose test (basal glucose <100 mg/dl; glucose 120 min <140 mg/dl). All the PCOS women participants in this investigation had hyperandrogenism and hyperinsulinemia. In addition, for the association study between insulin plasma levels and endometrial protein levels, four PCOS patients diagnosed as normo-insulinemic were included (insulin 120 min = 36.3 ± 3.7 μU/ml; basal glucose = 66.1 ± 2.0 mg/dl; glucose 120 min = 87.8 ± 6.6 mg/dl; HOMA = 1.61 ± 0.9).

The studied groups were endometria from control women obtained during the proliferative or the phase of the menstrual cycle (PE, n = 7; SE, n = 7, respectively) and from endometria obtained from PCOS (PCOSE h-INS, n = 7). Control endometria were obtained from hysterectomy for benign causes, and those from the phase were compared with PCOSE endometria, because of their similar morphology. None of the women, neither controls nor those with PCOS, had received hormonal therapy within 3 months prior to the recruitment into the study. Noyes criteria (Noyes et al., 1950) were used by an experienced pathologist to confirm, on the basis of histological dating, the endometrial phase in control and PCOS endometria. The diagnosis of PCOS was made according to the Rotterdam Consensus (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004) and the Androgen Excess Society criteria (Azziz et al., 2004) for the definition of PCOS. The exclusion criteria were women who presented with hyperprolactinemia (prolactin (PRL) >35 ng/ml), hypothyroidism (TSH >5 mIU/ml), androgen-secreting tumors (total testosterone >2 ng/ml; dehydroepiandrosterone sulfate (DHEAS) >3600 μg/dl), Cushing’s syndrome (urine cortisol concentration >50 μg/dl at 24 h and fasting plasma concentration of cortisol >25 μg/dl), congenital adrenal hyperplasia (17-OH progesterone >2.5 ng/ml), diabetes or treatment with hormones and/or ovulation induction. The reference values are from the Laboratory of Endocrinology and Reproductive Biology, University of Chile Clinical Hospital.

This investigation was approved by the Ethical Committees from the University of Chile Clinical Hospital, School of Medicine, University of Chile and informed written consent was obtained from all subjects.

**Antibodies and reagents**

Monoclonal antibodies for Rab8A, Rab10, N-WASP and β-actin were purchased from Abgent (CA, USA), Abcam (Cambridge, UK), Santa Cruz Biotechnology (CA, USA) and Sigma (MO, USA), respectively. Polyclonal antibody for WASP was obtained from Santa Cruz Biotechnology. Anti-mouse and anti-rabbit secondary antibodies were purchased from KPL (MD, USA). The protease inhibitor cocktail was obtained from Roche Mol Biochemicals (Mannheim, Germany). BCA Protein assay kit was from Pierce (Rockford, IL, USA) and labeled streptavidin biotin kit was purchased from Dako. Glucose measurement was assessed by the glucose oxidase colorimetric assay (Human GmBh Wiesbaden, Germany). Hormone determinations were assayed using commercial kits: serum testosterone (T), estradiol (E2) and progesterone (P4) by the solid-phase, competitive chemiluminescent enzyme immunoassay (Ortho-Clinical Diagnostics, Johnson & Johnson, UK), androstenedione (A4) by the radioimmunoassay (Siemens, CA, USA), and sex hormone-binding globulin (SHBG) and insulin concentration by the immulite and the solid-phase chemiluminescent immunoassays (Siemens, Llanberis, Gwynedd, UK), respectively.

**Western blot**

As previously reported (Fornes et al., 2010), the endometrial tissue was homogenized in a lysis buffer (Hepes 20 mM, EDTA 2 mM, EGTA 2 mM, Triton X-1%, PMSF 5 μM, Na3VO4 50 μM) containing protease inhibitor cocktail. After centrifugation at 10 000g for 20 min at 4°C, protein concentration was determined using the BCA protein assay kit (Pierce, IL, USA). Total proteins (50 μg) were denatured and fractionated using 8% one-dimensional SDS-PAGE and transferred to nitrocellulose membrane (BioRad, CA, USA). Membranes were blocked at room temperature for 2 h in Tris-Buffered Saline Tween-20 (TBST) (20 mM Tris, pH 7.6; 137 mM NaCl; 0.1% Tween 20) containing 10% non-fat dry milk (for all markers). Subsequently, the membranes were washed three times for 5 min each in TBST and then incubated overnight with antibodies against Rab8A (1:1000), Rab10 (1:1000), WASP (1:200) or N-WASP (1:100), while rocking at 4°C, and with antibody against human β-actin (1:15 000), then incubated for 1 h at room temperature. The
membranes were then washed three times for 5 min each with TBST, followed by incubation for 30 min at room temperature with anti-mouse IgG, peroxidase-conjugated species-specific (1:5000 for β-actin) or 1 h with anti-mouse or anti-rabbit IgG peroxidase-conjugated species-specific (1:10 000 for Rab8A, 1:3000 for Rab10, 1:1500 for WASP and 1:3000 for N-WASP), while rocking. Then after washing three times for 5 min each with TBST, the bound antibodies were detected with an enhanced chemiluminescence system from Pierce (Rockford, IL, USA). Band intensities were quantified by scanning densitometry utilizing the Un-SCAN-IT software Automated Digitizing System, version 5.1, normalized relative to β-actin and expressed as arbitrary units (AU).

### Immunohistochemistry

Immunohistochemistry assays for Rab8A and Rab10 were performed on 5 μm sections of formalin fixed paraffin-embedded endometrial biopsies. Tissue sections were deparaffinized in xylene and hydrated gradually through graded alcohols. The sections were incubated in antigen retrieval solution (100 mM Tris buffer, pH 9.5) at 100°C for 20 min. Endogenous peroxidase activity was prevented by incubating the samples in 0.3% hydrogen peroxide in PBS for 30 min. Non-specific antibody binding was prevented with a specific blocker of the Histostain Bulk kit for 1 h. A dilution of the primary antibody (1:100) was used. Negative controls were analyzed on adjacent sections and incubated without primary antibody, as well as with non-immune species-specific antisera.

The second antibody was a biotinylated anti-mouse/anti-rabbit immunoglobulin. The reaction was developed by the streptavidin-peroxidase system, and 3,3′-diaminobenzidine was used as the chromogen; the counterstain was carried out with hematoxylin. The analysis of slide was performed by the measurement of positive pixel intensity in an equal area in stroma, epithelia and stromal plus epithelial regions in different sections of the sample. The mean of these values was obtained per sample and studied group.

### Immunofluorescence

Deparaffinized and hydrated 5-μm tissue sections were incubated in the same conditions as for immunohistochemistry for the antigen retrieval and to prevent non-specific binding before incubation with rabbit polyclonal antibodies. For Rab8A, 1:3000 for Rab10, 1:1500 for WASP and 1:3000 for N-WASP, while rocking. Then after washing three times for 5 min each with TBST, the bound antibodies were detected with an enhanced chemiluminescence system from Pierce (Rockford, IL, USA). Band intensities were quantified by scanning densitometry utilizing the Un-SCAN-IT software Automated Digitizing System, version 5.1, normalized relative to β-actin and expressed as arbitrary units (AU).

### Acquisition and imaging analysis

The immunohistochemistry and immunofluorescence images were acquired with a Q Imaging camera Micropublisher 3.3 RTV mounted in an Olympus optical microscope BX-51 (PA, USA) and an objective lens Olympus type UplanFL N, 40X / 0.65 at 25°C. Image evaluation was performed with Image Pro Plus 6.2 Software, Media Cybernetics (MD, USA). All the images for each sample were evaluated with an intensity optical densitometry tool, which measures the positive pixels for immunostaining and immunofluorescence of the tissue.

### Statistical analysis

The number of subjects in this study was calculated assuming α = 0.05 and β = 0.20 and a difference between means of 0.25 and standard deviation of 0.2 according to our previous studies (Bacallao et al., 2008; Fornes et al., 2010). Since the distribution of the data was not parametric (assessed by Kolmogorov–Smirnov test), we used the Mann–Whitney test for comparison of the data between two of the studied groups. For the correlation study, the data showed a normal distribution and the Pearson test was used. P-values < 0.05 were considered significantly different. Statistical tests were performed using Graphpad Prism for Windows version 5.0 Software, Inc.

### Results

#### Clinical and endocrinological characteristics

Clinical and hormonal characteristics of control and PCOS women are shown in Table I. The age of the control group was higher than that of the women with PCOS because they belong to a group who underwent hysterectomy for benign uterus pathology. The mean of the body mass index (BMI) of the control group showed an overweight condition and some with a BMI >30 (BMI range: 23–32 kg/m²). Likewise, PCOS h-INS women exhibit an even higher BMI than the controls, which is inherent to the syndrome (Giudice, 2006); between 30 and 50% of PCOS patients are obese (Calle and Kaaks, 2004; Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004; Bhatia, 2005). In addition, all PCOS women presented a clinical hyperandrogenism accompanied by an excessive ovarian androgen production which, in addition to the decreased SHBG blood levels, leads to a significantly higher free androgen index (FAI) in PCOS women. A histological study of endometria from PCOS patients shows no differences compared with control women (data not shown), as we have reported recently (Fornes et al., 2010).

#### Tissue localization and protein levels of Rab8A and Rab10

The distribution of Rab8A and Rab10 proteins in endometrial tissue were analyzed by immunohistochemistry in PE, SE and PCOSE h-INS samples. No positive immunostaining was detected for Rab8A in any studied group, even with a high concentration of the primary antibody (1:50), whereas Rab10 showed a similar distribution between epithelial and stromal cells in PE. Nevertheless, a lower staining for Rab10 was detected in stromal cells of SE (90% decrease compared with PE; P < 0.05); additionally, at the epithelial level we found a polarized apical and basal staining compared with PE (Fig. 1A and B). Positive staining for Rab10 was observed in PCOSE h-INS, similar to PE in both cell compartments (P = 0.8530) (Fig. 1A and C).

When the samples were analyzed by western blot, no differences on Rab10 protein levels were observed between PE and SE samples (P = 0.1105), nor between PE and PCOSE h-INS (P = 0.7302) (Fig. 2). Nevertheless, Rab8A protein levels exhibited a decrease of 62.6% in SE compared with PE (P < 0.05); additionally, at the epithelial level we found a polarized apical and basal staining compared with PE (Fig. 1A and B). Positive staining for Rab10 was observed in PCOSE h-INS, similar to PE in both cell compartments (P = 0.8530) (Fig. 1A and C).

#### Expression of N-WASP and WASP in endometria

N-WASP protein expression was assessed by western blot in the normal endometrial cycle and PCOSE h-INS endometria. We found that SE showed an increase in N-WASP expression of 69.2% with respect to PE (P < 0.05) (Fig. 3A and B). Besides, a 61.5% lower content of N-WASP was observed in PCOSE h-INS compared with PE (P < 0.05) (Fig. 3A and B).

Expression levels of the WASP protein were similar to that of N-WASP in all groups. In SE, we found a near 2-fold increase in the
WITH respect to PE ($P < 0.05$) (Fig. 3C). On the other hand, PCOSE h-INS showed a reduction in WASP protein level of 48% compared with PE ($P < 0.05$) (Fig. 3A and C). The WASP immunofluorescence assays supported this result showing a 80% decrease in intensity in PCOSE h-INS compared with that in PE ($P < 0.05$) (Fig. 4A and B). Immunohistochemistry

**Table 1** Clinical and endocrine characteristics of control women during the PE and SE phases and patients with PCOS and hyperinsulinemia (h-INS).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Proliferative phase patients (7)</th>
<th>Secretory phase patients (7)</th>
<th>PCOS h-INS patients (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (range)</td>
<td>36.3 ± 1.8 (27–46)</td>
<td>38.2 ± 1.8 (30–45)</td>
<td>26.5 ± 0.7 (19–33)</td>
</tr>
<tr>
<td>BMI (kg/m²) (range)</td>
<td>26 ± 1.3 (23–32)</td>
<td>25 ± 1.8 (18–26)</td>
<td>34 ± 1.2* (28–42)</td>
</tr>
<tr>
<td>HOMA</td>
<td>2.1 ± 1.5</td>
<td>2.2 ± 1.9</td>
<td>3.9 ± 1.7*</td>
</tr>
<tr>
<td>LH (UI/l)</td>
<td>3.5 ± 0.1</td>
<td>5.1 ± 1.2</td>
<td>7.0 ± 0.8*</td>
</tr>
<tr>
<td>FSH (UI/l)</td>
<td>4.6 ± 0.1</td>
<td>2.8 ± 0.9</td>
<td>5.6 ± 0.7</td>
</tr>
<tr>
<td>$E_2$ (pg/ml)</td>
<td>73.8 ± 22.4</td>
<td>120.1 ± 37.7</td>
<td>63.1 ± 4.7</td>
</tr>
<tr>
<td>$P_4$ (ng/ml)</td>
<td>0.9 ± 0.5</td>
<td>6.9 ± 2.0</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>$A_4$ (ng/ml)</td>
<td>1.7 ± 0.3</td>
<td>1.8 ± 0.5</td>
<td>4.5 ± 1.6*</td>
</tr>
<tr>
<td>$T_t$ (ng/ml)</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.1*</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>60 ± 16</td>
<td>57.5 ± 7.5</td>
<td>23.4 ± 3.1*</td>
</tr>
</tbody>
</table>

The values are mean ± SEM. $^* P < 0.05$ between the proliferative phase and PCOS and hyperinsulinemic patients.

![Image of Immunohistochemical detection for Rab10 protein in paraffin wax sections of: (A) PE from control women (n = 7); (B) SE from control women (n = 7) and (C) PCOS women with hyperinsulinemia (PCOSE h-INS, n = 7). Positive staining was detected in epithelial and stromal cells of all samples of PE and SE groups. Nevertheless, a significant decrease in staining was detected in stromal cells from SE compared with PE ($P$-value < 0.05), whereas no differences were detected between the PE and PCOS groups. (D) As a negative control, the primary antibody was omitted. Arrows: epithelial glands. Asterisk: stromal compartment. Image magnification ×400.](image-url)

WASP with respect to PE ($P < 0.05$) (Fig. 3C). On the other hand, PCOSE h-INS showed a reduction in WASP protein level of 48% compared with PE ($P < 0.05$) (Fig. 3A and C).

The WASP immunofluorescence assays supported this result showing a 80% decrease in intensity in PCOSE h-INS compared with that in PE ($P < 0.05$) (Fig. 4A and B). Immunohistochemistry
and immunofluorescence assays for N-WASP were performed with low dilutions (up to 1:50) and results were similar to the negative controls in all studied groups (data not shown).

**Correlation between WASP protein levels and hyperinsulinemia**

Unpublished data from our group revealed that WASP protein levels in PCOS endometria from normo-insulinemic patients are 30% higher than in PE, which differs from the results obtained for PCOSE h-INS. Therefore, to determine if an association between plasma insulin levels and endometrial WASP protein levels exists in a group of PCOS women (with or without hyperinsulinemia, \( n = 11 \)), we assessed the correlation study of these parameters. We found a significant negative correlation (\( r = -0.8564, P < 0.05 \)), where PCOS patients with higher insulin levels had lower expression levels of the WASP protein in the endometrial tissue (Fig. 5). Correlation tests were also performed between FAI and all studied molecules, showing no association among them (Rab8A \( r = 0.2995, P = 0.7005 \); Rab10 \( r = 0.1518, P = 0.7452 \); N-WASP \( r = 0.6981, P = 0.1899 \); WASP \( r = 0.2375, P = 0.5088 \)).

**Discussion**

The PCOS syndrome is characterized by an excess ovary androgen production and in many women with this pathology, it is accompanied by an increment of LH/FSH ratio and insulin resistance with compensatory hyperinsulinemia, which affects the function of extra-reproductive organs such as the liver (Calle and Kaaks, 2004;
exhibited that testosterone, which is elevated in PCOS women, has a negative effect on the IRS-1 and GLUT4 protein expression in PCOS endometria (Maliqueo et al., 2003; Mozzanega et al., 2004; Bhatia, 2005). Nevertheless, it is known that downstream of AS160 other molecules exert their action, such as Rabs proteins that participate in the GLUT4 traffic, and cortical actin remodeling molecules that allow the exposition of GLUT4 in the cell membrane for glucose uptake (Jiang et al., 2002; Miinea et al., 2005; Ishikura et al., 2008).

In this work, we studied the localization and protein content of Rab8A and Rab10, identified as the major target for AS160 action in cell lines of muscular and adipose tissue (Ishikura et al., 2007; Sano et al., 2007, 2008), and of WASP, and N-WASP which are involved with the actin-remodeling in many cell types (Miinea et al., 2005; Samarim, 2005; Takenawa and Suetsugu, 2007).

In the endometrium during the menstrual cycle, the higher levels of Rab8A found in the phase compared with the phase could represent a necessary level of this molecule at this phase for the cellular vesicle trafficking that could include the GLUT4 traffic to the cell surface. However, although we cannot explain the increase of Rab8A in PCOS endometria from hyperinsulinemic women, we speculate that it could be a compensatory response to the impairment of the insulin signaling pathway, at least in this tissue (Fornes et al., 2010). On the other hand, the lower levels of the Rab8A protein in control phase endometria, might give Rab10 a major role in the vesicle trafficking at this time, which is in agreement with the apical and basal distribution of this molecule found in epithelial cells from endometria. Moreover, unpublished data from our group show a 2-fold increase of AS160 phosphorylation at a key site for GLUT4 traffic to the cell surface (Fornes et al., 2010). It has been reported that molecules such as N-WASP and WASP are responsible for the actin reorganization in many cellular models promoting cell motility and vesicle trafficking (Samarin, 2005; Takenawa and Suetsugu, 2007). On the other hand, studies on L6-myotubes and muscle cells from insulin-resistant hyperinsulinemic rats have demonstrated that hyperinsulinemia leads to a loss of cortical F-actin, that negatively affects glucose uptake at least in the muscle cell line model (McCarthy et al., 2006). In addition, free fatty acids have been related with the insulin-resistance condition in muscle cells from PCOS women, where the fatty acids can induce the...

**Figure 4** Immunofluorescense of WASP protein in PE endometria from control women (PE, n = 7) and PCOS patients with hyperinsulinemia (PCOSE h-INS, n = 7). A positive signal was detected principally in epithelial cells of control samples (A), whereas the intensity of WASP in PCOSE h-INS (B) was lower than in the controls. A negative control without the primary antibody was included (C). Semi-quantification of the intensity of WASP was assessed by densitometry analysis in equal areas of endometrial tissue for each group.

**Figure 5** Correlation between endometrial WASP protein levels and plasma insulin levels of PCOS patients with or without hyperinsulinemia. Five normo-insulinemic PCOS patients and six hyperinsulinemic PCOS, all with normal glycemia were included. A negative correlation was observed between WASP levels and levels of plasma insulin.

Diamanti-Kandarakis, 2008). In this context, it is known that women with PCOS and insulin resistance exhibit low SHBG plasma levels compared with normo-insulinemic women (Bhattacharya and Ghosh, 2009). In our study, all PCOS women exhibited an increase of LH with respect to FSH hormone levels. In addition, the hyperinsulinemia condition was present and they displayed a high FAI indicative of hyperandrogenemia. These hormonal features together with a decrease in plasma SHBG levels and a BMI corresponding to obesity, suggest an insulin resistance condition associated with PCOS in these women (Maliqueo et al., 2003; Mozzanega et al., 2004; Bhatia, 2005; Salley et al., 2007; Bhattacharya and Ghosh, 2009). On the other hand, no association was found between BMI and levels of the studied Rabs and WAVE family proteins (data not shown), which in part suggest that obesity could not be a factor influencing our results.

In this same kind of patients, we have recently demonstrated that the endometrial tissue exhibits changes in the insulin signaling pathway, including decreases in the total and phosphorylated content of IRS-1, in AS160 phosphorylation and in the GLUT4 content (Fornes et al., 2010). Moreover, other studies have demonstrated that testosterone, which is elevated in PCOS women, has a negative effect on the IRS-1 and GLUT4 protein expression in ex vivo endometrial tissue and adipocyte cells (Calle and Kaaks, 2004; Bhatia, 2005).
activation of kinases and phosphatases that can alter the normal insulin signaling within the cell (Corbould et al., 2005; Højlund et al., 2008).

Additionally, other experiments associated with this fact have demonstrated that exposition of L6-myotubes to ceramides leads to a reduction of cell surface GLUT4 translocation and glucose uptake, through inhibiting small G protein Rac activation, interfering with the insulin-dependent actin-remodeling (JeBailey et al., 2007). Moreover, in 3T3-L1 cells mutant forms of N-WASP lead reduced GLUT4 exposition in the cell surface (Jiang et al., 2002). In PCOS endometria from obese euglycemic women but with hyperinsulinemia, we found a decrease in the protein levels of N-WASP and WASP and showed that WASP levels are even lower in women with higher insulinemia. On the other hand, FAI values of PCOS patients showed no correlation with the levels of Rabs or the WAVES family proteins included in this study, suggesting that it is the hyperinsulinemia which in part affects the expression of these molecules. This may be true at least in the endometrial tissue, with a consequent impairment of actin-remodeling and GLUT4 exposition on the plasma membrane. Further studies are needed to identify the degree of GLUT4 exocytosis, free fatty-acid content and mRNA levels of N-WASP and WASP in this tissue, and to determine if the protein reduction is repeated in other insulin-responsive tissues such as skeletal muscle and/or adipose cells.

It is well established that PCOS patients exhibit an increment in the miscarriage rate compared with the normal population. However, pharmacological treatment has been shown to improve fertility in these women; insulin sensitizers such as metformin can reduce the early miscarriage risk in PCOS pregnant patients (Diamanti-Kandarakis, 2008). Insulin sensitizers lead to a decrease in the high insulin and androgen plasma levels that are implicated in the endocrine disorder characteristic of the PCOS syndrome (Bhatia, 2005; Diamanti-Kandarakis, 2008), and in a quiescence state in the endometrial tissue (Bacallao et al., 2008; Fornes et al., 2010). Molecular studies on the effects of tiazolidinediones show an augmentation of the activation of AKT and AS160 proteins in skeletal muscle from PCOS insulin resistance patients; these proteins are involved in GLUT4 translocation to the plasma membrane (Højlund et al., 2008). Moreover, a decrease in GLUT4 mRNA levels had been documented in endometria treated with testosterone in vivo, but it was reverted after treatment with metformin (Zhang and Liao, 2010). An impairment of insulin signaling in this tissue could affect the glucose uptake and compromise the energy requirement necessary for the reproductive functions of the endometrium.

In conclusion, the SE phase of the normal menstrual cycle is characterized by changes in expression of proteins involved in the plasma cell GLUT4 exposition, such as AS160 Rabs targets, and N-WASP and WASP, which might favor glucose uptake at this time. However, endometria from hyperandrogenic PCOS women with hyperinsulinemia exhibit a diminution of molecules that could compromise the cell surface GLUT4 exposure and the consequent glucose uptake in this tissue.

**Authors’ roles**

The roles of individual authors in this study were as follows: C.R. participated in all experimental procedures and the manuscript redaction. F.G. (histopathologist) performed the endometrial dating of control patients and PCOS women with hyperinsulinemia. D.V. conducted the clinical and surgical evaluation of the patients. C.R. participated in the discussion of the results and the revision of the manuscript. M.V. participated in the discussion of the results, the direction of this study and the revision of the manuscript.

**Acknowledgements**

We thank M. Maliqueo, PhD, for appreciated advice and M. Molina (Graphic Designer) for his support.

**Funding**

This work was funded by grant no.1095127 from the Fondo Nacional de Desarrollo Científico y Tecnológico, Chile (FONDECYT).

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