Role of the transcriptional factors FOXO1 and PPARG on gene expression of SLC2A4 in endometrial tissue from women with polycystic ovary syndrome

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Abstract

Fifty to seventy percent of patients with polycystic ovary syndrome (PCOS) present hyperinsulinemia. On the other hand, reports indicate that forkhead box class O 1 (FOXO1) and peroxisome proliferator-activated receptor- γ (PPARG) are involved in the insulin signaling pathway, regulating the gene expression of SLC2A4 (GLUT4). The negative effect of FOXO1 over PPARG transcription disappears when FOXO1 is phosphorylated (p-FOXO1) and excluded from the nucleus, whereas PPARG can suppress gene expression of SLC2A4. Scarce knowledge is available in endometrium of women with PCOS and hyperinsulinemia (PCOSE h-Ins) about the role of these factors. We aimed to evaluate whether the endocrine and metabolic status of PCOS modify the levels of gene and protein expression of FOXO1, PPARG, and SLC2A4 in the endometria from hyperinsulinemic PCOS women compared with controls. In endometria from control (CE, $n=7$) or PCOSE h-Ins ($n=7$), we determined the subcellular location and protein levels of p-FOXO1Ser319 and FOXO1/FOXO4 by immunohistochemistry and western blot respectively; gene and/or protein levels of PPARG and SLC2A4 were evaluated by RT-PCR and/or western blot. Cytoplasm location for FOXO1 and p-FOXO1Ser319 was immunodetected in both groups of endometria, showing significantly higher staining in PCOSE h-Ins for these proteins ($P<0.05$). In PCOSE h-Ins, gene and protein levels of PPARG were significantly higher than in CE, whereas SLC2A4 mRNA was decreased ($P<0.05$). In conclusion, the derepression of PPARG transcription by the high levels of p-FOXO1Ser319 could partially account for the lower levels of SLC2A4 found in PCOSE h-Ins, suggesting an alteration of the endometrial function in these patients.

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Introduction

Polycystic ovary syndrome (PCOS) is a disease characterized by an endocrine metabolic disorder and is considered to be the most common alteration among women in fertile age, having prevalence between 5 and 10% (Dunaif 1997, Salley *et al.* 2006). The women who suffer from this syndrome present ovarian dysfunction generating hyperandrogenism and/or hyperandrogenemia (Pugeat *et al.* 2000, Azziz *et al.* 2006). This syndrome is related to obesity in 30% of the cases (Calle & Kaaks 2004) and to insulin resistance in 60–75% of them (Froment *et al.* 2006, Giudice 2006). Therefore, these patients are at a high risk of developing cardiovascular diseases and diabetes mellitus (Ehrmann *et al.* 2006). PCOS has also been linked with infertility, abortions, and recurrent miscarriages; in fact, women with PCOS present a higher significant miscarriage rate

(40–50%) in contrast with normal women (20%; Porter & Scott 2005, Giudice 2006).

Besides the ovarian dysfunction in PCOS, other tissues of the organism are also affected, including the endometrium. To fulfill its function, the endometria need high supply of energy, particularly from glucose intake and its posterior metabolism. In this aspect, results from our laboratory and other groups indicate that some molecules involved in the insulin signaling pathway could be altered in the endometrium of women with PCOS (PCOSE; Mioni *et al.* 2004, Avellaira *et al.* 2006). In fact, we have previously shown that phosphorylation of AKT at Ser473 is increased in the PCOSE (Avellaira *et al.* 2006, Villavicencio *et al.* 2009).

In most tissues, glucose uptake is achieved by glucotransporters, some of which are insulin dependent, like SLC2A4 (GLUT4). It has been reported that in

PCOSE, there is a decrease in the transcript and protein of SLC2A4, particularly in the endometria from obese and hyperinsulinemic PCOS women (Mioni *et al.* 2004). The decreased levels of SLC2A4 may be due partially to changes in molecules that regulate the expression of this glucotransporter. In this regard, it is known that the peroxisome proliferator-activated receptor (PPAR) and the so-called forkhead box class O (FOXO) transcription factors are important to regulate the cell homeostasis of glucose (Armoni *et al.* 2007). PPARs are members of a superfamily of nuclear receptors, and three known PPAR isoforms – α , β/δ , γ – have been described (Yu & Reddy 2007). The isoform γ (PPARG) is expressed mainly in tissues that respond to insulin, having a role in regulating glucose intake (Jermendy 2007).

As mentioned, the FOXO proteins are part of the family of transcription factors forkhead-winged helix box. The members of this family include isoforms FOXO1, FOXO3a, and FOXO4. The protein FOXO1 is the most abundant isoform in insulin-sensitive tissues such as liver, pancreas, and adipose tissue (Gross *et al.* 2008). The function of FOXO1 is negatively regulated by AKT, exhibiting three sites for AKT phosphorylation, Thr24, Ser256, and Ser319. When phosphorylated, FOXO1 is located at the cytoplasm level where it is inactivated (Armoni *et al.* 2006, Lengyel *et al.* 2007), and degraded by the proteasome (Nakae *et al.* 2008). On its basal state (not phosphorylated), FOXO1 molecules are mainly detected at the nuclear level, where they bind directly to the promoter of PPARG, resulting in suppression of its transcription (Armoni *et al.* 2007). The action of PPARG as a transcription factor involves the heterodimerization with retinoid X receptor. Transcriptional activation occurs in a ligand-dependent manner, because the binding of ligand could allow the recruitment of a coactivator complex (Picard & Auwerx 2002, Yu & Reddy 2007, Ziouzenkova & Plutzky 2008), whereas, in the absence of ligand, corepressors are recruited (Ricote & Glass 2007). Therefore, according to the mechanism described above, PPARG regulates the expression of SLC2A4 by activating or repressing its transcription (Karnieli & Armoni 2008). So far, no information is available about the expression levels of these transcription factors involved in insulin signaling and regulation of SLC2A4 mRNA transcription in endometria from women bearing PCOS. Therefore, the aim of the present investigation was to determine whether the levels of the transcription factors FOXO1 and PPARG in PCOSE differ from those in control endometria.

Results

Clinical and metabolic characteristics of subjects

The clinical and metabolic characteristics of the two groups of women are summarized in Table 1. The higher body mass index observed in PCOS women group is

Table 1 Clinical and endocrine characteristics of control women (CE) and women with polycystic ovary syndrome (PCOS) and hyperinsulinemia (PCOSE h-Ins).

	CE	PCOSE h-Ins
Age (years)	39.72 ± 1.23	27.16 ± 0.62*
BMI	29.69 ± 1.95	32.71 ± 0.93
E ₂ (pg/ml)	73.78 ± 22.37	62.2 ± 3.45
P ₄ (ng/ml)	0.69 ± 0.28	0.612 ± 0.06
A ₄ (ng/ml)	1.57 ± 0.22	2.84 ± 0.19*
T (ng/ml)	0.44 ± 0.06	0.81 ± 0.05*
SHBG (mmol/l)	51.67 ± 9.53	24.24 ± 2.08*
FAI	3.73 ± 0.74	13.5 ± 1.58*

* $P < 0.05$ vs CE.

inherent to the syndrome. In addition, all the PCOS women included in this study presented with hyperinsulinism and hyperandrogenism, and the excessive ovarian androgen production besides the decreased sex hormone-binding globulin (SHBG) blood level leads to a significantly higher free androgen index in PCOS women.

Protein expression for phosphorylated and unphosphorylated forms of FOXO

We evaluated the protein levels of phosphorylated FOXO1 at Ser319 by immunohistochemistry, whereas, for the detection of unphosphorylated FOXO1, we used immunohistochemistry and western blotting. The antibody used for the detection of FOXO1 in the western blotting also recognizes FOXO4 isoform, although with a higher migration pattern than FOXO1. For the immunohistochemistry analysis, this antibody might recognize the two isoforms of FOXO. In the western blot analysis, no differences were observed between groups in the levels of FOXO1 protein (CE: 0.067 ± 0.01 versus PCOSE h-Ins: 0.088 ± 0.03 arbitrary unit (AU; Fig. 1A) or FOXO4 (CE: 0.042 ± 0.005 versus PCOSE h-Ins: 0.037 ± 0.006 AU; Fig. 1B). The levels of FOXO1 and FOXO4 were normalized to the levels of β -actin. Although no differences were obtained between FOXO1 and FOXO4 in the studied groups, the relative levels of FOXO1 were 60% higher than FOXO4 for CE and 100% higher for PCOSE h-Ins, suggesting a more important role for FOXO1 in these tissues.

Besides the assessment of the protein levels by western blot analysis, we were most interested in knowing the subcellular location of FOXO1/FOXO4 and p-FOXO1 Ser319. The immunohistochemical evaluation (Fig. 2) showed that in the PCOSE and hyperinsulinemia (PCOSE h-Ins), FOXO1/FOXO4 presented a strong staining in the cytoplasm of epithelial cells (Fig. 2C and E). The p-FOXO1Ser319 protein was located mainly in the cytoplasm of epithelial and stromal cells of endometria from control (Fig. 2D) and PCOS women (Fig. 2F). In the epithelial compartment, the protein levels of FOXO1 were significantly higher in the endometria of patients

Figure 1 Western blot analysis of protein levels of FOXO1 and FOXO4 in endometria from CE and PCOSE h-Ins. Equal amounts of protein (50 µg) were loaded in each lane. FOXO1 (A) was detected as a band with a molecular mass between 70 and 75 kDa, and FOXO4 (B) was detected as a band with a molecular mass of 65 kDa. Band intensities were quantified by scanning densitometry and normalized to intensities observed for β-actin as internal control. Lane 1, CE; lane 2, PCOSE h-Ins. The results are expressed as arbitrary units (AUs), and the values shown are means $\pm$ S.E.M. in CE ($n=7$) and PCOSE h-Ins ($n=7$).

with PCOS compared with controls ($P<0.05$; Fig. 2G). The same was observed for p-FOXO1Ser319, where a 40% of higher protein levels was observed ($P<0.05$; Fig. 2H). Nevertheless, in the stromal compartment, no differences were observed between the studied groups for the protein in its phosphorylated or nonphosphorylated state.

Gene and protein levels of PPARG

The next step was to evaluate the transcription of *PPARG* mRNA in both types of endometria. We observed that the PCOS hyperinsulinemic endometria exhibited significantly higher levels of *PPARG* mRNA compared with those found in control proliferative endometria ($P<0.05$; Fig. 3A). Similarly, the western blot analysis revealed a significant increase of PPARG protein levels

in the PCOSE h-Ins compared with controls ($P<0.05$; Fig. 3B). In addition, a comparison between the gene levels of *PPARG* in control proliferative and secretory phase was made. It was observed that the mRNA of *PPARG* is expressed in the endometrium of healthy women in both phases of the menstrual cycle, and found no significant differences between them (data not shown).

mRNA levels of SLC2A4

The RT-PCR analysis for *SLC2A4* showed a significant decrease in the levels of transcript in endometria from women with PCOS and hyperinsulinemia compared with control endometria ($P<0.05$; Fig. 4). These results are in agreement with the diminished protein levels of SLC2A4 observed in PCOSE h-Ins reported previously (Fornes *et al.* 2010).

In addition, we performed a correlation study between protein levels of PPARG and *SLC2A4* mRNA in PCOSE h-Ins patients, obtaining a negative and significant statistical correlation ($r=-0.78$; $P=0.037$), suggesting that high protein levels of PPARG cause a decrease in *SLC2A4* transcript.

Discussion

It is well known that a high percentage of women with PCOS present hyperinsulinemia besides their hyperandrogenism, which could alter the function of numerous tissues (Diamanti-Kandarakis & Papavassiliou 2006), including the endometrium. Our group has previously shown that the endometria from PCOS women exhibit several abnormalities, leading to failure in uterine receptivity (Quezada *et al.* 2006), endometrial homeostasis, and steroid bioavailability (Maliqueo *et al.* 2003b, Avellaira *et al.* 2006, Bacallao *et al.* 2008, León *et al.* 2008, Villavicencio *et al.* 2009). The present investigation aimed to gain knowledge about the regulation of the transcription of *SLC2A4* in PCOSE h-Ins. To our knowledge, this is the first study where the protein levels of the transcription factors PPARG and FOXO1 and its phosphorylated form were evaluated in endometrial tissue from PCOS women.

Little information is available related to the regulation of *SLC2A4* transcription in human endometrium. Experimental evidences show that in adipocytes (Armoni *et al.* 2007), the transcription factors FOXO1 and p-FOXO1Ser319 are involved in the insulin pathway by regulating the transcription of PPARG. As known, FOXO1 is located mainly at the nuclear level where it exerts a negative action on the transcription of PPARG by binding to the promoter. When FOXO1 is phosphorylated, it migrates to the cytoplasm and the transcription of PPARG proceeds. FOXO1 can be phosphorylated in several residues and by several kinases, including AKT

Figure 2 Immunohistochemical detection for FOXO1/FOXO4 and p-FOXO1Ser319 proteins in paraffin wax sections of proliferative endometria obtained from normal women (CE, $n=7$), and PCOSE with hyperinsulinemia (PCOSE h-Ins, $n=7$). Positive staining was detected in epithelial and stromal cells of all studied endometria for both antigens. As a negative control, the primary antibody was omitted. A, represents negative control for FOXO1/FOXO4; B, negative control for p-FOXO1Ser319; C, CE for FOXO1/FOXO4; D, CE for p-FOXO1Ser319; E, PCOSE h-Ins for FOXO1/FOXO4; F, PCOSE h-Ins for p-FOXO1Ser319; G, semiquantification of FOXO1/FOXO4; H, semiquantification of FOXO1Ser319. Magnification 400 $\times$. Scale bars represent 50 μ m. The immunostaining was measured by HScore (HS), expressed as $HS \pm S.E.M.$ * $P < 0.05$ versus CE compared with PCOSE h-Ins. Calculation of HScore is described in Materials and Methods.

and MAPK (Asada *et al.* 2007). In the present study, we analyzed the levels of basal FOXO1/FOXO4 and phosphorylated FOXO1 in Ser319, site of phosphorylation by activated AKT (Armoni *et al.* 2006, Nakae *et al.* 2008). We found higher levels of FOXO1/FOXO4 and p-FOXO1Ser319 particularly in epithelial cells of PCOSE h-Ins, as assessed by immunohistochemistry, where the phosphorylated form was located mainly in the cytoplasm, in agreement with previous reports in adipose tissue (Armoni *et al.* 2007). Accordingly, our previous investigations (Avellaira *et al.* 2006, Villavicencio *et al.* 2009) demonstrated an increase in p-AKTSer473 in PCOSE that could account for the high levels of p-FOXO1Ser319. Nevertheless, when the protein levels of FOXO1 were analyzed by western blotting, no differences were found between the studied groups, probably due to the different methodologies used in each case. As already mentioned, in control endometria, the nonphosphorylated form of

FOXO1/FOXO4 was located mainly at the nuclear level in accordance with previous reports, whereas, in PCOSE h-Ins, this protein was found mainly in the cytoplasm of cells. Currently, we have no explanation for this finding, although it can be speculated that the cytoplasm location of FOXO1/FOXO4 in the pathological endometria could prevent its function as a negative regulator of PPARG transcription. On the other hand, our data cannot discard a role for FOXO4 in the regulation of insulin action in human endometria; nevertheless, the higher levels of FOXO1 than FOXO4 detected by western blot suggest an important regulatory role for FOXO1 in the insulinsignaling pathway present in this tissue.

Therefore, all together, these data indicate a positive transcriptional regulation over PPARG, and indeed we found elevated levels of its mRNA, as well as the protein, in endometria from hyperinsulinemic PCOS women. In addition, we observed no changes in the levels of the transcript and protein of PPARG in control

Figure 3 (A) Semiquantitation of mRNA levels for the transcriptional factor *PPARG*. Conventional RT-PCR was performed in human endometria: control (CE) and polycystic ovarian syndrome with hyperinsulinemia (PCOSE h-Ins). Representative gel is shown (2% agarose gel electrophoresis, staining with ethidium bromide). Lane 1, negative control; lane 2, muscular tissue as positive control; lane 3, CE; and lane 4, PCOSE h-Ins. (B) Western blotting for *PPARG* in human endometrial tissue. Equal amounts of endometrial protein were loaded in each lane. The protein *PPARG* was detected as bands with molecular mass of 58 kDa. Lane 1, CE; lane 2, PCOSE h-Ins. The results are expressed as arbitrary units (AUs), and the values shown are mean $\pm$ S.E.M. in CE ($n=7$) and PCOSE h-Ins ($n=7$). * $P<0.05$ in CE compared with PCOSE h-Ins.

endometria throughout the menstrual cycle (data not shown), which agrees with previous publications (Ota *et al.* 2006).

It has been described that in cultures of adipocytes, *PPARG* is capable of producing an inhibition of the transcription of *SLC2A4* (Armoni *et al.* 2003). Therefore, the same situation could be occurring in the pathological endometria, where the decrease of *SLC2A4* mRNA observed in the PCOSE h-Ins could be partially caused

by the high levels of *PPARG* detected in these patients. These findings are in agreement with the negative correlation between these parameters observed in the present investigation.

Diverse studies have focused on the effects of thiazolidinediones (TZDs), synthetic hypoglycemic agents which are potent ligands of *PPARG*. The TZDs increase insulin sensitivity without increasing insulin secretion through the activation of multiple genes, including the upregulation of glucose transporter *SLC2A4* (Al-Khalili *et al.* 2005). Despite the presence of documented favorable reproductive and metabolic effects of TZDs in PCOS population, their tissue-specific cellular effects are less clear (Froment & Touraine 2006). Among these drugs, rosiglitazone represents a new generation of TZDs, which reduces the hyperandrogenism, besides the improvement of the fertility capacity, as well as, the metabolic profile in PCOS patients (Cataldo *et al.* 2001). Moreover, Jensterle *et al.* (2008) found in subcutaneous adipose tissue samples from women with PCOS that received either metformin or rosiglitazone for 6 months, a significant increase of *SLC2A4* mRNA expression in both groups. Interestingly, after treatment, the frequencies of menstrual bleeding were significantly higher in both groups, suggesting that TZD may exert a direct effect in the endometria, as it has been demonstrated in other reproductive tissues (Seto-Young *et al.* 2005). In addition, Legro *et al.* (2007) studied the effects of metformin and/or rosiglitazone in the endometria of hyperandrogenic PCOS patients and found an

Figure 4 Analysis of *SLC2A4* mRNA expression. Conventional RT-PCR was performed in human endometria: control (CE) and polycystic ovarian syndrome with hyperinsulinemia (PCOSE h-Ins). Representative gel is shown (2% agarose gel electrophoresis, staining with ethidium bromide). Lane 1, negative control; lane 2, muscular tissue as positive control; lane 3, CE; and lane 4, PCOSE h-Ins. The number of women evaluated was $n=7$ for each analyzed group. The results are expressed as mean $\pm$ S.E.M. RNA ribosomal 18S was used as the internal control: * $P<0.05$ versus CE.

improvement in the prevalence of the secretory morphology, not excluding a local effect of these compounds in the ovary or the endometria of PCOS patients.

In summary, in the present investigation, we report the expression levels of FOXO1/FOXO4, PPARG, and SLC2A4 in the PCOSE h-Ins. We also present evidence that the potential problems of the metabolism of insulin and glucose could be due at least in part to alteration in the expression levels of these proteins in the PCOSE h-Ins. Therefore, we can conclude that high levels of p-FOXO1Ser319 and cytoplasm localization of nonphosphorylated FOXO1 cause a derepression in the transcription of PPARG, showing not only higher levels of its mRNA, but also the protein, which might be suppressing the expression of *SLC2A4* gene, and consequently altering endometrial function in PCOS women.

Materials and Methods

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

Subjects

Human endometria were obtained with a Pipelle suction curette from the corpus of the uteri of 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 the load of glucose. The diagnosis of hyperinsulinemia was determined when the levels of insulin were 2 s.d.s of insulin concentration over the mean of the control group, as in previous studies (Maliqueo *et al.* 2003a, Fornes *et al.* 2010). The insulin values for the control group and PCOS h-Ins were 49.6 ± 11.6 and 150.5 ± 46.1 $\mu\text{IU/ml}$ respectively. All women had normal glycemic values in the oral tolerance glucose test (basal glucose <100 mg/dl; glucose 120 min <140 mg/dl). Also, all the PCOS women participants in this investigation had hyperandrogenism besides their hyperinsulinemia.

The studied groups were endometria from control women obtained during the proliferative phase of the menstrual cycle (CE, $n=7$) and endometria obtained from anovulatory PCOS women (PCOSE h-Ins, $n=7$). Control endometria were obtained from hysterectomy for benign causes, and they were selected in the proliferative phase because of the similar morphology between proliferative endometrium and PCOSE. None of the women, neither controls nor those with PCOS, had received hormonal therapy within 3 months prior to the recruitment into the study. The proliferative phase in CE and PCOSE was confirmed on the basis of histological dating and classification according to the Noyes criteria (Noyes *et al.* 1950) by an experienced pathologist.

The diagnosis of PCOS was made according to the Rotterdam Consensus (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004) and to the Androgen Excess

Society criteria (Azziz *et al.* 2006) for the definition of PCOS. The exclusion criteria were women who presented with hyperprolactinemia (prolactin >35 ng/ml), hypothyroidism (TSH >5 UI/l), androgen-secreting tumors (total testosterone >2 ng/ml; DHEAS >3600 $\mu\text{g/ml}$), Cushing's syndrome (urine cortisol concentration >150 $\mu\text{g}/24$ h and fasting plasma concentration of cortisol >25 $\mu\text{g/dl}$), congenital adrenal hyperplasia (17-OH progesterone >2.5 ng/ml) and women with diabetes or treatment with hormones and/or ovulation induction.

Reagents

The MABs for PPARG and β -actin were purchased from Abcam (Cambridge, MA, USA) and Sigma respectively. The polyclonal antibodies for FOXO1/FOXO4 and phosphorylated FOXO1 in Ser319 were obtained from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies (mouse monoclonal and rabbit polyclonal) were purchased from Amersham Biosciences (Amersham International). Protease inhibitor cocktail was obtained from Roche Molecular Biochemicals, BCA protein assay kit was obtained from Pierce (Rockford, IL, USA), and Histostain SP kit was obtained from Zymed Laboratories (San Francisco, CA, USA). TRIzol reagent and M-MLV RT were obtained from Invitrogen, and Taq DNA polymerase was obtained from Biotools (Madrid, Spain). Hormone determinations were assayed by commercial kits: serum testosterone, estradiol, and progesterone by solid-phase, competitive chemiluminescent enzyme immunoassay (Ortho-Clinical Diagnostics, Johnson & Johnson, High Wycombe, UK); androstenedione (A4) by RIA (Siemens, Los Angeles, CA, USA); SHBG concentration by Immulite and solid-phase chemiluminescent immunometric assay (Siemens, Llanberes, Gwynedd, UK).

Immunohistochemistry

Immunostaining for FOXO1/FOXO4 and p-FOXO1Ser319 was performed on 5- μm sections of formalin-fixed, paraffin-embedded endometrial biopsies. Tissue sections were deparaffinized in xylene and hydrated in a series of graded alcohol. The sections were incubated in antigen retrieval solution (10 mmol/l sodium citrate buffer, pH 6.0) at 96–98 °C for 20 min. Endogenous peroxidase activity was prevented by incubating the samples in 3% (v/v) hydrogen peroxide for 15 min. Nonspecific antibody binding was prevented with specific blocker of the Histostain SP kit. Primary antibody of FOXO1/FOXO4 (1:75) and p-FOXO1Ser319 (1:75) was applied to the samples and incubated overnight at 4 °C. Negative controls were analyzed on adjacent sections and incubated without primary antibody, as well as, with nonimmune species-specific antisera. The secondary antibody was a biotinylated antimouse/antirabbit immunoglobulin. The reaction was developed by the streptavidin-peroxidase system, and 3,3'-diaminobenzidine was used as the chromogen; counterstaining was carried out with hematoxylin. The slides were evaluated in an optical microscope Olympus BX51 (Olympus, Tokyo, Japan). The immunohistochemical evaluation for each protein was performed by a semiquantitative analysis described by Lessey *et al.* (1988) and validated in our

laboratory (Castro *et al.* 2002, Villavicencio *et al.* 2009) named HScore (histochemical score, HS), which corresponds to: $\Sigma[P]/(i+1)/100$, where $[P]$ is the percentage of positively stained cells and i is the intensity of the staining on a scale of 0–3 (0, no stain; 1, low intensity; 2, mid intensity; and 3, higher intensity). Each protein was evaluated in the functional layer by three independent observers blinded to patient category, and the positive staining was assessed in at least 3000 cells per sample.

Western blot analysis

The endometrial tissue was homogenized in a lysis buffer (HEPES 20 mmol/l, EDTA 2 mmol/l, EGTA 2 mmol/l, Triton 1%, phenylmethylsulphonyl fluoride 5 µmol/l, and Na₃VO₄ 50 µmol/l) containing protease inhibitor cocktail (Roche). After centrifugation at 10 000 *g* for 20 min at 4 °C, protein concentrations were determined using the BCA protein Assay kit (Pierce). Total proteins (50 µg) were denatured and fractionated using 8% one-dimensional SDS-PAGE, and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 h in TBST (20 mmol/l Tris, pH 7.6; 137 mmol/l NaCl; and 0.1% Tween 20) containing 10% (w/v) nonfat dry milk–TBST (for all markers). Subsequently, the membranes were washed three times for 5 min each in TBST, and then incubated with antibodies against PPARG (1:750) and FOXO1/FOXO4 (1:500) overnight with rocking at 4 °C, and against β-actin (1:15 000) for 1 h at room temperature. The membranes were then washed three times for 5 min each with TBST, followed by incubation for 1 h at room temperature with antimouse IgG peroxidase-conjugated species-specific (1:3000 for PPARG and 1:5000 for β-actin) or with antirabbit IgG peroxidase-conjugated species-specific (1:1500 for FOXO1/FOXO4), while rocking. After washing three times for 5 min each with TBST, the bound antibodies were detected with an enhanced chemiluminescence system (Amersham International) and analyzed by the program UN-SCAN-IT gel 4.1 (Silk Scientific Corporation, Orem, UT, USA). The protein levels were normalized by the levels of the protein β-actin and expressed as AUs.

RNA isolation and semiquantitative RT-PCR

Total RNA was isolated from endometrial tissue using TRIzol reagent according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically (A260:A280), while the integrity of the RNA was determined by electrophoresis on a formaldehyde agarose gel under denaturing conditions. The RNA was visualized by adding ethidium bromide (EtBr) to the sample before loading on the gel. The RNA was stored at –80 °C until use. Two micrograms of total RNA were digested with DNase I and transcribed into cDNA by RT with M-MLV RT using random primers in a total volume of 25 µl. The PCR amplifications were obtained using gene-specific primers (Table 2). Ribosomal RNA 18S was used as an internal control. Semiquantitative RT-PCRs were achieved in the exponential linear zone amplification for each gene studied. The PCR conditions for PPARG were 2 mmol/l MgCl₂, 0.20 mmol/l dNTPs, 0.750 U of *Taq* DNA polymerase, and 25 pmol each primer; the PCR conditions for

Table 2 Primer sequences used for PCR of cDNA for the analysis of peroxisome proliferator-activated receptor-γ (PPARG), SLC2A4, and RNA 18S.

Gene	Primers	Size (bp)
PPARG	5'-TCTCTCCGTAATGGAAGACC-3' (sense) 5'-GCATTATGAGACATCCCCAC-3' (antisense)	474
SLC2A4	5'-ATCCTGATGACTGTGGCTCTGCT-3' (sense) 5'-TCGTTCTCATCTGGCCCTAAATCA-3' (antisense)	433
rRNA 18 S	5'-GTAACCCGTTGAACCCCAT-3' (sense) 5'-CCATCCAATCGGTAGTAGCG-3' (antisense)	200

SLC2A4 were 2 mmol/l MgCl₂, 0.20 mmol/l dNTPs, 2 U of *Taq* DNA polymerase, and 30 pmol each primer. The PCR amplification was performed in the thermocycler, model PTC-100 (MJ Research Inc., Watertown, MA, USA) and Mastercycler Personal (Eppendorf AG, Foster City, CA, USA). The PCR products were electrophoretically resolved on 2% agarose gel and stained with EtBr. The bands were evaluated using an image analyzer UN-SCAN-IT gel 4.1 (Silk Scientific Corporation) and normalized relative to the ribosomal mRNA 18S PCR product.

Statistical evaluation

The number of subjects in this study was calculated, assuming $\alpha=0.05$ and $\beta=0.20$ and a difference between means of 0.25 and s.d. of 0.16 according to our previous studies (Bacallao *et al.* 2008, Fornes *et al.* 2010). The distribution of the data was analyzed by Kolmogorov–Smirnov test. The results were analyzed by Student's *t*-test or Mann–Whitney test. *P* values <0.05 were considered significant. Statistical tests were performed using Graphpad for Windows version 5.0 Software, Inc. For the correlation study, the data showed normal distribution, and Pearson's test was used. A *P* value <0.05 was considered significantly different.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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