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Expression of molecules associated with tissue homeostasis in secretory endometria from untreated women with polycystic ovary syndrome

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BACKGROUND: The hormonal alterations observed in women with polycystic ovary syndrome (PCOS) may promote implantation failure as well as disruption of their endometrial homeostasis. To evaluate cell survival of mid-secretory endometrium from untreated women with PCOS, we measured the expression of apoptosis and proliferation-related proteins. METHODS: A case–control study of 11 patients with PCOS and 11 fertile women in the Hospital Research Unit was performed. Endometrial samples were obtained from PCOS women (PCOSE) and fertile healthy women (CE) during the mid-secretory phase of the menstrual cycle. Protein expressions for Akt, p-AktSer473 and p-AktThr308, Bad, p-BadSer136, Bcl-2, Bax and pro-caspase-3/caspase-3, were assessed by western blot, and Ki67 and p-histone-3 (p-H3) by immunohistochemistry. RESULTS: In CE and PCOSE, a predominance of p-AktThr308 over p-AktSer473 is observed; p-BadSer136 expression is higher in PCOSE than in CE ($P < 0.05$). Also, Bcl-2 protein is overexpressed in PCOSE ($P < 0.05$), with no changes in Bax expression among the two groups, resulting in a significantly higher Bcl-2/Bax ratio in PCOSE than in CE ($P < 0.05$). No changes in the expression of caspase-3 are obtained between both groups of endometria. Furthermore, cell proliferation detected by the expression of Ki67 and p-H3 proteins is higher in the epithelia than the stroma of PCOSE versus CE ($P < 0.05$). CONCLUSION: The abnormal tissue homeostasis exhibited by the secretory endometrium from PCOS patients with spontaneous ovulation may interfere with their endometrial receptivity.

Key words: Akt signalling pathways/apoptosis-related proteins/cell survival/endometrium/polycystic ovary syndrome

Introduction

The human endometrium is one of the few tissues in which proliferation and apoptosis take place in a cyclic manner. Both processes are regulated mainly by changes in steroid hormone levels, and a tight balance between cellular proliferation and apoptosis must exist for the normality of endometrial function (Harada *et al*., 2004). Therefore, in endocrine-metabolic situations associated with abnormalities in plasma hormone concentrations, as in the case of polycystic ovary syndrome (PCOS), the processes occurring in the endometrium, including cell proliferation and differentiation and cell responses to biological stimuli, could be affected (Maliqueo *et al*., 2003).

Experimental evidences indicate that the endometrium from PCOS women behaves differently from that of normal women, with a potential association between PCOS, hyperplasia and endometrial carcinoma (Balen, 2001; Ehrmann, 2005). Also, a high number of PCOS patients present infertility (Franks, 1995; Porter and Scott, 2005), and when ovulation is restored pharmacologically or spontaneously, these patients exhibit a higher rate of miscarriage (Kastner *et al*., 1990; Connely and Lydon, 2000; Whiele and Manga, 1995).

Recently, we have observed a higher expression of steroid receptors and co-activators in the secretory endometria of PCOS patients who reactivated their ovulation spontaneously (Quezada *et al*., 2006), suggesting an increased sensitivity to steroid action. In addition, the persistence of epithelial progesterone receptor concomitantly with an aberrant expression of β_3-integrin subunit has been detected, indicating implantation failure in PCOS women. Nevertheless, no knowledge is available regarding tissue homeostasis in the endometria of those patients.

In the normal human endometrium, the pro-apoptotic and anti-apoptotic factors are under fine regulation that leads to tissue homeostasis. Hormonal alterations, like those observed in women with PCOS, could disrupt the expression of proteins that regulate cell survival. Apoptosis is importantly observed during the secretory and menstrual phases of the normal human

endometrium (Castro *et al*., 2002; Li *et al*., 2005). This process is controlled mainly by members of the Bcl-2 family, and in the case of endometria from PCOS women with no restoration of ovulation, a significant increase of the Bcl-2/Bax ratio has been reported (Maliqueo *et al*., 2003). However, up to now many studies have focused only on the Bcl-2/Bax ratio, disregarding other components of the apoptotic pathway such as the pro-apoptotic protein Bad of which no evidence exists in normal and PCOS endometrial tissue. In this regard, recent data in normal human endometrium indicate that the PI3K/Akt pathway could be an important regulating step of cell proliferation by means of the phosphorylation of Akt, its active form (Kayisli *et al*., 2004). Moreover, PI3K may form a complex with estrogen receptors, enhancing the kinase activity (Simoncini *et al*., 2000).

Phosphorylated Akt at Ser473 and Thr308 occurs in response to growth factors and other extracellular stimuli and are essential for maximal Akt activation (Alessi *et al*., 1996). Akt kinases regulate diverse cellular processes (Bellacosa and Larue, 2005); in particular, activated Akt is a well-established survival factor exerting anti-apoptotic activity (Whang *et al*., 2004). Akt phosphorylates and, consequently, inactivates the pro-apoptotic proteins Bad and procaspase-9 among other factors (Downward, 2004).

It has been shown that Ki67, a proliferation marker, exhibits high expression in the proliferative phase and low expression in the secretory phase of the normal menstrual cycle (Morsi *et al*., 2000; Vaskivuo *et al*., 2000; Mertens *et al*., 2002).

Since PCOS is an endocrine disorder, the hormonal milieu present in the endometrium from these patients could deregulate the endometrial homeostasis. Therefore, in the present investigation, we evaluated the expression of proteins related to cell survival associated with the Akt signalling pathway in the endometria from PCOS women with spontaneous ovulation obtained during the window of implantation.

Materials and methods

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

Reagents

The monoclonal antibodies for Ki67, Bcl-2, p-AktSer473 and β-actin were purchased from Oncogene (CA, USA), Calbiochem (CA, USA), BD Pharmingen (CA, USA) and Sigma (MO, USA), respectively. The polyclonal antibody for Bax was obtained from Dako (CA, USA), and polyclonal antibodies for pro-caspase-3, caspase-3 and Akt were purchased from BD Pharmigen; for Bad, p-BadSer136 from Cell Signalling Technology (CA, USA); for p-AktThr308 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); for p-histone-3 (p-H3) from Upstate (NY, USA) and for glycodelin from R&D Systems, Inc. (Minneapolis, MN, USA). All polyclonal antibodies were raised from rabbit.

Secondary antibodies [anti-mouse and anti-rabbit immunoglobulin G (IgG)] were purchased from Amersham Biosciences (Amersham International, Piscataway, NJ, USA). Protease inhibitor cocktail was obtained from Roche Mol Biochemicals (Mannheim, Germany) and bovine serum albumin (BSA) protein assay kit from Pierce (Rockford, IL, USA); labelled streptavidin biotin kit was purchased from Dako. Hormone determinations were assayed using commercial kits: serum testosterone (T), androstenedione (A_4) and progesterone (P_4) by radioimmunoassay (RIA) (Diagnostic System Laboratories, Webster, TX, USA); and sex hormone-binding globulin (SHBG) concentration by RIA (DPC, Los Angeles, CA, USA). Estradiol (E_2) was measured by electrochemiluminescence (Roche, Basel, Switzerland).

Subjects

The endometrial samples were obtained with a Pipelle suction curette from the corpus of the uteri from 56 women with PCOS. These women attended for the first time the Infertility Clinic of the San Borja Arriarán Clinical Hospital, School of Medicine, seeking pregnancy. From the 56 biopsies, 11 corresponded to endometria from mid-secretory phase of the menstrual cycle, coincident with the time of the window of implantation. The 11 women, belonging to a subgroup of PCOS patients, studied were nulliparous and presented a history of no previous abortions, and they had ovulated spontaneously according to plasma P_4 measurements >4 ng/ml. The diagnosis of PCOS was made according to the Rotterdam Consensus (The Rotterdam ESHRE/ASRM, 2004), considering two criteria out of three (oligo and/or anovulation; clinical and/or biochemical signs of hyperandrogenism; polycystic ovaries to ultrasonography). Hyperprolactinaemia, androgen-secreting tumours, Cushing's syndrome, congenital adrenal hyperplasia, attenuated 21-hydroxylase deficiency as well as thyroid disease were excluded by appropriate tests. Control endometrium (CE) was obtained from 11 healthy women with proven fertility during the mid-secretory phase of the menstrual cycle, at the time of bilateral tubal ligation at the San Borja-Arriarán Clinical Hospital, National Health Service (Santiago, Chile). None of these women had taken oral contraceptives or other medications for at least 3 months before starting the study. The endometrial samples were selected in the mid-secretory phase (during the window of implantation) on the basis of histological dating and classification according to Noyes criteria (Noyes *et al*., 1950) by an experienced histopathologist. Furthermore, the profile of glycodelin expression was similar in the two types of endometria, CE and PCOS endometrium (PCOSE), indicating normal response of endometrial cells to progesterone.

Western blot analysis

The endometrial tissue was homogenized in a lysis buffer (HEPES 20 mM, EDTA 2 mM, EGTA 2 mM, Triton 1%, PMSF 5 μM and Na_3VO_4 50 μM) containing protease inhibitor cocktail (Roche, IN, USA). 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, 100 μg for Bad and p-BadSer136 and 50 μg for total Akt, p-AktSer473 and p-AktThr308, Bcl-2, Bax, pro-caspase-3, caspase-3 and β-actin, were denatured and fractionated using 7.5% (Total Akt, p-AktSer473 and p-AktThr308) or 15% (Bcl-2, Bax, pro-caspase-3, caspase-3, Bad, p-BadSer136 and β-actin) one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Three gels were loaded with the twenty-two samples, running the same amount of CE and PCOSE samples in each gel. Six 7.5% and nine 15% gels were performed and all of them were transferred to nitrocellulose membrane (BioRad, CA, USA).

Membranes were blocked for 2 h in TBST (20 mM Tris, pH 7.6; 137 mM NaCl; 0.1% Tween 20) containing 10% non-fat dry milk (for the markers Bcl-2, Bax, pro-caspase-3, caspase-3) and for 1 h in TBST containing 5% non-fat dry milk or 5% BSA (for the markers Bad, p-Bad; Akt and p-Akt, respectively). Subsequently, the membranes were washed three times for 7 min each in TBST and then incubated with antibodies against human Bcl-2 (2 μg/ml), Bax (1:1000), Akt (0.5 μg/ml), p-AktSer473 (2 μg/ml), p-AktThr308 (1:200), Bad (1:500) and p-BadSer136 (1:300) overnight with rocking at 4°C; pro-caspase-3 and caspase-3 (1:500) for 2 h at room temperature; and with β-actin (1:15 000) for 1 h at room temperature. The ten

proteins were evaluated in fifteen different membranes. Each membrane was used to detect more than one protein, considering the antibody (monoclonal or polyclonal), the percentage of SDS–PAGE used and the molecular weight of each protein. β-Actin was determined in all the fifteen membranes. The membranes were then washed three times for 7 min each with TBST, followed by incubation for 1 h at room temperature with anti-mouse IgG, peroxidase-conjugated species-specific (1:5000 for p-AktSer473, Bcl-2 and β-actin) or anti-rabbit IgG peroxidase-conjugated species-specific (1:1000 for p-Bad; 1:2000 for Bad, Bax and caspase-3; 1:5000 for Akt and p-AktThr308), while rocking. After washing three times for 7 min each with TBST, the bound antibodies were detected with an enhanced chemiluminescence system (Amersham International). Band intensities were quantified by scanning densitometry utilising the UN-SCAN-IT software, Automated Digitizing System, version 5.1.

Immunohistochemistry

Immunostaining for Ki67, which is expressed in cycling cells (G1, G_2, S and M phase) (Chamlian and Taylor, 1970; Cheung, 2001), and for p-H3 whose expression is during the M phase of the cell cycle (Brenner *et al.*, 2003), was performed on 5-μm sections of formalin-fixed, paraffin-embedded endometrial biopsies.

Tissue sections were deparaffinized in xylene and hydrated gradually through graded alcohol. 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 0.2% hydrogen peroxide in methanol for 30 min. Non-specific antibody binding was prevented with 4% phosphate-buffered saline–BSA for 1 h. Primary antibody of Ki67 (monoclonal; 0.05 μg/ml) and p-H3 (polyclonal; 1/75) was applied to the samples and incubated overnight at 4°C. Negative controls were analysed on adjacent sections incubated without primary antibody, as well as, with a 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; counterstaining was carried out with haematoxylin. The slides were evaluated in a Nikon optical microscope (Nikon Inc., Melville, NY, USA).

The immunohistochemical evaluation was determined as the percentage of positive stained cells. In all cases, the proteins were evaluated by three independent observers and blinded to patient category, and the positive staining was assessed in at least 1000 cells per sample.

Statistical evaluation

The number of subjects in this study was calculated assuming an $\alpha = 0.05$ and $\beta = 20\%$ and a difference between mean of 0.2 and standard deviation of 0.165 according to our previous studies. The data were analysed using Kolmogorov–Smirnov test. Due to the normal distribution of the data, comparisons between groups were performed by parametric Student's *t*-test. *P*-values <0.05 were considered significant. Statistical tests were performed using SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical and endocrine characteristics

The clinical and endocrinological characteristics of the two groups of women are summarized in Table I. The age of the CE group was higher than that of the women with PCOS because they belong to the group of bilateral tubal ligation. The higher BMI observed in the group from PCOS women is inherent to the syndrome. The latter is in agreement with the fact that around 30% of PCOS patients are obese (Calle and Kaaks, 2004). In addition, all PCOS women presented hyperadrogenism, and the excessive ovarian androgen production besides the decreased SHBG blood level leads to a significantly higher free androgen index in PCOS women.

Table I. Clinical and hormonal characteristics of the endometria obtained control women (CE) and women with PCOS (PCOSE)

	CE (n = 11)	PCOSE (n = 11)
Age (years)	35.8 ± 1.6	29.9 ± 2.0
Body mass index (kg/m^2)	25.6 ± 1.0	29.7 ± 1.95
Sex hormone-binding globulin (nmol/l)	62.9 ± 1.84	38 ± 3.5
Testosterone (ng/ml)	0.47 ± 0.02	0.66 ± 0.05*
Free androgen index	2.64 ± 0.12	8.85 ± 3.5*
Estradiol (pg/ml)	96.1 ± 11	138.3 ± 25.09
Progesterone (ng/ml)	5.05 ± 0.68	4.76 ± 0.9

The values are mean ± SEM.
*$P < 0.05$ between CE and PCOSE.

Protein expression for total and phosphorylated forms of Akt

Western blot analysis was performed for total Akt and Akt phosphorylated in Ser473 and Thr308. No variations were observed in the endometrial expression of total Akt in normal and PCOS women [secretory phase (RU): CE = 2.40 ± 0.3; PCOSE = 2.25 ± 0.3]. Nevertheless, in the mid-secretory endometria from both groups, we detected an increased expression of p-AktThr308 compared with p-AktSer473 ($P < 0.05$) (Figure 1). No different signal intensities for p-AktSer473 and p-AktThr308 were observed between both groups of endometria (Figure 1).

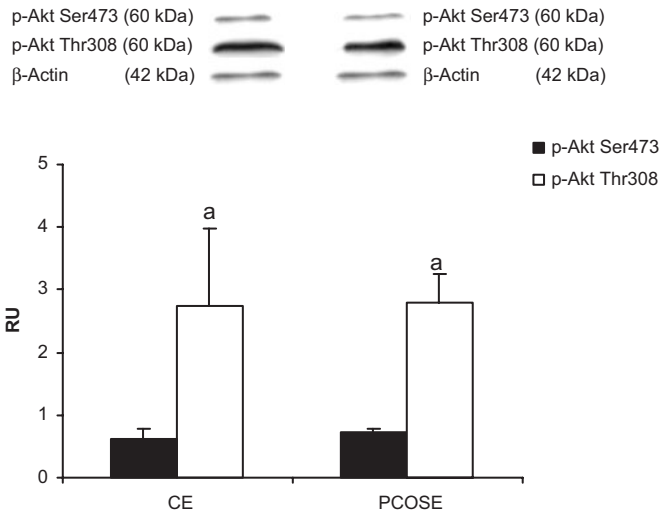

Figure 1. Western blot analysis of Akt phosphorylated forms, p-AktSer473 and p-AktThr308, in control (CE) and polycystic ovary syndrome endometrium (PCOSE) from the mid-secretory phase of the menstrual cycle. Equal amounts of endometrial protein were loaded in each lane, and p-AktSer473 and p-AktThr308 band intensities were quantified by scanning densitometry and normalized to intensities observed for β-actin as internal control. A representative gel of the media of bands obtained from PCOSE and CE is shown. The results are expressed as relative units (RU), and the values shown are mean ± SEM in CE (n = 11) and PCOSE (n = 11). [a]$P < 0.05$ between p-AktThr308 and p-AktSer473 in both groups.

Expression of proteins related to apoptosis

Protein expression evaluated by western blot of total Bad, p-BadSer136, pro-caspase-3, caspase-3, Bcl-2 and Bax was analysed in CE and PCOSE obtained during the window of implantation. Total Bad expression was significantly higher in PCOSE than in CE ($P < 0.05$) (Figure 2), but most importantly, an increased p-BadSer136 expression was obtained in PCOSE (45%; $P < 0.05$).

The expression of caspase-3 was performed using an antibody that recognizes both the unprocessed pro-caspase-3 and the 17 kDa subunit of active caspase-3. The inset in Figure 2 shows a band with a molecular mass of 32 kDa for pro-caspase-3 in all tissue samples, and an increase of this protein was observed in PCOSE with respect to CE ($P < 0.05$). A similar expression of caspase-3 between normal and PCOSE was detected during the mid-secretory phase.

The data obtained in relation to other proteins associated with cell survival, such as, Bcl-2 and Bax, are shown in Figure 3. The protein expression of Bcl-2 was significantly increased in PCOSE (3.5-fold; $P < 0.05$) compared with that in CE. Moreover, in PCOSE we found higher levels of Bcl-2 than Bax protein expression ($P < 0.05$); therefore, the Bcl-2/Bax ratio, which is a key factor in the regulation of apoptosis, is significantly higher in PCOSE than in CE (Bcl-2/Bax: CE = 2.06 ± 0.2; PCOSE = 4.32 ± 0.09; $P < 0.05$).

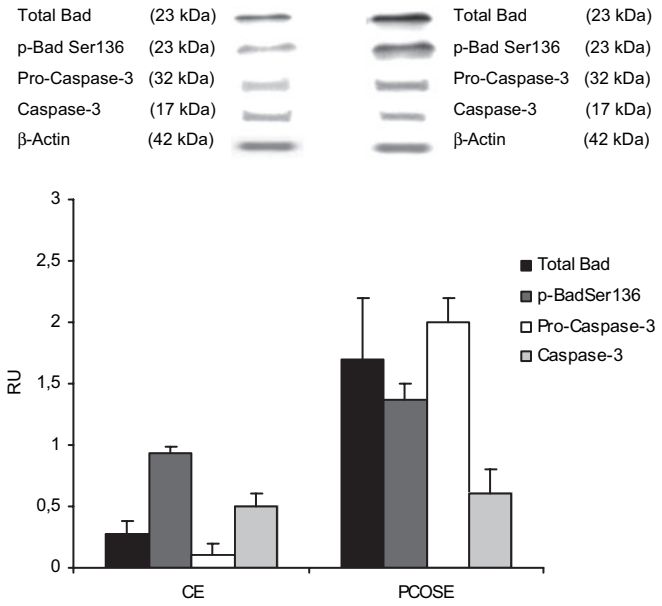

Figure 2. Western blot analysis of total Bad, p-BadSer136, pro-caspase-3 and caspase-3 in secretory endometria from control (CE) and PCOS (PCOSE) women from mid-secretory phase of the menstrual cycle. Equal amounts of endometrial protein were loaded in each lane, and total Bad, p-BadSer136, pro-caspase-3 and caspase-3 band intensities were quantified by scanning densitometry and normalized to intensities observed for β-actin as internal control. A representative gel of the media of bands obtained from PCOSE and CE is shown. The results are expressed as relative units (RU), and the values shown are means ± SEM in CE ($n = 11$) and PCOSE ($n = 11$). [a]$P < 0.05$ between total Bad, p-BadSer136 and pro-caspase-3 in both groups.

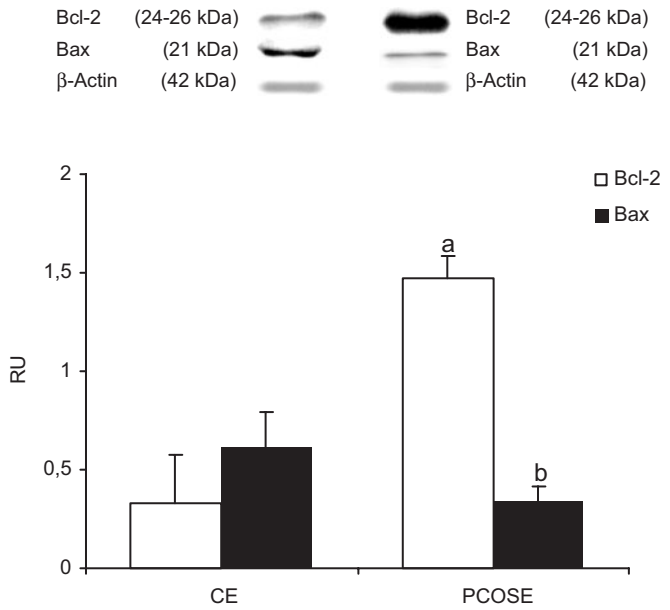

Figure 3. Expression of Bcl-2 and Bax proteins in normal (CE) and polycystic ovary syndrome endometrium (PCOSE) from mid-secretory phase of the menstrual cycle assessed by western blot analysis. Equal amounts of protein were loaded in each lane. Bcl-2 and Bax were detected as a band with a molecular mass of 24 and 21 kDa, respectively. Bcl-2 and Bax band intensities were quantified by scanning densitometry and normalized to intensities observed for β-actin as internal control. A representative gel of the media of bands obtained from PCOSE and CE is shown. The results are expressed as relative units (RU) and the values shown are means ± SEM in CE ($n = 11$) and PCOSE ($n = 11$). [a]$P < 0.05$ in PCOSE compared with CE; [b]$P < 0.05$ between Bcl-2 and Bax in PCOSE.

Expression of proteins related to cell proliferation

The Ki67 protein expressed in cycling cells was immunolocalized at the nuclear level of endometrial cells of the two groups of endometria studied, mainly at the epithelial level (Figure 4). Importantly, a significant increase in the percentage of epithelial

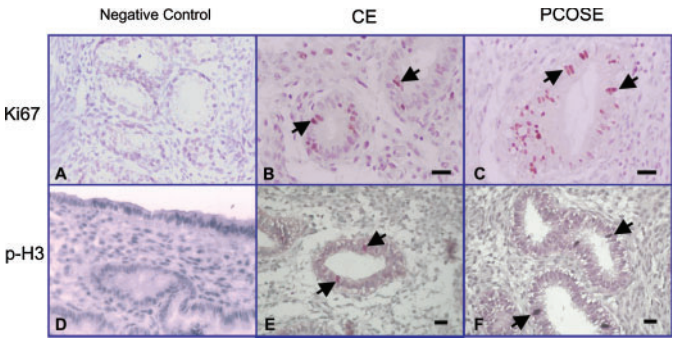

Figure 4. Immunohistochemical detection of Ki67 and phosphohistone 3 (p-H3) in paraffin sections of endometria obtained from control (CE) and polycystic ovary syndrome (PCOSE) women in mid-secretory phase of the menstrual cycle. Positive cells for Ki67 and p-H3 were detected in both cell types, epithelial and stromal, particularly at the nuclear level. A higher number of epithelial cells from PCOSE than CE show positive staining for both proteins. Arrows indicate positive nuclear staining for Ki67 and p-H3 in epithelial cells. Magnification ×400 in panels **A**, **B** and **C** and ×200 in panels **D**, **E** and **F**. Scale bars represent 30 μm.

cells with positive staining was detected in PCOSE with respect to CE (PCOSE = 30.3 ± 5.7%; CE = 1.1 ± 0.5%; $P < 0.05$). The endometrial cells also showed positive staining for p-H3 protein, particularly at the nuclear level of epithelial cells (Figure 4). The expression of p-H3 was during the M phase of the cell cycle, and thus the percentage of cells in mitosis was higher in PCOSE than in CE (PCOSE = 2.5 ± 0.1%; CE = 1.8 ± 0.09%; $P < 0.05$).

Discussion

Several reports indicate that the endometrium from PCOS women behaves differently from that of fertile cycling women (Regan *et al*., 1990; Tulppala *et al*., 1993; Sagle *et al*., 1998). As known, these patients are often infertile, mainly due to ovarian failure and recurrent miscarriage. The event of ovulation increases the chances to become pregnant in some PCOS patients. Nevertheless, spontaneous ovulation in oligomenorrhoeic PCOS women does not necessarily lead to an improvement of their endometrial receptivity (Quezada *et al*., 2006).

Previous studies in endometria from PCOS women who have not reactivated their ovulatory cycles have demonstrated unbalanced expression of proteins related to cell survival (Maliqueo *et al*., 2003). Therefore, in the current investigation, we addressed the question whether tissue homeostasis is abnormal in the secretory endometria from untreated PCOS women with spontaneous ovulation.

The regulation of proliferation and cell survival processes in the normal human endometrium is not completely understood, even though many proteins have been involved in the regulation of tissue homeostasis (Harada *et al*., 2004). Recent data indicate that the intracellular signalling pathway of PI3K/Akt could be an important regulating bridge of these processes. The Akt protein and its phosphorylated forms are activated by multiple growth factors and act as intracellular mediators downstream of PI3K (Kayisli *et al*., 2004). Even more, it has been reported that estrogens bound to its receptors may form a complex with PI3K, inducing an increase of the kinase activity, resulting in high levels of phosphorylated Akt (Simoncini *et al*., 2000). Therefore, in PCOS endometria from mid-secretory phase where estrogen action may be enhanced due to the overexpression of estrogen receptors and its co-activators (Quezada *et al*., 2006), the PI3K/Akt signalling pathway could importantly participate in the regulation of tissue homeostasis.

In the present work, we evaluated the two phosphorylated forms of Akt, Ser473 and Thr308, described to be important for maximal activation of Akt. Interestingly, we found a differential phosphorylation pattern dependent on the site of phosphorylation being p-AktThr308, the more abundant form in secretory endometria. The biological meaning of this finding is not understood at present. In addition, we have detected a higher expression of p-AktSer473 in endometria from proliferative phase than that from secretory phase (unpublished data), which is in agreement with a previous report in normal endometrium (Kayisli *et al*., 2004). Coincidentally, Yoshino *et al*. (2003) reported a diminished expression of p-AktSer473 in cultured stromal cells undergoing *in vitro* decidualization. This finding indicates an inverse relationship between cell differentiation and phosphorylated Akt.

Furthermore, the PI3K/Akt pathway may interfere with cell death by promoting direct phosphorylation of proteins involved in the apoptotic cascade, like the Bad protein. As known, apoptosis is an important process to maintain tissue homeostasis in the endometrium during the normal menstrual cycle. In this regard, Bad can be phosphorylated in Ser136 by Akt, promoting the kidnapping of this protein and preventing its interaction with other members of the Bcl-2 family, and hence, inhibiting the apoptotic process (Robert *et al*., 1997). Therefore, the overexpression of the phosphorylated form of Bad found in the endometrium from PCOS women in the current investigation is indicative of a diminished degree of apoptosis in this tissue. To the best of our knowledge, this is the first study to report the expression of the two phosphorylated forms of Akt and the expression of Bad and its phosphorylated form in endometria from control and PCOS women.

Moreover, the evaluation of other members of the Bcl-2 family, such as Bcl-2 and Bax in endometria from control and PCOS women, showed predominance of the anti-apoptotic protein Bcl-2 over the pro-apoptotic Bax in PCOSE. These data are in agreement with our previous results in proliferative endometria from normal (Castro *et al*., 2002) and PCOS patients (Maliqueo *et al*., 2003). Also, they are in accord with the findings that show an overexpression of estrogen receptors and its co-activators in both proliferative and secretory endometria from PCOS women (Quezada *et al*., 2006; Villavicencio *et al*., 2006). As reported, E_2 bound to its receptor regulates the endometrial expression of the Bcl-2 protein (Otsuki *et al*., 1994). The findings of the present investigation, in addition with the similar expression of caspase-3 found in both groups of secretory endometria, suggest prevalence of an anti-apoptotic condition in PCOSE. Despite the results of the present investigation and since the western blotting technique is unable to determine differences in expression between epithelial and stromal cell compartments, further studies using immunohistochemistry are needed to establish the expression of proteins related to cell survival in each endometrial cell type. The immunohistochemical analysis would allow a better understanding of the disruption of these processes in the secretory endometria of PCOS patients, although it was not possible in this study because of a lack of tissue.

Moreover, we found an increased proliferation rate in PCOSE, mainly at the epithelial level, represented by the high abundance of Ki67 and p-H3 detected in endometrial cells and a positive relationship between these two proliferation markers. Similar results have been reported by us in the PCOSE from proliferative phase (Maliqueo *et al*., 2003).

In summary, in the mid-secretory endometrium from untreated PCOS women, we found high abundance of Bad phosphorylated form, expression of the two phosphorylated forms of Akt, predominance of the anti-apoptotic protein Bcl-2 over Bax and normal amount of caspase-3, in addition to elevated proliferation rate. All together, these results indicate that PCOS endometrium from mid-secretory phase exhibits a scarce or absent apoptosis with increased cell proliferation, suggesting a deregulation in the control of tissue homeostasis.

Consequently, unbalanced or failures in the implantation process and maintenance of pregnancy could be observed in PCOS patients. In conclusion, we infer that the event of ovulation in these patients is not sufficient to re-establish their endometrial function.

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