Published in final edited form as:

Gynecol Oncol. 2009 October ; 115(1): 102–107. doi:10.1016/j.ygyno.2009.06.033.

Involvement of Akt, Ras and cell cycle regulators in the potential development of endometrial hyperplasia in women with polycystic ovarian syndrome

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Abstract

Objective—To examine whether the abundance, localization, and/or activity of cell cycle regulators CDK2, Cyclin E, p27, and survival proteins AKT and Ras in PCOS-associated endometria (with and without hyperplasia) differ from non-PCOS endometria.

Methods—The expression of CDK2, Cyclin E, p27, AKT and Ras was measured by immunohistochemistry and/or Western blot in 9 normal endometria (NE), 12 endometria from PCOS patients without endometrial hyperplasia (PCOSE), 7 endometria from PCOS women with endometrial hyperplasia (HPCOSE), and 9 endometria from patients with endometrial hyperplasia (HE). The activity of CDK2 was assessed by an *in vitro* kinase assay.

Results—CDK2, Cyclin E and p27 proteins were expressed mainly in the endometrial epithelial cells of the studied groups. No change in the activity of CDK2 was observed in total extracts obtained from the tissue samples. However, the nuclear expression of CDK2 in epithelial cells was slightly elevated in PCOSE and significantly increased in HPCOSE when compared to NE. Higher expression of p27 was detected in the cytoplasm of epithelial cells of PCOSE and HPCOSE when compared to NE. Also, we found an increment in Ser473-AKT phosphorylation and an over-expression of the Ras oncogene in endometria of patients with PCOS.

Conclusion—The PCOS condition is associated with increased Ser473-AKT phosphorylation, elevated expression of Ras, increased cytoplasmic abundance of p27, and increased nuclear

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygyno.2009.06.033.

abundance of CDK2 in the endometrial epithelial cells. These biological events could potentially provide a chance for endometrial cells from PCOS patients to exit the controlled cell cycle and become hyperplastic at a later stage.

Keywords

Cell cycle regulators; Survival proteins; Endometrium; Polycystic ovarian syndrome

Introduction

Polycystic ovarian syndrome (PCOS) is an endocrine disorder affecting 5–10% of women of reproductive age [1]. Women with PCOS are infertile [2] and have a predisposition to endometrial hyperplasia and endometrial cancer [3–7]. An international consensus group [8] proposed that PCOS can be diagnosed after the exclusion of other medical conditions causing irregular menstrual cycles and androgen excess, and the determination that at least two of the following are present: (i) oligo-ovulation or anovulation (usually manifested as oligomenorrhea or amenorrhea); (ii) elevated levels of circulating androgens or clinical manifestations of androgen excess; and (iii) polycystic ovaries.

It is well accepted that the endocrine metabolic condition inherent to PCOS affects endometrial homeostasis [9], and that it can lead to endometrial hyperplasia and/or endometrial cancer. Supporting this concept is the fact that 35.7% of women with untreated PCOS have endometrial hyperplasia, a phenomenon that generally precedes endometrial carcinogenesis [6,10–12].

Normal endometrial proliferation is regulated by interactions among cyclins, cyclin dependent kinases (CDKs), and CDK inhibitors [13–15]. The cyclin E/CDK2 complex is considered the rate-limiting activator of the G1-to-S cell cycle transition, and this process is blocked by CDK inhibitors [16,17]. In cancer cells, however, the CDK inhibitor p27 is frequently relocated from the nucleus to the cytoplasm upon AKT-mediated phosphorylation [18–21], and this phenomenon associates with enhanced survival and migratory capacity of the cells [22]. In endometrial cancer, abnormalities in the expression of cell cycle inhibitors and cell cycle stimulating proteins have been described [23–25], including the AKT signaling and the Ras oncogene, both important regulators of endometrial cell proliferation, apoptosis and survival [26–33].

Since in PCOS patients the altered hormonal environment deregulates endometrial homeostasis, which can lead to inadequate or pathologic endometrial growth [9], in the present study we examined whether the abundance, localization, and/or activity of cell cycle regulators CDK2, Cyclin E, p27, and survival proteins AKT and Ras in PCOS-associated endometria (with and without hyperplasia) differ from non-PCOS endometria.

Materials and methods

Serum testosterone, androstenedione and progesterone were assayed by radioimmunoassay (DSL Labs, Webster, TX). Sex hormone-binding globulin concentration was determined by radioimmunoassay (DPC, Los Angeles, CA). Estradiol was measured by electro-chemiluminescence (Roche, Basel, Switzerland). Monoclonal antibodies for Cyclin E, p27, p-AKT/Ser473 and Ras were from BD Transduction Labs (San Jose, CA), and for β -Actin was from Sigma (St. Louis, MO). Polyclonal antibodies for CDK2 and p-AKT/Thr308 were from Santa Cruz Biotechnology (Santa Cruz, CA), and for AKT from BD Transduction Labs. Secondary antibodies (mouse monoclonal and rabbit polyclonal) were from Amersham Biosciences (Piscataway, NJ). The protease inhibitor cocktail was from Roche

(Mannheim, Germany) and the BCA protein assay kit was from Pierce (Rockford, IL). The labeled streptavidin biotin kit was from Dako (Carpinteria, CA).

Subjects

The endometrial specimens ($n = 37$) were classified as follows: endometria obtained from women with proven fertility (NE, $n = 9$), endometria obtained from patients with PCOS (PCOSE, $n = 12$), endometria from PCOS women with endometrial hyperplasia (HPCOSE, $n = 7$), and endometria from patients with endometrial hyperplasia (HE, $n = 9$). The 4 groups of patients were accrued prospectively and each group was recruited independently. None of the women had received hormonal therapy or other medications within 3 months prior to recruitment into the study.

In women with PCOS (PCOSE and HPCOSE), endometria were obtained with a Pipelle suction curette from the corpus of the uteri. Endometria with hyperplasia from patients without PCOS were obtained at hysterectomy.

The diagnosis of PCOS was made according to the Rotterdam Consensus [8]. Hyperprolactinemia, androgen secreting tumors, Cushing's syndrome, congenital adrenal hyperplasia, attenuated 21-hydroxylase deficiency, as well as thyroid disease, were all excluded by appropriate tests. The diagnosis of endometrial hyperplasia in patients with and without PCOS was established by an experienced histopathologist according to the criteria of Kurman et al. [34]. The patients from the HPCOSE group had simplex (91%) and complex (9%) hyperplasia, without atypia. On the other hand, patients from the HE group had simplex (50%) and complex (50%) hyperplasia, without atypia. Simplex or complex hyperplasias were combined within the "endometrial hyperplasia" group (HPCOSE or HE), because no differences were found in the markers studied between endometria with simplex and complex hyperplasias in HPCOSE and HE groups.

Control endometria were obtained from fertile healthy women during the proliferative phase of the menstrual cycle at the time of bilateral tubal ligation. The endometria used all showed normal morphology. The controls were selected in the proliferative phase because of the similar morphologies among the proliferative endometrium of a normal patient and the endometrium of PCOS women (Supplementary Fig. 1). The proliferative phase was confirmed according to the histological criteria of Noyes [35].

Endometrial cancer samples ($n = 3$) were used to control for CDK2 kinase activity.

The clinical and endocrinological characteristics of the groups investigated are shown in Table 1. The intra- and inter-assay coefficients of variation of the hormonal measurements were, respectively, 7.0% and 11% for testosterone, 4.8% and 9.2% for progesterone, 3.8 and 7.9% for steroid hormone-binding globulin, 2.7% and 5% for estradiol, and 3.2% and 6.1% for androstenedione.

All protocols involved in this study were approved by the ethical committees of the San Borja-Arriarán Clinical Hospital and School of Medicine, University of Chile, and informed written consent from all subjects was obtained.

Immunohistochemistry

Immunostaining for CDK2, Cyclin E and p27 was performed on 5- μ m sections of formalin fixed paraffin-embedded endometrial biopsies. Tissue sections were deparaffinized in xylene, hydrated through graded alcohol, and 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 3% hydrogen peroxide for 30 min. Non-specific

antibody binding was prevented with 4% PBS-BSA for 1 h. Samples were exposed overnight at 4 °C to primary antibodies for Cyclin E (1:300), p27 (1:400) and CDK2 (1:1000). Negative controls were analyzed on adjacent sections incubated without primary antibody and using non-immune serum. The second antibodies were biotinylated anti-rabbit and anti-mouse immunoglobulins and the reaction was developed by the streptavidin–peroxidase system and 3, 3' diaminobenzidine; counterstaining was with hematoxylin. The immunochemical evaluation for each protein was done through a semiquantitative analysis named HScore (HS: histology score), described by Lessey et al. [36], and validated in our laboratory [37,38]. The HScore corresponds to: $[P] (i+1)/100$, where $[P]$ is the percentage of positively stained cells and i is the intensity of the staining on a scale of 1–3 (1 = low intensity; 2 = mid intensity; and 3 = higher intensity). In all cases, the antigen studied was evaluated in a Nikon optical microscope (Nikon Inc., Melville, NY) by 3 independent observers and blinded to patient category, and the positive staining was assessed in at least 1000 cells per sample.

Western blotting

The tissues were homogenized and lysed on ice using cell lysis buffer (20 mM Hepes, 2 mM EDTA, 2 mM EGTA, 1% Triton, 5 mM PMSF, 50 μ M Na_3VO_4) and protease inhibitor cocktail. After centrifugation at 10,000 $\times g$ for 20 min at 4 °C, protein concentrations were determined, and 50 μ g were denatured in Laemmli buffer, fractionated using 7.5% (AKT, p-AKT/Ser473 and p-AKT/Thr308), or 10% (CDK2, Cyclin E, p27 and Ras) one-dimensional-SDS-PAGEs, and transferred to nitrocellulose membranes (BioRad, CA). Blots were blocked for 1 h in TBS-T (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% non-fat dry milk. The blots were washed three 3 $\times$ 7 min in TBS-T and then incubated overnight at 4 °C with antibodies against human AKT (0.5 μ g/ml), p-AKT/Ser473 (2 μ g/ml), p-AKT/Thr308 (1:200), Cyclin E (1 μ g/ml), CDK2 (1:500) and p27 (1:2500), for 2 h at room temperature with anti-Ras (1:250), or for 1 h at room temperature with anti- β -Actin (1:15,000). The blots were washed with TBS-T, incubated for 1 h at room temperature with anti-mouse IgG, peroxidase-linked species-specific F(ab')₂ fragment (1:5000), or anti-rabbit IgG peroxidase-linked species-specific whole antibody (1:5000). Bound antibodies were detected by chemiluminescence (Amersham Biosciences). Band intensities were semiquantified by scanning densitometry utilizing the UN-SCAN-IT software (Silk Scientific, UT).

CDK2 activity

Tissues were homogenized and lysed on ice using cell lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin, 1 mM phenylmethyl-sulfonylfluoride, 50 mM sodium fluoride, and 1 mM activated Na_3VO_4). After centrifugation at 16,000 $\times g$ for 20 min at 4 °C, protein concentrations were determined, and 100 μ g of the lysates were incubated overnight at 4 °C with constant rotation in 0.5 ml of NP-40 lysis buffer with 1 μ g of anti-CDK2 antibody (M2; Santa Cruz Biotechnology). The mixture was incubated for 2 h at 4 °C with 25 μ l of protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology). Immunocomplexes were washed 3 times with lysis buffer and twice with kinase buffer [50 mM HEPES (pH 7.2), 10 mM MgCl_2 , 1 mM DTT, 1 mM sodium fluoride, and 10 mM β -glycerophosphate]. The beads were resuspended in 30 μ l kinase buffer containing 2 μ g of histone H1 (Upstate Cell Signaling Solutions, Lake Placid, NY), 5 μ M ATP (Upstate), and 5 μ Ci [γ -³²P] ATP (MP Biomedicals, Irvine, CA). The mixtures were incubated at 30 °C for 30 min and the reaction terminated with 30 μ l of 2 $\times$ electrophoresis sample buffer. The samples were boiled and separated on 12% SDS/PAGEs. The gels were stained with coomassie blue (Sigma) to visualize the histone H1 bands, dried, and autoradiographed.

Statistical analysis

The number of subjects in this study was calculated assuming $\alpha = 0.05$ and $\beta = 20\%$, and a difference between means of 0.3 and a standard deviation of 0.200 according to our previous studies. One-way ANOVA test was used. $P < 0.05$ was considered significant. Statistical tests were performed using SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL).

Results

Clinical and metabolic characteristics of the subjects

The ages of the group with NE are higher than those of PCOS women as the NE women belong to a group of bilateral tubal ligation; the higher body mass index in the PCOS women is inherent to the syndrome (Table 1). Also in women with PCOS an increase in ovarian androgen production and a decrease in serum concentration of sex-hormone-binding globulin leads to a higher free androgen index. In addition, the women from the PCOSE, HPCOSE and HE groups presented a higher relative hyper-estrogenism marked by a higher estradiol to progesterone ratio when compared to NE.

Cell cycle regulators

We studied the expression levels of Cyclin E and CDK2 by Western blot analysis, and the activity of CDK2 by an *in vitro* kinase assay in total extracts obtained from the tissue samples. There was a significant increase in CDK2 expression in HPCOSE patients with respect to the NE and PCOSE groups (Table 2), whereas no changes were detected in the activity of CDK2 in the four groups studied (Fig. 1). Furthermore, no variations were observed in the endometrial expression of Cyclin E and p27 in all analyzed endometria (Table 2).

Knowing that endometrial cancer originates from epithelial cells, we studied the immunolocalization of CDK2 and Cyclin E in endometria from all groups. The CDK2 and Cyclin E proteins were expressed mainly in the epithelial cells of the studied endometria (Figs. 2A–H). We observed a significant increase in CDK2 nuclear epithelial expression in HPCOSE and HE when compared to controls (Fig. 3A). Moreover, we detected a significant increase in Cyclin E nuclear epithelial expression in HE when compared to NE (Fig. 3B). No significant changes in nuclear expression of Cyclin E were detected in endometrial epithelial cells among NE, PCOSE and HPCOSE (Fig. 3B), and no changes were observed in the cytoplasmic staining of CDK2 and Cyclin E among the endometria (Figs. 3A, B).

We also observed that the expression of p27 in total endometrial extracts was similar in the 4 studied groups (Table 2), and positive p27 staining was observed in both nucleus and cytoplasm of epithelial and stromal cells in all samples (Figs. 2I–L). However, there was a significant higher expression of p27 in the cytoplasm of epithelial cells of PCOSE and HPCOSE when compared to NE (Fig. 3C), whereas the nuclear expression of p27 in the epithelial cells was similar in all groups studied.

Protein levels of total and phosphorylated forms of AKT

Western blot analysis was performed for total AKT and AKT phosphorylated on Ser473 and Thr308 (Figs. 4A, B). In PCOSE and HPCOSE we detected a significantly increased phosphorylation of AKT at Ser473 when compared to NE. No variations were observed among the groups in total AKT endometrial expression and in the phosphorylation of AKT at Thr308.

Protein levels of Ras

Significant higher levels of Ras were detected in PCOSE and HPCOSE when compared to NE (Figs. 4C, D).

Discussion

Endometrial growth in women with PCOS is influenced by androgens and estrogens without progestin opposition [6]. Thus, the endometrium in PCOS does not undergo secretory transformation, what consequently can lead to endometrial hyperplasia and neoplasia. Although several studies have supported an association between PCOS and endometrial carcinogenesis [3–7], the mechanism(s) underlying such relationship are unclear. Previous results from our laboratory showed that the onset of endometrial hyperplasia in PCOS endometrium could be linked to a dysregulation in cell proliferation in association with reduced apoptosis [9]. Thus, in this investigation, we addressed the question as to whether the abundance, localization and/or activity of cell cycle regulators CDK2, Cyclin E, p27, and survival proteins AKT and Ras in PCOS-associated endometria (with and without hyperplasia) differ from non-PCOS endometria.

We found the Ras protein over-expressed in PCOSE and HPCOSE. This is a relevant finding because not only does Ras activate pathways involved in cell growth control, but it also positively regulates the estrogen receptor that usually drives endometrial growth [39]. On the other hand, our results clearly show that the PCOS condition associates with a higher degree of p-AKT/Ser473 when compared to the levels observed in NE, without changes in the levels of total AKT and of p-AKT/Thr308. Since AKT provides a survival signal to the cells [27,40], this result could explain, at least in part, the higher proliferation and survival rate we previously found in the epithelial endometrial cells of women with PCOS [9]. It has been reported that estrogens bound to its receptors may form a complex with PI3K, that results in an increase of its kinase activity, leading to increased levels of phosphorylated AKT [41]. Therefore, in PCOS endometria where estrogen action may be enhanced due to the over-expression of estrogen receptors and of its coactivators [42], the PI3K/AKT pathway could participate in the deregulation of tissue homeostasis.

In regards to the cell cycle regulators studied, the results show that Cyclin E is mainly expressed in the endometrial epithelial cells of the four groups investigated. In particular, the higher expression of Cyclin E in the nuclear compartment of epithelial cells of HE when compared to other groups, suggest that Cyclin E does not seem to be deregulated in endometria of women with PCOS, and that nuclear Cyclin E expression can be a marker of endometrial hyperplasia in the absence of PCOS.

The progression from the G1 to the S phase of the cell cycle is driven by the kinase activity of the Cyclin E/CDK2 complex which phosphorylates key proteins leading to DNA synthesis [43,44]. The data obtained in this study show that CDK2 is mainly localized in epithelial endometrial cells together with Cyclin E, and coincides with the expression of the proliferation marker Ki67 shown in our previous study [9]. The expression of CDK2 in the nucleus of epithelial cells increased gradually from PCOSE to HPCOSE and HE, as compared to controls. This increase of CDK2 in the nuclear compartment may be related to the proliferative capacity of the epithelial cells. The nucleocytoplasmic trafficking of CDK2 is not completely understood, but it is generally accepted that it is the nuclear and not the cytoplasmic CDK2 which is involved in the phosphorylation of proteins needed for cell cycle progression [45]. The kinase activity of the Cyclin E/CDK2 complex is controlled by different mechanisms in the G0/G1 phase of the cell cycle [45]. It is possible that although the overall endometrial activity of CDK2 did not change among the studied groups, the activity of CDK2 within the nuclear compartment may have been increased in hyperplastic

endometrial tissues. It has been amply recognized that the Cyclin E/CDK2 complex can be inactivated by binding to CDK inhibitors such as p27, resulting in cell cycle arrest and decreased cell proliferation. The expression of p27 is usually decreased in endometrial cancer [14,46], and p27 has been shown to be involved in the progesterone-mediated suppression of endometrial growth [47]. Interestingly, when p27 is present in the nucleus, it acts as a blocker of cell proliferation by inhibiting the activity of the Cyclin E/CDK2 complex, whereas when it is present in the cytoplasm it appears to inhibit apoptosis [22]. In the present work, we observed that the total amount of p27 protein did not change in all analyzed groups as determined using Western blot analysis. However, using immunohistochemistry we detected an increase in the cytoplasmic expression of p27 in the epithelial cells from PCOSE and HPCOSE. In PCOS endometria, p27 could become less available in the nucleus where it is needed to block the activity of the Cyclin E/CDK2 complex, thus facilitating S phase progression. As it has been reported that estradiol may regulate the subcellular distribution of p27 [48], the observed changes in p27 localization in endometria from PCOS women could partly be explained by the hyper-estrogenism present in these patients. On the other hand it is known that the phosphorylation of p27 by AKT causes the cytoplasmic localization and targeted degradation of p27 favoring the progression of the cell cycle [18–21]. Thus, the high levels of phosphorylated and presumably active AKT in PCOS endometria could also contribute to the cytoplasmic localization of p27.

Despite the morphologic similarities between HPCOSE and HE, these two types of endometria have a different expression profile of regulatory molecules controlling cell proliferation and survival. We believe that while non-PCOS endometrial hyperplasia is triggered by the prolonged endometrial exposition to estrogen unopposed by progesterone, the endometrial hyperplasia in PCOS women could be influenced by various factors, among them androgens in addition to unopposed estrogens. Therefore, endometrial hyperplasia developed in PCOS women could be governed by molecular mechanisms different from those controlling the development of endometrial hyperplasia in patients without PCOS, that may be relevant when taking therapeutic decisions in both groups of patients.

We conclude that the endocrine metabolic condition inherent to PCOS affects the normal physiology of the endometrium at the level of tissue homeostasis and cell survival. The PCOS condition is associated with increased nuclear abundance of CDK2 in the endometrial epithelial cells, and increased Ser473-AKT phosphorylation, elevated expression of Ras and increased cytoplasmic abundance of p27 in endometrial tissue. These biological events could potentially provide a chance for endometrial cells from PCOS patients to exit the controlled cell cycle and become hyperplastic at a later stage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the women who donated tissue and blood. This work was supported by grant Nos. 1050098 and 1095127 from the Fondo Nacional de Desarrollo Científico y Tecnológico, PRE 070/2003 from the PLACIRH, and K22CA121991 from the National Cancer Institute.

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^{32}P -histone H1

Fig. 1.

The analysis of CDK2 kinase activity was performed in NE ($n = 9$), PCOSE ($n = 12$), HPCOSE ($n = 7$) and HE ($n = 9$). Cell extracts were prepared as described in Materials and methods. A portion of each extract, containing equal amounts of protein, was subjected to immunoprecipitation with anti-CDK2 antibody. Thereafter the sample was used to assay CDK2 kinase activity using histone H1 as substrate. The figure shows a representative image from the different groups of endometria studied. Endometrial tissue from patients with endometrial carcinoma ($n = 3$) was used as positive control [(+)].

Fig. 2.

Immunohistochemical detection of CDK2 (A–D), Cyclin E (E–H) and p27 (I–L) in paraffin wax sections of endometria from women with proven fertility in the proliferative phase (NE, $n = 9$), untreated PCOS women (PCOSE, $n = 12$), PCOS women with endometrial hyperplasia (HPCOSE, $n = 7$), or women with endometrial hyperplasia (HE, $n = 9$). Positive nuclear and cytoplasmic staining was detected in epithelial and stromal cells for CDK2, Cyclin E and p27. Arrows indicate positive staining for CDK2 and Cyclin E in the nuclei of epithelial cells, and arrowheads show the staining for p27 in the cytoplasm of epithelial cells. Magnification in the panels corresponds to $\times 400$ and the scale bar represents $10\ \mu\text{m}$. As a negative control, the primary antibody was omitted (insert in D).

Fig. 3. Semiquantitative evaluation of CDK2 (A), Cyclin E (B) and p27 (C) protein expression by HScore in epithelial cells [(nuclear compartment (NC) and cytoplasmic compartment (CC))] from the four groups studied. The values are expressed as HScore (HS) (mean $\pm$ SEM). Calculation of HScore is described in Materials and methods. * $P < 0.05$ vs. NE.

Fig. 4.

Results of Western blotting for phosphorylated AKT, total AKT, and Ras in NE, PCOSE, HPCOSE and HE. (A and C) Equal amounts of endometrial protein were loaded in each lane. p-AKT/Ser473, p-AKT/Thr308 and AKT were detected as bands with molecular mass of 60 kDa, and Ras was detected as a band with a molecular mass of 21 kDa. (B and D) p-AKT/Ser473, p-AKT/Thr308, AKT and Ras band intensities were semiquantified by scanning densitometry and normalized to β -Actin. The results are expressed as arbitrary densitometric units (AU), and the values shown are mean $\pm$ SEM in NE ($n = 9$), PCOSE ($n = 12$), HPCOSE ($n = 7$), and HE ($n = 9$). * $P < 0.05$ in PCOSE and HPCOSE compared to NE. # $P < 0.05$ in p-AKT/Ser473 vs. p-AKT/Thr308.

Table 1

Clinical and metabolic characteristics of healthy women (NE), women with PCOS (PCOSE), women with PCOS and endometrial hyperplasia (HPCOSE) and women with endometrial hyperplasia (HE).

	NE (n = 9)	PCOSE (n = 12)	HPCOSE (n = 7)	HE (n = 9)
Age (years)	36.9 ± 1.58	26.3 ± 0.88	30.8 ± 1.35	42.1 ± 2.14
BMI (kg/m ²)	25.5 ± 1.11	31.7 ± 1.35 *	32.0 ± 1.63 *	27.2 ± 2.14
A ₄ (ng/ml)	1.56 ± 0.22	2.27 ± 0.26	2.12 ± 0.56	1.30 ± 0.32
T (ng/ml)	0.35 ± 0.04	0.87 ± 0.09 *	0.70 ± 0.05 *	0.53 ± 0.03
SHBG (nmol/l)	52.8 ± 9.45	21.5 ± 3.29 *	30.0 ± 6.29 *	47.7 ± 1.67
FAI	2.61 ± 0.51	14.8 ± 3.19 *	10.5 ± 2.19 *	3.87 ± 0.34
E ₂ /P ₄ (ng/ml)	0.08 ± 0.00	0.14 ± 0.00 *	0.15 ± 0.01 *	0.17 ± 0.02 *

Abbreviation: body mass index (BMI), androstenedione (A₄), testosterone (T), sex-hormone-binding globulin (SHBG), free androgen index (FAI), estradiol (E₂), progesterone (P₄). FAI = ([T(ng/ml)]3.467/[SHBG](nmol/l)) 100.

Note. The values are mean ± SEM.

* *P* < 0.05 compared to NE.

Table 2

Western blotting analysis of Cyclin E, CDK2 and p27 expression in NE, PCOSE, HPCOSE and HE. Cyclin E, CDK2 and p27 band intensities were semiquantified by scanning densitometry and normalized to intensities observed for a β -Actin control.

	NE	PCOSE	HPCOSE	HE
CDK2/ β -Actin (AU)	1.09 $\pm$ 0.04	1.09 $\pm$ 0.05	1.30 $\pm$ 0.06*	1.21 $\pm$ 0.04
Cyclin E/ β -Actin (AU)	0.80 $\pm$ 0.05	0.90 $\pm$ 0.04	0.82 $\pm$ 0.09	0.77 $\pm$ 0.10
p27/ β -Actin (AU)	1.19 $\pm$ 0.17	1.34 $\pm$ 0.11	1.22 $\pm$ 0.10	1.14 $\pm$ 0.11

Note. The result are expressed as arbitrary densitometric units (AU), and the values shown are means $\pm$ SEM in NE ($n = 9$), PCOSE ($n = 12$), HPCOSE ($n = 7$), HE ($n = 9$).

* $P < 0.05$ vs. PCOSE and NE groups.