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The role of androst-5-ene-3β,17β-diol (androstenediol) in cell proliferation in endometrium of women with polycystic ovary syndrome

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ABSTRACT

Women with polycystic ovary syndrome (PCOS) show high prevalence of endometrial hyperplasia and adenocarcinoma. Endometrial proliferation is increased, evaluated by high levels of Ki67 (cell cycle marker) and low levels of p27 (negative regulator of cell cycle). Nevertheless, endometrial changes in cyclin D1 (positive regulator of cell cycle) in PCOS-women are not described. Androst-5-ene-3β,17β-diol (androstenediol), steroid with estrogenic activity present in endometria, could be related to increased endometrial cell proliferation. The objective of this study was to determine protein content of cyclin D1 and androstenediol levels in endometria from PCOS and control-women and to evaluate the possible mechanism favoring cell proliferation associated with hormonal characteristics of patients. Therefore, cyclin D1 protein content in PCOS-women and control-endometrial tissue were assessed by western blot and immunohistochemistry. The androstenediol levels were evaluated by ELISA. To further analyze the effect of steroids (androstenediol, 17β-estradiol, testosterone) in cell proliferation, levels of proteins cyclin D1, p27 and Ki67 were evaluated in an *in vitro* model of stromal endometrial cells T-HESC and St-T1b. An increase in cyclin D1 and androstenediol was observed in tissues from PCOS-women relative to control group ($p < 0.05$). In the *in vitro* model, androstenediol exerted increase in cyclin D1 ($p < 0.05$) and a decrease in p27 protein level ($p < 0.05$), while Ki67 in St-T1b cells increased under this stimulus ($p < 0.05$). Testosterone produces opposite effects in the levels of the above markers ($p < 0.05$). Therefore, the hormonal imbalance associated with this syndrome could alter endometrial tissue homeostasis, promoting cell proliferation. Androstenediol is a molecule that could be involved by stimulating proliferation, whereas testosterone elicits a role of cell cycle repressor.

1. Introduction

Polycystic ovarian syndrome (PCOS) is an endocrine-metabolic pathology, with a high prevalence in women during their fertile age, affecting 2–9% of this group [1–3]. This pathology has been related to the development of endometrial carcinoma [4–7] as PCOS is associated with the deregulation of endometrial cell proliferation. In fact, higher levels of the cell cycle marker Ki67 have been reported in endometria from PCOS women [8,9].

It is known that endometrial tissue is mainly regulated by steroids, particularly by estrogens that promote cell proliferation. In endometrium of women with PCOS, an increased sensitivity to estrogen is apparent, based mainly on the high levels of estrogen receptor protein alpha and the coactivators TIF2 and AIB1 [10–12]. Additionally, in PCOS-endometria, the synthesis of molecules with estrogenic activity such as androstenediol is favored as a result of the high enzymatic activity of 17β-hydroxisteroid dehydrogenase (HSD) [13].

Cell cycle is importantly regulated by the activity of cyclin-dependent kinases (CDKs). A critical point in the cycle progression through G1 phase is modulated by the expression of cyclin D1, which is associated with CDK4 [14]. The overexpression of cyclin D1 is one of the most commonly observed alterations in cancer [15–17], constituting a risk factor for early tumorigenesis, progression and metastasis [18–23].

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Subsequently, the G1/S transition requires the action of cyclin E associated to CDK2. At this point, the negative regulator of the cell cycle p27 acts by inhibiting cyclin E/CDK2 complex formation. The decrease of p27 protein levels in tumors may constitute a marker of poor prognosis [14,23]. Interestingly, in the endometria of women with PCOS, a diminution in protein levels of p27 has been detected [24].

One of the molecular markers of the cell cycle is Ki67, a non-histone molecule expressed during G1, S, G2 and M phases of the cycle, where peak levels occur in the M stage and disappears in G0 [25,26]. Although this molecule could participate in cell division, its specific role in this process is still elusive [25,27].

Consequently, based on previous reports that indicate an endometrial cell cycle disruption in women with PCOS, the following study evaluated the protein expression of cyclin D1 in endometria obtained from PCOS women and controls. Additionally, the protein levels of Ki67, cyclin D1 and p27 were assessed in an *in vitro* model of endometrial cells under steroid stimuli similar to the altered steroidogenic condition found in PCOS women.

2. Experimental

Hormonal levels were assayed using commercial kits: serum testosterone, androstenedione and progesterone by radioimmunoassay (RIA) (Diagnostic System Laboratories, Webster, TX, USA); sex hormone-binding globulin (SHBG) concentration by RIA (DPC, Los Angeles, CA, USA) and 17β-estradiol by electrochemiluminescence (Roche, Basel, Switzerland). The utilized antibodies for immunohistochemistry, immunocytochemistry and/or western blot were β-actin (A 5441, Sigma, USA); cyclin D1 (92G2 Cell Signaling Technology®, Danvers, MA, USA); p27 (3686 Cell Signaling Technology®, Danvers, MA, USA), Ki67 (AB9260 Millipore Corporation, Billerica, MA, USA) and the anti-rabbit secondary antibody (074-1806 KPL, Gaithersburg MD, USA). Immunohistochemistry and immunocytochemistry were performed with 3,3′diaminobenzidine (DAB K3467, Dako, Carpinteria, CA, USA), the mount media was Entellan new® (107961, Merck Millipore Corporation, Billerica, MA, USA).

2.1. Subjects

This is a case-control study set at a research unit, approved by the Ethical Committees of the Faculty of Medicine and Clinical Hospital of the University of Chile. Twenty-eight PCOS patients were included (case group) and 18 fertile healthy women (controls) recruited at the time of hysterectomy due to benign uterine pathology. Endometrial and blood samples were obtained from women during the proliferative phase of the menstrual cycle. Metabolic assays and hormonal studies for determining sex steroids (testosterone, progesterone and 17β-estradiol) and SHBG concentration were assessed. Endometria from PCOS women (PCOSE) were obtained with a Pipelle suction curette from the corpus of the uteri. All PCOS women participants in this investigation had clinical and/or biochemical hyperandrogenism. Control endometria (CE) 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 Noyes criteria [28] by an experienced pathologist. The diagnosis of PCOS was made according to the Androgen Excess Society criteria [29,30] for the definition of PCOS. The exclusion criteria were women who presented with hyperprolactinemia (PRL > 35 ng mL^{-1}), hypothyroidism (TSH > 5 UI L^{-1}), androgen secreting tumors (total testosterone > 2 ng mL^{-1}; dehydroepiandrosterone sulfate (DHEAS) > 3600 μg mL^{-1}), Cushing's syndrome (urine cortisol concentration > 150 μg/24 h, and fasting plasma concentration of cortisol between 5–25 μg dL^{-1}), congenital adrenal hyperplasia (17-OH progesterone > 2,5 ng mL^{-1}), 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.

2.2. Tissue preparation

Endometrial tissue samples were divided into three or more pieces. Two pieces of each sample were frozen in liquid nitrogen and maintained at −80 °C for western blotting; one piece was embedded in paraffin for immunohistochemistry.

2.3. Cell culture

The endometrial stromal cell lines T-HESC (ATCC, CRL-4003) [31] and St-T1b [32] were used for this study. Both cell lines were obtained from primary cultures of endometrial stroma, T-HESC of the midsecretory phase and St-T1b of the proliferative phase. Those cells were immortalized with telomerase gene (hTERT) transfection using a retroviral system [31,32]. The cells were propagated in DMEM HAM F12 medium without phenol red (Sigma–Aldrich Co, Saint Louis MO, USA) supplemented with 1.5 g/l sodium bicarbonate, 1X Insulin–Transferrin–Selenium (ITS) mixture, 500 ng/ml puromycin, 10 v/v fetal bovine serum (FBS) and cultured at 37 °C and 5% CO_2.

In experiments, cells were cultured for 24 h at a ratio of 800,000 cells/plate for western blot and 200,000 cells/plate for immunocytochemistry in 4-well Lab-Tek® II Chamber Slides™ and then were treated for 48 h with 100 nM of androstenediol, 17β-estradiol, testosterone or androstenediol plus testosterone in DMEM HAM F12 without serum. The cultures with no hormonal stimulation were used as the basal condition for the study.

2.4. Western blotting

The western blot was assessed as previously reported [8]. In brief, fresh tissue specimens and cell samples were homogenized and lysed on ice using a cell lysis RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5 v/v sodium deoxycholate, 1 v/v triton, 0.1 v/v SDS) containing a 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, IL, USA). Total proteins (40 μg from each sample) were denatured and fractionated using 10% one-dimensional-SDS–PAGE and transferred to nitrocellulose membrane (BioRad, CA, USA). Blots were blocked for 2 h in TBST (20 mM Tris, pH 7.6; 137 mM NaCl; 0.1 v/v Tween 20) containing 5 v/v nonfat dry milk. Subsequently, the blots were washed three times for 5 min each in TBST and then incubated overnight at 4 °C with antibodies against cyclin D1 (1:300) and p27 (1:500). β-Actin antibody (1:20,000) was incubated for 1 h at room temperature. The blots were washed three times for 5 min each with TBST, followed by incubation while rocking for 1 h at room temperature with anti-rabbit IgG peroxidase-linked species-specific whole antibody (1:5000) or anti-mouse IgG, peroxidase-linked species-specific *F* (ab′)2 fragment (1:10,000). After washing three times for 10 min each with TBST, the bound antibodies were detected with an enhanced chemiluminescence system (Amersham International, GE Healthcare, UK). Band intensities were quantified by scanning densitometry utilizing the UN-SCAN-IT software, Automated Digitizing System, version 5.1. The results were expressed as a ratio of the housekeeping gene β-actin.