Steroids 75 (2010) 810–817

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

The conversion of dehydroepiandrosterone into androst-5-ene-3β,17β-diol (androstenediol) is increased in endometria from untreated women with polycystic ovarian syndrome

F. Plaza[a], F. Gabler[b], C. Romero[a], D. Vantman[a], L. Valladares[c], M. Vega[a,*]

[a] Department of Obstetrics and Gynecology, School of Medicine, University of Chile, Clinical Hospital, Santos Dumont # 999, Santiago, Chile
[b] Department of Pathology, School of Medicine, University of Chile, San Borja Arriarán Clinical Hospital, Santa Rosa #1234, Chile
[c] Institute of Nutrition and Food Technology, University of Chile, Macul #5540, Chile

ARTICLE INFO

Article history:
Received 29 January 2010
Received in revised form 1 April 2010
Accepted 22 June 2010
Available online 8 July 2010

Keywords:
PCOS
Endometrium
Androstenediol
Dehydroepiandrosterone
Intracrinology
Steroid metabolism

ABSTRACT

The changes in endometrial homeostasis found in women with polycystic ovarian syndrome (PCOS) could be associated with alterations in the intracrine metabolism of steroid hormones. The uptake of dehydroepiandrosterone-sulphate (DHEA-S), precursor of the intracrine pathway, is achieved by transporters, such as organic anion transporter polypeptides (OATPs), and molecules with oestrogenic activity, such as androst-5-ene-3β,17β-diol (androstenediol), can be generated. We aimed to determine androstenediol generation and the expression of OATPs in human endometria throughout the menstrual cycle and in endometria from PCOS women. Endometrial samples were obtained from control women in the proliferative phase (control endometria (CEp), $n = 7$), secretory phase (CEs, $n = 7$), and from PCOS patients (PCOSEp, $n = 7$). The mRNA levels of OATP-B, OATP-D and OATP-E were measured by reverse transcriptase polymerase chain reaction (RT-PCR) and protein levels of OATP-E by immunofluorescence; 3β-hydroxysteroid dehydrogenase (HSD) by immunohistochemistry/Western blot; the metabolism of DHEA to androstenediol was evaluated by thin layer chromatography–high-performance liquid chromatography (TLC–HPLC). Lower levels of OATP-E transcript were obtained in PCOSEp ($p < 0.05$) compared with CEp, while OATP-E protein levels ($p < 0.05$) and DHEA conversion to androstenediol ($p < 0.01$) were higher in PCOSEp. Lower 3β-(hydroxysteroid dehydrogenase) HSD protein levels were found in PCOSEp ($p < 0.05$) (Western blot, immunohistochemistry). These results reveal a higher capacity of the endometria from PCOS women to metabolise DHEA to androstenediol, which, coupled with the high oestrogen sensitivity previously found in these endometria, may account for the increase in cell proliferation in PCOSEp already reported.

During the proliferative phase of the menstrual cycle, a predominance of the action of 17β oestradiol of ovarian origin is observed, which induces cell proliferation and growth of endometrial tissue [1,2]. In polycystic ovary syndrome (PCOS), a large percentage of women are anovulatory; therefore, their endometria are maintained at the stage of the proliferative phase [2]. PCOS is an endocrine-metabolic disorder that affects between 5% and 10% of women of childbearing age [3]. Currently, the diagnosis of this syndrome is based upon signs of clinical or biochemical hyperandrogenism and, also, if the patient presents oligo-ovulation or anovulation, and/or polycystic ovaries at ultrasonography [4,5].

Women with PCOS exhibit a greater plasma level of dehydroepiandrosterone-sulphate (DHEA-S) [6,7], infertility, ovarian dysfunction and insulin resistance with compensatory hyperinsulinaemia [3,8]. In addition, these women have a higher rate of recurrent abortions, hyperplasia and endometrial adenocarcinoma than control women [8–11]. In previous studies, we have described a high expression of oestrogen and androgen receptors and co-activators of steroid receptors, such as, AIB1 and ARA70 in the endometrium of women with PCOS, besides an increase in the intracellular steroid metabolism, all of which can be interpreted as an increased sensitivity to steroidal action in these endometria [12,13].

The intracrinology is defined as the ability of a peripheral non-steroidogenic cell to synthesise steroid hormones, which elicits their effect on the same cell. The precursor of these steroids is DHEA, synthesised and subsequently secreted from the adrenal gland into the circulation. Importantly, a number of peripheral organs,

* Corresponding author. Tel.: +56 2 9788304; fax: +56 2 7374555.
E-mail address: mvega@med.uchile.cl (M. Vega).

0039-128X/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2010.06.011

Table 1
Primer sequences for PCR of cDNA for the analysis of organic anion transporter polypeptide B (OATP-B), organic anion transporter polypeptide D (OATP-D), organic anion transporter polypeptide E (OATP-E) and rRNA 18s.

Gene	Primers	Sequence	PCR product size (bp)
OATP-B	Sense	5′-CAT GGG ACC CAG GAT AGG GCC AGC G-3′	718
	Antisense	5′-GGC CTG GCC CCA TCA TGG TCA CTG-3′	
OATP-D	Sense	5′-GCT GAG AAC GCA ACC GTG GTT CC-3′	163
	Antisense	5′-GAC TTG AGT TCA GGG CTG ACT GTC C-3′	
OATP-E	Sense	5′-GCC ATG CCA CTG CAG GGA AAT G-3′	291
	Antisense	5′-TTC TGG TAC ACC AAG CAG GAG CCC-3′	
rRNA 18S	Sense	5′-GTA ACC CGT TGA ACC CCA TT-3′	200
	Antisense	5′-CCA TCC AAT CGG TAG TAG CG-3′	

through their intracellular enzymatic machinery, are capable of transforming precursors to steroids with androgenic or oestrogenic activity [14–16].

DHEA enters the cell from circulation by passive diffusion; by contrast, its sulphated form needs special transporters such as some organic anion transporter polypeptides (OATPs). Many OATPs have been identified in various tissues, and the studies have been conducted mainly in kidney, placenta, brain and liver [17]. In fact, the mammary gland expresses transporter OATP-B and, to a lesser extent, OATP-D and OATP-E [18]. Furthermore, in the intestinal cell line Caco-2, it was observed that the transport capacity of oestrone 3-sulphate into the cell by OATP-B is higher compared with the other two transporters [19]. In human endometrium, the expression of these transporters has not been detected; however, the protein levels of OATP-D have been determined in rat endometrial tissue [20].

Once inside the cells, DHEA can be metabolised and converted into androstenediol by the activity of the enzyme 17β hydroxysteroid dehydrogenase (17β-HSD), which in turn can be converted into testosterone by the activity of the enzyme 3β-HSD [21–25]. Androstenediol has the ability to induce oestrogenic or androgenic activity by binding to their receptors, having a role in the proliferation of pathological tissues such as breast and prostate cancer, without the need to be metabolise to other steroids [23,26–29]. Androstenediol has less affinity for the androgen receptor than testosterone and dihydrotestosterone (DHT); on the other hand, ligands of oestrogen receptors such as oestradiol, oestrone and oestriol have more affinity than androstenediol [26,31,32]. Thus, the activity of androstenediol depends on the concentration of oestradiol, since high concentrations of 17β-oestradiol induce androstenediol to bind to androgen receptors, whereas a lower concentration of 17β-oestradiol enhances the binding of androstenediol to oestrogen receptors, as reported for breast cancer cells [29,30]. Therefore, in the present investigation, we aimed to study the transporter proteins involved in the entry of sulphated steroids to cells and the conversion of DHEA into androstenediol in control endometria during the menstrual cycle and whether in the PCOS endometria these parameters are modified.

1. Experimental

This investigation was approved by the University of Chile Clinical Hospital and School of Medicine, University of Chile Ethical Committees and informed written consent was obtained from all subjects, in agreement with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki).

1.1. Reagents

The polyclonal antibodies for OATP-E and 3β-HSD were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibody for β-actin was purchased from Sigma (MO, USA). The secondary antibody for goat-fluorescein isothiacyanate (FITC), mouse-horseradish peroxidase (HRP) and goat-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Amersham Biosciences (Piscataway, NJ, USA) and KPL (Gaithersburg, MD, USA), respectively. The Moloney-murine leukaemia virus (M-MLV) reverse transcriptase was obtained from Invitrogen (CA, USA); Taq DNA polymerase from Biotools (Madrid, Spain); diaminobenzidine (DAB) from DAKO (Carpinteria, CA, USA); BCA Protein Assay Kit from Pierce (IL, USA); β-nicotinamide adenine dinucleotide phosphate, reduced tetra (cyclohexylammonium) salt from Sigma (MO, USA); the TRIzol reagent from Invitrogen (CA, USA) and the Western blotting (WB) substrate from Pierce (IL, USA).

1.2. Subjects

The endometrial specimens ($n = 21$) were classified as follows: endometria obtained during the proliferative phase of the menstrual cycle (control endometria (CEp), $n = 7$), endometria obtained during the secretory phase of the menstrual cycle (CEs, $n = 7$) and endometria obtained from patients with PCOS (PCOSEp, $n = 7$). The tissues were obtained with a pipelle suction curette from the uterine corpus from women with PCOS. The diagnosis of PCOS was made according to the Rötterdam Consensus [4] and Excess Androgen Society [5], considering clinical and/or biochemical signs of hyperandrogenism and at least one of two other criteria: oligo- and/or anovulation and polycystic ovaries. Hyperprolactinaemia (prolactin (PRL) > 35 ng ml^{-1}), androgen-secreting tumours (total testosterone > 2 ng ml^{-1}; DHEA-S > 3600 μg ml^{-1}), Cushing's syndrome (urine cortisol concentration > 150 μg 24 h and fasting plasma concentration of cortisol > 25 μg dl^{-1}), congenital adrenal hyperplasia (17-OH progesterone > 2.5 ng ml^{-1}), attenuated 21-hydroxylase deficiency, diabetes and thyroid disease (thyroid stimulating hormone (TSH) > 5 U/l^{-1}) were excluded. Glucose and insulin levels were evaluated by an oral glucose tolerance test; we measured plasma glucose and insulin levels at 2 h post-load of glucose. Then, the diagnosis of hyperinsulinaemia was determined when levels of insulin were 2 SD (standard deviations) of insulin concentration over the mean of the control group, as in previous studies [33]. All women included in the study had normal glycaemia values during oral tolerance glucose test and the women with PCOS presented hyperinsulinaemia. Endometria from patients without PCOS (control endometria, CE) were obtained by hysterectomy from women with non-neoplasic pathology at hysterectomy. None of the women (control or PCOS) had received hormonal therapy, clomiphene citrate or insulin sensitisers within 3 months prior to recruitment into the study, and the endometria used in this study all showed normal morphology. The endometrial phases were evaluated by an experienced pathologist using the histological criteria of Noyes [34].

1.3. Tissue preparation

Endometrial tissue samples were divided into three pieces by the pathologist. Two pieces of each sample were frozen in liquid nitrogen and maintained at −80 °C for RT-PCR, WB and DHEA metabolism study. The third piece of sample was included in paraffin for morphological and protein studies by immunohistochemistry (IHC) and immunofluorescence (IF).

2. RNA isolation and semiquantitative RT-PCR

Total RNA was isolated from endometrial tissue (20–50 mg) using TRIzol reagent according to the manufacturer's instructions and as previously reported [35]. Total RNA was then reverse transcribed. The cDNA was subjected to PCR using specific primers for OATP-B, OATP-D and OATP-E; the rRNA 18S was used as an internal control (Table 1). Semiquantitative RT-PCRs were achieved in the exponential linear zone amplification for each gene studied. A total of 2 μg of total RNA were used for RT. The PCR condition was $MgCl_2$ 50 mM, dNTP 10 mM, Taq DNA polymerase 5 U μl^{-1} and 12.5 pmol, 10 pmol, 25 pmol and 5 pmol of the primer for OATP-B, OATP-D, OATP-E and rRNA 18 s, respectively. The cDNA of the mammary gland was used as positive control and water without cDNA as negative control. The PCR amplification was carried out in the Termocycler PTC-100 (MJ Research Inc, Watertown, MA, USA). The PCR products were resolved by electrophoresis on 1% agarose gel and stained with ethidium bromide. The bands were evaluated using the image analyser UN-SCAN-IT gel 4.1 (Silk Scientific Corporation, Orem, UT, USA) and normalised relative to rRNA 18S product. The results were expressed as the ratio of the transcript of OATP/rRNA 18S, as the mean of arbitrary units (AU). The bands detected by RT-PCR were sequenced by capillary electrophoresis (Macrogene Company Inc., Seoul, Korea); Basic Local Alignment Search Tool (BLAST) found 98%, 100%, 97% of identity for OATP-B, OATP-D and OATP-E, respectively.

2.1. Immunofluorescence

Immunofluorescence for OATP-E was performed on 5-μm sections of formalin-fixed paraffin-embedded endometrial biopsies. Tissue sections were deparaffinised in xylene and hydrated gradually through graded alcohols. The sections were incubated in antigen retrieval solution (10 mmol l^{-1} citrate buffer, pH 9.5) at 100 °C for 20 min. Non-specific antibody binding was prevented with 4% phosphate buffered saline–bovine serum albumin (PBS–BSA) for 1 h. Primary goat polyclonal antibody for OATP-E (1:75) was applied to the samples and incubated overnight at 4 °C. Negative controls were analysed on adjacent sections incubated without primary antibody. The second antibody was an FITC-donkey anti-goat (1:100). The counterstaining was carried out with Hoescht (1:500) for 1 min and 30 s. IF was evaluated by the program Image Pro Plus 6.2 (Silver Spring, MD, USA) in 10 microphotographs per sample, where each microphotograph was divided in areas of epithelia and stroma. The same assessment was made for the negative controls, and then the background of each sample was subtracted. The results were measured as the ratio of area of object of total selected area (per area (object/total)) and expressed as AU.

2.2. Immunohistochemistry

Immunostaining for 3β-HSD was assessed on 5-μm sections of formalin-fixed paraffin-embedded endometrial biopsies. The procedure was as previously described [36]. In brief, tissue sections were deparaffinised in xylene and hydrated gradually through graded alcohols. The sections were incubated in antigen retrieval solution (10 mmol l^{-1} citrate buffer, pH 9.5) at 100 °C for 20 min. The incubation of samples in 3% hydrogen peroxide for 15 min prevented endogenous peroxidase activity. Non-specific antibody binding was prevented with 4% PBS–BSA for 1 h. Primary goat polyclonal antibody of 3β-HSD (1:100) was applied to the samples and incubated overnight at 4 °C. Negative controls were analysed on adjacent sections incubated without primary antibody. The second antibody was a biotinylated rabbit anti-goat immunoglobulin (1:250). The reaction was developed by the streptavidin-peroxidase system, DAB was used as the chromogen; counterstaining was carried out with haematoxylin (1:5). The slides were evaluated by a Histochemical Score (HScore), a semiquantitative method described by Lessey et al. [37] and validated in our laboratory [13,38]. In all cases, the protein was evaluated in the functional layer by three independent observers and blinded to patient category, and the positive staining was assessed in at least 1000 epithelial cells and 1000 stromal cells per sample. The results were expressed as HScore (HS).

2.3. Western blot analysis (WB)

The frozen endometrial tissue (~30 mg) was homogenised in a lysis buffer (HEPES 20 mM, ethylene diamine tetraacetic acid (EDTA) 2 mM, ethylene glycol tetraacetic acid (EGTA) 2 mM, PSMF 15 μM, Triton X-100 1X, Na_3VO_4 0.4 mM) and a protease inhibitor cocktail (Roche, IN, USA). After centrifugation at 10 000 × *g* for 20 min at 4 °C, the protein concentration was measured using the BCA protein assay kit. For the evaluation, 50 μg of total protein were used; after denaturation, a one-dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis was run at 100 V for 2 h. The proteins were transferred to nitrocellulose membrane (BioRad, CA, USA) at 400 mA for 1 h. The membranes were blocked for 1 h in BSA fraction V 10% and then incubated with antibody against human 3β-HSD (1:600) overnight at 4 °C and with the secondary antibody anti-goat-HRP (1:10 000) for 1 h. To normalise, β-actin, antibody mouse anti-β-actin (1:15 000) were used for 1 h and anti-mouse-HRP (1:5000) for 30 min. The bound antibodies were detected with WB substrate. Band intensities were quantified by scanning densitometry using the UN-SCAN-IT software (Silk Scientific Corporation, USA) and the results were measured as protein level of 3β-HSD/protein level of β-actin, expressed as AU.

2.4. Metabolism of DHEA to androstenediol

The metabolism of DHEA was performed according to the technique of Jellinck et al. [39] Twenty to 30 mg of frozen tissue (−80 °C) were homogenised with phosphate buffer (100 mM KCl, 10 mM KH_2PO_4, 10 mM Na_2HPO_4, and 1 mM EDTA (pH 7.5), and then centrifuged at 1000 × *g* for 15 min to separate the aqueous phase (supernatant) from the organic phase. The proteins in the supernatant were quantified. A total of 100 μl of the organic phase was added to 50 μl Dulbecco PBS buffer, 50 μl of 1 mM nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and 50 μl of [^{3}H]-DHEA plus DHEA. The radioactivity measured from duplicate controls without samples was used for the subtraction of the background. In addition, we used two controls without NADPH to determine the effectiveness of the cofactor. The solution was incubated in a humid atmosphere of 5% CO_2 and air at 37 °C for 3 h with agitation. The reaction was stopped with acetone/ethyl acetate (1:2.5, v/v). The mixture was vortexed and then centrifuged, the supernatant was removed and dried with nitrogen and the residue was dissolved in 0.2 ml methanol. [^{3}H]-Androstenediol was characterised sequentially by thin layer chromatography (TLC) and with high-performance liquid chromatography (HPLC).

Table 2
Clinical and hormonal characteristics of patients.

	Cp	PCOSp	Cs
n	7	7	7
Age (years)	33.3 ± 1.6	27.2 ± 0.6	35.8 ± 2.7
BMI	26.9 ± 1.3	32.7 ± 0.9*	25.6 ± 1.2
E_2 (pg/ml)	73.8 ± 22.4	62.2 ± 3.5	125.2 ± 37.7
P_4 (ng/ml)	0.69 ± 0.28	0.61 ± 0.06	6.9 ± 2.0
T (ng/ml)	0.44 ± 0.06	0.81 ± 0.05*	0.49 ± 0.06
A_4 (ng/ml)	1.6 ± 0.2	2.8 ± 0.2*	2.1 ± 0.3
SHBG (nmol/l)	51.7 ± 9.5	24.2 ± 2.1*	57.5 ± 8.5
FAI	3.7 ± 0.7	13.5 ± 1.6*	3.3 ± 0.6
DHEA-S (ng/ml)	1312 ± 187.5	2273 ± 285.3*	1057 ± 198.7

Controls in proliferative phase (Cp), patients with polycystic ovary syndrome in proliferative phase (PCOSp) and controls in secretory phase (Cs), number of women by group (*n*), body mass index (BMI), 17β estradiol (E_2), progesterone (P_4), testosterone (T), androstenedione (A_4), steroid hormone binding globulin (SHBG), free androgen index (FAI), dehydroepiandrosterone-sulphate (DHEA-S). Values are expressed as mean ± S.E.M.

* $p < 0.05$ vs. Cp.

2.5. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC)

Separation by TLC was performed on silica-gel plates, using a solution of xylene–ethyl acetate–chloroform (40 ml–40 ml–20 ml). The identification of radioactive metabolites was monitored by the addition of non-radioactive steroids controls; it was resolved with sulphuric acid spray at 5% and subsequently exposed to 100 °C. To verify the identity of [^{3}H]-androstenediol, reverse-phase HPLC (RP-HPLC) was used. Samples were separated by TLC as described above, but without staining of standard compounds, and the region corresponding to androstenediol was eluted and analysed by RP-HPLC on a Merck-Hitachi liquid chromatograph (L-6200 pump, UV/vis detector). The detection of androstenediol was by dual-wavelength ultraviolet absorbance (240 nm), and the radioactivity was measured in a scintillation counter for liquid samples. The results were expressed as the mean of picomoles of androstenediol per mg of protein. The HPLC confirmed that the radioactivity measured in the scintillation counter corresponded to [^{3}H]-androstenediol elutes.

2.6. Statistical evaluation

The results were expressed as mean ± standard error of mean (S.E.M.). The number of subjects was calculated with $\alpha = 0.05$ and $\beta = 0.2$, difference between mean of 0.25 and SD of 0.16 according to our previous studies [35]. The distribution of the data was analysed by the Kolmogorov–Smirnov test. The results were analysed by Student's *t*-test or Mann–Whitney test. The statistical differences were considered with *p*-values less than 0.05. The evaluation was performed using Graph Pad 5.0 for Windows version 5.0 Software, Inc.

3. Results

3.1. Clinical and endocrine characteristics

Table 2 shows the hormonal profile of patients. The PCOS women present a higher body mass index (BMI) compared with controls, which is inherent to the syndrome. In fact, more than 30% of PCOS patients are obese [40]. All PCOS women showed hyperandrogenism expressed by an increase of testosterone, 4-androstene-3,17-dione (androstenedione), a decrease of SHBG blood levels and a high free androgen index (FAI) compared with control subjects. The DHEA-S blood level in PCOS women was significantly higher than the control group, although within normal range, which is characteristic of the syndrome [6].

Table 3
Semiquantitation of organic anion transporter polypeptide B (OATP-B), organic anion transporter polypeptide D (OATP-D) and organic anion transporter polypeptide E (OATP-E).

	CEp	PCOSEp	CEs
OATP-B/rRNA 18s	2.29 ± 0.22	1.80 ± 0.09	1.92 ± 0.57
OATP-D/rRNA 18s	0.65 ± 0.09	0.75 ± 0.04	1.01 ± 0.17
OATP-E/rRNA 18s	0.92 ± 0.15	0.20 ± 0.20*	1.44 ± 0.14*

Control in proliferative phase (CEp), polycystic ovarian syndrome in proliferative phase (PCOSEp) and control in secretory phase (CEs). The number of women evaluated was $n = 7$ for each of the analysed groups. The results are expressed as AU ± S.E.M. rRNA 18 s was used as the internal control.

* $p < 0.05$ vs. CEp.

3.2. Messenger RNA levels for OATP-B, OATP-D and OATP-E

We describe for the first time the expression of transporters OATP-B, D and E in human endometrium (Table 3). No differences were observed either between CEp and CEs for OATP-B ($p = 0.32$) and OATP-D ($p = 0.11$), or between CEp and PCOSEp for OATP-B ($p = 0.18$) and OATP-D ($p = 0.65$). Nevertheless, there was a significant increase ($p < 0.05$) in the level of mRNA for OATP-E during menstrual cycle progression. When comparing the level of transcripts of the three transporters between CEp and PCOSEp, it appears that the transporter OATP-E is the only one that modifies the level of its mRNA, being lower in PCOSEp ($p < 0.05$) (Fig. 1).

3.3. Protein levels of the transporter OATP-E

The presence and location of the transporter OATP-E in the endometrial cells of the studied groups were performed by IF. The staining was detected in a peripheral location in endometrial cells from both compartments, as shown in Fig. 2(A) and (B). In fact, the PCOSEp group exhibits a significant increase of fluorescence compared with the CEp group ($p < 0.05$) in the epithelial compartment. Although similar results were obtained between epithelia from proliferative and secretory phase of control endometria, likewise, no differences were observed in the stromal compartment between the studied groups.

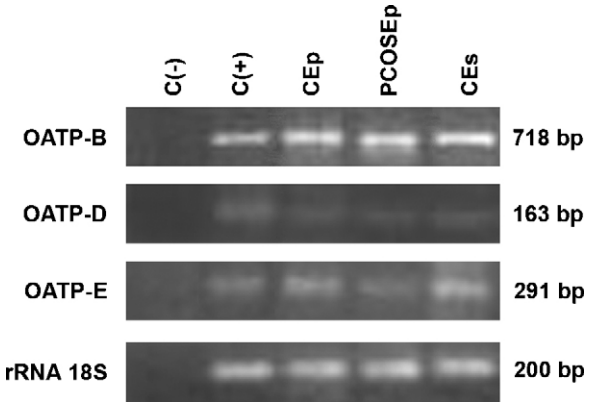

Fig. 1. Semiquantitation of organic anion transporter polypeptide B (OATP-B), D (OATP-D) and E (OATP-E). Conventional RT-PCR was performed in human endometria: negative control C(−), performed without cDNA; positive control C(+), performed using mammary gland cDNA; control endometria in proliferative phase (CEp); polycystic ovarian syndrome endometria in proliferative phase (PCOSEp) and control endometria in secretory phase (CEs). Representative gel (2% agarose gel electrophoresis, staining with ethidium bromide). rRNA 18 s was used as the internal control. The number of women evaluated was $n = 7$ for each of the analysed groups.

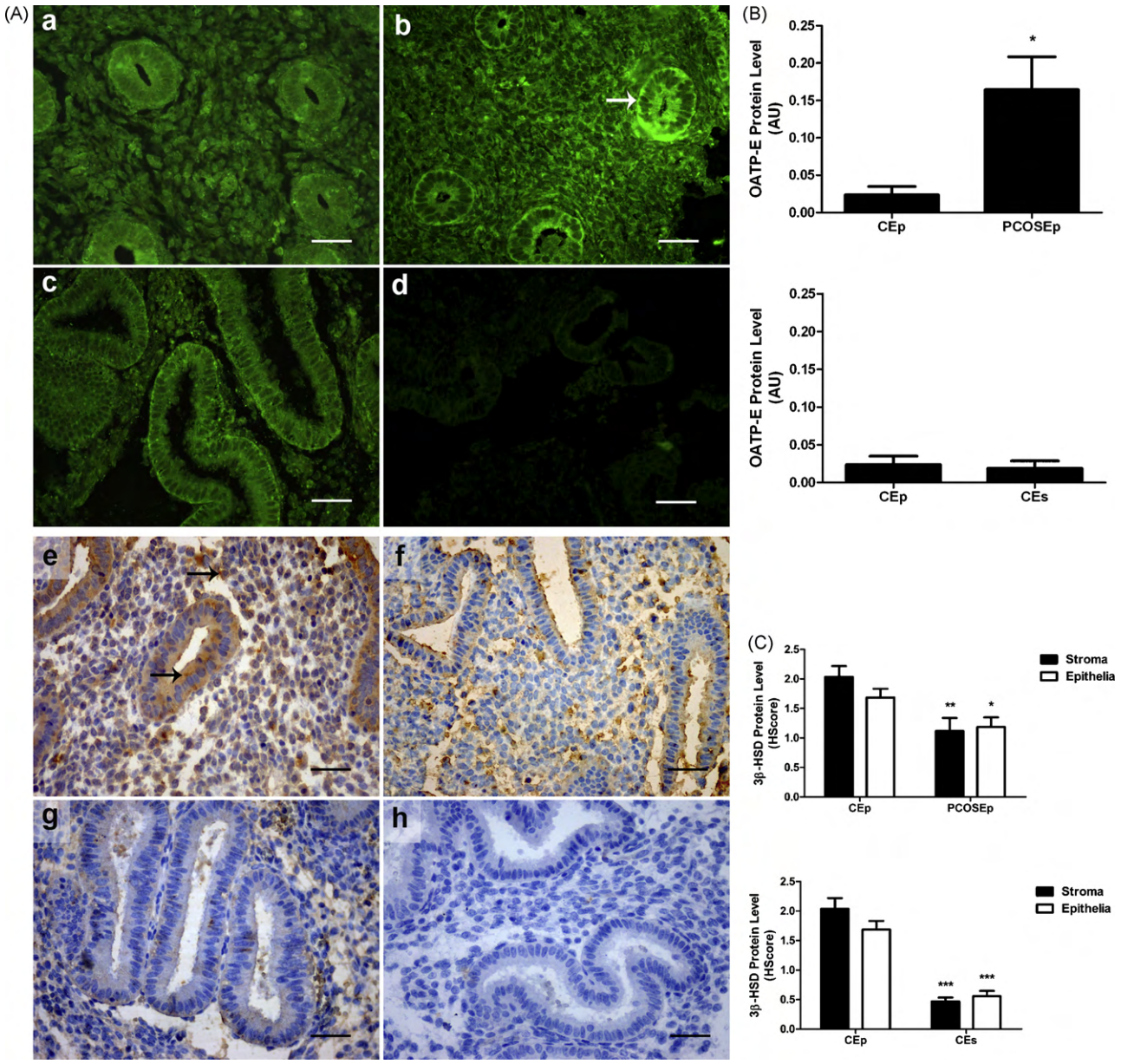

Fig. 2. (A) In a, b, c, d representative microphotographs of immunofluorescence detection of OATP-E in human endometria obtained from control in proliferative phase (a); polycystic ovarian syndrome in proliferative phase (b); control in secretory phase (c); the negative control (d) performed by omitting the primary antibody. The immunolocalization was in the cytoplasm towards the cell periphery. In e, f, g, h is shown representative microphotographs of the immunohistochemistry detection for 3β hydroxysteroid dehydrogenase (3β-HSD) in endometria of control proliferative phase (e); polycystic ovarian syndrome in proliferative phase (f); control in secretory phase (g); the negative control (h) performed by omitting the primary antibody. The positive staining was observed at the cytoplasm level and it was of granular type. The arrows indicate the positive immunostaining. Magnification in all panels is 400× and the scale bars represent 50 μm. The number of women evaluated was $n = 7$ for each of the analysed groups. (B) Semiquantitation of OATP-E protein. Endometria from control in proliferative phase (CEp); polycystic ovarian syndrome in proliferative phase (PCOSEp); control in secretory phase (CEs). The results were measured as the ratio of area of object of total selected area and expressed as AU ± S.E.M, as we described in Section 2. $p < 0.05$ vs. CEp. (C) Semiquantitation of 3β-HSD protein. The immunostaining was measured by HScore, expressed as HS ± S.E.M. $^{*}p < 0.05$ vs. CEp; $^{**}p < 0.01$ vs. CEp; $^{***}p < 0.001$ vs. CEp.

3.4. Protein levels of the enzyme 3β-HSD

The enzyme 3β-HSD plays an important role in the intracellular formation of androgens; therefore, we assessed the location and protein levels of the enzyme by IHC and WB analysis. IHC showed that the staining for 3β-HSD was homogeneous and of the granular type and was only detected at the cytoplasm level (Fig. 2(A) and (C)). In addition, the semiquantitative analysis indicated a decrease in the immunostaining during the menstrual cycle, with a significant difference between CEs and CEp ($p < 0.001$) in the glandular epithelium and in the stromal compartment. In PCOSEp, the immunostaining for 3β-HSD protein detected in the glandular epithelium and the stroma was significantly lower than in CEp (epithelium: $p < 0.05$; stroma: $p < 0.01$). These observations were confirmed by WB evaluation with significant differences between CEp and PCOSEp ($p < 0.05$), and between CEs and CEp ($p < 0.05$) (Fig. 3), showing higher protein levels in CEp.

3.5. Metabolism of DHEA to androstenediol

The formation of metabolites with oestrogenic and/or androgenic activities is relevant to the intracrinology of cells. To further understand the steroid action within endometrial cells, we evaluated the metabolism of DHEA to androstenediol by TLC–HPLC.

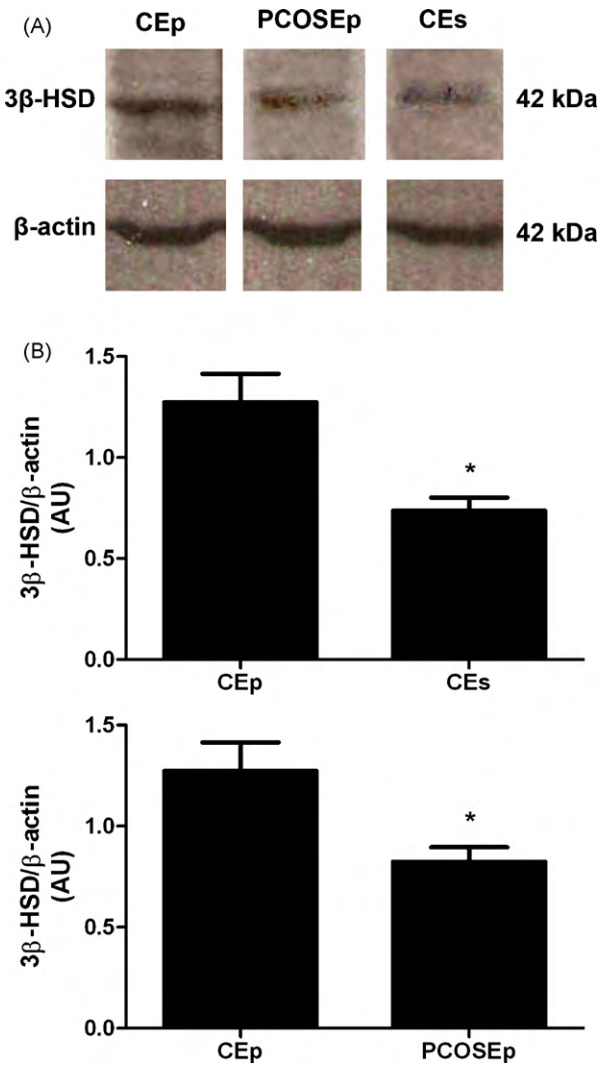

Fig. 3. Western blotting analysis of 3β hydroxysteroid dehydrogenase (3β-HSD) protein levels in human endometria. (A) Representative gels of 3β-HSD and β-actin bands of control endometria in proliferative phase (CEp), polycystic ovarian syndrome endometria in proliferative phase (PCOSEp) and control endometria in secretory phase (CEs). (B) 3β-HSD protein levels corrected by β-actin levels and expressed as AU ± S.E.M. $^{*}p < 0.05$ vs. CEp. The number of women evaluated was $n = 7$ for each of the analysed groups.

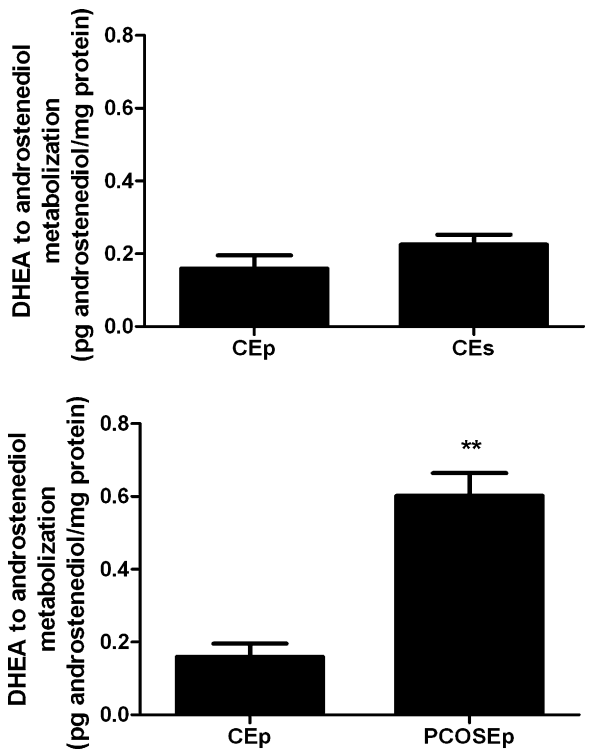

Fig. 4. Thin layer chromatography and high-performance liquid chromatography assays for the study of metabolism of DHEA to androstenediol performed in human endometria: control in proliferative phase (CEp), polycystic ovarian syndrome in proliferative phase (PCOSEp) and control in secretory phase (CEs). The results are expressed as pg of androstenediol/mg of protein ± S.E.M. $^{**}p < 0.01$ vs. CEp.

The data presented in Fig. 4 reveal that the PCOSEp group exhibited an increase in production by 275% of androstenediol than controls (PCOSEp: 0.6 ± 0.06 pmol androstenediol mg^{-1} protein vs. CEp: 0.16 ± 0.04 pmol androstenediol mg^{-1} protein; $p < 0.01$). In parallel, HPLC confirmed that androstenediol was the metabolite quantified from TLC.

4. Discussion

The human endometrium is a tissue highly sensitive to the effects of steroids, which lead to both morphological and molecular changes during the normal menstrual cycle. As known, the endometrium present changes in the levels of some proliferation/apoptosis-related proteins throughout the menstrual cycle. In fact, the proliferation rate is high during the first phase of the cycle under the effect of oestrogens, whereas, a cell-differentiation process occurs in the secretory phase induced by progesterone. Further, androgens exhibit a role in the modulation of cell survival [1,2,41]. Because the enzyme 3β-HSD has a key role in the formation of androgens in the cells, it is important to note that the protein levels of this enzyme changes during the normal menstrual cycle, being high during the proliferative phase and decreasing in the secretory phase, as observed in this study. Therefore, the data of the present investigation allow us to propose that in proliferative phase the metabolism of molecules with androgenic activity such as testosterone, androstenedione and 5α-DHT, could be increased, suggesting a positive effect on cell proliferation, besides the effect of ovarian oestrogens. This is consistent with a study, which indicates that androgens increase cell proliferation in the stromal compartment of human endometrial tissue [41]. Moreover, during the secretory phase, the decrease in protein levels of the enzyme may prevent the metabolic activity of molecules with androgenic activity. The protein levels of 3β-HSD decrease apparently associated with hormonal changes during the menstrual cycle, because there is information showing that steroid hormones have a regulatory role upon the enzyme. Indeed, in tissues such as placenta, 17β-oestradiol stimulates the transcription of the enzyme as well as androgens in the ovary of women with PCOS, but 17β-oestradiol has a repressor action in the transcription of 3β-HSD in human corpus luteum [42,43].

It is known that the sensitivity of endometrial tissue to steroid hormones, specifically to oestrogen together with the effect exerted by progesterone, induces cell proliferation and differentiation required for embryonic implantation during the secretory phase [1]. On the other hand, women with PCOS have higher prevalence of abortion and recurrent miscarriages, and to develop endometrial hyperplasia and adenocarcinoma [2,9]. Therefore, these findings indicate that women with PCOS, in addition to the anomalies present in ovarian function, can present alterations in the function of other tissues such as the endometrium.

In previous studies from our laboratory, a change in the homeostasis of the endometrial cells of these patients was reported, characterised by increased protein Ki67 and phospho-histone 3,

markers of cell cycle and mitosis, respectively [36,44]. Furthermore, in the endometrium of women with PCOS, an increased Bcl-2/Bax ratio [36,44,45] and increased levels of cyclin D1 protein (unpublished data), involved in the progression of G1 to S and decrease in p27, a cell-cycle inhibitor, were found [46]. These antecedents strongly suggest an increase in cell proliferation and a decrease in the process of apoptosis, which is reflected in the potential development of endometrial hyperplasia and adenocarcinoma in women with PCOS.

Alterations in the regulation of the cell cycle could be importantly related with changes in the intracellular concentration of steroid hormones and their action on the endometrial cells. This tissue, in women with PCOS, exhibits a high expression of oestrogen receptor α, androgen receptor and co-activators of steroid hormone receptors [12,13,47,48], which together with the results of the present study suggest that the sensitivity of the PCOS endometria to the action of steroid hormones is increased.

Some unpublished data from our laboratory show no differences in testosterone, androstenedione, oestradiol or progesterone levels between endometria from PCOS women and control subjects. Therefore, these patients have similar levels of circulating oestrogen or tissue concentration compared with controls, which lead us to believe that molecules generated by the intracrine metabolism that have oestrogenic action, such as androstenediol, could be acting upon the human endometrium. In fact, studies on cell lines of prostate cancer (LNCaP) and breast cancer (MCF-7), found that androstenediol is able to bind to the androgen and oestrogen receptors, stimulating cell proliferation. As reported, the binding of androstenediol to oestrogen or androgen receptors is dependent on oestrogen tissue concentration [28,29]. Therefore, if there is a high concentration of oestradiol, androstenediol preferentially binds to the androgen receptor and *vice versa*; when low levels of oestradiol are present, androstenediol binds to the oestrogen receptors. The high levels of endometrial androstenediol found in our model suggest the preferential binding to oestrogen receptors. Undoubtedly, the action at the cellular level depends on the interaction between the ligand, the receptors and co-regulators, which should be addressed in the future.

Androstenediol is a steroid that derives from the precursor DHEA of adrenal origin. In this study, we found significantly higher levels of circulating DHEA-S in PCOS patients compared with the control group, although within the normal range. These findings are consistent with those reported in the literature [6,7]. The high plasma concentration of DHEA-S, coupled with the high levels of the transporter OATP-E, suggest a potential higher uptake of DHEA-S into the endometrial cells from PCOS women, which will be discussed later. As mentioned, the DHEA-S requires transporters to enter cells, which until now have not been characterised in endometrial tissue. In fact, to our knowledge, this is the first study to describe the expression of OATPs in human endometrium throughout the menstrual cycle. In this study, we obtained transcripts of transporters OATP-B, OATP-D and OATP-E in human endometrium during the menstrual cycle and also those from women with PCOS. The OATP-E transporter transcript levels were the only one increased in the control endometrium in the secretory phase of the menstrual cycle. On the other hand, a lower transcript expression of OATP-E in the endometrium of PCOS women was observed compared with the control group. These differences in the mRNA expression could involve changes within the tissue protein levels of steroids receptors and co-regulators, where the action of testosterone increases and oestrogen decreases gene transcription [49]. This has been described for *oatp1* mouse kidney tissue but not in liver, suggesting a tissue-specific regulation. Importantly, in the present study, we found a high protein expression of the transporter OATP-E in the endometrium of women with PCOS. The discrepancies between transcript and protein levels are unclear, and further investigation is needed to elucidate this finding.

The data of this study suggest a greater uptake of sulphated steroids by the endometrial cells of women with PCOS in the glandular epithelial compartment, even though in the present investigation we did not measure the activity of transporters. Once DHEA-S enters the cell, it is the precursor of the metabolic intracrine pathway. In DHEA replacement therapy for postmenopausal women, endometrial atrophy persists after 12 months of treatment, probably due to the lack of metabolisation of this steroid [50]. Nevertheless, the changes observed in the PCOS endometria generate a high sensitivity of the steroids and an increase in the metabolisation of DHEA [12,13,35,51].

In our previous studies, it was determined that there is an increase in the activity of sulphatase, the enzyme that transforms DHEA-S into DHEA, in PCOS endometrium compared with controls [51]. These results indicate an increase in free DHEA and, therefore, the availability of this precursor to be metabolised by enzymes such as 17β-HSD and 3β-HSD. Previous studies from our group indicate that it is most likely that the metabolism of DHEA to androstenediol and of androstenedione to testosterone could be facilitated in women with PCOS, because an increase in the transcript of 17β-HSD type 1 vs. type 2 and reduced activity of 17β-HSD type 2 was detected [35,51]. Consistent with the above information, the results obtained in this investigation indicate that the metabolism of DHEA to androstenediol is favoured in patients with PCOS, probably due to the higher concentration of enzyme17β-HSD isoform 1 [35]. Therefore, we can speculate that, in the PCOS endometrium, a high tissue concentration of androstenediol may be generated, which could bind to the oestrogen receptor and co-activators, exerting an oestrogenic effect in the cells.

Another of the enzymes involved in this metabolic pathway is 3β-HSD, which converts DHEA to androstenedione and androstenediol to testosterone [43]. Our data in the endometrium of women with PCOS revealed that they have a lower protein expression of 3β-HSD compared with the endometrium of control women. These findings suggest that the amount of enzyme is limiting for the conversion of androstenediol to testosterone, allowing a greater availability of androstenediol. To address this point more precisely, future studies will be necessary to determine the enzymatic activity of 3β-HSD in this tissue. Consequently, previous data from our laboratory indicate that the PCOS endometrium has a high sensitivity to oestrogen action [12], which together with the results obtained in the present study, suggest a greater potential input of DHEA-S, an increased metabolism of DHEA to androstenediol as well as the limited transformation of androstenediol to testosterone in endometrial cells of patients with PCOS. We can infer that the high concentration of androstenediol in the endometrial cells of women with PCOS can bind to oestrogen receptors and promote the transcription of genes that regulate cell proliferation.

Finally, because we know that there is a high sensitivity of the endometrium to oestrogen and androgen action, our hypothesis has been focussed on the possible oestrogenic activity of androstenediol, based primarily on the known ability of oestrogen to regulate the cell cycle during the normal menstrual cycle. However, it is possible that androgens may also have a significant role in these processes, with androstenediol acting on the androgen receptor as well as other androgens with high activity such as DHT. Therefore, further work must not only focus on androstenediol and its role in the homeostatic alteration of endometrial tissue, but also on other metabolites with androgen action, such as DHT.

Acknowledgements

The authors thank M.D. Armando Cortinez and Emiliano Soto (University of Chile Medical School) for their role in the recruitment

of subjects and to Ketty Bacallao, PhD, for laboratory assistance. We are also grateful to the women who donated tissue. This study was supported by grant no. 1095127 from the Fondo Nacional de Desarrollo Científico y Tecnológico, Chile.

References

[1] Giudice L. Elucidating endometrial function in the post-genomic era. Hum Reprod Update 2003;9:223–35.
[2] Giudice L. Endometrium in PCOS: implantation and predisposition to endocrine CA. Best Pract Res Clin Endocrinol Metab 2006;20:235–44.
[3] Dunaif A. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. Endocr Rev 1997;18:774–800.
[4] Rötterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril 2004;81:19–25.
[5] Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. Positions statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. J Clin Endocrinol Metab 2006;91:4237–45.
[6] Kumar A, Woods K, Bartolucci A, Azziz R. Prevalence of adrenal androgen excess in patients with the polycystic ovary syndrome (PCOS). Clin Endocrinol 2005;62:644–9.
[7] Puurunen J, Piltonen T, Jaakkola P, Ruokonen A, Morin-Papunen L, Tapanainen JS. Adrenal androgen production capacity remains high up to menopause in women with polycystic ovary syndrome. J Clin Endocrinol Metab 2009;94:1973–8.
[8] Futterweit W. Review: polycystic ovary syndrome: clinical perspectives and management. Obstet Gynecol Surv 1999;54:403–13.
[9] Pillay OC, Wong Te Fong LF, Crow JC, Benjamin E, Mould T, Atiomo W, et al. The association between polycystic ovaries and endometrial cancer. Hum Reprod 2006;21:924–9.
[10] Elliott J, Hosford S, Demopoulos R, Perloe M, Sills E. Endometrial adenocarcinoma and polycystic ovary syndrome: risk factors, management, and prognosis. South Med J 2001;94:529–31.
[11] Cheung A. Ultrasound and menstrual history in predicting endometrial hyperplasia in polycystic ovary syndrome. Obstet Gynecol 2001;98:325–31.
[12] Quezada S, Avellaira C, Johnson M, Gabler F, Fuentes A, Vega M. Evaluation of steroid receptors, coregulators and molecules associated with uterine receptivity in secretory endometria from untreated women with polycystic ovary syndrome. Fertil Steril 2006;85:1017–26.
[13] Villavicencio A, Bacallao K, Avellaira C, Gabler F, Fuentes A, Vega M. Androgen and estrogen receptors and co-regulators levels in endometria from patients with polycystic ovarian syndrome with and without endometrial hyperplasia. Gynecol Oncol 2006;103:307–14.
[14] Labrie C, Bélanger A, Labrie F. Androgenic activity of dehydroepiandrosterone and androstenedione in the rat ventral prostate. Endocrinology 1988;123:1412–7.
[15] Labrie F, Bélanger A, Luu-The V, Labrie C, Simard J, Cusan L, et al. DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during. Aging Steroids 1998;63:322–8.
[16] Labrie F, Luu-The V, Lin SX, Simard J, Labrie C, El-Alfy M, et al. Intracrinology: role of the family of 17β-hydroxysteroid dehydrogenases in human physiology and disease. J Mol Endocrinol 2000;25:1–16.
[17] Mikkaichi T, Suzuki T, Tanemoto M, Ito S, Abe T. The organic anion transport (OATP) family. Drug Metab Pharmacokin 2004;19:171–9.
[18] Pizzagalli F, Varga Z, Huber RD, Folkers G, Meier PJ, St-Pierre MV. Identification of steroid sulfate transport processes in the human mammary gland. J Clin Endocrinol Metab 2003;88:3902–12.
[19] Sai Y, Kaneko Y, Ito S, Mitsuoka K, Kato Y, Tamai I, et al. Predominant contribution of organic anion transporting polypeptide oatp-b (oatp2b1) to apical uptake of estrone-3-sulfate by human intestinal caco-2 cells. Drug Metab Dispos 2006;34:1423–31.
[20] Adachi H, Suzuki T, Abe M, Asano N, Mizutamari H, Tanemoto M, et al. Molecular characterization of human and rat organic anion transporter OATP-D. Am J Physiol Renal Physiol 2003;285:F1188–97.
[21] Labrie F, Luu-The V, Bélanger A, Lin SX, Simard J, Pelletier G. Is dehydroepiandrosterone a hormone? J Endocrinol 2005;187:169–96.
[22] Adams J. Control of secretion and the function of C19-Δ5 steroids of the human adrenal gland. Mol Cell Endocrinol 1985;41:1–17.
[23] Poulin R, Labrie F. Stimulation of cell proliferation and estrogenic response by adrenal C19-Δ5-steroids in the ZR-75-71 human breast cancer cell line. Cancer Res 1986;46:4933–7.
[24] Simard J, Vincent A, Duchesne R, Labrie F. Full oestrogenic activity of C19-Δ5 adrenal steroids in rat pituitary lactotrophs and somatotrophs. Mol Cell Endocrinol 1988;55:233–42.
[25] Nakata T, Takashima S, Shiotsu Y, Murakata C, Ishida H, Akinaga S, et al. Role of steroid sulfatase in local formation of estrogen in postmenopausal breast cancer patients. J Steroid Biochem Mol Biol 2003;86:455–60.
[26] Mizokami A, Koh E, Fujita H, Maeda Y, Egawa M, Koshida K, et al. The adrenal androgen androstenediol is present in prostate cancer tissue after androgen deprivation therapy and activates mutated androgen receptor. Cancer Res 2004;64:765–71.
[27] Chang HC, Miyamoto H, Marwah P, Lardy H, Yeh S, Huang K, et al. Suppression of Δ5-androstenediol-induced androgen receptor transactivation by selective steroids in human prostate cancer cells. Proc Natl Acad Sci USA 1999;96:11173–7.
[28] Miyamoto H, Yeh S, Lardy H, Messing E, Chang C. Δ5-Androstenediol is a natural hormone with androgenic activity in human prostate cancer cells. Proc Natl Acad Sci USA 1998;95:11083–8.
[29] Andò S, De Amicis F, Rago V, Carpino A, Maggiolini M, Panno ML, et al. Breast cancer: from estrogen to androgen receptor. Mol Cell Endocrinol 2002;193:121–8.
[30] Adams J. Adrenal androgens and human breast cancer: a new appraisal. Breast Cancer Res Treat 1998;51:183–8.
[31] Paris F, Servant N, Térouanne B, Sultan C. Evaluation of androgenic bioactivity in human serum by recombinant cell line: preliminary results. Mol Cell Endocrinol 2002;198:123–9.
[32] Kuiper G, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997;138:863–70.
[33] Maliqueo M, Atwater I, Lahsen R, Pérez-Bravo F, Angel B, Sir-Petermann T. Proinsulin serum concentrations in women with polycystic ovary syndrome: a marker of β-cell dysfunction? Human Reprod 2003;18:2683–8.
[34] Noyes R, Hertig A, Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol 1975;122:262–3.
[35] Bacallao K, Leon L, Gabler F, Soto E, Romero C, Valladares L, et al. *In situ* estrogen metabolism in proliferative endometria from untreated women with polycystic ovarian syndrome with and without endometrial hyperplasia. J Steroid Biochem Mol Biol 2008;110:163–9.
[36] Avellaira C, Villavicencio A, Bacallao K, Gabler F, Wells P, Romero C, et al. Expression of molecules associated with tissue homeostasis in secretory endometria from untreated women with polycystic ovary syndrome. Hum Reprod 2006;21:3116–21.
[37] Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, McCarty KS. Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. J Clin Endocrinol Metab 1988;67:334–40.
[38] Castro A, Johnson MC, Anido M, Cortinez A, Gabler F, Vega M. Role of nitric oxide and bcl-2 family genes in the regulation of human endometrial apoptosis. Fertil Steril 2002;78:587–95.
[39] Jellinck PH, Kaufmann M, Gottfried-Blackmore A, McEwen BS, Jones G, Bulloch K. Selective conversion by microglia of dehydroepiandrosterone to 5-androstenediol—A steroid with inherent estrogenic properties. J Steroid Biochem Mol Biol 2007;107:156–62.
[40] Calle E, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nature Rev Cancer 2004;4:579–91.
[41] Maliqueo MA, Quezada S, Clementi M, Bacallao K, Anido M, Johnson C, et al. Potential action of androstenedione on the proliferation and apoptosis of stromal endometrial cells. Reprod Biol Endocrinol 2004;2:81–90.
[42] Thomas JL, Duax WL, Addlagatta A, Brandt S, Fuller RR, Norris W. Structure/function relationships responsible for coenzyme specificity and the isomerase activity of human type 1 3β-hydroxysteroid dehydrogenase/isomerase. J Biol Chem 2003;278:35483–90.
[43] Simard J, Ricketts ML, Gingras S, Soucy P, Feltus FA, Melner MH. Molecular biology of the 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase gene family. Endocr Rev 2005;26:525–82.
[44] Villavicencio A, Bacallao K, Gabler F, Fuentes A, Albornoz J, Casals A, et al. Deregulation of tissue homeostasis in endometria from patients with polycystic ovarian syndrome with and without endometrial hyperplasia. Gynecol Oncol 2007;104:290–5.
[45] Maliqueo M, Clementi M, Gabler F, Johnson MC, Palomino A, Sir-Petermann T, et al. Expression of steroid receptors and proteins related to apoptosis in endometria of women with polycystic ovary syndrome. Fertil Steril 2003;80:812–9.
[46] Villavicencio A, Goyeneche A, Telleria C, Bacallao K, Gabler F, Fuentes A, et al. Involvement of AKT, Ras and cell cycle regulators in the potential development of endometrial hyperplasia in women with polycystic ovarian syndrome. Gynecol Oncol 2009;115:102–7.
[47] Gregory CW, Wilson EM, Apparao KB, Lininger RA, Meyer WR, Kowalik A, et al. Steroid receptor coactivator expression throughout the menstrual cycle in normal and abnormal endometrium. J Clin Endocrinol Metab 2002;87:2960–6.
[48] Apparao KB, Lovely LP, Gui Y, Lininger RA, Lessey BA. Elevated endometrial androgen receptor expression in women with polycystic ovarian syndrome. Biol Reprod 2002;66:297–304.
[49] Hagenbuch B, Meier PJ. The superfamily of organic anion transporting polypeptides. Biochim Biophys Acta 2003;1609:1–18.
[50] Labrie F, Diamond P, Cusan L, Gómez JL, Bélanger A, Candas B. Effect of 12-month dehydroepiandrosterone replacement therapy on bone, vagina, and endometrium in postmenopausal women. J Clin Endocrinol Metab 1997;82:3498–505.
[51] León L, Bacallao K, Gabler F, Romero C, Valladares L, Vega M. Activities of steroid metabolic enzymes in secretory endometria from untreated women with polycystic ovary syndrome. Steroids 2008;73:88–95.