In situ estrogen metabolism in proliferative endometria from untreated women with polycystic ovarian syndrome with and without endometrial hyperplasia

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Keywords:
Steroid metabolic enzyme
PCOS
Proliferative endometrium
Endometrial hyperplasia

A B S T R A C T

The aim of the present investigation was to study whether the endocrinological status of women bearing polycystic ovarian syndrome (PCOS) affects the endometrial *in situ* steroid metabolism. For this purpose, we evaluated the mRNA levels (RT-PCR), and the activity of steroid metabolic enzymes: P450 aromatase, steroid sulfatase (STS), estrogen sulfotransferase (EST) and 17β-hydroxysteroid dehydrogenase (17β-HSD) in 23 samples of normal endometria (CE), 18 PCOS endometria without treatment (PCOSE), 10 specimens from PCOS women with endometrial hyperplasia (HPCOSE), and 7 endometria from patients with endometrial hyperplasia not associated to PCOS (EH). The data showed lower levels of STS mRNA for PCOSE and HPCOSE ($p < 0.05$, $p < 0.01$, respectively) and of EST for HPCOSE and EH compared to control ($p < 0.05$). However, higher levels for EST mRNA were obtained in PCOSE ($p < 0.05$) versus CE. The mRNA and protein levels for P450 aromatase were undetectable in all analyzed endometria. The relationship between the activities of STS and EST was lower in PCOSE and HPCOSE ($p < 0.05$) versus CE. The ratio betweeen the mRNA from 17β-HSD type 1/type 2 was higher in PCOSE ($p < 0.05$), whereas, a diminution in the 17β-HSD type 2 activity was observed in PCOSE ($p < 0.05$). These results indicate that the activity of enzymes related to the steroid metabolism in analyzed PCOSE differ from those found in the CE. Consequently, PCOSE may present an *in situ* deregulation of the steroid metabolism.

1. Introduction

Polycystic ovary syndrome (PCOS) is an endocrine-metabolic pathology. This disorder has uncertain aetiology, and affects between 6 and 10% of women in reproductive age. An international consensus group [1] proposed that the syndrome can be diagnosed after the exclusion of other medical conditions that cause irregular menstrual cycles and androgen excess. Therefore, the determination of at least two of the following should be present in PCOS oligoovulation or anovulation (usually manifested as oligomenorrhea or amenorrhea), elevated levels of circulating androgens (hyperandrogenemia) or clinical manifestations of androgen excess (hyperandrogenism), and polycystic ovaries as defined by ultrasonography [1].

The endometrium from PCOS women is different from normal endometrium, and is consistent with a poor reproductive potential, a higher rate of spontaneous miscarriage [2,3], and higher incidence of hyperplasia and carcinoma [4,5]. There are evidences that indicate that the increased prevalence of endometrial hyperplasia and carcinoma in PCOS women has been attributed to the persistent stimulation of endometrial tissue by estrogens without the progesterone-induced inhibition of proliferation and differentiation to secretory endometrium that occurs after ovulation [6]. On the other hand, a substantial proportion of PCOS women are overweight and many are obese [7]; and was reported that obesity, as well as, decreased concentrations of sex-hormone binding globulin (SHBG), are associated with endometrial cancer [8]. Besides, our group has reported the presence of higher levels of estrogen receptor alpha (ERα), the co-activators of steroid receptors AIB1 and ARA70 and increased levels of Ki67 in endometria from PCOS women respect to CE, which indicated higher estrogen sensitivity and greater levels of cells in the proliferative cell cycle in those endometria [9,10].

The *in situ* steroid hormone metabolism and synthesis have been considered to play an important role in the development and progression of cancer [11,12]. Therefore, it becomes important to analyze the expression of enzymes involved in the *in situ* endometrial steroid metabolism in an endocrine-metabolic pathology like

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PCOS, to understand the effect of its local hormonal environment. Some of the important enzymes related to steroid metabolism in human endometria are cytochrome P450 aromatase, steroid sulfatase (STS), estrogen sulfotransferase (EST) and isoforms 1 and 2 of 17β-hydroxysteroid dehydrogenase (17β-HSD).

The enzyme P450 aromatase converts androstenedione and testosterone, into estrone (E_1) and 17β-estradiol (E_2), respectively [13,14], whereas, STS, member of a superfamily of sulfatases, hydrolyzes biologically inactive estrogen sulfates (E_1-S or E_2-S) to free estrogens [15]. Another enzyme involved in the steroid metabolism is EST, member of a superfamily of steroid sulfotransferases, sulfonates estrogens to estrogen sulfates [16]. Both enzymes, STS and EST, are expressed in a variety of tissues, including normal human endometrium [17,18]. A recent report indicate that in human breast carcinoma, the balance between the levels of intratumoral STS and EST may play an important role in the regulation of the *in situ* estrogen levels [19]. The activities of STS and EST have also been examined in estrogen-dependent neoplasms such as endometrial carcinoma [20].

The interconversion of E_1 to E_2 is catalyzed by the isoforms of 17β-hydroxysteroid dehydrogenase (17β-HSD). There are at least 12 isoforms of this enzyme being the reduction reactions catalyzed by the isoforms with odd numbers and the oxidation reaction by the even numbers [21]. In fact, the enzyme 17β-HSD type 1 catalyzes the conversion of E_1 to E_2 and therefore, it generates the active estrogen; meanwhile, the isoform 2 catalyzes the formation of E_1 from E_2 [22].

Based on the overexpression of ERα and co-activators reported in PCOS endometria, as well as, the induction of proliferation exerts by estrogens in several tissues, the aim of the present study is to evaluate if the *in situ* metabolism of steroids is modified in endometria from PCOS women.

2. Materials and methods

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

2.1. Reagents

[2,4,6,7-3*H*(*N*)]-estrone (specific activity 80–100 Ci/mmol), [2,4,6,7-3*H*(*N*)]-estradiol (specific activity 115 Ci/mmol] and [6,7-3*H*(*N*)]-estrone sulfate (specific activity 50 Ci/mmol) were purchased from PerkinElmer (LAS, Shelton CT, USA). β-Nicotinamide adenine dinucleotide phosphate (NADP+) and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). TRIzol Reagent was obtained from Invitrogen Corp. (CA, USA); Revertaid H Minus M Mulv Reverse, DNase I and *Taq* DNA polymerase were obtained from Fermentas (Hanover, MD, USA). 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), SHBG concentration by RIA (DPC, Los Angeles, CA, USA) and E_2 by electrochemiluminescence (Roche, Basel, Switzerland).

2.2. Subjects

The endometrial specimens ($n = 58$) were divided into four groups as follows: endometria obtained from women with proven fertility obtained in proliferative phase of the menstrual cycle (CE, $n = 23$); endometrium obtained from patients with PCOS with no treatment (PCOSE, $n = 28$). The latter group was divided in two subgroups; one subgroup include endometria without hyperplasia (PCOSE, $n = 18$) and the other, with endometrial hyperplasia (HPCOSE, $n = 10$). Finally, the fourth group of endometria were specimens with endometrial hyperplasia not associated to PCOS (EH, $n = 7$). The control women, PCOS patients (PCOSE and HPCOSE) and patients with endometrial hyperplasia were accrued prospectively, and each group was recruited independently. Human endometria were obtained with a Pipelle suction curette from the corpus of the uteri of women with PCOS (PCOSE and HPCOSE). The diagnosis of PCOS was made according to the Rotterdam Consensus, considering two criteria out of three (oligo and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, polycystic ovaries). Hyperprolactinemia, androgen secreting tumors, Cushing's syndrome, congenital adrenal hyperplasia, attenuated 21-hydroxylase deficiency and thyroid disease, were excluded by appropriate tests. Endometria with hyperplasia from patients without PCOS were obtained at hysterectomy. The PCOS women were not consecutive patients, and the endometrial hyperplasia found in a subgroup of PCOS women was detected after the analysis of their biopsies. The diagnosis of endometrial hyperplasia in patients with and without PCOS was established according to Kurman et al. criteria [23] by an experienced histopathologist.

Control endometria were obtained from fertile healthy women during the proliferative phase of their menstrual cycle at the time of bilateral tubal ligation at the San Borja Arriaran Clinical Hospital of the University of Chile, National Health Service (Santiago, Chile). The proven fertility was determined by the following criteria: (a) regular menstrual cycles, (b) proven natural fertility by at least one pregnancy carried to term within normal range of time (1 year or less exposed to pregnancy risk), (c) no evidences of endocrinological diseases and (d) no ovarian abnormalities as assessed by transvaginal ultrasound.

None of the women had received hormonal therapy or other medications within 3 months prior to recruitment into the study, and all the endometria used in this study showed normal morphology. The controls were selected in the proliferative phase because of the similar morphology of the proliferative endometrium and PCOSE. The proliferative phase was confirmed according to the histological criteria of Noyes et al. [24].

2.3. Tissue preparation

Endometrial tissue samples were divided into five or more pieces by the pathologist. One piece of each sample was frozen in liquid N_2 and maintained at −80 °C for reverse transcription-polymerase chain reaction (RT-PCR). Another piece of sample was included in paraffin for morphologycal studies and three pieces of tissue were used for enzymatic activity assays, being each enzymatic activity performed in the same tissue sample.

2.4. RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from endometrial tissue using TRIzol Reagent according to the manufacturer's instructions. The concentration of RNA was determinated spectrophotometrically while the integrity was determined by electrophoresis on a formaldehyde agarose gel under denaturing conditions. The RNA was visualized by adding ethidium bromide (EtBr) to the sample before loading on the gel. The RNA was stored at −80 °C until use. Two micrograms of total RNA were digested with DNase I and transcribed into complementary DNA (cDNA) by reverse transcription with Revertaid H Minus M-Mulv Reverse by using random primers in a total volume of 25 μl. The PCR amplifications were obtained by using gene-specific primers (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Previous

Table 1
Primers sequences used for PCR of cDNA for the analysis of the enzymes P450 aromatase ($P450_{arom}$), steroid sulfatase (STS), estrogen sulfotransferase (EST), 17β-hydroxysteroid dehydrogenase (17β-HSD) type 1 and 2

Gene	Primers	Sequence	Denaturation	Annealing	Extention	No. of cycles
$P450_{arom}$	Sense Antisense	5′-TGG CTA CCC AGT GAA AAA GG-3′ 5′-TCA AAG CAC ATT TGG TGG AA-3′	94 °C/1 min	50 °C/1 min	72 °C/1.5 min	35
STS	Sense Antisense	5′-AAC TCA CTC AGC ACC TGG CA-3′ 5′-GGG AGG AAG ACC AGC CTC TT-3′	94 °C/1 min	52 °C/1 min	72 °C/1.5 min	28
EST	Sense Antisense	5′-CAA ATC CTG GAT CCT TTC CA-3′ 5′-TCC TGT CCA CAA GCT CCT CT-3′	94 °C/45 s	50 °C/1 min	72 °C/1.5 min	28
17β-HSD 1	Sense Antisense	5′-AGG CTT ATG CGA GAG TCT GG-3′ 5′-CAT GGC GGT GAC GTA GTT GG-3′	94 °C/1 min	52 °C/1 min	72 °C/1.5 min	35
17β-HSD 2	Sense Antisense	5′-CTG AGG AAT TGC GAA GAA CC-3′ 5′-GAA GTC CTT GCT GGC TAA CG-3	94 °C/1 min	55 °C/1 min	72 °C/1.5 min	30
GAPDH	Sense Antisense	5′-CCA CCA TGG AGA AGG CTG GG-3′ 5′-ATC ACG CCA CAG TTT CCC GG-3′	94 °C/45 s	55 °C/1 min	72 °C/1.5 min	22

studies performed by our group showed no changes in the mRNA levels of GAPDH in the endometrial tissues [17]. Semi-quantitative RT-PCRs were achieved in the exponential linear zone amplification for each gene studied. The PCR condition for P450 aromatase, STS, EST and GAPDH was 1.5 mM of $MgCl_2$, 0.25 mM of dNTPs, 0.625 U of *Taq* DNA polymerase and 0.4 μM of each primer; for 17β-HSD 1 and 2, was 3 mM of $MgCl_2$, 0.25 mM of dNTPs, 1.5 U of *Taq* DNA polymerase, and 0.4 μM of each primer. The PCR amplification was performed in the Thermocycler, model PTC-100 (MJ Research Inc., Watertown, MA, USA). The PCR products were electrophoretically resolved on 1% agarose gel and stained with ethidium bromide. The bands were evaluated using an image analyzer (Kodak 1D Image Analysis Software, Rochester, NY) and normalized relative to the GAPDH PCR product.

2.5. Enzymatic assay

Twenty-three endometrial cases of frozen tissues were available for the enzymatic assay. As mentioned before, the activity assays for the different enzymes were performed in the same endometrial sample. The optimal conditions for activity assays were previously established in endometrial tissue by our group and other investigators [17,25–27].

The activity of EST was assayed as previously described [17]. In brief, the tissue samples were homogenized at 4 °C in phosphate buffer [100 mM KCl, 10 mM KH_2PO_4, 10 mM Na_2HPO_4, and 1 mM EDTA (pH 7.5)]. Then, the samples were centrifuged for 15 min at 1000 × *g*. The upper layer was used as the enzyme source and to determine the concentration of protein with the BCA Protein Kit Assay (Thermo Fisher Scientific Inc., USA). Approximately 0.25 mg of protein was used in each assay. The protein was added to a mixture that contained 7 mM $MgCl_2$, unlabeled E_1 at 20 μM and [3*H*]-E_1 at 20 nM in the reaction buffer 50 mM Tris–HCl (pH 7.4). The reaction was started with the addition of the cofactor 3′-phosphoadenosine 5′-phosphosulfate (20 μM final concentration) in a final volume of 0.23 ml and incubated at 37 °C for 45 min. It was ended with the addition of 375 μl Tris–HCl 0.25 M (pH 8.7) to alkalinize the solution and followed by the addition of 4.0 ml of chloroform. Then, the reaction mixture was centrifuged at 600 × *g* for 5 min to separate E_1-S (product in aqueous phase) from E_1 (substrate in organic phase). The production of [3*H*]-E_1-S was determined in a liquid scintillation counter (Beckman, LC-6500).

The STS activity was assayed according to previous report [17]. Briefly, the enzyme solution (0.25 mg protein) was mixed with cold E_1-S at 20 μM and [3*H*]-E_1-S. The reaction mixture was incubated at 37 °C for 60 min in a shaking water bath. The enzyme reaction was ended with the addition of 0.375 ml Tris–HCl 0.25 M (pH 8.7) followed by 2.0 ml of toluene and mixed by vortex mixer for 1 min. Then, the reaction mixture was centrifuged at 600 × *g* for 5 min to separate E_1-S (aqueous phase) from E_1 (organic phase). The toluene layer was collected, and the tritium radioactivity, was measured in a liquid scintillation counter.

The enzymatic assay for the isoform type 2 of 17β-HSD was performed as described previously [17]. In brief, the reaction was carried out using 0.25 mg of protein homogenate in the reaction buffer Tris (pH 7.4, 100 mM). To this mixture was added [3*H*]-E_2 and unlabeled E_2 (5 μM) in a total volume of 0.23 ml. The assay was started by the addition of the cofactor NADP+ (1.5 mM) and carried out for 45 min at 37 °C. It was ended by the addition of 100 μl of 0.1 N NaOH and the samples were extracted in diethyl ether/ethyl acetate (9:1). The products were spotted on 20 cm × 20 cm thin layer chromatography (TLC) aluminum sheet pre-coated with silica gel (60 F254, Merck, Darmstadt, Germany) and were separated using chloroform/ethyl acetate (4:1). Excess unlabeled E_2 and E_1 were added to aid the visualization of steroid products with iodine vapors after TLC, and the spots were scraped into scintillation vials. Product formation was determined as the number of disintegrations per minute (dpm) in the spot product. No significant counts were identified in other sites of the plate. The obtained dpm was expressed as product formation.

The enzyme activity was calculated by subtracting from sample dpm, the background dpm (negative control, no enzyme source added); the specific activity was expressed as pmol product/mg protein × h, as used by other investigators [17,25].

2.6. Statistical evaluation

Data are expressed as mean ± S.E.M. The number of subjects in this study was calculated assuming an $\alpha = 0.05$ and $\beta = 20\%$ and a difference between mean of 0.25 and standard deviation of 0.200 according to our previous studies [10]. The data distribution were analyzed using Kolmogorov–Smirnov test. One-way ANOVA test was used, *p*-values less than 0.05 were considered significant. Statistical tests were performed using Graph Pad Prism 4.0.

3. Results

3.1. Clinical and endocrinological characteristics

The clinical and endocrinological characteristics of the four groups of women participants in this study are summarized in Tables 2 and 3. The CE group belongs to the group of bilateral tubal

Table 2
Clinical characteristics of women included in the study

	C	PCOS	HPCOS	EH
n	23	18	10	7
Age	37.1 ± 1.5	26.6 ± 1.0[bd]	29.4 ± 1.4[ac]	41.5 ± 4.1
BMI (kg/m^2)	25.6 ± 1.0	31.4 ± 1.6[a]	31.9 ± 2.0[a]	28.0 ± 2.0

Control (C), patients with polycystic ovarian syndrome without endometrial hyperplasia (PCOS) and with endometrial hyperplasia (HPCOS), patients without polycystic ovary syndrome and with hyperplasia (EH), number of women evaluated in the study (*n*), body mass index (BMI). Values are expressed as mean ± S.E.M. [a]$p < 0.05$ vs. C; [b]$p < 0.01$ vs. C; [c]$p < 0.05$ vs. EH; [d]$p < 0.01$ vs. EH.

ligation, showing a higher range of age than women with PCOS. The higher body mass index observed in the group from PCOS women is inherent to the syndrome, which is in agreement with the fact that around 30% of PCOS patients are obese [28]. In addition, all women with PCOS and PCOS with endometrial hyperplasia presented hyperandrogenism, and the excessive ovarian androgen production besides the decreased SHBG blood level, leads to a significantly higher free androgen index in PCOS women, as shown in Table 3. Otherwise, no important differences in E_2 and P_4 plasma concentrations were found in the four studied groups.

3.2. Messenger RNA levels for P450 aromatase, STS, EST, 17β-HSD type 1 and 2

The mRNA of the P450 aromatase enzyme was not detected in all analyzed endometria, even when the amounts of cDNA and primers were increased. Nevertheless, a band was obtained when corpus luteum was used as a positive control (data not shown). These data suggest that the levels of endometrial mRNA for P450 aromatase are very low, and probably are under the detection limit of the PCR. Therefore, we believe that P450 aromatase pathway does not have an important role in estrogen production in the groups of analyzed endometria.

On the other hand, the profile of STS and EST mRNA expression is presented in Fig. 1. Fragments of 376 bp for STS and 218 bp for EST were observed in the four groups of endometria studied. Interestingly, the mRNA levels for STS were significantly diminished in PCOSE and HPCOSE respect to CE ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 1A and B). The mRNA levels for EST were lower in HPCOSE and EH ($p < 0.05$) and higher in PCOSE ($p < 0.05$) respect to CE (Fig. 1A and C).

The analysis of gene expression for 17β-HSD revealed that fragments of 384 bp for 17β-HSD type 1 and 592 bp for the enzyme type 2 were detected in the four groups of studied endometria. The data show that the mRNA levels of the isoform type 1 were diminished in PCOSE and HPCOSE respect to CE ($p < 0.05$) (Fig. 2A and B). In the case of 17β-HSD 2 the PCOSE presented lower levels of mRNA respect to CE ($p < 0.05$) (Fig. 2A and C). Moreover, the ratio 17β-HSD type 1/type 2 was higher in PCOSE respect to CE ($p < 0.05$) and respect to HPCOSE ($p < 0.05$) (Fig. 3).

Table 3
Hormonal characteristics of control and women with polycystic ovarian syndrome with and without endometrial hyperplasia

	C	PCOS	HPCOS
n	23	18	10
P_4 (ng/ml)	0.9 ± 0.5	1.0 ± 0.3	1.55 ± 1.09
E_2 (pg/ml)	42.4 ± 17.1	63.1 ± 4.7	85.8 ± 9.6
A_4 (ng/ml)	1.7 ± 0.3	4.5 ± 1.6[a]	3.2 ± 0.7
T (ng/ml)	0.4 ± 0.1	0.9 ± 0.1[b]	0.8 ± 0.0[a]
SHBG (nmol/l)	60.4 ± 16.1	23.8 ± 3.1[b]	25.7 ± 5.5[a]
FAI	2.7 ± 0.7	15.0 ± 2.5[a]	11.9 ± 2.3[a]

Progesterone (P_4), estradiol (E_2), androstenedione (A_4), testosterone (T), sex-hormone binding globulin (SHBG), free androgen index obtained from testosterone total/SHBG (FAI), control women (C), patients with polycystic ovarian syndrome without endometrial hyperplasia (PCOS) and with endometrial hyperplasia (HPCOS), number of women evaluated in the study (*n*). The values are expressed as mean ± S.E.M.: [a]$p < 0.05$ vs. C; [b]$p < 0.01$ vs. C.

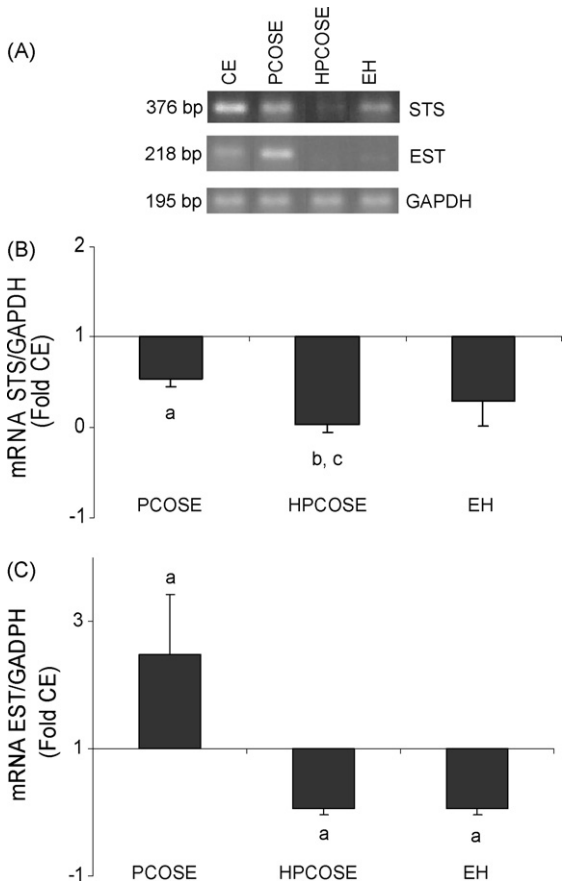

Fig. 1. Semi-quantitation of mRNA levels for the enzymes steroid sulfatase (STS) and estrogen sulfotransferase (EST). Conventional RT-PCR was performed in human endometria: control (CE), polycystic ovarian syndrome without endometrial hyperplasia (PCOSE) and with hyperplasia (HPCOSE) and from women with endometrial hyperplasia (EH). (A) Representative gel (2% agarose gel electrophoresis, staining with ethidium bromide). (B) mRNA for STS. (C) mRNA for enzyme EST. The number of women evaluated was $n = 6$ for all the analyzed groups. The results are expressed as mean ± S.E.M. GAPDH was used as the internal control: (a) $p < 0.01$ vs. CE, (b) $p < 0.01$ vs. CE and (c) $p < 0.01$ vs. ESOP.

3.3. Enzymatic activities for STS, EST and 17β-HSD type 2

A diminished STS activity was determined in PCOSE compared to CE ($p < 0.05$) (Table 4), similar to the profile of mRNA levels, whereas, in the other groups, the STS activity compared to control did not change. In the case of EST, a similar enzyme activity was observed in the four groups of studied endometria (Table 4). A decreased ratio between STS/EST was found in PCOSE and HPCOSE respect to CE ($p < 0.05$) (Fig. 4). On the other hand, the activity of the isoform type 2 of 17β-HSD was diminished in PCOSE respect to CE ($p < 0.05$) (Fig. 5). The latter suggest that in PCOSE may exist a low tendency to inactive estradiol.

4. Discussion

The presence of P450 aromatase in human endometria is discussed. Some reports have shown the expression of P450 aromatase mRNA in normal endometrium [29,30]; however, other investigations have not been able to detect the activity of this enzyme in

Table 4
Steroid sulfatase and estrogen sulfotransferase activities

	CE	PCOSE	HPCOSE	EH
STS (pmol/mg protein × h)	129.0 ± 13.0	67.2 ± 13.7[ab]	90.1 ± 10.6	174.2 ± 51.8
EST (pmol/mg protein × h)	2.1 ± 0.4	2.8 ± 0.3	1.7 ± 0.3	2.5 ± 2.3

Steroid sulfatase (STS), estrogen sulfotransferase (EST), control endometrium (CE), endometrium from patients with polycystic ovarian syndrome without endometrial hyperplasia (PCOSE) and with endometrial hyperplasia (HPCOSE) and endometrium from patients with endometrial hyperplasia not associated to PCOS (EH). The number of women evaluated was $n = 6$ for all the analyzed groups. The values are expressed as mean ± S.E.M.: [a] $p < 0.05$ vs. CE; [b] $p < 0.05$ vs. EH.

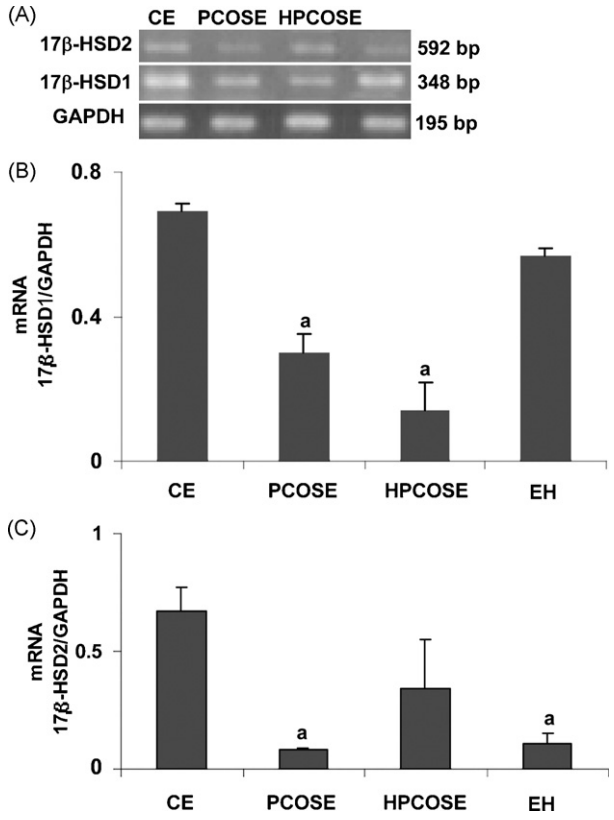

Fig. 2. Semi-quantitation of mRNA levels for enzymes 17β-hydroxysteroid dehydrogenase (17β-HSD) types 1 and 2. Conventional RT-PCR was made for human endometria: control (CE), of patients with polycystic ovarian syndrome without endometrial hyperplasia (PCOSE) and with hyperplasia (HPCOSE) and from women with endometrial hyperplasia (EH). (A) Representative gel of isoforms in tissues (2% agarose gel electrophoresis with ethidium bromide). (B) mRNA for enzyme 17β-HSD type 1. (C) mRNA for enzyme 17β-HSD type 2. The number of women evaluated was $n = 6$ for all the analyzed groups. The results are expressed as mean ± S.E.M. GAPDH was used as the internal control: (a) $p < 0.01$ vs. CE.

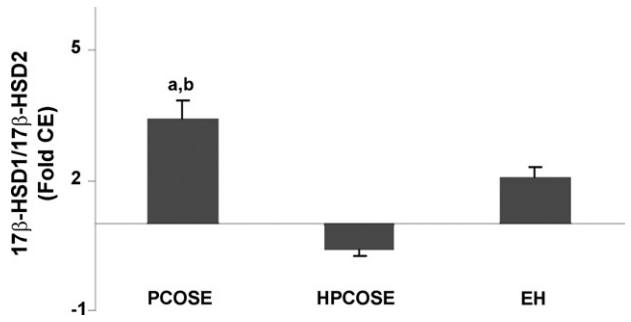

Fig. 3. Ratio between the mRNA levels for enzymes 17β-hydroxysteroid dehydrogenase (17β-HSD) types 1 and 2. The data are presented as fold vs. control (CE 0.9 ± 0.3), to which arbitrary value of 1 was assigned. The significance for PCOSE was (a) $p < 0.05$ vs. CE and (b) $p < 0.05$ vs. HPCOSE.

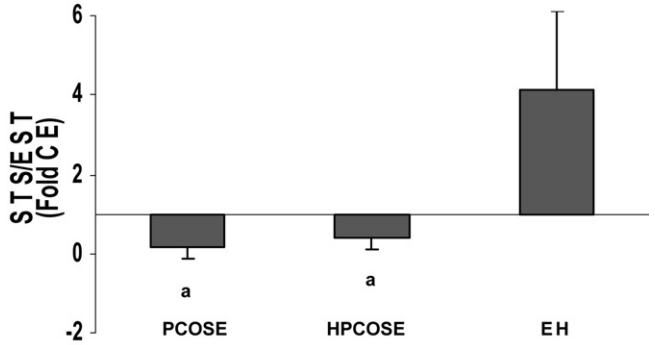

Fig. 4. Ratio between the activities of STS/EST. Both enzymatic activities were determined in endometria from the same women. Control (CE), with polycystic ovarian syndrome without endometrial hyperplasia (PCOSE), and with endometrial hyperplasia (HPCOSE) and from patients with endometrial hyperplasia (EH). The number of women evaluated was $n = 6$ for all the analyzed groups. The results are expressed as fold vs. control (CE: 140.1 ± 17.9 pmol/mg protein × h), to which an arbitrary value of 1 was assigned. (a) $p < 0.05$ vs. CE.

the human endometria [31,32]. The latter agrees with the results obtained in the present work, where nor the mRNA neither the protein for the P450 aromatase were determined in the analyzed endometria. Therefore, it is more likely that the P450 aromatase pathway could not be an important mechanism for the endometrial estrogen production in the analyzed conditions. Moreover, Watanabe et al. [33] reported the absence of endometrial activity for P450 aromatase in hyperplasia; in addition, Ma et al. [34] did not find differences in the immunostaining of the enzyme between normal and hyperplastic endometrium. These findings sustain the deficiency of functional significance of P450 aromatase for the progression to endometrial hyperplasia, in agreement with the data of the present study.

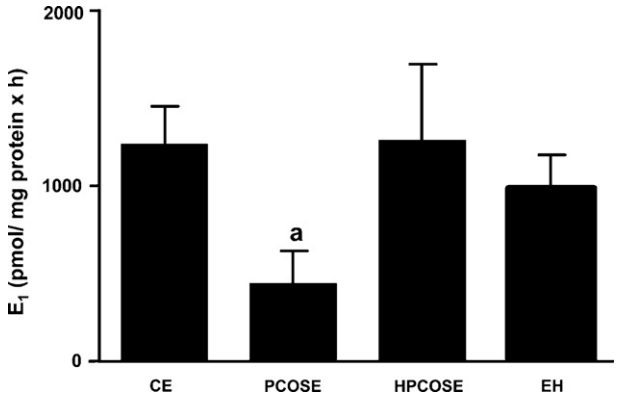

Fig. 5. Enzymatic activity for 17β-HSD type 2 in human endometrium. The activity was assessed in endometria from: control (CE), from polycystic ovarian syndrome patients without hyperplasia (PCOSE) and with endometrial hyperplasia (HPCOSE) and from women with hyperplasia without polycystic ovarian syndrome (EH). The number of women evaluated was $n = 6$ for all the analyzed groups. The results are expressed as specific activity (pmol product/mg protein × h) ± S.E.M: (a) $p < 0.05$ vs. CE.

On the other hand, solid experimental evidences describe the sulfatase pathway as fundamental in estrogen production in different tissues, including the endometrium [35]. This is in accordance with the data of the present investigation. In fact, in all the analyzed human endometria we detected the mRNA for steroid sulfatase, and most importantly, a higher STS activity than that of EST. These results coincide with those reported for breast cancer, where it has been suggested additional routes to the P450 aromatase pathway for the obtention of compounds with estrogenic activity [36]. In this context, it is known that the steroid sulfatase activity in breast cancer cells is at least 100 times greater than the P450 aromatase activity [36]. Besides, it is known that the sulfated estrogens are in higher concentrations in the circulation where they have an average life greater than the free estrogenic forms [37]. To our knowledge, this is the first study to address the activity of enzymes involved in steroid metabolism in proliferative endometrium from patients with disrupted endocrine-metabolic parameters such as PCOS, with and without endometrial hyperplasia and hyperplasia non-associated to PCOS. Nevertheless, it will be necessary to perform further studies with a greater number of patients to confirm the above data. Interestingly, the smaller relationship between steroid sulfatase and EST activities obtained in PCOSE and HPCOSE with respect to control samples, could be related to the fact that sulfated estrogens has been considered a reservoir for the formation of active estrogens, as described by Nakata et al. in breast cancer cells [36]. Nevertheless, the smaller activity of 17β-HSD type 2 found in PCOSE, indicate a smaller tendency to the formation of E_1 from E_2. Therefore, despite the lower activity of steroid sulfatase in PCOSE, the data of the present research suggest that the smaller activity of 17β-HSD type 2 potentially allows a greater *in situ* concentration of E_2. This fact is in agreement with the higher estrogenic action reported in PCOSE, partly represented by the overexpression of ERα and co-activators and also, by the greater proliferation observed in those endometria [9,10]. Likewise, the higher relationship between the mRNA for isoforms 1 and 2 of 17β-HSD in PCOSE also indicates a smaller tendency to the inactivation of E_2 in endometria from PCOS patients. Importantly, our group recently reported higher enzymatic activity of STS and lower activity of EST in PCOSE from women in the mid-secretory phase of the menstrual cycle compared to control secretory endometria [17]. This apparent discrepancy respect to proliferative endometria could reflect morphological, physiopathological and molecular differences between endometria from the two stages of the menstrual cycle [24,38].

It is important to note that the enzyme 17β-HSD type 2 could have a protective role in normal tissues [39], controlling the estrogenic effect through the *in situ* inactivation of E_2. Also, the deregulation of its activity, like the one observed in this work for PCOSE, could be related to the malignant transformation of the tissue [39]. In this regard, it is known that a diminution of the activity of 17β-HSD type 2 with respect to hyperplasia, takes place in endometrial cancer. In the present investigation we did observe a lower activity of this enzyme in endometrial hyperplasia versus control endometria (25%). In addition, evidences exist that in endometrial cancer the levels of the mRNA for 17β-HSD type 2 are inversely correlated with the concentration of E_2 [39]. Interestingly, in the present study we detected the presence of the mRNA and the activity for 17β-HSD type 2 in all the analyzed endometria, which contrasts with the data reported previously [39,40].

Furthermore, it is important to consider that in endometrial tissue other compounds with estrogenic activity could exist which have not been addressed in the present research, and could be partially responsible for the activity of ERα. In fact, it has been reported that in breast cancer cells, andro-5-ene-3,17-diol (Adiol) is an active estrogen and it is derived from dehydroepiandrosterone sulfate in a route that involves the activity of dehydroepiandrosterone sulfatase, but not of P450 aromatase [36].

In summary, the results of the present investigation indicate that PCOSE exhibit altered activities of the enzymes involved in the intracellular steroid metabolism. Consequently, endometria from PCOS women show deregulation of their *in situ* steroid metabolism. Also, the present data suggest that the four types of endometria differ in their *in situ* estrogen metabolism. Furthermore, our previous investigations reveal that CE, PCOSE, HPCOSE and EH display differences in the expression of steroid receptors and co-regulators [9] and also in the expression of regulatory molecules of the cell cycle [unpublished data]. Therefore, despite the morphologic similarity between CE and PCOSE and between HPCOSE and EH and despite the similar hormonal microenvironment in which PCOSE and HPCOSE are developed, these three types of endometria have a different molecular expression profile. So, 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; thus, this knowledge could be an important support when taking therapeutic decisions in both groups of patients.

Acknowledgments

The authors thank M.D. Teresa Sir-Petermann (School of Medicine, University of Chile) for her role in the recruitment of the subjects and Yenny Muñoz for laboratory assistance. We are also grateful to the women who donated tissue. This study was supported by grant nos. 1050098 from the Fondo Nacional de Desarrollo Científico y Tecnológico and AT 24050052 from the Comisión Nacional de Ciencia y Tecnología, Chile.

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