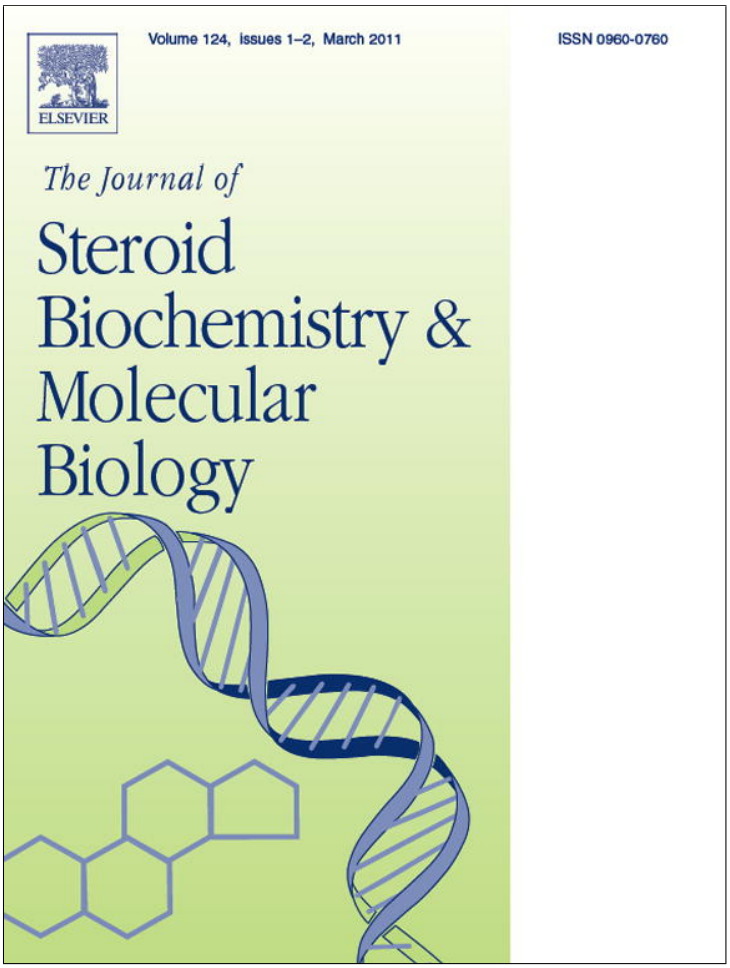

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Alterations of folliculogenesis in women with polycystic ovary syndrome

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ABSTRACT

The objective of the present study was to examine some factors involved in follicular development of women with polycystic ovary syndrome (PCOS). Women with PCOS showed increased levels of serum luteinizing hormone (LH) but decreased follicular production of progesterone and estradiol by pre-ovulatory follicles. The mRNA expression corresponding to steroidogenic acute regulatory protein (StAR), and 20alpha-hydroxysteroid dehydrogenase (20α-HSD) was increased, while that corresponding to cytochrome P450 aromatase (P450arom) was decreased in PCOS follicles as compared to controls. No changes in the mRNA expression for 3beta-hydroxysteroid dehydrogenase 2 (3β-HSD2), cytochrome P450 side-chain cleavage (P450scc), cytochrome P450 17 alpha hydroxylase/lyase (P450c17), cyclooxygenase 2 (COX2), and transcription factors (GATA-4 and GATA-6) were found. We conclude that despite the hyper-luteinized environment of PCOS follicles, these follicles produce lower levels of progesterone and estradiol, and that this is characterized by increased degradation of progesterone and decreased estradiol synthesis. Our data demonstrate that the synthesis of prostaglandin F2α (PGF2α) may be affected in PCOS-follicles and that the transcription factors GATA-4 and GATA-6 are present in PCOS-follicles but they are not involved in the abnormal transcription observed in the steroidogenic enzymes.

1. Introduction

It is known that folliculogenesis starts when follicles leave the pool of resting follicles in order to enter the growth phase. The early growing follicle undergoes a developmental process of cellular proliferation and differentiation and most of them fail to complete this maturation scheme and die in a process termed atresia [1]. Polycystic ovary syndrome (PCOS) is a heterogenous disease characterized by hyperandrogenemia, hirsutism, oligo- or amenorrhea and anovulation, and is frequently associated with hyperinsulinemia, insulin resistance syndrome, increased cardiovascular risk and diabetes mellitus [2,3]. The PCOS follicles are present in large numbers but they are arrested at an early to mid developmental state and fail to mature even when they are exposed to normal serum follicular stimulating hormone (FSH) levels [4–6]. This failure of PCOS follicles to respond to endogenous or exogenous gonadotropins and to mature suggests alterations in local modulators within the ovary further than those involved in the classical gonadotropin pathway. The present study was designed to investigate some parameters which may be involved in this failure.

GATA, a family of six proteins, is a new group of transcription factors that are important regulators of steroidogenesis [7]. Members belonging to this family, namely GATA-4 and GATA-6, are strongly expressed in the fetal and adult adrenals and gonads. Within the gonads, both GATA-4 and GATA-6 are expressed in the ovarian steroidogenic compartments [8,9]. Target genes for GATA factors are the main proteins and enzymes involved in the steroidogenic cascade as; 17 beta-hydroxysteroid dehydrogenase type 1 (17β-HSD, which synthesizes estradiol) [10], CYP19 (which encodes for estrogen-synthesizing enzyme P450 aromatase; P450arom) [11], human 3 beta-hydroxysteroid dehydrogenase gene (3β-HSD) [12], CYP17 (which encodes for 17 alpha-steroid hydroxylase) [7,13–15], CYP11A (which encodes for P450 side-chain cleavage; P450scc) [14], SULT2A1 (which encodes for dehydroepiandrosterone-sulfotransferase) [14], and for the steroidogenic acute regulatory protein (StAR) [7]. A recent report demonstrated that GATA-6 is up-regulated in theca cells from PCOS ovaries [15]. Although it has been well established that GATA factors are expressed in ovarian tissue, their functions remain

Abbreviations: PCOS, polycystic ovary syndrome; LH, luteinizing hormone; StAR, steroidogenic acute regulatory protein; 3β-HSD2, 3beta-hydroxysteroid dehydrogenase 2; 20α-HSD, 20alpha-hydroxysteroid dehydrogenase; P450arom, cytochrome P450 aromatase; P450scc, cytochrome P450 side-chain cleavage; P450c17, cytochrome P450 17 alpha hydroxylase/lyase; COX2, cyclooxygenase 2; FSH, follicular stimulating hormone; PGs, prostaglandins.

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Table 1
Primer sequences and reaction conditions used in the PCR amplification of the various cDNAs.

	Sense	Antisense	Reference
StAR	GGCATCCTTAGCAACCAAGA	TCTCCTTGACATTGGGGTTC	[34]
P450scc	AGCGATTCATTGATGCC	CTGGGTGTATATGTCAGCTTTA	[35]
3β-HSD2	ACACTTGTGCGTTAAGAC	CTGGGTTGACTGTAGAGAA	[35]
20α-HSD	GTAAAGCTTTAGAGGCCAC	CACCCATGGTTCTTCTCGG	[36]
P450c17	GTGGTTAAATGGACCCTG	CGAAGCACCTCTCGGA	[35]
P450arom	GAATATTGGAAGGATGCACAGACT	GGGTAAAGATCATTTCCAGCATGT	[37]
COX2	AAGCCTTCTCTAACCTCTCC	TAAGCACATCGCATACTCTG	[38]
GATA-4	CGGAGGGCGAGCCTGTGT	CCGCATTGCAAGAGGCCTGGG	[39]
GATA-6	GCGCGTGCCTTCATCAC	TCTGCGCCATAAGGTGGTAG	[39]
G6PDH	CAGCGCCTCAACAGCCACAT	AAGGGCTTCTCCACGATGATGC	[40]

controversial. For example, it has been reported that the lack or diminution of the GATA system results in cellular dedifferentiation and ovarian tumorigenesis [16,17], whereas it has also been reported that GATA-4 is over-expressed in gonadal tumors [9].

Prostaglandins (PGs) play a fundamental role in the reproductive system [18–20]. In ovarian tissue, PGs modulate ovarian functions, such as ovulation [21,22] and corpus luteum regression [23,24]. Moreover, PGs within the periovulatory follicle are essential for various female reproductive functions such as follicular development and maturation [25]. Dyslipidemia is frequently associated with PCOS [2,26,27] and circulating levels of PGs are altered in PCOS patients [28] and hyperandrogenized mice [29]. Taken together, these data led us to investigate whether the steroidogenic cascade is altered in pre-ovulatory follicles from PCOS patients. In addition, our studies focused on the role of GATA-4 and GATA-6 and the PG system as regulators of steroidogenesis. The PG system was studied by evaluating PGE and PGF2α production and the mRNA and protein expression of cyclooxygenase 2 (COX2), the limiting enzyme of PG synthesis.

2. Materials and methods

2.1. Patients

The aim of this study was to determine the follicular steroidogenesis pathway on infertile PCOS patients. We included patients with PCOS diagnosis ($n = 28$) who failed to be pregnant after six months of ovulation induction (low complexity treatment with clomiphene citrate, metformin and gonadotrophin therapy). PCOS was diagnosed on the basis of oligoamenorrhea and either clinical (hirsutism – according to Feriman and Gallwey [30] – and severe acne) or biochemical hyperandrogenism (high levels of total or free testosterone, androstenedione, or dehydroepiandrosterone sulphate). Exclusion criteria were serum creatinine >1.5 mg/dl, types 1 and 2 diabetes, pituitary insufficiency, persistent hyperprolactinemia, and congenital adrenal hyperplasia [31].

The control group ($n = 28$) consisted of young fertile women (with healthy children and normal control studies) enrolled in a protocol of voluntary oocyte donation. All patients were recruited from the Instituto de Ginecología y Fertilidad (IFER), Buenos Aires, Argentina, and were informed about the protocol and signed the corresponding consent before the study was started. We followed the ethical standards for human experimentation established in the Declaration of Helsinki Ethical and the protocol was approved by the IFER Research Ethics Committee (Project # 525, 09-01-2008). Both groups received a controlled ovarian hyperstimulation, using the antagonist GnRH protocol (with recombinant follicle stimulating hormone (rFSH) and human menopausal gonadotropin (hMG), 225 UI/day). Follicular development in both groups was monitored by ultrasound and when at least two follicles reached 18 mm in diameter (in general eight to ten days after the treatment) ovulation was triggered with 5000 IU human gonadotropin hormone (hCG). Thirty-four hours after hCG administration, before ovulation occurs, the peri-ovulatory follicles were collected by transvaginal via. This process was monitored by ultrasound of follicular development and endometrial thickness. In order to avoid any contaminations between samples only the first follicle from each ovary was collected. After that, the follicular fluid was separated from the follicle and both stored at −70 °C until assays. Blood from control and PCOS women was immediately processed to determine LH and FSH levels.

2.2. Serum levels of LH and FSH

Serum LH and FSH levels were evaluated by radioimmunoassay (RIA) as previously described [32]. RIA reagents were supplied by Diagnostic Products Corp. (Los Angeles, CA, USA). Results are expressed as IU/ml serum.

2.3. Levels of progesterone and estradiol in follicular fluid

Progesterone and estradiol levels from follicular fluid were determined by specific RIA as described before [33]. Both antisera were kindly provided by Dr G.D. Niswender (Colorado State University, Fort Collins, CO, USA). The progesterone antiserum

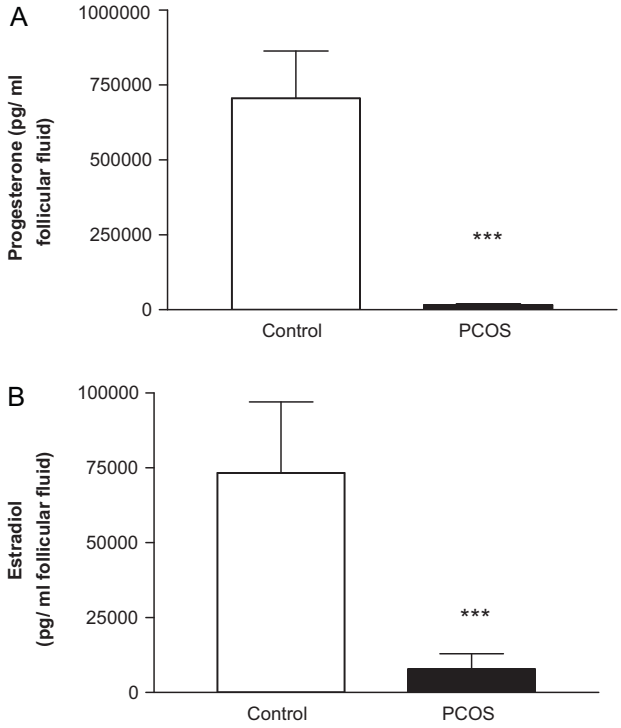

Fig. 1. Follicular progesterone and estradiol are diminished in PCOS. (A) progesterone, and (B) estradiol. Each column represents the mean + SEM from different women, PCOS patients $n = 28$, controls $n = 28$, $^{***}P < 0.001$ by Student's t-test.

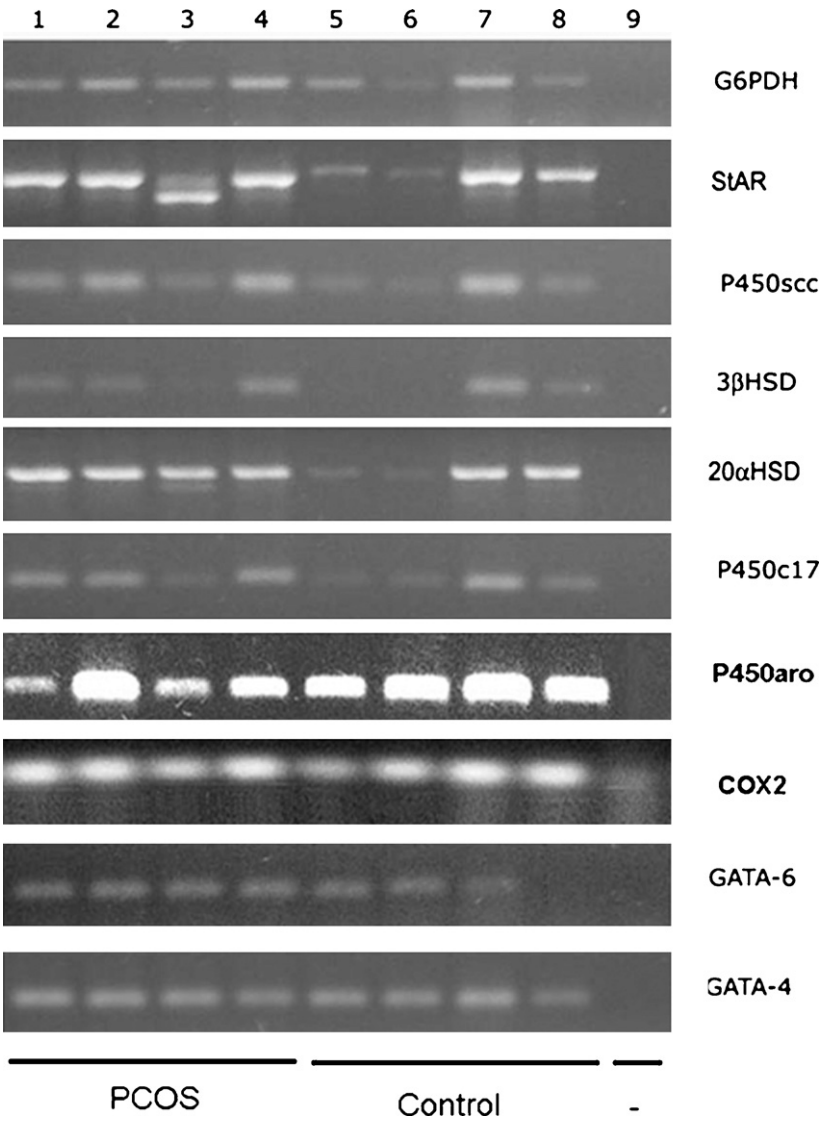

Fig. 2. A representative gel photograph quantified corresponding to mRNA from StAR, P450scc, 3β-HSD2, 20α-HSD, P450c17, P450arom, COX2, transcriptional factors GATA-4 and GATA-6 from control and PCOS follicles using the NIH image.

was highly specific for progesterone with low cross reactivity: <2.0% for 20 alpha-dihydro-progesterone and deoxycorticosterone, and 1.0% for other steroids normally present in serum. The sensitivity was 5–10 pg/tube. The estradiol antiserum showed low cross-reactivity; <1% for progesterone and testosterone, <5% for 17 estradiol estriol and <10% for estrone. Both results are expressed as pg/ml follicular fluid.

2.4. mRNA isolation and RT-PCR analysis

A semi-quantitative analysis of mRNA corresponding to the enzymes involved in follicular steroidogenesis was carried out in follicles both from the control group and the women with PCOS. Total RNA was prepared using TRIZOL Reagent (Invitrogen Life Technologies), following the manufacturer's instructions for RNA isolation. Total RNA (10 μg) was reverse-transcribed at 42 °C using random hexamer primers and *Moloney murine* leukaemia virus retrotranscriptase (Invitrogen/Life Technologies) in a 20 μl reaction mixture. Aliquots of the reverse transcription reaction mix cDNA corresponding to different quantities of cDNA for each reaction were amplified with specific primers as described in Table 1. The conditions and quantities of cDNA added were such that the amplification of the products was in the exponential phase and the assay was linear with respect to the amount of input cDNA. RNA samples were assayed for DNA contamination by performing different PCRs without prior reverse transcription. The PCR products were analyzed on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and photographed using a Kodak DC 290 Zoom Digital Camera. Band intensities of RT-PCR products were quantified using NIH Image software. Relative levels of mRNA were expressed as the ratio of signal intensity for the target genes relative to that for the housekeeping gene G6PDH used as control gene. The experiment was performed twice. Results are expressed as mRNA/G6PDH in arbitrary units.

2.5. Prostaglandin radioimmunoassay

PGE and PGF2α levels were measured in the follicular fluid from control women and women with PCOS. To extract PGs from the follicular fluid, the samples were first acidified to pH 3.0 with 1 M HCl and then extracted three times with 1 volume of ethyl acetate. Pooled ethyl acetate extracts were dried under a N_2 atmosphere and stored at −20 °C until prostaglandin RIA was performed. PGE and PGF2α were quantified by using the specific rabbit anti-

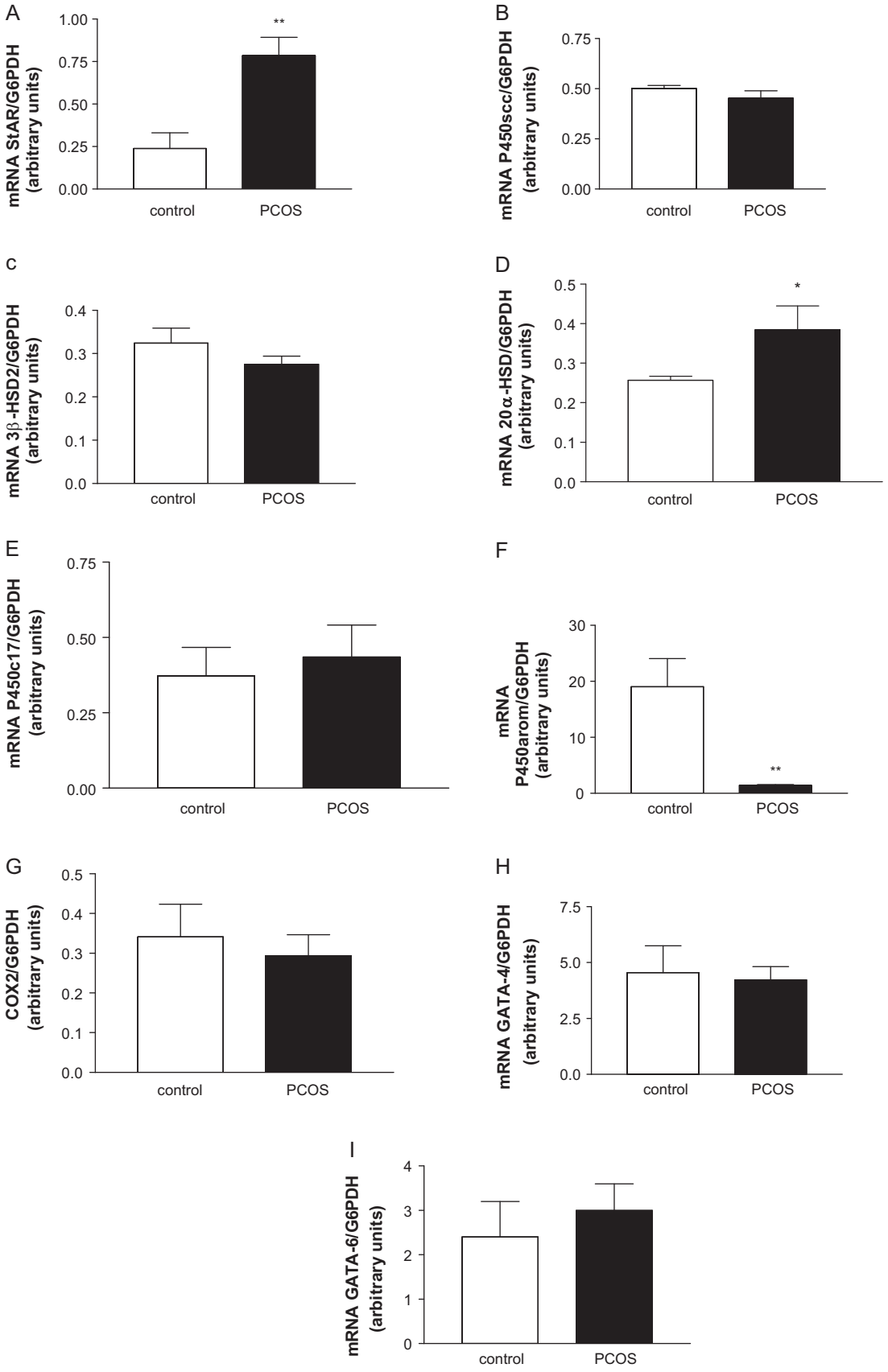

Fig. 3. The mRNA abundances corresponding to StAR and steroidogenic enzymes are altered in PCOS patients. (A) StAR, (B) P450scc, (C) 3β-HSD2, (D) 20α-HSD, (E) P450c17, (F) P450arom, and (G) COX2. Results are expressed in arbitrary units. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ by Student's *t*-test. Each column represents the mean ± SEM from different women from the control and PCOS groups.

serum from Sigma Chemical Co (St. Louis, MO, USA). Sensitivity was 10 pg/tube and cross-reactivity was <0.1% with other PGs. Cross-reactivity was 100% with PGE and PGF2α respectively. The experiment was performed twice. Results are expressed as pg/ml follicular fluid.

2.6. Level of proteins

Follicular protein content was determined by Bradford method [41].

2.7. Statistical analysis

Statistical analyses were carried out by using the Instant program (GraphPad software, San Diego, CA, USA). $P < 0.05$ was considered significant. Student's *t*-test followed by Welch correction was used for comparisons between values of groups. All results are presented as the mean ± SEM.

3. Results

3.1. Serum levels of LH and FSH

Serum LH levels were increased in women with PCOS as compared with controls (4.9 ± 2.1 IU/ml), whereas FSH levels from PCOS patients (5.7 ± 1.2 IU/ml) did not differ from those of control women (6.2 ± 1.9 IU/ml).

3.2. Levels of progesterone and estradiol in follicular fluid

Follicular progesterone and estradiol levels were diminished in PCOS patients when compared to controls (Fig. 1A and B, respectively).

3.3. mRNA abundances of the enzymes and transcriptional factors involved in ovarian steroidogenesis

The mRNA expressions of the enzymes involved in the ovarian steroidogenic cascade were evaluated by RT-PCR (Fig. 2). The mRNA abundances corresponding to StAR and 20α-HSD were increased in PCOS follicles as compared to controls (Fig. 3A and D, respectively), whereas the mRNA expression corresponding to P450arom was decreased (Fig. 3F). In addition, the mRNA abundances corresponding to P450scc, 3β-HSD2 and P450c17 from PCOS follicles did not differ from those of control follicles (Fig. 3B, C and E, respectively).

No changes in the mRNA abundance corresponding to COX2 (Fig. 3G) and transcription factors GATA-4 (Fig. 3H) and GATA-6 (Fig. 3I) were found in PCOS follicles when compared to controls.

3.4. Follicular levels of PGE and PGF-2α

Follicular PGE levels from PCOS patients were similar to those in the control group (Fig. 4A), whereas the PGF-2α levels were diminished as compared to controls (Fig. 4B).

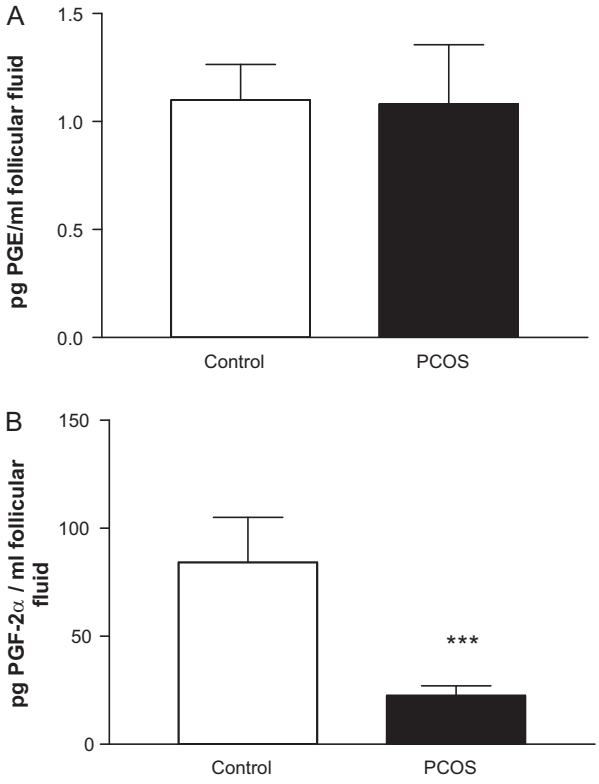

Fig. 4. Follicular prostaglandin E (PGE) (A) and (B) PGF-2α levels corresponding to control and PCOS patients. Each column represents the mean ± SEM from different women. ***$P < 0.001$ by Student's *t*-test.

4. Discussion

The present study was designed to investigate some intrinsic ovarian parameters suspected to be responsible for the failure of maturation of PCOS follicles. To this end, we assessed the mRNA expression for the protein StAR, the enzymes involved in the steroidogenic cascade (3β-HSD2, 20α-HSD, P450scc, P450c17, 17-βHSD5 and P450arom), the transcription factors GATA-4 and GATA-6, and COX2 by RT-PCR. We also analyzed the protein expression of COX2 and the production of PGE, PGF2α, progesterone and estradiol by pre-ovulatory follicles.

In agreement with previous findings [2,26,27], we found that women with PCOS showed increased levels of serum LH and enhanced mRNA abundances for StAR when compared with controls. These findings are also in accordance with the fact that gene expression for StAR, and consequently the up-take of cholesterol by the cell, is hormonally regulated by LH [42]. Paradoxically, PCOS follicles showed increased levels of serum LH and enhanced expression of mRNA for StAR, but lower progesterone and estradiol levels than healthy follicles. These apparently controversial results were clarified when the steroidogenic cascade and its complex network of regulations were analyzed. In fact, follicles from PCOS patients showed increased mRNA abundance for 20α-HSD, the enzyme that catabolizes progesterone to the inactive metabolite 20α-dihydroprogesterone. On the other hand, PCOS follicles showed decreased mRNA abundance for P450arom, the enzyme responsible for estradiol synthesis. It is important to point out that the lack of negative feedback on LH secretion mediated by estradiol and progesterone is considered the principal reason for the high serum LH concentrations in women with PCOS [26]. As previously described in ovarian epithelial [17], theca [15] and luteal cells [43] our data demonstrate for the first time that GATA-4 and GATA-6 are expressed in pre-ovulatory follicles from both control and PCOS women. However, the fact that the mRNA abundances for both transcription factors in PCOS follicles were similar to those in controls led us to conclude that neither GATA-4 nor GATA-6 are responsible for the altered gene expressions of the steroidogenic enzymes found in PCOS patients. Our finding that PCOS-follicles showed lower PGF2α levels than controls is in agreement with the fact that hyper-luteinization decreases PGF2α levels [44]. We may assume that in PCOS patients the increased LH levels are responsible for the decreased follicular production of PGF2α. Besides, it has been recently demonstrated that PGF2α up-regulates the promoter that controls the transcription factors for the gene expression of P450arom in human [45],

pig [46] and murine [43] luteal cells. We thus hypothesize that the hyperluteinized microenvironment of PCOS follicles leads to the decrease in PGF2α levels, which in turn, decrease the P450arom gene expression. Experiments are being designed to clarify this point.

5. Conclusions

The present study compiles novel evidences about the relationship between some factors involved in the regulation of pre-ovulatory follicles and how they are altered in PCOS. In fact, increased LH levels result in a hyperluteinized environment that would alter both the expression of the enzymes involved in the steroidogenic cascade (in a mechanism independent of GATA transcriptional factors) and the production of prostaglandins.

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