Elevated levels of arachidonic acid metabolites in follicular fluid of PCOS patients

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Abstract

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-age women usually accompanied by lipid metabolic disorders. However, it remains unknown whether arachidonic acid (AA) and its metabolites in follicular fluid (FF) were altered in PCOS patients. This study was intended to measure the levels of AA and its metabolites in the FF of non-obese PCOS patients that underwent *in vitro* fertilization (IVF) and to explore the possible causes of the alterations. Thirty-nine non-obese women with PCOS and 30 non-obese women without PCOS were enrolled. AA and its metabolites were measured by liquid chromatography-mass spectrometry. The levels of AA metabolites generated via cyclooxygenase-2 (COX-2) pathway and cytochrome P450 epoxygenase pathway but not lipoxygenase (LOX) pathway were significantly higher in the FF of PCOS patients. The metabolites generated via COX-2 pathway were significantly correlated with levels of testosterone and fasting insulin in serum. The *in vitro* study further demonstrated that insulin but not testosterone could promote the IL-1 β and hCG-induced COX-2 expression and prostaglandin E2 (PGE₂) secretion in primary human granulosa cells. In conclusion, there was an elevation in AA metabolites in FF of PCOS patients. Insulin played a pivotal role in the increased AA metabolites generated via COX-2, which could be interpreted as another novel molecular pathophysiological mechanism of PCOS.

Reproduction (2020) 159 159–169

Introduction

Polycystic ovary syndrome (PCOS) is a complex gynecological and endocrinological disorder affecting 5–10% of reproductive-age women (Azziz *et al.* 2004, Li *et al.* 2013, Zhuang *et al.* 2014). The clinical manifestations of PCOS patients include oligomenorrhea or chronic anovulation, hyperandrogenism, and polycystic ovarian morphology. Besides reproductive disorders, PCOS is often accompanied by several metabolic abnormalities, such as obesity, glucose intolerance, insulin resistance, and dyslipidemia.

Arachidonic acid (AA) is metabolized by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 epoxygenase (P450_{epo}) pathways that produce other key metabolites. For example, the COX pathway produces several prostaglandins (PGs) that affect oocyte maturation, ovulation, implantation, delivery,

and other reproductive processes (Tokugawa *et al.* 1998, Marions & Danielsson 1999, Schaiff *et al.* 2000), whereas three LOX enzymes (5-, 12-, 15-LOXs) metabolize AA to 5-, 12-, and 15-hydroxyeicosatetraenoic acids (5-, 12-, 15-HETEs), respectively.

Follicular fluid (FF) is produced by a complex process that involves the diffusion of serum proteins through the blood–follicle barrier, as well as the secretion of granulosa cells and theca cells (Gerard *et al.* 2002, Schweigert *et al.* 2006, Kolialexi *et al.* 2008, Rodgers & Irving-Rodgers 2010). FF provides a microenvironment for follicle development and oocyte maturation, and alterations in the composition of FF may affect oocyte development and maturation, fertilization, cleavage, and early embryo formation (Kim *et al.* 2006, Revelli *et al.* 2009, Von Wald *et al.* 2010). Recent studies have addressed the role of FF in the pathogenesis of PCOS (Sorensen *et al.* 2016, Zhu *et al.* 2016). Furthermore, a detailed

analysis of the FF revealed that the concentrations of fatty acids and amino acids were closely related to embryo development (Matoba *et al.* 2014). Other studies have identified an association between AA metabolites in the FF and oocyte developmental competence (Khajeh *et al.* 2017); however, the levels of AA and its metabolites in follicular fluid in PCOS patients remain unclear.

Taken collectively, there is a direct correlation between the microenvironment provided by the FF and embryo development. Epidemiological studies have shown that approximately 40% of women with PCOS are infertile (Teede *et al.* 2010) and undertake treatment of *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI). Compared with women without PCOS, a higher number of oocytes are retrieved in women with PCOS receiving controlled ovarian stimulation. However, PCOS patients usually exhibit a relatively lower oocyte utilization rate and high-quality embryo rate (RHQE) (Homburg *et al.* 1993, Heijnen *et al.* 2006), and an increased risk of adverse outcomes (Boomsma *et al.* 2006). Currently, there is no study that measures the levels of AA and its metabolic profile in the FF of patients with PCOS.

In the present study, we measured and compared AA and its metabolites in FF of PCOS and non-PCOS patients. Our primary aim in this study was to clarify whether there is an imbalanced state of AA and its metabolites in the follicular fluid of PCOS. A secondary aim of this study was to elucidate the possible etiology of the imbalanced AA metabolism in PCOS patients.

Materials and methods

Participants

Thirty-nine non-obese (BMI, <25 kg/m²) patients with PCOS were enrolled according to the revised Rotterdam consensus guidelines (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). According to three key clinical features of PCOS, namely oligo-/anovulation(OA), hyperandrogenism (HA) and polycystic ovary morphology (PCOM), the involved patients can be divided into four subtypes (HA+OA, *n*=5; HA+PCOM+OA, *n*=14; PCOM+OA, *n*=14; HA+PCOM, *n*=6) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004). Thirty non-obese patients without PCOS were enrolled as non-PCOS patients; these patients took IVF because of tubal factors (unilateral or bilateral tubal obstruction, adhesion, unilateral or bilateral salpingectomy, or tubal ligation). All patients were 20–35 years old with a history of infertility for over 1 year. The following patients were excluded from the study: (1) women who underwent unilateral oophorectomy; (2) women with a history of a uterine abnormality (malformed uterus, adenomyosis, submucous myoma, or intrauterine adhesion); (3) women or their partners with a diagnosis of an abnormal chromosome karyotype; and (4) women with a history of recurrent spontaneous abortions.

All procedures were performed at the Center for Reproductive Medicine, Renji Hospital, School of Medicine,

Shanghai Jiao Tong University. Written informed consent was obtained, and approval of the ethics protocol was granted from the Ethics Committee of Renji Hospital (Ethical Authorization Number: 2015030308).

Controlled ovarian hyperstimulation and FF collection

All patients were enrolled in a standardized gonadotropin-releasing hormone (GnRH) antagonist protocol (Shi *et al.* 2014, Zhu *et al.* 2016). In brief, patients received recombinant follicle-stimulating hormone (rFSH, Gonal-f; Merck Serono) on day 3 of spontaneous menses or day 3 of the cycle induced by oral contraceptive pills. Patients received the GnRH antagonist Cetrorelix (Merck Serono) at a daily dose of 250 µg by s.c. injection when there was at least one follicle with a mean diameter of ≥12 mm until the desired stage of follicular development was reached. Patients received human chorionic gonadotropin (hCG, Lvzhu, Zhuhai, China) to initiate ovulation when there was at least one leading follicle with a diameter of ≥18 mm. Transvaginal ultrasound-guided oocyte retrieval was performed 36 h later. The FF from the first leading oocytes without contaminating blood was collected immediately from the dominant follicles. The FF was centrifuged at 200 *g* for 5 min at room temperature, and 2 mL of the supernatant was collected and stored at –80°C until further analysis.

Clinical analyses

Hormone measurements

The blood samples were collected before gonadotropin use. The basal serum hormonal profiles of luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, estradiol (E₂), prolactin (PRL), and anti-Müllerian hormone (AMH) were determined using chemiluminescent assay kits (Beckman Access Health Company, Chaska, MN, USA). Serum fasting insulin and fasting plasma glucose were measured using a chemiluminescence assay kit (Beckman Access Health Co) and a standard glucose oxidase method (Roche), respectively. The homeostasis model of assessment for insulin resistance index (HOMA-IR) was calculated as fasting serum insulin (µIU/mL) × fasting plasma glucose (mmol/L)/22.5).

AA metabolite measurements

Standards and deuterated standards of AA, including PGD₂, PGE₂, PGF_{2α}, PGI₂, thromboxane B₂ (TXB₂), 5-HETE, 12-HETE, 15-HETE, and epoxyeicosatrienoic acids (EETs), were purchased from Cayman Chemicals. The solvents for sample preparation and mass spectrometry (MS), such as methanol, chloroform, and water, were purchased from Burdick & Jackson (Muskegon, MI, USA). Other HPLC quality solvents, such as methanol, 2-propanol, hexane, and acetonitrile, were purchased from either Fisher Chemical (Phillipsburg, NJ, USA) or EM Science (Gibbstown, NJ, USA).

Lipids were extracted from FF as previously described with minor modifications (Han *et al.* 2004, 2005), and AA metabolites were analyzed by a metabolomic method (Deems *et al.* 2007, Buczynski *et al.* 2009, Yang *et al.* 2009, Huang *et al.* 2014, Lu *et al.* 2017). After adding the mixture

of deuterated internal standards (0.5 ng/ μ L) to 500 μ L of the FF, the pH of the solution was adjusted to 3.0 using 1 N HCl. Liquid–liquid extraction of the mixture was carried out twice using hexane:methyl *t*-butyl ether (50:50, v/v). The samples were separated on a Phenomenex Kinetix C18 column (particle size, 3 μ m; length $\times$ internal diameter, 100 $\times$ 2.1 mm) using a Thermo Accela HPLC system at a flow rate of 0.4 mL/min using a gradient of mobile phase A (water:acetonitrile:formic acid 63:37:0.02, v/v/v) and mobile phase B (acetonitrile:isopropanol 50:50, v/v). MS analysis was carried out on a TSQ Vantage triple quadrupole mass spectrometer (San Jose, CA, USA). The mass spectrometer was operated in the negative-ion mode using multiple-reaction monitoring. Data acquisition and analysis were performed using Xcalibur software, version 2.0. The limit of detection and coefficient variations of our method are comparable to those reported in the literature (Huang *et al.* 2014, Wang *et al.* 2014). Specifically, the limit of sensitivity for most AA metabolites is in the pg/mL range. All the metabolites were detected with a precision of coefficient variation less than 20% and accuracy of relative error less than 25%.

Isolation and culture of primary granulosa cells

The granulosa cells (GCs) were isolated from the follicular fluid as described previously (Zhu *et al.* 2016). Briefly, follicular fluid from each patient was pooled and centrifuged. The purified pellet was obtained by density centrifugation with Ficoll-Paque (GE Healthcare) and then dispersed in 0.1% hyaluronidase (Sigma) at 37°C for 10 min. The cells were then cultured in Dulbecco's modified Eagle medium/Ham's F12 with 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Invitrogen, Life Technologies) and 100 μ g/mL streptomycin sulfate (Invitrogen, Life Technologies). The viable cells were seeded at 7×10^5 cells per well in a six-well culture plate. Three days after culture, the cells were seeded in phenol red and serum-free medium. The cells were incubated with insulin (100 nM), testosterone (100 nM), hCG (10 IU/L) and interleukin 1 β (IL-1 β , 1 ng/mL) for 24 h.

Reverse transcription and real-time quantitative PCR

Total RNA from cultured granulosa cells was extracted using a total RNA Kit (Foregene) according to manufacturer's instructions and was reverse transcribed to cDNA using the PrimeScript® RT kit (TaKaRa) with appropriate controls. Quantitative real-time PCR was performed and analyzed with ABI Prism System (Applied Biosystems) using SYBR® Premix (TaKaRa) in triplicate. Relative mRNA expression was calculated by the comparative cycle threshold method ($\Delta\Delta C_t$) with ACTB as the housekeeping gene. The primer sequences used of targeting genes were as follows:

COX-2: 5'-CTGGCGCTCAGCCATACAG-3' (forward)
and 5'-CGCACTTATACTGGTCAAATCCC-3' (reverse);
ACTB: 5'-GGGAAATCGTGCGTGACATTAAG-3' (forward)
and 5'-TGTGTTGGCGTACAGGTCTTTG-3' (reverse)

Western blot analysis

Total protein was extracted from cells using an ice-cold radio-immunoprecipitation assay lysis buffer (Cwbio) containing a phosphatase inhibitor (Active Motif) and a protease inhibitor cocktail (Roche). Protein (40 μ g) from each sample was electrophoresed in a 10% SDS-PAGE gel and then transferred onto a nitrocellulose blot. After 1 h of blocking with 5% nonfat milk, the blot was incubated overnight at 4°C with antibodies against COX-2 (1:1000, 320 μ g/L, Poteintech) and ACTB (1:1000, 430 μ g/L, Poteintech). On the second day, the blot was washed and then incubated with the respective secondary antibody conjugated to horseradish peroxidase (Poteintech) for 1 h. Bands with peroxidase activity were detected by an enhanced chemiluminescent detection kit (Merck Millipore) and visualized with a G-Box chemiluminescence image capture system (Syngene).

PGE₂ measurement

The culture medium of granulosa cells was collected. PGE₂ was measured using PGE₂ ELISA Kit (No. 514010, Cayman) following manufacturer's instructions. The intra-assay variation at 500 pg/mL and 62.5 pg/mL were 3.9 and 6.6 respectively. The inter-assay variation at 500 pg/mL and 62.5 pg/mL were 6.4 and 15.5 respectively.

Statistical analyses

Data were presented as means $\pm$ S.D. The Kolmogorov–Smirnov test was used to determine whether the continuous variables fit a normal distribution. The unpaired *t*-test was used to assess the differences in normally distributed variables. The non-parametric Mann–Whitney test was used for non-normal distribution variables between PCOS and non-PCOS groups. The correlation between the variables was performed using the Pearson analysis. Multiple linear regression analysis was performed to investigate the correlations between the serum testosterone, fasting insulin and AA metabolites. The Statistical Package for Social Sciences (SPSS, version 18.0 for Windows) was used for data analysis.

Results

Clinical characteristics of patients with PCOS that underwent IVF

The basal testosterone (1.30 ± 0.83 vs 0.75 ± 0.45 nmol/L, $P < 0.05$), basal LH (8.01 ± 4.94 vs 4.48 ± 1.44 IU/L, $P < 0.001$) and fasting insulin levels (14.17 ± 13.85 vs 6.13 ± 1.83 mIU/L, $P < 0.05$) in the serum were significantly higher ($P < 0.05$) in PCOS patients than in non-PCOS patients. Age (27.85 ± 3.86 vs 27.33 ± 3.49 years) and BMI (22.98 ± 3.30 vs 20.53 ± 2.65 years) were comparable in PCOS and non-PCOS patients, and there were no statistically significant differences in the FSH, E₂, AMH and fasting glucose levels between groups. The fasting insulin, basal testosterone and basal LH levels in the FF were also significantly higher in PCOS patients

than in non-PCOS patients (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). In non-PCOS patients, the average number of retrieved oocytes is 13.29 ± 1.26 , while in PCOS patients the average number of retrieved oocytes is 16.90 ± 1.15 ($P < 0.05$ vs non-PCOS).

Changes in the levels of AA and its metabolites in FF from PCOS patients that underwent IVF

Figure 1 shows representative liquid chromatography-mass spectrometry (LC-MS) of the FF from patients that underwent IVF. Complete LC-MS lipidomic datasets were obtained for the FF of patients with and without PCOS. The levels of free AA (F-C 20:4 n-6) and total AA (T-C 20:4 n-6) in follicular fluid from PCOS patients were comparable with non-PCOS group (Fig. 2). There was a significant elevation in the levels of AA metabolites generated via P450_{epo} (8,9-DHET and 11,12-DHET) in PCOS group than in non-PCOS group

Figure 1 Representative LC-MS chromatograms of follicular fluid (FF) specimens from patients that underwent IVF. Chromatograms of arachidonic acid metabolites in a standard mixture (A), FF from a PCOS patient (B), and an internal standard (C).

(Fig. 2). The levels of AA metabolites generated via the COX pathway (PGI₂, PGE₂, PGD₂, PGF_{2α} and TXB₂) were significantly higher ($P < 0.05$) in patients of PCOS group than in those of the non-PCOS group (Fig. 2). The levels of PGJ₂ and 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂), which were metabolized from PGD₂, were also significantly higher ($P < 0.05$) in patients of the PCOS group than in those of the non-PCOS group (Fig. 2). There were no statistically significant differences in the levels of metabolites generated via the LOX pathways between groups.

Correlation between the AA metabolites, testosterone and fasting insulin

In Pearson's correlation tests, the levels of AA metabolites generated via P450_{epo} (8,9-DHET, 11,12-DHET, and 14,15-DHET) in follicular fluid, the levels of AA metabolites generated via LOX (5-HETE and 15-HETE) in follicular fluid and the levels of AA metabolites generated via COX (PGJ₂, PGE₂, PGF_{2α} and TXB₂) in follicular fluid were significantly correlated with serum basal testosterone levels. The levels of AA metabolites generated via COX (PGJ₂, PGE₂, PGF_{2α} and TXB₂) were significantly correlated with serum fasting insulin levels.

Further multivariate linear regression analysis confirmed that only AA metabolites generated via COX (PGJ₂, PGE₂, and PGF_{2α}) in follicular fluid were significantly associated with basal testosterone after adjusting for basal LH and BMI. As for the associations between AA metabolites and fasting insulin, significant correlations were also observed in AA metabolites generated via COX (PGJ₂, PGE₂, and PGF_{2α}) adjusting for basal testosterone and BMI (Table 1).

Then the correlation between the levels of AA metabolites generated via P450_{epo}, LOX, and COX in follicular fluid with serum basal testosterone and fasting insulin levels were also analyzed in PCOS group and non-PCOS group by Pearson's correlation tests. The levels of TXB₂ generated via COX in follicular fluid were significantly correlated with serum basal testosterone levels in non-PCOS group. The levels of PGJ₂ generated via COX in follicular fluid were significantly correlated with serum basal testosterone levels in PCOS group. The levels of AA metabolites generated via P450_{epo} (5,6-EET, 11,12-EET) in follicular fluid, and the levels of AA metabolites generated via COX (15d-PGJ₂, PGE₂, and PGF_{2α}) in follicular fluid were significantly correlated with serum fasting insulin levels in non-PCOS group. The levels of AA metabolites generated via COX (PGJ₂, PGD₂, and PGE₂) in follicular fluid were significantly correlated with serum fasting insulin levels in PCOS group. Multivariate linear regression analysis showed that AA metabolites generated via COX (15d-PGJ₂, PGE₂ and PGF_{2α}) and via P450_{epo} (5,6-EET) in non-PCOS group, and only AA metabolites generated via COX (PGJ₂, PGD₂, and PGE₂) in PCOS group were

Figure 2 Levels of AA and its metabolites generated in the FF from patients with PCOS and without PCOS (non-PCOS). COX, cyclooxygenase; PGD₂, prostaglandin D₂; PGJ₂, prostaglandin J₂; 15d-PGJ₂, 15-deoxy-Δ^{12,14}-prostaglandin J₂; PGE₂, prostaglandin E₂; PGF_{2α}, prostaglandin F_{2α}; PGFM, prostaglandin F metabolite; PGI₂, prostaglandin I₂; and TxB₂, thromboxane B₂; LOX, lipoxidase; P450_{epo}, cytochrome P450 epoxygenase; EETs, epoxyeicosatrienoic acids; HETEs, hydroxyeicosatetraenoic acids. Data are mean ± S.E.M. *n* = 30 for non-PCOS and *n* = 39 for PCOS. **P* < 0.05, ***P* < 0.01 compared with group non-PCOS. The blue column represents non-PCOS and the red column represents PCOS.

Table 1 Correlation between AA metabolites, testosterone and fasting insulin.

	Basal testosterone (nmol/L)			Fasting insulin (μ U/L)		
	r^*	P^*	$P^\dagger$	r	P^*	$P^\dagger$
P450 metabolites						
5,6-EET	-0.063	0.622		-0.118	0.366	
8,9-EET	-0.038	0.764		-0.079	0.543	
11,12-EET	-0.037	0.770		-0.113	0.385	
14,15-EET	-0.103	0.419		-0.038	0.770	
5,6-DHET	0.195	0.122		-0.038	0.771	
8,9-DHET	0.283	0.024	0.313	-0.079	0.543	
11,12-DHET	0.321	0.010	0.675	-0.119	0.361	
14,15-DHET	0.252	0.045	0.550	-0.192	0.138	
20-HETE	0.227	0.072		0.132	0.310	
LOX metabolites						
5-HETE	0.272	0.030	0.966	0.020	0.878	
12-HETE	0.094	0.459		-0.099	0.446	
15-HETE	0.266	0.033	0.299	0.248	0.054	
LTB ₄	-0.027	0.830		0.020	0.879	
COX metabolites						
15-d-beta-12,14PGJ ₂	0.020	0.876		0.057	0.660	
PGJ ₂	0.400	0.001	0.032	0.431	0.001	0.013
PGD ₂	0.206	0.102		0.386	0.002	0.520
PGE ₂	0.328	0.008	0.027	0.455	0.000	0.004
PGF _{2a}	0.292	0.019	0.031	0.373	0.003	0.009
8-iso-PGF _{2a}	-0.072	0.570		0.040	0.757	
PGFM	0.139	0.272		0.091	0.483	
PGI ₂	0.167	0.187		0.286	0.025	0.829
TXB ₂	0.327	0.008	0.455	-0.083	0.527	

*Pearson's correlation tests. † Multivariate linear regression model.

significantly associated with basal fasting insulin after adjusting for basal LH and BMI (Table 2).

Effects of testosterone and insulin on hCG- and IL-1 β -induced COX-2 expression

The primary granulosa cells were obtained from non-PCOS patients. Treatment of human primary granulosa cells with IL-1 β and hCG for 24 h upregulated COX-2 expression significantly (Fig. 3A and B) and the coexistence of IL-1 β and hCG could further increase the expression of COX-2. Insulin alone could not induce the expression of COX-2 but could further upregulate the induction role of IL-1 β and hCG (Fig. 3A). Testosterone alone could not induce the expression of COX-2, and it had no effect on the induction role of IL-1 β and hCG either (Fig. 3B). The concentrations of COX-2 protein were further measured by Western blot, and the change of the protein level was same as the mRNA expression measured by real-time PCR (Fig. 3C and D).

Effects of testosterone and insulin on hCG and IL-1 β -induced PGE₂ secretion

The same treatment of human primary granulosa cells with testosterone and insulin with or without IL-1 β and hCG for 24 h described as before. Insulin and testosterone alone could not increase the concentration of PGE₂ in the culture medium, while IL-1 β and hCG alone could ($P < 0.05$). IL-1 β combined with hCG could

further increase the secretion of PGE₂ by the granulosa cells cultured *in vitro*. Only insulin, not testosterone, could further increase the stimulation role of IL-1 β combined with hCG (Fig. 3E and F).

Discussion

More than 40% of PCOS patients are infertile and require IVF/ICSI (Teede *et al.* 2010). Although the number of retrieved oocytes is higher in PCOS patients who undergo controlled ovarian stimulation, the oocyte utilization rate does not increase accordingly and IVF/ICSI outcomes are unsatisfactory (Boomsma *et al.* 2006, Heijnen *et al.* 2006). Emerging evidence suggests that FF provides a microenvironment for oocyte development and maturation, and the FF components might influence the oocyte development (Robker *et al.* 2009, Jungheim *et al.* 2011, Leroy *et al.* 2011). AA-derived metabolites, especially PGs, which play key roles in oocyte maturation, cumulus expansion and ovulation. To our knowledge, the profile of AA-derived metabolites in FF in PCOS patients has never been examined. Testosterone or insulin, or both was involved in regulating the concentration of AA-derived metabolites in human FF at the moment has not been reported either. This study provides an extensive profile of follicular AA metabolism in PCOS patients. We evaluated the AA metabolites via COX, P450_{epo}, and LOX in FF and first demonstrated the elevation of AA metabolites via COX (PGI₂, PGE₂, PGD₂, PGF_{2a}, TXB₂, PGJ₂, and 15d-PGJ₂) and P450_{epo}

Table 2 Correlation between AA metabolites, testosterone and fasting insulin in non-PCOS and PCOS patients separately.

	Basal testosterone (nmol/L)						Fasting insulin (μ U/L)					
	r^*	P^*	P^{**}	$r^{\dagger}$	$P^{\dagger}$	$P^{\dagger\dagger}$	r^*	P^*	P^{**}	$r^{\dagger}$	$P^{\dagger}$	$P^{\dagger\dagger}$
P450 metabolites												
5,6-EET	-0.047	0.821		-0.115	0.492		0.419	0.033	0.031	-0.244	0.158	
8,9-EET	-0.244	0.231		-0.120	0.472		0.019	0.928		-0.213	0.220	
11,12-EET	0.248	0.222		-0.139	0.404		0.439	0.025	0.094	-0.206	0.235	
14,15-EET	-0.203	0.320		-0.157	0.345		0.181	0.377		-0.113	0.516	
5,6-DHET	-0.127	0.536		0.245	0.139		0.331	0.099		-0.200	0.249	
8,9-DHET	-0.083	0.686		0.260	0.116		0.043	0.834		-0.265	0.124	
11,12-DHET	-0.047	0.820		0.297	0.070		0.065	0.751		-0.269	0.118	
14,15-DHET	0.100	0.627		0.275	0.095		-0.074	0.719		-0.288	0.094	
20-HETE	0.298	0.140		0.124	0.460		0.059	0.776		0.058	0.742	
LOX metabolites												
5-HETE	0.104	0.613		0.237	0.152		0.080	0.698		-0.114	0.516	
12-HETE	-0.022	0.914		0.105	0.531		0.144	0.484		-0.162	0.352	
15-HETE	0.102	0.620		0.239	0.148		0.168	0.413		0.203	0.242	
LTB ₄	-0.192	0.349		-0.083	0.621		0.354	0.076		-0.085	0.629	
COX metabolites												
15-d-beta-12,14PGJ ₂	0.329	0.101		-0.094	0.576		0.539	0.004	0.011	-0.041	0.815	
PGJ ₂	0.154	0.453		0.322	0.049	0.068	0.154	0.451		0.356	0.036	0.025
PGD ₂	0.279	0.167		0.092	0.583		0.343	0.086		0.344	0.043	0.006
PGE ₂	0.143	0.485		0.261	0.114		0.548	0.004	0.010	0.391	0.020	0.003
PGF _{2α}	0.136	0.508		0.215	0.194		0.471	0.015	0.044	0.293	0.088	
8-iso-PGF _{2α}	0.091	0.659		-0.165	0.322		0.195	0.339		0.022	0.899	
PGFM	0.123	0.548		0.105	0.529		0.271	0.181		0.035	0.843	
PGI ₂	0.329	0.101		0.015	0.927		0.222	0.275		0.250	0.148	
TXB ₂	0.433	0.027	0.051	0.212	0.200		0.299	0.138		-0.247	0.153	

*Pearson's correlation test model in non-PCOS patients. † Pearson's correlation test model in PCOS patients. ** Multivariate linear regression model in non-PCOS patients. †† Multivariate linear regression model in PCOS patients.

(8,9-DHET and 11,12-DHET) in PCOS patients when compared with non-obese control.

We previously reported the diminished AA metabolites in the serum of PCOS patients (Li *et al.* 2017) and elevated COX-2 expression in ovaries of PCOS rats (Huang *et al.* 2018). Here we further clarified that AA metabolites via COX-2 were also increased in FF of PCOS patients, which was consistent with the rat model results. While the levels of AA and its downstream metabolites in serum reflect the AA metabolism in the whole body, the AA in the ovaries of PCOS rats and in the FF of PCOS patients has more profound effects on the local metabolism and biological consequence in the ovaries. These differences between serum and FF in PCOS patients further indicate the important role of local AA metabolism in PCOS patients. Furthermore, we also clarified the elevation of AA metabolites via P450_{epo} in PCOS follicular fluid. Although the function of AA metabolites via P450_{epo} has not been clarified, our finding provides a new insight in the pathophysiology and potential treatment of PCOS patients in the future.

PGs played important roles in many processes of reproduction. For example, PGE₂ is an autocrine and paracrine mediator of oocyte maturation and cumulus expansion. PGE₂ participates in cumulus-oocyte coupling and cumulus cell expansion (Calder *et al.* 2001), thus enhancing the release of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus (Kim & Ramirez 1986). On the other hand, an increased PGE₂ level in

the FF may delay follicle development, and a very high PGE₂ level may be detrimental to oocyte maturation (Marei *et al.* 2010, Wang *et al.* 2012). Along with PGE₂, PGF_{2 α} is critical for ovulation because it increases collagenolysis and ovarian contractility. The increase in the PGF_{2 α} level in the ovary may serve to overcome the inability to ovulate in patients with PCOS. Besides PGE₂ and PGF_{2 α} , we also found that PGJ₂ was significantly elevated in PCOS patients, and it was closely correlated with serum insulin and testosterone. However, the role of PGJ₂ in granulosa cells remains to be studied. PGJ₂ is not stable *in vivo*, and it could be easily transformed to cyclopentenone PGs, including 9-deoxy- Δ 9,12,13,14-dihydro PGD₂ (Δ 12-PGJ₂) and 15d-PGJ₂ (Narumiya & Fukushima 1985). 15d-PGJ₂ is an endogenous ligand of peroxisome proliferator-activated receptor gamma (PPAR γ) that regulates inflammation (Jiang *et al.* 1998, Valledor & Ricote 2004), granulosa cell proliferation (Chen *et al.* 2015), steroid hormone biosynthesis (Komar 2005), and fibrosis (Iwase *et al.* 2009). By using 'one follicle – one retrieved oocyte – one resulting embryo' approach, Ciepiela *et al.* found that elevated concentrations of AA-derived metabolites in FF at the time of oocyte retrieval significantly decreased the ability of oocytes to form pronuclei after ICSI in non-PCOS patients (Ciepiela *et al.* 2015). The specific function of PGs in IVF/ICSI still needs further exploration.

Both hyperandrogenism and hyperinsulinemia are two common characteristics in PCOS patients

Figure 3 The induction role of insulin, testosterone, interleukin-1 β and hCG on COX-2 expression and PGE₂ production. The mRNA level of COX-2 with treatment of insulin, interleukin-1 β and hCG (A). The mRNA level of COX-2 with treatment of testosterone, interleukin-1 β and hCG (B). The protein level of COX-2 with treatment of testosterone, interleukin-1 β and hCG (C). The PGE₂ concentration of culture medium of granulosa cells treated with testosterone, interleukin-1 β and hCG (D). The medicine concentrations are as followed: insulin (100 nM), testosterone (100 nM), hCG (10 IU/L) and interleukin 1 β (1 ng/mL). Data are represented as mean $\pm$ s.e.m. $n=4$ for each experiment. Values with a different letter above are statistically significantly different ($P<0.05$). CTRL, control; INS, insulin; T, testosterone; IL1B, interleukin-1 β .

(Diamanti-Kandarakis & Dunaif 2012). Insulin resistance, characterized by compensatory hyperinsulinemia, is observed not only in peripheral tissues but also in ovarian granulosa cells in PCOS patients (Zhu *et al.* 2016). Previous studies have explored the role of insulin on COX-2 in other tissues but the results remain controversial. Some studies demonstrated that insulin could increase COX-2 expression, and might further augment the IL-1 β -induced COX-2 expression and PGE₂ production (Martins *et al.* 2010, Song *et al.* 2014). Meanwhile, there were also some studies that clarified the reduction role of insulin on COX-2 expression (Martins *et al.* 2008, Xu *et al.* 2018). These findings led us to speculate whether insulin and androgen were correlated with the increased AA metabolites.

Our study revealed that AA metabolites via COX-2 pathway (PGE₂, PGF_{2 α} , and PGJ₂) were significantly related with serum insulin and testosterone levels in the multivariate linear regression model, which indicates hyperinsulinemia and hyperandrogenism in PCOS might contribute to the enhanced AA metabolism through COX-2 pathway. Multivariate linear regression analysis showed that AA metabolites generated via COX (PGJ₂, PGD₂, and PGE₂) in PCOS group were significantly associated with basal fasting insulin level, not testosterone, after adjusting for basal LH and BMI in the groups separately. To clarify whether testosterone or insulin regulates COX₂ and PGE₂ expression, we designed an *in vitro* experiment using primary isolated granulosa cell.

Considering the chronic inflammation state of PCOS patients and the application of hCG in supra-ovulation, the *in vitro* study mimics the collaboration role of the inflammatory PCOS state. We treated granulosa cells together with IL-1 β , hCG, testosterone and insulin to figure out their induction role on COX-2. IL-1 β and hCG are known to induce COX-2 expression and PGs production in many different cells and tissues. Our study revealed that both IL-1 β and hCG could significantly induce the expression of COX-2 and the combined treatment of IL-1 β and hCG could further increase the expression of COX-2. However, neither insulin nor testosterone had an induction role on the expression of COX-2 without the IL-1 β and hCG treatment. Insulin, but not testosterone, could enhance the IL-1 β - and hCG-induced COX-2 expression in granulosa cells. These *in vitro* findings demonstrated the hyperinsulinemia in PCOS might be a causing factor in the increased prostaglandins in follicular fluid. Although we previously reported that androgens downregulate the levels of COX metabolites in the serum of patients with PCOS, there are several important differences between the previous and present studies. Firstly, the levels of AA metabolites in serum are contributed from many different cells, such as hepatocytes, endothelial cells, peripheral blood granulocytes, and mononuclear cells, whereas only ovarian cells contribute to the levels of these metabolites in the FF. The AA metabolites in follicular fluid specifically

reflect local ovarian state. Second, the patients in this study underwent gonadotropin stimulation, which upregulated the levels of PGs. Hyperinsulinemia could exaggerate the induction role of inflammation and LH on COX-2 expression and stimulate the granulosa cells producing more PGs, PGE₂ for example. Consistent with the correlation analysis, *in vitro* study provided evidence for the enhancement of insulin on the IL-1 β and hCG-induced COX-2 expression. Therefore, we put forward the elevated AA metabolites in PCOS follicular fluid and its close correlation with hyperinsulinemia for the first time.

Limitation

There are some limitations in this study. (1) Since the FF in this study are collected from more than one follicle, which may have been sub-par to the one follicle-one retrieved oocyte-one resulting embryo design. (2) Previously, we reported that there was no significant difference in serum AA concentration between lean control group and lean PCOS group (Li *et al.* 2017). In this study, there was no change in the concentration of AA in follicular fluid, but its metabolism was higher in non-obese PCOS group than BMI-matched control group, suggesting a high transfer of AA from circulation or increased hydrolysis of local phospholipids in the ovary. But lack of serum AA and its metabolites is a real shortage of this study. (3) We have not directly detected the activity of COX in primary isolated granulosa cells from PCOS patients and control. (4) Relatively small sample sizes, large data variations, may weaken statistical efficiency. (5) The *in vitro* experiment could not exactly mimic the *in vivo* effects of testosterone. Firstly, testosterone may exert its physiological effects through different signaling pathways (androgen receptor or membrane receptors) depending on the target tissue and microenvironments in the body (Goldman *et al.* 2017). Meanwhile, testosterone could metabolite to estrogen and have complicated functions *in vivo*. *In vitro* cell experiments could not fully mimic the *in vivo* condition and exert the actual action of testosterone in the body. Second, the metabolites or hormones in the follicular fluid reflect the changes in testosterone after it acts on various cells in ovarian tissue (e.g. theca cell, granulosa cell, etc.), while *in vitro* experiments only reflect the secretion changes of primary isolated granulosa cells after the stimulation of testosterone.

In conclusion, we demonstrated that under gonadotropin stimulation, the levels of AA metabolites generated via the COX-2 were elevated in the FF of patients with PCOS and for the first time reported the elevation of AA metabolites via P450_{epo} in PCOS patients. We also suggest the possible role of hyperinsulinemia on AA metabolism via COX-2 pathway in PCOS patients. Further studies are needed to define the potential mechanism and to further characterize the relationship

between PGs and follicle development in patients that undergo IVF. These results provide new insights on how to improve embryo health and IVF outcomes.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0136>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the National Key R&D Program of China (No. 2017YFC1001403 to Y S), National Natural Science Foundation of China under Grant (No. 81571499 to Y S, No. 81471029 to S L, No. 81671518 to W L), Clinical skills improvement project of major disorders hospital development center of Shanghai (No. 16CR1022A to Y S) and Shanghai Municipal Education Commission-Gaofeng Clinical Medicine Grant Support (Grant 20161413 to Y S).

Author contribution statement

Y S and H Y designed the study. Q Z, R H, Y L, J Y and W L collected patients' specimens and related information. S L, J Q, Y T and H Z contributed to conducting the experiments and analyzing the data. S L, J Q and H Y drafted and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

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Received 28 March 2019

First decision 8 May 2019

Revised manuscript received 17 November 2019

Accepted 26 November 2019