Altered endothelin expression in granulosa-lutein cells of women with polycystic ovary syndrome

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A B S T R A C T

Aims: To examine the levels of endothelin system components in granulosa lutein cells (GLCs) of women with PCOS and compare them to normally ovulating women undergoing In Vitro Fertilization (IVF).

Polycystic ovary syndrome (PCOS) is one of the most common endocrine-metabolic disorders in women of reproductive age. Endothelins are locally produced by endothelial and granulosa cells of the preovulatory follicle. Abnormal expression or production of endothelins may be a contributing factor in PCOS pathogenesis.

Main methods: Follicular aspirates containing GLCs were obtained from PCOS and normally ovulating patients undergoing oocyte retrieval during the IVF cycle. RNA was extracted and endothelin system components were quantified using real-time PCR. GLCs were cultured in basal media for 7 days, and then challenged with various luteinizing agents (luteinizing hormone, hCG, or forskolin) for 24 h.

Key findings: In GLCs from women with PCOS, Endothelin-1 mRNA expression was elevated (2.2-fold) as compared with normally ovulating women, whereas endothelin-2 mRNA was reduced (1.8-fold). ET receptors and endothelin-converting enzyme showed the same expression levels in the two groups. In vitro modeling showed that although the steroidogenic response was preserved in GLC, endothelin expression levels were not exhibited in vitro in their original pattern.

Significance: Dysregulation of ovarian endothelin expression may induce a pathologic ovulation pattern characteristic of PCOS.

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Introduction

Polycystic ovary syndrome (PCOS) is a clinical diagnosis characterized by the presence of two or more of the following features: chronic oligo-ovulation or anovulation, clinical or biochemical hyperandrogenism, and polycystic ovaries appearing on an ultrasound scan (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). PCOS affects 5 to 10% of women of childbearing age, and it is the most common cause of anovulatory infertility in developed countries (Asuncion et al., 2000). It has been proposed that PCOS results from abnormal regulation of steroidogenesis and specifically from androgen secretion by the ovary (Wickenheisser et al., 2000). Provocative tests using GnRH agonists have shown that the hyperandrogenism usually has an ovarian source (Rosenfield et al., 2000). PCOS is also associated with significant metabolic derangements such as hyperinsulinemia and insulin resistance (Legro et al., 2004). These metabolic derangements may predispose the patient to a range of diseases with attendant morbidity and mortality risks.

The prevalence of the metabolic syndrome (obesity, an abnormal lipo-protein profile, high blood pressure, and increased fasting glucose) is two to three times as high among women with PCOS compared with normal women matched for age and body-mass index (Apridonidze et al., 2005). Although ovulation induction treatments are successful in getting these women to ovulate, data from women with PCOS undergoing IVF treatment show that their oocytes are often of poor quality, leading to lower fertilization, cleavage, and implantation rates, and these women suffer from a higher miscarriage rate (Qiao and Feng, 2011). The pathophysiological characteristics of the polycystic ovary syndrome are not fully understood but are known to involve complex interactions among gonadotrophic peptides, ovarian cells and hormones, androgens, and insulin.

Endothelins (ETs) are a family of peptides involved in many physiological processes (Khimji and Rockey, 2010). Their involvement in PCOS was suggested by the observation that obese and non-obese women with PCOS have higher levels of circulating endothelin-1 compared with controls (Diamanti-Kandarakis et al., 2001). Endothelial cells produce endothelins, but endothelin-1 is also expressed in ovarian cells and the peptide is present in the follicular fluid of normal women (Magini et al., 1996; Mancina et al., 1997; Iwai et al., 1993). Moreover, the concentration of endothelin-1 is negatively correlated...
with follicle diameter (Plonowski et al., 1999). Another endothelin peptide was recently shown to have an important ovarian function: produced by the granulosa cells around the time of ovulation, endothelin-2 affects follicular rupture and corpus luteum formation (Ko et al., 2006; Klipper et al., 2010). The present study was undertaken to investigate whether women with PCOS have altered levels of the endothelin system constituents in their granulosa lutein cells (GLCs). We determined the mRNA levels of endothelin-1, endothelin-2, their converting enzyme, ECE-1, and the two receptor subtypes, ETα and ETβ, in freshly isolated GLCs from women with PCOS and compared them to GLCs from age-matched normally ovulating women. The patients underwent ovulation induction and ovum pick-up through an in-vitro fertilization cycle. We also examined the effects of lutetotropic agents on endothelin production and the steriodogenic response of cultured GLCs derived from PCOS and normally ovulating women.

Materials and methods

Subjects

Women with PCOS and normally ovulating women (12 and 14, respectively) were enrolled in this study. Diagnosis of PCOS was made according to the Rotterdam revised criteria (2004). The Hadassah Hebrew University Medical Center Institutional Review Board approved this study. All subjects gave written informed consent to participate in the study.

Treatment protocol

All patients were subjected to the long suppression protocol (Fleming et al., 1988). In brief, gonadotropin-releasing hormone agonist (3.75 mg Decapeptil; Ferring Pharmaceutical, Malmo, Sweden) was utilized for 14 days until ovarian depression was complete. Thereafter, recombinant FSH (75 IU Gonal F; Seeno, Israel) was administered, at a dose of 2 ampoules a day and subsequently adjusted according to the individual’s response. When >3 follicles reached >18 mm in diameter, 5000 IU of human chorionic gonadotropin (hCG) (Organon, Oss, Holland) was injected into the patient to induce ovulation. Transvaginal oocyte retrieval was performed using ultrasound probe guidance 36 h after hCG. All accessible follicles were harvested and oocytes were removed from follicular aspirates for further fertilization and embryo transfer. The residual follicular fluid aspirates containing GLCs were collected for further investigation.

Experiment protocol

Follicular fluids containing GLCs and erythrocytes were centrifuged. Follicular fluid was decanted, and erythrocytes were lysed using ACK solution (0.15 M NH4Cl, 1.0 mM KHCO3, and 0.1 mM EDTA) for 15 min at 37 °C. The cells were then washed 3 times with PBS (300 g, 4 min). Finally, cells were counted in a hemocytometer. A fraction of the freshly isolated cells were taken for RNA extraction using TRIzol reagent (Peqlab Biotechnologie GmbH, Erlangen, Germany). The remaining cells were placed in 6-well dishes (~1.5×10^6 cells per well). Cultures were maintained in humidified 95% air-5% CO2 at 37 °C. GLCs from six normally ovulating patients and GLCs from 7 women with PCOS were cultured in media containing 10% FCS. Following 7 days culture, cells were washed and challenged with either hCG (10 units/ml), 100 nM LH, 10 μM forskolin (FRS), or left without treatment. Twenty-four hours later, media were collected from each well for further progesterone analysis.

RNA isolation and real-time PCR

Total RNA was isolated from freshly isolated and cultured cells using Tri-Fast reagent (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the manufacturer’s instructions. Real-time PCRs were performed using the Mx3000P quantitative PCR system (Stratagene, Garden Grove, CA), with the SYBR Green I PCR kit with ROX passive reference (Eurogentec, Seraing, Belgium) as previously described (Klipper et al., 2009). Briefly, each real-time reaction (18 μl) contained the SYBR Green master mix that comprised the ROX 6-carboxy-X-rhodamine passive reference, 1.5 mM deoxynucleotide triphosphates, including deoxyuridine 5-triphosphate, 5 mM MgCl2, uracil N-glycosylase, and AmpliTaq HotGoldStar DNA polymerase, 0.5 μl of a 1:10,000 dilution of SYBR Green stock solution, 10 nM of each primer, and cDNA. The glyceraldehyde 3-phosphate dehydrogenase (GPDH) gene was used as the housekeeping gene. Dissociation curve analysis was performed after each real-time experiment to confirm the presence of only one product and the absence of the formation of primer dimers. The threshold cycle number (Ct) for each tested gene X was used to quantify the relative abundance of the gene; arbitrary units were calculated as 2−ΔCt=2−(Ct target gene − Ct housekeeping gene). Primers that were used were as follows: G3PDH, forward 5′-GGGAACATGTGGCCTGATG-3′ and reverse 5′-CGTTCAGCTAAGGGATGC-3′; ECE-1, forward 5′-GGGAAACATTCGCCAGCACA-3′ and reverse 5′-TGCAAGGCCCAGGAAAG-3′; ET-1, forward 5′-CTCCACCTGGCACAATCTTG-3′ and reverse 5′-TTCAGGCTCTGTTGCTGTGC-3′; ET-2, forward 5′-GCCAGGCTCTATCCTCAT-3′, reverse 5′-CCGATAAGGCGTGCTGTCC-3′; ETα, forward 5′-CCTCCTGGTTAACCATCAAA-3′ and reverse 5′-GTCTGCTTGCGAACATATTG-3′; ETβ, forward 5′-AGCCCTGGGACTTCGGAAGA-3′, reverse 5′-CCGCTCCGAGTTGCCAG-3′; STAR, forward 5′-TGGCATGGACACAGACTTGC-3′, reverse 5′-AGGAACCTTGTTGAGATGC-3′.

Hormonal assay

Progesterone concentrations were measured in the culture media by commercially available RIA Kits (Coat-A-Count Progesterone RIA, DPC, Los Angeles, CA, USA).

Statistical analysis

Data are not normally distributed; the non-parametric Mann Whitney U test was used to analyze data using SPSS software Version 14 for Windows. A value of P<0.05 was considered statistically significant. Results are presented as means±SEM.

Results

Clinical data

Age, body mass index (BMI), levels of gonadotrophins (FSH and LH) on day 3 of the cycle, total testosterone, estradiol (E2) on the day of hCG injection, the number of oocytes collected, and the number of embryos developed are presented in Table 1. As expected, LH levels are significantly higher in women with PCOS; this is in accordance with the syndrome’s characteristics (Tropeano et al., 1996). Although the BMI tended to be higher in women with PCOS, it did not reach statistical significance. Estradiol levels at the day of hCG injection and the number of ovum retrieved were not different between

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinical characteristics and cycle outcome in normal ovulatory and PCOS patients.</th>
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<tbody>
<tr>
<td></td>
<td>PCOS (n=14)</td>
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<tr>
<td>Age (years)</td>
<td>27.3±4</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>28±5.5</td>
</tr>
<tr>
<td>FSH (mIU/l)</td>
<td>4.9±1.5</td>
</tr>
<tr>
<td>LH (mU/l)</td>
<td>60.0±3.6</td>
</tr>
<tr>
<td>Total testosterone (nmol/l)</td>
<td>2.8±1.2</td>
</tr>
<tr>
<td>E2 level during ovulation (pmol/l)</td>
<td>9591±3659</td>
</tr>
<tr>
<td>No. of oocytes</td>
<td>20.6±9.6</td>
</tr>
<tr>
<td>No. of embryos</td>
<td>11.3±6.3</td>
</tr>
</tbody>
</table>

Data presented as mean±SD.
Expression of the endothelin system in GLCs from PCOS and normally ovulating women

We characterized the expression profile of the endothelin system in freshly collected GLCs. Quantitative RT–PCR analysis revealed that transcripts of endothelin-1, endothelin-2, ECE-1, and ET receptors A and B were present (Figs. 1 and 2) in both groups. The results show that although both endothelin-1 and endothelin-2 are expressed in human GLCs, endothelin-2 is the predominant endothelin isoform in human GLC regardless of the group of women. More specifically, it was expressed at least 10-fold higher than endothelin-1 in the GLCs from normal women and from women with PCOS. Interestingly, we observed here that the levels of endothelin-1 in the GLCs from ovulating follicles were significantly elevated in those from women with PCOS as compared with those from normally ovulating women (a 2.2-fold difference). Endothelin-1 expression was markedly and significantly elevated; this rise was observed in cells from both groups cultured in basal media (Fig. 1). ECE-1 mRNA is highly expressed in the GLCs from both groups (Fig. 2).

Similarly, both endothelin receptor subtypes (ET₁ and ET₂) were readily detected in freshly isolated GLCs from the two groups. However, the ET₁ receptor mRNA was 5-fold higher than ET₂ in GLCs. Unlike endothelin mRNA expression, which differed significantly between the groups, StAR transcripts were the same in both groups (Fig. 3).

Effects of gonadotropin withdrawal on cultured human GLCs

Cells were cultured in basal media for 7 days to examine the effects of gonadotropin deprivation in each group of patients. Data depicted in Fig. 4 show the precipitous decline in StAR mRNA after 7 days of culture without gonadotropins, as compared with cells obtained soon after (36 h) hCG treatment. The decline in steroidogenesis was similar in both groups. However, the mRNA from the two endothelins exhibited an opposite pattern after 7 days of culture. Endothelin-1 expression was markedly and significantly elevated; this rise was observed in cells from both groups cultured in basal media (Fig. 5). Interestingly, endothelin-2 levels declined in GLCs from normally ovulating women (3–4-fold decrease, P< 0.05) but did not change significantly in the PCOS group (Fig. 5).

Effect of luteotropic agents on steroidogenesis and endothelin expression in human GLCs in vitro

In order to examine the GLC response to luteotropic signals after 7 days of culture under basal conditions, cells from each group of patients were challenged with various luteinizing agents (luteinizing hormone, hCG, or FRS) for 24 h, as detailed in the Materials and methods (Figs. 6 and 7). All agents significantly enhanced progesterone production, with FRS being the most potent inducer (6–7-fold, Fig. 6). Induction of progesterone production and endothelin expression by GLCs was not statistically different between normally ovulating and PCOS women.

Luteotropic agents reduced the mRNA expression of the endothelin-1 gene in the GLCs of normally ovulating and PCOS women. Twenty-four hours treatment with FRS nearly eliminated endothelin-1 expression (a 10- and 20-fold decrease, respectively, for normal ovulatory and PCOS women, Fig. 7A). Endothelin-2 expression also tended to decrease in the presence of luteinizing agents (Fig. 7B). However, the magnitude was smaller than that observed for endothelin-1, with maximal inhibition not higher than twofold as compared with control media. Moreover, in cells from PCOS women, only FRS could significantly reduce Endothelin-2.
PCOS

N.S

P= 0.003

706

mation of atherosclerotic lesions (Maemura et al., 1992). Although

(Wild, 2002), including diabetes mellitus (Diamanti-Kandarakis, 2006;

but also a metabolic disorder associated with long-term health risks

associated with platelet aggregation, macrophage in

as a result of endothelial cell damage and is regulated by factors asso-

Talbott et al., 2001). One of the early signs of its vascular damage is

Talbott et al., 2007) and coronary artery disease (Cibula et al., 2000;

we compared the expression pro

1999; Korth et al., 1999; Mancina et al., 1997). In the current study,

(Apa et al., 1998; Choi et al., 2011; Haq et al., 1996; Karam et al.,

individual endothelin components in the human and primate ovary

syndrome or whether it results from its metabolic derangements.

PCOS (Diamanti-Kandarakis et al., 2001), it is not known whether

its effect on GLC functions in women with the polycystic ovary syn-

and age-matched normally ovulating women undergoing

follicular aspiration during an IVF cycle. Our findings also support

previous reports showing that endothelins are present in follicular

fluid (Gentili et al., 2001; Haq et al., 1996). Their levels in the follicu-

lar fluid are signi

higher than their levels in the serum (Choi

2011), suggesting that endothelins are produced endogenously. Howev-

however, their levels from hyper-stimulated IVF patients are too

scattered and thus endothelins are not suitable as a biomarker for

the ovulation process (Plonowski et al., 1999). Therefore, we evalu-

ated their expression levels in the GLCs. In comparing endothelin

system expression in GLCs from normal vs. PCOS women, we found

altered endothelin gene expression in patients suffering from this

syndrome. These differences might be related to the mechanism

underlying this syndrome.

Higher levels of endothelin-1, which were observed in the PCOS

group, may act as a negative regulator of granulosa and luteal cells.

In vitro cultures, using human granulosa or luteal cells, have shown

that endothelin-1 influences ovarian steroidogenesis and inhibits

Fig. 5. mRNA levels of endothelin-1 (A) and endothelin-2 (B) in freshly collected GLCs

(day 0) and after 7 days in culture (day 7). RNA extraction, cDNA synthesis, and real-time

PCR were as described in the Materials and methods. n.s — non significant statistically.

Fig. 6. Progesterone production by human GLCs from PCOS and normally ovulating

patients. After 7 days of culture, cells were further incubated for 24 h in the presence

of either 100 nM LH, hCG (10 units/ml), 10 μM forskolin (FRS), or media alone. At the

end of the incubation period, the media were collected and kept frozen until assayed

for progesterone using EIA. * denotes significant statistical difference (P<0.05) from

controls.
endothelin-2 was transiently expressed in GLCs from PCOS women. In rat and 2 action via receptor antagonists reduced follicle rupture and oocyte release (Bridges et al., 2010; Palanisamy et al., 2006). This implies that endothelin-2 mRNA expression level may explain why there was no significant reduction by the luteotrophic agents, suggesting aberrant endothelin-2 expression in GLCs from PCOS women. In rat and mouse models, endothelin-2 was transiently expressed in GLCs immediately before gonadotropin-induced ovulation (Al-Alem et al., 2007; Ko et al., 2006; Palanisamy et al., 2006). Blocking endothelin-2 action via receptor antagonists reduced follicle rupture and oocyte release (Bridges et al., 2010; Palanisamy et al., 2006). This implies that endothelin-2 acts by contracting the smooth muscle layer around the follicle, resulting in follicular rupture (Ko et al., 2006). Another, alternative mode of endothelin-2 action in ovulation was suggested, according to which, endothelin-2 mediates the induction of cGMP-dependent protein kinase II, a possible downstream target during the final stages of follicular wall breakdown (Palanisamy et al., 2006). In addition to its effects on follicular rupture, elevated endothelin-2 levels in the early corpus luteum may facilitate its maintenance (Klipper et al., 2010). Therefore, decreased production of endothelin-2 in women with PCOS might interfere with follicular rupture, the ovulatory process, and corpus luteum formation. In fact, scanning the ovaries of women with PCOS reveals multiple small, non-ruptured follicles (Chavez-Ross et al., 1997).

In accordance with findings reported previously, all endothelin pathway constituents in human GLCs were expressed in our samples, including ECE-1, the rate-limiting enzyme in producing active endothelins (Yoshioka et al., 1998), thus indicating that the ovary is a suitable site for endothelin action. ETa levels were significantly higher than ETb levels. This is in agreement with a recent study showing that ETa expression was more abundant in the granulosa cell layer (Choi et al., 2011). Which receptor type mediates endothelin-2 action in the ovary remains unclear. Although it was suggested that ETa could act by inducing smooth muscle constriction around the ovulating follicle (Ko et al., 2006), another study implied that ETb receptors in granulose cells mediate endothelin-2 induction of target genes during the final stages of follicular wall breakdown (Palanisamy et al., 2006). Whether ETa in human GLCs mediates granulosa-dependent functions of endothelins is not yet known.

In addition to the ovulatory process, gonadotropins induce and maintain the steroidogenic function in GLCs. One of the prominent proteins stimulated by LH/hCG is steroidogenic acute regulatory protein (StAR), which is responsible for translocating cholesterol into the mitochondria, leading to the production of their main steroid—progesterone (Miller and Strauss, 1999). GLCs derived from normally ovulating patients treated with controlled ovarian hyperstimulation exhibited a clear elevation in StAR gene activity in response to luteotropic agents (Amsterdam and Selvaraj, 1997; Christenson and Strauss, 2000). We showed here that StAR mRNA in GLCs from PCOS patients undergoing controlled ovarian hyperstimulation was not different from that found in normally ovulating women. These findings might be related to the similar progesterone concentrations measured in mature follicles from PCOS women and normally ovulating women undergoing IVF treatment cycles (Teissier et al., 2000). Only a few studies examined the levels of StAR expression in fresh GLCs from mature follicles from PCOS patients undergoing oocyte aspiration, and the results obtained were contradictory. Sander et al. concluded that women with PCOS exhibited enhanced mRNA abundance of StAR when compared with controls (Sander et al., 2011). Paradoxically, they found lower progesterone and estradiol levels than in healthy follicles. They hypothesized that increased degradation of progesterone to its inactive metabolite is responsible for this finding. In another study of PCOS and control patients the mean levels of StAR mRNA in granulosa cells of a small follicle were similar (Jakimiuk et al., 2001).

Prolonged culture of the GLCs in gonadotrophin-free medium was found to re-establish their responsiveness to FSH stimulation and to enhance the formation of both progesterone and estradiol upon stimulation with either FSH or hCG (Schipper et al., 1993). Using a similar model, we studied the steroid-synthesizing ability of these cells in vitro and their responses to luteotropic stimuli. StAR expression was elevated in GLCs in response to luteotropic signals such as LH/hCG/FSK (data not shown). A clear response was also evident at the functional level, since these signals increased progesterone production by the cells. Although we could restore the steroidogenic response after 7 days of deprivation, endothelin expression in the GLCs from
women with PCOS reexposed to luteotropic signals did not exhibit the original pattern of expression when purified immediately after hCG stimulation in vivo. This may suggest that the regulation of endothelin expression cannot be only explained by gonadotropins. Several studies found hypoxia as a critical factor for inducing endothelin-2 in the periovulatory granulosa cells of bovine and mice (Kim et al., 2009; Klipper et al., 2010; Na et al., 2008). Altered endothelin expression in PCOS has been reported in many studies, including those focusing on the role of hypoxia in the regulation of endothelin expression in human tissues. A recent study by Kim et al. (2009) demonstrated that hypoxia-inducible factors are critical for ovulation in mice. Endocrinology 2009;150(7):3392–400.

Kim J, Bagchi IC, Bagchi MK. Signaling by hypoxia-inducible factors is critical for ovulation in mice. Endocrinology 2009;150(7):3392–400.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2012.06.006.

References


