RESEARCH ARTICLE

Molecular Reproduction & Development 81:748–756 (2014)

Platelet-Derived Growth Factor BB and DD and Angiopoietin1 are Altered in Follicular Fluid from Polycystic Ovary Syndrome Patients

LEOPOLDINA SCOTTI,[1] FERNANDA PARBORELL,[1] GRISELDA IRUSTA,[1] IGNACIO DE ZUÑIGA,[2] CLAUDIO BISIOLI,[2] HERNAN PETTOROSSI,[2] MARTA TESONE[1,3], AND DALHIA ABRAMOVICH[1]*

[1] Instituto de Biología y Medicina Experimental—CONICET, Buenos Aires, Argentina
[2] Centro Médico PREGNA Medicina Reproductiva, Buenos Aires, Argentina
[3] Departamento de Química Biológica, Facultad de Ciencias Exactas, Universidad de Buenos Aires, Buenos Aires, Argentina

SUMMARY

Polycystic ovary syndrome (PCOS) is the most common endocrinological pathology among women of reproductive age, and is characterized by abnormalities in ovarian angiogenesis, among other features. Consistent with this association, follicular fluid (FF) concentration and ovarian expression of vascular endothelial growth factor (VEGF) are increased in PCOS patients. In this study, we examined the protein levels of platelet-derived growth factor (PDGF) BB and DD (PDGFBB and PDGFDD), angiopoietin 1 and 2 (ANGPT1 and ANGPT2), and their soluble receptor sTIE2 in FF from PCOS and control patients undergoing assisted reproductive techniques. We also analyzed the effect of FF from PCOS and control patients on tight and adherens junction protein expression in an endothelial cell line. PDGFBB and PDGFDD were significantly lower whereas ANGPT1 concentration was significantly higher in FF from PCOS patients than from control patients. No changes were found in the concentration of ANGPT2 or sTIE2. Expression of claudin-5 was significantly increased in endothelial cells incubated for 24 hr in the presence of FF from PCOS versus from control patients, while vascular-endothelial cadherin, β-catenin, and zonula occludens 1 expression were unchanged. The changes observed in the levels of PDGF isoforms and ANGPT1 may prevent VEGF-induced vascular permeability in the PCOS ovary by regulating endothelial-cell-junction protein levels. Restoring the levels of angiogenic factors may provide new insights into PCOS treatment and the prevention of ovarian hyperstimulation syndrome in affected women.

*Corresponding author:
Instituto de Biología y Medicina Experimental
Vuelta de Obligado
2490, C1428ADN
Buenos Aires, Argentina.
E-mail: dnabramovich@gmail.com

Grant sponsor: ANPCYT; Grant numbers: PICT 2011-2106, 2010-0248
Grant sponsor: Roemmers Foundation

Mol. Reprod. Dev. 81: 748–756, 2014. *© 2014 Wiley Periodicals, Inc.*

Published online 23 July 2014 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/mrd.22343

Received 27 February 2014; Accepted 15 May 2014

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrinological pathology among women of reproductive age, affecting more than 5% of the female population (Azziz et al., 2004; Carmina, 2012). PCOS is characterized by hyperandrogenism, anovulation, and oligo- or amenorrhea (Franks, 1995), and is associated with obesity and insulin

Abbreviations: ANGPT, angiopoietin; DHEA, dehydroepiandrosterone; ELISA, enzyme-linked immunosorbent assay; FF, follicular fluid; hCG, human chorionic gonadotropin; PCOS, polycystic ovary syndrome; PDGF, platelet-derived growth factor; OHSS, ovarian hyperstimulation syndrome; VEGF, vascular endothelial growth factor.

© 2014 WILEY PERIODICALS, INC.

resistance (Dunaif, 1997). Therefore, PCOS has implications for both reproductive function and long-term health. Within the ovary, stromal and theca interna hyperplasia leads to hypersecretion of androgens and the formation of multiple cysts (Amin et al., 2003). PCOS has also been characterized by abnormalities in ovarian angiogenesis (Kamat et al., 1995; Zaidi et al., 1995; Agrawal et al., 1998; Artini et al., 2006).

It is known that PCOS patients present increased follicular fluid (FF) concentration and ovarian expression of vascular endothelial growth factor (VEGF) (Kamat et al., 1995; Stanek et al., 2007), which is reflected in the their serum VEGF concentration (Agrawal et al., 1998; Abd El Aal et al., 2005; Artini et al., 2006). After human chorionic gonadotropin (hCG) administration during ovarian hyperstimulation in PCOS patients, VEGF concentration increases significantly, resulting in a higher risk of suffering from ovarian hyperstimulation syndrome (OHSS) (Agrawal et al., 1998; Pellicer et al., 1999).

The platelet-derived growth factor (PDGF) family plays a critical role in the recruitment of pericytes to newly formed vessels (Hoch and Soriano, 2003; Betsholtz, 2004). This family consists of five ligands—PDGFAA, PDGFBB, PDGFAB, PDGFCC, and PDGFDD—and two tyrosine kinase receptors—PDGFRα and PDGFRβ (Andrae et al., 2008). PDGFAA, PDGFCC, and PDGFAB bind to PDGFRα, whereas PDGFBB and PDGFDD bind to PDGFRβ. Endothelial cells express PDGFBB, while periendothelial cells (smooth muscle cells and pericytes) express PDGFDD and PDGFRβ (Andrae et al., 2008). Therefore, only PDGFRβ and its ligands are involved in vascular permeability. Interestingly, the PDGF pathway is required for theca-cell development and steroid production in the ovary (Schmahl et al., 2008). Indeed, we recently demonstrated that ovarian levels of both PDGFBB and PDGFDD are reduced in a rat OHSS model (Scotti et al., 2013).

Another family of angiogenic factors involved in the stabilization of new vessels is the angiopoietin/TIE2 family, which includes angiopoietin 1 (ANGPT1), angiopoietin 2 (ANGPT2), and their tyrosine kinase receptor, TIE2. ANGPT1, which activates TIE2, is involved in the maturation and stabilization of newly formed capillaries by regulating perivascular cell coverage of these capillaries (Suri et al., 1996). Although ANGPT2 also binds to TIE2 with the same affinity as ANPGT1, it does not activate signaling and is therefore considered a TIE2 antagonist that promotes vessel destabilization (Maisonpierre et al., 1997). Another natural antagonist is the soluble form of TIE2 (sTIE2), which traps ANGPT1 and impairs its binding to TIE2 (Yabkowitz et al., 1999). We and others have demonstrated the expression of both *ANGPT*s in the ovary in different species at both mRNA and protein levels (Maisonpierre et al., 1997; Goede et al., 1998; Hazzard et al., 1999; Abramovich et al., 2009). In a recent study, we reported that ANGPT1 and TIE2 protein levels are increased while ANGPT2 protein level is lower in the ovaries of dehydroepiandrosterone (DHEA)-induced PCOS rats (Abramovich et al., 2012).

Endothelial cell–cell junctions regulate the permeability and stability of blood vessels (Bazzoni and Dejana, 2004). The main endothelial cell junctions are tight and adherens junctions, both of which can transfer intracellular signals that control different endothelial-cell functions (Dejana et al., 2009). Claudins are the central components of tight junctions. Claudin 5 is an endothelial-specific member that intracellularly binds to members of the zonula occludens (ZO) protein family (Nitta et al., 2003). One of the endothelial-specific transmembrane proteins contributing to adherens junctions is vascular-endothelial (VE)-cadherin, which binds to cytoplasmic adapter molecules such as β-catenin (Dejana et al., 2009).

Junctional proteins undergo continuous rearrangement, even in the adult vasculature (Kametani and Takeichi, 2007). Several angiogenic and inflammatory agents further modulate vascular integrity (Dejana et al., 2009). The ovary, however, must tightly control these dynamic changes to the localization and expression of adhesion molecules during follicular development, ovulation, and corpus luteum formation (Groten et al., 2006; Rodewald et al., 2007).

PCOS patients have elevated concentrations of VEGF both in the serum and in the ovaries. Based on some of the symptoms associated with this disease, we hypothesized that other angiogenic factors besides VEGF may be altered in FF from PCOS patients. Accordingly, we analyzed the levels of PDGFBB and PDGFDD, ANGPT1 and ANGPT2, and sTIE2 in FF from PCOS and control patients undergoing assisted reproduction. We also studied the effect of FF from these patients on tight and adherens junction protein expression in an endothelial cell line based on the knowledge that these angiogenic factors regulate vascular permeability.

RESULTS

Concentrations of Some Soluble Angiogenic Protein Differ Between PCOS and Control Patients

We first corroborated that FF from PCOS patients had higher concentrations of VEGF than that from control patients; as previously reported, VEGF concentration was significantly higher in FF from the PCOS patients in our cohort. No differences were found in FF concentrations of progesterone or estradiol between the groups (Table 1).

Concentrations of PDGF proteins were measured from FF using Western blot whereas enzyme-linked immunosorbent assays (ELISAs) were used to quantify the levels of soluble members of the ANGPT family. Both PDGFBB and PDGFDD levels were significantly lower in PCOS patients than in control patients (Fig. 1). Conversely, ANGPT1 concentration was higher in FF from PCOS patients than control patients (Fig. 2A). The concentration of the two antagonists ANGPT2 and sTIE2 showed no changes (Fig. 2B and D). The ANGPT1/ANGPT2 ratio was significantly higher in FF from PCOS patients (Fig. 2C).

In PCOS patients, the concentration of ANGPT1 in FF positively correlated with the number of oocytes retrieved

TABLE 1. Progesterone, Estradiol, and VEGF Concentration in FF From Control and PCOS Patients

	Control	PCOS	*P* value
Progesterone (ng/ml)	22,498 ± 1,527	23,851 ± 2,275	>0.05
Estradiol (ng/L)	1,640 ± 137	1,808 ± 91	>0.05
VEGF (pg/ml)	1,020 ± 55	1,320 ± 135	<0.05

Progesterone and estradiol were measured by radioimmunoassay whereas VEGF was measured by ELISA. Data are expressed as the mean ± standard error of the mean. Statistical significance was defined as $P < 0.05$.

(Fig. 2E). No significant differences were found in the number of oocytes retrieved between groups, however (data not shown).

Effects of FF Angiogenic Factors on Intercellular Junctions Integrity of Endothelial Cells

The total levels of adherens junctions proteins (VE-cadherin and β-catenin) in EA.hy926 cells incubated in the presence of FF from control or PCOS patients were not different (Table 2). Levels of phospho-VE-cadherin and phospho-β-catenin also showed no changes between groups at any time point analyzed (data not shown). Conversely, levels of the tight junction protein claudin-5 were significantly increased in EAhy.926 cells exposed to FF from PCOS patients for 24 hr, but not earlier (Fig. 3). ZO1 concentrations, however, did not change at any time point analyzed (Table 2).

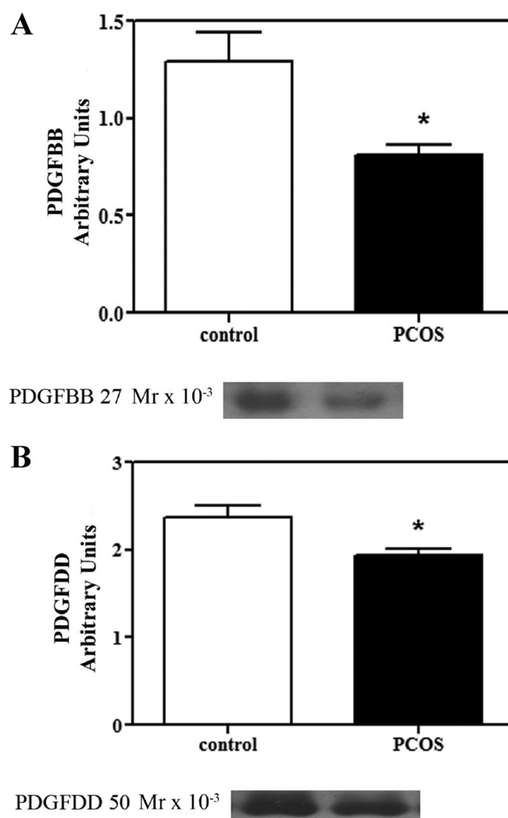

Figure 1. Expression of PDGF in FF from control and PCOS patients, as measured by Western blot. FF samples from control and PCOS patients were centrifuged for 20 min at 14,000 rpm, and the supernatant was diluted 1:10 in homogenization buffer. **A**: Densitometric quantification of PDGFBB ($P < 0.01$) and **B**: PDGFDD ($P < 0.05$). Data are expressed as arbitrary units ± standard error of the mean of three independent experiments ($P < 0.05$). Representative immunoblots are shown in the lower panel.

DISCUSSION

PCOS is a heterogeneous disease associated with alterations in ovarian angiogenesis. Women with this pathology present increased ovarian VEGF synthesis, vascular blood flow, and VEGF serum concentration (Kamat et al., 1995; Zaidi et al., 1995; Agrawal et al., 1998; Artini et al., 2006). In the present work, we describe a decrease in PDGFBB and PDGFDD versus an increase in ANGPT1 proteins in FF from PCOS patients undergoing assisted reproduction. We also found that, despite an increase in the concentration of VEGF in the ovary, the vascular permeability of endothelial cells exposed to these endothelial protein levels showed no changes when incubated with FF from PCOS versus control patients. These results suggest that other angiogenic systems, for example ANGPTs or PDGF, are compensating for the chronic increase in the concentration of this primary permeability factor.

Members of the PDGF system, especially PDGFRβ and its ligands PDGFBB and PDGFDD, constitute a family of angiogenic factors that help regulate permeability and vascular stability. PDGF signaling has been proposed as one of the factors involved in early folliculogenesis (Nilsson et al., 2006; Sleer and Taylor, 2007; Pinkas et al., 2008) since PDGFRβ has been detected in granulosa cells of primordial follicles from adults (Pinkas et al., 2008). Indeed, luteinizing hormone significantly increases *PDGFD* mRNA levels but suppresses *PDGFB* mRNA in human granulosa-luteal cells (Hwu et al., 2009). While PDGF concentrations in FF from OHSS patients are reported to be comparable to those of ascites fluid (Hwu et al., 2009; Chen et al., 2010), the decrease in PDGF levels found in the present work imply a distinct vascular etiology is underlying PCOS—one that may be related to the altered early folliculogenesis observed in PCOS patients. The concomitant reduction in PDGF levels and elevation in ovarian VEGF concentrations in PCOS patients are in agreement with changes reported for *Pdgfb* and *Pdgfrb* knock-out mice, which possess

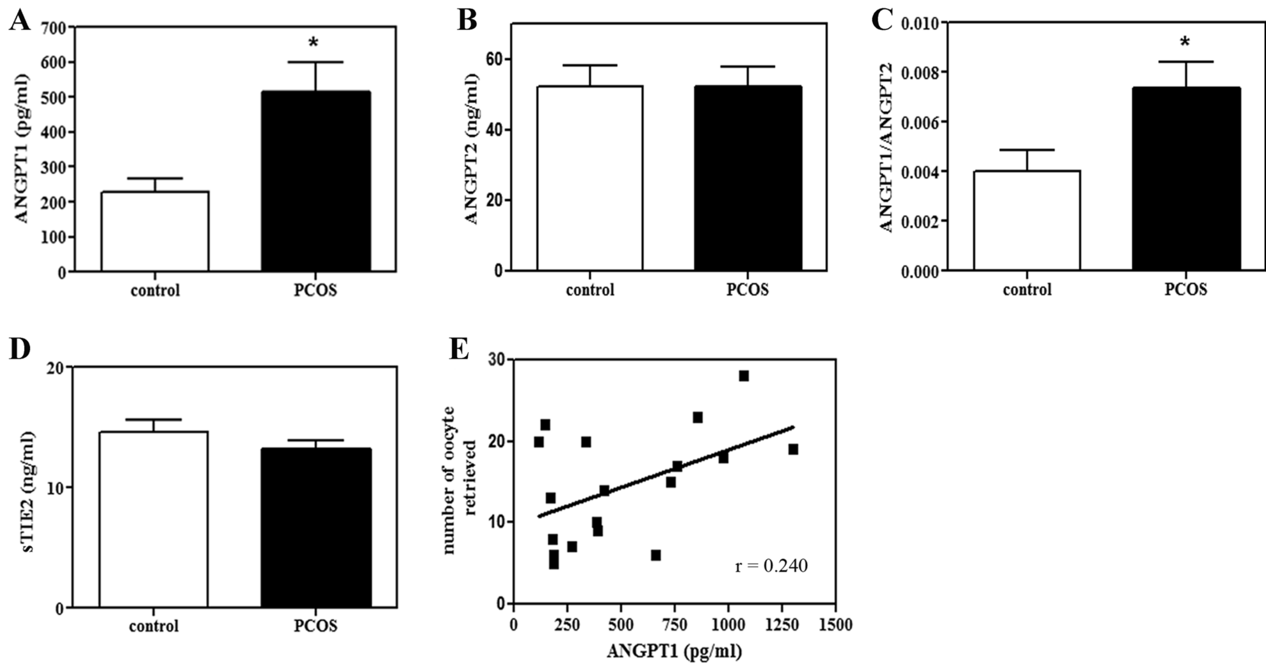

Figure 2. Concentration of the members of the ANGPTs/TIE2 system in FF from control and PCOS patients, as measured by ELISA. **A**: Concentration of ANGPT1 in FF (control: 201.1 ± 23.1 pg/ml; PCOS: 516.4 ± 81.7 pg/ml; $P < 0.05$). **B**: Concentration of ANGPT2 in FF (control: 52.2 ± 6.1 ng/ml; PCOS: 52.4 ± 5.3 ng/ml). **C**: ANGPT1/ANGPT2 ratio in FF from control and PCOS patients ($P < 0.05$). **D**: Concentration of sTIE2 in FF (control: 14.6 ± 1.0 ng/ml; PCOS: 13.2 ± 0.7 ng/ml). **E**: Correlation between the concentration of ANGPT1 in FF and the number of oocytes retrieved from PCOS patients (r = 0.240; $P < 0.05$).

elevated VEGF levels at late gestation (Hellstrom et al., 2001). Taken together, the changes observed in PCOS patients could thus be either a cause or a consequence of the alterations observed in angiogenic factors and/or signaling, although further studies are needed to elucidate this issue.

Among members of the ANGPT/TIE2 family, we and others have demonstrated the importance of the ANGPT system in follicular development and corpus-luteum formation in many species (Maisonpierre et al., 1997; Goede et al., 1998; Parborell et al., 2008; Nishigaki et al., 2011;

TABLE 2. Levels of Intercellular Junction Proteins in Cultured Endothelial Cells after FF Exposure[a]

	6 hr	12 hr	24 hr
VE-cadherin			
Control	0.74 ± 0.10	0.59 ± 0.03	0.64 ± 0.02
PCOS	0.95 ± 0.07	0.52 ± 0.05	0.57 ± 0.07
β-catenin			
Control	2.78 ± 0.43	1.22 ± 0.03	1.35 ± 0.06
PCOS	2.69 ± 0.22	1.25 ± 0.07	1.36 ± 0.03
Zonula occludens 1 (ZO1)			
Control	0.47 ± 0.08	0.58 ± 0.02	1.07 ± 0.17
PCOS	0.67 ± 0.08	0.51 ± 0.03	1.11 ± 0.17

[a]Presented in arbitrary units ± standard error of the mean.

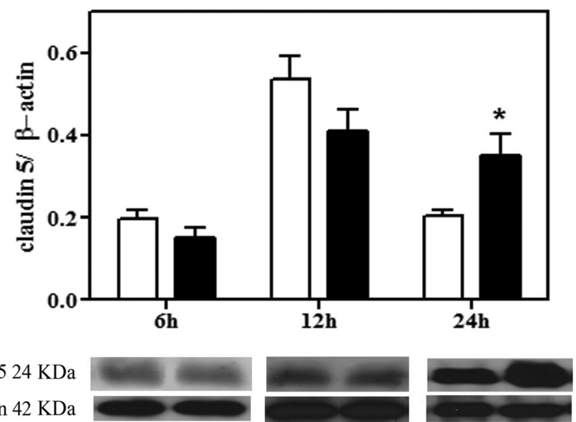

Figure 3. Effect of FF from control and PCOS patients on endothelial cell–cell tight junction proteins. Monolayers of endothelial cells were incubated at 37°C with FF from either control or PCOS patients (25% of FF in culture medium without fetal bovine serum) for 6, 12, or 24 hr. Upper panel: Levels of claudin 5 in endothelial cells incubated with FF for 6, 12, and 24 hr. Data are expressed as arbitrary units ± standard error of the mean, after normalization to β-actin. Lower panel: Representative immunoblots from control patients and PCOS patients.

Parborell et al., 2011). In humans, ANGPT2 concentrations and the ANGPT2/ANGPT1 ratio in FF increase with follicle volume, suggesting that the change in ANGPT1 and ANGPT2 levels may be associated with follicular growth and angiogenesis during the pre-ovulatory period (Nishigaki et al., 2011). We previously reported an increase in the ovarian ANGPT/TIE2 system in a DHEA-induced PCOS rat model (Abramovich et al., 2012), which is consistent with the findings we report herein that ANGPT1 and the ANGPT1/ANGPT2 ratio are increased in FF from PCOS patients whereas the absolute concentration of antagonists ANGPT2 and sTIE2 showed no changes. ANGPT1 normally stabilizes the permeability of blood vessels, thus protecting them from VEGF-induced permeabilization (Thurston et al., 1999; Gamble et al., 2000). Thus in PCOS patients, the increase in the FF ANGPT1/ANGPT2 ratio may be part of a compensatory response to the effect of elevated ovarian VEGF levels. More interestingly, the positive correlation between FF ANGPT1 concentration and the number of oocytes retrieved suggests that the change in ANGPT1/ANGPT2 ratio may be a biomarker that is related to the regulation of follicle development and ovulation in PCOS patients undergoing assisted reproduction.

Angiogenic factors such as VEGF, ANGPTs, and PDGFs have been shown to be involved in the dynamic regulation of the proteins that control vascular permeability, which depends on the integrity of endothelial cell–cell junctions (Harhaj et al., 2002; Dejana et al., 2009). One of the mechanisms by which VEGF can promote endothelial permeability is the phosphorylation of VE-cadherin, which leads to its internalization and endocytosis (Esser et al., 1998). In the present work, we observed no difference in the levels of VE-cadherin and phospho-VE-cadherin in an endothelial cell line incubated with FF from control and PCOS patients. Mechanistically, as ANGPT1 is able to prevent VE-cadherin phosphorylation (Gavard and Gutkind, 2008) and even to dephosphorylate VE-cadherin (Gamble et al., 2000), the increased concentration of ANGPT1 found in FF from PCOS patients could be reversing any increased VEGF-triggered phosphorylation of VE-cadherin (Gamble et al., 2000). Similarly, no differences were found in the other junction proteins analyzed (ZO1, β-catenin, and phospho-β-catenin), suggesting some compensatory activity. The levels of endothelial claudin-5, however, were elevated after incubation with FF from PCOS patients.

PCOS patients have an increased risk of developing OHSS after gonadotropin stimulation in assisted reproductive protocols (Peitsidis and Agrawal, 2010). OSHH is characterized by a rapid shift of fluid from the vascular bed to the extravascular space, which can be triggered by hCG stimulation (Gomez et al., 2010) followed by VEGF-dependent vascular permeabilization (McClure et al., 1994; Garcia-Velasco et al., 2004). hCG can stimulate *VEGF* expression, which in turn decreases the expression of claudin-5, thus contributing to VEGF-induced vascular permeability—at least in human umbilical-vein endothelial cells (HUVECs) (Rodewald et al., 2009). In PCOS patients, FF VEGF concentration is increased, reflecting an increase in ovarian VEGF expression (Kamat et al., 1995; Stanek et al., 2007). This PCOS-related elevation does not influence phospho-VE-cadherin levels, but did increase claudin-5 abundance in endothelial cells co-incubation with FF, suggesting the influence of a compensatory mechanism involving other angiogenic/inflammatory factors present in FF that serve to suppress any excessive VEGF-triggered permeability. Thus, the delicate balance of angiogenic factors expressed by a PCOS ovary may be altered after gonadotropin stimulation, leading to a decrease in the levels of endothelial junction proteins and an increase in vascular permeability that, in turn, increases the risk of OHSS in PCOS patients (Levin et al., 1998; Soares et al., 2008; Rodewald et al., 2009).

In summary, this is the first study to provide evidence of an alteration in the PDGF and ANGPT/TIE2 proteins in FF from PCOS patients. Despite the higher concentration of VEGF and the expected low levels of endothelial junction proteins in an endothelial cell line incubated with FF from PCOS women, VE-cadherin, β-catenin, and ZO1 levels were not altered whereas claudin-5 increased. Therefore, we hypothesize that the changes seen in the levels of PDGFs and ANGPT1 may prevent VEGF-induced vascular permeability in the ovary by regulating the abundance of endothelial-cell junction proteins. Restoration of angiogenic factor concentrations may provide new approaches to treatment of PCOS symptoms and prevention of OSHH in PCOS patients.

MATERIALS AND METHODS

Subjects and Collection of FF

FF was collected from 46 patients, aged 30–40 years old, undergoing artificial reproduction at the Reproductive Medicine Center Pregna (Buenos Aires, Argentina). Written, informed consent was given by all the patients before recruitment. The study was approved by the Ethics Committee of the Institute of Biology and Experimental Medicine (IByME, Buenos Aires, Argentina).

Patients excluded from the study included those with pelvic pathologies, such as endometriosis, uterine fibroids, or pelvic inflammatory disease. The patients included were classified into two groups: control ($n = 27$) and PCOS ($n = 19$), based on criteria published in the 2003 Rotterdam Consensus (2004). Grouped patients were matched for age and body-mass index.

hCG (10,000 UI/ml Pregnyl®, Schering Plough, Buenos Aires, Argentina) was injected when follicles reached 17 mm in diameter. Oocytes were retrieved under vaginal ultrasound guidance 34 hr later. During oocyte retrieval, the FF from all 16- to 20-mm follicles of each patient was pooled. Only macroscopically clear fluids, indicating lack of contamination, were considered; FF samples with visible blood were excluded from the study. The FF was centrifuged immediately at 2,000*g* for 10 min to remove cellular components and debris, and then transferred to sterile polypropylene tubes. The supernatant was stored at −80°C until assayed.

Measurement of VEGF, ANGPT1, ANGPT2, and Soluble TIE2

Commercial ELISA kits were used to measure FF concentrations of VEGF, ANGPT1, and sTIE2 (Quantikine; R&D Systems Inc., Minneapolis, MN) and ANGPT2 (Sigma–Aldrich, St Louis, MO). Intra-assay and inter-assay coefficients of variation were, respectively, 6.7% and 8.8% for VEGF; 11.5% and 18% for ANGPT1; 10% and 12% for ANGPT2; and 9% and 8% for sTIE-2.

Radioimmunoassay

Progesterone and estradiol concentrations in FF were examined by radioimmunoassay. Labeled steroids were added as internal standards, and the percentage of recovery was 60–80%. Progesterone and estradiol concentrations were measured using specific antibodies supplied by Dr. G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO). Under these conditions, the intra- and inter-assay coefficients of variation were, respectively, 8.0% and 14.2% for progesterone and 7.2% and 12.5% for estradiol.

Endothelial Cell–Cell Junction Protein Quantification

To assess the levels of endothelial cell–cell junction proteins in the presence of FF from control or PCOS patients, we used the EA.hy926 endothelial cell line, kindly provided by Dr. Gareth Owen (Pontifical Catholic University of Chile). This cell line was obtained by hybridizing HUVECs with the A549/8 human lung carcinoma cell line (Dr. Cora-Jean S. Edgell from the University of North Carolina, Chapell Hill, NC). Although used for in vitro models of angiogenesis, this cell line has maintained the phenotype of endothelial cells, with highly differentiated functions that are characteristic of the human vascular endothelium, while offering the advantage of immortality and stability through passage number (Edgell et al., 1983, 1990). EA.hy926 cells express endothelin-1, Weibel-Palade bodies, prostacyclin, factor VIII-related antigen, and endothelial adhesion molecules ICAM-1 and VCAM-1, which are characteristics of pure endothelial cultured cells (Edgell et al., 1983, 1990; Suggs et al., 1986; van Oost et al., 1986; Emeis and Edgell, 1988; Saijonmaa et al., 1991; Thornhill et al., 1993). EA.hy926 cells are also preferred as a homogeneous experimental model because they provide more consistent responses to specific variables and greater reproducibility of data (Eremeeva and Silverman, 1998).

EA.hy926 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Life Technologies, Grand Island, NY; Sigma–Aldrich) with 10% fetal bovine serum (FBS), in the presence of 100 U/ml penicillin G and 100 mg/ml streptomycin sulfate, at 37°C with 5% CO_2. EA.hy926 cells were detached by trypsinization, resuspended in the same medium, plated at a density of 1×10^5 cells per well of a 24-well plate, and grown to confluence. Cells were then incubated at 37°C with 25% FF from either control or PCOS patients for 6, 12, or 24 hr without FBS. After incubation, the culture medium was removed, cells were washed twice with ice-cold phosphate-buffered saline, and cell pellets were stored at −80°C until assayed.

Western Blot

FF was centrifuged for 20 min at 14,000 rpm, and then the supernatant was diluted 1:10 in homogenization buffer containing 20 mM Tris-HCl [pH 8], 137 mM NaCl, 1% Nonidet P-40, and 10% glycerol, supplemented with protease inhibitors (0.5 mM phenylmethanesulfonylfluoride, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM N-*p*-tosyl-lysine chloromethyl ketone, and 0.025 m L-1-tosylamide-2-phenyl-ethylchloromethyl ketone) and phosphatase inhibitors (25 mM sodium fluoride; 0.2 mM sodium orthovanadate, and 10 mM β-glycerophosphate). EA.hy926 cells were lysed in the same homogenization buffer. The cell lysates were centrifuged at 10,000*g* for 10 min at 4°C, then protein concentration was measured in the supernatant by the Bradford assay.

After boiling for 5 min, 20 μg of protein was applied to an SDS-polyacrylamide gel and electrophoresed at 25 mA for 1.5 hr. The resolved proteins were transferred for 2 hr onto nitrocellulose membranes. The blots were preincubated in blocking buffer (5% non-fat milk, 0.05% Tween-20 in 20 mM TBS pH 8.0) for 1 hr at room temperature and incubated overnight with appropriate primary antibodies (anti-PDGFBB, 1:100 [AbCam, Cambridge, MA]; anti-PDGFDD, 1:1,000; anti-actin B, 1:3,000; anti-VE-cadherin, 1:200; anti-β-catenin, 1:2,000 [Santa Cruz Biotechnology, Inc., Dallas, TX]; anti-claudin 5, 1:2000 [Invitrogen Corp., Carlsbad, CA]; anti-ZO1, 1:2000; anti-phospho-VE-cadherin, 1:1,000 [Millipore Corp., Billerica, MA]; and anti-phospho-β-catenin, 1:1,000 [Cell Signaling Technology, Inc., MA]) in blocking buffer at 4°C. Proper loading of secreted FF proteins was evaluated by staining the membranes with Ponceau S (Scotti et al., 2013). Loading of endothelial samples was normalized to actin B (n = 7 per group per gel). The protein levels were quantified by densitometry using Scion Image for Windows (Scion Corporation, Worman's Mill, CT).

Data Analysis

Data are expressed as the mean ± standard error of the mean. Differences between groups were tested for significance using the independent samples *t*-test for parametric variables. The Pearson correlation coefficient was used to assess the relationship between the parameters analyzed. Analysis of data was achieved using the statistical program Prism v5.0. Statistical significance was defined as $P < 0.05$.

ACKNOWLEDGMENT

This study was supported by ANPCYT (PICT 2011-2106 2010-0248) and Roemmers Foundation.

REFERENCES

Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. 2004. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod 19:41–47.

Abd El Aal DE, Mohamed SA, Amine AF, Meki AR. 2005. Vascular endothelial growth factor and insulin-like growth factor-1 in polycystic ovary syndrome and their relation to ovarian blood flow. Eur J Obstet Gynecol Reprod Biol 118:219–224.

Abramovich D, Rodriguez CA, Hernandez F, Tesone M, Parborell F. 2009. Spatiotemporal analysis of the protein expression of angiogenic factors and their related receptors during folliculogenesis in rats with and without hormonal treatment. Reproduction 137:309–320.

Abramovich D, Irusta G, Bas D, Cataldi NI, Parborell F, Tesone M. 2012. Angiopoietins/TIE2 system and VEGF are involved in ovarian function in a DHEA rat model of polycystic ovary syndrome. Endocrinology 153:3446–3456.

Agrawal R, Sladkevicius P, Engmann L, Conway GS, Payne NN, Bekis J, Tan SL, Campbell S, Jacobs HS. 1998. Serum vascular endothelial growth factor concentrations and ovarian stromal blood flow are increased in women with polycystic ovaries. Hum Reprod 13:651–655.

Amin AF, Abd el-Aal DE, Darwish AM, Meki AR. 2003. Evaluation of the impact of laparoscopic ovarian drilling on Doppler indices of ovarian stromal blood flow, serum vascular endothelial growth factor, and insulin-like growth factor-1 in women with polycystic ovary syndrome. Fertil Steril 79:938–941.

Andrae J, Gallini R, Betsholtz C. 2008. Role of platelet-derived growth factors in physiology and medicine. Genes Dev 22: 1276–1312.

Artini PG, Monti M, Matteucci C, Valentino V, Cristello F, Genazzani AR. 2006. Vascular endothelial growth factor and basic fibroblast growth factor in polycystic ovary syndrome during controlled ovarian hyperstimulation. Gynecol Endocrinol 22: 465–470.

Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. 2004. The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab 89:2745–2749.

Bazzoni G, Dejana E. 2004. Endothelial cell-to-cell junctions: Molecular organization and role in vascular homeostasis. Physiol Rev 84:869–901.

Betsholtz C. 2004. Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev 15:215–228.

Carmina E. 2012. PCOS: Metabolic impact and long-term management. Minerva Ginecol 64:501–505.

Chen SU, Chou CH, Lin CW, Lee H, Wu JC, Lu HF, Chen CD, Yang YS. 2010. Signal mechanisms of vascular endothelial growth factor and interleukin-8 in ovarian hyperstimulation syndrome: Dopamine targets their common pathways. Hum Reprod 25:757–767.

Dejana E, Tournier-Lasserve E, Weinstein BM. 2009. The control of vascular integrity by endothelial cell junctions: Molecular basis and pathological implications. Dev Cell 16:209–221.

Dunaif A. 1997. Insulin resistance and the polycystic ovary syndrome: Mechanism and implications for pathogenesis. Endocr Rev 18:774–800.

Edgell CJ, McDonald CC, Graham JB. 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci USA 80:3734–3737.

Edgell CJ, Haizlip JE, Bagnell CR, Packenham JP, Harrison P, Wilbourn B, Madden VJ. 1990. Endothelium specific Weibel-Palade bodies in a continuous human cell line, EA.hy926. In Vitro Cell Dev Biol 26:1167–1172.

Emeis JJ, Edgell CJ. 1988. Fibrinolytic properties of a human endothelial hybrid cell line (Ea.hy 926). Blood 71:1669–1675.

Eremeeva ME, Silverman DJ. 1998. Rickettsia rickettsii infection of the EA.hy 926 endothelial cell line: Morphological response to infection and evidence for oxidative injury. Microbiology 144:2037–2048.

Esser S, Lampugnani MG, Corada M, Dejana E, Risau W. 1998. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J Cell Sci 111: 1853–1865.

Franks S. 1995. Polycystic ovary syndrome. N Engl J Med 333:853–861.

Gamble JR, Drew J, Trezise L, Underwood A, Parsons M, Kasminkas L, Rudge J, Yancopoulos G, Vadas MA. 2000. Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions. Circ Res 87:603–607.

Garcia-Velasco JA, Zuniga A, Pacheco A, Gomez R, Simon C, Remohi J, Pellicer A. 2004. Coasting acts through downregulation of VEGF gene expression and protein secretion. Hum Reprod 19:1530–1538.

Gavard J, Gutkind JS. 2008. VE-cadherin and claudin-5: It takes two to tango. Nat Cell Biol 10:883–885.

Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG. 1998. Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest 78:1385–1394.

Gomez R, Soares SR, Busso C, Garcia-Velasco JA, Simon C, Pellicer A. 2010. Physiology and pathology of ovarian hyperstimulation syndrome. Semin Reprod Med 28:448–457.

Groten T, Fraser HM, Duncan WC, Konrad R, Kreienberg R, Wulff C. 2006. Cell junctional proteins in the human corpus luteum: Changes during the normal cycle and after hCG treatment. Hum Reprod 21:3096–3102.

Harhaj NS, Barber AJ, Antonetti DA. 2002. Platelet-derived growth factor mediates tight junction redistribution and increases permeability in MDCK cells. J Cell Physiol 193:349–364.

Hazzard TM, Molskness TA, Chaffin CL, Stouffer RL. 1999. Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval. Mol Hum Reprod 5:1115–1121.

Hellstrom M, Gerhardt H, Kalen M, Li X, Eriksson U, Wolburg H, Betsholtz C. 2001. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. J Cell Biol 153: 543–553.

Hoch RV, Soriano P. 2003. Roles of PDGF in animal development. Development 130:4769–4784.

Hwu YM, Li SH, Lee RK, Lin MH, Tsai YH, Yeh TS. 2009. Luteinizing hormone increases platelet-derived growth factor-D gene expression in human granulosa-luteal cells. Fertil Steril 92:2065–2068.

Kamat BR, Brown LF, Manseau EJ, Senger DR, Dvorak HF. 1995. Expression of vascular permeability factor/vascular endothelial growth factor by human granulosa and theca lutein cells. Role in corpus luteum development. Am J Pathol 146:157–165.

Kametani Y, Takeichi M. 2007. Basal-to-apical cadherin flow at cell junctions. Nat Cell Biol 9:92–98.

Levin ER, Rosen GF, Cassidenti DL, Yee B, Meldrum D, Wisot A, Pedram A. 1998. Role of vascular endothelial cell growth factor in Ovarian Hyperstimulation Syndrome. J Clin Invest 102: 1978–1985.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55–60.

McClure N, Healy DL, Rogers PA, Sullivan J, Beaton L, Haning RV Jr, Connolly DT, Robertson DM. 1994. Vascular endothelial growth factor as capillary permeability agent in ovarian hyperstimulation syndrome. Lancet 344:235–236.

Nilsson EE, Detzel C, Skinner MK. 2006. Platelet-derived growth factor modulates the primordial to primary follicle transition. Reproduction 131:1007–1015.

Nishigaki A, Okada H, Tsuzuki T, Cho H, Yasuda K, Kanzaki H. 2011. Angiopoietin 1 and angiopoietin 2 in follicular fluid of women undergoing a long protocol. Fertil Steril 96:1378–1383.

Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, Tsukita S. 2003. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 161:653–660.

Parborell F, Abramovich D, Tesone M. 2008. Intrabursal administration of the antiangiopoietin 1 antibody produces a delay in rat follicular development associated with an increase in ovarian apoptosis mediated by changes in the expression of BCL2 related genes. Biol Reprod 78:506–513.

Parborell F, Abramovich D, Irusta G, Tesone M. 2011. Angiopoietin 1 reduces rat follicular atresia mediated by apoptosis through the PI3K/Akt pathway. Mol Cell Endocrinol 343:79–87.

Peitsidis P, Agrawal R. 2010. Role of vascular endothelial growth factor in women with PCO and PCOS: A systematic review. Reprod Biomed Online 20:444–452.

Pellicer A, Albert C, Mercader A, Bonilla-Musoles F, Remohi J, Simon C. 1999. The pathogenesis of ovarian hyperstimulation syndrome: In vivo studies investigating the role of interleukin-1beta, interleukin-6, and vascular endothelial growth factor. Fertil Steril 71:482–489.

Pinkas H, Fisch B, Rozansky G, Felz C, Kessler-Icekson G, Krissi H, Nitke S, Ao A, Abir R. 2008. Platelet-derived growth factors (PDGF-A and -B) and their receptors in human fetal and adult ovaries. Mol Hum Reprod 14:199–206.

Rodewald M, Herr D, Fraser HM, Hack G, Kreienberg R, Wulff C. 2007. Regulation of tight junction proteins occludin and claudin 5 in the primate ovary during the ovulatory cycle and after inhibition of vascular endothelial growth factor. Mol Hum Reprod 13:781–789.

Rodewald M, Herr D, Duncan WC, Fraser HM, Hack G, Konrad R, Gagsteiger F, Kreienberg R, Wulff C. 2009. Molecular mechanisms of ovarian hyperstimulation syndrome: Paracrine reduction of endothelial claudin 5 by hCG in vitro is associated with increased endothelial permeability. Hum Reprod 24: 1191–1199.

Saijonmaa O, Nyman T, Hohenthal U, Fyhrquist F. 1991. Endothelin-1 is expressed and released by a human endothelial hybrid cell line (EA.hy 926). Biochem Biophys Res Commun 181:529–536.

Schmahl J, Rizzolo K, Soriano P. 2008. The PDGF signaling pathway controls multiple steroid-producing lineages. Genes Dev 22:3255–3267.

Scotti L, Abramovich D, Pascuali N, de Z I, Oubina A, Kopcow L, Lange S, Owen G, Tesone M, Parborell F. 2013. Involvement of the ANGPTs/Tie-2 system in ovarian hyperstimulation syndrome (OHSS). Mol Cell Endocrinol 365:223–230.

Sleer LS, Taylor CC. 2007. Cell-type localization of platelet-derived growth factors and receptors in the postnatal rat ovary and follicle. Biol Reprod 76:379–390.

Soares SR, Gomez R, Simon C, Garcia-Velasco JA, Pellicer A. 2008. Targeting the vascular endothelial growth factor system to prevent ovarian hyperstimulation syndrome. Hum Reprod Update 14:321–333.

Stanek MB, Borman SM, Molskness TA, Larson JM, Stouffer RL, Patton PE. 2007. Insulin and insulin-like growth factor stimulation of vascular endothelial growth factor production by luteinized granulosa cells: Comparison between polycystic ovarian syndrome (PCOS) and non-PCOS women. J Clin Endocrinol Metab 92:2726–2733.

Suggs JE, Madden MC, Friedman M, Edgell CJ. 1986. Prostacyclin expression by a continuous human cell line derived from vascular endothelium. Blood 68:825–829.

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD. 1996. Requisite role of

angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 87:1171–1180.

Thornhill MH, Li J, Haskard DO. 1993. Leucocyte endothelial cell adhesion: A study comparing human umbilical vein endothelial cells and the endothelial cell line EA-hy-926. Scand J Immunol 38:279–286.

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM. 1999. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science 286: 2511–2514.

van Oost BA, Edgell CJ, Hay CW, MacGillivray RT. 1986. Isolation of a human von Willebrand factor cDNA from the hybrid endothelial cell line EA.hy926. Biochem Cell Biol 64: 699–705.

Yabkowitz R, Meyer S, Black T, Elliott G, Merewether LA, Yamane HK. 1999. Inflammatory cytokines and vascular endothelial growth factor stimulate the release of soluble tie receptor from human endothelial cells via metalloprotease activation. Blood 93:1969–1979.

Zaidi J, Campbell S, Pittrof R, Kyei-Mensah A, Shaker A, Jacobs HS, Tan SL. 1995. Ovarian stromal blood flow in women with polycystic ovaries–a possible new marker for diagnosis? Hum Reprod 10:1992–1996.