Signal mechanisms of vascular endothelial growth factor and interleukin-8 in ovarian hyperstimulation syndrome: dopamine targets their common pathways

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BACKGROUND: Ovarian hyperstimulation syndrome (OHSS) is a serious complication of ovarian stimulation with massive ascites, pleural effusion and hemoconcentration. The pathophysiological signal mechanisms of OHSS are still unclear and merit further investigation.

METHODS: Various angiogenic cytokines of follicular fluid and ascites of patients with risk of OHSS were measured, and examined for inducing endothelial permeability. These include vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-8, basic fibroblast growth factor, tumor necrosis factor-α, IL-1α, IL-1β and platelet-derived growth factor. We explore the molecular signal pathways of major contributing cytokines in granulosa-lutein cells and endothelial cells possibly involved in OHSS.

RESULTS: Neutralizing antibodies of VEGF or IL-8 significantly decreased follicular fluid- and ascites-induced endothelial permeability. Human chorionic gonadotrophin induced VEGF secretion of granulosa-lutein cells through the Sp1 and CREB dependent pathways. IL-8 activated CXCR1/2 of endothelial cells leading to VEGF receptor (VEGFR)-2 transactivation. Both VEGF and IL-8 of follicular fluid enhanced endothelial permeability via VEGFR-2-mediated Rho/Rock activation, actin polymerization and phosphorylations of VE-cadherin and occludin, resulting in opening of adherens junctions and tight junctions. Dopamine (2 μM) inhibited follicular fluid-induced VEGFR-2 signals and endothelial permeability, without diminishing migration and tube formation.

CONCLUSIONS: Our results suggest that VEGF and IL-8 secreted from corpora luteae may play major roles in OHSS. Delineation of signal pathways would be helpful for treatment. Dopamine may block VEGF- and IL-8-induced endothelial permeability by inhibiting common VEGFR-2 dependent signals.

Key words: signal pathway / IL-8 / ovarian hyperstimulation syndrome / vascular endothelial growth factor

Introduction

Ovarian hyperstimulation syndrome (OHSS) is a potential complication of ovarian stimulation in in-vitro fertilization treatments (Schenker and Weinstein, 1978; Delvigne and Rozenberg, 2003; Chen et al., 2008a). A critical condition may develop with massive ascites, pleural effusion, hemoconcentration, oliguria and thrombosis. The underlying cause is an increase in the vascular permeability of enlarged ovaries and other mesothelial surfaces with acute fluid shift out of the intravascular space (Alvarez et al., 2007a; Villasante et al., 2007). Angiogenic cytokines including vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-8, basic fibroblast growth factor (bFGF), tumor necrotic factor-α (TNF-α), IL-1α and IL-1β, etc. produced by multiple corpora luteae may be involved in OHSS (Elchalal and Schenker, 1997; Stanek et al., 2007; Stocco et al., 2007; Varnagy et al., 2009). VEGF is thought to be an attributing factor (McClure et al., 1994; Levin et al., 1998; Manau et al., 2007). However, the effects of remaining angiogenic cytokines on OHSS are vague.

The development of OHSS following ovarian stimulation with gonadotropins is mainly associated with the administration of human
chorionic gonadotrophin (hCG), as the syndrome rarely develops if it is withheld (Nargund et al., 2007; van de Lagemaat et al., 2009; Vardhana et al., 2009). In addition, OHSS becomes more severe after pregnancy occurs. It had been shown that expressions of VEGF mRNA and secretion of VEGF protein were positively regulated by hCG in granulosa-lutein cells (Neulen et al., 1995; Chen et al., 2008b). However, the signal pathway of hCG-mediating VEGF secretion is still unclear (Stocco et al., 2007).

Lysophosphatidic acid (LPA) had been detected to exist at considerable amounts in follicular fluid of pre-ovulatory follicles (Tokumura et al., 1999). In the previous study, we found that LPA mediated IL-8 and IL-6 expressions of granulosa-lutein cells through LPA receptors, mitogen activating protein kinase (MAPK) and nuclear factor (NF)-κB dependent pathways. LPA-induced IL-8 and IL-6 increased angiogenesis and permeability of endothelial monolayer (Chen et al., 2008b). However, the signals of these vasoactive cytokines in endothelial cells involved in OHSS remain elusive.

Conservative treatments with fluid, electrolyte and albumin supplements or paracentesis are primarily used for OHSS (Delvigne and Rozenberg, 2003; Chen et al., 2008a). However, these methods may be not effective for a life-threatening OHSS. The change of endothelial permeability may be determined by inter-endothelial junctions (Chiba et al., 2006; Petreaea et al., 2007; Villasante et al., 2008; Dejana et al., 2008; Rodewald et al., 2009). A better understanding of the pathophysiological and signal mechanisms would be helpful for optimal treatments.

Dopamine, a catecholamine neurotransmitter, is synthesized from the amino acid tyrosine. It is found in the central nervous system, in the periphery at the sympathetic nerve endings, in chromaffin tissues and in mucosal tissues (Chakroborty et al., 2009). Dopamine is present in measurable quantities (1 μM) at nerve synapses which are often adjacent to blood vessels in normal adult tissues (Basu et al., 2001). It has been demonstrated that dopamine receptors are present on endothelial cells and dopamine might exert anti-tumor effect by inhibiting tumor angiogenesis (Chakroborty et al., 2009). The effects of dopamine on the follicular fluid-induced angiogenesis deserve further investigation.

In this study, we explore the effects of angiogenic cytokines of follicular fluid and ascites from patients with risk of OHSS on the endothelial permeability. We examine the molecular signal pathways in granulosa-lutein cells and endothelial cells possibly involved in OHSS. Furthermore, we investigate the potential chemotherapeutics that target these signals, for reducing endothelial permeability.

Materials and Methods

Reagents

Human VEGF, IL-1α, IL-1β, IL-6, IL-8, TNF-α, bFGF and platelet-derived growth factor (PDGF) neutralizing antibodies were obtained from R&D Systems (Minneapolis, MN, USA). Chemical inhibitors of signal mediators including MDL12, H89, U73122, LY294002, SB203580, PD98059, SP600125, staurosporine, CBO-P11, repertaxin, C3 excoyme and Y-27632 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Antibodies of Sp1, MAP response element binding protein (CREB), CXCR1, CXCR2, Rho, VE-cadherin and occludin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies of phospho-Sp1 (T453) and phospho-VE-cadherin (Y731) were from Abcam Biotechnology (Cambridge, MA, USA). Antibodies of phospho-CREB (Ser133), and phospho-VEGFR-2 (Tyr951) were obtained from R&D Systems.

Follicular fluid and ascites

This study was approved by the ethics committee of the National Taiwan University Hospital. Follicular fluid was obtained from patients (n = 22) undergoing controlled ovarian stimulation and transvaginal aspiration of follicles for in-vitro fertilization with more than 20 follicles. To obtain the follicular fluid and avoid contamination from blood and flush medium during oocyte retrieval, only the follicular fluid from the first retrieved follicle (mean diameter of 18–20 mm) from bilateral ovaries was collected. The presence or absence of blood contamination was determined by visual inspection, and samples with blood stain were discarded. Meticulous care was taken to include only uncontaminated samples. Ascites was obtained from patients (n = 8) undergoing paracentesis due to severe OHSS. The obtained fluid was centrifuged at 350 g for 5 min to remove cells and frozen at −70 °C for subsequent assays.

Granulosa-lutein cell culture

The granulosa-lutein cells were collected from follicular aspirates of patients with more than 20 follicles. Follicular fluid from all follicles was collected and then centrifuged at 350 g for 5 min. The cells were resuspended in 10 ml of hepes-buffered human tubal fluid (HTF) medium. The cell suspensions were layered onto 10 ml Ficoll (Sigma-Aldrich). After centrifugation at 450 g for 15 min, the interphase cells were collected. The granulosa-lutein cells were treated with 80 IU/ml hyaluronidase in 1 ml HTF for 10 min for dispersion of cells. The cells were washed and suspended in RPMI containing 10% fetal bovine serum, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.25 mg/ml amphotericin B. The granulosa-lutein cells were seeded in a flask. On the second day, the cells were washed to remove remaining red blood cells, as these did not adhere to the plastic surface. The cells were then incubated at 37 °C in a humidified atmosphere with 5% CO₂ in air. Three days after collection, the cultured granulosa-lutein cells were used for experiments.

Enzyme immunoassay

Levels of free VEGF-A, IL-1α, IL-1β, IL-6, IL-8, TNF-α, bFGF or PDGF of follicular fluid, ascites and supernatant of granulosa-lutein cell culture were determined using enzyme immunoassay (EIA) kits (R&D Systems). The intra- and inter-assay coefficients of variation for VEGF-A were 5.1 and 6.2%, respectively; for IL-1α were 1.4 and 4.3%, respectively; for IL-1β were 8.5 and 8.4%, respectively; for IL-6 were 2.0 and 3.8%, respectively; for IL-8 were 6.5 and 6.1%, respectively; for TNF-α were 8.6 and 7.0%, respectively; for bFGF were 8.7 and 13.4%, respectively; and for PDGF were 8.7 and 12.0%, respectively.

Human umbilical vein endothelial cells permeability assay

Human umbilical vein endothelial cells (HUVEC) were isolated from fetal umbilical cord after Cesarean section and cultured in endothelial cell culture medium (Sigma-Aldrich) with 10% fetal bovine serum. Horseradish peroxidase diffusion through HUVEC monolayer was measured as previously described (Essler et al., 1999), with some modifications. HUVEC were cultured in trans-well chambers (0.4 μm pore-size polycarbonate filters; Costar Corp., Cambridge, MA, USA). After reaching confluence, the medium was replaced with the indicated conditions (0.3 ml in the upper chamber and 1 ml in the lower chamber). Horseradish peroxidase molecules (Type VI-A, 44 kDa; Sigma-Aldrich) at a concentration of 0.126 μM were added to the upper compartment. After incubation for
1 h, the medium in the lower compartment was collected and assayed for enzymatic activity using a photometric guaiacol substrate assay (Sigma-Aldrich). The reaction was allowed to proceed for 15 min at room temperature, and absorbance was measured at 450 nm.

RNA interference
Small interfering RNA duplexes (siRNA) of hCG/LH receptor gene (SC-40105) were purchased from Santa Cruz Biotechnology. Negative control siRNAs (Invitrogen Corporation, Carlsbad, CA, USA) with sequences that did not target any gene product was used for control. Liphilized siRNA duplex was resuspended in the RNase-free water to form a 10 μM solution in a 10 μM Tris–HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA buffered solution. The siRNA was incubated for 15 min at room temperature to allow complex formation between siRNA and TransFast™ Transfection Reagent (Promega, Southampton, UK). Granulosa-lutein cells (2.5 × 10^7/ml in 6-cm dish) were transfected with siRNA in serum-free Opti-MEM (Invitrogen Corporation, Carlsbad, CA, USA) at a concentration of 1 μM and centrifuged at 30,000 g for 25 min at 4°C. The specificity of the above decoys was verified with control scrambled decoys (Chen et al., 2007).

Western blotting
The granulosa-lutein cells were lysed in a lysis buffer. The cell lysates were centrifuged at 30,000 g for 1 h at 4°C. The cell lysates were侦鼠 with 5% fat-free milk for non-phosphorylated form of the targeted genes, thus inhibiting gene transactivation. The sequences of the phorothioate oligodeoxynucleotides for various decoys were 5′-CGC GGG GCA TG-3′ for Sp1; 5′-ATA CGG GGG GCA TG-3′ for p53; 5′-ATT CCG GGG GCA TG-3′ for Sp1; and 5′-TGA CGT CAT GAC GTC TCA-3′ for cAMP response element (CRE). For transfection of granulosa-lutein cells, the AP-1, p53, Sp1 or CRE decoys at the concentration of 25 nM by incubation for 1 h at 37°C. The cells were then changed for culture medium and incubated for 24 h at 37°C prior to experiments.

Double-stranded oligodeoxyxynucleotides decoys
The procedures of oligodeoxyxynucleotides decoy assay were performed based on previous description (Wu et al., 2007). Synthetic double-stranded oligodeoxyxynucleotides were used as ‘decoy’ cis elements to block the binding of nuclear factors to promoter regions of the targeted genes, thus inhibiting gene transactivation. The sequences of the phosphorothioate oligodeoxynucleotides for various decoys were 5′-TGT CTG ACT CAT GTC-3′ for activator protein 1 (AP-1); 5′-TAC AGA ACA TGT CTA AGC ATG CTG GGG-3′ for p53; 5′-ATT CCG GGG GGG GCA TG-3′ for Sp1; and 5′-TGA CGT CAT GAC GTC ATG ACG TCA-3′ for cAMP response element (CRE). For transfection of granulosa-lutein cells, the AP-1, p53, Sp1 or CRE decoys at the concentration of 1 μM were mixed with the Transfast transfection reagent (Promega) for 15 min and then incubated with the cells in a serum-free medium. The specificity of the above decoys was verified with control scrambled decoys (Chen et al., 2008b).

GTP-Rho pull-down assay
Active Rho was determined by GTP-Rho pull-down assay as described previously (Shimizu et al., 2007), with some modifications. Briefly, HUVEC that treated with different conditions were lysed with cold lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 10% glycerol, 50 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 50 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml each of leupeptin and pepstatin and 1% Nonidet P-40). The cell lysates were centrifuged, 30,000 g, at 4°C for 20 min and the supernatants were collected. A half volume of supernatants were incubated with 20 μg of glutathione S-transferase/Rho-binding domain fusion protein conjugated with glutathione beads (Thermo Fisher Scientific Inc., Rockford, IL, USA) for 2 h at 4°C. The beads bound with Rho-GTP were washed twice with lysis buffer, and were then boiled in SDS-PAGE loading buffer. Rho-GTP was detected by western blotting. Precipitated proteins were analyzed by 12% SDS-PAGE and immunoblotting. Total Rho in the other half volume of supernatants was detected as input control.

Actin stain and fluorescence microscopy
Stain of filamentous actin (F-actin) was performed based on previous description (Eiselein et al., 2007). HUVEC on collagen-coated trans-well membranes were washed with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde for 20 min. They were then washed three times in PBS, and permeabilized with 0.1% Triton-X-100. Fluorescence isothiocyanate-conjugated phalloidin (Invitrogen), diluted in PBS (2 μl/ml), was then applied to the specimens in the dark for 1 h. The specimens were mounted with 10% glycerol. The images were viewed using a fluorescence microscope (Nikon, Tokyo, Japan).

HUVEC migration assay
A total of 2 × 10^5 HUVEC in 200 μl of culture medium were added to the upper chamber of the 24-well Millicell inserts (8 μm pore; Millipore Corporate, Bedford, MA, USA), with 500 μl of culture medium added to the lower chamber. After 1 h for cell attachment, the medium was changed to serum-free medium in the upper chamber and to the indicated conditioned medium in the lower chamber. Following incubation for 6 h to allow endothelial cells to migrate across the membrane, the inserts were then disassembled. The upper wells were fixed with 1% formaldehyde solution. The cells remaining on the upper surface of the filter were removed using a rubber scrapper. Cells migrating across the filter onto the lower surface were counted under a microscope (×20).

HUVEC capillary tube formation assay
HUVEC (5 × 10^4) in the medium of indicated conditions were plated on a growth factor-reduced Matrigel coated 24-well plates. Following incubation for 6 h to allow the tube formation, the wells were observed under a phase-contrast microscope (×20). The tube formation was defined as the formation of capillary-like structure with a complete ring composed by endothelial cells (Tamilarasan et al., 2006). To quantify tube formation, six random fields were imaged and the mean value was calculated.

Statistics
In this study, each experiment was repeated at least three times on different occasions. Data were presented as mean ± standard deviation (SD). The data were examined with one-way analysis of variance, followed by Tukey test for multiple comparisons. Significance level was set as P < 0.05 by two-tailed test. SAS software version 8.01 (SAS Institute Inc., Cary, NC, USA) was used for calculation.

Results

Angiogenic factors of follicular fluid and ascites inducing endothelial permeability
Using EIA, we measured the concentrations of various angiogenic cytokines in the follicular fluid and ascites (Table I). The levels of
these cytokines in the follicular fluid were comparable to those of ascites. Using HUVEC permeability assay, with pre-incubation of neutralizing antibodies of various cytokines, the follicular fluid-induced permeability was reduced, to different degrees (Fig. 1A, n = 22). When the endothelial permeability induced by follicular fluid was defined as 100%, the relative permeability was significantly reduced to 43.6 ± 10.7% by VEGF antibody and to 33.6 ± 25.4% by IL-8 antibody. The differences were not significant when follicular fluid was pretreated with antibodies of IL-6, bFGF, TNF-α, IL-1α, IL-1β or PDGF. The comparable results were found in the experiments of ascites (Fig. 1B, n = 8). The relative permeability was significantly diminished to 45.6 ± 19.3% by VEGF antibody and to 53.8 ± 14.6% by IL-8 antibody.

We further performed the HUVEC permeability assay of the blank (controlled) medium and co-incubation studies. We found that follicular fluid significantly enhanced endothelial permeability, compared with the controlled medium. By co-incubation of VEGF and IL-8 neutralizing antibodies with follicular fluid, the relative permeability was remarkably decreased to 9.8 ± 6.0% for the follicular fluid-induced permeability. The relative permeability was not significantly different between the blank medium group and the co-incubation group (Fig. 1C, n = 22). The comparable results were found in the experiments of ascites. Ascites significantly led to endothelial hyperpermeability, compared with the controlled medium. By co-incubation of VEGF and IL-8 antibodies with ascites, the relative permeability was remarkably decreased to 12.8 ± 7.4% for the ascites-induced relative permeability. The relative permeability was not significantly different between the blank medium group and the co-incubation group (Fig. 1D, n = 8).

**Signal transduction pathways involved in hCG-inducing VEGF secretions in granulosa-lutein cells**

Using western blot analyses, we verified that cultured granulosa-lutein cells expressed LH/hCG receptor and the LH/hCG receptor siRNA...
effectively inhibited its expression (Fig. 2A, left panel). We found that hCG induced VEGF secretion of granulosa-lutein cells via LH/hCG receptor, as evidence of a significant decrease of VEGF secretion by treatment with LH/hCG receptor siRNA (Fig. 2A, right panel). We investigated the signal mechanism using chemical inhibitors of adenylate cyclase (MDL12) and protein kinase A (PKA) (H89) and phospholipase C (PLC) (U73122). The data showed that MDL12, H89 and U73122 significantly inhibited hCG-mediated VEGF secretions of granulosa-lutein cells (Fig. 2B). The signal mediators involved in hCG-enhanced VEGF secretion were further explored using inhibitors of PI3K/Akt (LY294002), MAPK/p38 (SB203580), MAPK/ERK (PD98059), JNK (SP600125) and PKC (staurosporine). The results revealed that staurosporine significantly diminished hCG-enhanced VEGF secretion (Fig. 2C).

We further applied the AP-1, p53, Sp1 and CRE decoy oligodeoxynucleotides strategy to ascertain the transcription factors involved. We found that Sp1 and CRE decoys, but not AP-1 or p53 decoys, significantly inhibited hCG-induced VEGF secretion in granulosa-lutein cells (Fig. 2D). Using western blotting and densitometry, we explored signal cascades of hCG inducing Sp1 and CREB activation. We found that hCG induced phosphorylation of Sp1. The phosphorylation was significantly decreased by pretreatment with PLC inhibitor (U73122) and PKC inhibitor (staurosporine) (Fig. 2E). We observed that hCG induced phosphorylation of CREB. The phosphorylation was significantly diminished by adenylate cyclase inhibitor (MDL12) and PKA inhibitor (H89) (Fig. 2F).

These results indicate that via LH/hCG receptor, hCG induces VEGF secretion of granulosa-lutein cells through PLC/PKC/Sp1 and adenylate cyclase/PKA/CREB signal pathways.
Signaling pathways of VEGF and IL-8 of follicular fluid inducing endothelial permeability

Using western blotting and densitometry, we found that follicular fluid strongly induced VEGFR-2 phosphorylation of endothelial cells. The phosphorylation was significantly reduced by pretreatment with IL-8 antibody or VEGF antibody (Fig. 3A). We further verified that follicular fluid induced phosphorylations of CXCR1/2 of endothelial cells. The phosphorylations were decreased by pretreatment of IL-8 antibody (Fig. 3B). The inhibitor of CXCR1/2 (repertaxin) diminished IL8-induced VEGFR-2 phosphorylation (Fig. 3C). These data suggest that through CXCR1/2, IL-8 of follicular fluid transactivates VEGFR-2 of endothelial cells.

Using Rho-GTP pull-down assays, we further demonstrated that follicular fluid strongly induced Rho-GTP expression of endothelial cells. The follicular fluid-induced Rho-GTP expression was significantly reduced when the follicular fluid was pre-incubated with either IL-8 antibody or VEGF antibody. In addition, the follicular fluid-induced Rho-GTP expression was significantly inhibited when the endothelial cells were pretreated with the inhibitor of VEGFR-2 (CBO-P11) (Fig. 3D).

Using western blotting, we verified that follicular fluid induced phosphorylations of VE-cadherin and occludin of endothelial cells. The follicular fluid-induced phosphorylations of VE-cadherin and occludin of endothelial cells were significantly reduced when the follicular fluid was pre-incubated with either IL-8 antibody or VEGF antibody. In addition, the phosphorylations were significantly inhibited by inhibitors.

**Figure 3** Signaling of VEGF and IL-8 of follicular fluid inducing endothelial permeability.

The pooled follicular fluid sample was used for experiments. (A) Follicular fluid (FF) induced VEGFR-2 phosphorylation of endothelial cells in both VEGF and IL-8 dependent manners, examined by western blotting. Data were measured as relative density (RD) of p-VEGFR-2/VEGFR-2. Data are compared between the follicular fluid-treated-only group and different neutralizing antibody groups. *P < 0.05; n = 3. (B) IL-8 of follicular fluid induced phosphorylations of CXCR1 (left panel) and CXCR2 (right panel) of endothelial cells. *P < 0.05; n = 3. (C) Through CXCR1/2, IL-8 of follicular fluid induced VEGFR-2 phosphorylation of endothelial cells. HUVEC were pretreated with repertaxin (20 μM) for 1 h prior to follicular fluid stimulation. *P < 0.05; n = 3. (D) Through VEGFR-2, follicular fluid activated Rho signaling of endothelial cells. CBO-P11 (1 μM). Data were measured as relative density of RhoA/Total Rho. *P < 0.05; n = 3. (E) Through Rho/ROCK signals, follicular fluid induced phosphorylation of VE-cadherin. C3 exozyme (1 μg/ml); Y-27632 (5 μM). *P < 0.05; n = 3. (F) Through Rho/ROCK signals, follicular fluid induced phosphorylation of occludin of endothelial cells. *P < 0.05; n = 3. (G) HUVEC permeability assays. HUVEC were pretreated with indicated chemical inhibitors for 1 h prior to follicular fluid stimulation. Data are compared between the follicular fluid-treated-only group and different inhibitor groups. *P < 0.05; n = 5.
of Rho (C3 exozyme) and ROCK (Y-27632) (Fig. 3E and F). With HUVEC permeability assay, we further demonstrated that follicular fluid-induced endothelial permeability was significantly reduced by repertaxin, CBO-P11, C3 exozyme and Y-27623 (Fig. 3G).

Using actin stain, we found that follicular fluid remarkably induced actin polymerization and increased paracellular gap formation of endothelial cells (Fig. 4B), in comparison with the morphology of endothelial cells in culture medium (Fig. 4A). The follicular fluid-induced polymerization of actin was partially inhibited when follicular fluid was pre-incubated with either VEGF antibody (Fig. 4C) or IL-8 antibody (Fig. 4D). The follicular fluid-induced actin polymerization was also extensively diminished when endothelial cells were pretreated with inhibitors of VEGFR-2 (CBO-P11), Rho (C3 exozyme) and ROCK (Y-27632) (Fig. 4F–H).

Effects of dopamine on the follicular fluid-induced endothelial permeability and other steps of angiogenesis

Using HUVEC permeability assay, we found that the follicular fluid-induced endothelial permeability was diminished by dopamine treatment in a dose-dependent mode. At 2 μM, dopamine significantly reduced the relative permeability to 50 ± 3.8% when the follicular fluid treatment control was defined as 100% (Fig. 5A). With western blotting, we found that dopamine inhibited follicular fluid-induced phosphorylation of VEGFR-2 of endothelial cells (Fig. 5B). We further observed that dopamine reduced follicular fluid-induced phosphorylations of VE-cadherin and occludin of endothelial cells (Fig. 5C and D).

We explored the effects of dopamine on other steps of angiogenesis. We found that at the lower doses (0.5, 2 μM), dopamine did not inhibit follicular fluid-induced migration and capillary tube formation of endothelial cells. But it significantly blocked these actions at the higher dose (10 μM) (Fig. 5E and F).

The representation of possible signal pathways of VEGF and IL-8 in granulosa-lutein cells and endothelial cells contributing to the mechanisms of OHSS and dopamine targeting the common VEGFR-2 signaling is schematically summarized in Fig. 6.

Discussion

Compared with the controlled blank medium, both follicular fluid and ascites significantly enhanced endothelial permeability. The results of individual neutralizing antibody and co-incubation studies suggest that among various angiogenic cytokines of follicular fluid and ascites, VEGF and IL-8 may play major roles in inducing endothelial hyperpermeability of OHSS. We further delineate the signal pathways of VEGF and IL-8 in granulosa-lutein cells and endothelial cells possibly involved in OHSS. We find that hCG induces VEGF secretion of granulosa-lutein cells through Sp1 and CREB dependent pathways. Using neutralizing antibodies, we find that both VEGF and IL-8 contribute to follicular fluid-induced VEGFR-2 phosphorylation, activation of Rho, actin polymerization and phosphorylations of VE-cadherin and occludin of endothelial cells. IL-8 of follicular fluid acts on CXCR1/2 of HUVEC and leads to VEGFR-2 transactivation. The possible

![Figure 4](image-url)

**Figure 4** Effect of VEGF and IL-8 of follicular fluid on actin polymerization of endothelial cells. The pooled follicular fluid sample was used for experiments. (A) HUVEC were cultured in endothelial cell culture medium with 10% fetal bovine serum. The HUVEC were stained for actin filaments and visualized by a fluorescent microscope (×40). (B) HUVEC were cultured with follicular fluid (FF) for 1 h, and then were stained. (C) Follicular fluid was pre-incubated with VEGF antibody (Ab) (2 μg/ml) for 30 min. HUVEC were then cultured for 1 h. (D) Follicular fluid was pre-incubated with IL-8 antibody (2 μg/ml), and then used for HUVEC culture. (E) Follicular fluid was co-incubated with both VEGF antibody and IL-8 antibody prior to use for HUVEC culture. (F) HUVEC were pretreated with CBO-P11 (1 μM) for 1 h, and then were cultured with follicular fluid for 1 h. (G) HUVEC were pretreated with C3 exozyme (1 μg/ml) prior to culture with follicular fluid. (H) HUVEC were pretreated with Y-27632 (5 μM) before culture with follicular fluid. Illustrations shown are representative from five independent experiments.
mechanisms may involve IL-8-inducing VEGF release by HUVEC or IL-8-transactivating VEGFR-2 directly, which merits further investigation.

We use the strategy of chemical inhibitors for clarification of the signal cascade of both VEGF and IL-8 of follicular fluid in inducing endothelial permeability. With inhibitors of VEGFR-2, Rho and Rock, the results suggest that through a common VEGFR-2 signal, both VEGF and IL-8 of follicular fluid induce endothelial permeability via activation of Rho/Rock resulting in polymerization of actin and phosphorylations of VE-cadherin and occludin with opening of adherence and tight junctions. The step-by-step strategies using both neutralizing antibodies and chemical inhibitors facilitate delineation of the signal pathway. van den Driesche et al. (2008) found that hCG could augment hypoxia inducing factor-1α (HIF-1α) and VEGF expressions under hypoxic conditions. Recently, Rodewald et al. (2007, 2009) suggest that hCG may increase endothelial permeability by up-regulating VEGF in granulosa-lutein cells which causes reduction in endothelial specific tight junction protein, claudin 5, expression. The clarification of pathophysiological mechanisms would be helpful to find therapeutic agents for the targeted treatment of OHSS.

To reduce the secretion of IL-8 or VEGF from corpora luteae may be an approach to treat OHSS. In our previous study, we found that LPA induced IL-8 secretion of granulosa-lutein cells through NF-κB dependent pathway (Chen et al., 2008b). Non-steroidal anti-inflammatory drugs (NSAIDs) have been recently shown to suppress the activity of NF-κB and the expressions of its related genes (Takada et al., 2004). Indomethacin had been used as an inhibitor of prostaglandin synthesis to treat OHSS (Schenker and Polishuk, 1976); however, the efficacy data remains controversial. Katz et al. (1984) reported that indomethacin effectively treated OHSS. However, Borenstein et al. (1989) found no clinical improvement of ascites in severe OHSS using indomethacin. The effect of

Figure 5 Effects of dopamine on the follicular fluid-induced endothelial permeability and other steps of angiogenesis.

The pooled follicular fluid sample was used for experiments. (A) HUVEC permeability assay. HUVEC were pretreated with dopamine (DA) of indicated doses (0.5, 2 or 10 μM) for 1 h prior to follicular fluid (FF) treatments. We chose the doses of dopamine for experiments with reference of the previous study by Basu et al. (2001). Data are compared between the follicular fluid-treated-only group and different dopamine dosage groups. *P < 0.05; n = 5. (B) Effect of dopamine on the follicular fluid-induced phosphorylation of VEGFR-2 of endothelial cells, examined by western blotting. Data were measured as relative density (RD) of p-VEGFR-2/VEGFR-2. Data are compared between the follicular fluid-treated-only group and different dopamine dosage groups. *P < 0.05; n = 3. (C) Effect of dopamine on the follicular fluid-induced phosphorylation of VE-cadherin of endothelial cells. *P < 0.05; n = 3. (D) Effect of dopamine on the follicular fluid-induced phosphorylation of occludin of endothelial cells. *P < 0.05; n = 3. (E) Effect of dopamine on the follicular fluid-induced HUVEC migration. *P < 0.05; n = 5. (F) Effect of dopamine on the follicular fluid-induced HUVEC capillary tube formation. The tube formation was assessed under a phase-contrast microscope (× 20). Six random fields were examined and the mean value was calculated. *P < 0.05; n = 5.
We demonstrate that the follicular fluid- or ascites-induced endothelial permeability in vitro is significantly inhibited by pretreatment with both IL-8 and VEGF neutralizing antibodies. In the experiments of recombinant VEGF and endothelial cells, Villasanti et al. (2007) found that anti-human VEGF antibody neutralized VEGF actions of endothelial cells. To use specific antibodies for neutralizing VEGF or IL-8 may be another approach to treat OHSS. Avastin, an antibody of VEGF, is used in clinical cancer therapy with its effect of anti-angiogenesis (Ma and Waxman, 2008). The effect of avastin in the management of severe OHSS may deserve further investigation.

On the other hand, angiogenesis is essential for establishment of pregnancy, and VEGF plays an important role (Plaisier et al., 2009). Significant potential limitations for a VEGF neutralizing antibody such as risks for implantation and maintenance of pregnancy should be concerned.

On the basis of the results of co-incubation studies, the effects of both VEGF and IL-8 of follicular fluid on endothelial permeability appear additive. These findings suggest that the drug targeting the common VEGFR-2 signals may block the endothelial hyperpermeability induced by both VEGF and IL-8. It has been found that in a mouse cancer model, administration of dopamine blocked VEGF/VEGFR-2-related angiogenesis and endothelial permeability via dopamine receptor (DR)-2 (Basu et al., 2001). In the present study, we demonstrate that dopamine inhibits follicular fluid-induced VEGF signaling of endothelial cells and diminishes follicular fluid-induced permeability and other steps of angiogenesis with a dose-dependent manner. Dopamine may block not only VEGF, but also IL-8-induced endothelial permeability through inhibition of common VEGFR-2-mediated pathways. We further find that at the lower dose (2 μM), dopamine inhibited permeability without affecting migration and capillary tube formation of endothelial cells. At the higher dose (10 μM), dopamine hindered all of these steps.

Gomez et al. (2006) first investigated the effect of cabergoline, a DR-2 agonist, on OHSS in the rat model. They found that cabergoline reduced vascular permeability without affecting luteal angiogenesis. In the human application, Alvarez et al. (2007a) reported that cabergoline improved ascites by reducing vascular permeability and decreased the incidence of moderate OHSS. However, the incidence of severe OHSS was not significantly decreased. They further reported no difference between the groups with and without cabergoline treatments regarding fertilization, implantation and pregnancy rates of a small number of cases (Alvarez et al., 2007b). The effect of dopamine or DR-2 agonists in the management of OHSS merits further studies. As both VEGF and IL-8 participate in angiogenesis and implantation of pregnancy (Velez et al., 2008; Chen et al., 2008c), potential limitations for DR-2 agonists regarding risks for implantation and maintenance of pregnancy should be considered.

Doxycycline, a derivative of tetracycline, has been recently shown to inhibit tumor angiogenesis (Moses et al., 2006). Fainaru et al. (2008a) found that doxycycline inhibited vascular permeability by targeting the adherens junction of endothelial cells via inducing the VE-cadherin expression and decreasing its phosphorylation. They further reported that doxycycline inhibited peritoneal vascular leakage and ascites accumulation in the hyperstimulated mice (Fainaru et al., 2008b). The value of doxycycline in preventing or treating human OHSS warrants further studies.
Conclusions
Among various angiogenic cytokines, VEGF and IL-8 secreted from corpora lutea may play major roles in inducing endothelial permeability of OHSS. Delineation of signal pathways of VEGF and IL-8 in granulosa-lutein cells and endothelial cells would facilitate to find possible medicines targeting at the various sites. IL-8 and VEGF increase endothelial permeability through common VEGFR-2-mediated signals. Dopamine inhibited VEGFR-2 and, in a low dose, reduces permeability without affecting migration and capillary tube formation of endothelial cells. Angiogenesis is essential in establishment of pregnancy in that VEGF, placental growth factor, bFGF, IL-8, and angiopoietins, etc. may be involved (Plaisier et al., 2007, 2009; Velez et al., 2008; Chen et al., 2008c). To find the targeting medicines for endothelial hyperpermeability in OHSS and to investigate their effects on angiogenesis of pregnancy merits further studies.

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References


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