Effects of hypoxia-inducible factor-1α on endometrial receptivity of women with polycystic ovary syndrome

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Abstract. Embryo implantation is associated with an hypoxic endometrial microenvironment. Hypoxia-inducible factor-1α (HIF-1α) is activated under hypoxic conditions. In the present study, the expression pattern of HIF-1α in endometrial tissue was investigated and its effects on endometrial receptivity in patients with polycystic ovary syndrome (PCOS) were examined. A total of 81 patients were enrolled for in vitro fertilization and embryo transfer. They were divided into PCOS (n=40) and Control groups (n=41); both groups were further divided based on body weight (overweight and normal weight subgroups). The expressions of HIF-1α, vascular endothelial growth factor (VEGF) and glucose transporter protein (GLUT)-1 and GLUT4 were determined by reverse transcription-quantitative polymerase chain reaction and immunohistochemistry. The results demonstrated that mRNA and protein expression levels of HIF-1α and VEGF in the PCOS group were significantly lower compared with expression levels in the Control group. However, there were no statistically significant differences in the expression levels of GLUT1 and GLUT4 between groups. In patients with PCOS, GLUT1 and GLUT4 were mainly localized in the nuclei and cytoplasm, but not in the cell membrane. Overweight patients had the lowest expression levels of HIF-1α, VEGF and GLUT1 expression compared with normal weight patients. In conclusion, HIF-1α may be involved in the molecular mechanisms of endometrial dysfunction in women with PCOS, particularly in those who are overweight. HIF-1α might therefore be a novel target for improving the endometrial receptivity and successful embryo implantation in PCOS women.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects 5-10% of 20-37 years old women (1). PCOS is characterized by anovulation, hyperandrogenemia and polycystic ovaries. Up to 60% of all patients with PCOS are overweight or obese (2); recent studies have demonstrated the negative effects of increased body weight on reproductive outcomes (3,4). In the past few decades, advances in assisted reproductive techniques allowed for the selection of high quality embryos, but the implantation rates for patients with PCOS remained low in clinical settings (5,6). Poor reproductive outcomes in patients with PCOS are probably associated with decreased endometrial receptivity (7). Therefore, it is important to improve the endometrial receptivity of women with PCOS.

During the window for embryo implantation, a rich vascular network is necessary to supply nutrients and oxygen for cell proliferation and implantation of the blastocyst (8). At this time, the endometrium microenvironment is considered to be hypoxic (9). Hypoxic conditions activate hypoxia-inducible factor (HIF)-1, which is an oxygen-sensitive transcription factor. HIF-1 is a heterodimer, which comprises α and β subunits (10). The HIF1-β subunit is stably expressed, whereas the expression of HIF-1α is regulated by oxygen concentration (11,12). HIF-1α is rapidly degraded through the ubiquitin-proteasome pathway under physiological conditions. Under hypoxic conditions, HIF-1α is stabilized and is involved in the regulation of cellular processes, including angiogenesis, glucose metabolism and cell differentiation (13,14). Previous studies have reported that HIF-1α expression in the endometrium occurs exclusively during the secretory and menstrual phase, which may be associated with the physiological changes associated with menstruation (15).

Vascular endothelial growth factor (VEGF) is an important mediator of angiogenesis (16). Glucose transporter protein (GLUT) is responsible for glucose uptake and storage in the endometrium (17-19). VEGF and GLUT are targets of HIF-1α and their expression is necessary for endometrial receptivity (20). The implantation window is in the mid-secretory phase of human endometrium, that is, 7-10 days following

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luteinizing hormone (LH) surge (21). The role of HIF-1α in the regulation of endometrial receptivity is yet to be confirmed. In the present study, the expressions of HIF-1α and its target genes VEGF, GLUT1 and GLUT4 were investigated in the endometrium of women with and without PCOS during the implantation window, and the effects of body weight on their expression levels were also examined.

Materials and methods

Subjects. The present study recruited 40 women with PCOS and 41 women with tubal blockage who served as controls. Women with PCOS were divided into overweight (OW)-PCOS (n=22) and normal-weight (NW)-PCOS (n=18) subgroups. Women in the control group were also divided into OW-Control (n=21) and NW-Control (n=20) subgroups. All participants were referred to the Reproductive Medicine Center, Yantai Yuhuangding Hospital (Yantai, China) for in vitro fertilization pre-embryo transfer. The patients were 20-37 years old with normal basal serum follicle stimulating hormone levels and antral follicle counts (AFC). None of the patients had taken oral contraceptives or other medicines in the preceding 3 months. Hysteroscopic examination was performed to exclude endometrial diseases in all participants. Patients with endometriosis, intrauterine adhesions, endometrial polyps, recurrent history of miscarriage and those with a recurrent implantation failure were excluded from the present study. PCOS was diagnosed according to the 2003 Rotterdam Consensus (22). Overweight and/or obesity were defined according to the 2000 World Health Organization and The International Obesity Task Force diagnostic criteria for the Asia-Pacific population (23). Clinical data, including age, duration of infertility, body weight, height, waist-to-hip ratio (WHR), basal serum follicle-stimulating hormone, LH, total testosterone (TT), fasting glucose (mmol/l), fasting insulin (mIU/l), estradiol (E2) and progesterone (P) levels following human chorionic gonadotropin (HCG) administration during controlled ovarian hyperstimulation (COH), number of oocytes, endometrial thickness and high-quality embryo rate were recorded. Body mass index (BMI) was calculated as weight/height² (kg/m²) and the homeostatic model assessment-insulin resistance (HOMA-IR) was calculated as [(fasting basal blood glucose)(fasting basal insulin)]/22.5 (24). This study was approved by The Ethics Committee of Yantai Yuhuangding Hospital (Yantai, China), and written informed consent was received from each patient prior to enrolment in the study.

Ovarian stimulation. The gonadotropin-releasing hormone antagonist (GnRH-ant) protocol was used for COH (25). On day 3 of menstruation, 125-225 IU/day of recombinant FSH (Puregon; Merck Sharp & Dohme-Hoddesdon, UK) was used for ovarian stimulation, according to the patient's age, BMI, anti-Müllerian hormone, infertility duration and AFC. A 0.25 mg dose of GnRH antagonist (Orgalutran; Merck Sharp & Dohme-Hoddesdon) was administered daily when the follicle diameter reached 12 mm until the day of HCG (Beijing Saisheng Pharmaceutical Co, Ltd., Beijing, China) administration. Highly purified human menopausal gonadotropin (Menopur; Ferring Pharmaceuticals, Saint-Prex, Switzerland) was administered at the late follicle phase. HCG (6,000 IU) was administered for final oocyte maturation when at least two leading follicles were ≥18 mm in size, and oocytes were retrieved following 34-36 h. All patients received 600 mg/day progestogen (Utrogestan; Besins Manufacturing Belgium, France) for luteal phase support.

Specimens. Endometrial tissues were collected from patients with a curette on day 5 following the oocyte pick-up, certain tissues were fixed in 4% formaldehyde for 12 h at room temperature for paraffin embedding; the other tissues were washed in phosphate-buffered saline and stored immediately at -70°C for RNA isolation.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), as described previously (26). A BioWaveI spectrophotometer (Biochrom, Ltd., Cambridge, UK) was used to evaluate the concentration and purity of the RNA (A260/A280 ratio). cDNA was produced by reverse transcription using a PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. qPCR was performed in 20 µl reactions using 1 µl of cDNA, 10 µM of each primer and 2X Platinum SYBR Green qPCR Supermix-UDG (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), following the manufacturer's protocol. The following gene-specific primers were purchased from Invitrogen (Thermo Fisher Scientific, Inc.) (27): HIF-1α, forward 5'-CATCAGCTATT TGCGTGAGGACGAA-3', reverse 5'-AGCAATTCATCTGTGCTTTTCATGC-3'; VEGF, forward 5'-CTCGGTTGACATCTTCAGGATCC-3', reverse 5'-GAAGC-TCACTCTCTCCATATGCTGGC-3'; GLUT1, forward 5'-GCCATTTGCGCTT-3', reverse 5'-GACGTAGGACACACAGTTGC-3'; GLUT4, forward 5'-CTGGCCCTACAGTGGCTAC-3', reverse 5'-TCTGGCGCTTCAGATCTTCT-3'; GAPDH, forward 5'-GGGAACCTGTGCGGTGAT-3', reverse 5'-GAGTGGGTGTGCGGTGTTGA-3'. PCR thermocycling was performed at 95°C for 30 sec, followed by 45 cycles at 95°C for 5 sec and at 60°C for 60 sec.

Specificity of the PCR reactions was assessed by melting curve analysis. Correct melting temperatures were obtained for all products. The relative gene expression levels in each sample were normalized to the expression levels of GAPDH, which was used as the housekeeping gene, and were analyzed using the 2^ΔΔCt method (28,29). This normalization could account for the inherent variability in the efficiency of the reverse transcription reactions. Each set of RT-qPCR reactions was repeated three times.

Hematoxylin and eosin (H&E) and immunohistochemical staining of endometrial sections. H&E staining was used for examination of the morphological characteristics of the endometrium and for demonstration of tissue integrity (30). Briefly, the tissues were hydrated in H2O for 30 sec. Then the slides were immersed in hematoxylin (Beyotime Institute of Biotechnology, Shanghai, China; cat. no. CO015-1) for 30 sec and rinsed in H2O for 1 min. Subsequently, the slides were stained with 1% eosin Y solution (Beyotime Institute of Biotechnology; cat. no. CO015-2) for 30 sec and dehydrated in 95 and 100% alcohol.
Immunohistochemical staining of 4 µm thick sections of the formaldehyde-fixed and paraffin-embedded endometrial tissue was performed, as described previously (31). Briefly, the tissue sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked by incubation of sections in 3% hydrogen peroxide in methanol for 10 min at room temperature. Following three washes in PBS, the sections were placed in a blocking solution sheep heat-inactivated serum (Beyotime Institute of Biotechnology; cat. no. C0265) for 30 min at room temperature to block nonspecific binding sites. Subsequently, sections were incubated with the following primary antibodies: Rabbit polyclonal anti-HIF-1α (1:700; Abcam, Cambridge, UK, cat. no. ab85886), rabbit polyclonal anti-VEGF (1:200; Abcam; cat. no. ab46154), rabbit monoclonal anti-GLUT1 (1:150; Abcam; cat. no. ab150299) and rabbit polyclonal anti-GLUT4 (1:400; Abcam; cat. no. ab654), overnight at 4˚C. The sections were washed three times in PBS and were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:50, Beyotime Institute of Biotechnology; cat. no. A0208) at 37˚C for 1 h. 3,3-diaminobenzidin was used as the chromogen and counterstaining was done with hematoxylin.

Sections were examined with an Olympus CX31-LV320 light microscope (Olympus Corporation, Tokyo, Japan) at x20 magnification. Reactions demonstrating the specificity of primary antibodies were also carried out by omission of the antibodies in the incubating medium; no immunoreactivity was observed in these sections (data not shown). Experiments were repeated at least three times. The immunostaining density of HIF-1α, VEGF, GLUT1 and GLUT4 was evaluated using a semi-quantitative method, as previously described (32). The Image-Pro Plus 6.0 software (Media Cybernetics company, USA) was used to calculate the integrated optical density (IOD) and areas, three fields were examined in per slide. Data are presented as mean density (IOD/area) to indicate the relative expression (33).

Statistical analysis. Data analysis was performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Data were expressed as the mean ± standard deviation (or standard error of the mean for the RT-qPCR experiments). Inter-group differences with respect to normally distributed variables were assessed with Student’s t-test; differences pertaining to non-normally distributed variables were assessed with the Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical and COH characteristics of patients. Serum levels of LH, TT, fasting insulin, fasting glucose and HOMA-IR in patients with PCOS were significantly higher than those in the controls (P<0.05; Table I). No significant inter-group differences were observed with respect to E2, P level on HCG day and high-quality embryo rate. OW-PCOS patients exhibited higher BMI, WHR, fasting insulin and HOMA-IR compared with NW-PCOS patients. In the control group, only BMI and WHR were significantly different between the OW-Control and NW-Control groups (P<0.05; Table II).

Endometrial mRNA expression levels of HIF-1α, VEGF, GLUT1 and GLUT4 during the implantation window. In both the PCOS and the Control groups, mRNA expression levels of HIF-1α were notably higher compared with the mRNA expression levels of of VEGF, GLUT1 and GLUT4. mRNA expression
levels of HIF-1α and VEGF in patients with PCOS were significantly lower compared with the respective expression levels in the Control patients (P<0.05; Fig. 1A). GLUT4 mRNA expression levels were very low in both groups and there was no statistically significant inter-group differences with respect to GLUT1 and GLUT4 expression levels (P>0.05; Fig. 1A).

Association between HIF-1α, VEGF, GLUT1 and GLUT4 mRNA expression levels with body weight. mRNA expression levels of HIF-1α and VEGF in OW patients were significantly lower than in the NW patients in both the PCOS and Control groups (P<0.05; Fig. 1B and C, respectively). GLUT1 mRNA expression levels were significantly lower in OW patients compared with expression in the NW-Controls (Fig. 1C; P<0.05); no statistically significant differences in GLUT4 expression levels were identified between OW and NW patients with or without PCOS (P>0.05; Fig. 1B and C, respectively).

Endometrial histology and protein expression levels of HIF-1α, VEGF, GLUT1 and GLUT4 during implantation window. The histological stage of endometrium in PCOS and control groups was in secretory phase according to the Noyes standard (Fig. 2) (34). In the secretory endometrium, strong brown immunostaining for HIF-1α and VEGF were observed in the nuclei of epithelial and stroma cells in the PCOS and control groups. Positive cytoplasmic and nuclear immunostaining for GLUT1 and GLUT4 was detected in the epithelial cells of OW-PCOS and NW-PCOS patients. However, these were localized mainly in the cell membrane of cells in the OW-control and NW-control group (Fig. 2).

Semi-quantitative IOD protein expression levels of HIF-1α and VEGF in PCOS samples were significantly lower compared with expression levels in the controls (P<0.05; Fig. 3A). No

Table II. Clinical characteristics of overweight and normal-weight subgroups of the PCOS and controls.

<table>
<thead>
<tr>
<th>Variable</th>
<th>NW-PCOS (n=18)</th>
<th>OW-PCOS (n=22)</th>
<th>NW-Control (n=20)</th>
<th>OW-Control (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>30.94±2.65</td>
<td>31.18±2.75</td>
<td>30.65±2.94</td>
<td>31.57±1.77</td>
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<tr>
<td>Infertility duration (year)</td>
<td>3.25±1.68</td>
<td>4.48±3.17</td>
<td>4.60±3.22</td>
<td>2.93±2.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.64±1.73</td>
<td>28.66±2.27*</td>
<td>22.0±1.97</td>
<td>27.94±2.27*</td>
</tr>
<tr>
<td>WHR</td>
<td>0.82±0.01</td>
<td>0.93±0.04*</td>
<td>0.83±0.01</td>
<td>0.89±0.02*</td>
</tr>
<tr>
<td>FSH (mIU/ml)</td>
<td>5.47±1.68</td>
<td>6.00±2.45</td>
<td>6.67±1.33</td>
<td>6.31±1.69</td>
</tr>
<tr>
<td>LH (mIU/ml)</td>
<td>10.89±6.64</td>
<td>8.46±4.48</td>
<td>5.85±1.78</td>
<td>5.49±2.51</td>
</tr>
<tr>
<td>TT (ng/ml)</td>
<td>0.44±0.25</td>
<td>0.43±0.18</td>
<td>0.29±0.20</td>
<td>0.30±0.15</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>5.11±0.43</td>
<td>5.11±0.21</td>
<td>4.75±0.77</td>
<td>4.70±0.67</td>
</tr>
<tr>
<td>Fasting insulin (mIU/ml)</td>
<td>9.72±3.99</td>
<td>16.78±5.91*</td>
<td>7.73±2.83</td>
<td>8.25±2.12</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.22±0.95</td>
<td>3.84±1.41*</td>
<td>1.63±0.64</td>
<td>1.73±0.52</td>
</tr>
<tr>
<td>Endometrium thickness on the day of HCG administration (mm)</td>
<td>10.31±2.30</td>
<td>10.52±1.99</td>
<td>10.60±1.53</td>
<td>10.57±1.77</td>
</tr>
<tr>
<td>Number of oocytes</td>
<td>11.00±5.52</td>
<td>11.00±5.85</td>
<td>11.00±4.07</td>
<td>8.95±4.34</td>
</tr>
<tr>
<td>High-quality embryo rate (%)</td>
<td>70.5±24.4</td>
<td>61.4±20.42</td>
<td>73.62±20.70</td>
<td>68.0±22.85</td>
</tr>
</tbody>
</table>

*P<0.05 vs. NW-PCOS. *P<0.05 vs. NW-Control. BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; HCG, human chorionic gonadotropin; HOMA-IR, homeostasis model assessment-insulin resistance; NW, normal weight; OW, overweight; TT, total testosterone; WHR, waist-to-hip ratio.
statistically significant inter-group differences were identified with respect to GLUT1 and GLUT4 immunostaining (P>0.05; Fig. 3A).

Association between body weight and protein expression levels of HIF-1α, VEGF, GLUT1 and GLUT4. Semi-quantitative protein expression levels of HIF-1α and VEGF in OW patients were significantly lower compared with expression levels in the NW patients in both groups (P<0.05; Fig. 3B and C). Protein expression levels of GLUT1 were significantly lower in OW-Control patients compared with expression levels in the NW-Control group (Fig. 3C; P<0.05). No statistically significant differences in GLUT4 protein expression levels were identified between the two subgroups of patients with or without PCOS (P>0.05; Fig. 3B and C, respectively).

Discussion

Human embryogenesis takes place in a hypoxic environment. At the time of embryo implantation, cell proliferation and implantation of the blastocyst requires an increasing supply of nutrients and oxygen, which promotes the establishment of the vascular network at the implantation site. Hypoxia-induced synthesis of HIF-1α modifies the endometrial microenvironment and contributes to an improvement in uterine receptivity (35,36). In the present study, quantitative mRNA and semi-quantitative protein expression levels of HIF-1α were determined in the endometrium during the implantation window. To the best of our knowledge, this is the first study to investigate the role of HIF-1α in determining endometrial receptivity for embryo implantation.
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References


