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Effects of Metformin on the Expression of GLUT4 in Endometrium of Obese Women with Polycystic Ovary Syndrome[1]

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ABSTRACT

The objective was to explore the effects of metformin on the expression of endometrial glucose transporter 4 (GLUT4) and analyze the related factors of GLUT4 in patients with polycystic ovary syndrome (PCOS). This study included 20 obese patients with PCOS (PCOS group) and 20 obese patients who had infertility caused by oviducal or pelvic factors but had no PCOS (control group). Follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol-2 (E_2), testosterone (T), fasting serum glucose (FSG), fasting insulin serum (FINS), homeostasis model assessment-insulin resistance (HOMA-IR), and endometrial GLUT4 expression were determined in the two groups. In PCOS group, patients were given 500 mg of metformin three times per day for 3 mo, and then the parameters above were determined again and compared with that before metformin treatment. The parameters above also were compared between PCOS and control groups. The correlation of GLUT4 with its related factors was analyzed. The levels of T, FINS, and HOMA-IR were higher in PCOS group than in the control group ($P < 0.01$). The levels of protein and mRNA of endometrial GLUT4 were lower in the PCOS group than in the control group ($P < 0.001$). The expression of protein and mRNA of endometrial GLUT4 increased after metformin treatment ($P < 0.001$). HOMA-IR was negatively correlated with GLUT4 expression ($P = 0.027$). In patients with PCOS, the levels of protein and mRNA of endometrial GLUT4 were lower compared with that in non-PCOS women, and HOMA-IR was strongly associated with endometrial GLUT4 expression. Metformin may up-regulate endometrial GLUT4 expression to improve endometrial IR.

glucose transporter 4, insulin resistance, metformin, polycystic ovary syndrome

INTRODUCTION

Polycystic ovary syndrome (PCOS), one of the most common endocrine diseases, affects 5%–10% of childbearing-age women and 75% of anovulatory infertility is associated with PCOS [1, 2]. The patients with PCOS have not only endocrine abnormality and reproductive dysfunction but also metabolic disorder. Insulin resistance (IR) and second hyperinsulinism are physiopathological characteristics of PCOS [3]. Both obese and nonobese patients with PCOS have IR compared with normal individuals with matched age and body weight, and obesity aggravates IR [4–6]. In PCOS women of childbearing age, the cause of infertility is due to ovulation failure, and induced ovulation is often adopted. Although the ovulation rate may reach 80%, the pregnancy rate is only 30%–40% [7]. It is believed that in PCOS, low pregnancy rate is related to high androgen, hyperinsulinism, and IR, and metabolic disorder may affect endometrial metabolism and function [8]. Endometrial cell differentiation depends on sufficient glucose metabolism. Since insulin-mediated glucose transport is performed with glucose transporter 4 (GLUT4) as a carrier, the reduction of endometrial GLUT4 leads to endometrial IR, which may impair endometrial cell metabolism in patients with PCOS [9]. Due to endometrial IR in PCOS, a euglycemic agent is used in the treatment of PCOS [10]. Metformin, a kind of common drug, can improve insulin sensitivity, decrease hyperinsulinemia and androgen levels, and promote ovulation in patients with PCOS [11, 12]. However, little research has been done on whether metformin can improve endometrial IR in patients with PCOS. The purpose of this study was to explore the effects of metformin on endometrial GLUT4 expression and the correlation of GLUT4 with its related factors in patients with PCOS.

MATERIALS AND METHODS

All study methods were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All the subjects enrolled in the study gave written formal consent to participate.

Subjects

Between January and June 2011, 20 obese patients with PCOS who underwent, in our center, diagnostic curettage due to menstrual disorder or infertility were designated PCOS group, with a mean age of 28.64 ± 5.19 yr (range: 23–39 yr). At the same period, 20 obese patients who had infertility caused by oviducal or pelvic factors but had no PCOS were designated the control group, with a mean age of 29.29 ± 5.21 yr (range: 24–40 yr). The patients who had endocrine disorders or who had taken hormone drugs within the previous 3 mo were excluded from this study.

PCOS was diagnosed according to the corrected diagnostic criteria at the Rotterdam meeting in 2003 [13]. Obesity was diagnosed according to the obese criteria (body mass index [BMI] $\geq$ 25) redefined by the International Obesity Task Group for Asian Region in 2000 [14]. IR was diagnosed according to homeostasis model assessment-insulin resistance (HOMA-IR) ([FINS {mU/L} × FSG {mmol/L}]/22.5) > 2.69 calculated by the Chinese Diabetes Prevention Group [15].

Research Design

In all patients, fasting venous blood was collected for Days 2–5 of the menstrual cycle in patients with menstruation or when B-ultrasound showed no dominant follicles in patients with amenorrhea to determine follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol-2 (E_2), testosterone (T), fasting serum glucose (FSG), and fasting insulin serum (FINS) levels.

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Since endometrial morphology in the proliferative phase is similar in the PCOS and control groups, endometrial samples in the proliferative phase were collected (Days 3–7 after menstruation in patients with menstruation and Days 3–7 after progesterone withdrawal bleeding in patients with amenorrhea) under sterile conditions. Some samples were fixed with 10% formaldehyde solution and embedded in paraffin, followed by immunohistochemistry. Other samples were washed twice with Hanks balanced salt solution and stored at −80°C for RT-PCR.

In the PCOS group, 500 mg of metformin was given three times per day for 3 mo from Days 2 to 5 after menstruation or withdrawal bleeding. Three months later, parameters above were determined again.

Apparatus and Reagents

DXI800 luminescence analyzer and kits for blood reproductive hormone and insulin determination were provided by Beckman Coulter. The Hitachi 7170A automatic biochemistry analyzer was purchased from Hitachi. The glucose kit was from Shanghai Biological Engineering Co., Ltd. GLUT4 rabbit anti-polyclonal antibody was from Santa Cruz Biootechnology, Inc. Streptavidin peroxidase and DAB kits were purchased from Beijing Zhongshan Golden Bridge Co., Ltd. Trizol extraction kit was from Gibco. RT-PCR was from Promega. Primers of GLUT4 and GAPDH were synthesized by Shanghai Sangon Biotechnology Co., Ltd. Metformin was from Shanghai Squibb Pharmaceutical Co., Ltd.

Immunohistochemistry

Immunohistochemical streptavidin-peroxidase (SP) method: After routine deparaffinage and gradient alcoholic dehydration, samples were incubated in 3% H_2O_2 at room temperature for 20 min to block endogenous peroxidase. Samples were washed with PBS (pH 7.2), then placed into citrate buffer solution (pH 6.0) for 15 min. Goat serum was added to block endogenous biotin; 20 min later, GLUT antibody (1:100) was added at 4°C overnight. After samples were washed with PBS, biotin-labeled secondary antibody was added at 37°C for 20 min, followed by addition of horseradish peroxidase-labeled streptavidin at 37°C for 20 min. After DAB coloration, hematine counterstaining, dehydration, transparency, and mounting, samples were observed under microscope. The cells with brown particles in the cell membrane or cytoplasm were regarded as positive cells: 0% of cells positive (-, negative), <25% of cells positive (+, weakly positive), 25%–50% of cells positive (++, moderately positive), >50% of cells positive (+++, strongly positive). Negative and weakly positive were regarded as low expression, and moderately and strongly positive were regarded as high expression. PBS served as negative control. Photography was performed with a Leica imaging system, followed by semiquantitative analysis and measurement of optical density values.

Reverse Transcription-Polymerase Chain Reaction

Total endometrial RNA was extracted with Trizol solution and then quantitated with ultraviolet spectrophotometry. Reverse transcription was carried out with 2 mg of RNA to synthesize cDNA. After dilution to 1:10, 2 μl of cDNA was used as template to perform PCR amplification with CAPDH as internal control. GLUT4 upstream primer was 5-GCCCGAAAGAGTC TAAAG-3, and downstream primer was 5-AGAGCCACGGTCATCAAG-3, with a fragment length of 407 bp. The CAPDH upstream primer was 5-CAAGGTCATCCATGACAACTTTG-3, and the downstream primer was 5-GTCCACCACCCTGTTGCTGTAG-3, with a fragment length of 541 bp. PCR conditions were as follows: denaturing at 94°C for 5 min, at 94°C for 30 sec, at 56°C for 30 sec, at 72°C for 45 sec, for 30 cycles; finally, elongation at 72°C for 10 min. PCR products (10 μl) underwent 1.5% agarose gel electrophoresis followed by ultraviolet imagery. The integral optical density was measured with Gel-Pro Analyzer 4.0 software (Shanfu Science Instrument Co., Ltd, Shanghai, China) in triplicate. The average ratio of three integral optical density values of the target gene to GAPDH served as the relative expression level of the target gene.

Statistical Analysis

Statistical treatment was performed with SPSS 13.0 software (SPSS Inc., Chicago, IL). Data were expressed as mean ± SD. These data were normally distributed. An independent samples *t*-test was used in the comparison of the BMI, reproductive hormone levels, and metabolic parameters between the prior-treatment PCOS group and the control group. A paired *t*-test was used in the comparison of the BMI, reproductive hormone levels, and metabolic parameters between the prior- and posttreatment PCOS groups. An independent samples *t*-test was used in the comparison of GLUT-4 protein and mRNA levels between the PCOS groups (including prior- and posttreatment PCOS groups) and the control group. A paired *t*-test was used in the comparison of GLUT-4 protein and mRNA levels between the prior- and posttreatment PCOS groups. Bivariant correlation analysis was used in the correlations of GLUT4 with its related factors. Statistical significance was established at $P < 0.05$.

TABLE 1. BMI and the levels of reproductive hormone and metabolic parameters in prior-treatment PCOS group and control group (mean ± SD, n = 20 for each group).

Parameter	PCOS group	Control group	*P* values
BMI (kg/m^2)	29.28 ± 3.80	28.86 ± 2.43	0.532
FSH (pmol/L)	5.42 ± 2.00	5.72 ± 2.14	0.425
LH (pmol/L)	8.87 ± 4.43	7.58 ± 3.48	0.243
E2 (pmol/L)	65.92 ± 38.95	69 ± 30.75	0.192
T (ng/ml)	0.777 ± 0.320	0.426 ± 0.147	0.006
FSG (mmol/L)	5.42 ± 0.65	5.02 ± 0.42	0.126
FINS (mU/L)	20.75 ± 13.31	8.69 ± 4.54	<0.001
HOMA-IR	4.87 ± 3.56	1.86 ± 1.24	<0.001

RESULTS

BMI and the Levels of Reproductive Hormone and Metabolic Parameters in the Prior-Treatment PCOS Group and Control Group

The levels of T, FINS, and HOMA-IR were higher in the prior-treatment PCOS group than in the control group, with significant differences. However, there were no significant differences in BMI and the levels of FSH, LH, E_2, and FSG between the two groups (Table 1).

BMI and the Levels of Reproductive Hormone and Metabolic Parameters in the Prior and Posttreatment PCOS Groups

BMI and the levels of LH, T, FINS, and HOMA-IR were decreased in the PCOS group after 3-mo metformin treatment, significant differences. However, there were no significant differences in the levels of FSH, E_2, and FSG between the prior- and posttreatment PCOS groups (Table 2).

GLUT4 Protein and mRNA Expression in the Prior- and Posttreatment PCOS and Control Groups

In the three groups, there was GLUT4 protein expression in endometrial epithelial cells, but little GLUT4 protein expression in stromal cells. GLUT4 protein expression in endometrial epithelial cells was significantly lower in the prior-treatment PCOS group than in the control group. After 3-mo metformin treatment, GLUT4 protein expression in endometrial epithelial cells was significantly increased compared with that of the

TABLE 2. BMI and the levels of reproductive hormone and metabolic parameters in prior- and posttreatment PCOS groups (mean ± SD, n = 20 for each group).

Parameter	Prior-treatment group	Posttreatment group	*P* values
BMI (kg/m^2)	29.28 ± 3.80	27.78 ± 3.43	0.043
FSH (pmol/L)	5.42 ± 2.00	4.71 ± 1.62	0.870
LH (pmol/L)	8.87 ± 4.43	6.74 ± 4.67	0.009
E2 (pmol/L)	65.92 ± 38.95	61.54 ± 33.76	0.467
T (ng/ml)	0.777 ± 0.320	0.646 ± 0.266	0.046
FSG (mmol/L)	5.42 ± 0.65	5.35 ± 0.82	0.132
FINS (mIU/L)	20.15 ± 13.31	12.83 ± 9.69	<0.001
HOMA-IR	4.87 ± 3.56	3.07 ± 1.84	<0.001

TABLE 3. Average integral optical density of positive area in the three groups (mean ± SD, n = 20 for each group).

Parameter	PCOS groups		Control group
	Prior-treatment	Posttreatment	
GLUT4 protein	97.32 ± 5.07	114.35 ± 5.07[b]	130.34 ± 22.39[b,c]
GLUT4 mRNA	0.596 ± 0.128	0.696 ± 0.108[a]	0.803 ± 0.149[b,c]

[a,b] Compared with prior-treatment PCOS group, [a]$P < 0.05$ and [b]$P < 0.01$.
[c] Compared with posttreatment PCOS group, $P < 0.01$.

prior-treatment PCOS group, but was still significantly lower than that of the control group (Table 3 and Fig. 1).

In the three groups, a GLUT4-specific gene band occurred in 407 bp, an internal control GAPDH-specific fragment occurred in 541 bp. A densitometric scan of electrophoretic band indicated that GLUT4 mRNA expression in endometrial epithelial cells was significantly lower in the prior-treatment PCOS group than in the control group. GLUT4 mRNA expression was negatively correlated with HOMA-IR ($r = 0.495$, $P = 0.027$), but was not correlated with other parameters ($r = -0.122$–0.264, $P > 0.05$). After 3-mo metformin treatment, GLUT4 mRNA expression in endometrial epithelial cells was significantly increased compared with that of the prior-treatment PCOS group, but was still significantly lower than that of the control group (Table 3 and Fig. 2).

DISCUSSION

The PCOS pathogeny has not been well understood. It is considered to be associated with genetic and environmental factors [16]. Patients with PCOS have not only a reproductive disorder, but also long-term risks such as type 2 diabetes mellitus, hyperlipemia, and cardiovascular diseases caused by high androgen and IR. Compared with a control group, obese patients with PCOS had high androgen and hyperinsulinemia. Metformin can decrease hyperinsulinism, androgens, and BMI, which is conducive to the recovery of reproductive function and avoidance of long-term metabolic syndrome [17, 18]. Metformin inhibits hepatic gluconeogenesis, decreases intestinal glucose absorption, increases glucose utilization of surrounding tissue, and promotes the binding of insulin to its receptors, which increases insulin sensitivity and decreases insulin level. Hyperinsulinemia can activate cytochrome P450C17α in the ovary and adrenal gland to synthesize and release androgen; while metformin can decrease hyperinsulinism, so can a decrease in androgen [19].

GLUT4, the only insulin-related glucose transporter protein, is closely associated with glycometabolism. GLUT4 is mainly distributed in cells to transport glucose, playing an important role in glucose balance. It was previously believed that GLUT4 mainly existed in skeletal muscle, cardiac muscle, and adipose tissue. The latest study indicates that GLUT4 also exists in other tissues [20]. Mioni et al. [11] first reported the evidence for the presence of GLUT4 in the endometrium. Since differentiation and maturation of endometrial cells depend on adequate glucose metabolism, GLUT4 plays an important role in the maturation process of endometrial glandular tissue [9, 21]. In patients with PCOS, metabolic abnormalities may affect endometrial metabolism, which decreases pregnancy rate. It is reported that insulin can stimulate glucose oxidation in human endometrium, which suggests insulin is related to endometrial metabolic activity [22]. After the binding of insulin to its receptor, self-phosphorylation of insulin allows GLUT4 to transfer onto the cell membrane, followed by glucose transmembrane transport, which provides energy for the biological function of cells. We confirmed the presence of GLUT4 protein and mRNA in the endometrium in both groups, which is consistent with the results reported by Wang et al. [8] and Mioni et al. [9]. Many studies suggest that the decreased expression and weakened translocation of GLUT4 is closely related to IR in muscle cells and fat cells [3, 23–25]. This study indicated that GLUT4 was negatively correlated with HOMA-IR ($r = 0.495$, $P = 0.027$), which is consistent with the results reported by Mioni et al. [9]. This result supports that the reduction of endometrial GLUT4 expression is closely related to hyperinsulinism. However, whether the reduction of GLUT4 expression is related to endometrial cell function remains unclear. Fornes et al. [26] recently investigated insulin receptor substrates IRS-1- and AS160-related protein expression in the endometrium and found that IRS-1 and AS160 expression was decreased in patients with PCOS and hyperinsulinemia, which led to a reduction of the expression and translocation capacity of GLUT4, affecting endometrial glucose metabolism.

In the patients with PCOS, the level of androgen is higher. At present, there are different reports about the correlation between GLUT4 expression and androgen level. Sato et al. [27] have reported that androgen can regulate insulin-mediated GLUT4 expression. Another study has indicated that androgen level is not correlated with endometrial GLUT4 expression [9], which is consistent with our result. The correlation between androgen and GLUT4 expression and its mechanism at the molecular level remain to be further investigated.

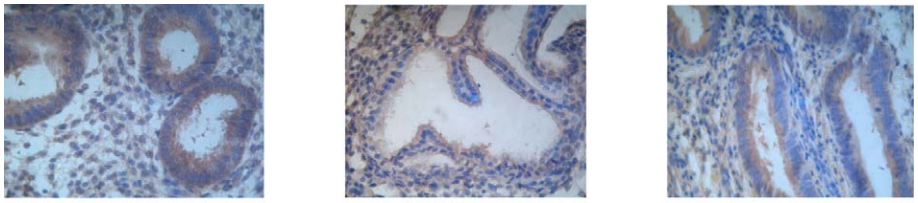

FIG. 1. Immunohistochemical staining indicates GLUT4 expression in the PCOS and control groups. **A**) In the control group, GLUT4 is strongly positive in endometrial epithelial cells and weakly positive in stromal cells (SP staining ×400). **B**) In the prior-treatment PCOS group, GLUT4 is weakly positive in both endometrial epithelial cells and stromal cells (SP staining ×400). **C**) In the posttreatment PCOS group, GLUT4 is moderately positive in endometrial epithelial cells and weakly positive in stromal cells (SP staining ×400).

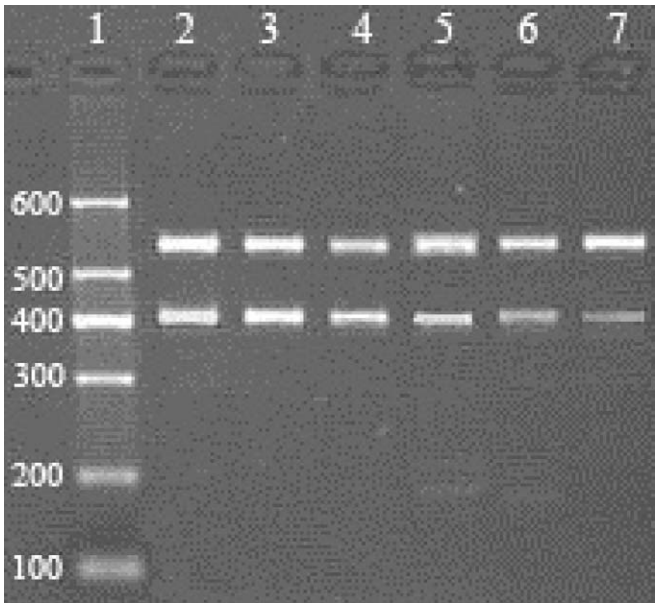

FIG. 2. GLUT4 mRNA bands on gel electrophoresis of the three groups. Lane 1: Mark (relative molecular mass [bp]); lanes 2 and 3: control group; lanes 4 and 5: posttreatment PCOS group; lanes 6 and 7: prior-treatment PCOS group.

The patients with PCOS often have obesity. There have been different reports regarding whether obesity leads to decreased GLUT4 expression. It is reported that GLUT4 expression in endometrial cells is closely related to obesity [9, 28, 10]; it is also reported that decreased GLUT4 expression in fat tissue is not correlated with obesity in patients with PCOS [29]. In this study, all subjects were obese, so the correlation between obesity and endometrial GLUT4 expression failed to be observed, which will be further explored by us.

In this study, compared with the prior-treatment PCOS group, endometrial GLUT4 protein and mRNA expression was significantly increased after a 3-mo metformin treatment ($P < 0.001$, $P = 0.04$). It is reported that hyperglycemia and hyperinsulinemia may allow GLUT4 expression to decrease in fat cells, and metformin can prevent this process and promote GLUT4 translocation [30]. GLUT4, the final step of the insulin signal transduction pathway, can transport glucose into cells, so the increase in GLUT4 expression suggests the improvement of tissue insulin sensitivity [10]. The present study indicates that endometrial GLUT4 expression still failed to reach normal level in patients with PCOS after 3-mo metformin treatment, which may be related to the following factors: 1) beside the insulin signal pathway, AMPK pathway is also associated with endometrial GLUT4 expression and translocation; 2) GLUT4 translation and its posttranslational level, protein synthesis, and degradation are regulated by many factors; 3) besides systemic IR, endometrial autocrine and paracrine also affect endometrial metabolism, GLUT4 translocation, and expression [8]; and 4) the most appropriate course and dose of metformin treatment are unclear.

The limitations of this study were that the quantitative analysis of GLUT4 protein with Western blot was not done, and we did not observe whether endometriual function is improved with the increase in endometrial GLUT4 expression after metformin treatment.

In summary, this study confirms the presence of GLUT4 protein and mRNA in endometrial proliferative phase cells of both the PCOS group and the control group, and GLUT4 expression was significantly lower in the PCOS group than in the control group. We infer that the reduction of GLUT4 expression is closely related to endometrial IR. The reduction of endometrial GLUT4 expression decreases glucose transmembrane transport, affects cellular glucose utilization, and leads to endometrial dysbolism with embryo implantation failure and abortion. After 3-mo metformin treatment, endometrial GLUT4 protein and mRNA expression was increased in patients with PCOS. We infer that metformin may up-regulate endometrial GLUT4 expression to improve endometrial IR in the patients with PCOS.

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