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Metformin inhibits estradiol and progesterone-induced decidualization of endometrial stromal cells by regulating expression of progesterone receptor, cytokines and matrix metalloproteinases

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ABSTRACT

Background: Polycystic ovary syndrome (PCOS) is a serious threat for reproductive-aged women. Metformin has been used for the treatment of PCOS. However, its molecular mechanism in decidualization process of PCOS has not been well featured.

Methods: RT-qPCR analysis was used to detect expression patterns of progesterone receptor (PGR), estradiol receptor alpha (ERα), Cytokeratin 8 and Vimentin in endometrial tissues of PCOS and non-PCOS patients. RT-qPCR assay was also employed to determine mRNA expression of prolactin, Insulin-like growth factor-binding protein 1 (IGFBP-1), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP9). Cytokine secretion were measured by matching ELISA kits. Protein expression of p-ERK1/2, ERK1/2, p-p38 MAPK, p38 MAPK, and PGR (PGRA and PGRB) was tested by western blot assay.

Results: PGR expression was upregulated in PCOS patients. Metformin alleviated estradiol (E2) and progesterone (P4) (EP)-induced decidualization of endometrial stromal cells. Abnormal cytokine secretion was observed in EP-stimulated endometrial stromal cells in the absence or presence of metfromin. Metformin suppressed EP-induced MMP-2 and MMP-9 upregulation. Metformin alleviated EP-triggered p38 MAPK inactivation and PGR (PGRA and PGRB) expression. Metfromin had no effect on ERK1/2 signaling in EP-stimulated endometrial stromal cells.

Conclusion: Metformin alleviated EP-induced decidualization of endometrial stromal cells by modulating secretion of multiple cytokines, inhibiting expression of MMP-2 and MMP-9, activating p38-MAPK signaling and reducing PGR expression, providing a deep insight into the molecular basis of metfromin therapy for PCOS patients.

1. Introduction

Polycystic ovary syndrome (PCOS) is an endocrine metabolic disorder disease with an incidence of 5%–20% in all reproductive-aged women worldwide [1,2]. PCOS is characterized by infertility, polycystic ovarian morphology, hyperandrogenism and ovulatory dysfunction [2–4]. PCOS is correlated with an increased risk of pregnancy complications, endometrial cancer, venous thromboembolism and cardiovascular events [2–4]. The united diagnosis is difficult for PCOS patients due to its multifarious phenotypes and there is no available cure for PCOS [5].

Progesterone (P4) and estradiol (E2), two vital steroid hormones, play central roles in female reproductive processes such as menstrual cycle, implantation, and pregnancy establishment/maintenance [6,7]. P4 exerts its regulatory function on reproductive processes by progesterone receptor (PGR), which contains PGRA and PGRB two major isoforms [8]. E2 functions as an agonist of estrogen receptor (ER), which is composed of ERα and ERβ two subtypes [7]. PGR has been demonstrated to be abnormally expressed in PCOS patients and PCOS-like rat uteri [9–12]. Also, previous findings revealed that PGR was a target of metformin and metformin inhibited expression and secretion of decidualization markers such as IGFBP1 and prolactin [11,13]. Metformin, a versatile drug, has been used for the treatment of type 2 diabetes, insulin resistance syndromes and PCOS [14,15].

Decidualization, a differentiation process of endometrial stromal fibroblasts into secretory decidual cells, offers a nutritive and immunoprivileged matrix for pregnancy maintenance and successful embryo implantation [16,17]. Decidualization of endometrium is characterized by immune cell recruitment, endometrial stromal cell decidualization, vascular reconstruction, and plentiful molecule

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production [18]. Decidualization progression depends on P4 signaling activation, PGR upregulation and cyclic adenosine monophosphate (cAMP) convergence increase [18,19].

In the present study, decidualization models were firstly established by stimulating endometrial stromal cells of non-PCOS patients with E2 and P4 (EP) for over 10 days, which was supported by a previous finding [20]. Then, the molecular mechanism of metformin in EP-induced decidualization of endometrial stromal cells was further explored.

2. Population, materials and methods

2.1. Clinical samples and reagents

Endometrial tissues were obtained from non-PCOS (n = 10) or PCOS patients (n = 10) by pipelle, tubal ligation, or hysterectomy at the Center of Reproduction Medicine, the Affiliated Wuxi Matemity and Child Health Care Hospital of Nanjing Medical University. PCOS was diagnosed according to the Rotterdam criteria of the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine. All non-PCOS patients had regular menstrual cycles. Neither non-PCOS nor PCOS patients have suffered from any steroidal or hormonal therapies within three months before surgery. The age range of patients was 20 to 50 year-old with the average age of 34.90 ± 6.21 and 31.10 ± 6.47 in non-PCOS and PCOS groups, respectively. Specimens were snap frozen in liquid nitrogen and stored at −80 °C refrigerator for following RNA analyses. Our study was conducted with written informed consent form all patients and the authorization of Ethical and Scientific Committees of Maternal and Child Health Hospital of Wuxi. This study was approved by the institutional Review Board of the Affiliated Wuxi Matemity and Child Health Care Hospital of Nanjing Medical University. Estradiol, progesterone and metformin were purchased from Selleck Chemicals Co.ltd. (Houston, Texas, USA). The information of non-PCOS and PCOS patients was provided in Table 1.

2.2. Endothelial stromal cell isolation and culture

Endothelial stromal cells were isolated from endometrial tissues of non-PCOS patients by the method as previously described [13,21]. Briefly, tissues were minced into small pieces, treated with collagenase (Sigma-Aldrich, St. louis, MO, USA), separated using a 40 μm fiter and then cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and passaged twice to remove contaminating cells by microscope. Then, cells were plated in 60 mm petri dishes and continued to cultured till 80–90% confluency. Next, cells (10^6/well) were seeded into 6 well plates and then treated with EP or metformin, alone or in combination, for 14 days. Cell purity was evaluated by flow cytometry using anti-CD45 (for leucocytes) and anti-CD31 (for endothelial cells), and found to be > 98% (data not shown). For the passage of endothelial stromal cells, cells were digested with 0.25% Trypsin-EDTA solution (Thermo Scientific) until most cells became detached and then centrifuged at 400 *g* for 5 min after the addition of 5 ml culture medium, followed by the resuspension of cell pellet using the complete medium.

Table 1
Clinical characteristics of the study subjects.

Group	Case	Age	Histology	PCOS	Diagnosis
non-PCOS	N1	35	SE	NO	Ovarian cyst
non-PCOS	N2	29	PE	NO	Undesired fertility
non-PCOS	N3	44	PE	NO	Volunteer
non-PCOS	N4	28	SE	NO	Volunteer
non-PCOS	N5	38	PE	NO	Fibroids
non-PCOS	N6	42	SE	NO	Fibroids
non-PCOS	N7	31	PE	NO	Undesired fertility
non-PCOS	N8	36	PE	NO	Volunteer
non-PCOS	N9	40	SE	NO	Volunteer
non-PCOS	N10	26	PE	NO	Menorrhagia
PCOS	P1	26	SE	PCOS	HA, A , PCO
PCOS	P2	23	SE	PCOS	Hirsutism, OA, PCO
PCOS	P3	35	SE	PCOS	HA, hirsutism, OA, PCO
PCOS	P4	22	PE	PCOS	HA, hirsutism, acne, A,PCO
PCOS	P5	27	SE	PCOS	HA, OA, PCO
PCOS	P6	34	PE	PCOS	HA, OA, PCO
PCOS	P7	32	PE	PCOS	HA, acne, A, PCO
PCOS	P8	32	PE	PCOS	HA, acne, OA, PCO
PCOS	P9	41	PE	PCOS	HA, acne, OA, PCO
PCOS	P10	39	PE	PCOS	HA, hirsutism, OA, PCO

PE, proliferative phase; SE, secretory phase; HA, biochemical hyperandrogenism; OA, oligo-amenorrhea; A, amenorrhea; PCO, polycystic ovaries.

2.3. RT-qPCR assay

Total RNA was isolated from endometrial tissues and endometrial stromal cells using Trizol reagent (Invitrogen). The amount and quality of RNA was determined using NanoDrop ND 1000 instrument (NanoDrop Technologies, Wilmington, DE, USA). Then, RNA (about 1 μg, 2 μl/25 μl reaction solution) was reversely transcribed into cDNA first strand using random primers, M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) and RNase inhibitor (Thermo Scientific, Fair Lawn, NJ, USA). Finally, RNA expression of PGR, ERα, cytokeratin 8, vimentin, prolactin, Insulin-like growth factor-binding protein 1 (IGFBP-1), matrix metalloproteinase-2 (MMP-2) and, matrix metalloproteinase-9 (MMP9) were determined using specific quantitative primers and SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with cDNA (2 μl) as the template and GAPDH as the endogenous control. Quantitative primer sequences were presented as below: PGR, 5′-GAAGGGCTACGAAGTCAAA-3′ (forward) and 5′-GCAG CAATAACTTCAGACATC-3′ (reverse); ERα, 5′-TTCACACCAAAGCCTC GGG-3′ (forward) and 5′-TGCAGCAGCATCAGCGGAC-3′ (reverse); cytokeratin 8, 5′-TGGGCAGCAGCAACTTTC-3′ (forward) and 5′-AGGCG AGACTCCAGCTCTAC-3′ (reverse); vimentin, 5′-GAGAACTTTGCCGTT GAAGC-3′ (forward) and 5′-GCTTCCTGTAGGTGGCAATC-3′ (reverse); prolactin, 5′-GGGTTCATTACCAAGGCCATC-3′ (forward) and 5′-TTCA GGATGAACCTGGCTGAC-3′ (reverse); IGFBP-1, 5′-TTGGGACGCCATC AGTACCT-3′ (forward) and 5′-TTGGTGACATGGAGAGCCTTC-3′ (reverse); MMP2, 5′-TTGATGGCATCGCTCAGATC-3′ (forward) and 5′-TGTCACGTGGCGTCACAGT-3′ (reverse); MMP9, 5′−CCACCACAAC ATCACCTATTGG-3′ (forward) and 5′- GCAAAGGCGTCGTCAATCA-3′ (reverse); GAPDH, 5′-ACCCAGAAGACTGTGGATGG-3′ (forward) and 5′-TCTAGACGGCAGGTCAGGTC-3′ (reverse). The expression levels of genes were determined using $2^{-\triangle\triangle Ct}$ method with GAPDH as the internal control. Moreover, the mRNA levels of genes in experimental group was normalized by control group.

2.4. Western blot assay

Total protein was extracted from endometrial stromal cells using RIPA Lysis and Extraction Buffer (Thermo Scientific) containing Protease and Phosphatase Inhibitors (Roche Diagnostics, Mannheim, Germany) and quantified using BCA Protein Assays (Thermo Scientific). Then, equivalent protein (50 μg) was resolved on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto PVDF membranes (Millipore, Billerica, MA, USA). Next, the membranes were sequentially blocked with 5% non-fat milk, probed with primary antibodies against p38 MAPK (#9212, 1:1000, Cell Signaling Technology, Danvers, MA, USA), phospho-p38 MAPK (#9211, 1:1000, Cell Signaling Technology), phospho-p44/42 MAPK (ERK 1/2, #9101, 1:1000, Cell Signaling Technology), p44/42 MAPK (ERK 1/2, #9102, 1:1000, Cell Signaling Technology), Progesterone Receptor A/B (#3176, 1:1000, Cell Signaling Technology) and GAPDH (#2118, 1:1000, Cell Signaling

Technology). After probed with Anti-rabbit IgG HRP-linked secondary antibody (#7074, 1:5000, Cell Signaling Technology) for 1 h, the membranes were incubated with SignalFire™ ECL Reagent (#6883, Cell Signaling Technology) for 5 min to detect specific protein expression, which were further quantified by Quantity One SoftwareVersion 4.1.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. ELISA assay

Secreted levels of prolactin, IGFBP-1,Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-1 beta (IL-1β), Granulocyte-macrophage colony-stimulating factor (GM-CSF), RANTES (also named as C-C motif Chemokine ligand 5 (CCL5)) and monocyte chemoattractant protein 1 (MCP1) into cell culture supernatant were determined using matching ELISA kits for prolactin (ab226901, Abcam), IGFBP-1 (ab100539, Abcam), IL-6 (ab46042, Abcam), IL-8 (ab46032, Abcam), IL-1β (ab214025, Abcam), GM-CSF (ab100529, Abcam) RANTES (ab100633, Abcam) and MCP-1 (ab100586, Abcam) according to the manufacturer's instructions. The detection zone of each ELISA kit was listed as follows: 39.06 pg/ml - 2500 pg/ml for prolactin, 2.74 pg/ml -2000 pg/ml for IGFBP-1, 1.56 pg/ml - 50 pg/ml for IL-6, 62.5 pg/ml - 2000 pg/ml for IL-8, 14.06 pg/ml - 900 pg/ml for IL-1β, 2.05 pg/ml - 500 pg/ml for GM-CSF, 2.74 pg/ml - 2000 pg/ml for RANTES, 2 pg/ml - 500 pg/ml for MCP-1.

2.6. Statistical analysis

All data were exhibited as mean ± standard deviation from more than triple individual experiments. Differences between two groups were analyzed using Student's *t*-test. *P* values less than 0.05 were denoted as statistically significant.

3. Results

3.1. PGR expression was upregulated in endometrial tissues of PCOS patients

Firstly, expression pattern of PGR in endometrial tissues of PCOS (n = 10) and non-PCOS patients (Normal, n = 10) was examined by RT-qPCR assay. RT-qPCR results showed that PGR mRNA level was markedly upregulated in endometrial tissues of PCOS patients compared with non-PCOS patients (Fig. 1A). Previous findings indicated that ERα, cytokeratin 8 (an epithelial cell marker), and vimentin (a mesenchymal/stromal cell marker) were abnormally expressed in the uterus of PCOS patients and PCOS-like rats [11,22,23]. Thus, expression patterns of ERα, cytokeratin 8, and vimentin in PCOS patients and non-PCOS patients were examined to further demonstrate the right diagnose of PCOS patients. Results showed that mRNA level of ERα was strikingly elevated in the endometrial tissues of PCOS patients as compared to that in non-PCOS patients (Fig. 1B). Also, mRNA level of vimentin was noticeably increased, and cytokeratin 8 level was significantly reduced in endometrial tissues of PCOS patients (Fig. 1C and D). In a word, our study indicated that PGR was upregulated in endometrial tissues of PCOS patients.

3.2. Metformin inhibited EP-induced decidualization of endometrial stromal cells

Previous studies manifested that PGR was a target of metformin and metformin curbed decidualization in PCOS-like rats and human endometrial stromal cells [11,13]. Thus, endometrial stromal cells were stimulated with estradiol (10 nM) and progesterone (1 μM) for more than 10 days to establish decidualization models. As presented in Fig. 2A and B, EP stimulation induced a striking upregulation of IGFBP1 and prolactin (two markers of decidualization) mRNA levels in a time-dependent manner in endometrial stromal cells. However, the introduction of metformin markedly inhibited EP-mediated promotive effect on IGFBP1 and prolactin expression in endometrial stromal cells (Fig. 2A and B). Also, the secretion levels of IGFBP-1 and prolactin into media were markedly elevated on day 10 and day 14 in endometrial stromal cells after EP stimulation relative to untreated control cells (Fig. 2C and D). Additionally, lower IGFBP-1 and prolactin secretion was observed in EP-treated endometrial stromal cells after the introduction of metformin (Fig. 2C and D). However, metformin treatment had no effect on the expression and secretion of IGFBP-1 and prolactin in endometrial stromal cells without EP stimulation. In other words, these results indicated that metformin alleviated EP-induced decidualization of endometrial stromal cells.

3.3. Metformin inhibited decidualization by regulating cytokine secretion in EP-stimulated endometrial stromal cells

Some cytokines, growth factors and matrix metalloproteinases (MMPs) have been reported to be involved in decidualization of PCOS [20,24,25]. Our study showed that the secretion of IL-6 and MCP-1 was notably reduced in EP-treated endometrial stromal cells versus Control group (Fig. 3A and D). But, the inhibitory effect of EP on IL-6 and MCP-1 secretion was notably alleviated by metformin, presented by increased IL-6 and MCP-1 secretion in EP-treated endometrial stromal cells following the administration of metformin (Fig. 3A and D). Conversely, EP treatment induced the upregulation of IL-8, IL-1β and GM-CSF secretion, while this effect was enhanced by metformin in endometrial stromal cells (Fig. 3B, C and F). Also, a notable increase of RANTES secretion was observed in EP-treated endometrial stromal cells relative to control group (Fig. 3E). But, RANTES secretion was markedly reduced in EP-treated endometrial stromal cells in response to metformin administration (Fig. 3E). However, metformin treatment alone did not affect the secretion of these factors (Fig. 3A-F). In a word, these data proclaimed that metformin might affect decidualization by regulating secretion of multiple cytokines in EP-stimulated endometrial stromal cells.

3.4. Metformin weakened EP-induced decidualization by inhibiting expression of MMP-2 and MMP-9 in endometrial stromal cells

A earlier finding pointed out that secretion of MMP-2 and MMP-9 was elevated in endometrial stromal cells upon decidualization [26]. Hence, MMP-2 and MMP-9 expression was further detected in endometrial stromal cells under the treatment of EP or metformin. As displayed in Fig. 4A and 4B, EP stimulation induced the upregulation of MMP-2 and MMP-9 expression, while this effect was attenuated following the introduction of metformin. Also, no obvious change of MMP-2 and MMP-9 levels was observed in endometrial stromal cells treated with metformin alone compared with untreated control cells (Fig. 4A and 4B). In summary, these results implied that metformin weakened EP-induced decidualization by inhibiting expression of MMP-2 and MMP-9 in endometrial stromal cells.

3.5. Metformin weakened EP-induced decidualization by activating p38-MAPK signaling and reducing PGR expression in endometrial stromal cells

ERK1/2 and p38 MAPK signaling pathways have been demonstrated to be implicated in decidualization progression in mice and human endometrial stromal cells [27,28]. Thus, protein levels of p-ERK1/2, ERK1/2, p-p38 MAPK and p38 MAPK were measured to test whether ERK1/2 and p38 MAPK signaling pathways were involved in mediating decidualization responses of EP and metformin in human endometrial stromal cells. As presented in Fig. 5A-C, EP treatment resulted in the reduction of p-p38 MAPK level in endometrial stromal cells, indicating that EP inhibited the activation of p38 MAPK signaling pathway. However, p-p38 MAPK level was notably upregulated in EP-treated endometrial stromal cells upon metformin administration (Fig. 5A-C),

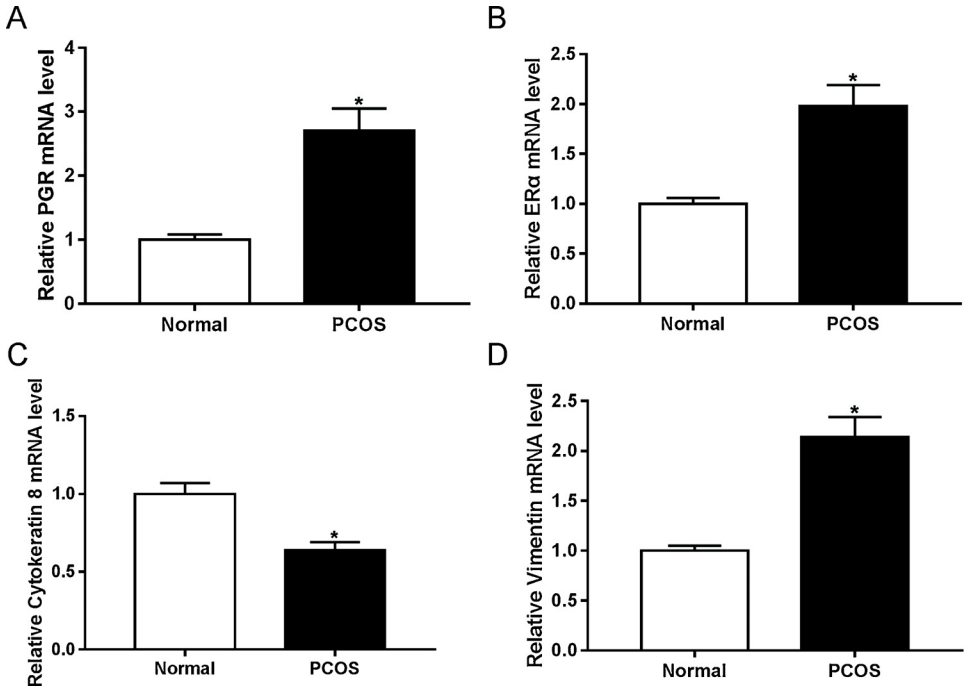

Fig. 1. PGR expression was upregulated in PCOS patients. RT-qPCR assay was performed to determinate mRNA expression of PGR (A), ERα (B), cytokeratin 8 (C) and vimentin (D) in endometrial tissues of PCOS and non-PCOS patients. * $P < 0.05$.

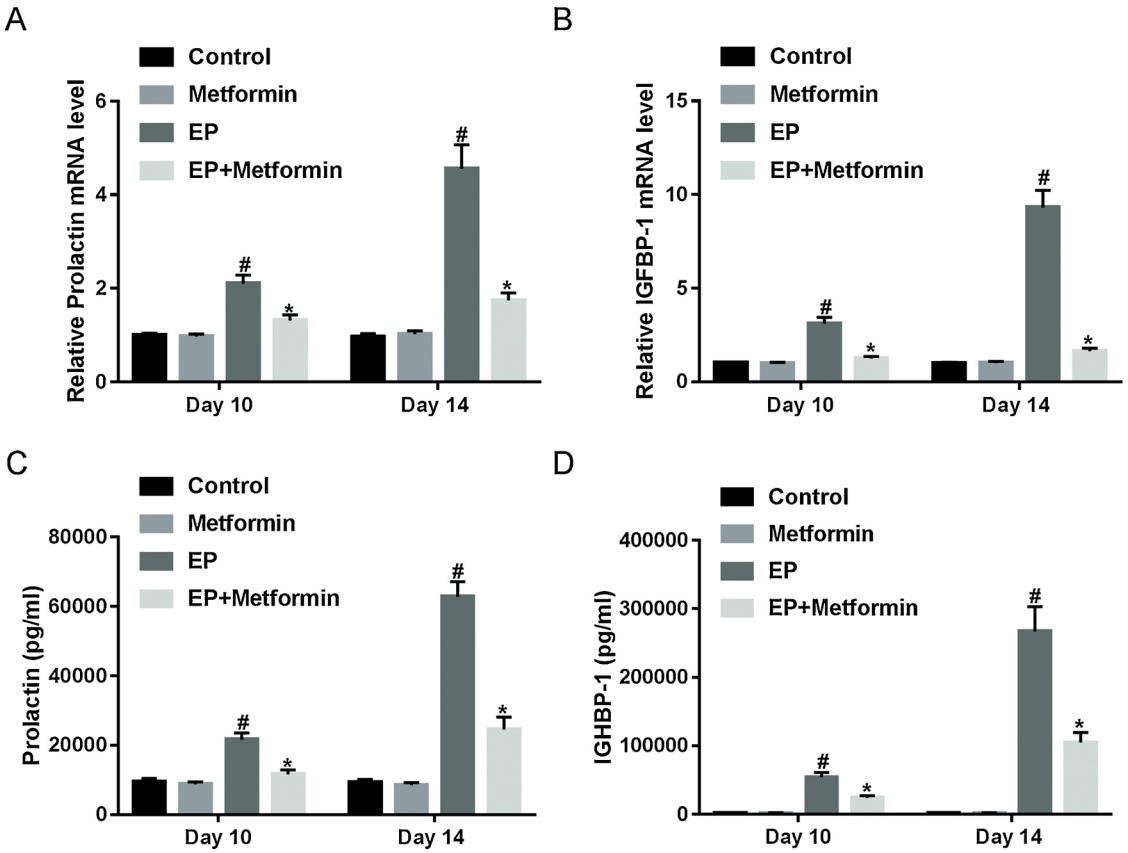

Fig. 2. Metformin alleviated EP-induced decidualization of endometrial stromal cells. (A–D) Endometrial stromal cells were treated with or without estradiol (10 nM) and progesterone (1 μM) in the absence or presence of metformin (1 mM) for 10 days or 14 days. Then, mRNA levels of IGFBP-1 and prolactin in endometrial stromal cells were examined by RT-qPCR assay (A and B). Secreted protein levels of IGFBP-1 and prolactin into media were tested by matching ELISA kits (C and D). #* $P < 0.05$.

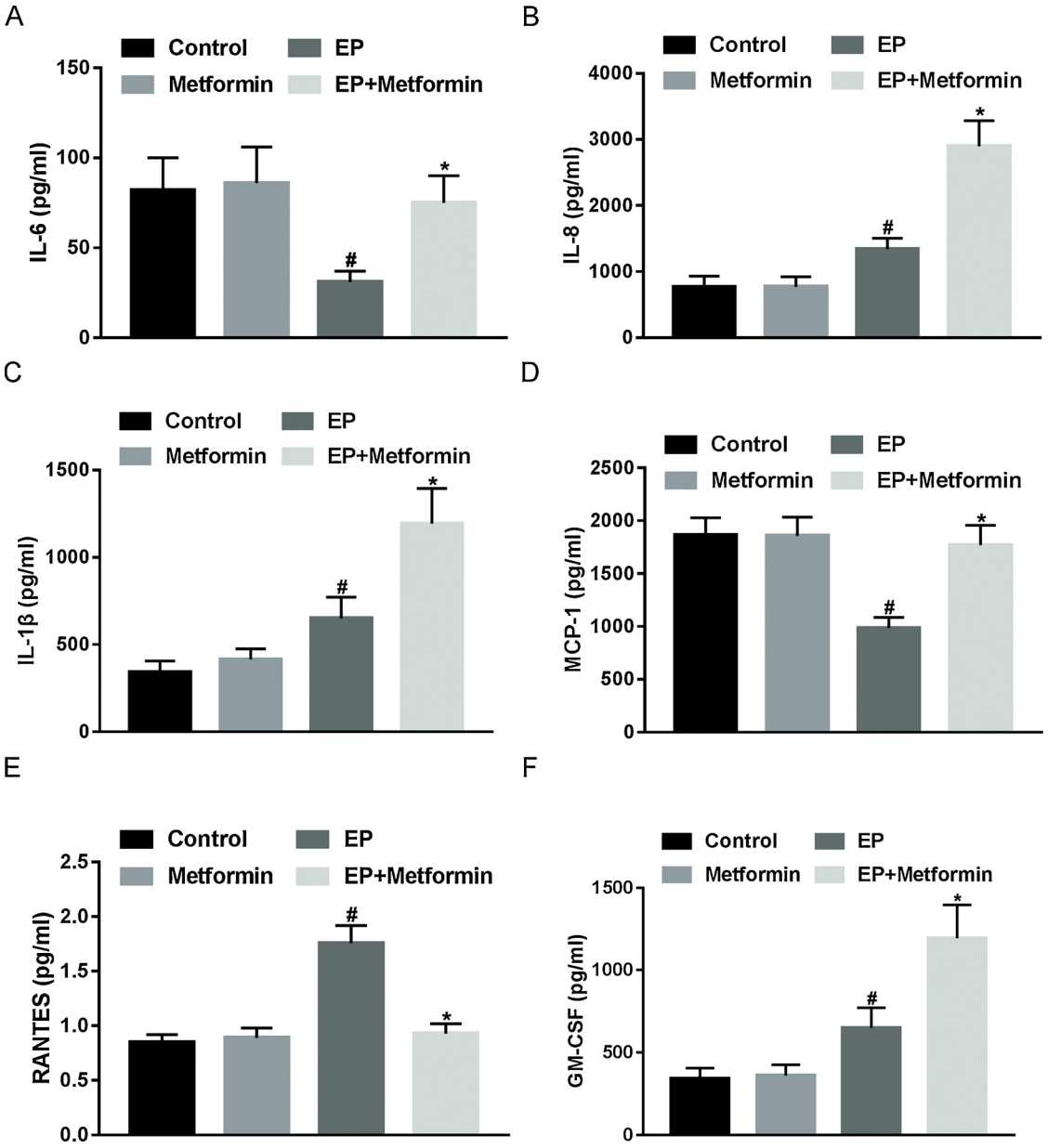

Fig. 3. Metformin inhibited decidualization by regulating secretion of multiple cytokines in EP-stimulated endometrial stromal cells. (A–F) Endometrial stromal cells were treated with or without estradiol (10 nM) and progesterone (1 μM) for 10 days or 14 days in the absence or presence of metformin (1 mM). Secreted protein levels of IL-6, IL-8, IL-1β, RANTES and GM-CSF into media were detected by corresponding ELISA kits. #* $P < 0.05$.

hinting that the introduction of metformin could abolish EP-induced inactivation on p38 MAPK signaling pathway. But, no obvious change of p-ERK1/2 and ERK1/2 levels was observed in endometrial stromal cells in response to EP treatment alone or along with metformin (Fig. 5A-C). These data hinted that p38 MAPK signaling pathway but not ERK1/2 pathway played a central role in EP and metformin-mediated decidualization responses. Furthermore, we further validated that PGR including PGRA and PGRB was highly expressed in EP-treated endometrial stromal cells, and metformin could markedly abate EP-induced PGR (PGRA and PGRB) expression in endometrial stromal cells.

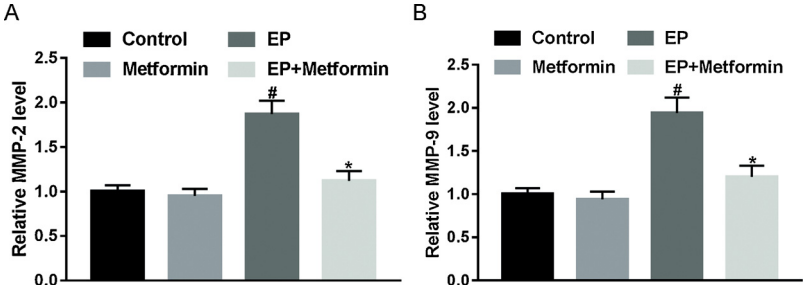

Fig. 4. Metformin weakened EP-induced decidualization by inhibiting expression of MMP-2 and MMP-9 in endometrial stromal cells. (A and B)Endometrial stromal cells were treated with or without estradiol (10 nM) and progesterone (1 μM) in the absence or presence of metformin (1 mM) for 10 days or 14 days, followed by the measurement of MMP-2 and MMP-9 mRNA level via RT-qPCR assay. #* $P < 0.05$.

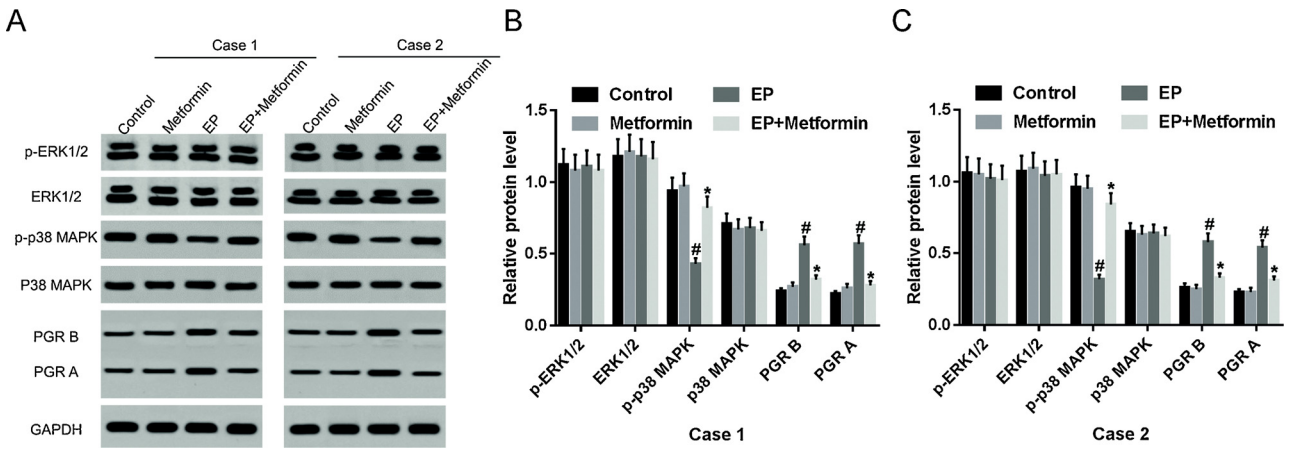

Fig. 5. Metformin suppressed EP-induced decidualization by activating p38-MAPK signaling and reducing PGR expression in endometrial stromal cells. (A–C) Endometrial stromal cells were treated with or without estradiol (10 nM) and progesterone (1 μM) in the absence or presence of metformin (1 mM) for 10 days or 14 days. Then, expression profiles of p-ERK1/2, ERK1/2, p-p38 MAPK, p38 MAPK, PGRA and PGRB were determined by western blot assay. Case 1 and Case 2 represented randomly selected two endometrial stromal cell lines. #* $P < 0.05$.

Additionally, metformin treatment alone had no impact on p38 MAPK pathway, ERK1/2 pathway and PGR expression in endometrial stromal cells (Fig. 5A-C). In summary, these data disclosed that metformin weakened EP-induced decidualization by activating p38-MAPK signaling and reducing PGR expression in endometrial stromal cells.

4. Discussion

PCOS is responsible for about 30% of infertility in couples seeking treatment [5]. Endometrial dysfunction plays a central role in PCOS-related infertility [29]. A previous report pionted out that PGR dysregulation was a leading cause of uterine pathophysiology [6]. Hence, expression pattern of PGR in endometrial tissues of PCOS patients and non-PCOS patients was examined. Results showed that PGR expression was notably upregulated in endometrial tissues of PCOS patients, which was in line with earlier findings [9,10]. However, Artimani et al. showed that PGR mRNA level was notably reduced in granulosa cells of PCOS patients [12]. Also, we further demonstrated that ERα and vimentin expression was markedly upregulated and cytokeratin 8 expression was strikingly reduced in endometrial tissues of PCOS patients.

A prior study pointed out that metformin suppressed PGR expression and corrected the abnormal expression of PGR-targeted genes (Ihh, Ptch, Fkbp52, and Ncoa2) in PCOS-like rat uterus [11]. Additionally, prior studies revealed that metformin therapy has a central influence on endometrial function in PCOS-like rats [11,30] and PCOS patients [31,32]. Insulin-like growth factor (IGF) binding protein 1 (IGFBP-1) and prolactin have been identified as markers for decidualization of human endometrial stromal cells [33,34]. Hence, IGFBP-1 and prolactin were employed to assess the effect of metformin on decidualization in EP-stimulated human endometrial stromal cells. Results displayed that expression and secretion of IGFBP-1 and prolactin was both remarkably increased in human endometrial stromal cells following the treatment of EP, meaning that EP-induced PCOS decidualization models were successfully established. Moreover, we further demonstrated that metformin treatment notably inhibited EP-induced decidualization in endometrial stromal cells, which was also supported by a previous study [13]. However, metformin treatment alone did not influence the decidualization of endometrial stromal cells.

Previous studies indicated that some cytokines, growth factors and matrix metalloproteinases (MMPs) played vital roles in the decidualization responses of PCOS [20,24,25]. For instance, cytokines such as IL-6, IL-8, TNFα, RANTES and MCP-1 (CCL-2) was highly expressed in the uterus of PCOS-like rats and endometrium of PCOS patients [22,30,35]. Also, RANTES, IL-1β, IL-8, MMP-2 and MMP-9 facilitated decidualization of endometrial stromal cells [36–38]. Additionally, EP treatment induced reduced secretion of IL-6, MCP-1 and GM-SCF, and increased secretion of IL-8, RANTES and MMP-2 in endometrial stromal fibroblasts of non-PCOS patients and PCOS patients with normal decidualization responses to E2P4 [20]. In the present study, we demonstrated that secretion of IL-6 and MCP-1 were downregulated and secretion of IL-8, IL-1β, RANTES and GM-SCF were upregulated in endometrial stromal cells following the stimulation of EP. Moreover, metformin adminstration resulted in the elevation of IL-6, IL-8, IL-1β, MCP-1 and GM-SCF secretion together with the decrease of RANTES secretion in EP-induced PCOS decidualization models. Also, metformin treatment alone had no much impact on the secretions of these factors in endometrial stromal cells. However, a report pointed out that levels of IL-8 and IL-1β were markedly upregulated in EP-stimulated endometrial stromal cells after short-term (48 h) metformin exposure, but an unconspicuous increase of IL-8 expression and a notable elevation of IL-1β expression was observed in EP-treated endometrial stromal cells following the long-term (14 day) treatment of metformin [13]. Also, a finding revealed that metformin treatment induced the reduction of IL-1β, IL-8, IL-6, MCP-1 and MMP-2 expression in uterus of insulin- and hCG-induced PCOS rats [30].

Also, MMP-2 and MMP-9 expression was elevated in response to EP treatment in endometrial stromal cells, which is in line with a report showing that secretion of MMP-2 and MMP-9 was elevated in endometrial stromal cells upon decidualization [26]. Additionally, the introduction of metformin induced a notable reduction in expression of MMP-2 and MMP-9 in EP-induced PCOS decidualization models. Similar results were obtained in human umbilical vein endothelial cells [39]. And, no notable change of MMP-2 and MMP-9 levels was noticed in endometrial stromal cells treated with metformin alone. However, previous studies showed that metformin inhibited IL-1β-induced IL-8 production in endometrial stromal cells [40]. IL-1β notably enhanced MMP-9 expression, but not MMP-2 expression in first-trimester human decidual cells stimulated with estradiol (E2) alone or along with medroxyprogesterone acetate (MPA) [41], hinting the close association among merformin, metalloproteinases and cytokines. Our study provides the in vitro data showing that endothelial stromal cells have an aberrant decidualization response to EP stimulation, concomitant with abnormal cytokine, chemokine and MMP release-creating a microenvironment conducive to recruiting migratory immune cells.

Prior reports also showed that metformin could inhibit leptin-induced MMP-2 expression by interfering with the PKC/MAPK/NF-κB pathways in human vascular smooth muscle cells [42]. Also, Hwang et al. pointed out that metformin could suppress phorbol-12-myristate-

13-acetate (PMA)-induced MMP-9 upregulation and activation by inactivating PKCα/ERK and JNK/AP-1-signalling pathways in human fibrosarcoma cells [43]. Moreover, metformin inhibited MMP-2 and MMP-9 activation by activating AMPK/p53 pathway in melanoma and human umbilical vein endothelial cells [44,45]. Additionally, Li et al. suggested that metformin could reduce MMP-2 and MMP-9 expression by regulating AMPK/mTOR/autophagy pathway in endothelial progenitor cells [46]. In a word, these data promoted us to suppose that JNK, AMPK, p53, mTOR or autophagy pathway might play vital roles in metformin-mediated decidualization process of endometrial stromal cells or anti-PCOS responses.

Then, we further demonstrated that EP treatment inhibited activation of p38 MAPK signaling pathway, while this effect was abrogated by metformin in endometrial stromal cells. Also, an earlier study presented that expression of p38 MAPK and p-p38 MAPK was strikingly reduced in PCOS-like rats relative to control rats, and the downregulation of p-p38 MAPK protein expression but not p38 MAPK could be reversed by metformin [11]. However, ERK1/2 signal was not altered in endometrial stromal cells in response to EP treatment alone or along with metformin. Moreover, metformin treatment alone had no effect on p38 MAPK and ERK1/2 pathways in endometrial stromal cells without EP stimulation. Furthermore, we further validated that PGR (PGRA and PGRB) was highly expressed in EP-treated endometrial stromal cells, and metformin could markedly abate EP-induced upregulation of PGR (PGRA and PGRB) expression in endometrial stromal cells.

E2 and P4 exerted different effects on the pro- and anti-inflammatory immune responses in vitro and in vivo [47–49]. Estradiol at the concentration less than 10 nM enhanced pro-inflammatory pathways including IL-6 and IL-1β, while high concentration of estradiol exhibited reverse effects, and progesterone could inhibit both pro- and anti-inflammatory cytokine production at low or high concentration [47–49]. However, EP stimulation could strongly suppress the innate immune responses of monocytes, which are the main source of TNF and IL-6 [50]. Moreover, IL-1β stimulated IL-8 expression in endometriotic stromal cells [40]. These data might can explain why IL-6 level was downregulated and IL-8 and IL-1β levels were upregulated in response to EP stimulation. Previous studies showed that metformin also inhibited progesterone signaling and restored p38 MAPK signaling to exert its anti-PCOS effect [11]. The inhibition of progesterone signaling and the activation of p38 MAPK signaling could induce the production of IL-6, IL-8 and IL-1β [51–57], which might can explain why metformin stimulation can induce the upregulation of IL-6, IL-8 and IL-1β levels in EP-treated cells. The responses of cytokine production and secretion were very complicated and associated with multiple biological processes.

5. Conclusion

Collectively, our results indicated that metformin hampered EP-induced decidualization by altering expression of multiple cytokines, MMP-2, MMP-9 and PGR via regulating p38 MAPK signaling pathway, deepening our understanding on molecular basis of metformin for PCOS therapy.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgement

Not applicable.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.10.128.

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