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Metformin promotes progesterone receptor expression via inhibition of mammalian target of rapamycin (mTOR) in endometrial cancer cells

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ABSTRACT

Progesterone has been used in the hormonal treatment of endometrial cancer (EC) for many years, but the response rates are unsatisfying. The down-regulated progesterone receptor (PR) is the main reason for treatment failure. The insulin-like growth factor (IGF) system is related to EC risk, and IGF-I can inhibit PR transcription in breast cancer. Recent evidence suggests that metformin-combined oral contraceptives may reverse progesterone-resistant atypical endometrial hyperplasia, but the mechanism is unclear. We attempt to investigate the interaction of metformin, PR and IGF-II expression, and identify whether metformin can enhance the antitumor effect of medroxyprogesterone acetate (MPA) using Ishikawa and HEC-1B EC cell lines. We found that both IGF-I and IGF-II inhibit PR A/B mRNA and protein expression, whereas metformin markedly promotes PR expression. In parallel, IGF-II increases phosphorylation of AKT and p70S6K, while metformin increases AMPK phosphorylation and decreases p70S6K phosphorylation. The effects of metformin on PR A/B and p70S6K are partially reversed by an AMPK inhibitor. Furthermore, metformin synergistically antiproliferates MPA in two cell lines, with the peak synergy occurring with 10 μM metformin combined with 1 μM MPA (CI = 0.20448 for Ishikawa, CI = 0.12801 for HEC-1B). Our results demonstrate that metformin promotes PR expression, which can be inhibited by overexpressed IGF-II in EC. This effect is partially mediated through activating AMPK followed by inhibiting the overactivated mTOR pathway.

1. Introduction

In Western countries, endometrial cancer (EC) is the most common gynecological malignancy, accounting for 6% of all cancers in women [1,2]. Approximately 80% of EC patients are diagnosed in Stage I and are usually cured with hysterectomy [3]. However, a subset of young women present with EC in a setting of obesity, irregular menses, chronic anovulation and polycystic ovarian syndrome (PCOS). This group of women poses a therapeutic dilemma, since preservation of fertility is a major concern for these individuals. Thus, reproductive-sparing treatment is crucial in this scenario.

Endometrial carcinogenesis is related to estrogen overexposure without progesterone modulation. The role of progesterone in the endometrium is primarily to induce cellular differentiation and to antagonize estrogen-mediated cell proliferation [4]. Progesterone and its synthetic form (medroxyprogesterone acetate, or MPA) have been used for the treatment of EC in advanced or recurrent cases, and in those who wish to preserve their fertility [5,6]. Progesterone binds to its receptor and activates the transcription of several genes which are involved in cross-talk with other signaling pathways, such as growth factors and cytokines [7]. The antitumor effect of progesterone is in its binding to the human progesterone receptors (hPR-A, hPR-B), belonging to the steroid hormone superfamily of nuclear receptors [8]. Unfortunately, PR expression decreases during EC progression, resulting in the loss of progesterone-regulated growth inhibition [9]. Down-regulated progesterone receptors frequently lead to carcinogenesis and treatment failure, as evidenced by the overall response rate of PR-rich or PR-poor tumors (72% vs 12%, respectively) [5]. Unfortunately, progesterone treatment also leads to depletion of PRs within the target tissue.

Accumulating evidence indicates that obesity, diabetes and insulin resistance are strong risk factors for EC, and the insulin-like growth factor (IGF) system plays a vital role in carcinogenesis and disease progression [10]. IGF-II and IGF-IR (IGF-I receptor) were found to be much higher in EC than in normal endometrium [11]. Both IGF-I and IGF-II are mitogenic and antiapoptotic. IGF-IR binds to the ligands IGF-II, IGF-I, or insulin, triggering autophosphorylation. This in turn leads to activation of distinct signaling pathways, including the phosphatidylinositol 3-kinase (PI3K)-AKT/mammalian target of rapamycin (mTOR) pathway [12]. On the contrary, phosphatase and tensin homolog deleted on chro-

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mosome ten (PTEN) exerts its tumor-suppressive function through its activity as a phospholipid phosphatase, leading to inhibition of PI3K signaling and inactivation of downstream kinases such as AKT and mTOR. Unfortunately, loss of PTEN is found in 30–83% of EC, which leads to overactivation of the mTOR pathway, ultimately contributing to dysregulation of cell proliferation, growth, differentiation, and survival [13]. A recent study suggests that IGF-I inhibits PR gene transcription via the PI3K/AKT/mTOR pathway in breast cancer [14]. Thus, IGF-I may weaken the antitumor effect of progesterone through reduction of PR levels in breast cancer, although this has not been identified in EC.

A recent case report has shown that combination therapy with metformin and oral contraceptives may reverse progesterone-resistant atypical endometrial hyperplasia [15]. Therefore, metformin may enhance the effect of progesterone on atypical endometrial hyperplasia, but this mechanism is unclear. The mechanism of action of metformin is by activating AMP-activated protein kinase (AMPK) via Germline mutation in serine/threonine kinase 11 (STK11, also called LKB1), the kinase responsible for phosphorylating and activating AMPK [13]. This process leads to the regulation of multiple signaling pathways involved in cellular proliferation, including the mTOR pathway. Loss of LKB1 expression has been documented in up to 65% of ECs, which stimulates mTOR pathway overactivation in ECs [16,17].

Based upon the preceding evidence, we investigated the interaction of metformin, IGF-II and PR expression, explored the cell signaling pathway targets, and identified whether metformin can enhance the antitumor effect of MPA using Ishikawa and HEC-1B EC cell lines.

2. Materials and methods

2.1. Cell lines and reagents

The human EC cell lines Ishikawa (IK, well differentiated) and HEC-1B (moderately differentiated), generously provided by Prof. Wei LH (Perking University People's Hospital, China), were maintained in phenol red-free DMEM/F12 with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. The cell cultures were routinely passaged every 3–5 days. Metformin, MPA (medroxyprogesterone17-acetate), dextran-coated charcoal were purchased from Sigma. Insulin-like growth factor-I (IGF-I) and IGF-II were purchased from Sigma and R&D System, respectively. Compound C (AMPK inhibitor) was purchased from Calbiochem. MPA was diluted in DMSO as a stock solution of 20 mM.

2.2. Real-time RT-PCR

The IK and HEC-1B cells were plated at 2×10^5 cells/well in 6-well plates for 24 h and then were treated with metformin (1 μM, 10 μM, and 100 μM) in the presence or absence of Compound C (1 μM) in phenol red-free DMEM/F12 containing 3% steroid-stripped FBS (DCC-FBS) (using dextran-coated charcoal) for 72 h, or were treated with increasing concentration of IGF-II (5, 10 ng/ml) in phenol red-free DMEM/F12 without FBS for 48 h. Total RNA was extracted from cells with Trizol reagent (Invitrogen) according to the manufacturers' protocol. RNAs were subjected to DNaseI digestion to avoid possible genomic DNA contamination, and then reverse transcribed with oligo-dT primers and M-MLV Reverse Transcriptase (Promega). Real-time PCR was carried out using SYBR green sequence detection reagents (Takara) in a 20 μl reaction, which contains 1 μl of cDNA, 10 μl of Mix, 0.4 μl of Rox and 1 μl of 5 μM each primer. Primer sequences are as follows: PRG(f): 5′-CAGATGCTGTATTTTGCACCTGAT-3′, PRG(r): 5′-CTTCTTGGCTAACTTGAAGCTTGA-3′. PRB(f): 5′-CGGACACCTTGC CTGAAGTT-3′, PRB(r): 5′-CAGGGCCGAGGGAAGAGT-3′. GAPDH(f): 5′-CAGTCAGCCGCATCTTCTTTT-3′, GAPDH(r): 5′-GTGACCAGGCG-CCCAATAC-3′. PRG includes PRA and PRB. The PCR cycling conditions were as follows: 95 °C for 30 s followed by 40 cycles of two steps at 95 °C for 5 s, 60 °C for 31 s. Fluorescent signals were detected using an ABI 7500 (Applied Biosystems), and the accumulation of PCR product was measured in real time as the increase in SYBR green fluorescence. Real-time PCR was performed in triplicate of each sample. The obtained PGR and PRB mRNA levels were acquired by normalizing the threshold cycle (Ct) of PR to the Ct of GAPDH. The relative levels of mRNA were compared and expressed as the ratio to the control subjects.

2.3. Western immunoblotting

The IK and HEC-1B cells were plated at 2×10^5 cells/well in 6-well plates for 24 h, and then were treated with metformin (1 μM, 10 μM, and 100 μM) in the presence or absence of Compound C (1 μM) in phenol red-free DMEM/F12 containing 3% steroid-stripped FBS (DCC-FBS) (using dextran-coated charcoal) for 72 h, or were treated with increasing concentration of IGF-II (5, 10 ng/ml) in phenol red-free DMEM/F12 without FBS for 48 h to observe the change of PR protein levels. To investigate the relevant signaling targets, the IK and HEC-1B cells were plated at 2×10^5 cells/well in 6-well plates for 24 h, and then were serum-starved for an additional 24 h before metformin or IGF-II treatment. First, cells were treated with 10 mM of metformin for 0, 1, 3, 6, and 8 h to observe the AMPK and P70S6K activation. Next, cells were treated with 100 ng/ml of IGF-II for 0, 15, 30, 60, 120 min to observe the AKT activation. Finally, cells were treated with IGF-II (100 ng/ml) in the presence or absence of metformin for 30 min to observe the P70S6K activation. Cell lysates were prepared in RIPA buffer (1% NP40, 0.5 sodium deoxycholate and 0.1% SDS). Twenty micrograms of protein extract was subjected to 10% SDS-PAGE and subsequent electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and 0.1% Tween for 1 h at room temperature with constant agitation, and then incubated with a primary antibody (1:1000; CST) overnight at 4 °C. After having been washed three times for 5 min each with PBST, the membrane was incubated with a secondary HRP-linked antibody (1:2000; CST) for 2 h. After the membrane was finally washed three times for 5 min each with PBST, bands were visualized by enhanced chemiluminescence (ECL) reagents according to the manufacture's instruction (Pierce Chemical Co.). After developing, the membrane was stripped and re-probed using antibody against GAPDH (1:1000, CST) and either pan-S6K or pan-AMPK to confirm equal loading. The relative protein were normalized to GAPDH and expressed as the ratio to the nontreatment control subjects. Protein bands, including GAPDH, were quantified by densitometry with the Quantity One imaging program (Bio-Rad, Hercules, CA).

2.4. Cell proliferation assays

The cell proliferation assays were performed by BrdU (5-bromodeoxyuridine)-ELISA Kit (Roche). The IK and HEC-1B cells were plated into 96-well plates at a concentration of 8×10^3 cells/well and 1×10^4 cells/well, respectively. Twenty-four hours after plating, cells were serum-starved for an additional 24 h, and then were treated with increasing concentration of metformin (0.1 μM, 1 μM, 10 μM, and 100 μM) in the absence or increasing concentration of MPA (0.1, 1, and 10 μM) for 72 h. The effect of metformin and MPA was calculated as a percentage of control cell growth obtained from PBS or DMSO treated cells grown in the same 96-well plates. In order to assess the role of AMPK, cells were treated with metformin with or without AMPK inhibitor (Compound C, 0.5, 1 μM) for 72 h. Assays were performed