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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

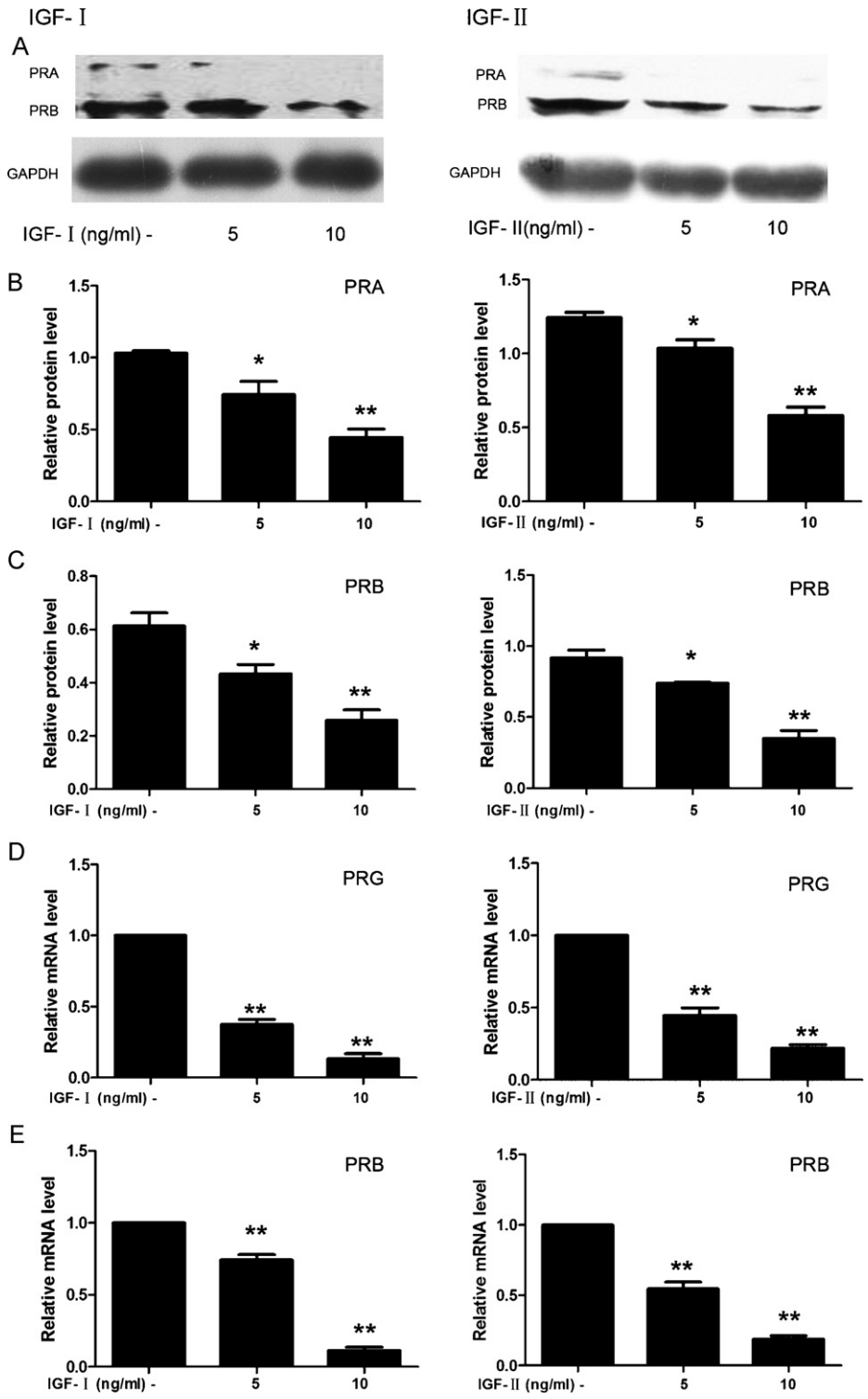

Fig. 1. IGF-I (left) and IGF-II (right) down-regulate PR mRNA and protein levels in Ishikawa cell lines. Western blot analysis (A and B) and real-time RT-PCR (D and E) were performed to detect of PRA/B protein and mRNA levels in Ishikawa cells (*$p < 0.05$, **$p < 0.01$, one-way ANOVA). (A) Ishikawa cells were stimulated for 48 h with increasing concentrations of IGF-I (0, 5, and 10 ng/ml) and IGF-II (0, 5, and 10 ng/ml). Cell lysates were immunoblotted with anti-PR antibody. GAPDH was used as a loading control. This figure represents three independent experiments. (B) and (C) Graphic representation of data in panel A after densitometry and correction for GAPDH expression. PRA (B) and PRB (C) protein levels in IGF-I or IGF-II treated samples were compared with controls. Values are means ± S.E. of three independent experiments, each in duplicate. (D) and (E) Ishikawa cells were stimulated for 48 h with increasing concentrations of IGF-I (0, 5, and 10 ng/ml) and IGF-II (0, 5, and 10 ng/ml). PR mRNA levels in each sample were calculated from a standard curve and normalized using GAPDH mRNA levels. Values are means ± S.E. of three independent experiments, each in duplicate.

under serum-free conditions. DNA synthesis was monitored based on the incorporation of BrdU into DNA, which was detected by immunoassay according to the manufacturer's instruction. Briefly, after incubation, the cells were reincubated with 10 μl/well BrdU labeling solution for additional 2 h at 37 °C, then labeling medium was removed, 200 μl/well FixDenat was added, and the cells were incubated for 30 min at 20 °C. Next, FixDenat solution was removed thoroughly and cells were incubated with 100 μl/well anti-BrdU-

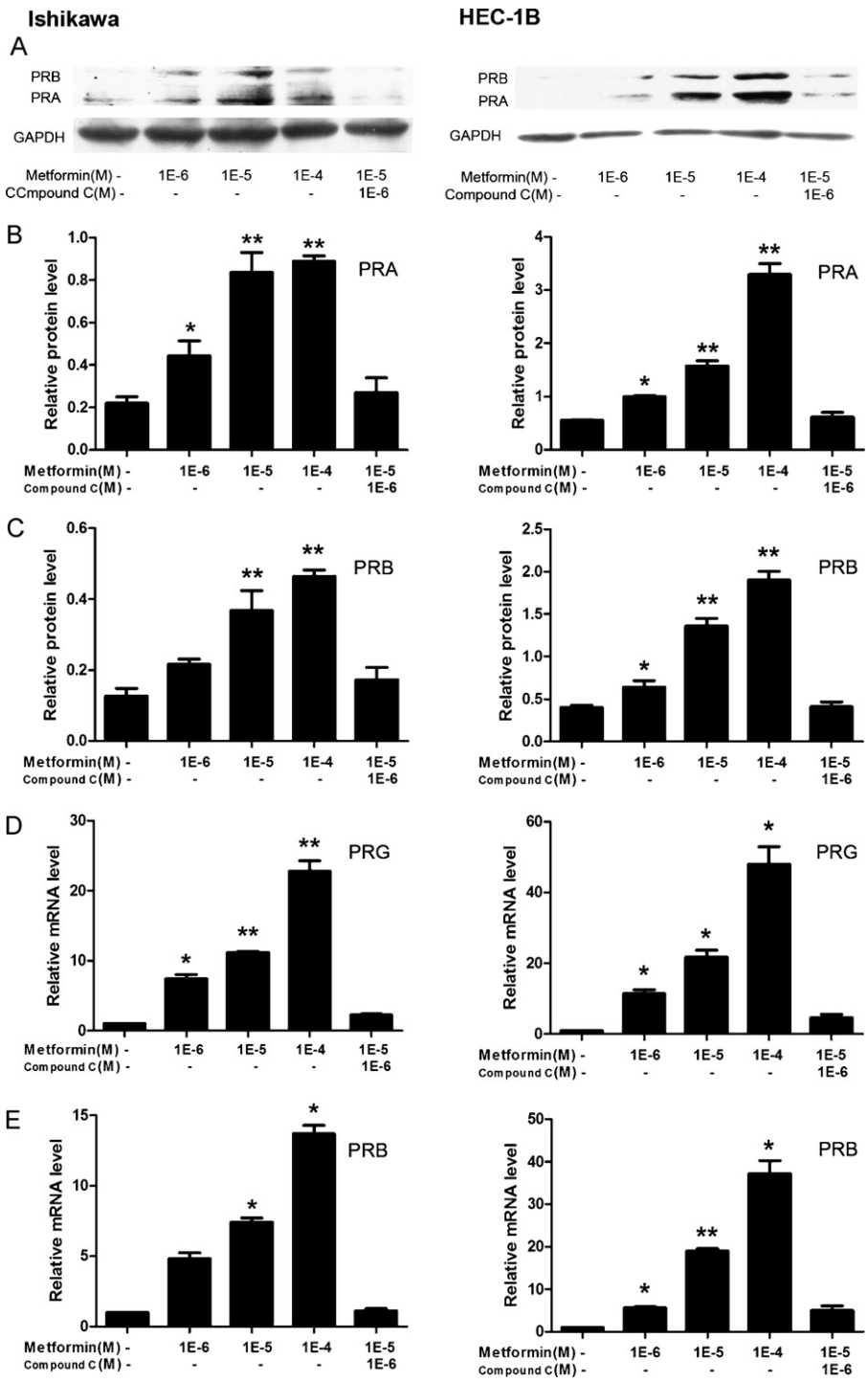

Fig. 2. Metformin up-regulates PR protein and mRNA levels, which depend on AMPK activation in Ishikawa (left) and HEC-1B (right) cells. (A) Ishikawa and HEC-1B cells were stimulated for 72 h with increasing concentrations of metformin (0, 1E−6, 1E−5, and 1E−4) with and without Compound C (AMPK inhibitor). Cell lysates were immunoblotted with anti-PR antibody. GAPDH was used as a loading control. This figure represents three independent experiments. (B) and (C) Graphic representation of data in panel A after densitometry and correction for GAPDH expression. PRA (B) and PRB (B) levels in metformin-treated samples were compared with controls. Values are means ± S.E. of three independent experiments, each in duplicate. (*$p < 0.05$, **$p < 0.01$, one-way ANOVA). (D) and (E) Ishikawa and HEC-1B cells were stimulated for 72 h with increasing concentrations of metformin (0, 1E−6, 1E−5, and 1E−4) with and without Compound C. Total mRNA was isolated and PRG/B mRNA levels were measured by real-time RT-PCR. PRG/B mRNA levels in each sample were calculated from a standard curve and normalized using GAPDH mRNA levels. Values are means ± S.E. of three independent experiments, each in duplicate (*$p < 0.05$, **$p < 0.01$, one-way ANOVA).

POD working solution for 90 min at 20 °C, then antibody conjugate was removed and cells were rinsed three times with washing solution. Last, after removal of washing solution, with 100 μl/well substrate solution was added and cells were incubated at 20 °C for 20 min, then with 25 μl of 1 M H_2SO_4 was added and cells were incubated for 1 min on the shaker at 300 rpm, the absorbance of the samples was measured in an ELISA reader at 450 nm (reference wavelength: 690 nm). Each experiment was performed in triplicate and repeated three times to assess for consistency of the results. We also compared our results using BrdU technique with MTT assay and confirmed the validity of our finding (data not shown).

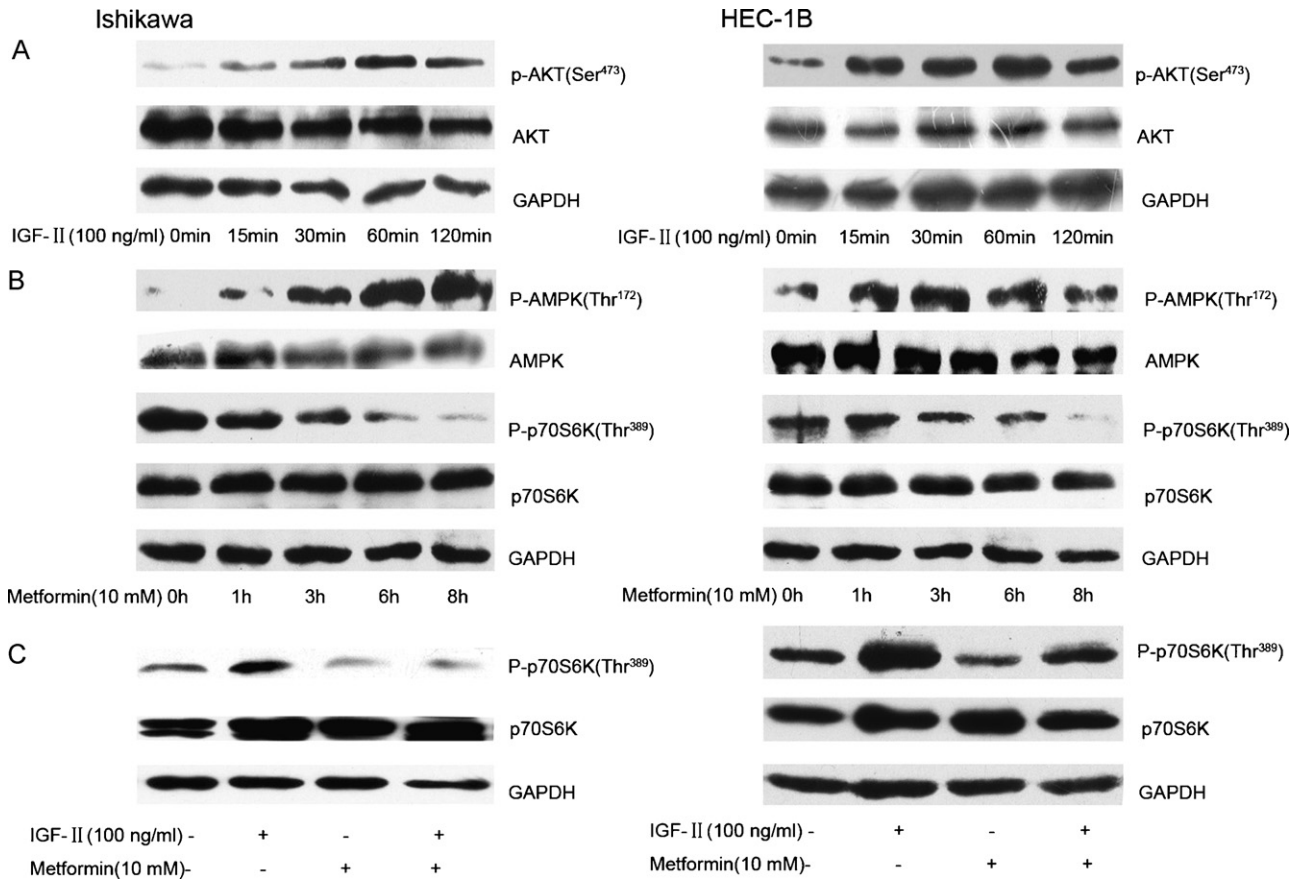

Fig. 3. Metformin activates AMPK and inhibits mTOR signaling pathway in Ishikawa (left) and HEC-1B (right) cell lines. (A) In both EC cell lines Ishikawa and HEC-1B, IGF-II increased phosphorylation of AKT in a time-dependent manner. Ishikawa and HEC-1B cells were serum-starved for 48 h followed by treatment with IGF-II (100 ng/ml) for indicated time intervals. Untreated cells are denoted as "0″. Total protein was immunoblotted using specific antibodies for phosphorylated AKT (p-AKT-Ser^{173}) and total AKT. (B) Time-dependent changes in phosphorylation of AMPK, p70S6K following exposure to metformin. Metformin treatment increases phosphorylation of AMPK and induces dephosphorylation of S6K in Ishikawa and HEC-1B cells. Cells were serum-deprived for 48 h followed by treatment with 10 mM of metformin for indicated time intervals. Untreated cells are denoted by "0″. Total protein was immunoblotted by using specific antibodies for phosphorylated AMPK (pAMPK-Thr^{172}) and phosphorylated p70S6K. The membranes were reblotted using total AMPK and p70S6K antibody as a control. (C) Stimulation of phosphorylate-PS6K by IGF-II; the activation can be attenuated by metformin. Cells were serum-deprived for 48 h followed by treatment with IGF-II (100 ng/ml) in the presence and absence of metformin (10 mM) for 30 min. Untreated cells are denoted by "0." All blots represent three independent experiments.

2.5. Synergistic effect assay

The effect of the combination of metformin and progesterone on cell proliferation was assessed by calculating combination index (CI) values using Calcusyn Software (Biosoft). Derived from the median-effect principle of Chou and Talalay, the CI provides a quantitative measure of the degree of interaction between two or more agents [16]. Combination index (CI) over 1.1 indicates antagonistic, 0.9–1.1 additive, 0.7–0.9 moderately synergistic, 0.3–0.7 synergistic, and <0.3 strongly synergistic.

2.6. Statistical analysis

All data are presented as mean ± S.E. The data were analyzed by one-way ANOVA using SPSS (version 13.0), and value of $p < 0.05$ was considered significant.

3. Results

3.1. IGF-I and IGF-II down-regulate PR mRNA and protein levels while metformin up-regulates PR levels

At 48 h treatment, IGF-I and IGF-II significantly decreased PRA/B mRNA and protein levels in IK and HEC-1B cell lines ($p < 0.05$, Fig. 1). The maximal reduction of PR protein levels occurred at 10 ng/ml of IGF-II ($p < 0.01$), the physiologic concentration in females. This finding suggests that IGF-II has a down-regulatory effect on PR levels in EC cells.

At 72 h, metformin significantly increased PRA/B mRNA and protein levels in two cell lines ($p < 0.05$ for 10 μM, $p < 0.01$ for 100 μM, Fig. 2). Additionally, this effect was partly attenuated by Compound C after 72 h of combined metformin and Compound C ($p > 0.05$ vs the control group) (Fig. 2).

3.2. IGF-II activates the PI3K/AKT/mTOR pathway, while metformin activates AMPK followed by inhibition of the mTOR pathway

After 24 h of serum starvation, treatment of IK and HEC-1B cells with IGF-II produced a dramatic increase in AKT and p70S6K phosphorylation in the two cell lines (Fig. 3A and C). Pan-AKT and pan-p70S6K expressions were not affected by IGF-II.

After metformin treatment of IK and HEC-1B cell lines, increased phosphorylation of AMPK and decreased phosphorylation of p70S6K were observed over time (Fig. 3B). Pan-AMPK and pan-p70S6K expressions were not affected by metformin. We also observed that the p70S6K phosphorylation increased after IGF-II treatment, and that metformin attenuated this effect (Fig. 3C).

Prior studies argue that p70S6K is a downstream target of the mTOR pathway via IGF-II, which activates AKT and p70S6K phos-

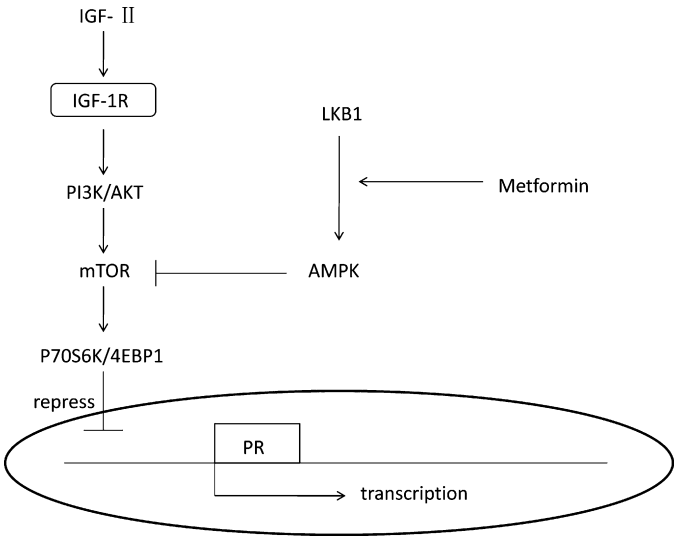

Fig. 4. Interaction between the metformin and mTOR pathways, and illustration of the regulation of PR expression.

phorylation, resulting in mTOR pathway activation [18]. Metformin increases AMPK phosphorylation, subsequently decreasing phosphorylation of the P70S6K protein, which results in mTOR pathway inhibition. Therefore, metformin might actually inhibit the activation of mTOR pathway as induced by IGF-II (Fig. 4).

3.3. Metformin has a synergistic effect with MPA on anti-proliferation

IGF-II promoted proliferation ($p < 0.05$, Fig. 5B), while metformin inhibited proliferation of both cell lines at 72 h, with the most significant effect at a concentration of 100 μM ($p < 0.05$, Fig. 5A). Metformin's metabolic action requires AMP kinase; we hypothesized that the anti-proliferative effects of metformin involve the same mechanism. Compound C is an ATP-competitive inhibitor of AMPK. We found that the anti-proliferative effect of metformin was partly rescued by Compound C (combination group compared with control group, $p > 0.05$, Fig. 5A). This suggests that AMPK pathway activation is required for metformin-induced anti-proliferation.

Based upon the assumption that metformin promotes PR expression (Fig. 2), we questioned whether metformin could improve the anti-proliferative effect of MPA alone. We demonstrated that MPA coincubated with metformin has an increased antiproliferative effect compared with either agent alone ($p < 0.01$). The most significant effect was found with the combination of 10 μM metformin and 1 μM MPA, in both cell lines (Fig. 5C, $p < 0.01$).

A synergistic effect was noted between metformin and MPA with peak synergy at a concentration of 10 μM metformin + 1 μM MPA (CI = 0.20448 for IK, CI = 0.12801 for HEC-1B, Table 1). The mean IC_{50} values of metformin for IK and HEC-1B were 21.4 μM and 18.9 μM, respectively. The mean IC_{50} values of MPA for IK and HEC-1B were 1.64 μM and 2.09 μM, respectively (Table 2).

Table 2
IC_{50} of metformin and MPA in IK and HEC-1B cell lines.

Cell lines	Metformin IC_{50} (M)	MPA IC_{50} (M)
Ishikawa	2.14E−5	1.64E−6
HEC-1B	1.89E−5	2.09E−6

Note: The values of IC_{50} were calculated by the Chou and Talalay method.

4. Discussion

This study is the first to identify the function of metformin in promoting PR expression in EC cells. Conversely, IGF-I and IGF-II inhibit PR expression. We demonstrate that metformin promotes PR expression via by AMPK activation, which is followed by mTOR pathway inhibition in EC cells. We also show that metformin synergistically enhances the anti-proliferative effect of MPA on EC cells.

4.1. IGF-II overexpression and PR down-regulation promote cell proliferation in EC

EC is strongly associated with obesity and diabetes. The IGF system (IGF-I, IGF-II, IGF-I R and IGF-II R) has also been linked with obesity, diabetes, hyperinsulinemia, and several human malignancies, including EC [19]. Several prior studies have shown that IGF-II and IGF-IR were upregulated in EC [11]. IGF-II exerts its mitogenic potential via IGF-IR, a tyrosine kinase membrane receptor, which triggers PI3K signaling and activation of downstream kinases such as AKT and mTOR [20]. mTOR is up-regulated in many cancers as a result of genetic alterations or aberrant activation of components of the PI3K/AKT pathway, contributing to dysregulation of cell proliferation, growth, differentiation and survival [12,13,20]. The PI3K/AKT/mTOR pathway can be inhibited by phospholipid phosphatases, as PTEN, resulting in tumor suppression [21,22]. Loss of PTEN, together with overexpression of IGF-II and IGF-IR, leads to overactivation of the mTOR pathway in EC, which ultimately results in increased proliferation and resistance to apoptosis [23]. Our results confirm that IGF-II markedly promotes cell proliferation in EC (Fig. 5B).

Progesterone normally limits growth and proliferation of EC. The binding of progesterone to PRA and PRB, and subsequent transport from the cytosol into the nucleus, results in cross-talk with growth factor and cytokine signaling pathways [4]. After entering into the nucleus, PR mediates transcription of genes such as the cyclin-dependent kinase inhibitors p21 and p27, significantly reducing cell proliferation [24]. Our data provides the first evidence that IGF-I and IGF-II reduces PR mRNA and protein levels in EC cells (Fig. 1). Unlike progestins, IGF-I and IGF-II do not utilize proteasomes in PR down-regulation. Cui et al. reported that IGF-I inhibits PR gene transcription via the PI3K/AKT/mTOR pathway [14]. IGF-II has a similar biological effect to IGF-I in EC-activation of

Table 1
Effect of metformin and MPA combination in IK and HEC-1B cell lines according to the Chou and Talalay method.

Cell lines	Schedule	FA	CI	Effect
Ishikawa	1E−6M Met + 1E−7M MPA	0.35687	0.73327	Moderately synergistic
	1E−6M Met + 1E−6M MPA	0.51283	0.56070	Synergistic
	1E−5M Met + 1E−7M MPA	0.51918	0.40478	Synergistic
	1E−5M Met + 1E−6M MPA	0.62608	0.20448	Strongly synergistic
HEC-1B	1E−6M Met + 1E−7M MPA	0.36048	0.69778	Synergistic
	1E−6 MMet + 1E−6M MPA	0.50980	0.47193	Synergistic
	1E−5M Met + 1E−7M MPA	0.54990	0.27757	Strongly synergistic
	1E−5M Met + 1E−6M MPA	0.65192	0.12801	Strongly synergistic

Note: Met indicates metformin, FA denotes the growth effect of drug-treated cells compared with control cells and CI denotes the combination index.

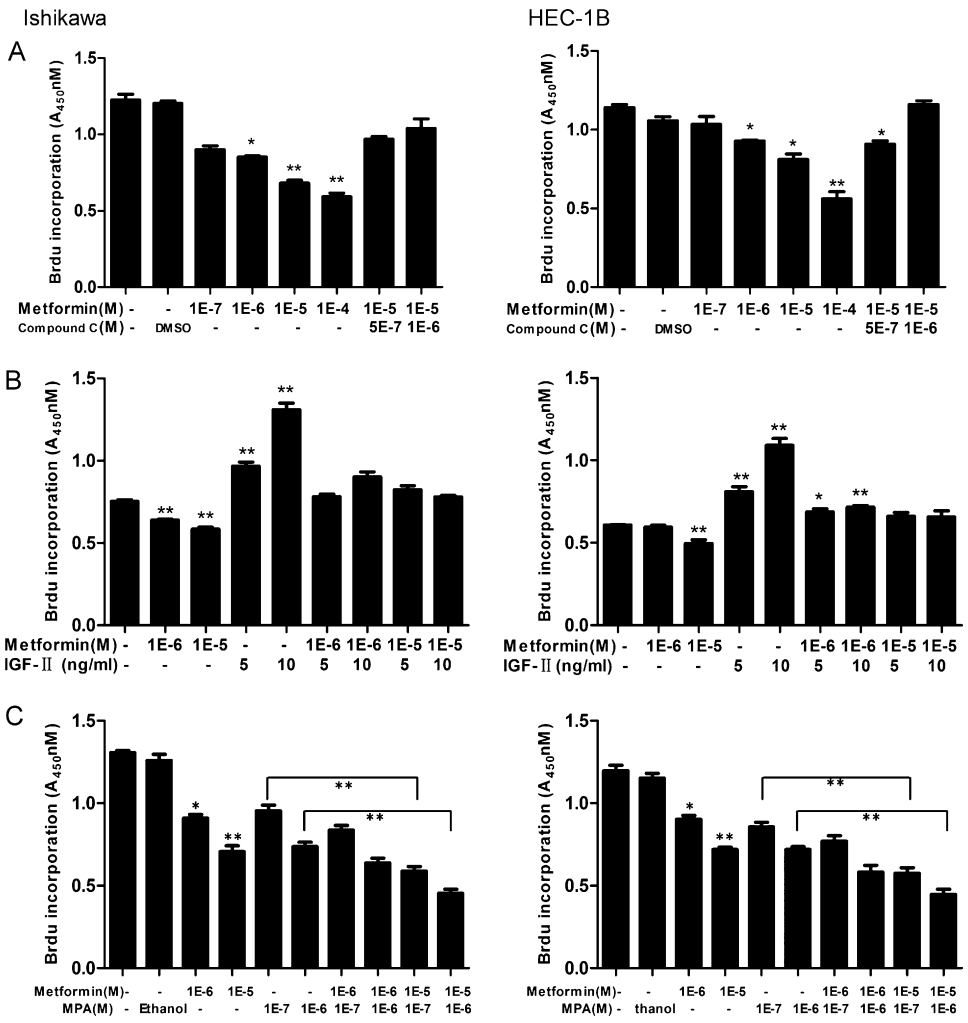

Fig. 5. Effect of metformin, IGF-II and MPA on cell proliferation of Ishikawa (left) and HEC-1B (right) EC cells under serum-free conditions. The results are shown as the mean ± S.E. of triplicate samples and represent three independent experiments. (*$p < 0.05$, **$p < 0.01$, one-way ANOVA). (A) Metformin inhibits Ishikawa and HEC-1B cell proliferation, and the inhibition can be partially rescued by Compound C. Ishikawa and HEC-1B cells were treated in the presence of varying concentrations of metformin with or without Compound C for 72 h, and then cell growth was measured by BrdU. (B) IGF-II promotes Ishikawa and HEC-1B cells proliferation but the promotion can be partially attenuated by metformin. Ishikawa and HEC-1B cells were treated with different concentrations of IGF-II, with or without metformin for 48 h and cell proliferation measured by BrdU. (C) Both metformin and MPA inhibit cell proliferation of Ishikawa and HEC-1B cells, and metformin coincubation with MPA shows increased cell proliferation inhibition as compared to each agent alone. Ishikawa and HEC-1B cells were treated with metformin with and without MPA for 72 h, and then cell proliferation measured by BrdU.

the PI3K/AKT/mTOR pathway (Fig. 3); therefore we suggest that IGF-II may down-regulate PR mRNA and protein levels via the mTOR pathway. IGF-II expression is up-regulated in EC, contributing to the reduction of PR levels and activity during EC progression.

4.2. AMPK is a possible therapeutic target in EC

Metformin's immediate downstream target is AMPK; AMPK activation triggers the regulation of multiple downstream pathways, including the mTOR. AMPK mediates its effect on cell growth through inhibition of mTOR, via phosphorylation of the tuberous sclerosiscomplex (TSC2), a subunit of the larger TSC1/TSC2 (hamartin/tuberin) complex that negatively regulates mTOR signaling. This in turn affects cellular transcription and translation via downstream molecular effectors 4E-BP1 and p70S6K [25–27]. Our results confirm that metformin attenuates IGF-II promotion of cell proliferation (Fig. 5B) and metformin's inhibition of cell proliferation may result from AMPK activation, which attenuates mTOR overactivation (caused by IGF-II and IGF-IR overexpression in EC). Because AMPK inhibits mTOR signaling downstream of AKT, AMPK activation is thought to be a possible therapeutic target for cancers with activated AKT signaling. Metformin-induced AMPK activation may be a particularly compelling anti-cancer strategy for EC, given the high prevalence of PTEN mutations in EC which lead to constitutive AKT expression.

On the other hand, PI3K signaling through AKT can modulate glucose transporter expression, stimulating glucose capture by hexokinase and increasing phosphofructokinase activity [28,29]. The PI3K/AKT/mTOR pathway plays a critical role in both growth control and glucose metabolism. Thus, the regulation of glucose uptake and utilization by EC cells may also be responsible for the anti-tumorigenic benefit of metformin. Future studies are needed to elucidate the relationship between metformin cell signaling and glucose metabolism.

4.3. Metformin promotes PR expression through mTOR pathway inhibition: MPA combined with metformin may be a novel treatment strategy in EC

Our data suggest that metformin promotes PR mRNA and protein expression in EC cell lines, and are partly reversed by AMPK inhibitor Compound C, implying that metformin promotes PR

expression via AMPK pathway activation. A possible explanation is that IGF-I and IGF-II decrease PR expression via the PI3K/AKT/mTOR pathway. Because PR receptor plays an important anti-proliferative effect and IGF-II suppresses PR expression, IGF-II attenuates progesterone's antitumor function in EC cells. However, metformin can reverse this effect by up-regulating PRs (Fig. 4). Furthermore, we verified that metformin combined with MPA increases the inhibitory effect on EC cell proliferation compared with either agent alone and that metformin has a synergistic effect with MPA on antiproliferation in EC (Table 1).

In clinical pharmacokinetics, therapeutic levels in healthy volunteers ranged from 0.5 to 2.0 mg/l (peak plasma levels 0.6–1.8 μg/ml), and other investigators calculated 20 μM as a clinically equivalent dose *in vitro* [30,31]. Although metformin has been previously shown to inhibit cell proliferation *in vitro* in epithelial cancer, the doses used were supertherapeutic compared to those used in diabetics [32]. Cells were cultured in high concentrations of glucose and the interference from serum has not been eliminated in cell proliferation assays, which would be the reason for the effective dose *in vitro* higher than clinical equivalent dose. However, these interferences have been avoided in our study. The IC_{50} value of metformin was approximately 20 μM (Table 2) and the effective doses of metformin in combination treatment (metformin combined with MPA) were less than 20 μM, which corresponds to the clinically equivalent dose.

5. Conclusion

Our results demonstrate that metformin promotes PR expression via inhibition of the mammalian target of rapamycin (mTOR) pathway in EC cells and identified that metformin had a synergistic antitumor effect with MPA in EC. Because PR expression is important in EC prevention and in successful progesterone therapy, the combination of metformin and MPA may be a potentially effective way to control EC, especially in PR poor cancers. We hope that our data will provide a scientific foundation for future studies using the combination of metformin and MPA in EC therapy. Further evaluation of combination therapy in preclinical experiments is one of our future research goals. Since obesity and diabetes have been linked to an increased mortality risk from EC, and metformin with progesterone has been shown to have synergistic antitumor potential, metformin combined with MPA may emerge as an effective therapy choice in the treatment and long-term management of EC.

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