Metformin regulation of progesterone receptor isoform-B expression in human endometrial cancer cells is glucose-dependent

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Abstract. Metformin (MET) constitutes the first-line treatment against type 2 diabetes. Growing evidence linking insulin resistance and cancer risk has expanded the therapeutic potential of MET to several cancer types. However, the oncostatic mechanisms of MET are not well understood. MET has been shown to promote the expression of progesterone receptor (PGR) and other antitumor biomarkers in patients with non-diabetic endometrial cancer (EC) and in Ishikawa EC cells cultured in normal glucose (5.5 mM) media. Therefore, the present study aimed to assess the effects of MET on EC cells under conditions simulating diabetes. Ishikawa cells treated with 10 nM 17β-estradiol (E2) and/or 100 µM MET and exposed to normal and high (17.5 mM) concentrations of glucose were evaluated for proliferative and PGR expression status. Under normal glucose conditions, MET attenuated E2-induced cell proliferation and cyclin D1 gene expression, and increased total PGR and PGR-B transcript levels. MET inhibited Ishikawa cell spheroid formation only in the absence of E2 treatment. In E2-treated cells under high glucose conditions, MET showed no effects on cell proliferation and spheroid formation, and increased total PGR but not PGR-B transcript levels. Transfection with Krüppel-like factor 9 small interfering RNA increased PGR-A transcript levels, irrespective of glucose environment. Medroxyprogesterone acetate downregulated PGR-A expression more effectively with metformin under high compared with normal glucose conditions. To evaluate the potential mechanisms underlying the targeting of PGR by MET, E2-treated cells were incubated with MET and the AMPK inhibitor Compound C, or with the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), under normal glucose conditions. Compound C abrogated the effects of MET on PGR-B while AICAR increased PGR-B transcript levels, albeit less effectively compared with MET. The present results demonstrate the glucose-dependent effects of MET on PGR-B isoform expression, which may inform the response to progestin therapy in diabetic women with EC.

Introduction

Endometrial cancer (EC) is the sixth most commonly occurring cancer in women, with an incidence rate rising to 3.5% of all new cancer cases and representing 2% of all cancer deaths in the USA (1). While the 5-year survival rate for patients with EC in economically advantaged countries is high (~80%) compared with those for patients with other female-associated types of cancer, such as breast and ovarian cancer, the prognosis for women with EC is dismal in other parts of the world where treatment options are limited. The metabolic disorders type 2 diabetes and obesity are increasingly linked to an increased risk of cancer, including EC (2-4). The estimated type 2 diabetes incidence rate for 2019 is 9.3% globally (5). Furthermore, 35-40% of adults are obese in the USA and the worldwide prevalence of obesity has tripled to 13% over a timespan of 40 years (6). The staggering financial and personal burdens of cancer underscore the need for the development of novel therapeutic and preventative strategies that are clinically effective and financially deliverable to the general population.

Metformin (1,1-dimethylbiguanide hydrochloride; MET) constitutes the first-line treatment for type 2 diabetes. Previous studies have highlighted the therapeutic value of MET as an adjuvant treatment in breast cancer (7). EC is pathologically akin to breast cancer since it is steroid hormone-driven, tightly linked to abnormal glucose metabolism and insulin signaling, and exhibits a complex, multi-factorial origin. In breast cancer cells, MET has been shown to inhibit cell proliferation, invasion and inflammation, promote apoptosis and exert a cytotoxic effect on cancer stem cells (8,9). Nevertheless, the anti-breast cancer benefits of MET have been inconsistent and appear to be dependent on context and the duration of exposure. In triple
negative breast cancer cell lines for example, glucose levels in the diabetic range diminished the effects of MET on cell proliferation, cell death and cell cycle arrest (10). Conversely, glucose starvation enhanced the inhibitory effect of MET on the mTOR pathway, a downstream target of AMP-activated protein kinase (AMPK) and an activator of growth factor signaling in breast cancer cells (11). Moreover, breast cancer cells chronically exposed to MET acquired resistance to the drug, which was accompanied by changes in the expression of key genes and resulted in the generation of a metastatic stem-cell like phenotype (12).

In a previous study, it was shown that the short-term treatment of non-diabetic and obese women with MET during the pre-surgical window between diagnosis and hysterectomy promoted the expression of antitumor biomarkers; this effect was recapitulated in Ishikawa human endometrial cancer cells treated with a physiological dose of MET under normal glucose conditions (13). While these results support the ability of MET to reduce EC risk and development, meta-analyses of current clinical data indicate conflicting MET outcomes, with some showing no effect (14) and others showing inhibitory effects (15). In light of the findings for breast cancer, a strong rationale exists for clarifying the ambiguities regarding the context and dosage at which MET exerts its optimal therapeutic effects in EC to achieve its potential for clinical use.

In humans treated with a single 500-ng dose of MET, the blood concentration of MET 2.4 h post-oral intake was 4 µM, as measured by high-pressure liquid chromatography (16). In mice, the oral administration of MET at 50 mg/kg body weight resulted in a MET concentration of 10-70 µM in the portal vein and 10-40 µM in the plasma, as measured using 14C-radiolabeled MET (17). Thus, studies that use mM concentrations of MET may not be physiologically relevant. In the present study, estrogen-treated Ishikawa EC cells were characterized for their response to 100 µM MET and glucose concentrations typifying the non-diabetic and diabetic ranges, using progesterone receptor (PGR) gene expression as the primary outcome. The present findings demonstrate the glucose-dependency of the MET-mediated effects on PGR isoform B (PGR-B) expression, which may be relevant when assessing the response to progestin-based therapy of diabetic and non-diabetic patients with EC and other gynecological pathologies.

Materials and methods

Cell culture and treatments. The Ishikawa human endometrial epithelial carcinoma cell line (a gift from Dr Bruce Lessey, Greenville Health System; Prisma Health) was authenticated and propagated as previously described (13). Cells were initially grown in Minimal Essential Media (MEM; Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and containing phenol red and 1% antibiotic-antimycotic solution (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator (5% CO2/95% air) at 37°C. In subsequent experiments incorporating various treatments, cells were incubated in either phenol red-free MEM or phenol red-free Dulbecco’s modified Eagle’s medium (DMEM)/Nutrient Mixture F-12 (DMEM/F-12), each supplemented with charcoal-stripped 10% FBS and 1% antibiotic-antimycotic solution and referred to hereinafter as MEM-FBS and DMEM-FBS, respectively. MEM which contains 5.5 mM glucose represents the normal glucose environment while DMEM with 17.5 mM glucose, typifies a high glucose environment.

MET (Sigma-Aldrich; Merck KGaA) was dissolved in phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc.) and initially evaluated at final concentrations of 10 or 100 µM, which approximate the physiological range found in patients treated with MET for type 2 diabetes (18). 17β-estradiol (E2; Sigma-Aldrich; Merck KGaA) was dissolved in dimethyl sulfoxide (DMSO), further diluted in PBS and used at final concentrations of 0.1 or 10 nM. Medroxyprogesterone acetate (MPA; Sigma-Aldrich; Merck KGaA) was dissolved in DMSO, further diluted in PBS and used at a final concentration of 1 µM. The AMPK activator, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; Sigma-Aldrich; Merck KGaA) was dissolved in PBS and used at a final concentration of 500 µM. Compound C (dorsomorphin; Sigma-Aldrich; Merck KGaA), an AMPK inhibitor, was dissolved in DMSO, further diluted in PBS and used at a final concentration of 5 µM. In all treatment protocols where DMSO was added to solubilize E2, MPA and compound C, the same amount of DMSO was added to the control treatment. The various treatment strategies are summarized in Fig. 1.

Cell proliferation assay. Cells were seeded onto 96-well culture dishes at a density of 1x10⁴ cells/well in MEM-FBS or DMEM-FBS. After 24 h, cells were pre-treated with E2 (0.1 or 10 nM) or vehicle control (DMSO-PBS) and incubated for another 24 h at 37°C. E2 was removed with a change of media and cells were then treated with MET (10 or 100 µM) or vehicle control (PBS) twice at 24-h intervals at 37°C. Cells were collected 24 h after the final MET treatment. Cell viability was evaluated by the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) metabolic assay according to the manufacturer’s instructions. Absorbance at 450 nm was assessed using a CLARIOstar plate reader (BMG Labtech GmbH) and the mean optical density from n=4 wells/treatment group was calculated. The cells treated with and without E2 (10 nM) and in the presence or absence of MET (100 µM) were collected and their cyclin D1 (CCND1) transcript levels were measured by reverse transcription-quantitative (q)PCR.

Spheroid-formation assay. Ishikawa cells were plated in 24-well ultra-low attachment plates (Corning Inc.) at a seeding density of 8x10⁴ cells/well. The plating medium consisted of phenol red-free MEM or phenol red-free DMEM-F12 and was supplemented with B27 (Invitrogen; Thermo Fisher Scientific, Inc.), human basic fibroblast growth factor (20 ng/ml; Invitrogen; Thermo Fisher Scientific, Inc.), human epidermal growth factor (20 ng/ml; Invitrogen; Thermo Fisher Scientific, Inc.), heparin (10 µg/ml; Sigma-Aldrich; Merck KGaA), antibiotic-antimycotic solution (1% v/v) and gentamicin (100 µg/ml; Sigma-Aldrich; Merck KGaA). Cells were treated with PBS alone or with MET (100 µM) in the presence or absence of E2 (10 nM) at seeding and incubated in a humidified incubator (5% CO2/95% air) at 37°C. Culture media, as appropriate for each treatment group, were replenished with 62.5 µl medium representing 12.5% of the initial plating volume, on incuba-
On day 3. On day 5, non-adherent spheroids, designated as endospheres, with diameters >60 µm were counted manually using an Olympus IMT-2 inverted microscope (Olympus Corporation). In order to assess the self-renewal capacity of the primary endospheres, non-adherent spheroids from the first plating (passage 1, P1) were collected, dissociated into single-cell suspensions with 0.05% trypsin (Invitrogen; Thermo Fisher Scientific, Inc.), filtered using a 40-µm sieve, and re-plated in MEM or DMEM-F12 supplemented as described for the plating medium, in ultra-low attachment plates with no further E2 or MET treatments. Endospheres with diameters >60 µm, designated as passage 2 (P2), were counted after incubation for 5 days, with addition of fresh media (MEM or DMEM) on day 3.

RNA isolation and gene expression assay. Ishikawa cells were plated in 6-well plates (Falcon) at a density of 1.5x10^5 cells/well in MEM-FBS or DMEM-FBS for 24 h and treated with various reagents, following the timeline summarized in Fig. 1. For all RNA analyses, cells were collected 24 h after the final MET treatment. RNA isolation, preparation of cDNAs and qPCR analyses were performed as previously described (19), using the geometric mean of β-actin and TATA-binding protein mRNAs as normalization controls. Primers (Table I), designed to span introns, were synthesized by Integrated DNA Technologies, Inc. qPCR was conducted using the CFX96™ Real-Time PCR System (Bio-Rad Laboratories, Inc.) under the following thermocycling conditions: 30 sec at 95˚C, followed by 39 cycles of 5 sec at 95˚C and 30 sec at 60˚C, and a melt curve protocol of 5 sec at 65˚C with a gradual increase in temperature to 95˚C by 0.5˚C increments. Normalized mRNA expression was calculated with CFX Manager Software version 3.1.1517.0823 (Bio-Rad Laboratories, Inc.) using the 2^(-ΔΔCq) method (20).
described protocols (21). Briefly, cells were seeded in 6-well plates at a density of 1.5x10^5 cells/well in MEM-FBS or DMEM-FBS without added antibiotic for 24 h. Cells were then transfected with Lipofectamine® 2000/OPTI-MEM I (Invitrogen; Thermo Fisher Scientific, Inc.) containing 50 µM siKLF9 RNA pools (cat. no. L-011223-00-0005) or non-targeting (scrambled control, cat. no. D-001810-01-05) siRNAs (GE Healthcare Dharmacon, Inc.). After 6 h of transfection, cells were incubated for 24 h in MEM-FBS or DMEM-FBS containing 1% antibiotic-antimycotic solution, and then treated with E2 (10 nM) for 24 h, followed by MET (100 µM) twice at 24-h intervals at 37°C. RNA isolation and gene expression analysis of cells collected 24 h after the last MET treatment were performed by the aforementioned method.

Statistical analysis. Data are presented as the mean ± SEM and were analyzed for statistical differences between two groups using Student's t-test, and among three or more groups using two-way analysis of variance followed by Tukey's post hoc test. Analyses were performed using SigmaStat (version 3.5; Systat Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

MET inhibits proliferation of Ishikawa cells in normal but not high glucose conditions. Previous studies showed that MET decreased the viability of EC cells exposed to E2 (22,23); however, those studies used supra-physiological MET concentrations of >5 mM, which do not reflect the systemic µM concentrations detected in patients receiving MET (18). In order to evaluate whether physiologically relevant concentrations of MET act directly on E2-responsive EC cells to influence their viability, the well-differentiated Ishikawa endometrial carcinoma cell line was treated with E2 and exposed to MET in normal and high glucose media. Without MET, 10 nM E2-treated cells under normal glucose conditions exhibited increased cell viability (Fig. 2A); this was decreased to basal levels when 10 or 100 µM MET was added. By contrast, the viability of E2-treated cells was not influenced by MET in the high glucose medium (Fig. 2B). Notably, treatment with 10 µM MET in the absence of E2 increased cell viability in the presence of a high concentration of glucose. Gene expression analyses for the proliferation marker cyclin D1 showed that E2 induced CCND1 transcript levels in the absence of MET and that MET reduced these E2-induced effects under normal glucose conditions. Similar effects of MET were not observed in high glucose conditions (Fig. 2C).

MET inhibits Ishikawa sphere formation in normal but not high glucose conditions. Spheres formed from Ishikawa cells grown under non-adherent conditions are considered to constitute EC stem-like cells, based on their characterized genotype and phenotype (24,25). In other types of tumor, MET has been shown to selectively target cancer stem cells by decreasing sphere formation in vitro and tumor formation in vivo (26). To assess whether MET influences the formation of endometrial spheres under normal or high glucose conditions, Ishikawa cells that normally grow as monolayers in plastic culture dishes (Fig. 3A, top panel) were plated in low-attachment culture dishes to enable the formation of endospheres (Fig. 3A, bottom panel) and treated with 100 µM MET in the presence or absence of 10 nM E2. Without E2, MET significantly inhibited the growth of primary endospheres (P1; >60 µm diameter) under normal glucose conditions; however, this effect of MET was lost with the addition of E2 (Fig. 3B). By contrast, MET with and without E2, showed no inhibitory effect on basal P1-endosphere formation under high glucose conditions (Fig. 3C). P1-endospheres were collected and re-plated in low
attachment plates in normal or high glucose conditions without further MET or E2 treatments. P2-endospheres exhibited increased formation efficiency (10-12 vs. 2-3% for P1) when normalized to initial seeding density, indicating self-renewal (Fig. 3D and E). Moreover, P2-endospheres grown in normal and high glucose conditions exhibited responses to MET without and with added E2 that were comparable with those observed for the respective P1-endospheres.

**MET-induced changes in total PGR and PGR-B transcript levels are dependent on glucose.** We previously showed that MET inhibited the expression of the antitumor biomarker PGR in EC cells in vivo (protein) and in vitro (transcript) (13). Transcript levels of total PGR and its antiproliferative isoform PGR-B were evaluated in Ishikawa cells in response to 100 µM MET, following a 24-h pre-treatment with E2. MET significantly increased total PGR transcript levels in normal and high glucose conditions by 5- and 15-fold, respectively, relative to the corresponding non-MET treated levels (Fig. 4A). Interestingly, while MET increased the levels of PGR-B transcripts by 25-fold in cells grown in normal glucose + E2, no comparable effects on PGR-B were elicited by MET in cells incubated under high glucose + E2 (Fig. 4B). Given that total PGR transcript levels are the sum of those for PGR-A + PGR-B isoforms, the relative changes in PGR-A and PGR-B expression under normal glucose + E2 and high glucose + E2 conditions were determined from the PGR-B/PGR transcript expression ratios. Under normal glucose conditions, the increase in PGR expression with MET + E2 was due to increased PGR-B transcript levels (Fig. 4C, left panel). Under high glucose conditions, MET decreased the ratio of PGR-B/PGR transcripts in the presence or absence of E2, suggesting that levels of PGR-A transcripts are increased by treatment with MET under high glucose conditions (Fig. 4C, right panel).

**Reduced KLF9 expression promotes PGR-A transcription in E2/MET-treated cells independent of glucose environment.** Our previous study showed that human endometrial tumors display lower levels of the tumor suppressor protein KLF9 compared with those in the adjacent non-tumor endometrial tissue (21). These findings suggest that loss of KLF9 expression is a feature of EC cells and that the antiproliferative effects of MET in the context of normal glucose (Fig. 2A and C) may be partly due to the induction of KLF9 expression. To test the latter hypothesis, KLF9 transcript levels and those of its family member Krüppel-like factor 4 (KLF4) were measured in E2/MET-treated cells. Under normal glucose conditions, MET or E2 alone had no effect on KLF9 transcript levels (Fig. 5A). With MET-treatment, KLF9 mRNA levels were modestly decreased by E2, which contrasted with the increase noted under high glucose conditions (Fig. 5A). KLF4 mRNA levels were unresponsive to MET, alone or with E2, in normal and high glucose conditions (Fig. 5B).

We previously reported that in E2-treated Ishikawa cells, KLF9 siRNA increased total PGR transcript levels (27). In the context of E2/MET treatments (Fig. 1), the transfection of Ishikawa cells with KLF9 siRNA promoted total PGR expression in both normal and high glucose culture conditions (Fig. 6). Parallel changes in PGR-B transcript levels were not observed when KLF9 expression was reduced, indicating increased levels of PGR-A transcripts.

**MPA-mediated downregulation of PGR expression is greater with MET treatment under high compared with normal glucose conditions.** Treatment with progestins can often lead to resistance over time due to progestin-mediated downregulation and/or desensitization of its receptor (28,29). To determine if MET alters the progestin sensitivity of EC cells by inhibiting PGR downregulation, cells were treated...
with MPA after exposure to E2/MET in normal and high glucose environments. KLF9 mRNA levels were not altered by MPA co-treatment under normal or high glucose conditions. Total PGR mRNA levels were significantly decreased by MPA under high glucose but not normal glucose conditions (Fig. 7). Notably, no corresponding changes in PGR-B transcript levels were noted under either glucose condition. These results suggest that MET promotes the MPA-induced downregulation of PGR-A mRNA under high glucose conditions.

**MET effects on PGR gene expression are partly AMPK-dependent.** In EC cells, supra-physiological doses of MET have been shown to activate AMPK, leading to inhibition of the mTOR signaling pathway and resulting in decreased cellular protein synthesis and proliferation (30-32). To determine whether the effects of physiologically relevant doses of MET on PGR signaling under normal glucose conditions are mediated by the AMPK pathway, PGR expression levels in the presence of the AMPK inhibitor Compound C (33) were evaluated. Consistent with the aforementioned data (Fig. 4A and B), the MET-induced increase in transcript levels for total PGR, while significant, was less robust than that for PGR-B (~5-fold vs. ~12-fold). Moreover, while there was a marked reduction in PGR-B expression with Compound C, no significant reduction was found in total PGR expression (Fig. 8A). When cells were treated with AICAR, an AMPK-activator (34), instead...
of MET, transcript levels of PGR-B but not total PGR were increased (Fig. 8B), albeit the fold increase (~4-fold) was lower compared with that achieved with MET (~25-fold; Fig. 4B). However, AICAR demonstrated a broader range of gene targets with increased levels of estrogen receptor 2 (ESR2), KLF9 and KLF4 (Fig. 8B), which were not significantly affected by MET (Fig. 5 for KLF9 and KLF4; ESR2, data not shown) and by the inhibition of MET-mediated AMPK signaling (Fig. 8A).

Discussion

MET as an adjuvant in cancer treatments is increasingly gaining support, based on population studies demonstrating a reduction in cancer incidence and mortality in patients with type 2 diabetes taking MET (7). Nevertheless, the benefit of MET in the management of EC remains largely unknown, given limited clinical studies to date and the lack of an established causal relationship between physiologically relevant MET exposure and the EC genotype and phenotype in patients within normal and diabetic glucose ranges. The present study compared the proliferation, stem cell-like growth/self-renewal and expression of selected genes in the well-differentiated Ishikawa EC cell line treated with E2 and exposed to physiologically-relevant MET doses (µM) in the presence of normal and high concentrations of glucose. The results showed that MET reduced EC cell numbers and spheroid formation under normal glucose conditions, and that these MET-elicited changes were lost when glucose levels...
transcript levels in EC cells is partly
isoform transcript levels. Given acetate; siKLF9, siRNA targeting Krüppel-like factor 9.
expression by MET is mediated by AMP-activated protein kinase and other yet unknown pathways (5). Lines with an arrowhead and with a blocked bar represent pathway promotion and inhibition, respectively. MET, metformin; E2, 17β-estradiol; PGR, progesterone receptor; MPA, MPA, medroxyprogesterone acetate; siKLF9, siRNA targeting Krüppel-like factor 9.

were elevated. The reduction in CNND1 transcript levels and the inhibition of stem cell-like activity with MET in normal but not high glucose conditions, respectively indicate that MET has inhibitory effects on EC cell cycle progression and metastatic potential (25,35), which predict better outcomes with MET in non-diabetic patients with EC compared with their diabetic counterparts. Moreover, it was found that while MET increased total PGR transcript levels, irrespective of the level of glucose exposure, MET preferentially induced PGR-B transcript levels under normal glucose conditions, and conversely, those of PGR-A under high glucose conditions. These findings suggest that in non-diabetic compared with diabetic patients with EC, MET may elicit distinct effects on progestin signaling, which differ according to their mediation by PGR-B and PGR-A isoforms. A role for MET in progestin signaling is supported by a previous study demonstrating that the PI3K/AKT/mTOR pathway promotes progestin resistance in EC cells (22). Furthermore, the present study showed that the ability of MET to enhance progestin signaling via the induction of PGR-B transcript levels in EC cells is partly but not entirely dependent on AMPK activation. The present results indicate that the direct effects of MET on EC are cellular context-dependent, and suggest that the potential use of MET as an adjuvant in EC management involving progestin therapy may involve its induction of PGR-B expression via AMPK and other yet unknown signaling pathways.

The present study modeled the effects of MET in non-diabetic and diabetic patients with EC using MEM (normal glucose) and DMEM (high glucose), respectively. While MEM and DMEM have differences in several constituents, including their levels of vitamins and amino acids, the major difference is their respective glucose concentrations, which we hypothesize to have more consequential effects on cellular metabolic status. In a previous study to evaluate the effects of MET in EC cells, MEM and DMEM were similarly used to mimic normal and hyper-insulinemic conditions (36). However, the present results are likely to be more physiologically relevant, since the MET doses used were in the µM range rather than the supra-physiological (mM) doses used in earlier studies (22,23). The present findings align with those reported in triple-negative breast cancer cell lines wherein the antiproliferative effects of MET were enhanced and inhibited in glucose-starved and glucose-excess conditions, respectively (10). The present study also found that the formation of EC cell spheroids, referred to as endospheres and considered to display cancer stem cell-like activity and metastatic potential (25,35), was differentially responsive to MET, dependent on cellular glucose. MET alone was shown to inhibit endosphere growth (P1) and regeneration (P2) under normal glucose conditions. By contrast, under high glucose conditions, Ishikawa spheroid formation was unresponsive to MET, paralleling the lack of MET-elicted antiproliferative response for these cells. MET-induced inhibition of spheroid growth in the presence of a normal glucose concentration has been similarly demonstrated in breast, ovarian and colon cancer cell lines (37). Currently, there is no explanation for the E2-induced abrogation of the effects of MET on the formation of spheroids under normal glucose conditions, given that endometrial epithelial stem/progenitor cells are reported to lack estrogen receptor expression (38).

The in vivo relevance of PGR signaling as a MET target is supported by our previous study showing the induction of total PGR protein levels in the endometrial tumors of non-diabetic patients with EC following short-term pre-surgical MET treatment (13). The present study reports the novel finding that changes in glucose conditions modify the effects of MET on PGR signaling. While MET increased total PGR transcript levels, irrespective of the glucose environment, MET under normal glucose conditions preferentially induced PGR-B isoform transcript levels, whereas under high glucose conditions it increased PGR-A isoform transcript levels. Given the pro-differentiation activity of PGR-B and the function of PGR-A as a repressor of PGR-B transcriptional activity (39), changes in their expression ratios can significantly impact...

Figure 9. Schematic diagram summarizing the proposed effects of MET on endometrial carcinoma cells under normal and high glucose conditions. Pathways (1) and (2) are influenced by MET under normal but not high glucose conditions. The MET-induced increase of PGR-B transcript levels is lost in Ishikawa cells under normal or high glucose conditions when KLF9 expression is reduced via pathway (3); instead, induction of PGR-A transcript levels is observed. Pathway (4) depicts MET-mediated inhibition of PGR-A expression in response to MPA administration under high glucose. Induction of PGR-B expression by MET is mediated by AMP-activated protein kinase and other yet unknown pathways (5). Lines with an arrowhead and with a blocked bar represent pathway promotion and inhibition, respectively. MET, metformin; E2, 17β-estradiol; PGR, progesterone receptor; MPA, MPA, medroxyprogesterone acetate; siKLF9, siRNA targeting Krüppel-like factor 9.
EC phenotype and gene signaling networks. Thus, the loss of inhibitory effects of MET on cell proliferation and endosphere formation in the presence of high concentrations of glucose may reflect the less-differentiated state of EC cells as a consequence of predominant PGR-A isoform activity. Another study also reported the upregulation of PGR-B mRNA and protein levels by MET, albeit at supra-physiological doses, in Ishikawa and HEC-1A cells under normal glucose conditions (40). Given that MET-responsive Ishikawa cells express PGR, we postulate that this mechanism of MET will not be applicable to triple-negative breast cancer cells, further affirming the pleiotropic context-dependent signaling of MET in distinct cancer types. While the PGR-A and PGR-B protein levels corresponding to changes in the PGR-B and PGR-A isoform transcript levels were not quantified in the present study, previous studies (27,41,42) have demonstrated that changes in the respective PGR transcript levels are closely recapitulated in their protein levels and transcriptional activity.

The influence of the glucose environment on the ability of MET to modify EC cell response, based on two parameters related to PGR signaling, namely KLF9 expression and response to MPA administration, was evaluated. Our previous study showed that KLF9 is a PGR-B interacting protein (43,44) and that human EC tumors demonstrate loss of KLF9 expression (21). The present study found that the transfection of Ishikawa cells with siKLF9, under conditions resulting in the reduction of KLF9 protein levels (13), caused an increase in total PGR transcript levels without a corresponding change in PGR-B transcript levels, under normal and high glucose conditions. The indicated increase in PGR-A isoform transcript levels with reduced KLF9 expression suggests that in more advanced tumors, in which KLF9 expression is already significantly attenuated, MET may not positively affect EC outcome, irrespective of the glucose environment. In the context of MPA administration and a high glucose environment, MET appeared to amplify the reduction of PGR-A isoform levels, given the lack of change in PGR-B isoform transcript levels. Whether the increase in PGR-B relative to PGR-A expression with MPA/MET treatments is functionally consequential to EC remains to be determined. However, in a previous study the treatment of Ishikawa cells expressing only PGR-B with progesterin resulted in complete growth inhibition, while those expressing only PGR-A showed only 50% growth inhibition (45). Thus, by favoring the expression of PGR-B over PGR-A under high glucose conditions, MET may synergize with MPA to increase progesterin-sensitivity and thereby favor cell differentiation at the expense of cell proliferation, with significant relevance to progesterin therapy for patients with EC.

Using two pharmacological agents, namely Compound C and AICAR, to inhibit and stimulate AMPK-signaling, respectively, a substantial reduction (~90%) of PGR-B expression with Compound C and more modest induction of PGR-B transcript levels with AICAR were demonstrated. Indeed, the induction of PGR-B transcript levels by AICAR (~5-fold) is ~2-fold lower in magnitude than that by MET (12-15-fold), suggesting that MET may utilize other pathways to promote PGR-B transcript levels. In this regard, Compound C has been reported to inhibit the activities of other kinases, in addition to AMPK, suggesting a role for MET in the regulation of multiple kinase activities in target cells. The ability of AICAR to increase ESR2, KLF9 and KLF4 transcript levels in the absence of similar effects by MET under the same experimental conditions (Fig. 5; data not shown for ESR2) may be attributable to pathways other than AMPK that have been implicated in AICAR signaling (46,47). Taken together, the present results are suggestive of AMPK-dependent and -independent actions of MET on EC cells, as has been shown for other systems such as T cells, retinal epithelial cells and mouse embryonic fibroblasts (46,48), which may contribute to the preferential inductive effect of MET on PGR-B transcript levels.

In conclusion, the present study underscores the context-dependent effects of MET in its selective targeting of PGR-B isoform transcript levels in EC cells (Fig. 9). Given the current global pandemic of diabetes and the increasing focus on MET as a viable adjuvant for cancer management, it is essential to understand the context underlying the favorable effects of MET on EC. The present data indicate that the PGR signaling pathway may constitute an important and novel MET target to increase the progesterin sensitivity of EC cells. This may provide an opportunity for the early prevention of EC and other progesterin-dependent gynecological pathologies in women with increased risks.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

RCMS and MCV supervised the study; SJUS, RCMS and MCV conceived and designed the experiments; SJUS performed the experiments; SJUS and IA analyzed the data; SJUS, RCMS and MCV wrote the manuscript; SJUS, IA, RCMS and MCV revised the manuscript. All authors read and approved the final manuscript.
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