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The anti-diabetic drug metformin inhibits vascular endothelial growth factor expression via the mammalian target of rapamycin complex 1/hypoxia-inducible factor-1α signaling pathway in ELT-3 cells

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ABSTRACT

The aim of this study was to elucidate whether metformin can regulate the expression of vascular endothelial growth factor (VEGF) in rat-derived uterine leiomyoma cells (ELT-3 cells). In vitro studies were conducted using ELT-3 cells. Under normoxic conditions, metformin suppressed VEGF protein levels in the supernatant and cells in a dose-dependent manner. In hypoxia-mimicking conditions, VEGF and hypoxia-inducible factor-1α (HIF-1α) proteins were both highly expressed and were suppressed by the metformin treatment. Metformin did not affect HIF-1α mRNA levels, which indicated that its effects occurred at the post-translational level. Metformin inhibited mammalian target of rapamycin complex 1 (mTORC1) activity by phosphorylating the mTORC1 component raptor. This study revealed the anti-angiogenic activity of metformin in ELT-3 cells by suppressing the expression of VEGF via the mTORC1/HIF-1α pathway. These results indicate that metformin may represent an effective alternative in the future treatment of uterine leiomyomas.

1. Introduction

Uterine leiomyomas or fibroids are the most common gynecological benign myometrial tumors in women of reproductive age. These tumors cause health concerns, such as profuse menstrual bleeding and pelvic discomfort, and are the primary indication for hysterectomy (Walker and Stewart, 2005). Moreover, leiomyomas have been linked to 10% of infertility cases (Kolankaya and Arici, 2006) and negatively impact assisted reproductive technology and perinatal outcomes (Levy et al., 2012).

Angiogenesis is the physiological process that involves in the growth of new blood vessels from pre-existing vessels, and is essential for the growth of tumors. The precise mechanisms underlying leiomyoma angiogenesis remain unclear; however, previous studies demonstrated that disordered angiogenesis and the dysregulation of angiogenic promoting growth factors were key features in the pathophysiology of leiomyomas (Fleischer et al., 2008; Tal and Segars, 2014). Many factors, including growth factors and hormones, are involved in tumor angiogenesis. Of these, vascular endothelial growth factor (VEGF), a secreted protein that acts as a potent mitogen for vascular endothelial cells, is one of the main regulatory factors (Ferrara, 2004). VEGF levels were previously shown to be higher in leiomyoma tissue than in the adjacent myometrium (Gentry et al., 2001; Hague et al., 2000), and the current medications for leiomyomas, such as gonadotrophin-releasing hormone agonists and selective progesterone receptor modulators, have displayed suppressive effects on the expression of VEGF (Di Lieto et al., 2005; Xu et al., 2006). Therefore, VEGF is regarded as a potent target in the treatment of leiomyomas.

VEGF is chiefly induced under hypoxia and is regulated by the heterodimeric transcription factor, hypoxia-inducible factor-1 (HIF-1) (Forsythe et al., 1996). Prolylhydroxylases (PHDs) immediately hydroxylate the O_2-dependent subunit of HIF-1, HIF-1α in the presence of oxygen, which leads to its rapid proteasomal degradation. In contrast, HIF-1α is stabilized through PHD impairments under hypoxic conditions, and is then translocated into the nucleus, in

Abbreviations: VEGF, vascular endothelial growth factor; HIF-1, hypoxia inducible factor-1; PHD, prolylhydroxylase; HRE, hypoxia response element; PI3K, phosphatidyl inositol 3-kinase; mTORC1, mammalian target of rapamycin complex 1; mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; TSC2, tuberous sclerosis 2; FBS, fetal bovine serum; DMSO, Dimethyl sulfoxide; $CoCl_2{\cdot}6H_2O$, cobalt(II) chloride hexahydrate; GPR10, G-protein receptor 10.

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which it dimerizes the HIF-1β unit and reacts with the hypoxia response element (HRE) (Shofield and Ratcliffe, 2004). Although the inhibition of PHDs under hypoxic conditions is the leading mechanism for the accumulation of HIF-1α, the extent of de novo synthesis is also important (Garcia-Maceira and Mateo, 2009). The synthesis of HIF-1α is up-regulated in response to growth factors through activation of the phosphatidyl inositol 3-kinase (PI3K)/AKT/ mammalian target of rapamycin complex 1 (mTORC1) pathway (Karar and Maity, 2011; Skinner et al., 2004). The activation of mTORC1, a multi-protein complex of the serine/threonine kinase mammalian target of rapamycin (mTOR), was previously shown to be essential for the expression of HIF-1α (Skinner et al., 2004). Leiomyoma tissue is known to be severely hypoxic (Mayer et al., 2008), has dysregulated mTORC1 activity (Crabtree et al., 2009) and up-regulated HIF-1αmRNA levels (Hou et al., 2013); therefore, the mTORC1/HIF-1α pathway could play an essential role in leiomyoma angiogenesis.

Metformin is a biguanide drug that is widely prescribed to patients with type 2 diabetes mellitus with limited side effects (Bailey et al., 1996). Epidemiological studies revealed that the risk of cancer was lower for metformin users than for insulin users (Libby et al., 2009), while others demonstrated the anti-proliferative effect of metformin on tumors both in vitro and in vivo, including those of breast, colon, and lung cancers (Sahra et al., 2010). A recent study showed the anti-angiogenic effect of metformin in a rodent model of ovarian cancer (Rattan et al., 2011). One of the possible mechanisms supporting these anti-tumor effects of metformin is the direct action of the AMP-activated protein kinase (AMPK)/mTORC1 pathway (Zakikhani et al., 2006). Metformin-induced AMPK activation inhibits mTORC1 by phosphorylating tuberous sclerosis 2 (TSC2) and/or raptor, a component of mTORC1 (Gwinn et al., 2008).

Previous studies have shown that diabetes mellitus is associated with uterine leiomyomas (Okolo, 2008); however, there have been no reports regarding the effect of metformin on fibroids in diabetes women. We previously demonstrated the anti-proliferative and pro-apoptotic effects of metformin in uterine leiomyoma cells via the activation of AMPK (Li et al., 2013). In the present study, we further investigated the effects of metformin on VEGF using rat-derived leiomyoma cells. The molecular mechanisms underlying the actions of metformin in the mTORC1/HIF-1α pathway were also demonstrated.

2. Materials and methods

2.1. Chemical reagents and antibodies

All cell culture media and reagents were from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Gibco BRL (Grand Island, NY, USA). Metformin (1,1-dimethylbiguanide hydrochloride) was purchased from Alexis Biochemicals (San Diego, CA, USA) and rapamycin from Cell Signaling Technology (Danvers, MA, USA). Dimethyl sulfoxide (DMSO) and cobalt(II) chloride hexahydrate ($CoCl_2{\cdot}6H_2O$) were from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were purchased: an anti-VEGF antibody from Abcam (Cambridge, MA, USA); anti-HIF-1α antibody (H1alpha 67) from Novus Biologicals (Littleton, CO, USA); anti-phospho-mTOR (Ser^{2448}) antibody, anti-mTOR (7C10) antibody, anti-phospho-raptor (Ser^{792}) antibody, anti-raptor (24C12) antibody, anti-phospho-AMPKα (Thr^{172}) (40H9) antibody, anti-AMPKα (23A3) antibody, anit-phospho-p70S6 kinase (The^{389}) antibody, anti-phospho-S6 ribosomal protein ($Ser^{235/236}$) (91B2) antibody, anti-S6 ribosomal protein (5G10) antibody, and anti-PHD-2/Egln1 (D31E11) antibody from Cell Signaling Technology; anti-β-actin antibody from Sigma-Aldrich; and peroxidase-conjugated secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and cell treatment

Eker rat-derived leiomyoma cell line ELT-3 cells were a kind gift from Dr. Cheryl L. Walker (M.D. Anderson Cancer Center, University of Texas, Smithville, TX, USA). Leiomyomas in Eker rats share phenotypic, biochemical, and genetic characteristics with human leiomyomas (Walker et al., 2003). Eker rat leiomyomas lack TSC2, the well-established mTORC1 repressor; therefore, they have dysregulated mTORC1 activity, similar to human leiomyomas (Crabtree et al., 2009). These cells were maintained in DMEM:Ham's F12 (1:1) medium supplemented with 10% FBS as previously described (Howe et al., 1995a, 1995b) in an atmosphere of 37 °C with 5% CO_2. Cells were cultured in 10-cm dishes containing 10 ml of the medium. After attachment for 24 hours and growth to 60–70% confluence, the cells were treated with metformin or rapamycin, an mTORC1 inhibitor. A stock solution of 1 M metformin was dissolved and diluted with double-distilled water to obtain final concentrations of 0.5–2.5 mM. Rapamycin was stocked at a concentration of 100 μM (dissolved in DMSO) and was diluted with DMSO for the final doses of 10–50 nM (0.1% DMSO). Control cells were treated with an equal amount of either double-distilled water or DMSO. $CoCl_2{\cdot}6H_2O$ was used for the hypoxia mimicking conditions. Of the divalent metals that act as hypoxic mimetics, $CoCl_2$ has been shown to increase the stability of HIF-1α, a consequence of antagonizing Fe^{2+}, which is required for oxygen to interact with PHDs and lead HIF-1α to degradation (Yuan et al., 2003). $CoCl_2{\cdot}6H_2O$ was added 30 minutes after the metformin treatment to obtain a final concentration of 150 μM. $CoCl_2{\cdot}6H_2O$ was made fresh for every experiment and dissolved in double-distilled water.

2.3. Assessment of secreted VEGF

ELT-3 cells were treated with metformin at the concentration and time courses indicated. Supernatant medium was collected and VEGF concentrations were measured using commercially available sandwich ELISA kits (VEGFA Rat ELISA kit; Abcam) following the manufacturer's instructions. All experiments were carried out in triplicate and the results were expressed as light absorbance at 450 nm.

2.4. SDS-PAGE and Western blot analysis

ELT-3 cells were treated with metformin or rapamycin, washed with ice-cold PBS, and then collected with PBS containing 0.02% EDTA. The pelleted cells were homogenized with 40 μl of lysis buffer consisting of M-PER Mammalian Protein Extraction Reagent (Thermo Scientific Inc., Rockford, IL, USA) plus 0.1% protease inhibitor cocktail. Protein concentrations in the supernatant were quantified using the Bradford protein assay dye reagent concentrate (Bio-rad Laboratories; Hercules, CA, USA) following the manufacturer's instructions. Equal amounts of proteins (40 μg of total proteins or 10 μg of nuclear proteins) were fractionated on 10% polyacrylamide gels (Super Sep™ Ace; Wako, Osaka, Japan) by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immun-Blot™ PVDF; Bio-Rad Laboratories Inc., Hercules, CA, USA). The membranes were incubated with primary antibodies diluted in either the blocking solution or 5% bovine serum albumin (BSA). After washing, the membranes were incubated for 60 minutes at room temperature with HRP-conjugated secondary antibodies diluted in 5% non-fat milk or 5% BSA solution. The immunoblots were visualized with the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA). The relative density of the specific bands was quantified using the image detection software, Image J (Rasband WS, 1997–2012: http://imagej.nih.gov/ij/) when required. The experiments were repeated three times with different cell preparations.