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Research Paper

Metformin Ameliorates Uterine Defects in a Rat Model of Polycystic Ovary Syndrome

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ABSTRACT

Adult rats treated concomitantly with insulin and human chorionic gonadotropin exhibit endocrine, metabolic, and reproductive abnormalities that are very similar to those observed in polycystic ovary syndrome (PCOS) patients. In this study, we used this rat model to assess the effects of metformin on PCOS-related uterine dysfunction. In addition to reducing androgen levels, improving insulin sensitivity, and correcting the reproductive cycle, metformin treatment induced morphological changes in the PCOS-like uterus. At the molecular and cellular levels, metformin normalized the androgen receptor-mediated transcriptional program and restored epithelial–stromal interactions. In contrast to glucose transport, uterine inflammatory gene expression was suppressed through the PI3K–Akt–NFκB network, but without affecting apoptosis. These effects appeared to be independent of AMPK subunit and autophagy-related protein regulation. We found that when metformin treatment partially restored implantation, several implantation-related genes were normalized in the PCOS-like rat uterus. These results improve our understanding of how metformin rescues the disruption of the implantation process due to the uterine defects that result from hyperandrogenism and insulin resistance. Our data provide insights into the molecular and functional clues that might help explain, at least in part, the potential therapeutic options of metformin in PCOS patients with uterine dysfunction.

1. Introduction

Polycystic ovary syndrome (PCOS) is a common and multifactorial disease that affects approximately 4%–18% of all reproductive-aged women in the world (Moran et al., 2011). In the clinic, hyperandrogenism and insulin resistance appear to be the major etiological drivers for reproductive and metabolic abnormalities in women with PCOS (Rosenfield and Ehrmann, 2016). While it is believed that anovulation is a main reason for infertility in PCOS patients, accumulating evidence from clinical studies also indicates that the impairment of endometrial function likely causes recurrent pregnancy loss, premature delivery, endometrial hyperplasia, and carcinoma in women with PCOS (Goodarzi et al., 2011; Palomba et al., 2015; Shao et al., 2014). Additionally, several lines of evidence suggest that the systemic low-grade inflammation that often coincides with PCOS compromises multiple aspects of fertility (Repaci et al., 2011). Although the precise mechanisms of hyperandrogenism and insulin resistance-induced inflammation in the endometrium are not completely understood, in vivo and in vitro studies have demonstrated associations between the dysregulation of inflammation-related molecules in numerous endometrial cell lines and under PCOS conditions (Matteo et al., 2010; Orostica et al., 2016; Piltonen et al., 2013; Piltonen et al., 2015). Due to the clinically heterogeneous characteristics of this syndrome, its treatment remains complex with variable responses among PCOS patients (Palomba et al., 2015).

Metformin, an oral biguanide insulin-sensitizing drug, is the most widely used treatment for type 2 diabetes mellitus and PCOS worldwide (Naderpoor et al., 2015; Nestler, 2008; Pernicova and Korbonits, 2014). The primary actions of this drug are either to increase insulin sensitivity by inhibiting gluconeogenesis and stimulating glucose uptake and utilization in the liver, skeletal muscles, adipocytes, and ovaries or to increase cellular levels of AMP-activated protein kinase (AMPK) by

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inhibiting mitochondrial complex 1 and subsequently activating the AMPK signaling pathway (Foretz et al., 2014; Sivalingam et al., 2014). Indeed, these postulated molecular mechanisms have been demonstrated in human endometrial carcinoma tissues in vivo and in different endometrial cancer cells in vitro (Shao et al., 2014). Moreover, previous studies by us and others have also reported that metformin can improve endometrial receptivity, enhance endometrial vascularity and blood flow, and revert endometrial hyperplasia and carcinoma into normal endometria in addition to improving hyperandrogenism and insulin resistance in some women with PCOS (Jakubowicz et al., 2001; Li et al., 2014; Palomba et al., 2006). These results clearly suggest that metformin is a promising drug for treating PCOS patients with uterine dysfunction. However, these clinical studies have provided very limited insight into the anatomical, molecular, and functional metformin-induced alterations in the uterus under physiological and pathological conditions, especially the reproductive disturbance associated with PCOS.

The impairment of the androgen–androgen receptor (AR) signaling pathway is associated with PCOS patients with reproductive dysfunction (Cloke and Christian, 2012). Although previous studies have demonstrated that androgen modulates the expression of an array of genes in the mouse uterus, including *Wnt4*, *Wnt5a*, *Wnt7a*, *Cdh1*, *Vcl*, *Igf1*, *Prl8a2*, *Prlr*, *Foxa2*, *Fgf7*, and *Hgf* (Simitsidellis et al., 2016), it remains unclear which downstream targets of this pathway actually contribute to the uterine abnormalities associated with PCOS. Similar to our understanding of the transcriptional actions of AR activation in the uterus, the actions of insulin and insulin-like growth factor-1 – through the phosphatidylinositide-3-kinase (PI3K)–Akt signaling pathway – appear to also modulate endometrial cell survival, proliferation, and metabolism under physiological and pathological conditions, including PCOS (Li et al., 2016b; Shao et al., 2014). It has been shown that nuclear factor kappa B (NFκB) and Forkhead family of transcription factors such as Forkhead box O1 (FoxO1) are the key targets of activated Akt (Brunet et al., 1999; Dan et al., 2008). Furthermore, activation of AMPK, which interacts with alternative PI3K–Akt signaling pathways, is the hallmark of metformin action in several tissues and cell types (Foretz et al., 2014; Shao et al., 2014).

The aim of this study was to investigate the impact of therapeutic doses of metformin on uterine cells and reproductive function, including implantation and pregnancy, under conditions of hyperandrogenism and insulin resistance. We used a rat model in which PCOS-like features can be induced by a combination of insulin and human chorionic gonadotropin (hCG) (Chen et al., 2009; Damario et al., 2000; Li et al., 2013; Lima et al., 2006; Poretsky et al., 1992; Zhang et al., 2016). In this model, the AR-regulated transcriptional program, PI3K–Akt–NFκB–FoxO1, and the AMPK signaling pathways were measured, including implantation-related gene signature expression. We also evaluated the effects of metformin treatment on epithelial-stromal interactions, the levels of inflammation-related molecules, cell apoptosis, and autophagy in the uterus.

2. Materials & Methods

2.1. Ethics Statement

All treatments and animal care procedures were performed according to the National Institute of Health guidelines on the care and use of animals and were approved by the Animal Care and Use Committee of the Heilongjiang University of Chinese Medicine, China (HUCM 2015–0112).

2.2. Reagents, Antibodies, and Primers

Human recombinant insulin (Humulin NPH) was from Eli Lilly Pharmaceuticals (Giza, Egypt), and hCG was from NV Organon (Oss, Holland). Metformin and 3,3-diaminobenzidine tetrahydrochloride (DAB), anti-mouse IgG horseradish peroxidase (HRP)-conjugated goat (A2304), and anti-rabbit IgG HRP-conjugated goat (A0545) secondary antibodies were from Sigma-Aldrich (St. Louis, MO). The primary antibodies used for Western blot and immunohistochemical analyses in the present study, their dilution, and sources are listed in Table S1. Alexa Fluor 594-conjugated goat polyclonal anti-mouse IgG was from Invitrogen (Sollentuna, Sweden). The avidin-biotinylated-peroxidase complex detection system (ABC kit) was from Vector Laboratories Inc. (Burlingame, CA). A detailed list of primers is provided in Table S2.

2.3. Experimental Animals and In Vivo Treatment

Female Sprague–Dawley rats (n = 76) were aged 70 days at the onset of experiments and were obtained from the Laboratory Animal Centre of Harbin Medical University, Harbin, China (License number SCXK 2013-001). Animals were kept in groups with free access to food and water and a controlled temperature of 22 ± 2 °C with a 12 h light/dark cycle. All rats used in this study needed to have normal estrous cycles prior to treatment, and these were confirmed by examination of vaginal smears under a light microscope for two sequential cycles (about 8–10 days). Animal numbers in the experimental groups/subgroups are indicated in Figs. S1A, S5A, and S6A.

Rats were randomly divided into control (saline treatment) and experimental (PCOS-like) groups. They were either treated with an equal volume of saline as controls or insulin plus hCG to induce a combination of hyperinsulinemia and hyperandrogenism (Fig. S1A). The doses and treatment protocols for insulin and hCG were as described previously (Zhang et al., 2016). Briefly, insulin was started at 0.5 IU/day and gradually increased to 6.0 IU/day between the first day and the 22nd day to induce hyperinsulinemia and insulin resistance, and 3.0 IU/day hCG was given to induce hyperandrogenism. Animals were treated with twice-daily subcutaneous injections until the end of the experiment. Rats with repeated insulin injection have been shown to suffer no hypoglycemic episodes (Bogovich et al., 1999; Damario et al., 2000; Poretsky et al., 1992). On the 23rd day, each group of rats was divided into two subgroups of ten rats each. For treatment, metformin was dissolved in saline and given orally at 500 mg/kg by a cannula (Fig. S1A). The dose of metformin used in this study was equivalent to that used in the treatment of PCOS patients (Elia et al., 2009; Motta, 2010). After the animals were anesthetized, trunk blood was collected and the uteri were removed, stripped of fat and connective tissue, and weighed. One side of the uterus in each animal was immediately frozen in liquid nitrogen and stored at −70 °C for subsequent Western blot and quantitative real-time PCR analysis. The other side was fixed in 4% formaldehyde and neutral buffered solution for 24 h at 4 °C and embedded in paraffin for histochemical analysis.

2.4. Identification of Estrous Cycle Stage

Estrous cycles were monitored daily by vaginal lavage according to a standard protocol (Feng et al., 2010). None of the insulin + hCG-treated rats with prolonged estrous cycles were included in the study. Our study found that some insulin + hCG-treated rats with prolonged estrous cycles remained the hyperandrogenic condition, but they did not exhibit any sign of insulin resistance. The mechanisms responsible for metformin-rescued uterine defects in these rats are required for further investigation.

2.5. Assessment of Embryo Implantation Site and Fertility

Female rats were mated with fertile males of the same strain to induce implantation (Fig. S5A) and pregnancy (Fig. S6A). To identify the implantation sites, rats were injected intravenously with a Chicago Blue B dye solution (1% in saline) and sacrificed 10 min later. Uteri were dissected and assessed for clearly delineated blue bands as evidence of early implantation sites as described previously (Wang et al.,

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