EBioMedicine 18 (2017) 157–170

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

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

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ARTICLE INFO

Article history:
Received 25 January 2017
Received in revised form 14 March 2017
Accepted 14 March 2017
Available online 18 March 2017

Keywords:
Metformin
Hyperandrogenism
Insulin resistance
Molecular mechanism
PCOS
Rat uterus

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

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http://dx.doi.org/10.1016/j.ebiom.2017.03.023

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 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.,

2004). In some pregnant rats, the pup number, sex ratio, and body weight were monitored.

2.6. Oral Glucose Tolerance Tests (OGTT)

The OGTT was performed in live rats on day 57 as described previously (Zhang et al., 2016).

2.7. Measurement of Hormone Level

Serum concentrations of gonadotropins (follicle stimulating hormone and luteinizing hormone), steroid hormones (17β-estradiol, progesterone, testosterone, and androstenedione), sex hormone-binding globulin, and insulin were determined using enzyme-linked immunosorbent assay (ELISA) kits (Cloud-Clone Corp., Houston, TX). Homeostatic model assessment of insulin resistance was calculated to assess changes in insulin sensitivity (DeUgarte et al., 2005; Matthews et al., 1985). The intra- and inter-assay coefficients of variation are listed in Supplemental Table 3.

2.8. Histology and Immunostaining

Uterine tissues were fixed in 10% formalin, embedded in paraffin, and sectioned for hematoxylin and eosin (H&E) staining according to standard procedures. The areas of endometrium and myometrium in three uterine sections (at 2× magnification) per rat were measured and calculated using Micro Image (Olympus, Japan) by investigators blinded to group assignment. Immunohistochemistry and immunofluorescence were performed according to previously described methods (Li et al., 2015a; Li et al., 2015b). After incubation with the primary antibody (Supplemental Table 1) overnight at 4 °C in a humidified chamber, the sections were stained using the ABC kit followed by a 10-min treatment with DAB (SK-4100, Vector Laboratories). The other half of the uterine sections were incubated with primary antibody in 0.01 M Tris-buffered saline supplemented with Triton X-100 (TBST) containing 5% nonfat milk overnight at 4 °C, and a secondary antibody was applied at room temperature for 1 h. After the sections were washed with TBST, they were re-suspended in mounting medium containing DAPI (4′,6′-diamidino-2-phenylindole; Vector Laboratories). Sections were imaged on a Nikon E-1000 microscope (Japan) and photomicrographed using Easy Image 1 (Bergström Instrument AB, Sweden). All morphological and immunohistochemical assays were performed by at least two researchers in an operator-blinded manner.

2.9. Western Blot and Quantitative Analysis

A detailed explanation of the Western blot analysis protocol has been published (Li et al., 2015b; Zhang et al., 2016). Equal amounts (30 μg) of protein isolated from whole uterine tissue were resolved on 4–15% TGX stain-free gels (Bio-Rad Laboratories GmbH, Munich, Germany) and transferred onto PVDF membranes. The membranes were probed with the primary antibody (Supplemental Table 1) in TBST containing 5% non-fat dry milk followed by HRP-conjugated secondary antibody. When necessary, the PVDF membranes were stripped using Restore PLUS Western blot stripping buffer (Thermo Scientific, Rockford, IL) for 15 min at room temperature, washed twice in TBST, and then re-probed. Ultraviolet activation of the Criterion stain-free gel on a ChemiDoc MP Imaging System (Bio-Rad) was used to control for proper loading. Band densitometry was performed using Image Laboratory (Version 5.0, Bio-Rad). Each sample was repeated twice independently.

2.10. Quantitative Real-time PCR Analysis

qRT-PCR was performed with a Roche Light Cycler 480 sequence detection system (Roche Diagnostics Ltd., Rotkreuz, Switzerland) as previously described (Lima et al., 2006; Zhang et al., 2016). The PCR parameters were set according to the manufacturer's protocols, and amplifications were performed with a SYBR green qPCR master mix (#K0252, Thermo Scientific, Rockford, IL). Total RNA was prepared from the frozen whole uterine tissues. Single-stranded cDNA was synthesized from each sample (2 μg) with M-MLV reverse transcriptase (#0000113467, Promega Corporation, WI) and RNase inhibitor (40 U) (#00314959, Thermo Scientific). cDNA (1 μl) was added to a reaction master mix (10 μl) containing 2× SYBR green qPCR reaction mix (Thermo Scientific) and gene-specific primers (5 μM each of forward and reverse primers). All primers (Supplemental Table 2) were designed to exclude the amplification of genomic DNA. Amplification quality was validated by analysis of the melting curve. All reactions were performed six times, and each reaction included a non-template control. The CT values for both *GAPDH* and *U87* mRNA were not significantly different in any of the groups. The results for target genes were expressed as the amount relative to the average CT values of *GAPDH* + *U87* in each sample. Relative gene expression was determined with the $2^{-\Delta\Delta CT}$ method, and the efficiency of each reaction – as determined by linear regression – was incorporated into the equation.

2.11. Statistical Analysis

Data are represented as the means ± SEM. Statistical analyses were performed using SPSS version 21.0 statistical software for Windows (SPSS Inc., Chicago, IL). The normal distribution of the data was tested with the Shapiro–Wilk test. Differences between groups were analyzed by one-way ANOVA followed by Tukey's post hoc test for normally distributed data or the Kruskal–Wallis test followed by the Mann–Whitney *U* test for skewed data. For the OGTT studies, data were analyzed using one-way ANOVA repeated measures followed by Tukey's post hoc test. All p-values < 0.05 were considered statistically significant.

3. Results

3.1. Hyperandrogenism, Arrested Estrous Cycle and Insulin Resistance are Disturbances That Mimic PCOS Phenotype in Insulin + hCG-treated Rats

Our results showed that the insulin + hCG-treated rats had increased levels of gonadotropins, steroid hormones, and free androgen index, as well as decreased glucose tolerance and insulin sensitivity (Table 1 and Fig. S1B). Furthermore, these rats did not have a normal estrous cycle and remained at the metestrus and diestrus stages. These findings demonstrate that the development of hyperandrogenism combined with insulin resistance is coupled with reproductive disturbances that mimic PCOS.

We observed that in PCOS-like (insulin + hCG-treated) rats treated with metformin, body weight was reduced and levels of 17β-estradiol, total testosterone, and fasting glucose were suppressed compared to PCOS-like rats treated with saline (Table 1). Furthermore, the OGTT showed that PCOS-like rats treated with metformin were significantly more glucose tolerant compared to PCOS-like rats treated with saline. Our data showed that metformin improved peripheral insulin sensitivity and resulted in a regular estrous cycle in the PCOS-like rats (Table 1).

3.2. Uterine Morphology in Rats Treated With Metformin

Histological analysis of the uterus (Fig. 1A–D) showed that the luminal epithelial cells remained cuboidal with similar thickness in control rats treated with saline or metformin (Fig. 1A1 and B1) and in PCOS-like rats treated with metformin (Fig. 1D1). In contrast, PCOS-like rats treated with saline displayed greater numbers of luminal epithelial cells along with the formation of multiple cell layers (Fig. 1C1). In addition, the numbers of glands and gland conglomerates were increased in PCOS-like rats treated with saline (Fig. 1C2) compared to control rats treated with saline (Fig. 1A2) or metformin (Fig. 1B2).

Table 1
Effects of metformin on the reproductive, endocrine and metabolic alterations in control and insulin + hCG-treated rats.

	Saline (n = 10)	Met (n = 10)	Insulin + hCG (n = 10)	Insulin + hCG + Met (n = 10)
Reproductive characteristics				
BW (kg)	0.293 ± 0.006	0.304 ± 0.010	0.335 ± 0.007[a,d]	0.302 ± 0.006[f]
Ovaries (g)	0.086 ± 0.007	0.084 ± 0.006	0.138 ± 0.015[a,c]	0.111 ± 0.010
Ovaries/BW (g/kg)	0.296 ± 0.027	0.277 ± 0.017	0.408 ± 0.039[d]	0.369 ± 0.033
Uteri (g)	0.437 ± 0.035	0.481 ± 0.022	0.516 ± 0.053	0.426 ± 0.041
Uteri/BW (g/kg)	1.489 ± 0.118	1.594 ± 0.084	1.535 ± 0.142	1.409 ± 0.131
n (%) of present estrous cycle	10 (100)	10 (100)	0 (0)	10 (100)
Biochemistry				
FSH (ng/ml)	3.57 ± 0.03	3.67 ± 0.05	3.85 ± 0.05[a]	3.72 ± 0.06
LH (ng/ml)	3.52 ± 0.07	3.60 ± 0.06	3.93 ± 0.03[a,c]	3.80 ± 0.12[a]
LH/FSH	0.99 ± 0.02	0.99 ± 0.02	1.02 ± 0.01	1.02 ± 0.02
E2 (ng/ml)	1.17 ± 0.01	1.11 ± 0.01[b]	1.25 ± 0.03[a,c]	1.14 ± 0.01[e]
P4 (ng/ml)	3.87 ± 0.03	3.86 ± 0.01	3.83 ± 0.03	3.84 ± 0.02
Total T (ng/ml)	2.26 ± 0.03	2.21 ± 0.02	2.35 ± 0.03[b,c]	2.21 ± 0.01[e]
A4 (nmol/l)	5.06 ± 0.45	7.48 ± 0.99	8.04 ± 0.86[b]	7.71 ± 0.62
DHT (pg/ml)	114.92 ± 6.43	144.98 ± 12.43	156.21 ± 11.07[b]	149.21 ± 7.50
E2/Total T	0.52 ± 0.01	0.50 ± 0.01	0.53 ± 0.01	0.52 ± 0.01
T/DHT	0.020 ± 0.001	0.016 ± 0.002	0.016 ± 0.001	0.015 ± 0.001[b]
Total T/A4	0.49 ± 0.06	0.36 ± 0.06	0.31 ± 0.03	0.30 ± 0.02[b]
SHBG (ng/ml)	4.72 ± 0.05	4.68 ± 0.03	4.69 ± 0.03	4.74 ± 0.03
FAI	47.87 ± 0.54	47.17 ± 0.53	50.06 ± 0.66[b,c]	46.52 ± 0.32[e]
Fasting glucose (mmol/l)	4.09 ± 0.17	4.16 ± 0.16	5.29 ± 0.27[a,c]	4.48 ± 0.22[f]
OGTT (mmol/l)				
Glucose 30 min	6.70 ± 0.10	6.81 ± 0.28	9.16 ± 0.63[a,c]	7.03 ± 0.36[e]
Glucose 60 min	6.01 ± 0.25	5.92 ± 0.27	6.66 ± 0.33	6.72 ± 0.36
Glucose 90 min	4.72 ± 0.14	4.75 ± 0.22	5.71 ± 0.27[a,d]	5.51 ± 0.18[b,d]
Glucose 120 min	4.20 ± 0.14	4.42 ± 0.19	5.24 ± 0.20[a,d]	4.86 ± 0.15[b]
AUC glucose	8.43 ± 0.17	8.51 ± 0.24	10.54 ± 0.29[a,c]	9.21 ± 0.27[e]
Fasting insulin (pg/ml)	2.69 ± 0.03	2.69 ± 0.05	2.96 ± 0.05[a,c]	2.84 ± 0.05
HOMA-IR	0.49 ± 0.02	0.50 ± 0.02	0.70 ± 0.04[a,c]	0.57 ± 0.03[f]

BW, body weight; Met, metformin; hCG, human chorionic gonadotropin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, 17β-estradiol; P4, progesterone; T, testosterone; A4, androstenedione; DHT, 5α-dihydrotestosterone; SHBG, sex hormone-binding globulin; FAI, free androgen index, FAI = [T (pg/ml) × 100]/SHBG (pg/ml); OGTT, oral glucose tolerance tests; AUC, area under the curve, AUC = 0.5 × [BG0 + BG30]/2 + 0.5 × [BG30 + BG60]/2 + 0.5 × [BG60 + BG120]/2; HOMA-IR, Homeostasis model assessment of insulin resistance, HOMA-IR = fasting blood glucose (mmol/l) × fasting serum insulin (μU/ml)/22.5.
Values are Mean ± SEM. The multiple comparisons between data were performed using one-way ANOVA and Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.
[a] $p < 0.01$ versus Saline group.
[b] $p < 0.05$ versus Saline group.
[c] $p < 0.01$ versus Metformin group.
[d] $p < 0.05$ versus Metformin group.
[e] $p < 0.01$ versus Insulin + hCG group.
[f] $p < 0.05$ versus Insulin + hCG group.

Metformin treatment reduced the outer uterine diameter in PCOS-like rats compared to the other three groups (Fig. 1D), but quantitative assessment of endometrial and myometrial components indicated that the areas of endometrium and myometrium were decreased in the PCOS-like rats treated with metformin compared to control rats treated with metformin and PCOS-like rats treated with saline (Fig. 1E). We also found that the luminal epithelial cell numbers and cell layers (Fig. 1D1) as well as the numbers of glands and gland conglomerates (Fig. 1D2) were significantly decreased in PCOS-like rats treated with metformin compared to PCOS-like rats treated with saline. However, Western blot analysis showed no statistical differences in the expression of uterine cell marker proteins, including cytokeratin 8, platelet derived growth factor receptor β, and α-smooth muscle actin, between any of the four groups (Fig. 1F).

3.3. Correction of AR-dependent Epithelial-stromal Interactions by Metformin

We showed that AR protein levels were increased in PCOS-like rats compared to control rats and that metformin supressed uterine AR protein levels in PCOS-like rats (Fig. 2A). We found that metformin altered *Vcl*, *Igf1*, *Foxa2*, and *Hgf* mRNA expression in the uterus of control rats (Fig. 2B). Of note, there was considerable heterogeneity in the gene expression patterns in the same experimental groups with metformin treatment. For instance, *Wnt7a*, *Cdh1*, *Igf1*, *Prl8a2*, *Prlr*, and *Fgf7* mRNAs were increased, whereas *Vcl* and *Foxa2* mRNAs were decreased in the PCOS-like rats treated with metformin compared to PCOS-like rats treated with saline (Fig. 2B). Wnt–β-catenin signaling has been suggested to have an important role in regulating uterine cell growth and differentiation (van der Horst et al., 2012). We found that while β-catenin protein was predominantly expressed in glandular epithelial cells in all groups, the amount of β-catenin protein was decreased in the stromal cells by metformin in both control rats and PCOS-like rats compared to those treated with saline (Fig. 2C). It is likely, therefore, that the regulation of downstream targets of the androgen–AR signaling pathway is cell type-dependent in the uterus.

3.4. Metformin Induces PI3K–Akt-mediated Regulation of Glucose Transporters (Glut)

We further performed a Western blot analysis to measure the expression of several proteins that are involved in the insulin-mediated PI3K-Akt signaling pathway in the uterus after metformin treatment (Fig. 3). There was no significant difference in insulin receptor (IR) α, IRβ, insulin degrading enzyme, p85-PI3K, or p-Akt (Ser473) expression between saline-treated and metformin-treated rats. However, quantitative protein data indicated that the phosphorylation of p-Akt (Thr308) was significantly decreased in rats treated with metformin alone compared to rats treated with saline. In line with the results from a human endometrial tissue culture study, we also found that metformin

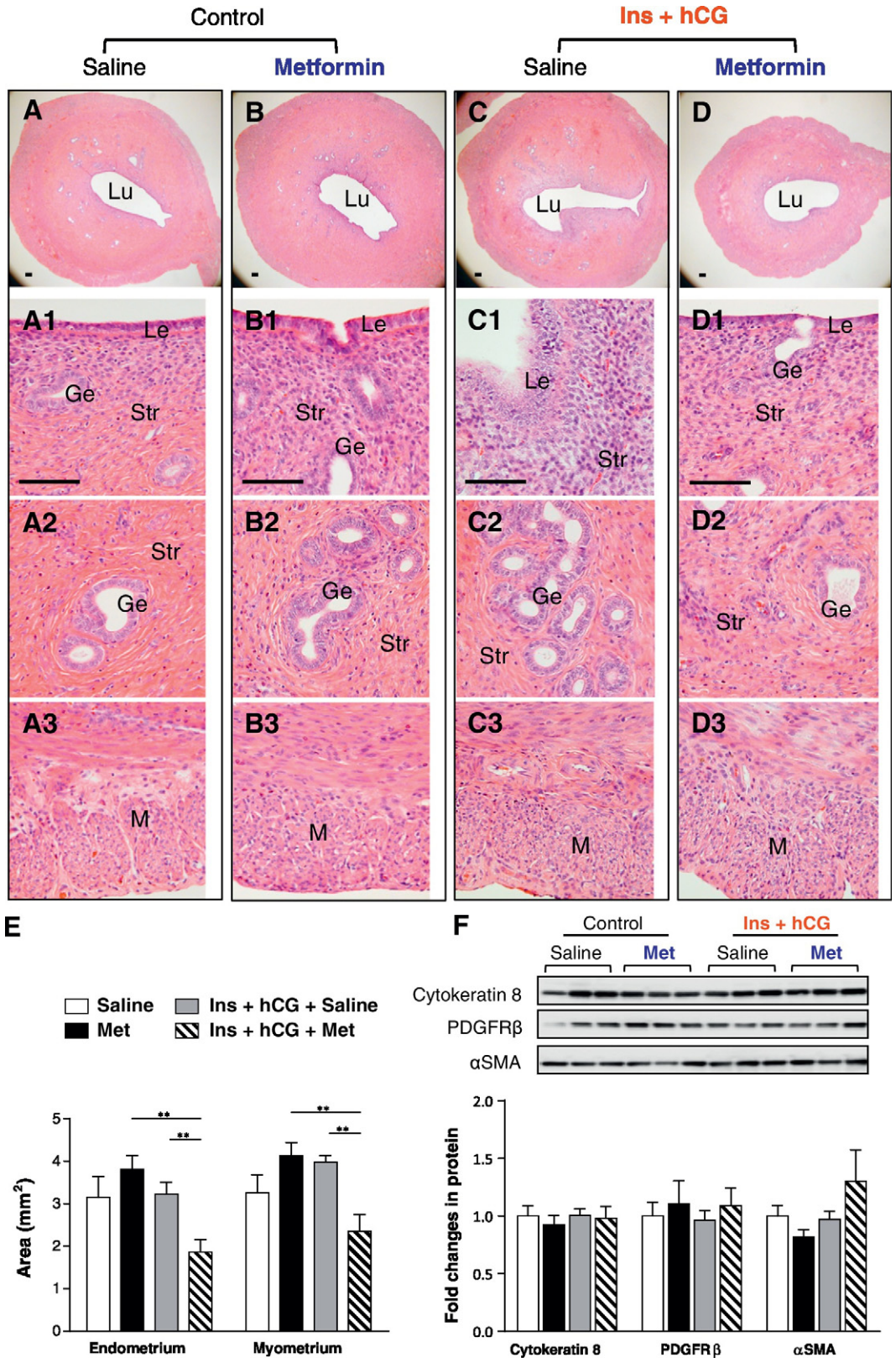


Fig. 1. Uterine morphology and cell-specific protein expression in the insulin + hCG-treated and control rats after metformin treatment. A–D, All representative photomicrographs are of cross sections of the middle zone. Uterine tissues from control rats treated with saline (A–A3) or metformin (B–B3) and from insulin + hCG-treated rats treated with vehicle (C–C3) or metformin (D–D3) were fixed in formalin and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin. Representative images (n = 10/group) are shown. Lu, lumen; Le, luminal epithelial cells; Ge, glandular epithelial cells; Str, stromal cells; Myo, myometrium. Scale bars (100 μm) are indicated in the photomicrographs. E, Quantification of the endometrial and myometrial areas (n = 10/group). Values are expressed as means ± SEM. **p < 0.01. F, Western blot analysis of protein expression in the rat uterus was performed. Representative images and quantification of the densitometric data (n = 9/group) of cytokeratin 8, PDGFRβ, and αSMA are shown. Statistical tests are described under Material and Methods.

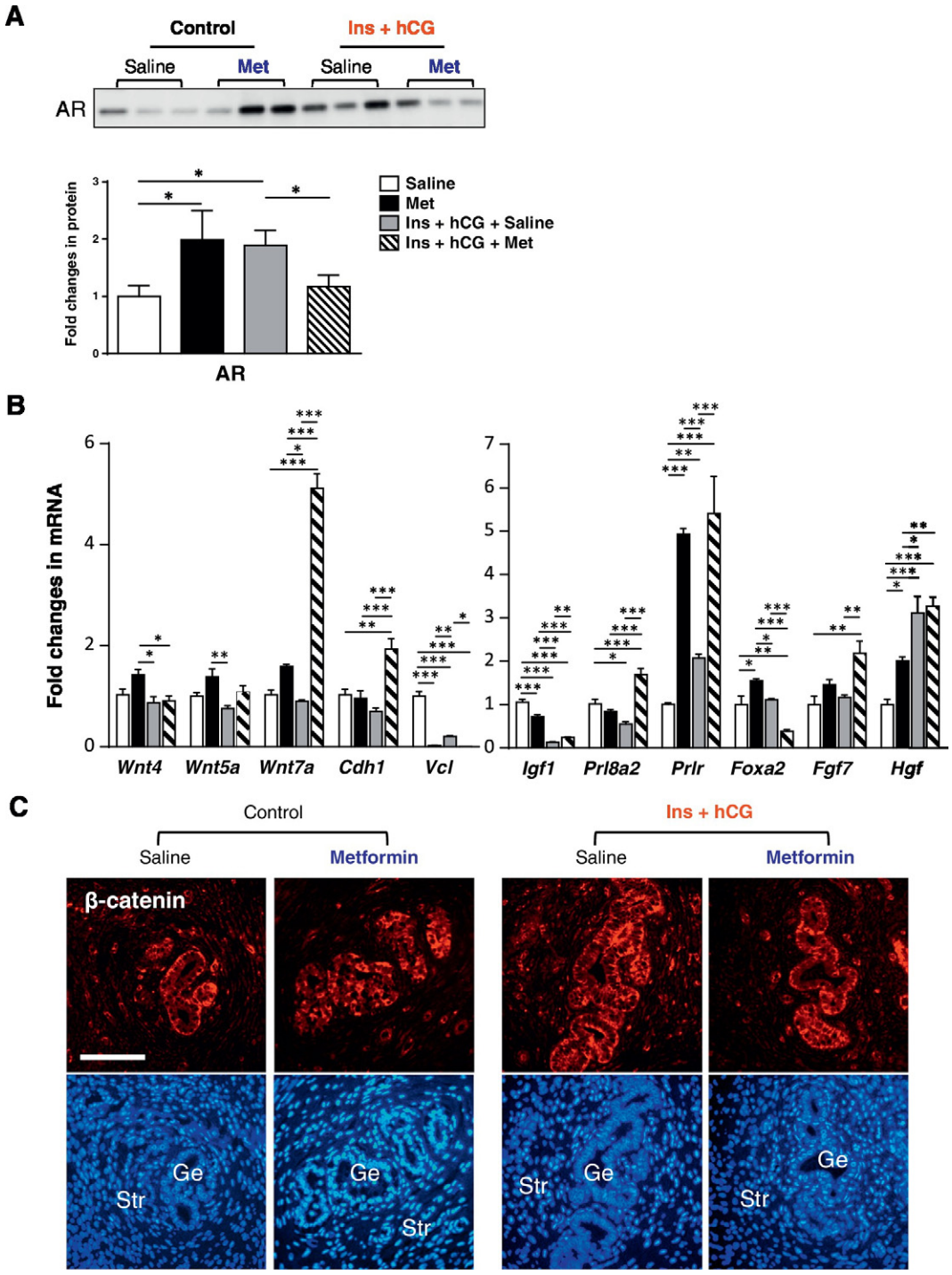


Fig. 2. Effects of metformin on AR protein expression and the expression of genes related to epithelial–stromal interactions in the rat uterus. A, Western blot analysis of protein expression in the rat uterus was performed. Representative images and quantification of the densitometric data (n = 9/group) of AR are shown. B, Uterine tissues from control rats treated with vehicle or metformin and insulin + hCG-treated rats treated with vehicle or metformin (n = 6/group) were analyzed for mRNA levels of *Wnt4*, *Wnt5a*, *Wnt7a*, *Cdh1*, *Vc1*, *Igf1*, *Prl8a2*, *Prlr*, *Foxa2*, *Fgf7*, and *Hgf* by qRT-PCR. The mRNA level of each gene relative to the mean of the sum of the *Gapdh* and *U87* mRNA levels in the same sample is shown. Values are expressed as means ± SEM. Statistical tests are described under Material and Methods. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. C, Immunofluorescence detection of β-catenin in control rats treated with vehicle or metformin and in insulin + hCG-treated rats treated with vehicle or metformin. Representative images (n = 5/group) are shown. Cell nuclei were counterstained with DAPI (blue, lower panel). Ge, glandular epithelial cells; Str, stromal cells. Scale bars (100 μm) are indicated in the photomicrographs.

treatment increased p110-PI3K protein levels but decreased pan-Akt protein levels in PCOS-like rats. We next determined the expression of glycogen synthase kinase-3 (Gsk3) and different Glut isoforms in the uterus. Although there was no statistical difference in Gsk3 expression between any of the groups (Fig. 3), quantitative data indicated that *Glut2*, *6*, *7*, and *12* mRNAs were increased and *Glut8*, *9*, and *13* mRNAs were decreased in rats treated with metformin alone compared to rats treated with saline (Fig. S2). Among the 12 Glut genes, *Glut1*, *3*, *5*, *6*, and *10* mRNAs were increased in PCOS-like rats treated with metformin (Fig. S2).

To further assess the effect of metformin in uterine cell apoptosis and survival, we examined the expression of FoxO1, activated caspase3,

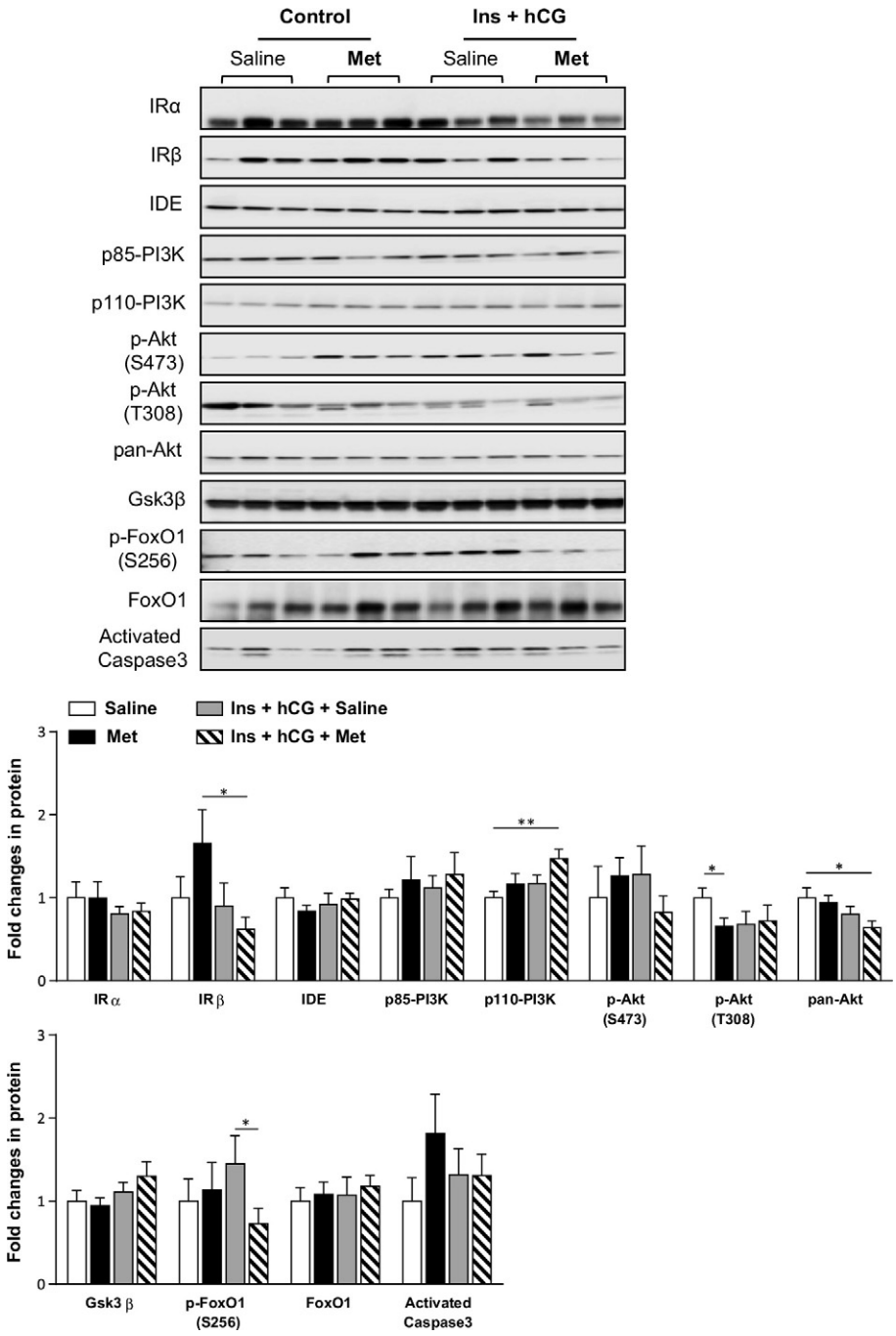


Fig. 3. Effects of metformin on the insulin-mediated PI3K–Akt signaling pathway and its downstream protein expression in the rat uterus. Western blot analysis of protein expression in the rat uterus was performed. Representative images and quantification of the densitometric data (n = 9/group) of IRα, IRβ, IDE, p85-PI3K, p110-PI3K, p-Akt (Ser473), p-Akt (Thr308), pan-Akt, Gsk3β, p-FoxO1 (S256), FoxO1, and activated caspase3 are shown. Values are expressed as means ± SEM. Statistical tests are described under Material and Methods. *$p < 0.05$; **$p < 0.01$.

and cytochrome *c*. While a reduction of FoxO1 protein levels was detected in PCOS-like rats treated with metformin compared to those treated with saline, treatment with metformin failed to alter activated caspase3 protein levels and cytoplasmic cytochrome *c* localization (data not shown) in either controls or PCOS-like rats (Fig. 3).

3.5. Mechanisms of Metformin-regulated Uterine Inflammation

We also profiled the expression of genes that are involved in the inflammatory process in the rat uterus by qRT-PCR. Quantitative data indicated that *Ccl5*, *Orm1*, *Tnfaip3*, *Il6*, *Il8*, and *Mmp2* mRNA levels were decreased in control rats treated with metformin compared to control rats treated with saline. Similarly, treatment with metformin compared to treatment with saline reduced *Orm1*, *C4a/b*, *Tnfa*, *Tnfaip3*, *Il1b*, *Il6*, *Il8*, and *Mcp1-3* mRNA levels in PCOS-like rats (Fig. 4). We also found that *Orm1*, *Tnfa*, *Tnfaip3*, *Il8*, and *Mmp2/3* mRNA levels were up-regulated in the rat uterus under hyperandrogenism alone or combined with insulin resistance (Fig. S3).

We next focused on studying the mechanism of metformin-induced regulation of inflammation-related molecules in the rat uterus, and we performed a Western blot analysis and immunochemistry (Fig. 5) to measure the expression of several proteins that belong to the NFκB, an inflammatory transcription factor, signaling system. While the inhibitor of NFkB kinase complex β (IKKβ) protein level was increased in PCOS-

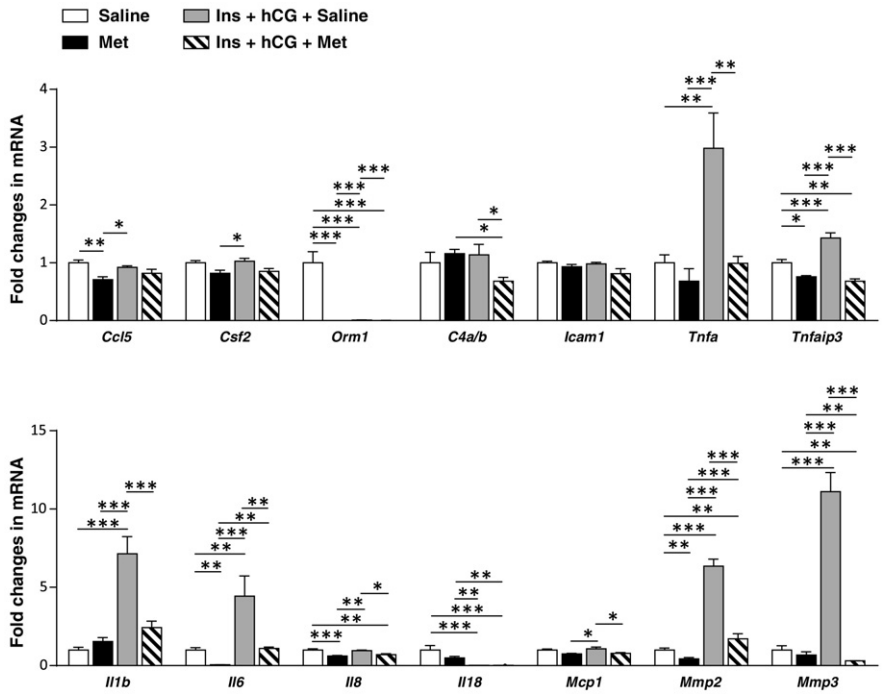


Fig. 4. Effects of metformin on inflammatory gene expression in the rat uterus. Uterine tissues from control rats treated with vehicle or metformin and from insulin + hCG-treated rats treated with vehicle or metformin (n = 6/group) were analyzed for mRNA levels of *Ccl5*, *Csf2*, *Orm1*, *C4a/b*, *Icam1*, *Tnfa*, *Tnfaip3*, *Il1b*, *Il6*, *Il8*, *Il18*, *Mcp1*, *Mmp2*, and *Mmp3* by qRT-PCR. The mRNA level of each gene relative to the mean of the sum of the *Gapdh* and *U87* mRNA levels in the same sample is shown. Values are expressed as means ± SEM. Statistical tests are described under Material and Methods. *p < 0.05; **p < 0.01; ***p < 0.001.

like rats compared to control rats, treatment with metformin increased both IKKβ and IκBα protein levels in PCOS-like rats (Fig. 5A). However, we observed that there was no significant difference in phosphorylated NFκB (p65) or NFκB expression between any of the groups. While NFκB protein was detected in the epithelial and stromal cells in all groups (Fig. 5B and C), we observed nuclear NFκB immunoreactivity only in luminal epithelial cells in PCOS-like rats treated with saline (Fig. 5B, arrowhead). In contrast to luminal epithelial cells, treatment with metformin significantly increased NFκB immunoreactivity in the nuclei of glandular epithelial cells in insulin + hCG-treated rats (Fig. 5C, blue arrowhead).

3.6. The Influence of Metformin on Mitochondrial and Autophagy-related Protein Expression

We investigated whether metformin could alter the expression of mitochondria-specific proteins or AMPK subunits (α, β, and γ) in the rat uterus. We observed increased voltage-dependent anion channel (VDAC) and pyruvate dehydrogenase expression in PCOS-like rats compared to control rats. We also found that treatment with metformin increased VDAC expression and decreased superoxide dismutase 1 (SOD1) in PCOS-like rats compared to control rats (Fig. 6A). Further, quantitative protein measurements indicated that there were no significant differences in AMPKα and AMPKβ protein expression (Fig. S4A) or AMPKα phosphorylation and localization (Fig. S4B) between any of the groups. However, as a control experiment for metformin treatment, we found that metformin indeed increased AMPKα protein expression and phosphorylation in the PCOS-like rat liver (data not shown).

We sought to determine whether metformin could regulate autophagy-related protein expression in the rat uterus. We observed no differences in Beclin-1, LC3A, or Atg3 protein expression between any of the groups, but we observed significantly increased protein expression of LC3B and Atg12 in PCOS-like rats compared to control rats (Fig. 6B). Metformin treatment decreased Atg5 and Atg7 protein expression in control rats and PCOS-like rats, but there were no significant differences in autophagy-related protein levels between PCOS-like rats treated with metformin or saline (Fig. 6B).

3.7. Metformin Partially Prevents Implantation Failure

To better understand the functional consequence of metformin treatment on implantation under conditions of hyperandrogenism and insulin resistance, we examined whether the rate of uterine implantation is altered by metformin in PCOS-like rats. Although different implantation numbers were detected, control rats treated with metformin or saline displayed similar implantation rates (Fig. 7A). However, no implantation occurred in PCOS-like rats (0% implantation), but this was rescued to a large extent by treating these rats with metformin (Fig. 7A). We subsequently examined whether the rescuing effect of metformin on implantation in PCOS-like rats might be due to normalization of key genes that are involved in implantation. The expression of *Prl*, *Igfbp1*, *Lif*, *Il11*, *Spp1*, *Maoa*, *Ednrb*, *Hoxa10*, *Hoxa11*, *Hbegf*, and *Krt13* mRNAs was similar between rat uteri with implantation under both normal conditions with metformin treatment and under conditions of hyperandrogenism combined with insulin resistance (Fig. 7B). Of note, even with metformin treatment, PCOS-like rats without implantation exhibited decreased expression of *Prl*, *Lif*, *Maoa*, and *Sgk1* mRNAs and increased expression of *Pc6*, *Ednrb*, *Lrh1*, and *Hbegf* mRNAs (Fig. 7B). Furthermore, our data showed that there were no significant differences in body mass or ovarian or uterine weights between PCOS-like rats treated with saline or with metformin (Fig. S5B). Although the number of corpora lutea in PCOS-like rats was higher than in control rats, the number of follicles was similar between all groups (Fig. S5C). These data suggest that implantation failure is likely due to uterine dysfunction in PCOS-like rats and not to ovulation rate.

3.8. Metformin Affects Offspring Body Weight During Postnatal Development

To gain more insight into the action of metformin on pregnancy outcome in rats under conditions of hyperandrogenism and insulin

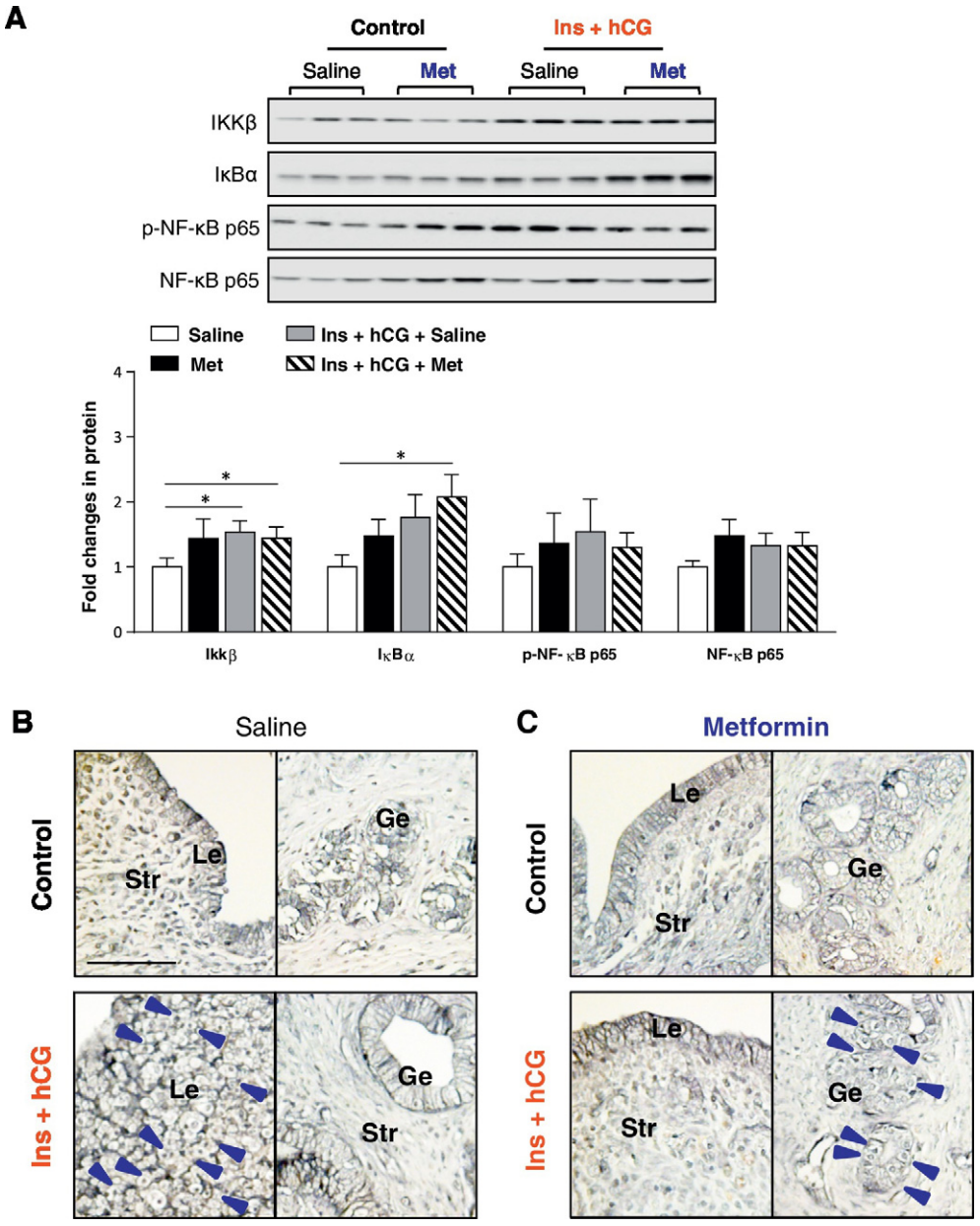


Fig. 5. Effects of metformin on the NF-κB signaling pathway and NF-κB translocation in the rat uterus. A, Western blot analysis of protein expression in the rat uterus was performed. Representative images and quantification of the densitometric data (n = 9/group) of IKKβ, IκBα, p-NF-κB (p65), and NF-κB (p65) are shown. Values are expressed as means ± SEM. Statistical tests are described under Material and Methods. *$p < 0.05$. B, Immunohistochemical detection of NF-κB (p65) in control rats treated with vehicle or metformin and insulin + hCG-treated rats treated with vehicle or metformin. Representative images (n = 10/group) are shown. Blue arrowheads indicate the positive immunostaining of NF-κB (p65) in the cell nuclei. Le, luminal epithelial cells; Ge, glandular epithelial cells; Str, stromal cells. Scale bars (100 μm) are indicated in the photomicrographs.

resistance, we measured the number of pups and the sex ratio, as well as the pups' body weight during postnatal development. The number of pups and the sex ratio in each litter were not significantly different between any of the groups (Fig. S6B). Interestingly, we found that the pups born to control rats treated with metformin had lower body weight at postnatal day 4, 7, 14, 28, and 35 compared to those from control rats treated with saline (Fig. S6C). We found that the pups born to PCOS-like rats treated with metformin only had decreased body weight at postnatal day 21 compared to those born to control rats treated with saline (Fig. S6C).

4. Discussion

Using a PCOS-like rat model, we show here that long-term treatment with metformin is associated with anti-androgenic and anti-inflammatory effects in the rat uterus. Importantly, we demonstrate that the sustained benefit of metformin is to rescue implantation failure in some PCOS-like rats by modulating the expression of key implantation-related genes in the uterus and not by improving ovarian function (e.g., follicular development and ovulation). We show that metformin alters uterine cell morphology through the suppression of circulating testosterone and uterine AR levels and through regulation of androgen–AR-mediated epithelial growth factors and epithelial–stromal interactions and that it inhibits insulin resistance-induced uterine inflammation through the downregulation of PI3K–Akt–NFκB-mediated inflammation-related gene expression. Given that metformin sufficiently suppresses hyperandrogenism and insulin resistance, exploring these mechanisms will help to optimize therapeutic treatment with metformin in PCOS patients with uterine dysfunction.

The process of implantation and pregnancy requires the coordinated interaction of uterine epithelial, stromal, and smooth muscle cells,

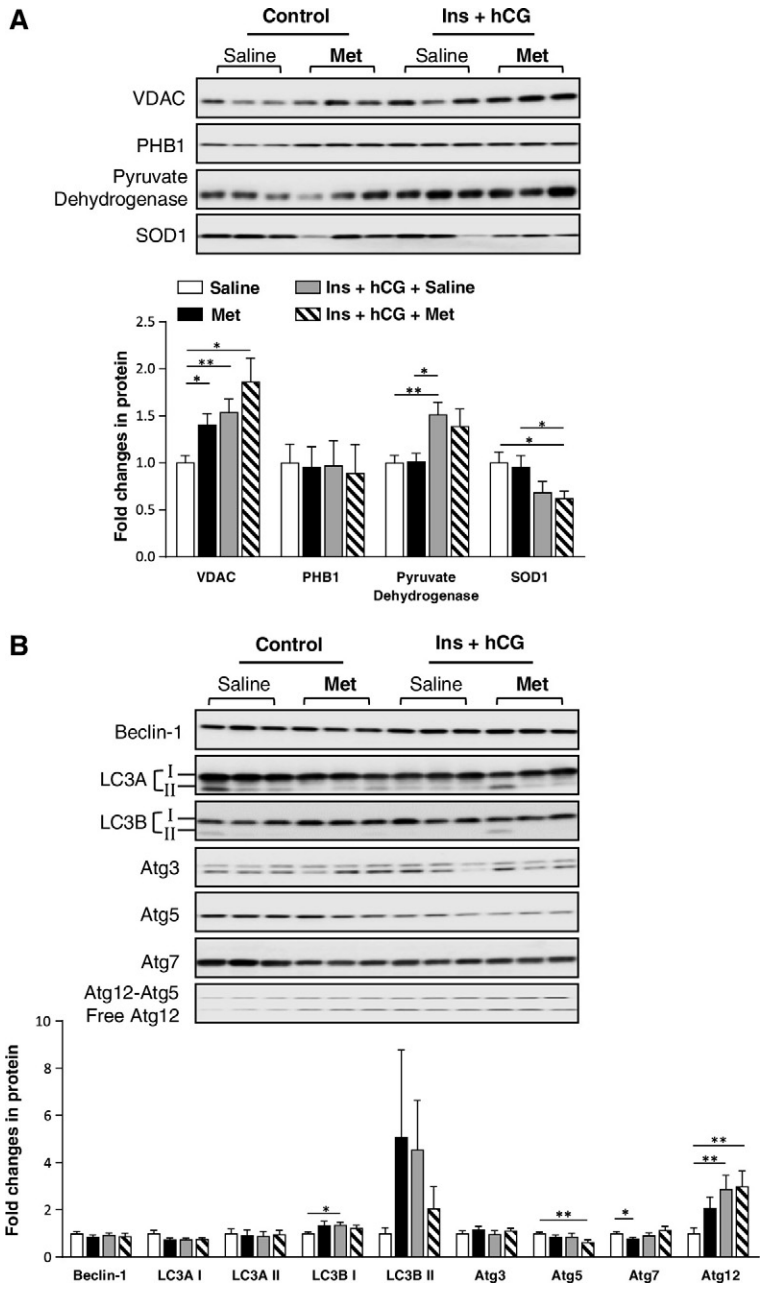
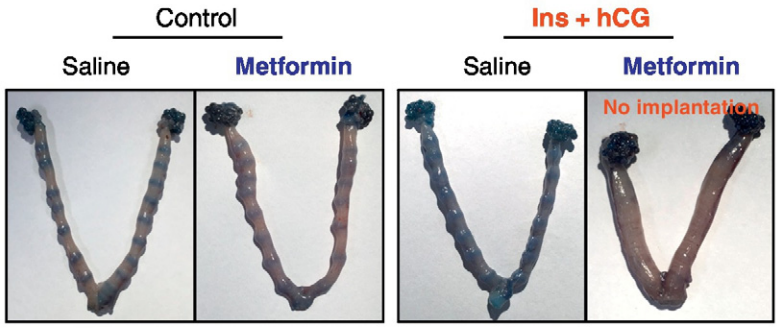


Fig. 6. Effects of metformin on mitochondrial and autophagy-associated protein expression in the rat uterus. Western blot analysis of protein expression in the rat uterus was performed. A, Representative images and quantification of the densitometric data (n = 9/group) of mitochondrial marker proteins (VDAC, PHB1, Pyruvate dehydrogenase, and SOD1). B, Representative images and quantification of the densitometric data (n = 9/group) for autophagy-associated proteins (B, Beclin-1, LC3A, LC3B, Atg3, Atg5, Atg7, and Atg12). Values are expressed as means ± SEM. Statistical tests are described under Material and Methods. *p < 0.05; **p < 0.01.

which not only drive their own set of distinct signaling pathways but also act together to regulate networks of signaling pathways (Cha et al., 2012; Zhang et al., 2013). In this study, PCOS-like rats exhibited high AR protein levels and increased numbers of glands and gland conglomerates in the uterus along with implantation failure. These findings are consistent with a recent study showing that chronic treatment with DHT increases AR-dependent epithelial cell proliferation in association with uterine gland formation in ovariectomized mice (Simitsidellis et al., 2016). Furthermore, the inhibitory effect of metformin on uterine AR expression in PCOS-like rats has confirmed its regulatory pattern as described previously in endometrial tissues collected from PCOS patients (Ito-Yamaguchi et al., 2015; Li et al., 2015a; Zhang and Liao, 2010). While activation of AR with DHT regulates epithelial growth factors and epithelial-stromal interactions in the mouse uterus (Simitsidellis et al., 2016), our current work has revealed that among the common regulatory genes metformin regulates DHT-induced alterations of *Wnt7c* and *Vcl* gene expression in the PCOS-like rat uterus.

It has been shown that inflammation is activated and increased in different endometrial cells and in the serum of PCOS patients (Liu et al., 2015; Matteo et al., 2010; Orostica et al., 2016; Piltonen et al., 2013; Piltonen et al., 2015), and the present study shows that the expression of an array of inflammation-related genes is dramatically altered in the PCOS-like rat uterus. For instance, uterine *TNFα* and *Il-6* gene expression is increased in PCOS-like rats, confirming that TNFα and Il-6 play inflammatory roles in the endometrial function in PCOS patients (Orostica et al., 2016; Piltonen et al., 2013, 2015). We also show that the anti-inflammatory effect of metformin is via the PI3k-Akt-NFκB signaling pathway. Similar to human endometrial stromal cells in vitro (Ferreira et al., 2014; Germeyer et al., 2011; Takemura et al., 2007) and PCOS patient sera in vivo (Victor et al., 2015), we show

A



	Saline	Met	Ins + hCG + Met
n	8	13	15
≥ 9 implantation sites	8 (100%)	10 (76.92%)	7 (46.67%)
< 9 implantation sites	0	3 (23.08%)	3 (20.00%)
No implantation	0	0	5 (33.33%)

B

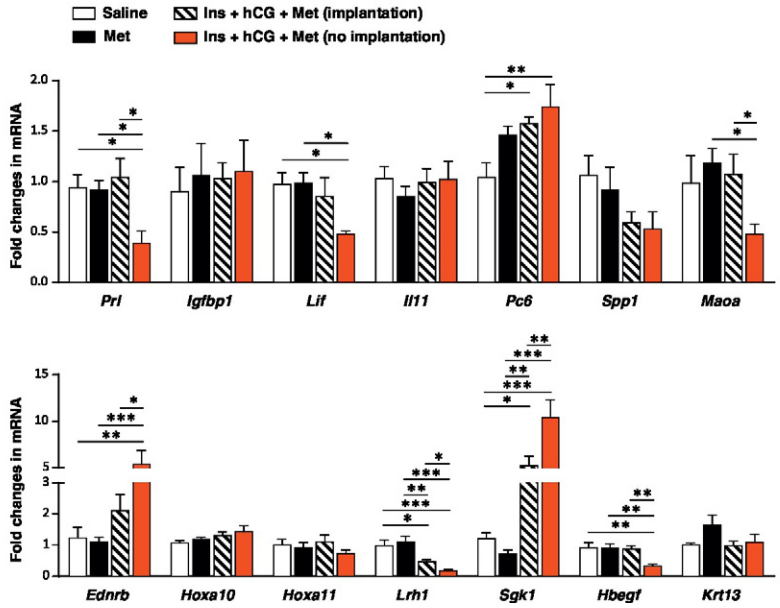


Fig. 7. Effects of metformin on uterine implantation rate and implantation-related gene expression in the rat uterus. A, Female rats were mated individually with fertile male rats according to their estrous cycle stage. Representative photomicrographs of uteri collected on day 6 after pregnancy with implantation sites visualized by Chicago Blue B dye injection. Because the insulin + hCG-treated rats without a normal estrous cycle display implantation failure, these rats treated with saline were excluded from the analysis. B, Uterine tissues (n = 7/group, except insulin + hCG-treated rats with metformin but no implantation, n = 5) were analyzed for mRNA levels of *Prl*, *Igfbp1*, *Lif*, *Il11*, *Pc6*, *Spp1*, *Maoa*, *Ednrb*, *Hoxa10*, *Hoxa11*, *Lrh1*, *Sgk1*, *Hbegf*, and *Krt13* by qRT-PCR. The mRNA level of each gene is shown relative to the mean of the sum of the *Gapdh* and *U87* mRNA levels in the same sample. Values are expressed as means ± SEM. Statistical tests are described under Material and Methods. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

that treatment with metformin suppresses PI3k–Akt-mediated increases in *TNFα*, *Il1b*, *Il6*, and *Il8* gene expression in the PCOS-like rat uterus. Indeed, metformin is capable of inhibiting uterine *Mcp1* and *Mmp2* gene expression, which is elevated in PCOS patients (Piltonen et al., 2015; Repaci et al., 2011). Furthermore, like the activation of NFκB signaling that leads to increased inflammation in ovarian granulosa cells and in serum in PCOS patients (Liu et al., 2015; Zhao et al., 2015), uterine cell-specific nuclear translocation of NFκB is different between PCOS-like rats treated with metformin compared to those treated with saline. Taken together, our results suggest that PI3K–Akt–NFκB-induced suppression of inflammation could be one of the key mechanisms through which metformin acts in the uterus. Interestingly, although acute inflammation is likely to induce autophagy in the mouse uterus (Park et al., 2016), and impaired endometrial autophagy has been observed in PCOS patients (Sumarac-Dumanovic et al., 2016), we failed to show that uterine cells capable of mitochondrial-dependent regulation of autophagy as judged by an increase in LC3II protein levels are sensitive to metformin treatment in PCOS-like rats. Further investigations are needed to elucidate if the autophagy contributes to uterine dysfunction under PCOS conditions.

The causes for implantation failure in PCOS patients might be due to endometrial defects as indicated by the dysregulation of the expression of proteins required for implantation in the human endometrium (Piltonen, 2016). A major finding of our study is that metformin treatment reverses endocrine (hyperandrogenism), metabolic (insulin resistance), and reproductive (absence of estrous cycle) abnormalities, and it also partially reverses the implantation failure to a large extent in most of the PCOS-like rats (Fig. 7A). A number of molecules in the uterus have been shown to be required for implantation, and aberrations in these molecules cause reproductive failure (Cha et al., 2012; Zhang et al., 2013). Together with studies showing that endometrial decidualization is impaired in PCOS patients (Piltonen et al., 2015) and that several implantation-related genes (e.g., *Lif*, *Ihh*, *Sgk1*, *Msx1*, *Hand2*, and *Muc1*) are dysregulated in the DHEA-treated mouse uterus (Li et al., 2016a), we

found that metformin fails to correct the abnormal levels of some of the implantation-related genes (*Lif*, *Pc6*, and *Sgk1*) in the PCOS-like rat uterus. Moreover, PCOS-like rats with implantation failure despite metformin treatment exhibited significant dysregulation of uterine *Prl*, *Maoa*, *Ednrb*, and *Hbegf* mRNA expression compared with control and PCOS-like rats with implantation. MAOA, EDNRB, and HBEGF all contribute to the enhancement of endometrial receptivity (Gibson et al., 2016), vascular permeability, and blood flow (Keator et al., 2011; Paria et al., 2001; Zhang et al., 2011). Although our study was not designed to determine whether aberrant regulation of *Maoa*, *Ednrb*, and *Hbegf* gene expression reduces endometrial receptivity and blood flow in PCOS-like rats, our results combined with a clinical study indicating the beneficial effect of metformin on endometrial receptivity and blood flow in PCOS patients (Palomba et al., 2006) led us to speculate that metformin improves uterine receptivity and blood flow through the correction of aberrant *Maoa*, *Ednrb*, and *Hbegf* gene expression under hyperandrogenism and insulin resistance conditions and that this is important for the establishment of implantation and subsequent fertility. Indeed, several clinical studies have reported that endometrial MAOA and HBEGF expression is decreased in infertile women (Leach et al., 2012; Vargas et al., 2012).

Our findings also indicate that the failure of implantation is not due to disturbed follicular development or anovulation in PCOS-like rats treated with metformin. Because clinical studies have reported that metformin therapy improves endometrial receptivity and reduces the risk of miscarriage and premature birth in PCOS patients (Feng et al., 2015; Palomba et al., 2006), the present study provides molecular evidence for treatment with metformin in PCOS patients with endometrial-induced infertility. However, it cannot be discounted that treatment of control rats with metformin contributed to reduced body weight in the offspring during their development. Whether such a reduction of body mass will lead to alteration of reproductive or other functions in adults warrants further investigation.

Previous results, including reports from our group, have indicated a significant difference in endometrial AMPKα expression and activation between non-PCOS and PCOS patients (Carvajal et al., 2013; Li et al., 2015b), but our current findings suggest that AMPKα is expressed in the rat uterus but its protein level is not changed in PCOS-like rats compared to control rats. Moreover, unlike what is seen in humans, we show that the uterine AMPKα activation is much more stable in PCOS-like rats. It has been suggested that increased expression and activation of AMPK is one of the crucial cellular mediators responsible for the effects of metformin treatment in several tissues and cell types (Foretz et al., 2014). Although others have reported that treatment with metformin enhances endometrial AMPKα expression and activation in PCOS patients with hyperinsulinemia (Carvajal et al., 2013), the present study does not support the hypothesis that uterine AMPKα expression and activation are preferentially regulated by metformin in rats with hyperandrogenism and insulin resistance. While the reasons for different regulation and activation of uterine AMPKα by metformin between humans and rats are not clear, it is tempting to speculate from these observations that the regulation of uterine AMPK is species specific and that the effects of metformin in the rat uterus are the same as in other tissues and cells (Foretz et al., 2014) and are independent of AMPK expression and activation.

In conclusion, the present study provides the comprehensive examination of the beneficial consequences of therapeutic doses of metformin in the PCOS-like rat uterus (Fig. 8). Strategies to treat PCOS-related reproductive disturbances, including long-term treatment with metformin, remain unstandardized. Therefore, whether similar molecular mechanisms and improvement in uterine function as a result of metformin therapy occur in PCOS patients who are predisposed to infertility needs to be answered by an appropriate controlled clinical trial.

Funding Sources

This work was supported by the Swedish Medical Research Council (grant number 10380), the Swedish Federal Government under the LUA/ALF agreement (grant number ALFGBG-147791), the Jane and Dan Olsson's Foundation, the Hjalmar Svensson Foundation, and the Adlerbert Research Foundation to HB and RS as well as the National Natural Science Foundation of China (grant number 81303118 to YZ, and grant number 81572555 to XL), the Project of Young Innovation Talents in Heilongjiang Provincial University (grant number UNPYSCT-

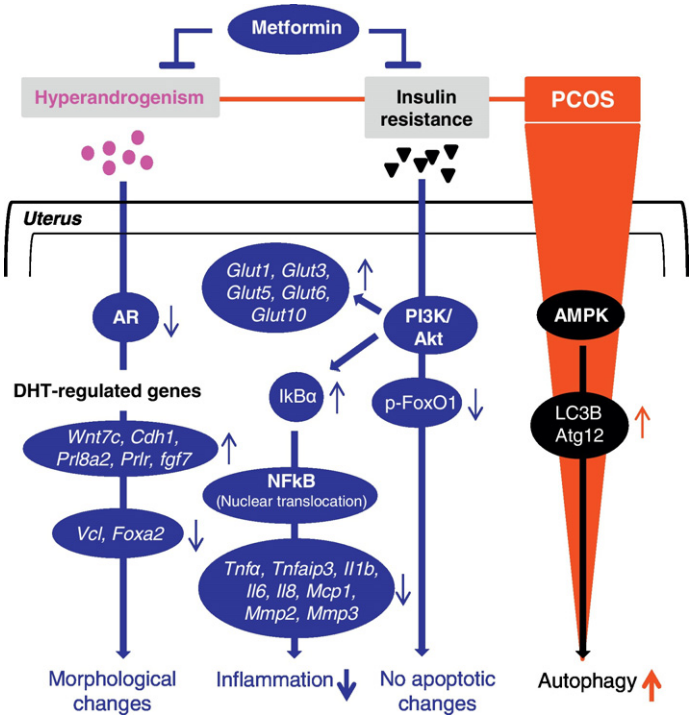


Fig. 8. Schematic representation of the actions of metformin in the PCOS-like rat uterus.

2015121), the Project of Innovation Talents (Young Reserve Talents) in Harbin city (grant number 2015RAQYJ089), and the Project of Excellent Innovation Talents by Heilongjiang University of Chinese Medicine to YZ.

Conflicts of Interest

The authors indicate no potential conflicts of interest.

Author Contributions

RS conceived the idea, designed the project and protocols, and developed the collaborations. YZ, MH, FM, XS, HX, JZ, PC, NM, XL, WL, and RS performed the experiments and analyzed the results. YZ, MH, and RS wrote the manuscript. MB, RS, and HB edited the manuscript. XL, XKW, MB, RS, and HB provided scientific oversight and guidance and edited the manuscript. YZ, MH, RS, and HB are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2017.03.023.

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