RESEARCH

Uterine progesterone signaling is a target for metformin therapy in PCOS-like rats

Min Hu1,2,*, Yuehui Zhang1,3,*, Jiaxing Feng3,*, Xue Xu3, Jiao Zhang4, Wei Zhao3, Xiaozhu Guo3, Juan Li1,2, Edvin Vestin1, Peng Cui1,5,6, Xin Li1,2,8, Xiao-ke Wu3, Mats Brännström9, Linus R Shao1 and Häkan Billig1

1Department of Physiology/Endocrinology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
2Department of Traditional Chinese Medicine, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China
3Department of Obstetrics and Gynecology, Key Laboratory and Unit of Infertility in Chinese Medicine, First Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin, China
4Department of Acupuncture and Moxibustion, Second Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin, China
5Department of Integrative Medicine and Neurobiology, State Key Lab of Medical Neurobiology, Shanghai Medical College and Institute of Acupuncture Research (WHO Collaborating Center for Traditional Medicine), Institute of Brain Science, Fudan University, Shanghai, China
6Institute of Integrative Medicine of Fudan University, Shanghai, China
7Department of Gynecology Obstetrics and Gynecology, Hospital of Fudan University, Shanghai, China
8Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai, China
9Department of Obstetrics and Gynecology, Sahlgrenska University Hospital, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Correspondence should be addressed to L R Shao: linus.r.shao@fysiologi.gu.se
*(M Hu, Y Zhang and J Feng contributed equally to this work)

Abstract

Impaired progesterone (P4) signaling is linked to endometrial dysfunction and infertility in women with polycystic ovary syndrome (PCOS). Here, we report for the first time that elevated expression of progesterone receptor (PGR) isoforms A and B parallels increased estrogen receptor (ER) expression in PCOS-like rat uteri. The aberrant PGR-targeted gene expression in PCOS-like rats before and after implantation overlaps with dysregulated expression of Fkbp52 and Ncoa2, two genes that contribute to the development of uterine P4 resistance. In vivo and in vitro studies of the effects of metformin on the regulation of the uterine P4 signaling pathway under PCOS conditions showed that metformin directly inhibits the expression of PGR and ER along with the regulation of several genes that are targeted dependently or independently of PGR-mediated uterine implantation. Functionally, metformin treatment corrected the abnormal expression of cell-specific PGR and ER and some PGR-target genes in PCOS-like rats with implantation. Additionally, we documented how metformin contributes to the regulation of the PGR-associated MAPK/ERK/p38 signaling pathway in the PCOS-like rat uterus. Our data provide novel insights into how metformin therapy regulates uterine P4 signaling molecules under PCOS conditions.

Introduction

Polycystic ovary syndrome (PCOS) is a clinically and etiologically heterogeneous hormone-imbalance disorder that is associated with multiple reproductive and metabolic abnormalities (Rosenfield & Ehrmann 2016). Women suffering from PCOS present with arrested folliculogenesis and chronic anovulation-linked infertility (Azziz et al. 2016, Rosenfield & Ehrmann 2016), and they also have more adverse reproductive risk as evidenced
by an increase in the prevalence of implantation failure, recurrent miscarriage, spontaneous abortion, premature delivery, endometrial carcinoma (Goodarzi et al. 2011, Shao et al. 2014b, Palomba et al. 2015). In addition to the ovarian dysfunction (Azziz et al. 2016, Rosenfield & Ehrmann 2016), it is assumed that the impairment of endometrial function also contributes to PCOS-associated infertility (Evans et al. 2016). Although progesterone (P4)-based oral contraceptive therapy is often efficacious (Vrbikova & Cibula 2005, Lopes et al. 2014), perturbations in endometrial P4 signaling that result from attenuated responsiveness and resistance to P4 are common in the endometrium of a PCOS patient (Li et al. 2014a, Piltonen 2016). P4 resistance is a condition in which tissues and cells do not respond appropriately to P4 (Chrousos et al. 1986), and this is evidenced by endometriosis and endometrial hyperplasia that may progress to endometrial carcinoma despite supplementation with P4 or its analogs (Gunderson et al. 2012, Shao et al. 2014a). Gene profiling experiments have shown that different endometrial genes are likely to act in concert in this abnormal condition in PCOS patients (Kim et al. 2009, Savaris et al. 2011); however, how changes in the expression of P4 signaling molecules contribute to the P4 resistance in a PCOS patient’s endometrium is poorly understood.

P4 is an essential contributing factor in female reproductive tissues that regulates multiple physiological processes such as the menstrual cycle, implantation, pregnancy maintenance and labor initiation (Evans et al. 2016). There are two major progesterone receptor (PGR) isoforms, PGRA and PGRB, both of which are involved in a common P4 signaling pathway for uterine cell-specific proliferation and differentiation (Li et al. 2014a, Patel et al. 2015). P4 binding activates both PGR isoforms and leads to translocation from the cytosol to the nucleus followed by binding to the P4-responsive elements of the target genes, resulting in alterations of PGR-targeted gene expression depending on the recruitment of co-regulators (Patel et al. 2015). It has been reported that endometrial PGR expression is elevated in PCOS patients who have anovulation compared to PCOS patients who still ovulate and to non-PCOS patients (Quezada et al. 2006, Margarit et al. 2010). Additionally, PGR activity is also modulated by the cytoplasmic mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway (Gellersen & Brosens 2014, Patel et al. 2015). While high levels of ERK1/2 expression and activation reflect the P4-PGR signaling-induced decidualization status in human and rodent uteri (Thienel et al. 2002, Lee et al. 2013, Tapia-Pizarro et al. 2017), it remains to be determined whether suppression of MAPK/ERK signaling occurs in the endometrium and whether such dysregulation can negatively impact uterine function under PCOS conditions.

Metformin is an anti-diabetic drug that is a clinically approved treatment in PCOS patients worldwide (Naderpoor et al. 2015). Several diverse molecular mechanisms of metformin have been demonstrated in human endometrial carcinoma tissues in vivo and in different endometrial cancer cells in vitro (Shao et al. 2014b), and metformin’s therapeutic effects on endometrial function are evidenced by improvement of endometrial receptivity, enhancement of endometrial vascularity and blood flow and reversion of endometrial hyperplasia and carcinoma into normal endometria in some women with PCOS (Jakubowicz et al. 2001, Palomba et al. 2006, Li et al. 2014b). Our recent studies using a PCOS-like rat model found that chronic treatment with metformin has significant anti-androgenic and anti-inflammatory impacts in the uterus (Zhang et al. 2017). Given the central role of P4 signaling in uterine implantation (Patel et al. 2015, Evans et al. 2016) and the ability of metformin to rescue implantation failure in some PCOS-like rats by modulating the expression of multiple implantation-related genes in the uterus in vivo (Zhang et al. 2017), we speculated that the beneficial effects of metformin might be mechanistically linked to the uterine P4 signaling pathway under pathological conditions such as PCOS. To address this hypothesis, we analyzed PCOS-associated PGR isoform expression and the MAPK signaling network in human and rat uterine tissues. By combining a PCOS-like rat model (Zhang et al. 2016) and in vitro tissue culture approach (Li et al. 2015), we aimed to determine whether metformin directly reverses aberrant PGR-targeted and implantation-related gene expression in the PCOS-like rat uterus.

Materials and methods

Study approval

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

Experimental animals and tissue preparations

Adult female Sprague–Dawley rats (n=134) were obtained from the Laboratory Animal Centre of Harbin Medical University,
Harbin, China (License number SCXK 2013-001). Animals were housed in the animal care facility with free access to food and water and a controlled temperature of 22°C±2°C with a 12 h light/darkness cycle. Estrous cycles were monitored daily by vaginal lavage according to a standard protocol (Feng et al. 2010). All rats (70 days old) with the different stages of estrous cycle used in this study were confirmed by examination of vaginal smears under a light microscope for two sequential cycles (about 8–10 days). Any PCOS-like (insulin + hCG-treated) rats that exhibited prolonged estrous cycles (more than 5 days) were excluded from the study.

Experiment 1: Rats were randomly divided into control (saline treatment, \(n=20\)) and experimental (PCOS-like, \(n=20\)) groups. The experimental group was treated with insulin plus hCG to induce a PCOS-like metabolic and reproductive phenotype, and the control rats were treated with an equal volume of saline (Zhang et al. 2016, 2018). In brief, insulin was started at 0.5IU/day and gradually increased to 6.0IU/day between day 1 and the day 22 to induce hyperinsulinemia and insulin resistance, and 3.0IU/day hCG was given on all 22 days to induce hyperandrogenism. Animals were treated with twice-daily subcutaneous injections until the end of the experiment. Rats with repeated insulin injections have not shown any hypoglycemic episodes (Poretsky et al. 1992, Bogovich et al. 1999, Damario et al. 2000, Zhang et al. 2018). Detailed analysis of endocrine and metabolic parameters as well as the uterine morphology in these animals has been reported previously (Zhang et al. 2016).

On day 23, each group of rats was divided into two subgroups of 10 rats each (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). For treatment, metformin was dissolved in saline and given as a daily oral dose of 500 mg/kg by a cannula. The treatment time and tissue collection are described in our previous study (Zhang et al. 2017).

Experiment 2: Rats were randomly divided into control (saline treatment, \(n=21\)) and experimental (PCOS-like, \(n=15\)) groups and treated as described in Experiment 1 (Supplementary Fig. 1B). After metformin treatment, control and PCOS-like rats were mated with fertile males of the same strain to induce implantation, which was determined by the presence of a vaginal plug (day 1 of pregnancy). The rats were killed between 08:00 and 09:00 h on day 6 of pregnancy. To identify the implantation sites, rats were injected intravenously with a Chicago Blue B dye solution (1% in saline) and killed 10 min later. Uteri were dissected and assessed for clearly delineated blue bands as evidence of early implantation sites as described previously (Zhang et al. 2017).

Experiment 3: Rats were divided into control (saline treatment, \(n=9\)) and experimental (PCOS-like, \(n=39\)) groups and treated as described in the Experiment 1. On the 23rd day, the PCOS-like rats were divided into four subgroups and treated daily with P4 (4 mg/kg), RU486 (6 mg/kg), or both for 3 days. For treatment, P4 and RU486 were dissolved in 100% ethanol and resuspended in sesame oil. All subcutaneous injections were in a volume of 100 µL. An equal volume of 100% ethanol and sesame oil was injected into both healthy control rats and PCOS-like rats as experimental controls (Supplementary Fig. 1C). The pharmacological doses and treatment time intervals of P4 and RU486 were chosen on the basis of previous studies (Knox et al. 1996, Kim et al. 2006).

After dissection, the uterine horns were trimmed free of fat and connective tissue. One side of the uterus in each animal was fixed in 10% neutral formalin solution for 24 h at 4°C and embedded in paraffin for histochemical analysis. The other side was immediately frozen in liquid nitrogen and stored at −70°C for Western blot and quantitative real-time PCR (qRT-PCR) analysis.

Detailed description of the methods including the primary in vitro tissue culture and treatment, morphological assessment and immunostaining, protein isolation and Western blot analysis, RNA extraction and qRT-PCR analysis, and measurement of biochemical parameters used in this study are provided in Supplementary files.

Statistical analysis

GraphPad Prism was used for statistical analysis and graphing. For all experiments, \(n\)-values represent the number of individual animals. Data are represented as the means ± S.E.M. Statistical analyses were performed using SPSS, version 24.0 statistical software for Windows (SPSS). The normal distribution of the data was tested with the Shapiro–Wilk test. Differences between groups were analyzed by one-way ANOVA or two-way ANOVA, and this was 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. All \(P\) values less than 0.05 were considered statistically significant.

Results

Metformin alters PGR isoform and PGR-targeted gene expression in PCOS-like rats

The insulin + hCG-treated rats exhibit reproductive disturbances that mimic human PCOS (Zhang et al.
2016, 2017). Prompted by these findings, we set out to investigate the impact of P4 signaling in this model. First, we showed that although the ratio of PGRA to PGRB was not significantly different between control and PCOS-like rats, the PCOS-like rats had increased levels of uterine PGRA and PGRB (Fig. 1A). While PGR immunoreactivity was primarily evidenced in control rat uterine luminal and glandular epithelia as well as in the stroma, the immunoreactivity of luminal epithelial PGR expression was associated with increased numbers of luminal epithelial cells and increased immunoreactivity of PGR in the stroma in PCOS-like rats (Fig. 1B). Metformin treatment did not significantly affect PGR isoform expression in control rats and PCOS-like rats compared to those rats treated with saline (Fig. 1A). However, we found that PGR immunoreactivity was decreased in the luminal and glandular epithelia by metformin treatment in both control rats and PCOS-like rats compared to those treated with saline (Fig. 1B). Conversely, intense immunoreactivity of PGR expression was detected in the stroma located close to the luminal epithelia in control and PCOS-like rats treated with metformin (Fig. 1B). In contrast to the epithelia and stroma, no significant changes in PGR expression in the myometrium were found in any of the groups (data not shown). Because a large body of evidence indicates that regulation of P4 signaling results in changes in the expression of several PGR-targeted genes in the uterus (Bhurke et al. 2016), we profiled the expression of genes that are indicators for PGR activity in the rat uterus by qRT-PCR. Quantitative data indicated that Smo, and Nr2f2 mRNA levels were increased in PCOS-like rats compared to control rats treated with saline. In contrast,
the Fkbp52 mRNA level was decreased in PCOS-like rats compared to control rats (Fig. 1C). We next determined the actions of metformin treatment on PGR-targeted gene expression and showed that Ptch, Fkbp52 and Ncoa2 levels were increased in PCOS-like rats treated with metformin compared to PCOS-like rats treated with saline, while Smo and Nr2f2 mRNA levels were decreased on PCOS-like rats treated with metformin compared to those treated with saline (Fig. 1C).

Metformin partially prevents implantation failure in parallel with regulation of PGR isoform and PGR-targeted gene expression in PCOS-like rats

Metformin has been shown to partially rescue the disruption of the implantation process in PCOS-like rats (Zhang et al. 2017), and the altered endocrine and metabolic parameters in these animals are shown in Supplementary Table 4. After metformin treatment, total testosterone levels, the ratio of total testosterone to androstenedione and fasting insulin levels were all significantly higher in PCOS-like rats where implantation did not occur compared to those with implantation, as was insulin resistance as assessed by the homeostasis model assessment of insulin resistance, mirroring the endocrine and metabolic abnormalities in PCOS patients (Azziz et al. 2016, Rosenfeld & Ehrmann 2016). Of note, PCOS-like rats that failed to implant embryos also exhibited decreased P4 levels. These data suggest that implantation failure in PCOS-like rats treated with metformin is due not only to hyperandrogenism and insulin resistance, but also to impairment of P4 signaling in the uterus. Further morphological characterization of metformin-treated PCOS-like rats with no implantation revealed the infiltration of immune cells into the glandular epithelial cell layer in a similar manner to when hormone imbalances were studied in a previous report (Wira et al. 2005) (Supplementary Fig. 3, black arrowheads). To determine how impairment of P4 signaling causes implantation failure, we subsequently analyzed PGR isoform and PGR-targeted gene expression in PCOS-like rats with no implantation. Although treatment with metformin increased PGR isoform expression in control and PCOS-like rats, neither the PGRA nor PGRB protein level was altered between PCOS-like rats with implantation and with failed implantation (Fig. 2A). As shown in Fig. 2B, while PGR protein was expressed in the deciduating stroma at the site of implantation in all groups, PGR immunoreactivity was increased in the stroma of the inter-implantation region in control rats treated with metformin. Furthermore, we found that the immunoreactivity of PGR was increased in the epithelia in PCOS-like rats without implantation despite metformin treatment. Thus, metformin appeared to participate in the regulation of uterine PGR expression in a cell type-specific manner in PCOS-like rats before and after implantation. qRT-PCR data indicated that Ihh and Ncoa2 mRNAs were increased and that Ptch and Fkbp52 mRNAs were decreased in metformin-treated PCOS-like rats with no implantation compared to control rats treated with saline or metformin and to metformin-treated PCOS-like rats with implantation (Fig. 2C).

Metformin directly regulates PGR isoform, PGR-target and implantation-related gene expression in vitro

Based on these in vivo observations, we asked whether the effect of metformin was direct or indirect in the PCOS-like rat uterus. In vitro uterine tissue culture experiments revealed that Pgr and Pgrb, mRNA levels were higher in PCOS-like rats compared to control rats, in agreement with alteration of PGR isoform protein expression (Fig. 1C). Furthermore, metformin treatment increased Pgr and Pgrb mRNA levels in control and PCOS-like rats in a time-dependent manner (Fig. 3A). Consistent with the in vivo effects of metformin in PCOS-like rats (Fig. 1C), Ihh, Smo and Nr2f2 mRNA levels were increased in the PCOS-like rat uterus compared to the control rat uterus and were downregulated by metformin treatment in vitro. While the Hand2 mRNA level was upregulated by metformin treatment at 48h and 72h, we detected the upregulation of Ptch, Fkbp52 and Ncoa2 mRNA levels in the PCOS-like rat uterine tissues over a 72-h course after metformin treatment (Fig. 3A).

The expression of a number of implantation-related genes has been reported to be regulated by metformin treatment in PCOS-like rats during implantation (Zhang et al. 2017). These previous observations prompted further analysis of implantation-related gene expression by metformin treatment in vitro. In contrast to the different regulation patterns of Spp1, Lrh1, Sgk1 and Krt13 mRNAs under in vivo and in vitro conditions, the in vitro responses of uterine Prl, Igfbp1, Il11, Pc6, Maoa, Ednrb, Hoxa10, Hoxa11 and Hbegf mRNA levels to metformin (Fig. 3B) were coincident with the in vivo regulation of the expression pattern of these genes (Zhang et al. 2017). Our data indicated that metformin directly upregulates uterine Prl, Maoa, Ednrb and Hbegf mRNA levels in PCOS-like rats during implantation in vivo.
To ascertain whether the modulation of uterine gene expression is P4 mediated and PGR dependent in PCOS-like rats, insulin + hCG-treated rats were injected subcutaneously with P4 and/or RU 486 for three days. As shown in Fig. 4, the increased PGR isoform protein levels (Fig. 1A) were confirmed by analysis of Pgr and Pgrb mRNA expression in the PCOS-like rat uterus. Although treatment with P4 and/or RU486 did not significantly affect Pgr mRNA expression, we found that Pgrb mRNA levels were decreased in PCOS-like rats compared to those rats with no treatment (Fig. 4). Among seven PGR-targeted genes (Fig. 1C), we found that Ptc, Hand2 and Fkbps2 mRNA levels were increased and that Ihh, Smo and Nr2f2 mRNA levels were decreased in PCOS-like rats treated with P4 compared to those rats with no treatment. We also observed that treatment with RU486 alone or combined with P4 reversed the changes in Smo, Hand2 and Fkbps2 mRNA levels in PCOS-like rats (Fig. 4). No significant differences of uterine Ncoa2 mRNA expression were observed in PCOS-like rats regarding the different treatments. Based on our current experimental approaches, it is likely that another regulatory mechanism contributes to the metformin-induced upregulation of Ncoa2 mRNA levels in PCOS-like rats.

**Metformin regulates the MAPK signaling pathway in PCOS-like rats before and after implantation**

In an attempt to understand the changes in PGR activation and function observed in PCOS patients...
(Patel et al. 2015), we performed a Western blot analysis to measure the expression of several proteins that are involved in the MAPK signaling pathway in the uterus after metformin treatment. As shown in Fig. 5A, there was no significant difference in p-c-Raf, p-MEK1/2, p-ERK1/2, p-p38 MAPK or p38 MAPK expression between saline-treated and metformin-treated rats. Quantitative protein data indicated that the expression of p-p38 MAPK and p38 MAPK was significantly decreased in PCOS-like rats compared to control rats. Nevertheless, metformin treatment only reversed p-p38 MAPK protein expression in PCOS-like rats.

We next assessed whether the MAPK/ERK signaling pathway contributes to uterine implantation in control and PCOS-like rats treated with metformin. As shown in Fig. 5B, although the p-MEK1/2 level was decreased in...
control rats treated with metformin compared to control rats treated with saline, no significant difference in p-c-Raf, p-ERK1/2, ERK1/2, p-p38 MAPK or p38 MAPK expression between these two groups was found. Furthermore, our data showed that p-c-Raf, p-MEK1/2 and p-ERK1/2 protein levels were downregulated in PCOS-like rats treated with metformin regardless of the occurrence of implantation. We also found that after metformin treatment PCOS-like rats with implantation exhibited decreased p-p38 MAPK, but not p38 MAPK, expression.

Upregulation of estrogen receptor (ER) expression in PCOS-like rats can be suppressed by metformin

Because estrogen-ER signaling regulates uterine PGR expression and activity (Li et al. 2014a, Patel et al. 2015) and because increased circulating E2 in PCOS-like rats can be inhibited by metformin treatment (Zhang et al. 2017), we sought to determine whether ER subtypes (ERα and ERβ) are involved in the regulation of aberrant PGR expression in PCOS-like rats and, if so, if metformin possibly alters ER subtype expression. Our data showed that PCOS-like rats exhibited increased Esr1 (ERα) and Esr2 (ERβ) mRNA levels, which were suppressed by metformin treatment. As shown in Fig. 6A, while nuclear ERα immunoreactivity was detected in the epithelia and stroma in control rats treated with saline (Fig. 6B1), immunoreactivity of ERα was increased in the glandular epithelia and stroma in PCOS-like rats (Fig. 6D1). Furthermore, treatment with metformin led to decreased ERα immunoreactivity in control (Fig. 6C1) and PCOS-like rats (Fig. 6E1). No obvious difference in ERα immunoreactivity was observed...
in the myometrium in any of the groups (Fig. 6B2, C2, D2 and E2). We also found that ERβ was mainly co-localized with ERα in the epithelia and stroma but not in the myometrium in control and PCOS-like rats regardless of the different treatments. Furthermore, with metformin treatment, we noted a significant increase in uterine Esr1 and Esr2 mRNAs in PCOS-like rats without implantation (Fig. 7A). Immunofluorescence staining revealed that, overall, immunoreactivities of both ERα and ERβ were diminished in the decidualizing stroma at the site of implantation (Fig. 7B1, C1 and D1), in the epithelia and stroma of the inter-implantation region (Fig. 7B2, C2 and D2) in control rats treated with saline or metformin (Fig. 7B2 and C2), and in the inter-implantation site of PCOS-like rats treated with metformin (Fig. 7D2) compared to those rats before implantation (Fig. 6B1, C1, D1, E1, B2, C2, D2 and E2). Interestingly, PCOS-like rats with no implantation exhibited sustained nuclear ERα immunoreactivity in the glandular epithelia and stroma (Fig. 7E1).

**Differential cell-specific expression of phospho-histone H3 in PCOS-like rats treated with metformin**

As previously demonstrated (Avellaira et al. 2006), p-histone H3 is of special interest because the endometrium of PCOS patients displays high levels of p-histone H3, which is associated with cellular processes such as mitosis (Brenner et al. 2003). Quantitative assessment of p-histone H3 indicated that no significant change in p-histone H3 immunoreactivity was present in the epithelia or stroma in any of groups (Supplementary Fig. 4E); however, metformin treatment decreased p-histone H3 immunoreactivity in the myometrium in PCOS-like rats compared to those treated with saline. Of note, intensely p-histone H3-positive stromal cells close to the luminal and glandular epithelia were found in PCOS-like rats treated with metformin (Supplementary Fig. 4D2). Similarly, p-histone H3 immunoreactivity was significantly increased in the stroma at the inter-implantation sites in PCOS-like rats treated with metformin.
independently of implantation (Supplementary Fig. 5E). In PCOS-like rats without implantation, p-histone H3 immunoreactivity was often detected in the luminal epithelia (Supplementary Fig. 5D1), although this was not statistically significant compared to PCOS-like rats with implantation (Supplementary Fig. 5E). It is thus likely that the regulation of mitotic activity by metformin is cell type dependent in the uterus.

Discussion

Reproductive dysfunction and infertility manifest noticeably in PCOS patients (Evans et al. 2016). In striking contrast to the attention given to hyperandrogenism and insulin resistance in women with PCOS, the aberrant P4 signaling pathway resulting in uterine P4 resistance has received much less attention (Li et al. 2014a, Piltonen 2016). This study is the first to show that the therapeutic effects of metformin on the regulation of uterine function in PCOS-like rats is mediated through P4 signaling.

Elucidating the regulation of endometrial PGR levels under PCOS conditions is important clinically. Our data show that increased PGR expression is paralleled with elevated ER expression in PCOS-like rats. This expression pattern is associated with an increased circulating E2 level (Zhang et al. 2016), suggests that E2-ER signaling...
contributes to the upregulation of PGR under PCOS conditions in vivo. Similar to PCOS patients (M Hu et al. unpublished observations), PCOS-like rats also displayed high levels of PGR isoforms and ER subtypes in the uterus. The induction of implantation is required for the activation of PGR, and implantation subsequently alters gene expression in the endometrium (Gellersen & Brosens 2014, Patel et al. 2015); however, PGR-targeted gene expression in PCOS patients and PCOS-like rats has only been demonstrated to a limited degree. The current study shows that significantly decreased Fkbp52 gene expression parallels increased expression of Ihh, Smo and Nr2f2 mRNAs without changes in Ncoa2 mRNA in PCOS-like rats. In addition, we also found that abnormal expression of PGR-target genes, including Fkbp52 and Ncoa2, is retained in PCOS-like rats with implantation failure. This is supported by in vivo studies showing that mice lacking Fkbp52 (Yang et al. 2006, Tranguch et al. 2007) or Ncoa2 (Mukherjee et al. 2006, 2007) demonstrate the absence of decidualization after P4 supplementation due to diminished P4 responsiveness. Previous studies have reported that women with endometriosis and endometrial hyperplasia/carcinoma who develop P4 resistance have low levels of PGR expression (Gunderson et al. 2012, Shao et al. 2014a). Although it is currently unclear why differences exist in the regulation of uterine PGR expression between different diseases with P4 resistance, it is likely that uterine P4 resistance...
in PCOS-like rats is due to impaired PGR activity rather than PGR expression.

Defects in PGR isoform-specific P4 signaling in the mouse uterus can give rise to distinct phenotypes of uterine impairment and implantation failure (Li et al. 2014a). Here, we observed no changes in total Pgr mRNA levels but a reduction of Pgrb mRNA levels in PCOS-like rat uterus after treatment with P4 and/or RU486. This suggests that uterine Pgrα mRNA levels are increased. Meanwhile, several PGR-target genes (e.g., Ihh, Smo, Ptc, Nr2f2, Hand2 and Fkbp52) are significantly altered in PCOS-like rats after P4 treatment. Thus, we speculate that changes in these P4-dependent PGR-target gene expression in PCOS-like rat uterus might be accounted for by an increase in Pgrα mRNA expression. Studies of mutant mice lacking specific PGR isoform will clarify the functional differences between the two PGR isoforms in the progression of PCOS-induced uterine dysfunction.

P4-mediated and PGR-dependent regulation of ERK1/2 expression plays a critical role in humans and rodents during endometrial decidualization and implantation (Thienel et al. 2002, Lee et al. 2013, Tapia-Pizarro et al. 2017), but such regulation under PCOS conditions has not previously been reported. The inhibition of ERK1/2 expression and activation has been reported in ovarian granulosa and thecal cells in PCOS patients (Nelson-Degrave et al. 2005, Lan et al. 2015), and we have previously shown that the expression and activation of uterine ERK1/2 is suppressed in rats treated with insulin and hCG to induce the PCOS phenotype (Zhang et al. 2016). The present study supports and extends this work. Here, we observed no changes in p-ERK1/2 or ERK1/2 expression in the rat uterus after prolonged treatment with insulin and hCG. However, we observed that the levels of p-c-Raf and p-MEK1/2, two upstream regulators of ERK1/2, were significantly decreased in PCOS-like rats after uterine implantation, establishing a tight link between different MAPK/ERK signaling molecules. Our data suggest that regulation of uterine ERK1/2 expression in vivo is time dependent (Thienel et al. 2002, Lee et al. 2013, Tapia-Pizarro et al. 2017), which is similar to the regulation of PGR isoforms and PGR-targeted gene expression. The MAPK/ERK/p38 signaling pathway contributes to the regulation of inflammation and cytokine production (Cuadrado & Nebreda 2010, Arthur & Ley 2013), and the dysregulation of inflammation-related molecules is associated with PCOS conditions (Matteo et al. 2010, Piltonen et al. 2013, 2015, Orostica et al. 2016). Furthermore, like the activation of NFκB signaling that induces the transcriptional levels of inflammation-related gene expression in ovarian granulosa cells and in serum in PCOS patients (Liu et al. 2015, Zhao et al. 2015), our previous study has shown that the sustained metformin treatment markedly suppresses uterine inflammatory gene expression,
especially the Il-6 and TNAF α mRNAs that are associated with inhibition of nuclear NFκB translocation in PCOS-like rats (Zhang et al. 2017). Importantly, p38 can antagonize ERK1/2 signaling mediated by protein phosphatase 2A and consequently down-regulate inflammatory cytokine and chemokine production (Cuadrado & Nebreda 2010), and the anti-inflammatory effects of MAPK/p38 are involved in the regulation of NFκB activity (Arthur & Ley 2013). These observations further indicate that metformin inhibits NFκB-driven inflammatory processes through p38 activation rather than through ERK1/2 inhibition in the PCOS-like rat uterus.

The results of the present study permit us to draw the following conclusions (Fig. 8). (1) With sustained low levels of P4, the expressions of both uterine PGR isoforms are elevated in PCOS-like rats in vivo. This is positively associated with the high levels of ERs in PCOS-like rats. Consistent with mouse-knockout studies, altered expression of Fkbp52 and Ncoa2, two genes that contribute to uterine P4 resistance, is seen in PCOS-like rats before and after implantation. (2) Metformin directly suppresses uterine PGR isoform expression along with the correction of aberrant expression of PGR-targeted and implantation-related genes in PCOS-like rats. Abnormal cell-specific regulation of PGR and ER, paralleling the aberrant expression of PGR-targeted and implantation-related genes, is retained in PCOS-like rats with implantation failure. (3) Increased PGR expression is associated with inhibition of the MAPK/ERK/p38 signaling pathway, and the primary effect of metformin treatment is to restore the MAPK/p38 signaling pathway in the PCOS-like rat uterus. Taken together, our findings provide support for metformin therapy in the improvement of P4 signaling in PCOS-like rats with uterine dysfunction and for its clinical relevance in the treatment of PCOS patients with P4 resistance.

Acknowledgements
The authors thank the Centre for Cellular Imaging at the University of Gothenburg and the National Microscopy Infrastructure (grant number VR-RFI 2016-00968) for providing assistance in microscopy.

References

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0086.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
Research reported in this publication 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 H B and L R S) as well as the National Natural Science Foundation of China (grant number 81774136), the Project of Young Innovation Talents in Heilongjiang Provincial University (grant number UNPYSCT-2015121), the Project of Innovation Talents (Young Reserve Talents) in Harbin city (grant number 2015RAQY089), and the Project of Excellent Innovation Talents by Heilongjiang University of Chinese Medicine (to Y 2).
Knox KL, Ringstrom SJ, Szabo M, Perlyn CA, Sutandi S & Schwartz NB 1996 RU486 on an estrogen background blocks the rise in serum follicle-stimulating hormone induced by antisem to inhibit or ovariectomy. Endocrinology 137 1226–1232. (https://doi.org/10.1210/endo.137.4.8625893)
Lan CW, Chen MJ, Tai KY, Yu DC, Yang YC, Jan PS, Yang YS, Chen HF & Ho MN 2015 Functional microarray analysis of differentially expressed genes in granulosa cells from women with polycystic ovary syndrome related to MAPK/ERK signaling. Scientific Reports 5 14994. (https://doi.org/10.1038/srep14994)
Piltonen TT, Chen JG, Khatun M, Kangasniemi M, Liakka A, Spitzer T, Tran N, Heddleston H, Irwin JC & Giudice LC 2015 Endometrial stromal fibroblasts from women with polycystic ovary syndrome have impaired progesterone-mediated decidualization, aberrant cytokine profiles and promote enhanced immune cell migration in vitro.


Received in final form 9 March 2018
Accepted 13 March 2018
Accepted Preprint published online 13 March 2018