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Effect of the interaction of metformin and bone morphogenetic proteins on ovarian steroidogenesis by human granulosa cells

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ABSTRACT

In the present study, we studied the effects of metformin and its interactions with the actions of bone morphogenetic proteins (BMPs) on ovarian steroidogenesis. It was revealed that metformin treatment enhanced progesterone production by human granulosa KGN cells and rat primary granulosa cells induced by forskolin and FSH, respectively. In human granulosa cells, it was found that metformin treatment suppressed phosphorylation of Smad1/5/9 activated by BMP-15 compared with that induced by other BMP ligands. Moreover, metformin treatment increased the expression of inhibitory Smad6, but not of that Smad7, in human granulosa cells, while metformin had no significant impact on the expression levels of BMP type-I and -II receptors. Thus, the mechanism by which metformin suppresses BMP-15-induced Smad1/5/9 phosphorylation is likely, at least in part, to be upregulation of inhibitory Smad6 expression in granulosa cells. The results suggest the existence of functional interaction between metformin and BMP signaling, in which metformin enhances progesterone production by down-regulating endogenous BMP-15 activity in granulosa cells.

1. Introduction

Metformin is an insulin-sensitizing agent and has been widely used for patients with type II diabetes. Metformin increases insulin sensitivity by decreasing glucose production in the liver and increasing glucose uptake in muscle. In addition to its anti-diabetic effects, metformin has been shown to exert beneficial effects on polycystic ovary syndrome (PCOS) and protective effects against cardiovascular diseases and cancers [1]. PCOS, which affects women of reproductive age, is characterized by the defect of ovulation and excess of androgens with manifesting menstrual irregularity, hirsutism and infertility [2]. Patients with PCOS also have increased risk factors for cardiovascular diseases just as shown in the patients with metabolic syndrome [3]. It has been considered that the existence of insulin resistance is highly involved in the pathogenesis of PCOS [4]. Therefore, clinical attention has been paid to the effects of metformin, which is linked to the improvement of insulin resistance, on changes of reproductive function in patients with PCOS.

On the other hand, it has been demonstrated that various growth factors expressed in the ovary play crucial and unique roles in integrating female reproduction in autocrine and/or paracrine fashion [5]. The growth factors, including bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs), mutually interact with gonadotropin actions in the ovary, leading to normal process of folliculogenesis and the following ovulation. Among these, the BMP system in ovarian follicles can mainly regulate the activity of FSH receptor (FSHR) signaling in granulosa cells [5,6].

In the present study, the effect of metformin on ovarian

Abbreviations: ActRII, activin type-II receptor; ALK, activin receptor-like kinase; AMPK, AMP-activated protein kinase; BMP, bone morphogenetic protein; BMPRII, BMP type-II receptor; FSH, follicle-stimulating hormone; FSK, forskolin; FSHR, FSH receptor; GCs, granulosa cells; GDF, growth and differentiation factor; IGF, insulin-like growth factor; 3βHSD, 3β-hydroxysteroid dehydrogenase; MAPK, mitogen-activated protein kinase; Met, Metformin; PCOS, polycystic ovary syndrome; P450arom, P450 aromatase; P450scc, P450 steroid side-chain cleavage enzyme; StAR, steroidogenic acute regulatory protein.

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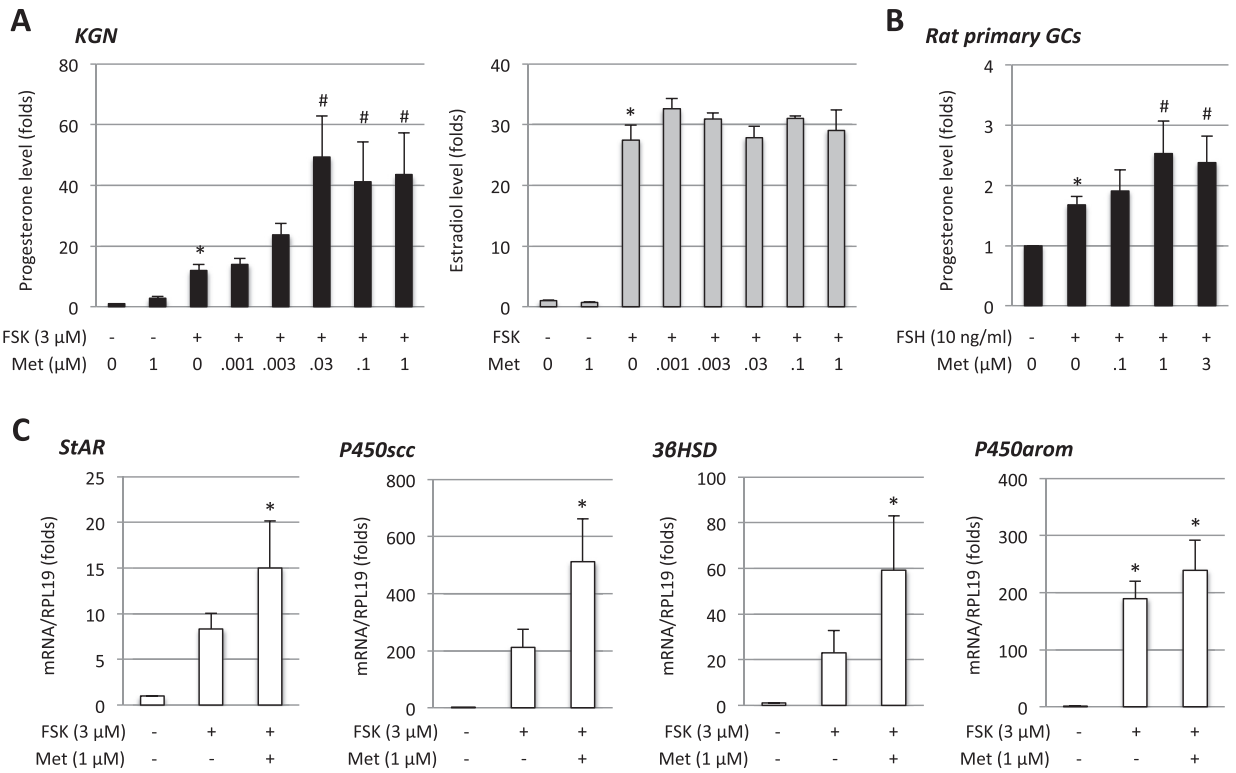

Fig. 1. Effects of metformin on steroidogenesis by human and rat granulosa cells. A, B) Conditioned media were collected from (**A**) human granulosa KGN cells and (**B**) rat primary granulosa cells (GCs) treated in a serum-free condition with the indicated concentrations of metformin either alone or in combination with FSK (3 μM) for 24 h or FSH (10 ng/ml) for 48 h, respectively, and the levels of progesterone and estradiol production were measured by CLIA and expressed as fold changes. **C**) Total cellular RNA was extracted from KGN cells treated with FSK (3 μM) either alone or in combination with metformin (1 μM) in a serum-free condition for 24 h, and mRNA expression levels of steroidgenetic genes including StAR, P450scc, 3βHSD and P450arom were determined by quantitative PCR. The expression levels of mRNA were normalized by RPL19 level and expressed as fold changes. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA. $^{*}P < 0.05$ vs. control groups; and $\#P < 0.05$ vs. the groups treated with FSK or FSH alone.

steroidogenesis and the functional interaction between metformin and BMP activity were investigated by utilizing the KGN cell line and rat primary granulosa cells. Actually, it has been recognized that treatment of PCOS patients with metformin alleviates hyperandrogenism and restores the menstrual cycle and ovulatory process [7]. However, the mechanisms underlying the effects of metformin on reproductive function and steroidogenesis have yet to be clarified. Here, it was uncovered that metformin is directly and functionally involved in progesterone production by modulating the BMP system in granulosa cells.

2. Materials and methods

2.1. Reagents and supplies

1:1 mixture of Dulbecco's Modified Eagle's Medium/Ham's F-12 medium (DMEM/F12), HEPES buffer, McCoy's 5A and Medium 199 were purchased from Invitrogen Corp. (Carlsbad, CA); 4-androstene-3,17-dione, diethylstilbestrol (DES), 3-isobutyl-1-methylxanthine (IBMX), ovine pituitary FSH, forskolin (FSK), and penicillin-streptomycin were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO); recombinant human BMP-2, -4, -6, -7, -9 and -15 were purchased from R&D Systems Inc. (Minneapolis, MN); and metformin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Cell preparations of KGN and rat granulosa cells

The KGN cells, originated from the human ovarian granulosa-like tumor cell line [8,9], were cultured in DMEM/F12 supplemented with 10%FCS and penicillin-streptomycin at 37 °C in an atmosphere of 5%CO_2. Primary culture of rat granulosa cells (GCs) was obtained from the ovaries of female Sprague-Dawley rats (Charles River, Wilmington, MA) exposed to DES-containing capsules (10 mg/tube) for 3 days by puncturing ovarian follicles with a 27-gauge needle and by filtering the cell suspension (BD Falcon, Bedford, MA) [10], and then the isolated granulosa cells were cultured in a serum-free McCoy's 5A medium supplemented with antibiotics at 37 °C with 5%CO_2 conditions. The protocol for animal experiments (OKU-2016065) was approved by Okayama University Institutional Animal Care and Use Committee.

2.3. Assays for estradiol and progesterone

KGN cells (1×10^5 viable cells/ml) were cultured in serum-free DMEM/F12 with androstenedione (100 nM; a substrate for aromatase) in 12-well plates, and then treated with FSK (3 μM) either alone or in combination with the indicated concentrations of metformin for 24 h. Rat granulosa cells (1×10^5 viable cells/0.2 ml) were cultured in serum-free McCoy's 5A with androstenedione (100 nM) in 96-well plates, and then treated with FSH (10 ng/ml) either alone or in combination with the indicated concentrations of metformin for 48 h. A range of metformin concentrations from 10 nM to 10 μM was screened, and by preliminary experiments, 1–3 μM of metformin was shown to elicit considerable changes in steroidogenesis. On the basis of results of our earlier *in vitro* experiments on the same culture conditions [11–15], each concentration of FSK (3 μM), FSH (10 ng/ml) and BMP ligands (100 ng/ml)

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