Actin Filaments are Necessary for FSH-Induced CYP19A1 Transcription in Bovine Granulosa Cells in vitro

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ABSTRACT

Estrogen biosynthesis is catalyzed by the Aromatase (P450arom), which is derived from CYP19A1. We investigated the relationship between actin filament rearrangement, CYP19A1 expression and estradiol bio-synthesis in bovine granulosa cells in vitro. Both bovine fibroblasts and granulosa cells were starved in serum-free culture medium for 24 hours. Serum-starvation disassembled stress fibers in fibroblasts, but not in granulosa cells. Additionally we added Y27632 and CB to medium to de-polymerize the actin filaments, while LPA and insulin were used to re-polymerize actin filaments. A real-time RT-PCR analysis revealed that, FSH induced transcription of CYP19A1 mRNA encoded aromatase was inhibited significantly as actin filaments were de-polymerized by both Y27632 and CB, whereas, restored as actin filaments were re-polymerized by both LPA and insulin. However, further study shows that, statistically there were no differences in the expression of aromatase proteins analyzed by western-blot, during the process of actin filament rearrangement. ARIA analysis also indicated that the secretions of estradiol by bovine granulosa cells were not changed significantly. The results suggested that serum-starvation could not induce actin filaments depolymerization in bovine granulosa cells, while chemically induced actin filament rearrangement influenced the expression of aromatase in mRNA level. But there were no differences in protein level and biological activity of bovine granulosa cells in vitro.


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INTRODUCTION

Follicle-stimulating hormone (FSH) is essential for the follicular maturation, generation of oocytes and estrogen secretion. Receptors for FSH (FSHRs) are located exclusively on granulosa cells in females (Hunzicker-Dunn et al., 2006; Talukder et al., 2014; Zhao et al., 2014). In ovarian granulose cells activation of the FSHR, upon binding to FSH or the FSH agonist pregnant mare's serum gonadotropin (PMSG), initiates a signaling cascade (Dupakuntla et al., 2012.). The signaling cascade regulates transcription of different genes, including rate-limiting enzyme cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1) (Sewer and Li, 2013), which is necessary for estrogen biosynthesis (Nelson et al., 1999). Aromatase (P450 arom) which catalyzes estrogen biosynthesis (Simpson et al., 1994; Michael et al., 1995) is a product of the CYP19A1 gene (Nelson et al., 1993). In the ovaries, aromatase converts testosterone to estradiol at smooth endoplasmic reticulum (Duarte et al., 2012; Young and McNeilly, 2010).

Furthermore, different researches indicates that, the actins filaments are a kind of cytoskeleton which play an important role in gene expression and cell signaling transduction (Murti et al., 1992; Fernandez-Valle et al., 1997; Grummt, 2006; Wolyniak and Sundstrom, 2007). The Actins filaments are also supposed to be playing a very vital role in sustaining the integrity of the cytoskeleton to maintain a proper cell shape, in addition to intra-cellular distribution as well as movement of various organelles, secretory proteins and hormones (Sims et al., 1992; Koukouritaki et al., 1996; Simon and Pon, 1996).

In previous researches, chemical-induced disruption of the actin filaments has been reported to inhibit LH-
stimulated progesterone production in ovarian and avian preovulatory granulosa cell models (Iczkowski and Hertelendy, 1991; Aharoni et al., 1993). In other steroidogenic cell models, such as adrenal, luteal, and MA-10 tumor Leydig cells, trophic hormones or cAMP analogs consistently promote rearrangement of the actin cytoskeleton, causing cells to round in shape (Nan et al., 2006), and disruption of the actin cytoskeleton often abrogates steroidogenesis, although in other instances, steroid genesis was enhanced (Nagy and Freeman, 1990; Gregoraszczuk and Stoklosowa, 1997; Karlsson et al., 2010). However, the relationships between actin filament rearrangement and estradiol biosynthesis are poorly understood.

The present study was design to know the inter-relationships between actin filament rearrangement and estradiol biosynthesis in bovine granulosa cells in vitro. For this purpose, we examined the transcription of CYP19A1, the expression of aromatase protein, and the secretion of estradiol during the process of actin filament rearrangement in bovine granulosa cells.

**MATERIALS AND METHODS**

All chemicals used during the study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA), otherwise specified specifically.

**Collection of the bovine fibroblasts and granulosa cells:** Experimental design is presented in Table 1. Fibroblasts were used in this study, because actin filaments in fibroblasts will depolymerize in the absence of serum (Hall, 1998; Hanna et al., 2001). Bovine skin fibroblasts were collected and culture as described by (Arat et al., 2002). Ovaries were collected from adult cows, at a local slaughterhouse and were transported to the laboratory at 35°C in PBS containing penicillin (100 IU ml⁻¹) and streptomycin (100 mg ml⁻¹). Granulosa cells were collected by aspiration using (20 gauge) needle from small follicles as described by (Sekar et al., 2000).

**Cell culture and fluorescent labeling of filamentous actin:** Both fibroblast and granulosa cells were cultured as described by Gutierrez et al. (1997). In brief, cells were cultured in DMEM/F12 (Gibco BRL) with containing Sodium bicarbonate (10 mmol l⁻¹), Sodium selenite (4 ng ml⁻¹), protease-free bovine serum albumin (0.1%), Penicillin (100 U ml⁻¹), Streptomycin (100 mg ml⁻¹), transferrin (2.5 mg ml⁻¹), nonessential amino acid mix (1.1 mmol l⁻¹), IGF-I, (10 ng ml⁻¹), testosterone (T, 10 ng ml⁻¹), protease-free bovine serum albumin (0.1%), and with or without FSH and chemicals as mentioned in experimental design (Table 1).

**Cells were fixed with 4% formaldehyde in PBS for 30 min, followed by permeabilization of the cells with 0.2% Triton X-100 in PBS for 15 min, and then incubated with 2% BSA in PBS for 30 min. Cells were incubated with FITC-phalloidin (1:100) for 1h and DAPI (1:1000) for 3 min in a humidified chamber. Cover slips were washed three times with PBS and mounted on glass microscope slides using polyvinyl alcohol alcohol medium containing 100 mg/ml DABCO (1,4-diazabicyclo [2.2.2] octane) as antifading reagent. Slides were visualized using the Olympus (FV1000) inverted laser scanning confocal microscope with Olympus Fluoview Ve3.0 Viewer software.**

**Real-time PCR:** Total mRNA was extracted using Trizol (TaKaRa Biotech, Dalian, Liaoning, China) from different groups. Total mRNA as template was reverse transcribed usingQuantscript RT Kit (TIANGEN Biotech, Beijing, China). RT- PCR analysis was performed using iQ SyBR Green supermix with ROX (Bio-Rad Laboratories, Hercules, CA, USA) in a MyIq single-color detection system (Bio-Rad Lab). PCR primers (SUN Biotech, Beijing, China) contained the following sequences bovine CYP19A1, forward GCATGCGGTACCAGCCCGTT and reverse CCCCCGGCCCCAAGGCGAAAT; bovine FSHR, forward CCAACCCTTCTCTATGCC and reverse TGGGCTTGCACCTCATAGCA. GAPDH was used as a housekeeping gene.

**Immunoblotting:** Immuno blotting analyses were performed using the protocols as described by (Wang et al., 2012). After blocking the membranes were incubated with primary antibodies (4°C, overnight) against Aromatase or β-actin. Then for 1h with secondary antibodies HRP-conjugated (Zhongsan biotechnology, Beijing, China) (1:5000) at room temperature, the protein bands were visualized by enhanced chemiluminescence detection reagents (Applygen Technologies Inc., Beijing, China) and X-Omat BT film (Kodak, Rochester, NY, USA), according to the manufacturer’s instructions.

**Immunossays:** Spent culture media obtained at the indicated time were assayed for E2 using RIA kits from (Biotech Research Institution of North China, Beijing, China). The intra- and inter-assay coefficients of variability were less than 9 and 6%, respectively. Assay sensitivity was 0.3 ng/µg of proteins.

**Statistical analysis:** Statistical analysis was performed by analysis of variance (ANOVA) using SPSS 16.0 software. Data presented are the mean±SEM, and P<0.05 was considered statistically significant.

**Table 1: Experimental design**

<table>
<thead>
<tr>
<th>Series number</th>
<th>Objects of analysis</th>
<th>Types of cells</th>
<th>Serum</th>
<th>Hormones and chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>actins filaments</td>
<td>Fibroblasts</td>
<td>Serum</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>actins filaments, transcription of CYP19A1, FSHR, expression of aromatase protein and Secretion of Estradiol.</td>
<td>Granulosa cells</td>
<td>No serum</td>
<td>FSH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Actin filament depolymerized granulosa cells</td>
<td>No serum</td>
<td>LPA+ FSH</td>
</tr>
</tbody>
</table>

FSH: follicle-stimulating hormone; CB: cytochalasin B; LPA: lysophosphatidic acid; Ins: insulin.
RESULTS

Effect of serum deprivation on actin filaments polymerization: The depolymerization of the actin filaments in the fibroblast and granulose cells are shown in Fig 1. The fibroblasts cells in the presence of the serum shows abundant of the stress fiber (Fig. 1a), but when the cells were cultured in the serum free culture, the exhibition of the stress fibers were decreased (Fig. 1b). Granulose cell cultured in the medium containing serum, there were no effects on the depolymerization of the actin filaments (Fig. 1c). But unlike the fibroblast cells in which the serum deprivation causes the depolymerization of the actin filaments, in granulose cells the serum deprivation did not affect polymerization of the stress fiber (Fig. 1d).

Effect of FSH on actin cytoskeleton rearrangement in bovine granulosa cells in vitro: The granulosa cells were cultured in serum deprived medium containing the FSH. Actin cytoskeleton morphological changes induced by hormone i.e. follicle-stimulating hormone (FSH) are shown in Fig. 2A. When cells were culture without FSH (Fig. 2Aa) and with FSH (Fig. 2Ab) there were no affect on the depolymerization of the actin filaments. The FSH treatment only causes some edge ruffling of the actin filaments with no obvious effect.

Different chemicals were used to increase or decrease the depolymerization of the actin filaments according to the experimental design in table-1. Y27632 and cytochalasin B (CB), which are believed to cause the depolymerization of the actin filaments, significantly stimulated the depolymerization of the actin filaments in the presence of the FSH (Fig. 2Ba&b). The insulin and PLA are used for the repolymerization of the actin filaments. The addition of the PLA and insulin to culture medium cause the repolymerization of the actin filaments (Fig. 2Bc &d) in the presence of FSH.

Expression of the mRNA of CYP19A1 and FSHR: A real-time RT-PCR showed that the transcription of mRNA for CYP19A1 and FSHR genes in bovine granulosa cells Fig 3 and Fig 4 respectively. The relative amount of CYP19A1 and FSHR mRNAs were normalized to the commonly used housekeeping gene, GAPDH. Significant influence of hormonal treatment and actin cytoskeleton rearrangement on the transcription of CYP19A1 gene was found (Fig. 3A).

In our experiment, addition of FSH significantly increased the transcription of CYP19A1, while actin filament depolymerization caused a significant reduction in FSH induced CYP19A1 transcription, but actin filament repolymerization had the positive effect (Fig. 3A). However, there were no significant changes in the transcription of FSHR under FSH induction and actin cytoskeleton rearrangement (Fig. 3B).

Aromatase protein expression: SDS PAGE–western blotting (Fig. 4) demonstrated that FSH significantly induced aromatase protein expression in bovine granulosa cells under different treatment. However, under both actin filament depolymerization and repolymerization procedure, there were no differences in the expression of aromatase proteins induced by FSH.
Fig. 3: Transcription of CYP19A1, FSHR mRNAs (real-time RT-PCR) and E2 (RIA) in bovine granulosa cells cultured with or without hormones and actin filament-related substances. A) Shows transcription of CYP19A1 in different treated groups. B) FSHR transcription in different treated groups. C) Shows E2 production (RIA) in different treated groups. Values are presented as mean±SEM. Different superscripts indicate significantly (P<0.05) differences between corresponding groups.

Fig. 4: Accumulation of key enzyme for estrogens biosynthesis (Aromatase) granulosa cells in different Groups (SDS PAGE–western blotting).

Production of the estriols: Fig. 3C showed the release of estriol by cultured bovine granulosa cells. Exposure to FSH stimulated estriol release. However, actin filament depolymerization and repolymerization did not modify FSH action on estriol release.

DISCUSSION

Actin filaments, which are an important component of the eukaryotic cytoskeleton, are capable of fastidious shift from polymerization and depolymerization dynamics. This dynamic equilibrium is important in many fundamental cellular events (Huber et al., 2013). Previous studies indicate that the actin cytoskeleton rearrangement often changes the progress of steroidogenesis (Nagy and Freeman, 1990; Iczkowski and Hertelendy, 1991; Karlsson et al., 2010). However, it is not sure ifactin filament rearrangement influences the estradiol biosynthesis. In this study, we attempted to mimic in vitro the effect of declining actin filaments. Our results clearly show that a decrease in CYP19A1 mRNA transcription in granulosa cells in the absence of actin filaments. This data thus support a vital link between changes in actin filaments and estradiol secretion: a chemical-induced disruption in actin filaments can specifically inhibit CYP19A1 transcription.

An important observation in the current investigation is the apparent essential role of actin filaments for CYP19A1 transcription. When actin filaments were depolymerized, expression of CYP19A1 mRNA level decreases. Even FSH could not prevent this loss of CYP19A1 gene transcription (Fig. 3A). These results also suggest that the FSH does not stimulate CYP19A1 after the removal of the actin filaments is not because of the absence/loss FSH receptors (Fig. 3B).

Equally important is the finding that actin filaments in bovine granulosa cells did not depolymerized, treated in the absence of serum. This is in contrast with data obtained from bovine fibroblasts (Fig. 1), and human intestinal microvascular endothelial cells (Hall, 1998; Hanna et al., 2001; Rafiee et al., 2004). Sasson et al. (2002) incubated human granulosa cells in serum-free medium for 24h found no effect on actin filaments healthy cells. All of these indicate that actin filaments in granulosa cells may be insensitive to serum-starvation. FSH apparently promoted aromatase protein expression and estradiol secretion despite inhibition of CYP19A1 mRNA levels. It is interesting that the FSH did not induced transcription of the CYP19A1 mRNA, but both aromatase protein levels and estradiol accumulation increased. Similarly, in previous studies during the period of IGF-I or IGF-I and insulin withdrawal in culture medium, P450arom mRNA abundance did not increased while secretion of estradiol increased by bovine granulosa cells (Silva and Price, 2002). The possible reason may be that, the state of actin filaments polymerization affects the prototype of gene expression. It was also reported in 1997 that, some gene that is involved in the myelinization of some nerve cells, have a specific pattern of expression, mediated by the cytocalasin D-mediated depolymerization in Schwann cells (Fernandez-Valle et al., 1997). Actin is capable of initiating the transcription process when its bind with a type of nuclear myosin interacting with RNA polymerases and other enzymes, which are important for transcription process (Grummt, 2006). However, at present, the exact reason how actin’s state of polymerization affects CYP19A1 mRNA transcription is not clear which still would require further investigation.

Conclusion: It was observed that unlike bovine fibroblasts, serum-starvation could not induce actin filaments depolymerization in granulosa cells. Our results suggested that actin filaments played significant roles in
controlling the transcription of CYP19A1 chemical-induced actin filament rearrangement affected FSH induced CYP19A1 transcription, but not the accumulation of aromatase protein.

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**Author’s contribution:** ZM designed and supervised the study YG and IUH conducted the experiments and SHK worked on data analysis and manuscript writing.

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