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GRK-6 mediates FSH action synergistically enhanced by estrogen and the oocyte in rat granulosa cells

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ABSTRACT

Estrogen is known to play a pivotal role in granulosa cell responses to follicle-stimulating hormone (FSH) that is critical for the establishment of dominant follicles and subsequent ovulation in mammals. Thus, elucidating the cellular and molecular mechanisms that regulate FSH activity is important to understand female fertility. We previously discovered that the oocyte is required for estrogen to exert its positive effects on FSH activity in rat granulosa cells. This finding supports the new concept that estrogen action in granulosa cells is mediated by the oocyte. In the current study, we explored the underlying mechanism. In the presence of oocytes, estrogens enhanced FSH-induced increases in aromatase, steroidogenic acute regulatory protein and FSH receptor mRNA expression as well as cAMP production. However, as forskolin did not mimic FSH activity this indicated that coexistence of estrogen/oocytes increases FSH activity at a site upstream of adenylate cyclase in granulosa cells. We therefore sought a possible involvement of the autoregulatory molecules for FSH receptor, G protein-coupled receptor kinases (GRKs) and ß-arrestins in enhancing FSH activity in response to the estrogen/oocyte co-treatment in granulosa cells. Among the seven known GRK and two ß-arrestin molecules, we found that estrogens with oocytes suppressed FSH-induced GRK-6 mRNA expression. Consistent with this finding, transfecting granulosa cells with small interfering RNA of GRK-6 significantly increased FSH induction of aromatase mRNA, suggesting that endogenous GRK-6 plays an inhibitory role in FSH-induced aromatase mRNA expression. Consequently, these findings strongly suggest that GRK-6 is involved in the mechanism by which estrogen and oocytes synergistically augment FSH activity in granulosa cells.

1. Introduction

A fundamental concept in female reproductive biology is that FSH is essential for dominant follicle formation and no other ligand by itself can serve in this regulatory capacity. Therefore, to understand follicle dominance one must understand the mechanisms governing FSH action and sensitivity in target granulosa cells. In any given ovarian cycle, the cohort of follicles that respond to FSH and develop into dominant follicles destined for ovulation is tightly controlled, such that the number of ovulated oocytes in a cycle is appropriate for a particular species. Given that FSH is the predominant factor that promotes follicle dominance and that the entire ovary is theoretically exposed to the same levels of FSH, the local governance of the sensitivity of a particular follicle to FSH is an important element in the determination of dominant preovulatory follicles.

In this context, it has been demonstrated previously that estrogen acts as a physiological regulator of FSH action in the rat based on *in vivo* studies using the immature hypophysectomized diethylstilbestrol (DES)-primed rats [1,2] and on *in vitro* studies using primary cultures of rat granulosa cells from these animals [3,4]. Based on these classic observations, it is now widely accepted that the activation of estrogen signaling pathways in the granulosa cells enhances FSH action. A particularly important action of estrogen is the enhancement of FSH-induced P450 aromatase (P450arom) activity, resulting in the production of more estrogens. This feed-forward attribute of estrogen activity may perpetuate continuous growth of the selected dominant follicles in the face of declining circulating FSH levels.

Abbreviations: DES, diethylstilbestrol; FSHR, follicle-stimulating hormone receptor; GRK, G protein-coupled receptor kinase; IBMX, 3-isobutyl-1-methylxanthine; P450arom, P450 aromatase; siRNA, small interfering RNA; StAR, steroidogenic acute regulatory protein.

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We previously discovered that the oocyte is required for estrogen to enhance FSH action in rat granulosa cells [5] and proposed the new concept that estrogen action in granulosa cells is mediated by the oocyte. While it has been known that oocytes play essential roles in the regulation of the function of granulosa cells throughout the course of folliculogenesis, our finding has extended the role of the oocyte to include the role of estrogen mediating in the enhancement of FSH action in granulosa cells. The question is how oocytes and estrogen act synergistically to enhance FSH action in granulosa cells.

It is well known that G-protein-coupled receptor (GPCR) signaling is subject to regulation by the GPCR-kinase (GRK)/ß-arrestin system [6–9]. The general mechanism by which the GRK/ß-arrestin system causes receptor desensitization involves the following steps (i) upon binding of a ligand to its GPCR, the cytoplasmic domain of the GPCR is phosphorylated by members of the GRK family, desensitizing the receptor by causing the uncoupling of the receptor from G proteins; (ii) after phosphorylation of the GPCR, ß-arrestins can then interact with and internalize GPCRs by a clathrin-mediated mechanism [10,11]. The role of the GRK/ß-arrestin system has been extended to the FSH receptor (FSHR) by studies that have used various strategies in rat primary sertoli cells, a sertoli cell line (MSC-1) and cell lines engineered to overexpress FSHR. However, the role of GRK/ß-arrestin system in the ovary remains to be investigated.

In the current study, we have tested our hypothesis that the GRK system is involved in the cellular and molecular mechanisms by which oocytes and estrogen act to enhance FSH action in granulosa cells. We provide the first analysis of the ovarian GRK, which is implicated in the mediation of the FSHR activity regulated by estrogen and oocytes.

2. Materials and methods

2.1. Primary culture of granulosa cells and co-culture with oocytes

Silastic capsules containing 10 mg of DES were implanted in 22-day-old female SD rats to increase the number of granulosa cells. After 4 days of DES exposure, ovarian follicles were punctured with a 28-gauge needle, and the isolated mixture of granulosa cells and oocytes was cultured in serum-free McCoy's 5A medium supplemented with penicillin–streptomycin at 37 °C. For indicated experiments, granulosa cells were separated from oocytes by filtering the oocyte/granulosa cell suspension through an additional 40-μm nylon mesh (BD Falcon) that allowed granulosa cells but not oocytes to pass through [12,13]. The animal protocols were approved by Okayama University Institutional Animal Care and Use Committee.

2.2. Measurements of cAMP

To assess cellular cAMP synthesis, granulosa cells (1×10^5) were cultured in 96-well plates with or without 20 oocytes with 200 μl of serum-free McCoy's 5A medium containing 0.1 mM of IBMX (specific inhibitor of phosphodiesterase activity). The ratio of the oocyte and granulosa cell numbers in this co-culture system was determined based on our previous finding [12]. FSH (30 ng/ml) was added to the culture medium either alone or in combination with 5×10^{-8} M of DES and estradiol. After 48-h culture with indicated treatments, the conditioned medium was collected and stored at −80 °C until assay. The extracellular contents of cAMP were determined by an enzyme immunoassay (Assay Designs, Ann Arbor, MI) after acetylation of each sample with assay sensitivity of 0.039 nM.

2.3. Cellular RNA extraction, RT and quantitative real-time PCR

Granulosa cells (5×10^5) with or without oocytes (10^2) were cultured in 12-well plates with 1 ml of serum-free McCoy's 5A medium. FSH (30 ng/ml) was added to the culture medium either alone or in combination with 5×10^{-8} M of DES and estradiol. After 48-h culture, total cellular RNA was extracted using TRIzol (Invitrogen Corp.). The extracted RNA (1 μg) was subjected to an RT-PCR reaction. PCR primer pairs are listed in Supplemental Table 1. The primer set for GRK-6 was designed to detect both splicing variants of GRK-6a and -6b mRNAs; the latter of which is only 2-bp longer than the former [14], thus their PCR products are indistinguishable by the agarose gel elctrophoresis. For the quantification of P450arom, FSHR and GRK-6 mRNA expression, real-time PCR was performed using a LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co., Tokyo, Japan). PCR primer pairs for real-time PCR are listed in Supplemental Table 2. Accumulated levels of fluorescence for each product were analyzed by the second derivative method (Roche Diagnostic Co.). The expression levels of target gene mRNA were normalized by RPL19 mRNA level in each sample.

2.4. Transient transfection

Granulosa cells (5×10^5) with or without oocytes (10^2) were cultured in 12-well plates in 1 ml of serum-free medium. After 1-h preculture, cells were transiently transfected with 10 μM GRK-6 siRNA or control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using Fugene 6 for 3 h. Cells were then treated with FSH (30 ng/ml) either alone or in combination with 5×10^{-8} M of DES or estradiol for 48 h. After 48-h culture, total cellular RNA was isolated and stored at −80 °C until assay.

2.5. Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The data were subjected to ANOVA to determine differences (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). If differences were detected by ANOVA, Fisher's PLSD test was used to determine which means differed (StatView 5.0 software). P values <0.05 were accepted as statistically significant.

3. Results

We reported previously that oocytes must be present in the cell cultures for DES to augment FSH activity in primary rat granulosa cells [13]. Fig. 1A shows not only the confirmation of our previous finding but also new data demonstrating that a physiological estrogen, estradiol, plays a role in evoking positive estrogen response in P450arom mRNA expression in rat granulosa cells in the presence, but not absence, of oocytes. Likewise, StAR and FSHR mRNA expression in granulosa cells was also increased by estrogen treatments only when the cells were co-cultured with oocytes (Fig. 1B and C). These results suggested that estrogen augments FSH action in granulosa cells in cooperation with oocytes.

Next we examined the effects of estrogens on FSH-induced cAMP synthesis by granulosa cells. DES and estradiol increased FSH-induced cAMP production in the presence of oocytes, although neither of the estrogens had a significant effect on basal or FSH-induced cAMP synthesis by granulosa cells in the absence of oocytes (Fig. 2A). In contrast, DES and estradiol failed to increase forskolin-induced cAMP synthesis by granulosa cells regardless of co-culture with oocytes (Fig. 2B). These data suggested that the stimulatory