Testosterone Potentially Triggers Meiotic Resumption by Activation of Intra-Oocyte SRC and MAPK in Porcine Oocytes

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ABSTRACT

The role of androgen and androgen receptors (ARs) in males has been well established. This steroid and its receptor also exist in follicles, but their functions are still unclear. In this study, using a culture system containing a low dose of hypoxanthine, we revealed the positive contribution of testosterone to oocyte meiotic resumption. By performing ultracentrifugation to allow clear visualization of porcine germinal vesicles, our results provide evidence that mitogen-activated protein kinase (MAPK) in the oocyte itself but not in cumulus cells was activated before germinal vesicle breakdown (GVBD) after testosterone treatment. We further explored the signal cascade of testosterone-triggered GVBD and showed significant contributions of AR to testosterone-induced MAPK activation and GVBD. By using a potent and selective inhibitor of SRC and detecting activation of the kinase, we found that testosterone activated SRC in oocytes but not in cumulus cells and that SRC (as an essential upstream molecule of MAPK) mediated this testosterone- and AR-promoted reinitiation of meiosis. The present findings propose an undefined signaling pathway and suggest the potential competence of testosterone for meiotic resumption in mammalian oocytes.

INTRODUCTION

In mammals, oocytes are arrested at the diplotene stage of the first meiotic prophase, which is termed the germinal vesicle (GV) stage. In vitro, fully grown immature oocytes restart their meiotic process when removed from the inhibitory environment of follicles or when stimulated by gonadotropins [1, 2], as indicated by GV breakdown (GVBD). Although roles of androgen and androgen receptor (AR) in males are well established [3, 4], the functions of this steroid and its receptor that also exist in follicles [5] are still unclear or controversial [6]. Recent reports provided evidence that the steroid (especially testosterone) was involved as a novel and obligatory candidate in meiotic resumption in lower vertebrates [7–9] and in mice [5, 8, 10, 11] and that progesterone promotes oocyte maturation in porcine cumulus-enclosed oocytes (CEOs) [12]. However, using in vitro culture models of rat and mouse follicle-enclosed oocytes (FEOs) and CEOs, results by Motola et al. [13] and by Tsafriri et al. [14] opposed major functioning of these steroids during oocyte meiotic resumption in mammals. Results from our laboratory (described herein) also revealed an ineffectiveness of testosterone in oocyte meiotic resumption induction in porcine CEOs. Moreover, the classic understanding during the past decades has suggested a negative role for testosterone in oocyte maturation in various conventional culture models [15–18]. These findings laid the groundwork for controversy about the actual roles of the steroid. Therefore, we explored the elusive functions of androgens in the meiotic cell cycle.

It has been shown that mitogen-activated protein kinase (MAPK) has crucial roles in regulating the meiotic cycle of oocytes. The most widely studied MAPKs are 42-kDa and 44-kDa MAPK isoforms (MAPK1 and MAPK3, respectively), which are activated by many upstream signals such as gonadotropin [2, 19, 20], epidermal growth factor (EGF) [21, 22], and insulin [23]. The timing of this kinase activation and its signaling events vary in different species and culture systems. In lower vertebrates such as Xenopus, the progesterone-induced meiotic resumption is mediated by intra-oocyte MAPK activation [24, 25]. In gonadotropin-induced, spontaneous, and inhibition maturation (intact follicle) models, MAPK activation in cumulus cells rather than in oocytes exerts essential functions during mammalian oocyte meiotic resumption [2, 26–29]. The detected activation of intra-oocyte MAPK occurred just after or simultaneously with GVBD, and MAPK activation did not seem to be required for meiotic GVBD [28–34]. In contrast, for the first time (to our knowledge) we provide evidence herein that testosterone activated MAPK in the oocyte before GVBD and promoted meiotic resumption, demonstrating the potential function of intra-oocyte MAPK in mammals.

It has been shown that steroid receptors mediate functions of their ligands in a transcription-independent manner in amphibians and mammals [7, 8, 10, 35] and that AR is effective by binding to SRC homology domains of SRC and phosphatidylinositol 3-kinase (PI3K) in many cases [36–38]. Therefore, we used a culture model to investigate the response of AR to the administration of testosterone and the downstream events. We found that a regular response of AR in denuded oocytes (DOs) but not in CEOs occurred after treatment with testosterone, followed by mediation of intra-oocyte SRC but not PI3K, leading to different effects of androgen on meiotic resumption. To our knowledge, these data reveal a previously unrecognized role of intra-oocyte AR, with subsequent activation of SRC and MAPK before GVBD in oocytes after testosterone stimulation.
MATERIALS AND METHODS

Chemicals

All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Stock solutions of dihydrotestosterone (DHT) (10 mM; International Laboratory, San Bruno, CA) and flutamide (10 mM) were prepared in ethanol and tissue culture medium-199 (TCM-199; Gibco, Grand Island, NY), respectively. Stock solutions of U0126 (10 mM; Calbiochem, La Jolla, CA) and PP2 (Calbiochem) were prepared in dimethyl sulfoxide (DMSO). Hypoxanthine solution was prepared in TCM-199 and stored at 4°C no longer than 1 wk before use. All stock solutions were frozen at −20°C in dark boxes. The chemicals were diluted and supplied to the culture medium approximately 1 h before culture.

Collection and Preparation of Porcine Oocytes

Porcine ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 2 h in a thermost bottle containing warm (30–35°C) saline (0.9% [w/v] sodium chloride supplemented with 40 IU/ml of penicillin G and 50 μg/ml of streptomycin sulfate). The contents of follicles 2–6 mm in diameter were aspirated using an 18-gauge needle fixed to a 20-ml disposable syringe and pooled in 50-ml conical tubes (Falcon, Franklin Lakes, NJ). After sedimentation, the sediment was washed with 20 mM Hepes-buffered TCM-199 (H-TCM-199; Life Technologies, Inc., Grand Island, NY) supplemented with penicillin G (100 IU/ml), streptomycin sulfate (100 μg/ml), BSA (fraction V, 4 mg/ml; Calbiochem), and hypoxanthine (4 mM). The H-TCM-199 was supplemented with the meiotic inhibitor hypoxanthine to prevent premature meiotic progression during oocyte collection, and subsequent removal of the inhibitor did not change the rate of maturation and development (data not shown).

Oocytes with at least four layers of intact compact cumulus cells were recovered under a stereomicroscope and were transferred to 35-mm Petri dishes (Falcon) containing culture medium and subjected to three washes before final culture. When DOs were needed for culture, CEOs were mechanically denuded in 0.5 ml of H-TCM-199 containing 300 IU/ml of hyaluronidase using a vortex instrument to remove all cumulus cells surrounding the oocytes.

Oocyte Culture

Culture of CEOs and DOs was performed in four-well dishes (Nunc, Roskilde, Denmark) at 39°C in an atmosphere of 5% carbon dioxide in air and saturated humidity. In this study, we used one basic culture medium and four culture models. The basic culture medium was TCM-199 supplemented with 0.1% polyvinyl alcohol (w/v), 3.05 mM d-glucose, 0.91 mM sodium pyruvate, 75 mM of penicillin, and 50 μg/ml of streptomycin. 1. The 4 culture models are as follows: 1) The normal maturation model used basic culture medium supplemented with 0.57 mM cysteine and 1.5 mM hypoxanthine; model B (for CEOs) used basic maturation medium supplemented with 0.57 mM cysteine, and 4 mM hypoxanthine. 2) The EGF-induced maturation model used basic culture medium supplemented with 0.57 mM cysteine and 10 ng/ml of EGF. 3) The inhibition maturation model used basic culture medium supplemented with 0.57 mM cysteine and 10 ng/ml of EGF. 4) The inhibition maturation model used basic culture medium supplemented with 0.57 mM cysteine and 10 ng/ml of EGF. 5) The low dose of testosterone, all culture media without testosterone were supplemented with the same volume of ethanol unless otherwise specified.

Microinjection of Anti-AR Antibody into Oocytes

Microinjection was performed using an Eppendorf microinjector and was completed within 30 min. Anti-AR antibody (0.5 mg/ml in PBS [pH 7.4]) was injected into the cytoplasm of oocytes at GV stages as previously reported [40, 41]. A microinjection volume of mixture of petroleum jelly and paraffin (9:1). Cells were observed under a confocal laser scanning microscope (Zeiss LSM 510; Carl Zeiss, Oberkochen, Germany) as soon as possible. Nonspecific staining was determined by substituting primary antibodies with normal rabbit IgG.

Immunofluorescent Staining of AR in Oocytes

Immunofluorescent staining of AR in oocytes. After removing the zona pellucida in acidic Tyrode medium (pH 2.5), oocytes were fixed with 4% paraformaldehyde in PBS (pH 7.4) for at least 30 min at room temperature. Cells were permeabilized with 1% Triton X-100 overnight at 37°C, followed by blocking in 1% BSA-supplemented PBS for 1 h and incubation overnight at 4°C with a rabbit anti-AR antibody (Santa Cruz Biochemicals, Santa Cruz, CA), diluted 1:200. After three washes in PBS containing 0.1% Tween 20 and 0.05% Triton X-100 for 5 min each, the oocytes were labeled with secondary fluorescein isothiocyanate (FITC)-conjugated antibody diluted 1:100 for 1 h at room temperature. The nuclear status of oocytes was evaluated by staining with 10 μM of propidium iodide for 5 min. Following extensive washing, samples were mounted between a coverslip and glass slide supported by four columns of mixture of petroleum jelly and paraffin (9:1). Cells were observed under a confocal laser scanning microscope (Zeiss LSM 510; Carl Zeiss, Oberkochen, Germany) as soon as possible. Nonspecific staining was determined by substituting primary antibodies with normal rabbit IgG.

Electrophoresis and Western Blot Analysis

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Statistical Analysis

All percentages from three repeated experiments are expressed as mean ± SEM. All frequencies were subjected to arcsine transformation. The transformed data were statistically compared by ANOVA using SPSS 10.0 software (SPSS Inc., Chicago, IL), followed by Student-Newman-Keuls test. Differences of P < 0.05 were considered statistically significant.

RESULTS

Testosterone Has No Effect on GVBD of CEOs or DOs in Normal, EGF-Induced, or Inhibition Maturation Models

To investigate the effect of testosterone on meiotic resumption, oocytes were incubated in normal, EGF-induced, and inhibition maturation models (as already described in Oocyte Culture), followed by GVB M examination. As shown

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Testosterone stimulates DOs but not CEOs to undergo GVBD in the low dose of hypoxanthine (HX) maturation model. A) The CEOs were cultured in the low dose of hypoxanthine maturation model. B) The DOs were cultured in the low dose of hypoxanthine maturation model. Testosterone was supplemented in each group in concentrations ranging from 0 to 1500 nM. Approximately 50 oocytes were included in each group. The rate of GVBD was assessed by orcein staining at 24 h of culture. Data are given as percentage of GVBD (mean ± SEM of three independent experiments). Elevated rates of GVBD indicated that testosterone promoted meiotic resumption of DOs rather than CEOs at its optimal concentration of ~600 nM in this culture model.

Testosterone Promotes GVBD of DOs but not CEOs in the Low Dose of Hypoxanthine Maturation Model

Because testosterone did not have a stimulatory effect on meiotic resumption in CEOs or DOs in any of the three models, we formulated the following hypothesis: As a weak promoter of meiosis reinitiation (if so), testosterone can function only in a mild adequate culture model in which the steroid has enough “strength” to overcome endogenous inhibition to promote GVBD. In this model, other meiotic promoters should not exert their full functions to mask the weak effect of testosterone.

To test this possibility, we used the low dose of hypoxanthine maturation model described herein to explore the effect of testosterone. Similar to the results already described, no effect of testosterone was detected in CEOs (Fig. 1A), with maximal GVBD of 36.4%. In contrast, testosterone facilitated meiotic resumption in DOs at an optimal concentration of 600 nM, which stimulated 66.4% of oocytes to undergo GVBD (Fig. 1B). When higher concentrations of this steroid were used, there was no dose-dependent effect during culture. Like the other models, when the concentration was raised to 1.5 μM, testosterone seemed to have an inhibitory effect on GVBD in DOs and in CEOs (Fig. 1, A and B). Therefore, these data revealed distinct responses of meiotic resumption to the stimulation of testosterone between CEOs and DOs in this model.

Testosterone Activates MAPK in Oocytes but Not in Cumulus Cells Before GVBD

To further investigate the mechanism of testosterone-induced GVBD in DOs in this model, MAPK phosphorylation in cumulus cells and DOs was evaluated. Intra-oocyte MAPK was activated before GVBD after testosterone treatment. As shown in Figure 2A, MAPK was not phosphorylated at 0.5, 4, or 8 h in DOs (lanes 3–5). When the culture was prolonged to 12 h or longer, activated MAPK was found in these DOs (lanes 6–8). Nonetheless, testosterone did not further activate MAPK in cumulus cells collected from CEOs at any time point before GVBD (Fig. 2B). As a positive control, Figure 2B shows that the activation of MAPK in cumulus cells still occurred in the presence of EGF or FSH. We subsequently examined MAPK activation in oocytes derived from CEOs in the other three culture models, and the results are shown in Figure 2C. The blots of p-MAPK were almost undetectable in normal, EGF-induced, and inhibition maturation models, confirming that intra-oocyte MAPK was not activated before GVBD in any of the CEOs. These results supported a potential mechanism that explains testosterone-induced GVBD through activation of intra-oocyte MAPK that is independent of the surrounding somatic cells (theca, mural, and cumulus cells).

Before finalizing this result, we must rule out the possibility that, although most DOs remain at the GV stage at 16 h in this model, there are a few DOs that might have undergone GVBD; therefore, activated MAPK would derive from these GVBD oocytes rather than from the stimulation of testosterone. To ensure that the protein samples were from DOs before GVBD, ultracentrifugation was used, and DOs with visible GVs (Fig. 2D) were then selected under a microscope for Western blots. We consistently detected activated MAPK in GV-stage oocytes in the testosterone-treated group but not in the control group (Fig. 2E), indicating that this steroid activated intra-oocyte MAPK before GVBD.

Extending the evidence in a previous study [10] that little testosterone metabolism occurred in mammalian oocytes, we used nonhydrolyzable DHT to validate the effect of testosterone. Similar to testosterone, DHT also promoted GVBD and activated MAPK (Fig. 2, F and G).

U0126 Blocks Testosterone-Promoted GVBD and Inhibits Testosterone-Induced Activation of Intra-Oocyte MAPK

Because testosterone promoted GVBD and activated intra-oocyte MAPK, we next determined the relationship between testosterone, MAPK, and GVBD. U0126 (8 μM), the specific inhibitor of MEK (also named MAP2K, the kinase upstream of MAPK), was added to the culture media. As shown in Figure 3A, phosphorylation of MAPK did not occur in the U0126-treated group, indicating that U0126 inhibited testosterone-induced MAPK activation in oocytes. Testosterone-promoted
GVBD was also effectively blocked by U0126 at a concentration of 8 μM (Fig. 3B), indicating that active MAPK in oocytes was indispensable for testosterone-induced meiotic resumption. Therefore, these results proposed a cascade that induces the activation of intra-oocyte MAPK and subsequent GVBD by testosterone.

Localization and Expression of AR in Oocytes and Cumulus Cells and Localization of Activated MAPK in Cumulus Cells

Based on the distinct effects of testosterone on meiotic maturation between CEOs and DOs, we speculated that there would be different roles of AR in oocytes of CEOs compared with DOs. We examined the localization of the receptor in cumulus cells and oocytes by immunofluorescent staining. Figure 4A shows obvious staining of AR in cumulus cells, and the signals were almost entirely localized in the nucleus. However, signals of AR were localized in both the GV (nucleus) and the cortex in oocytes treated with testosterone (see Discussion). In addition, by using Western blot, we detected a noticeable AR blot in cumulus cells derived from 300 CEOs, while the blot in the same number of DOs was much weaker. When 500 DOs were used, the blot began to become clearer (Fig. 4B).

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In addition, we tested the localization of active MAPK (p-MAPK) in cumulus cells. As seen in Figure 4C, p-MAPK in cumulus cells was exclusively localized in the cytoplasm rather than in the nucleus, which was reversed from the localization...
of AR (Fig. 4A, cumulus cells of CEOs). These differences indicated that there is little possibility that these two molecules could interact in cumulus cells because of the spatial separation between AR and MAPK.

**Different Responses of AR in Oocytes Between DOs and CEOs**

With these results, the distinct responses between CEOs and DOs to testosterone needed further investigation. For this aim, the CEOs and DOs were incubated in the same culture media supplemented with or without testosterone, and the signals of AR in oocytes of the two groups were compared. At the GV stage, there was no difference between the distribution of AR in DOs and CEOs in the absence of testosterone. As shown in Figure 5 (A1 and B1), AR displayed a scattered distribution in the two kinds of oocytes. However, after treatment with testosterone, the scattered ARs within oocytes in DOs regularly aggregated in the cortical cytoplasm and the GV (nucleus), which was strikingly different from CEOs, in which no response of AR redistribution occurred after treatment with testosterone (Fig. 5, A2, A2’, B2, and B2’). Subsequently, at the GVBD stage in DOs, we also found traces of AR shortly after GVBD (Fig. 5B3), while in the oocytes derived from CEOs, we did not obtain this result (Fig. 5A3). These findings indicated that oocytes derived from CEOs and DOs displayed distinct responses to testosterone stimulation during the meiotic cell cycle.

**Testosterone-Promoted GVBD and Activation of Intra-Oocyte MAPK Are Mediated by AR**

Because AR was expressed in oocytes and moved following stimulation with testosterone—and because studies [10, 38, 44–46] have shown that steroid-mediated signaling is regulated by their receptors—it was necessary to determine whether the testosterone-induced meiotic reinitiation is mediated by AR. Using the classic AR antagonist flutamide, we explored the contribution of the receptor. After treatment with flutamide, most DOs failed to complete GVBD, which was significantly different from the control group (Fig. 6A). Furthermore, by blocking the functions of AR via injecting its antibody into the cytoplasm of DOs, we obtained similar data supporting our speculation. As shown in Figure 6A, only 29% of DOs in the antibody-injected group underwent GVBD, while in the IgG-injected (control) group, the GVBD rate (63.3%) was roughly equivalent to that of the control group (66.4%). These results demonstrated that classic AR is required for testosterone-induced GVBD.

To further test the roles of AR in the MAPK cascade, we determined MAPK activity in the flutamide-treated group. As expected, p-MAPK was almost undetectable after administration of flutamide (Fig. 6B), suggesting that activation of intra-oocyte MAPK was AR dependent. Based on these results, we theorized a signaling pathway that induces meiotic resumption by AR, through which testosterone activates MAPK in oocytes and then causes GVBD.

**Roles of SRC in Testosterone-Induced GVBD**

PP2 but not LY294002 blocks testosterone-promoted GVBD and inhibits testosterone-induced activation of intra-oocyte MAPK. As already mentioned, SRC and PI3K are two potential candidates mediating testosterone- and AR-triggered meiotic resumption. To determine whether these two are involved, PP2 (a selective inhibitor of activity in SRC family tyrosine kinases [SFKs]) and LY294002 (a specific inhibitor of PI3K) were used to test the roles of SRC and PI3K. After treatment with PP2, the GVBD rate declined significantly compared with that in the control group (Fig. 7A). Consistently, activated MAPK in oocytes was almost undetectable in the PP2-treated group (Fig. 7B). Nevertheless, LY294002 did not seem to have an effect on GVBD percentage (Fig. 7A) or on MAPK activity at concentrations ranging from 0.1 to 50 μM (Fig. 7C). These observations showed that SRC rather than PI3K mediates the androgen-triggered resumption of meiosis.

**Testosterone activates SRC in oocytes but not in cumulus cells before GVBD, and the activation can be inhibited by PP2.** To further explore the definitive roles of SRC in the pathway, we tested SRC activity in cumulus cells and oocytes after treatment with testosterone. As shown in Figure 7D, activated SRC in oocytes was not detected as early as at 8 h, and the activity remained stable during the incubation period; as we predicted, SRC activation was inhibited by PP2 (Fig. 7E). Using CEOs, activity of the kinase in cumulus cells was also observed (Fig. 7F): activated SRC was present in cumulus cells (control lane), and its activity did not change after treatment with testosterone (test lane). These results indicated that SRC in the oocyte but not in cumulus cells is responsible for the testosterone-triggered GVBD.
molecules. In a flutamide-supplemented group, no activated SRC was detected after treatment with testosterone, suggesting that SRC is downstream of testosterone and AR, whereas U0126 as a specific inhibitor of MEK did not inhibit SRC activation, indicating that SRC is upstream of MAPK in the pathway (Fig. 7G).

Combined administration of PP2 and U0126 fails to exert a more forceful inhibitory effect on GVBD. To further define the linear relationship, we tested GVBD percentage in the presence of both flutamide and U0126. As shown in Figure 7A, there was no significant difference between combined administration and single administration, confirming that testosterone and AR, SRC, and MAPK have roles in the same signaling pathway.

Taken together, the data not only revealed a linear relationship between SRC and an SRC-mediated cascade but also positioned SRC between AR and MAPK. Therefore, a pathway that is triggered by testosterone is demonstrated in mammalian oocyte meiosis resumption. Through AR, testosterone activated intra-oocyte SRC and MAPK and subsequently promoted GVBD.

**DISCUSSION**

**Culture Models**

Androgens had long been considered to have negative, or at least not positive, roles in mammalian oocyte meiotic resumption [15–18] until they were recently shown to have a role in promoting the release of meiotic inhibition in mammals [5, 8, 10, 11]. In contrast, using in vitro culture models of rat and mouse FEOs and CEOs, other investigators have questioned the meiosis-stimulating competence of the steroids [13, 14]. In this study, by comparing the effects of testosterone on porcine oocyte meiotic maturation in different culture models, we found that testosterone promoted GVBD of DOs rather than CEOs, which indicates that androgen exerts a positive stimulatory effect on meiotic resumption in mammals. However, the underlying mechanism of the testosterone-induced meiotic resumption remains unclear.

As receptors of FSH, LH, or EGF do not exist in oocytes, their ligands cannot bind to their receptors. Thus, these promoters (FSH, LH, or EGF) will not mask the effect of testosterone in this model. Therefore, DO culture in a low dose of hypoxanthine not only allows testosterone to exert its subtle effect but also enables us to detect increased GVBD caused by intra-oocyte factors. In addition, this model switches off the endogenous steroids produced by theca and granulosa cells [5, 47, 48], providing a clear background for investigating the effect of exogenous testosterone on oocyte meiosis. Furthermore, because of the much lower activity of transcription and translation in oocytes vs. cumulus cells, the nongenomic effects of testosterone and AR were detected clearly and impartially.
These results also indicate a regulatory balance between higher and lower concentrations of testosterone existing within the oocyte. In addition to the requirement of a mild environment (e.g., the low dose of hypoxanthine maturation model), the effects of testosterone on meiotic maturation mainly rely on its concentration. When an optimal concentration of testosterone was used, the promoting effect of meiotic resumption appeared, while when a high concentration of testosterone was used, the inhibitory effects described in previous reports [15–18] dominated. However, because the androgen concentration we used in this study is higher than the physiological level in the ovary, the meiotic promotion by testosterone does not completely represent its physiological roles, but the results may suggest the potential competence of this steroid within oocytes.

Intra-Oocyte MAPK Is Activated Before GVBD in Testosterone-Treated DOs

In contrast to the traditional understanding that MAPK is activated just after or simultaneously with GVBD and that its activation does not seem to be required for GVBD in mammals [29–31, 33, 34], we demonstrated for the first time (to our knowledge) that testosterone activated intra-oocyte MAPK before GVBD and promoted GVBD. However, MAPK in cumulus cells did not seem to contribute to the resumption of testosterone-treated oocytes, as androgen did not elevate GVBD rates in all CEO culture groups, nor did it further activate MAPK in cumulus cells.
Testosterone Exerts Its Effect via AR in Oocytes

As shown in this study, distinct responses of AR occurred after treatment with testosterone in CEOs and DOs. By using the AR antagonist flutamide and antibody injection to eliminate intra-oocyte AR, followed by determination of GVBD rate and MAPK activation, our data showed that testosterone-induced GVBD was mediated by AR. However, it is still puzzling why AR in cumulus cells was not involved in meiotic resumption. It is possible that testosterone is being metabolized by surrounding granulosa cells before it reaches the oocyte, although the exact events in cumulus cells are subjects for future investigation. Herein, we found that AR was located exclusively in the nuclei of cumulus cells, indicating that these receptors exert their functions mainly through genomic rather than nongenomic action [49]. In contrast, in the DO culture model there were two obvious kinds of AR responses in the oocyte after administration of testosterone: some ARs translocated into the GV, while others migrated to the cortex. These data indicate that the intra-oocyte ARs tend to branch out to perform specific nongenomic action for classic signaling events in the cortex close to the membrane, in addition to their genomic action in the nucleus, which is supported by a recent study [49] proposing a model for genomic and nongenomic effects of AR. This is also supported by an earlier study [50] demonstrating that a small fraction of steroid receptors may be localized outside the nuclei in the vicinity of the cytoplasmic membrane that is poised to trigger the pathway that transduced signals from the membrane to the nucleus, in addition to the main function of steroid hormone receptors localized in the nucleus that were responsible for the transcriptional effects. Evidence from a recent study [51] revealed a definitive mechanism of AR shuttling between the nucleus and the cytoplasm, a finding that has stimulated our future exploration of the exact molecular behavior of AR.

An alternative view relates to the synergy that exists between gonadotropin, androgen, and AR in the regulation of meiotic resumption in mammals. A recent study [35] showed that blockade of androgen production significantly reduced gonadotropin-induced oocyte maturation and delayed ovulation; FSH receptor expression was markedly reduced in AR−/− mice [52]. Because of the dominant roles of gonadotropin in the regulatory cooperation, we barely detected a weak effect of androgens and AR in our study. In addition, it has been reported that the SRC and MAPK pathway alone was insufficient to promote complete maturation, which further suggests that some other pathways cooperate in this process in oocytes [53].

Testosterone-Induced MAPK Activation and GVBD Are Mediated by SRC

SRC and PI3K are two important signaling molecules that contain SRC homology domains and can interact with AR [36–38]. SRC family tyrosine kinases [7, 53–56] and PI3K [57–59] have essential roles in meiotic progression and activation of the MAPK cascade. In this study, by using a selective inhibitor of SFK activity (PP2) and a specific inhibitor of PI3K, we found that SRC but not PI3K was involved in testosterone-induced meiotic resumption. To explore detailed molecular events, we focused on the roles of SRC in this signaling cascade. PP2 effectively blocked testosterone-induced activation of MAPK and subsequent GVBD, confirming that SRC kinase mediates this pathway and is upstream of MAPK in the cascade. Moreover, the fact that activated SRC was detectable after administration of testosterone—and this activation did not occur in the presence of flutamide—provides evidence that SRC is located downstream of testosterone and AR in the cascade. Indeed, as pivotal molecules in the signaling network, SFKs are involved in regulating many cellular processes [60, 61], and these different actions may be due to the interactions of SRC with different receptors, resulting in subsequent various events in cells. In AR-negative COS-1 cells, transfection of AR is necessary to induce the activity of c-SRC/Raf/MAPK in response to synthetic androgen R1881 [36], which proves the ability of androgen to activate the MAPK cascade. Notably, the mild activation of MAPK in constitutively active SRC in Xenopus oocytes indicates that SRC serves as a weaker promoter in meiotic resumption or that other kinases may cooperate with it to regulate cell cycle events [53]. In this study, we elucidated the role of testosterone in meiotic resumption and propose a signal cascade in the porcine oocyte. By acting on its receptor AR, testosterone activates intra-oocyte SRC and MAPK and subsequently promotes GVBD.

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ANDROGEN IN OOCYTE MEIOSIS


