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ORIGINAL PAPER

Testosterone upregulation of tissue type plasminogen activator expression in Sertoli cells

tPA expression in Sertoli cells

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Abstract Our previous studies have demonstrated that tissue type plasminogen activator (tPA) might be involved in matrix degradation of blood-testis barrier in rat. In this study, we have further investigated the effect of testosterone (T) on tPA production in rat Sertoli cells. Our results showed that Sertoli cells isolated from rat testes at various ages in vitro secreted tPA in an age-dependent manner. The tPA activity was detected on day 20 after birth, and reached maximum on day 60. The Sertoli cells isolated from the testes on day 20 were then cultured in the presence or absence of testosterone, FSH, and forskolin, the tPA activities were upregulated by T, FSH and forskolin. Addition of H89 or U0126, both inhibited the testosterone-, FSH-, and forskolin-induced tPA expression. It is suggested that FSH- and testosterone-stimulated tPA expression in Sertoli cells may be via PKA and ERK signal transduction. Furthermore, we have observed that testosterone stimulated tPA secretion at all the stages of spermatogenesis (II–VI, VII–VIII, IX–XII and XIII–I), the highest stimulation of tPA activity was observed at stages VII–VIII. This study further suggests that testosterone-induced tPA activity in the Sertoli cells might be related to the function of blood-testis barrier opening and/or closing.

Keywords Testosterone · Tissue-type plasminogen activator · Blood-testis barrier · PKA · ERK pathway

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Introduction

In mammals, spermatogenesis takes place in seminiferous tubules, where Sertoli cells create a unique microenvironment necessary for normal germ cell development [1]. Plasminogen activator (PA) system is an important regulator mainly produced by Sertoli cells and involved in many biological processes as a general proteolytic system, such as spermatogenesis, oogenesis, embryo implantation, fibrinolysis, angiogenesis, inflammation, and tumor metastasis [2–16]. There are two forms of PA, tissue type PA (tPA) and urokinase type PA (uPA) characterized in mammals. They have different catalytic and antigenic properties and are encoded by two distinct genes [17], but share ability to cleave plasminogen to form active protease plasmin. It has been reported that low level of uPA activity was detected under basal condition in cultured Sertoli cells, whereas tPA activity was induced in a large amount in response to FSH stimulation [18–21]. PAI-1 is a primary physiologic inhibitor of naturally occurring plasminogen activators [22, 23], known to form an SDS-stable complex with tPA [24].

Function of these proteases and anti-proteases in testis is not yet understood. Due to their stage-specific production and their different regulation by gonadotropins [18–20] and cell–cell interactions [25], it has been suggested that PA in seminiferous epithelium might play a role in tissue remodeling and cell migration which take place during release of preleptotene spermatocytes from basement membrane[26] and opening tight junctions between neighboring Sertoli cells allowing migration of zygotene spermatocytes to adluminal compartment of seminiferous epithelium [27]. PA system may also play a role in process of spermiation [28], the detachment of residual bodies from the mature

spermatids [29], and the residual body phagocytosis by Sertoli cells [30].

Increasing evidence has shown that androgen plays a crucial role in development of male reproductive organ, and maintaining male fertility and sexual function. High level of intra-testicular testosterone secreted by Leydig cells is necessary for spermatogenesis. Testosterone level is low before puberty, and increases at puberty initiated by FSH regulation [31]. Tight junctions form during puberty in coincidence with increase in testosterone and its receptor expression in Sertoli cells [32]. In addition, the seminiferous epithelium at stage VII–VIII is sensitive to testosterone [33]. During the stages VII–VIII, there have two physiological processes: the first, blood-testis barrier must regularly break down and spermatogonia reform as premeiotic germ cells moving from the basal to adluminal compartment [34]; the second process is spermiation, the final mature spermatoza released from Sertoli cells into tubule lumen [3]. These two physiological processes are androgen-dependent [35], requiring proteases to disassembly and assembly tight junction and adherens junction [36, 37].

Our previous evidences have demonstrated that tPA and its inhibitor PAI-1 are expressed in testes of rat [38] and monkey [39] and might be involved in regulation of spermatogenesis [18–20]. In this study, we have designed in vitro rat Sertoli cell experiments to further investigate testosterone regulation of tPA activity in the Sertoli cells.

Results

Expression of tPA in rat Sertoli cells in a stage-dependent manner

Since testosterone production is high at puberty, it is interesting to comparatively look at changes in tPA expression in the Sertoli cells isolated from various ages of development. Therefore, we prepared Sertoli cells from rat testes on day 1(*a*), 20(*b*), 40(*c*), 60(*d*), and 90(*e*) after birth. As shown in Fig. 1, the tPA signal in the cells was increased gradually in an age-dependent manner. There was no tPA activity observed in the Sertoli cells on day 1. The Sertoli cells expressed measurable amount of tPA activity on day 20 when the rat was in a pre-puberty. A stronger tPA activity was detected in the Sertoli cells on day 60 when mature spermatoza appeared. The tPA activity measured in the Sertoli cell condition media was consistent with that of its expression observed by the immunocytochemistry. These data indicate that the immature Sertoli cells do not express tPA, only matured Sertoli cells are capable of expressing tPA activity.

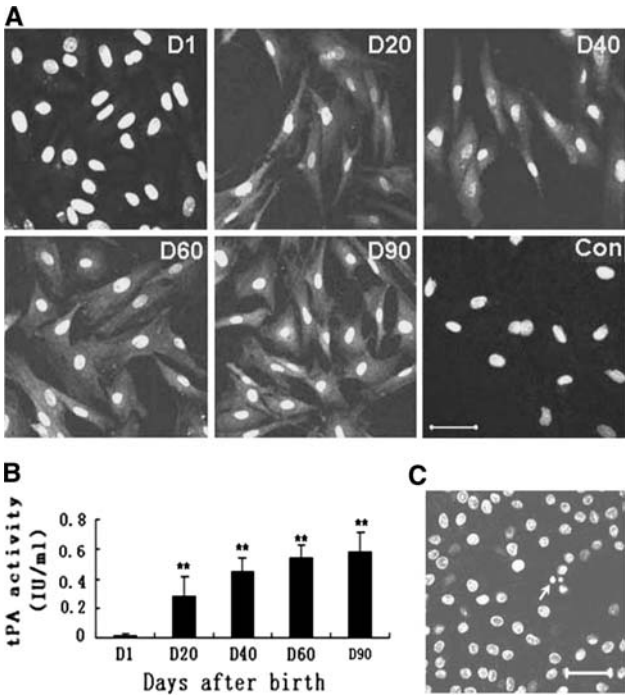


Fig. 1 Stage-dependent expression of tPA in rat primary Sertoli cells. The Sertoli cells were isolated from the rat testes on day 1, 20, 40, 60 and 90 after birth and cultured in DMEM plus F-12 medium. By the end of 24 h incubation in the serum free culture, the media were collected for tPA chromogenic analysis; The cells were prepared for immunohistochemistry. (**a**): Confocal immunohistochemistry. The cells were fixed in the mixture of methanol and acetone, and incubated in the primary antibody of tPA at 4°C and then with the flurescein isothiocyanate conjugated antimouse IgG. The green was the positive signal. The nuclei were stained in red using PI. Bar, 50 μm. (**b**): tPA chromogenic analysis. Data are presented as mean ± SEM ($n = 3$). Bar with ** is significantly different ($P < 0.01$). (**c**): The primary Sertoli cells were isolated from rat at 60 day after birth. The white arrow indicates germ cells. The purity was more than 90%. Bar, 50 μm

Effect of testosterone on tPA expression and secretion in Sertoli cells

In order to investigate the possible effect of testosterone on tPA expression and secretion in vitro, we isolated the Sertoli cells from rat testes on day 20 after birth. 0, 10 nM, 50 nM, 100 nM, and 200 nM testosterone was respectively added to the serum-free medium. The cultured cells and the media were collected, respectively, 24 h later, the expression of tPA in the cells and tPA activity in the media were analyzed, respectively. As shown in Fig. 2a, the Sertoli cells weakly expressed tPA in the control, with an increasing testosterone concentrations in the culture, the tPA signal was dose-dependently stimulated. The content of tPA in the condition media (Fig. 2b) was consistent with its expression observed by confocal immunocytochemistry.

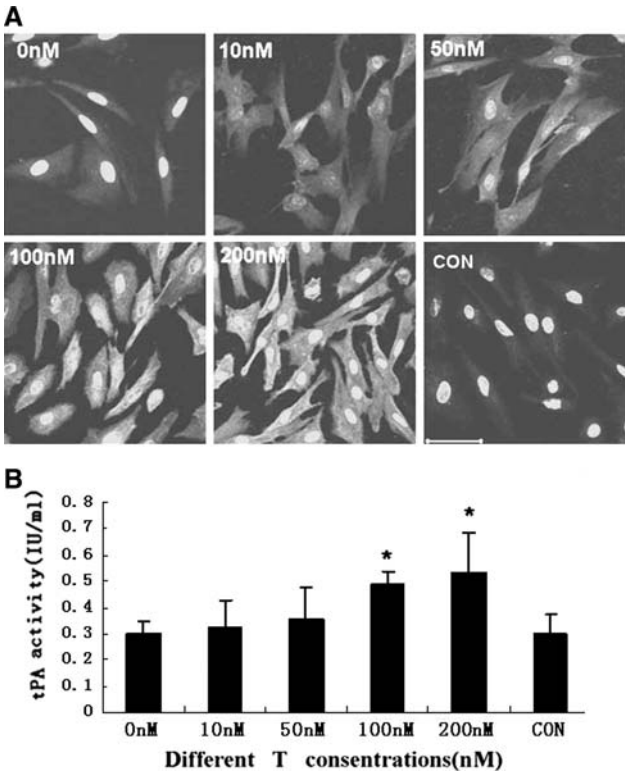


Fig. 2 Effect of testosterone on tPA expression in cultured rat primary Sertoli cells. The Sertoli cells were isolated from the rat testes on day 20 after birth and cultured in DMEM plus F-12 medium. The cells were further incubated with different doses of testosterone (0, 10 nM, 20 nM, 50 nM, 100 nM, 200 nM) in the serum free medium for 24 h, the media were collected for processing tPA chromogenic analysis; the cells were prepared for tPA immunohistochemistry. Con shows media with solubilization agent of testosterone(alcohol in PBS). (**a**): Confocal for tPA. The cells were fixed in the mixture of methanol and acetone, and incubated in the primary antibody of tPA at 4°C and then with the flurescein isothiocyanate conjugated antimouse IgG. The green was the positive signal. The nuclei were stained in red using PI. Bar, 50 μm. (**b**): tPA chromogenic activity assay. Data are presented as mean ± SEM ($n = 3$). Bar with * is significantly different ($P < 0.05$)

Effect of H89 and U0126 on testosterone-induced tPA expression

In order to examine the signal pathways of the testosterone-regulated tPA expression, we isolated the primary Sertoli cells from rat testes on day 20, and cultured in DMEM/F-12 medium with 10% FBS for 24 hours. After changed with the serum-free medium, the cells were treated with the ERK inhibitor, U0126 (20 μM) or PKA inhibitor, H89 (20 μM) for 30 min, then FSH (20 μg/ml), testosterone (100 nM), forskolin (5 mM), a PKA activator, were added to the culture media, respectively, and further incubated for 24 h. The cells and media were collected, respectively. The doses of PKA, FSH and FK [19] and the various inhibitors [40] used in the experiments was based on our previous studies and no obvious toxic effect of U0126 and H89 on Sertoli cells was observed**.** As shown in Fig. 3, FSH, testosterone or forskolin up-regulated tPA expression. The earlier treatment of the cells with U0126 and H89, significantly inhibited tPA expression. The medium tPA activities were consistent with its expression observed by the immunocytochemistry.

As shown in Fig. 4, the phosphorylated ERK was obviously up-regulated by the treatment of the cells with testosterone for 10 min, and the testosterone-induced ERK activation was remarkably inhibited by the addition of ERK or PKA inhibitor. The profile of the phosphorylated ERK activation in the cells was coincident with that of tPA secretion in the media, further confirming the specificity of testosterone-induced ERK activation.

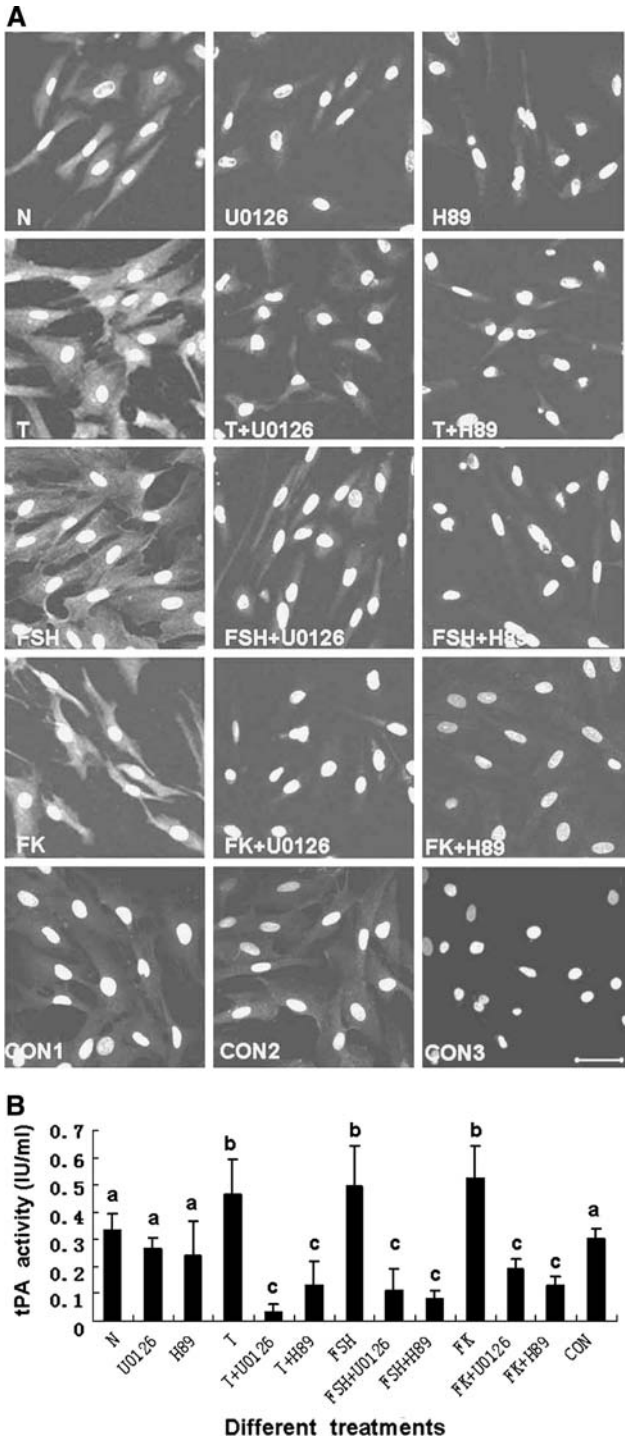
Testosterone stimulates tPA secretion in cultured rat seminiferous epithelium in a stage-dependent manner

Looking at a possible effect of testosterone on tPA secretion in seminiferous epithelium, we dissected the rat epithelium for stages II–VI, VII–VIII, IX–XII, XIII–Iinto segments respectively, and incubated with 100 nM testosterone for 24 h. The conditioned media were collected and the content of tPA activity in the media was measured. As shown in Fig. 5, testosterone stimulated tPA secretion at the all stages of the segments, the prominent peak of tPA activity, however, was detected at stage VII–VIII when blood-testes barrier was opening and spermiation was occurring, implying that testosterone upregulated-tPA secretion might be involved in the regulation of processes of blood-testis barrier and spermiation.

Discussion

In this study, we have demonstrated that testosterone stimulates tPA expression and activity in the Sertoli cells, a prominent tPA peak was detected at stages VII–VIII of rat seminiferous segments when the blood-testis barrier was opening and spermiation was occurring, implying that the increased tPA secretion by testosterone might be related to regulation of blood-testis barrier function.

We, further, prepared Sertoli cells from testes of rats at various ages, and compared the secretion ability of tPA in the presence of testosterone at different physiological conditions. Sertoli cells isolated from adult rat testes have been reported to be contaminated with germ cells [41], since contaminating germ cells adhered to Sertoli cells surface. We chosen the Sertoli cells at the specific age on day 20 used for the most culture studies. Rat Sertoli cells at this age have pre-matured and are easy to prepare more



◀ **Fig. 3** U0126 and H89 inhibit FSH-, testosterone (T)- and forskolin (FK)-induced tPA expression in cultured primary Sertoli cells. The Sertoli cells were isolated from the rat testes on day 20 after birth and cultured in DMEM plus F-12 medium. FSH (20 μg/ml), testosterone (100 nM) and FK (5 mM) were added to the serum free culture medium for 24 h. 30 min before the hormones, U0126 (20 μM), an ERK inhibitor and H89 (20 μM), a PKA inhibitor were also added to the cell culture. By the end of incubation, the media were collected for tPA chromogenic activity analysis; the cells were prepared for the immunohistochemistry, (**a**) Immunohistochemistry for tPA. The cells were fixed in the mixture of methanol and acetone, and incubated in the primary antibody of tPA at 4°C and then with the flurescein isothiocyanate conjugated antimouse IgG. The green was the positive signal. The nuclei were stained in red using PI. N indicates normal without any treatment. CON1 indicates negative control with DMSO(solubilization agent of U0126), CON2 indicates negative control with ddH_2O (solubilization agent of H89), and CON3 shows the control without primary antibody. Bar, 50 μm. (**b**) tPA chromogenic activity analysis. N shows normal without any treatment. CON indicates negative control with solubilization agent (DMSO). Data are presented as mean ± SEM ($n = 3$). Bar with a, b, and c indicate significantly different ($P < 0.05$)

pure Sertoli cells at this stage [25]. By the age on day 60 mature sperms could be observed in the testis [42]. Using the method previously reported, up to 95% pure Sertoli cells could be obtained from the testes under 20-day old immature rat [25], and the purity of Sertoli cells isolated from 60 day rat is more than 90% purity, which was evaluated with the cell specific expression of Wilms' tumor gene (WT1) located in nuclei of Sertoli cells [43] (Fig. 1c).

Because germ cells are capable of expressing PAI-1 activity, therefore the measured tPA activity in Fig. 1 in the matured Sertoli cells isolated from the testes of 40, 60, and 90 day may be underestimated, because of more germ cell contamination. Anyhow, our data clearly showed that the testicular Sertoli cells on day 20 start to express and secret tPA. It has been reported that when the animal reaches to its mature age, a peak level of testosterone in vivo could be induced by pituitary LH/FSH stimulation [31]. Evidence also showed that with spermatogenesis mature on day 60, testosterone production from the testis reaches another peak level [31, 34], that may be important for further increasing tPA expression and secretion in the Sertoli cells. These data suggest that in vivo increasing endogenous testosterone produced by Leydig cells is related to the Sertoli cell tPA production, which might be associated with the function of blood testis barrier opening and closing. Evidence showed that rat and mouse epithelium at stages VII–VIII secrets higher level of PA [19, 44, 45], when both spermiation in Sertoli cells and release of preleptotene spermatocyte from the basal to the adluminal compartment occur. Tissue PA is sensitive to gonadotropin, GnRH, forskolin and cAMP stimulation [18, 20, 21, 25]. FSH is also capable of stimulating tPA activity at stages IX–XII [19], when the sperm residual bodies are absorbed in the Sertoli cells [19]. In the present study, we observed that addition of testosterone to the Sertoli cell culture stimulated tPA secretion, predominantly at stages VII–VII, and IX–XII. It has been reported that TGF-β3 regulates anchoring junction dynamics in the seminiferous epithelium of the rat testis via the Ras/ERK signaling pathway [46] and blood-testis barrier dynamics via the P38 MAPK [47]. Our preliminary experiment showed that TGF-β3 is important in regulation of tPA expression

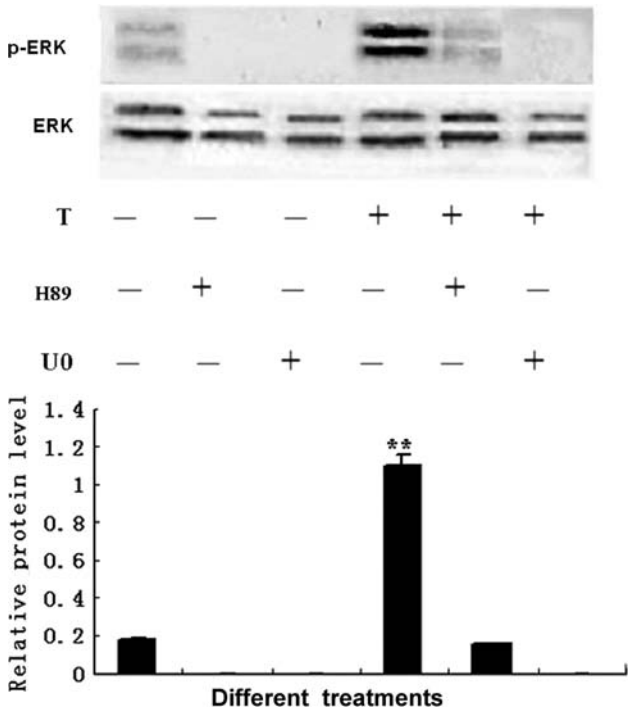


Fig. 4 U0126 and H89 inhibit testosterone-induced ERK activation in cultured primary Sertoli cells. The Sertoli cells were isolated from the rat testes on day 20 after birth and cultured in DMEM plus F-12 medium. Testosterone (100 nM) was added to the cell culture. Thirty minutes before the hormone, U0126 (20 μM), an ERK inhibitor and H89 (20 μM), a PKA inhibitor were also added to the serum free cell culture. The cells were further cultured in the serum free medium for 10 min, the cells were collected for ERK western blot analysis. Bar with ** is significantly different ($P < 0.01$)

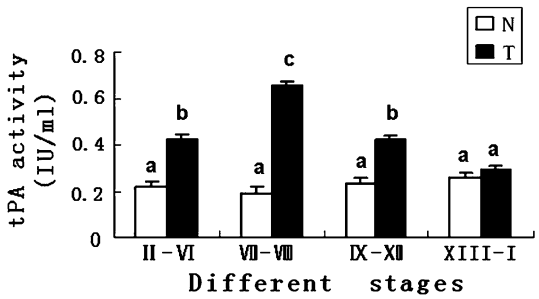


Fig. 5 Effect of testosterone on tPA secretion in cultured rat seminferous tubules. The rat seminiferous tubule segments at stage II–VI, VII–VIII, IX–XII and XIII–I were micro-dissected from the adult rat testes and cultured in DMEM plus F-12 medium with 0.1% FBS. Testosterone (100 nM) was added to culture and incubated for 24 h, the media were collected for the tPA chromogenic activity analysis. Bar with a, b and c indicate significantly different ($P < 0.05$). N shows normal without any treatment. T indicates media with testosterone. Note that testosterone stimulates tPA secretion of the segments at all the stages, the highest stimulation of tPA secretion was observed at stage VII–VIII

(unpublished observation). The study has been doing with tPA and TGF-β3 in regulating sertoli cell anchoring junctions by using the tight junction permeability assay technique to directly look at the changes in permeability of junctions induced by TGF-β3 via tPA expression in Sertoli cells.

Our previous studies showed that rat and monkey Sertoli cells expressed and in vitro secreted only tPA, which is regulated by various hormones, while germ cells express PAI-1 and uPA [20]. The tPA could form complex with PAI-1. The tPA and PAI-1 complex activities in the segments at different stages have no obvious changes detected by reverse fibrin overlay technique [19], therefore the increasing tPA activity observed in Fig. 5 may be not because of effect of the PAI-1 activity secreted from germ cells at different stages.

Having a more closer look at the possible molecular mechanism of testosterone action on tPA expressing in Sertoli cells, we have established various rat primary Sertoli cell cultures and examined their expression and possible signal pathway in various physiological conditions. We have examined PI3K-Akt and ERK signal pathway in the monkey testis. Testosterone treatment of monkeys in vivo has no obvious effect on p-Akt, but ERK activation. Therefore, we designed in vitro experiments to further examine the ERK signal pathway. The results showed that addition of an ERK inhibitor, U0126, or a PKA inhibitor, H89 to the Sertoli cell culture, the testosterone-, FSH- and forskolin-stimulated tPA secretion was completely inhibited. Furthermore, testosterone is also capable of activating ERK phosphorylation in the Sertoli cells. After treatment with its inhibitor U0126, the testosterone-induced ERK activation was completed blocked, indicating that testosterone-increased tPA expression in the Sertoli cells is regulated through PKA-dependent ERK activation.

In summary, we provide further evidence to show that testosterone-upregulated tPA activity might play an essential role in the processes of blood-testis barrier opening/closing and extra-cellular matrix degradation in Sertoli cells. However, direct evidence of involvement of the PA system in the above processes should be investigated.

Materials and methods

Materials and reagent

Dulbecco's Modified Eagle's medium, ERK1/2 inhibitor (U0126), trypsin (type I), collagenase (type V), Hyaluronidase and DNaseI were purchased from Sigma (St. Louis, MO). Ham's F-12 nutrient mixture was from Invitrogen Corporation (Grand Island, NY). Polyclonal anti-phospho-ERK1/2 (9101), anti-ERK1/2 (9102) antibodies were obtained from Cell Signaling Technology

(Beverly, MA). Monoclonal anti-tPA antibody was purchased from Biopool (Umea, Sweden). Testosterone undecanoate (TU) was purchased from Xianju Pharmaceutical Co, China.

Animals

Male Sprage Dawley rats at various ages were obtained from the Experiment Animal Center, Chinese Academy of Sciences (Beijing, China) and maintained under standard conditions with free water and food. The rats were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the protocols had the approval of the Institutional Committee on Animal Care and Use. The testes were taken and decapsulated for Sertoli cells isolation.

Isolation and culture of rat primary Sertoli cells

The methods used for the isolation of Sertoli cells from the testes of rat have been previously reported [25] with miner modifications. The cells were isolated from the rat testes on day1, 20, 40, and 90 after birth. The testes were decapsulated, the seminferous tubules were obtained. The tubules were washed twice in PBS. After centrifugation at 300×*g* for 3 min, the sedimentation was incubated with collagenase (0.5 mg/ml) for about 5 min to disperse the seminferous tubules. Centrifugation at 300×*g* for 3 min and the sedimentation was then washed twice again in PBS to remove the possibly remained blood and Leydig cells. The tubular pieces were then performed with the second digestion by addition of 0.05% trypsin for about 5 min and the fetal bovine serum (FBS) was added to stop the enzyme digestion. After filtration through a 100-mesh filter and centrifugation, the cells were collected and resuspended in the culture medium (DMEM plus F12 with 10% FBS, 30 mg/ml penicillin, and 50 mg/ml strepromycin). The cells were washed and seeded onto10 × 10 mm cover-slips placed in 6-well plates (2×10^5 cells/cover-slip) for the confocal immunohistochemistry or in the 6-well plates (2×10^6 cells/well) for protein extraction and analysis. The cells were incubated at 33°C in a humidified atmosphere of 5% CO2 and 95% air for 24 h and then the cells were gently washed to remove unattached germ cells. After additional 24 h culture, the medium was changed with serum free medium in the presence or absence of testosterone, FSH or foskolin with or without U0126 (20 μM), H89 (20 μM) for further 24 h incubation. By the end of incubation, the cells and media were collected respectively and processed for various analyses. We use WT1 antibody to examine the purity of Sertoli cells.

Microdissection and culture of staged seminiferous tubule segments

The methods used for microdissection and culture of the staged seminiferous segments have been previously described [19] with miner modifications. After de-capsulation of adult rat testes the seminiferous tubule segments for stages of II–VI, VII–VIII, IX–XII and XIII–I were microdissecteed respectively under a transillumination microscope in a dish containing PBS. The isolated tubule segments (2 mm in length) at various stages were respectively transferred into 96-well culture plate and incubated at 33°C with 5% CO_2 for 24 h in 100 μl culture medium (DMEM plus F12 medium with 100 IU/ml penicillin, and 100 μg/ml strepromycin).

Confocal immunohischemistry

Sertoli cells for the various experiments were cultured on cover-slips. After thrice washed in PBS, the cells were fixed in freshly prepared mixture of methanol and acetone (1:1) for 30 min, followed by washes in PBS, and incubated in 10% normal horse serum. Then the primary antibodies of tPA (1:50) was added at 4°C overnight. Flurescein isothiocyanate conjugated anti-mouse or anti-rabbit IgG (1:200) was used at room temperature for 1 h. After three washes in PBS, the cove-slips were incubated in propidium iodide (PI) for 10 min to dye the nuclei. Finally, the slides were analyzed by confocal laser scanning microscope (Carl Zeiss Inc.,Thornwood,NY).

Western blotting

The collected cells were incubated in RIPA lysis buffer (50 nM Tris–HCL, pH 7.4, 150 mM NaCl, 1%NonidetP-40, and 0.1%SDS), supplemented with protease inhibitors (PMSF) and phosphatase inhibitors (1 mM NaF). The supernatants from centrifugation (12000×*g*, 15 min) were collected, and the total protein concentration was determined by spectrophotometer. After boiled at 98°C for 5 min, 50 μg total protein per lane was separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The nitrocellulose sheet was incubated in 5% nonfat milk for 1 h at room temperature, and then exposed in the primary antibodies: ERK1/2(diluted 1:1000) and p-ERK1/2 (diluted 1:500) at 4°C overnight. After washes in PBST three times, the membranes were incubated in the corresponding peroxidase-conjugated second antibodies for 1 h at room temperature, washes it again with PBST. The bands were visualized by Supersignal West Pico

Chemiluminescent Substrate (Pierce, USA).Band intensities were determined by Quantity One software (Bio-Rad, USA).

tPA chromogenic activity assay

The cultured cell supernatant was obtained by centrifuge at 3,000×*g* for 15 min at 4°C to remove debris. The supernatants were collected and the enzymatic activity of tPA was assessed by a chromogenic substrate assay method following the procedure as described [48].

Data analyses and statistics

Experiments were repeated at leastthrice. The results are represented as the means ± SEM. Statistical analysis was used SPSS13.0 and One way ANOVA for analyzing the data in different groups. Probability values <0.05 or 0.01 were considered as significant. For the immunocytochemistry, one representative picture from three similar results was presented.

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