Estrogen and androgen regulate actin-remodeling and endocytosis-related genes during rat spermiation

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ABSTRACT

Spermiation, the sperm release process, is imperative to male fertility and reproduction. Morphologically, it is characterized by removal of atypical adherens junctions called ectoplasmic specializations, and formation of transient endocytic devices called tubulobulbar complexes requiring cytoskeleton remodeling and recruitment of proteins needed for endocytosis. Earlier, estrogen administration to adult male rats was seen to cause spermiation failure due to disruption of tubulobulbar complexes. This was accompanied by reduction in intratesticular testosterone levels and increase in intratesticular estrogen along with deregulation of genes involved in cytoskeleton remodeling (*Arpc1b*, *Evl* and *Capg*) and endocytosis (*Picalm*, *Eea1* and *Stx5a*). In the present study, we aim to understand the role of estrogen and androgen in regulating these genes independently using seminiferous tubule culture system treated with estrogen, androgen or agonists and antagonists of estrogen receptors. We find that transcripts of *Arpc1b*, *Evl* and *Picalm* are responsive to estrogen while those of *Picalm*, *Eea1* and *Stx5a* are responsive to androgen. We also find that the estrogen regulation of *Arpc1b* and *Evl* is mediated through estrogen receptor β and that of *Picalm* occurs through estrogen receptors α and β. Localization of these proteins at or in the vicinity of tubulobulbar complexes reveals that ARPC1B, EVL, PICALM, EEA1 and STX5A seem to be involved in spermiation. Thus, estrogen and androgen regulate specific genes in seminiferous tubules that could play a role in spermiation.

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1. Introduction

The mammalian testis is known to be under the influence of gonadotropins and testosterone which, through the secretion of various local factors, orchestrate spermatogenesis. Testosterone secreted by Leydig cells controls germ cell development in two ways, namely, by its action on Sertoli cells to create a favorable environment for germ cell maturation and by its conversion to estrogen by aromatase (Sharpe et al., 1992, for review see reference O'Shaughnessy, 2014). One such step of spermatogenesis that is known to be regulated by androgen and estrogen is the sperm release process, termed spermiation (D'Souza et al., 2005; Saito et al., 2000).

During spermiation mature spermatids are released from the seminiferous epithelium into the lumen of the tubule. In rats, this occurs during stage VIII of the seminiferous cycle. Stages VII and VIII are known to be particularly androgen dependent and expression of androgen receptors is maximal during stages VII–VIII (Hill et al., 2004). Suppression of androgen and FSH led to spermiation failure in adult rats (Saito et al., 2000). However, the testis also synthesizes estrogen and contains estrogen receptors. Transcript levels of estrogen receptor (ER) α are high during stages VIII–XIV while those of ERβ have similar levels across all stages (Bois et al., 2010). The importance of estrogen was appreciated in our earlier study when disturbance of the androgen/estrogen levels caused by exogenous 17β-estradiol (E2) administration also led to spermiation failure which was accompanied with increase in intratesticular 17β-estradiol (iE) and a concomitant reduction of intratesticular testosterone (iT) and FSH (D'Souza et al., 2005).

Morphologically, spermiation involves removal of the ectoplasmic specialization (ES), an actin-based structure aiding in adherence of spermatids to Sertoli cells, formation of tubulobulbar complexes (TBCs), transient attachment structures aiding in endocytosis of junctional molecules. The ES consists of hexagonally packed actin bundles while TBCs have branched actin networks with continuous endocytosis, thus making these structures a hub of actin reorganization (for reviews see O'Donnell et al., 2011, Russell, 1979,

1991, 1993). Also, the main function of TBCs is the removal of excess spermatid cytoplasm as well as recycling of junctional molecules generally present at the ES thus highlighting the importance of endocytic proteins at TBCs during spermiation (Russell, 1991; Young et al., 2009). In our earlier study of exogenous E2 administration, spermiation failure was caused by disruption of TBCs (D'Souza et al., 2009). Gene expression profiling in the testes of treated rats compared to controls revealed differentially regulated genes. Among them were genes involved in actin remodeling, namely, *Arpc1b* (Actin related protein complex 2/3 subunit 1B), *Evl* (Ena-VASP like protein), *Capg* (Capping protein-gelsolin like) and those involved in endocytosis and intracellular transport, namely, *Picalm* (Phosphatidylinositol binding clathrin assembly protein), *Eea1* (Early endosomal autoantigen 1), *Stx5a* (Syntaxin 5a) (Balasinor et al., 2010).

ARPC1B is one of the seven subunits of Arp2/3 complex that initiates formation of new actin filaments in the presence of nucleation-promoting factors (see Goley and Welch, 2006 for review). EVL belongs to the Ena/VASP family and is known for its 'filament-elongating' property at filopodial tips where it causes actin polymerization (see Sechi and Wehland, 2004 for review). CAPG belongs to the gelsolin/vilin family of actin-regulatory proteins and is known to cap barbed ends of actin thus keeping actin filament growth in check (Southwick and DiNubile, 1986). PICALM is an adaptor protein involved in clathrin internalization machinery during endocytosis (Tebar et al., 1999). EEA1, present on early endosomes, regulates endocytic membrane fusion (Mu et al., 1995). STX5A is a t-SNARE (Target Soluble NSF Attachment Protein Receptor) involved in ER-Golgi transport (Dascher et al., 1994). Based on these known functions in other systems, these proteins may have a role during spermiation and TBC formation.

Although, the *in vivo* exogenous E2 model has brought forth these differentially regulated genes that may be involved in spermiation, it is still unclear as to whether these genes are affected by the observed increase in iE or decrease in iT. Delineating this using an *in vivo* system is difficult due to the interplay of various hormones and secondary hormonal changes brought about by feedback mechanisms on the hypothalamo–pituitary–gonadal axis. Therefore, the purpose of our work was to study the effect of estrogen and androgen separately on gene expression in rat seminiferous tubules. For this, we used an adult rat seminiferous tubule culture system in which basic interactions between germ cells and Sertoli cells are largely preserved (Chapin et al., 2001). We also describe localization of these proteins in adult rat testis under normal and failed spermiation conditions.

2. Materials and methods

2.1. Animals

Male Holtzman rats were obtained from the institute's animal house facility. These animals were maintained at 22 °C in a fixed 14-h light, 10-h dark cycle with free access to food and water. The institutional animal ethics committee approved the use of animals for the study.

2.2. Exogenous estradiol treatment and tissue collection

17β-Estradiol (Sigma-Aldrich, St. Louis, MO, USA) was administered to adult male rats at a dose of 100 μg/kg body weight daily for 3, 5, 7 or 10 days. The drug was suspended in saline and administered by subcutaneous route as described earlier (D'Souza et al., 2005). Control animals received only saline. Control and treated groups consisted of six animals each. For F-actin and immunofluorescence studies, animals were perfusion fixed with warm (37 °C) 4% paraformaldehyde (PFA) prepared in phosphate buffered saline (PBS-0.01M phosphate buffer containing 0.154 M NaCl (pH 7.4))

through the heart for 30 min, stabilized with 30% sucrose, placed in Tissues Tek optimum cutting temperature compound, and snap frozen in liquid nitrogen and stored at –80 °C to be used later for cryosections. For gene and protein expression studies, the animals were euthanized by cervical dislocation; testes were excised, frozen in liquid nitrogen and stored at –80 °C for subsequent RNA extraction.

2.3. Antibodies

Antibodies to ARPC1B (1:100, catalog no. ab99314), EVL (1:500, catalog no. ab108406), CAPG (1:400, catalog no. ab155688) were from Abcam (Cambridge, MA, USA); PICALM (1:200, catalog no. HPA019053), STX5A (1:100, catalog no. WH0006811M1), β-actin (1:200, catalog no. A5441, mouse monoclonal), β-actin (1:300, catalog no. A2668, rabbit polyclonal) and GAPDH (1:1000, catalog no.) were from Sigma. Anti-EEA1 (1:25, catalog no.07–1820) was from Millipore (Temecula, CA, USA). Anti-GM-130 (mouse monoclonal 1:100) was from BD Biosciences (San Jose, CA, USA). Anti-STX5A was mouse monoclonal while anti-EVL was rabbit monoclonal. The rest were rabbit polyclonal unless mentioned otherwise. Secondary antibodies (goat anti-mouse and goat anti-rabbit) for immunofluorescence were conjugated to Alexa flour 488 or 596 (Molecular Probes, Eugene, Oregon, USA) and for Western blot were conjugated to horseradish peroxidase (Sigma).

2.4. Western blotting

Total testicular protein was extracted in radioimmunoprecipitation assay lysis buffer (RIPA) (150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid, 10% sodium dodecyl sulfate) containing protease inhibitors. Forty micrograms of protein was resolved on 12% SDS–PAGE and transferred onto nitrocellulose membrane (GE, Amersham, Buckinghamshire, UK). The blots were washed with PBS, blocked with 5% nonfat dry milk and incubated with a mixture of primary antibodies in 5% nonfat dry milk at 4 °C overnight. The membranes were washed with 0.01 M PBS containing 0.1% Tween 20 and incubated with appropriate secondary conjugated to horseradish peroxidase (Sigma), developed with ECL Plus (GE) or Femto reagent (Pierce Biotechnology, Rockford, IL, USA). The bands were visualized and quantified by densitometry.

2.5. Immunofluorescence and confocal studies

Five-micrometer cryo-sections were taken (Leica Biosystems, Nussloch, Germany) of the testes that were fixed with 4% PFA. They were allowed to air dry and were then post fixed in chilled acetone and dried. They were rehydrated in PBS and antigen retrieval was carried out using 10 mM sodium citrate (pH 6.0), followed by washing with PBS. The sections were blocked with 5% BSA and incubated with appropriate primary antibody overnight at 4 °C. Sections were washed in PBS and incubated with appropriate secondary antibody labeled with Alexa fluor. Sections were counterstained with 4′, 6′-diamidino-2-phenylindole (DAPI; Calbiochem, La Jolla, CA, USA) and mounted in Vecta Shield (Vector Laboratories, Burlingame, CA, USA). The negative controls contained normal mouse and rabbit IgG at equivalent concentration as that of primary antibody. Fluorescent images were captured using the LSM510-Meta confocal system (Carl Zeiss, Jena, Germany) and image acquisition settings were kept constant throughout all experiments for each immunolocalized protein. Images were captured after normalization against negative control. For double staining, the multitrack option was used to eliminate cross talk of chromophores. For fluorescence intensity measurement, the region corresponding to TBCs (in the concavity of step 19 spermatids) at stage VIII was selected and intensity within region of interest was measured in green (actin) and red (protein of interest) channels