17 β -Estradiol induces Akt-1 through estrogen receptor- β in the frog (*Rana esculenta*) male germ cells

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Abstract

Several lines of evidence support the key role of estrogens in male fertility. Here, we investigate the regulation of the serine/threonine kinase Akt-1 in the frog (*Rana esculenta*) testis during the annual sexual cycle and, whether 17 β -estradiol (E₂) exerts a role in the Akt-1 activity. Akt-1 has been shown to be the mediator of growth factor-dependent cell proliferation, survival, and metabolism in a variety of cell types.

First, we demonstrate by immunohistochemistry, the presence of estrogen receptor- β (ER β), and Akt-1 in the spermatogonia (SPG), spermatocytes (SPC), and spermatids (SPT). Western-blot analysis revealed that ER β isoform (molecular weight 55 kDa) was highly expressed in May (reproductive period) with respect to January and November (winter stasis); in parallel, Akt-1 (molecular weight 60 kDa) is highly phosphorylated (Ser-473) during the period of active spermatogenesis (May) compared with the winter stasis (January and November). In addition, *in vitro* experiments demonstrate that E₂ treatment induces the activation of Akt-1, and this effect is counteracted by the anti-estrogen ICI 182-780. In conclusion, our data show that E₂ induces Akt-1 phosphorylation (Ser-473) possibly via ER β in frog (*R. esculenta*) male germ cells.

Reproduction (2006) 132 477–484

Introduction

Estrogens play a pivotal role both in the regulation of male fertility, also they have profound influence on prenatal development, especially on sexual differentiation (O'Donnell *et al.* 2001). In adult males, estrogens are synthesized mainly in the testis, where they are formed from testosterone by the enzyme P450 aromatase. In various species, including humans, P450 aromatase has been found in germ cells (Nitta *et al.* 1993, Levallet *et al.* 1998). Estrogens exert their cellular effects through estrogen receptor (ER) that exist at least in two subtypes, ER α and ER β (Green *et al.* 1986, Mosselman *et al.* 1996). These two subtypes of ER have similar high affinities for 17 β -estradiol (E₂). ER β , but not ER α , is expressed in lizard (*Podarcis sicula*), mouse, and human mitotic and meiotic spermatogenic cells (Jefferson *et al.* 2000, Aschim *et al.* 2004, Chieffi & Varriale 2004, Selva *et al.* 2004, Vicini *et al.* 2006), and its downregulation strongly correlates with human testicular seminomas (Hirvonen-Santti *et al.* 2003, Pais *et al.* 2003). Although spermatogenesis is clearly

disrupted in mice deficient in ER α (ER α knockout, ER α KO), the lack of germ-cell development and the reduction in mature spermatids in the epididymis can be primarily attributed to compromise fluid resorption due to defective efferent ductule function (Hess *et al.* 1997). Recent investigations of mice, deficient in aromatase (cyp 19 gene knockout, ArKO) (Robertson *et al.* 1999), have provided direct evidence for a physiological role of estrogens in male reproductive organs suggesting additional direct effects of estrogens on spermatogenesis. In addition, it has been shown that anti-estrogens have detrimental effects on the morphogenesis and function of the male reproductive system (Oliveira *et al.* 2001, 2002).

Estrogens regulate differentiation, proliferation, and survival in different cell types activating rapidly, a kinase cascade, which in turn promotes transcription of the immediate early genes (Watters *et al.* 1997, Revelli *et al.* 1998). In addition to the mitogenic role exerted by E₂ through the c-Src/p21^{ras}/MAP kinase pathway, it has been reported that E₂ rapidly stimulates a p85-regulated PI3-kinase and Akt as well as the S-phase entry of cells

(Migliaccio *et al.* 1996, Simoncini *et al.* 2000, Castoria *et al.* 2001, Acconcia *et al.* 2005). The serine/threonine kinase Akt or protein kinase B (PKB) is a downstream effector of phosphatidylinositol 3 (PI 3)-kinase. It was shown to be the mediator of growth factor-dependent cell survival in a variety of cell types (for reviews, see Datta *et al.* 1999, Kandel & Hay 1999); in fact, Akt inactivates several pro-apoptotic molecules, including Bad, caspase-9, forkhead transcription factors, I κ B kinase, and p53 (through MDM2-mediated phosphorylation), resulting in enhanced cell survival (Kandel & Hay 1999). In addition, Akt has been implicated in executing many of the metabolic functions of insulin and growth factors, such as protein and lipid synthesis, carbohydrate metabolism, and transcription (for review, see Kandel & Hay 1999). The kinase activity of Akt is constitutively activated in human cancer because of mutation/deletion of the tumor suppressor PTEN, a phospholipid phosphatase that negatively regulates Akt activation (for review, see Simpson & Parsons 2001, Di Vizio *et al.* 2005).

Three major isoforms of Akt/PKB, termed Akt-1/PKB α , Akt-2/PKB β , and Akt-3/PKB γ , encoded by three separate genes with >85% sequence identity, have been found in mammalian cells. All Akt/PKB isoforms are assumed to have identical or similar substrate specificity (for reviews, see Alessi & Cohen 1998, Kandel & Hay 1999). Among the three Akt isoforms, Akt1 is the predominantly expressed one in most tissues.

Previously, we have demonstrated that E₂ induces Akt-1 activity in the lizard (*Podarcis s. sicula*) testis (Russo *et al.* 2005). *Rana esculenta* spermatogenesis is characterized by an annual gonadal cycle with a slow progression of germ cells during the winter stasis and an increase of spermatogonial proliferation in spring concomitantly with E₂ peak (Chieffi *et al.* 2000a,b). Interestingly, it has been demonstrated that E₂ induces spermatogonial proliferation by inducing ERK1/2 activation (Chieffi *et al.* 2000b). In this report, we have investigated the variations of the Akt-1 activity during the annual sexual cycle of the spermatogenesis of frog *R. esculenta*, and the role exerted by E₂ in the Akt-1 activation through the ER β .

Materials and Methods

Animals

Male frogs (*R. esculenta* L.) have been captured in the vicinity of Naples. Animals were killed by decapitation under anesthesia with MS222 (0.05% in aqueous solution, Sigma) and immediately the testes were removed and stored at -80 °C until processed for or quickly prepared for histological examinations. The animal experimentations described herein were conducted in accordance with accepted standards of animal care and the Italian regulations for the welfare of animals

used in experimental studies. The study was approved by our institutional committee on animal care.

Protein extract preparations

The frozen frog testes were homogenized directly into lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton-X-100 (1:2 weight/volume), 1 mM phenylmethylsulfonyl fluoride (PMSF) 1 μ g aprotinin, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate, (Sigma), and clarified by centrifugation at 14 000 g for 10 min. Protein concentrations were estimated using a modified Bradford assay (Bio-Rad).

Antibodies

Antibodies were purchased from the following sources: (1) polyclonal anti-phospho-Akt-1 kinase (Ser-473) antibody (#4058, Cell Signaling, MA, USA); (2) polyclonal anti-Akt-1 antibody (#9272, Cell Signaling, MA, USA); (3) polyclonal rabbit antibody anti-estrogen receptor β (#sc-8974, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); (4) monoclonal anti- β -tubulin (#T-4026, Sigma).

Western-blot analysis

Proteins, 50 μ g, were boiled in Laemmli buffer for 5 min before electrophoresis. The samples were subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membrane (Immobilon Millipore Corporation, Bedford, MA, USA); complete transfer was assessed using pre-stained protein standards (Bio-Rad). The membranes were treated for 2 h with blocking solution (5% non-fat powdered milk in 25 mM Tris, pH 7.4; 200 mM NaCl; 0.5% Triton-X-100, TBS/T) and incubated for 1 h at room temperature with the primary antibody: (1) against phospho-Akt-1 (diluted in the ratio, 1:1000); (2) against Akt-1 (1:2000); (3) against ER β (1:500); and (4) against β -tubulin (1:1000). After washing with TBS/T and TBS, membranes were incubated with the Horseradish peroxidase-conjugated secondary antibody (1:3000) for 45 min (at room temperature) and the reaction was detected with ECL system (Amersham).

Immunohistochemistry

Frog testes, rapidly removed and fixed in Bouin's fluid, were dehydrated in ethanol series and cleared in xylene. For each paraffin-embedded sample, a 4 μ m thick serial sections mounted on slides were dewaxed in xylene and brought through ethanol to deionized distilled water. Ten sections/animal per month have been examined. The

endogenous peroxidases were quenched by incubation of sections in 0.1% sodium azide with 0.3% hydrogen peroxide for 30 min at room temperature; non-specific binding were blocked by incubation with non-immune serum (1% Tris-BSA for 15 min at room temperature).

All sections were pretreated with 0.5% trypsin in 0.1% HCl for 30 min at 37 °C to unmask antigen. Before the immunohistochemical staining, sections were incubated in a 750 W microwave oven for 15 min (three cycles of 5 min) in 10 mM buffered citrate, pH 6.0, to complete antigen unmasking.

The standard streptavidin-biotin-peroxidase complex procedure was used (Dako, Corp. Denmark). Anti-P-Akt-1 (Ser-473), anti-Akt-1, and anti-ER β antibodies were used at a dilution of 1:200, 1:400, and 1:200 respectively. The peroxidase activity was developed with the use of a filtered solution of 5 mg 3-3'-diaminobenzidine tetrahydrochloride dissolved in 10 ml Tris buffer (0.05 M, pH 7.6) and 0.03% H₂O₂. For nuclear counterstaining, Mayer's hematoxylin was employed. Sections were mounted on a synthetic medium.

The following controls were performed: (1) omission of the primary antibody; (2) substitution of the primary antiserum with non-immune serum (Dako, Corp. Denmark), diluted 1:500 in blocking buffer, (3)

addition of the target peptide used to produce the antibody (10^{-6} M); no immunostaining was observed after any of the control procedures.

In vitro experiments

Protein extracts were prepared from frog ($n=15$ /month) testes, collected each month, were considered to detect Akt-1 immunoreactivity, and used for western-blot analysis.

To determine E₂ effect on germ cells, 50 animals have been captured in February, testes were removed and deprived of albuginea membranes, and then placed in cold KRB. After washing in KRB at 20–22 °C, ten testes (time 0) have been homogenized directly into lysis buffer for protein extraction, while 60 testes have been incubated at 20–22 °C in KRB containing E₂ (10^{-7} M), or E₂ (10^{-7} M) + ICI 182–780 (10^{-6} M), or ICI 182–780 (10^{-6} M) for 20, 40, and 60 min. Ten testes/time per treatment have been processed for western-blot analysis.

Statistics

Statistical analysis for the significance was performed using ANOVA followed by Duncan's test for multigroup comparisons.

Figure 1 Immunocytochemistry for ER β proteins in the *Rana esculenta* testis showing positive reaction in the spermatogonia (SPG), spermatocytes (SPC), spermatids (SPT), Sertoli cells (Ser), and interstitial tissue (*) during: (A) the reproductive period (May); (B) post-reproductive period (September); and (C) the winter stasis (January); (D) control section in which symbol are the same as those used to show positive reactions. Bar = 25 μ m.

Figure 2 Western-blot detection of ER β proteins in the testicular extracts of *Rana esculenta* during different months. Proteins (50 μ g/lane per month) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibody raised against (A) ER β (Santa Cruz Biotechnology, Inc., CA, USA). A specific band was observed sizing 55 kDa by comparison with the mouse testicular extract positive control and co-migrating size markers (Bio-Rad). (B) β -Tubulin (Sigma) was used to assess the equal amounts of protein. The blots are representative of three separate assays. (C) The amount of ER β was quantitated using ImageQuant 5.2 Program and normalized by total β -tubulin. The values shown represent the mean $\pm$ S.E.M. of three separate assays.

Results

ER β localization in the *R. esculenta* testis during the annual cycle

In order to precisely determine the cells producing ER β , we used immunohistochemistry approach to localize ER β protein in frog *R. esculenta* testis. Since the ER β sequence is not known in the frog, we used an antibody from human N-terminal sequence conserved in mouse and rat.

Immunohistochemistry analysis was performed on serial sections and revealed the presence of ER β protein in this organ. The immunopositivity was found throughout the year in the germinal epithelium and the interstitial tissue. In particular, in spring (reproductive period), and in the post-reproductive period

(September), among the germ cells, the positivity was intense and localized in the spermatogonia (SPG), cysts of spermatocytes (SPC), spermatids (SPT), and Sertoli cells (Ser) (Fig. 1A and B), while the abundant spermatozoa (SPZ) remained negative. The positivity was observed also in the interstitial tissue (Fig. 1B). In January, the frog testis showed all stages of spermatogenesis, but the spermatogonial mitotic index was rather low and there were several cysts of degenerating spermatocytes, spermatids, and few spermatozoa (Chieffi *et al.* 2000a). The ER β immunopositivity was not intense in SPG, SPC, and SPT (Fig. 1C). Specificity of antibody binding is indicated by addition of the target peptide used to produce the antibody (Fig. 1D).

In addition, to confirm the antibody specificity, western-blot analysis was performed in which, a specific band of 55 kDa was detected in the frog testis extracts when compared with that of the mouse, used as positive control (Fig. 2A) (Selva *et al.* 2004, Vicini *et al.* 2006). The 55 kDa band was the only one detected in the frog testis lysates throughout the annual cycle, with difference in expressions in the months observed (Fig. 2A), in fact, an increased expression in May (reproductive period) with respect to other months considered (January, September, and November). In addition, β -tubulin western-blot was used to assess equal amounts of protein (Fig. 2B).

Localization of Akt-1 proteins during the annual cycle

Immunohistochemistry analysis was utilized to localize the serine/threonine kinase Akt-1 in the *R. esculenta* testis. This method revealed that Akt-1 proteins were present in frog testis. In the germinal epithelium, the immunopositivity was found throughout the year in the SPG, SPC, and SPT (Fig. 3A). In particular, during spring, the seminiferous tubules became active (reproductive period) and increased numbers of P-Akt-1 (Ser-473) immunopositive SPG, SPC, and SPT were observed (Fig. 3B) with respect to the winter stasis (Fig. 3C). Specificity of antibody binding is indicated by addition of the target peptide used to produce the antibody (Fig. 3D).

Akt-1 activity during the annual cycle

The Akt-1 isoform was characterized by western-blot. A specific band of 60 kDa was detected during the annual cycle (Fig. 4B) with a different phosphorylation status on Ser-473; in fact, a strong phosphorylation was present during the period of active spermatogenesis (May) with respect to the winter stasis (January and November) (Fig. 4A). In addition, β -tubulin western-blot was used to assess equal amounts of protein (Fig. 4C).

Figure 3 (A) Immunocytochemistry for Akt-1 proteins in the *Rana esculenta* testis shows immunopositive spermatogonia (SPG), spermatocytes (SPC), and spermatids (SPT) in November. (B) Immunocytochemistry for P-Akt-1 (Ser-473) proteins in the *R. esculenta* testis shows immunopositive SPG, SPC, and SPT during the reproductive period (May) and (C) during the winter stasis (January); (D) control section in which symbol are the same as those used to show positive reactions. Bar=25 μ m.

In vitro E₂ effects on Akt-1 activity

Total proteins isolated from *R. esculenta* testes incubated with E₂, E₂+ICI 182–780, and ICI 182–780 were analyzed by western-blot analysis. During February, testes incubated with E₂ alone showed an increase of Akt-1 activity after 20, 40, and 60 min as compared with control (Fig. 5, lanes 1, 2, 3, and 4). The increase of the Akt-1 activity was more pronounced after 20 min, whereas testes incubated with E₂+ICI 182–780 after 20 min, and with ICI 182–780 after 20 min showed a lower activity, relative to the sample with E₂ treatment alone (Fig. 4, lanes 5 and 6; 40 and 60 min, not shown). In addition, β -tubulin western-blot was used to assess equal amounts of protein (Fig. 5C).

Discussion

The present results demonstrate that Akt-1 is present in frog germ cells and may suggest a biological role for these enzymes in germinal epithelium proliferation and

survival. Moreover, there were seasonal changes, which correlate with both steroidogenic and spermatogenic activity; in fact, the variations of Akt-1 phosphorylation fit well with the annual plasma E₂ concentration (Chieffi *et al.* 2000b). The E₂ profile shows a progressive increase during the period when spermatogonial mitosis is reinitiated (May); this corresponds to a high level of Akt-1 activation and the continuous recruitment of new sets of SPG undergoing mitosis (Chieffi *et al.* 2000a,b). Previously, it has been shown that E₂ treatment induces the proliferation of SPG, possibly via the activation of ERK1/2 and this effect is counteracted by the anti-estrogen ICI 182–780 (Chieffi *et al.* 2000b).

In vitro experiments and western-blot analysis reveals that in testes, Akt-1 activity increases after 20 min of E₂ incubation; this effect is counteracted by the anti-estrogens ICI 182–780. It has also been reported that Akt-1 is activated by E₂ in cell lines derived from human mammary cancer and vascular endothelial cells through the formation of ER/Src/p85-regulated PI3-kinase complex promoting the S-phase entry (Simoncini *et al.*

Figure 4 Western-blot detection of Akt-1 proteins in the testicular extracts of *Rana esculenta* during different months. Proteins (50 µg/lane per month) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibody raised against (A) phospho-Akt-1 (Ser-473, Cell Signaling, MA, USA), and (B) Akt-1 protein (Cell Signaling, MA, USA). A specific band was observed sizing 60 kDa by comparison with co-migrating size markers (Bio-Rad). (C) β-Tubulin (Sigma) was used to assess the equal amounts of protein. The blots are representative of three separate assays. (D) The amount of activated Akt-1 was quantitated using ImageQuant 5.2 Program and normalized by total Akt-1. The values shown represent the mean $\pm$ S.E.M. of three separate assays.

2000, Castoria *et al.* 2001). Recently, it has been demonstrated that E_2 acts as a germ cell survival factor in the human testis *in vitro* (Pentikainen *et al.* 2000). In addition, it has also been demonstrated that Akt/PTEN signaling mediates estrogen-dependent proliferation of primordial germ cells (PGCs) *in vitro* through the somatic cells of the gonadal ridges, stimulating the transcription of the *Steel* gene and the production of c-Kit ligand in gonadal somatic cells responsible for the observed stimulation of PGCs (Moe-Behrens *et al.* 2003).

In the testes, the effect of E_2 could be mediated by Leydig and/or Sertoli cells, which possess both $ER\alpha$ and $ER\beta$, or through $ER\beta$ located in germ cells. In fact, the presence of $ER\beta$ has been demonstrated in the present

Figure 5 (A) Western-blot detection of Akt-1 proteins in the testis of *Rana esculenta*. Testes have been incubated with KRB (Cont), E_2 (for 20, 40, and 60 min), E_2 + ICI 182–780 (for 20 min), ICI 182–780, (for 20 min). Proteins (50 µg/lane per month) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibody raised against (A) phospho-Akt-1 (Cell Signaling, MA, USA), and (B) Akt-1 protein (Cell Signaling, MA, USA). A specific band was observed sizing 60 kDa by comparison with co-migrating size markers (Bio-Rad). (C) β-Tubulin (Sigma) was used to assess the equal amounts of protein. The blots are representative of three separate assays. (D) The amount of activated Akt-1 was quantitated using ImageQuant 5.2 Program and normalized by total Akt-1. The values shown represent the mean $\pm$ S.E.M. of three separate assays.

and previous works on SPG, SPC, SPT, and Sertoli cells of human (Aschim *et al.* 2004), mouse (Jefferson *et al.* 2000, Selva *et al.* 2004, Vicini *et al.* 2006), rat (Saunders *et al.* 1998, van Pelt *et al.* 1999), and lizard (Chieffi & Varriale 2004) testes. In addition, several steroid hormones exert rapid effects on cells by interacting with specific receptors present on the surface (Revelli *et al.* 1998) or cross-talking with pathways activated by growth factors (Smith 1998). Alternatively, estrogens synthesized by the germ cells, could act in an intracrine or paracrine fashion, providing a local source of estrogen involved in controlling the complex process of spermatogenesis. The presence of cytochrome P450 aromatase in rat germ cells confirms the existence of an additional source of estrogens in the testis (Nitta *et al.* 1993, Levallet *et al.* 1998). In the testis, germ cells undergo a complex program of proliferation and differentiation to form mature sperms (Chaganti & Houldsworth 2000). Correct

proliferation and apoptosis is required to regulate the size of cell lineages and the timing of differentiation (Matsui 1998, Chaganti & Houldsworth 2000). In vertebrates, germ-cell proliferation seems to depend on a network of factors interacting locally, such as stem-cell factor, c-kit receptor (Sorrentino *et al.* 1991, Loveland & Schlatt 1997, Munsie *et al.* 1997), platelet-derived growth factor (Li *et al.* 1997), and E₂ (Chieffi *et al.* 2000b, 2002). Further studies are needed to clarify the signal transduction pathways involved in their activation and the downstream targets of their action. The spatial and temporal interaction of these factors is an intriguing aspect of the testicular activity that opens multiple ways for future researches.

In conclusion, using the frog model, which is characterized by an annual gonadal cycle, we show that Akt-1 is present and phosphorylated in germ cells and E₂ induces Akt-1 activity possibly via ER β in the testis. This action may have a crucial role/s in mechanisms related to germ cells proliferation, differentiation, and survival. These results may also provide a tool for the understanding of the cellular and molecular mechanisms involved in pathological conditions such as infertility and testicular tumorigenesis.

In addition, we suggest that seasonal system may represent important model to study the various steps of spermatogenesis progression, since each step of this cascade of event can be analyzed separately at different time. The species in which the spermatogenesis regulation is continuous, only pharmacological stimulations can be used to modulate the seminiferous epithelium, while in seasonal system the physiological progression allows to investigate each step separately.

Acknowledgements

This work was supported by Grant of Second University of Naples (2005) (P. C.). We thank Fabrizio Fiorbianco (www.studiociotola.it) for skilful technical assistance with the artwork. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 12 January 2006

First decision 27 March 2006

Revised manuscript received 29 April 2006

Accepted 28 June 2006