Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (*Rana esculenta*) testis

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Abstract

Several lines of evidence support a key role of estradiol-17 β (E₂) in male fertility. We have used a non-mammalian vertebrate model, the frog *Rana esculenta*, to investigate the regulation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) activity in the testis during the annual sexual cycle and to study whether E₂ exerts a role in spermatogenesis through the regulation of ERK1/2 activity.

ERK1/2 proteins are present in the cytoplasm and nucleus of the primary and secondary spermatogonia (SPG), and in the nucleus of primary spermatocytes. The

annual E₂ profile shows a progressive increase during active spermatogenesis with a peak in the month of June. In parallel, ERK1/2 are highly phosphorylated during the period of active spermatogenesis (from April to July) compared with the regressive period (September/October) and winter stasis (from November to March). E₂ treatment induces the proliferation of primary SPG, possibly via the activation of ERK1/2, and this effect is counteracted by the anti-estrogen ICI 182-780.

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Introduction

Male mice homozygous for a mutation in estrogen receptor (ER) α isoforms (ER knockout) are infertile, indicating an essential role for ER-mediated processes in the regulation of male reproduction (Eddy *et al.* 1996, Couse & Korach 1999, Gustafsson 1999). In fetal and adult rat testes, the expression of ER β mRNA as well as ER β protein is present in the germinal compartment in type A spermatogonia (SPG), pachytene spermatocytes (SPC), round spermatids, and in the Sertoli cells (Saunders *et al.* 1998, Shughrue *et al.* 1998, Van Pelt *et al.* 1999). In rats, gonocyte proliferation is induced by estradiol (E₂) (Li *et al.* 1997). Furthermore, a reduction in testicular estrogen levels inhibits spermatid maturation (Tsutsumi *et al.* 1987a,b). Normal spermatogenesis has been reported in hypophysectomized rats receiving follicle-stimulating hormone (FSH) and E₂ (Steinberger & Duckett 1967). It has been demonstrated that E₂ is capable of potentiating the stimulatory effects of FSH on N-cadherin mRNA in cultured mouse Sertoli cells (MacCalman *et al.* 1997).

Newbold & McLachlan (1985) reported that prenatal or early postnatal treatment with estrogens has detrimental effects on the morphogenesis and function of the male reproductive system. In addition, knockout mice that lack a functional aromatase enzyme and are therefore unable to convert C₁₉ steroids (androgens) to C₁₈ steroids (estrogens) show a progressively disrupted spermatogenesis (Robertson *et al.* 1999).

Steroid hormones regulate differentiation and proliferation in different cell types also by the activation of the kinase cascade which, in turn, promotes transcription of the immediate early genes (Watters *et al.* 1997). Mitogen-activated protein kinases (MAPK) occupy a focal point in signal transduction, mainly by activating gene transcription, including c-Myc, Ets1, Elk1 and c-Jun via translocation into the nucleus (Karin 1995, Waskiewicz & Cooper 1995, Cobb 1999). Among the members of this family the most extensively studied are p44 and p42 MAPK, also known as extracellular signal-regulated kinases (respectively ERK1 and ERK2), Jun amino-terminal kinase (JNK1/2), and p38. ERKs are expressed

ubiquitously and are closely related to the yeast protein kinases involved in pheromone-induced mating (Nielsen 1993). For example, it has been shown that 11-ketotestosterone induces ERK phosphorylation in sea star spermatogonia (Walker *et al.* 1998), and that JNK1 has a different activity in the lizard (*Podarcis s. sicula*) testis during the annual reproductive cycle (Chieffi *et al.* 1999).

Previous studies have demonstrated that ERs are expressed in the testis of the frog, *Rana esculenta* (Fasano *et al.* 1989) and that E₂ induces spermatogonial proliferation (Minucci *et al.* 1997). *R. esculenta* spermatogenesis is characterized by a slow progression of the germ cells during winter stasis and spermatogonial proliferation in spring and summer (spermatid and spermatozoa appearance) (Rastogi *et al.* 1976). Interestingly, E₂ peaks concomitantly with spermatogonial proliferation (Varriale *et al.* 1986, Minucci *et al.* 1997).

In this report, we have shown the variations in ERK (ERK1/2) activity during the annual sexual cycle in the testis of the frog, *Rana esculenta*, and have demonstrated that E₂ induces spermatogonial multiplication through the MAPK (ERK1/2) pathway, suggesting that these enzymes play a key role in the regulation of testicular epithelium proliferation.

Materials and Methods

Animals

Male frogs (*Rana esculenta*) were captured in the vicinity of Naples. They were killed by decapitation under anesthesia with MS222 (0.05% in aqueous solution; Sigma Chemical Co., St Louis, MO, USA), and the testes were removed immediately and either stored at -80 °C until processed or prepared quickly for histological examination.

Protein extract preparations

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, w/v), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg 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, Melville, NY, USA).

Antibody

The antibodies were purchased from the following sources: (1) polyclonal anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody (no. 9101S; New England Biolabs, Beverly, MA, USA), raised in rabbits, (2) polyclonal anti-ERK1 (no. sc-94-G; Santa Cruz Biotechnol-

ogy Inc., Santa Cruz, CA, USA) raised in rabbits against epitope corresponding to an amino acid sequence conserved in frog, chicken, murine and human, (3) monoclonal anti-β-tubulin (clone no. TUB 2.1, no. T-4026; Sigma) and (4) mouse monoclonal antibody against recombinant proliferating cellular nuclear antigen (PCNA; Dako Corp., Copenhagen, Denmark).

Western blot analysis

Forty micrograms total protein extracts were boiled in Laemmli buffer (Laemmli 1970) for 5 min before electrophoresis. The samples were subjected to SDS-PAGE (11% polyacrylamide) under reducing conditions. After electrophoresis, proteins were transferred to nitrocellulose membrane (Immobilon Millipore Corporation, Bedford, MA, USA); complete transfer was assessed using prestained 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 and 0.5% Triton X-100; TBS/T), and then the membranes were incubated for 1 h at room temperature with the primary antibody, (1) against phospho-ERK1/2 (diluted 1:2000), (2) against ERK1/2 (diluted 1:2000) and (3) against β-tubulin (diluted 1:1000). After washing with TBS/T and TBS, membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:2000) for 45 min (at room temperature) and the reactions were detected with an enhanced chemiluminescence system (Amersham Life Sciences, Amersham, Bucks, UK).

ERK1/2 immunocytochemistry

Frog testes rapidly removed and fixed in Bouin's fluid were dehydrated in an ethanol series and cleared in xylene. For each paraffin-embedded sample, 4 µm serial sections mounted on slides were dewaxed in xylene and brought through ethanols to deionized distilled water. Ten sections per animal per month were 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 was blocked by incubation with non-immune serum (1% Tris-bovine serum albumin (BSA) for 15 min at room temperature).

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

The standard avidin-biotin-peroxidase complex (ABC) procedure was used (Hsu *et al.* 1981). Anti ERK1/2 antibodies were used at a dilution of 1:400. Negative controls were performed using normal mouse serum

instead of the primary antibody. 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 with a synthetic medium.

Controls

The following controls were performed: (1) omission of the primary antibody and (2) substitution of the primary antiserum with non-immune serum (Dako Corp.) diluted 1:500 in blocking buffer; no immunostaining was observed after any of the control procedures.

Kinase assay

Testes were lysed with 1% NP-40 lysis buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.4 mM EDTA, 0.1 mg/ml PMSF, 2 mg/ml leupeptin, 2 mM Na vanadate, 10 mM NaF, 10 mM Na pyrophosphate and 2 µg/ml aprotinin). Protein concentration was estimated using a modified Bradford assay (Bio-Rad). Four hundred micrograms total proteins from each sample were immunoprecipitated with anti-ERK1 rabbit polyclonal antibodies (sc-94-G) at 4 °C for 2 h. The immunoprecipitates were washed once with lysis buffer and twice with assay buffer. To test kinase activity, the immunoprecipitates were incubated for 30 min at 30 °C in 30 µl assay buffer (20 mM Hepes, pH 7.5, 2 mM sodium vanadate, 10 mM Mg acetate, 0.1 mg/ml PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin and 100 mM ATP) supplemented with 8 µg myelin basic protein (MBP) and 8 µCi [γ -³²P]ATP. The reaction was terminated by the addition of 30 µl SDS buffer and proteins were separated by 14% SDS-PAGE. ³²P-Labeled bands were revealed by autoradiography of the dried gel and quantified on a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

Seasonal cycle and in vivo experiments

Total protein extracts were prepared from frog ($n=15$ /month) testes collected each month for 1 year and subjected to Western blot to detect ERK1/2.

To determine the effect of E₂ on germ cell proliferation, 30 animals were captured during February and divided into three experimental groups. The control group ($n=10$) was injected with 100 µl amphibian Krebs–Ringer bicarbonate buffer (KRB), pH 7.4. One group ($n=10$) was injected with 100 µl KRB containing 270 ng E₂, and one group ($n=10$) was injected with 100 µl KRB containing 270 ng E₂+100 µl KRB containing 6 µg ICI 182–780 (Zeneca, Macclesfield, Cheshire, UK). Animals received a single injection in the dorsal sac and were killed 24 h after the injection.

PCNA immunocytochemistry

For assessment of cell proliferation, PCNA immunohistochemistry was performed. Five micrometer paraffin sections were incubated with mouse monoclonal antibody against recombinant PCNA (Dako Corp.) at a dilution of 1:400 with 10% BSA, followed by incubation with horse anti-mouse IgG (1:1000) and ABC. The chromogen was developed in the same solution as described above. PCNA labeling in primary SPG (I SPG) was counted and a comparison among control, E₂-treated and E₂ plus ICI 182–780-treated animals was performed. At least four sections, 5 µm apart, from each frog testis ($n=10$ animals per experimental group) were used for quantitative analysis. The proliferative index of I SPG was expressed as the number of PCNA immunopositive per total I SPG counted per 40 sections multiplied by 100.

Radioimmunoassay (RIA)

E₂ was extracted by diethyl ether using specific antiserum (Sorin-Biomedica kit, Torino, Italy) and measured by RIA in 2 ml of two pools of plasma. Antiserum raised against E₂ did not cross-react with 15 α -hydroxyestriol, estriol, estrone, cortisol and cholesterol. Serial dilutions of plasma samples paralleled the standard curve. Intra- and interassay coefficients of variation were 5 and 10%; sensitivity was 5 pg/tube.

Statistics

Statistical analysis was performed using the analysis of variance test.

Results

Expression of ERK1/2 proteins during the annual cycle

We first addressed whether ERK1/2 proteins were expressed in frog testis. Immunocytochemistry was performed on serial sections and immunopositivity was found throughout the year in the germinal epithelium localized in the cytoplasm and nucleus of I SPG and secondary (II) SPG, and in the nucleus of primary SPC (I SPC) (Fig. 1a).

It is important to note that the antiserum used in the present study fulfils the criteria of specificity for investigating the distribution of immunoreactive materials. In particular, immunoadsorption tests revealed that the labeling was totally blocked by preincubation with 10^{−6} M of the cognate peptide (Fig. 1b), but was not affected by the same concentrations of non-related proteins. The antibodies used recognize amino acidic residues conserved in the frog.

The ERK isoforms were characterized by Western blot, and specific bands of 44 and 42 kDa were detected during the annual cycle (Fig. 2a) with a different activation status.

Figure 1 Immunocytochemistry for ERK1/2 proteins in the *R. esculenta* testis showing (a) positive reaction in cytoplasm and nuclei of I SPG (arrowheads) and II SPG (solid arrows) and in nuclei of I SPC (open arrows) in April and (b) control section (for details see Materials and Methods). Symbols are the same as those used to show positive reactions.

In fact, strong phosphorylation was present during the period of active spermatogenesis (from April to July) in contrast to the post-breeding period (September/October) and winter stasis (from November to March) (Fig. 2a). β -Tubulin Western blot was used to assess equal amounts of protein (Fig. 2a). Moreover, we also performed an ERK1 kinase assay in three different months: February, May and September. We found that ERK1 activity in May (active spermatogenesis) was high, in September (regressive period) it was low, whereas in February (winter stasis) it showed intermediate levels. The variations of activity fit well with the ERK1/2 phosphorylation status (Fig. 3).

Hormonal cycle

Plasma levels of E_2 were found at low levels during the autumn and winter and increased from April until July ($P < 0.01$, Duncan's test). The highest level was observed

in July ($P < 0.001$ vs March, Duncan's test), and decreased dramatically in September, reaching baseline values (Fig. 2b).

Effect of E_2 on ERK1/2 phosphorylation in vivo

The kinase assay (utilizing MBP as substrate) confirmed that *in vivo* treatment with E_2 in February increased ERK1/2 activity after 24 h compared with controls; this effect was counteracted by ICI 182-780 treatment (Fig. 4).

In order to assess the primary spermatogonial proliferation we utilized the PCNA as an early molecular marker (Fig. 5a). In fact, using a monoclonal antibody against the PCNA isoform, Western blot analysis showed a specific band of 36 kDa (data not shown). Treatment with E_2 increased the PCNA-immunopositive I SPG after 24 h compared with controls. This effect was counteracted by ICI 182-780 treatment (Fig. 5b).

Discussion

The present results have demonstrated that ERKs are present in frog germ cells, and suggest a role for these enzymes in germinal epithelium proliferation. However, the variations in the expression of ERK proteins and of their state of activation do not always correlate. For example, in the month of March, testes express high amounts of ERK1/2 proteins and low levels of phosphorylation of these proteins. *Vice versa*, in the months of June and July while the amount of ERK proteins decreases, the state of activation remains elevated.

Another interesting observation is that from April to July the level of activation of ERKs remains steadily elevated. On the one hand, the high level of ERK activation could be ascribed to the continuous recruitment of new sets of cells undergoing mitosis, but also to a different regulation of the kinase activity, especially in the months of March, June and July.

Since the changes in the ERKs state of activation also correlate with spermatogenetic activity, it is likely that these enzymes play a key role in the regulation of testicular epithelium proliferation.

In testicular germ cells, ERKs were predominantly localized to the nuclei, where they might also regulate gene transcription and substrate phosphorylation which, in turn, regulate cell proliferation. It has recently been reported that MAP-2, a possible substrate for ERKs, is abundant in rat testis and is localized both in the cytoplasm and, in the case of the human testis, in the nuclei of germ cells (Loveland *et al.* 1996). The lack of ERK staining in tubules of the testes of azoospermic men is a further indication of the possible role of ERKs in the maturation and differentiation of germ cells (Luconi *et al.* 1998).

Figure 2 (a) Western blot detection of ERK1/2 proteins in the testicular extracts of *R. esculenta* during the annual cycle. Proteins (40 µg per lane per month) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and then incubated with antibody raised against (1) phospho-ERK1/2 (P-ERK1/2) and (2) ERK1/2 protein. Two specific bands were observed at 44 and 42 kDa by comparison with co-migrating size markers. β-Tubulin was used to assess the equal amounts of protein. The blots are representative of four separate assays. (b) Annual profile of plasmatic E₂ in *R. esculenta*. Data are expressed as means ± S.E.M.

The variations in ERK1/2 phosphorylation following seasonal changes also correlate with the annual plasma E₂ concentration. It has recently been demonstrated that E₂ treatment induces the proliferation of rat gonocytes (Li *et al.* 1997), and of frog and sea star SPG (March & Walker 1995, Minucci *et al.* 1997, Walker *et al.* 1998).

In vivo experiments suggest that E₂ treatment induces ERK1/2 activation and a role for this hormone in the regulation of I SPG multiplication. In fact, the ability of these cells to undergo mitotic division is strongly inhibited when pretreatment with an anti-estrogen (ICI 182-780) is performed, as confirmed using PCNA as a molecular marker of I SPG proliferation (Schlatt & Weinbauer 1994, Chieffi *et al.* 2000). It has been demonstrated in *in vitro*

experiments that the primary spermatogonial mitotic index strongly increases in the frog (*R. esculenta*) testes incubated with E₂ but not in E₂ plus the anti-estrogen tamoxifen-treated testes (Minucci *et al.* 1997). Gill-Sharma *et al.* (1993) showed that the anti-estrogen tamoxifen has effects on the seminiferous tubule and impaired spermatogenesis may have been due to disturbed functioning of Sertoli cells. It is important to note that the E₂ treatment was carried out in February when the I SPG mitotic proliferation had not yet started and the endogenous E₂ concentration was at basal level.

It has also been reported recently that E₂, like several growth factors, activates the mitogenic c-Src/p21^{ras}/MAPK signal transducing pathway (Migliaccio *et al.* 1996,

Figure 3 (a) ERK1 kinase activity in three different months: February, May and September, corresponding respectively to winter stasis, breeding period and regressive period. ERK1 kinases were immunoprecipitated from whole extracts obtained each month, and equal amounts of protein were added to MBP in the presence of [γ - 32 P]ATP, as described in Materials and Methods. The samples were then subjected to SDS-PAGE and autoradiography. (b) The values shown represent the mean $\pm$ S.E. of four separate assays using a phosphoimager. IP, immunoprecipitation.

Revelli 1998), suggesting a role of E_2 in the regulation of cell proliferation, although via an as yet unknown mechanism. In the testes, the effects of E_2 could be mediated by Leydig and/or Sertoli cells, which possess ERs, or through putative receptors located in germ cells. In fact, the presence of ER β has been demonstrated in the SPG, SPC, spermatids and Sertoli cells of fetal and adult rat testis (Saunders *et al.* 1998, Shughrue *et al.* 1998, Van Pelt *et al.* 1999). In addition, several steroid hormones exert rapid effects on cells by interacting with specific receptors present on the surface (Pappas 1995, Collins & Webb 1999) or cross-talking with pathways activated by growth factors (Smith 1998). For example, it has been demonstrated that E_2 stimulates tyrosine phosphorylation of several sperm proteins (Luconi *et al.* 1999). Alternatively, estrogens synthesized by the germ cells could act in an intracrine or paracrine fashion, providing a local source of

Figure 4 (a) ERK1 kinase activity in the testis of *R. esculenta* injected with KRB (Cont), E_2 and E_2 +ICI 182–780 (ICI) and killed after 24 h. ERK1 kinases were immunoprecipitated from whole extracts, and equal amounts of protein were added to MBP in the presence of [γ - 32 P]ATP, as described in Materials and Methods. The samples were then subjected to SDS-PAGE and autoradiography. (b) The values shown represent the mean $\pm$ S.E. of four separate assays using a phosphoimager.

estrogens 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).

The ability to translocate from one cell compartment to another in response to extracellular signals is a common feature of MAPKs (Bokemeyer *et al.* 1996). Indeed, it has been shown that they are capable of translocation not only to the nucleus (Bokemeyer *et al.* 1996) but also to the cell surface membranes (Gonzales *et al.* 1993). Since MAPK

Figure 5 (a) Immunocytochemistry for PCNA in the testis of the frog *R. esculenta* treated with E₂ shows immunopositive I SPG (arrowheads). (b) Evaluation of the PCNA-immunopositive I SPG in the *R. esculenta* testis injected with KRB (Cont), E₂ and E₂+ICI 182–780 (ICI) and killed after 24 h (for details see Materials and Methods). Four randomly chosen sections of frog testes were evaluated. Values represent the means ± S.E.M. of number of PCNA immunopositive/total I SPG counted per 40 sections, multiplied by 100. The significance of differences was evaluated at $P < 0.01$.

substrates may be located in different (and multiple) cellular compartments, it is conceivable that MAPKs could translocate to multiple cell structures. One possibility is modulation of AP-1 activity, as estrogens are known to induce c-fos and c-jun gene expression (Weisz & Bresciani 1993). Activation of the MAPK pathway could serve to potentiate these effects, either by acting directly on c-fos or c-jun, or by regulating the expression of the corresponding genes (Deng & Karin 1994).

In vertebrates, germ cell proliferation seems to depend on a network of factors interacting locally, such as stem cell factor, c-kit receptor (Loveland & Schlatt 1997, Munsie *et al.* 1997), gonadotropin-releasing hormone-like sub-

stances (Di Matteo *et al.* 1988), platelet-derived growth factor (Li *et al.* 1997) and E₂ (Minucci *et al.* 1997). Although further studies are needed to clarify the signal transduction pathways involved in their activation and the downstream targets of their action, the present findings shed new light in the effort to clarify the molecular mechanisms involved in the process of fertilization. The spatial and temporal interaction of these factors is an intriguing aspect of testicular activity.

In conclusion, using the frog model, which is characterized by a discontinuous spermatogenesis, we show for the first time the involvement of MAPK activation in the regulation of germinal epithelium maturation. In addition, we have shown evidence of a potential mitogenic role of E₂, via ERK1/2 activation in frog testis. 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.

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