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Estrogen receptor β localization in the lizard (*Podarcis s. sicula*) testis

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Summary

There is increasing evidence that 17β-estradiol is necessary for normal male fertility. The aim of the present study was to characterize estrogen receptor β (ERβ) expression in a non-mammalian vertebrate model, the lizard (*Podarcis s. sicula*) testis. Immunocytochemical analysis shows that ERβ proteins are present among germ cells in the nucleus of the spermatogonia, in primary spermatocytes and spermatids. Western blot analysis with antibodies against the ERβ gene product revealed an isoform with a specific weight of 55 kDa. In conclusion, the widespread expression of ERβ in the *Podarcis s. sicula* testis is consistent with a role for estrogens in modulating spermatogenesis in the male.

Keywords: Estrogen receptor, Fertility, Proliferation, Spermatogenesis, Testis

Introduction

It is well known that androgens and gonadotrophins play crucial roles in spermatogenesis and in the development and function of the male reproductive system. However, the role of estrogens in male reproduction remains to be elucidated. Male mice homozygous for a mutation in estrogen receptor (ER) α isoform (ER knockout; ERKO) are infertile and there is evidence that genetic defects in ERα in human males result in decreased sperm counts, indicating an essential role for ER-mediated processes in the regulation of male reproduction (Smith *et al*., 1994; Eddy *et al*., 1996; Couse & Korach, 1999). In rats, gonocyte proliferation is induced by 17β-estradiol (Li *et al*., 1997). It is clear that the exposure of the developing male to exogenous estrogens either *in utero* or neonatally can result in a range of abnormalities of reproductive development and function (Newbold & McLachlan, 1985). Furthermore a reduction in testicular estrogen levels inhibits spermatid maturation (Tsutsumi *et al*., 1987*a*, *b*). In addition, knockout mice that lack a functional aromatase enzyme (ArKO) and are therefore unable to convert C_{19} steroids (androgens) to C_{18} steroids (estrogens) show progressively disrupted spermatogenesis (Robertson *et al*., 1999). However, the finding of a second estrogen-specific receptor (referred to as ERβ) in 1996 (Kuiper *et al*., 1996) has prompted research into new sites of actions of estrogens. In fetal and adult rat testes expression of ERβ mRNA as well as ERβ protein is localized in type A spermatogonia, pachytene spermatocytes, round spermatids and Sertoli cells (Saunders *et al*., 1999; Shughure *et al*., 1998 ; Van Pelt *et al*., 1999; Gustafsson, 1999).

The reptiles occupy a phylogenetic position of great interest. For this reason, the present study has focused specifically on the pattern of expression of ERβ in the testis of lizard (*Podarcis s. sicula*). We have found that immunoreactivity specific for a peptide in lizard ERβ is present in the nuclei of multiple testicular cell types including Sertoli cells and certain germ cell types. We conclude that estrogens could have a significant role in normal testicular function and that steroid binding to ERβ could have direct effects on germ cell function and maturation.

Materials and methods

Animals and cells

Male lizards (*Podarcis s. sicula*) were captured in the vicinity of Naples. Animals were killed by decapitation under anaesthesia with MS222 (0.05% in aqueous solution, Sigma Chemical, St Louis, MO) and immediately

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the testes were removed and stored at 80 °C until processed or quickly prepared for histological examination.

The human prostate cell line EPN (Sinisi *et al*., 2002) used in these studies was grown in keratinocyte-SFM medium (Gibco-BRL, Milan, Italy).

Protein extract preparations

Frozen lizard 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 phenylmethylsulfonylfluoride (PMSF) 1 µg aprotinin, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate (Sigma Chemical. St Louis, MO), and clarified by centrifugation at 14 000 × 10 min. Protein concentrations were estimated using a modified Bradford assay (Bio-Rad, Melville, NY).

Antibody

Antibody was purchased from the following source: polyclonal rabbit antibody anti-estrogen receptor β (ERβ, rat COOH-terminal; # PA1-313, ABR, Inalco, Milan).

Western blot analysis

Fifty micrograms of proteins 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, Bedford, MA); complete transfer was assessed using prestained protein standards (Bio-Rad, Melville, NY). 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 then incubated for 1 h at room temperature with the primary antibody against ERβ (diluted 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 detected with an enhanced chemiluminescence (ECL) system (Amersham Life Science, UK).

Immunocytochemistry

Lizard testes, rapidly removed and fixed in Bouin's fluid, were dehydrated in ethanol series, and cleared in xylene. For each paraffin-embedded sample, 4 µm thick 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 unmask antigen. Before 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, to complete antigen unmasking.

The standard streptavidin–biotin–peroxidase complex procedure was used (Dako, Denmark). Anti-ERβ antibodies were used at a dilution of 1:400. Peroxidase activity was developed with the use of a filtered solution of 5 mg of 3-3′-diaminobenzideine tetrahydrochloride dissolved in 10 ml of Tris buffer (0.05 M, pH 7.6) and 0.03% H_2O_2.

Controls

The following controls were performed: (1) omission of the primary antibody; (2) substitution of the primary antiserum with non-immune serum (Dako, Denmark) diluted 1:500 in blocking buffer, (3) preincubation with 10^{-6} M of the cognate peptide. No immunostaining was observed after any of the control procedures.

Results and discussion

In order to determine precisely the cells producing ERβ, we used an immunocytochemical approach to localize ERβ protein in the lizard *Podarcis s. sicula* testis. Since the ERβ sequence is not known in the lizard, we used an antibody from rat COOH-terminal sequence conserved in human and rat.

Immunocytochemical analysis was performed on serial sections and revealed the presence of ERβ protein in this organ. Immunopositivity was found throughout the year in the germinal epithelium. The positivity was localized in the nucleus of spermatogonia, spermatocytes, spermatids and Sertoli cells (Fig. 1A) while spermatozoa (SPZ) remained negative. The antiserum used in this study fulfils the criteria of specificity. In particular, immunoadsorption tests revealed that the labelling was totally blocked by preincubating antibodies with 10^{-6} M of the cognate peptide (Fig. 1B).

In addition, Western blot analysis was used to confirm the antibody specificity, which detected a specific band of 55 kDa in the lizard testis extracts as compared with the human prostatic EPN cells used as positive control (Chieffi *et al*., 2003). The band was detected throughout the annual cycle, with minor

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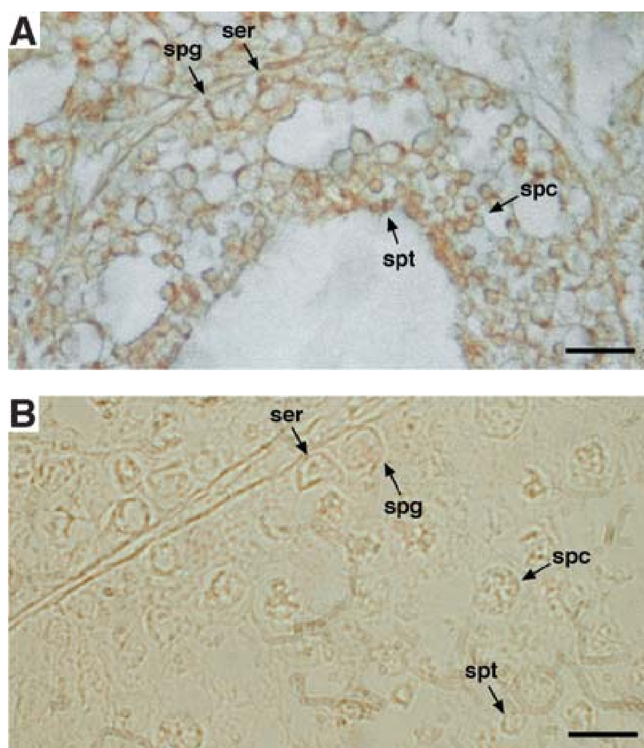

Figure 1 Immunocytochemistry for ERβ in the testis of *Podarcis s. sicula* showing: (A) positive reaction in the nucleus of spermatogonia (spg), spermatocytes (spc), spermatids (spt) and Sertoli cells (ser) throughout the year. Scale bar represents 100 μm. (B) Control section incubated with excess (10^{-6} M) ERβ. Symbols are the same as those used to show positive reactions. Scale bar represents 50 μm.

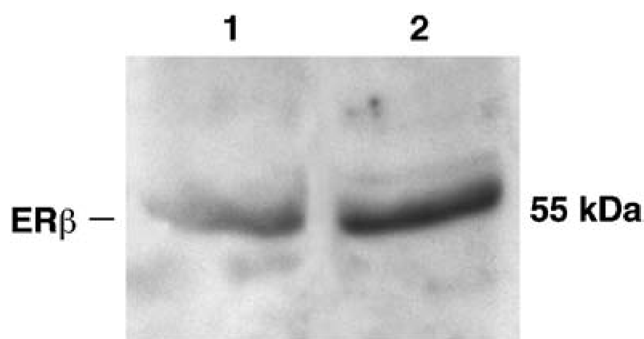

Figure 2 Western blot detection of ERβ protein in cytosolic testicular extracts of *Podarcis s. sicula*. Proteins (50 μg per lane per month, March) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and then incubated with antibody anti-ERβ (for details see Materials and Methods). Lane 1, a specific band of 55 kDa (ERβ) was detected by comparison with a positive control (lane 2, protein extracts of prostate cell line EPN) and by comparison with co-migrating size markers (Bio-Rad, Melville, NY). The blot is representative of three separate experiments.

variations in the different months (data not shown) (Fig. 2).

The presence of ERβ in Sertoli and germ cells suggests that estrogens might have a direct influence on germ cell function and maturation. The detection of ERβ protein in multiple lizard testicular cell types is consistent with the detection of specific ERβ protein in the testes of man, monkey and rat (Mosselman *et al*., 1996; Pelletier *et al*., 1999; Kuiper *et al*., 1996). For many years it has been accepted that testosterone exerts a fundamental regulatory effect on spermatogenesis indirectly via stage-specific effects on Sertoli cells due to the absence of AR from germ cells (Sharpe, 1994). This interpretation will now need to be reconsidered in view of the presence of ERβ in germ cells, and other findings showing the expression of aromatase in spermatocytes, spermatids and spermatozoa (Nitta *et al*., 1993; Levallet *et al*., 1998; Carreau *et al*., 2002). These developments raise the possibility that some of the effects of testosterone on spermatogenesis may occur directly on germ cells after the conversion of testosterone to estradiol, suggesting the existence of an additional local source of estrogens in the testis (Weniger, 1993).

The role of estrogens in the testis is still largely unknown. Previous studies have demonstrated that estrogens induce the proliferation of rat gonocytes, and of frog (*Rana esculenta*) and lizard (*Podarcis s. sicula*) SPG; this effect is blocked by an estrogen receptor antagonist (Minucci *et al*., 1997; Li *et al*., 1997; Chieffi *et al*., 2000, 2002). Recently, reports have clearly documented that estradiol induces spermatogonial multiplication through the ERK1/2 pathway in frog (*Rana esculenta*) and lizard (*Podarcis s. sicula*) (Chieffi *et al*., 2000, 2002). In addition, it has been demonstrated that estradiol acts as a germ cell survival factor in the human testis *in vitro* (Pentikainen *et al*., 2000).

In conclusion, ERβ is expressed in multiple germ cells. Estrogens capable of activating the receptor are present in the testis and can be produced locally from testosterone via the action of germ cell aromatase. These findings are consistent with a role for estrogens in the regulation of spermatogenesis.

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