Expression of oestrogen receptor beta (ER β) occurs in multiple cell types, including some germ cells, in the rat testis

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Abstract

The identification of a second oestrogen receptor (beta) has prompted a re-evaluation of the potential sites of action of oestrogens. The aim of the present study was to characterise immunoexpression of ER β expression in the testis to complement earlier data which had demonstrated that expression of ER α is confined to testicular interstitial Leydig cells. In all testes studied, including those from both fetal (day 20.5 p.c.) and adult rats, ER β was found to be expressed in multiple cell types. Sertoli cell nuclei were immunopositive at all ages. In

adult testes expression in Sertoli cells was not stage dependent and was unaffected by ablation of Leydig cells. In fetal testes ER β was also expressed in peritubular cells, fetal Leydig cells and gonocytes. In the pubertal and adult testis ER β was detected in the nuclei of spermatogonia and most pachytene spermatocytes. Weak immunopositive staining was present in the cytoplasm of spermatocytes undergoing the second meiotic division. In conclusion the widespread expression of ER β in the testis is consistent with a role for oestrogens in modulating spermatogenesis, and hence fertility, in the male.

Introduction

The observations that prenatal exposure to the potent oestrogen, diethylstilbestrol (DES) can have adverse effects on sperm count and formation of the male genital tract (Stillman 1982) have prompted the controversial suggestion that inappropriate exposure to oestrogens during life may contribute to adverse trends in male reproductive health (Sharpe & Skakkeback 1993; Toppari *et al.* 1996). A role for oestrogens in normal male fertility has been inferred from the finding that adult male mice in which normal expression of oestrogen receptor (ER α) has been disrupted (ERKO, Eddy *et al.* 1996) are infertile and evidence that genetic defects in ER α in human males results in decreased sperm counts (Smith *et al.* 1994). However the finding of a second oestrogen-specific receptor (referred to as ER β) in 1996 (Kuiper *et al.* 1996) necessitates a re-evaluation of the sites of action(s) of oestrogens. In the male a comparison of the sites of expression of mRNAs encoding the two receptors by reverse transcription PCR (Kuiper *et al.* 1997; Li *et al.* 1997) has revealed that multiple tissues in the male tract express both receptor types though this approach gives no indication of cell specific distribution in complex tissues such as the testis.

In a preliminary survey of sites of expression of ER β of using a specific polyclonal antiserum we had noted expression of this receptor in adult rat Sertoli cells (Saunders *et al.* 1997) which we have shown previously not to express ER α (Fisher *et al.* 1997). The present study has focused specifically upon the pattern of expression of ER β in the testis of rats using tissue from fetal, neonatal, immature and adult animals (both

control and after Leydig cell ablation). To our surprise we have found that immunoreactivity specific for a peptide in rat ER β is present in the nuclei of multiple testicular cell types including Sertoli cells, peritubular cells, Leydig cells and certain germ cell types in all ages of rat examined. We conclude that oestrogens could have a significant role to play in normal testicular function throughout life and that steroids binding to ER β could have direct effects on germ cell function and maturation.

Materials and Methods

Tissue collection

Adult female rats were placed in individual cages with male rats and checked for the presence of copulating plugs each morning. The day when the plug was found was taken as day 0.5 post coitum (p.c.). Pregnant females were killed on day 20.5 p.c. by inhalation of carbon dioxide and subsequent cervical dislocation, fetuses were examined under a dissecting microscope and the testes recovered. Testes obtained from fetal rats and those who were 3, 5, 7, 10, 14, 18 and 21 days of age were immersion fixed in Bouins for 5–6h before being transferred into 70% v/v ethanol for storage. Testes from adult rats were obtained following perfusion fixation with Bouins fixative (Millar *et al.* 1993). After fixation, testes were processed into paraffin wax, 5 μ m thick sections were cut and mounted on coated slides, dewaxed and rehydrated prior to immunohistochemistry as described previously (Millar *et al.* 1993). To induce complete testosterone withdrawal, rats were

administered a single intraperitoneal injection of 75 mg/kg ethane dimethane sulphonate (EDS) in 3:1 (v/v) water:dimethyl sulphoxide and perfusion-fixed 6 days later. This treatment results in destruction and removal of all of the Leydig cells within 30–36h (Sharpe *et al.* 1990).

Immunohistochemistry

ER β was immunolocalised to testicular cells using rabbit polyclonal anti-peptide IgGs raised against a specific peptide located in the D region of rat ER β as described in detail in Saunders *et al.* (1997). IgGs were diluted 1/100 to 1/250 and incubated for 48h at 4°C on sections that had been subjected to microwave antigen retrieval (Shi *et al.* 1993). To detect bound antibody, sections were incubated with biotinylated swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) diluted 1:500 in NSS-TBS (normal swine serum (NSS) 0.5 parts: normal rat serum 0.5 parts: 5 parts Tris buffered saline (TBS) containing 5% (w/v) BSA, 30 min), washed in TBS (twice, 5 min each) and incubated with horseradish peroxidase-avidin biotin complex (Dako) for 30 min. After additional washes in TBS (twice, 5 min each) bound antibodies were visualized using 0.05% (w/v) 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Sigma Chemical Co. Poole, Dorset) in 0.05M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide. Sections were lightly counterstained with haematoxylin. Oestrogen receptor alpha (ER α) was immunolocalised using a mouse monoclonal antibody directed against the A/B domain of the receptor according to the methods described in Fisher *et al.* (1997). The antibody was obtained from Novocastra (Newcastle, UK).

Results

Fetal and neonatal testes

On day 20.5 of fetal life ER β was immunolocalised to multiple cell nuclei within the testis including clumps of fetal Leydig cells (Fig 1a, L) and nuclei of cells closely applied to the outside of the seminiferous cords (Fig 1a, b white arrows) which may be Leydig cell precursors or peritubular myoid cells. Within the seminiferous cords the intensity of immunopositive signal in Sertoli cell nuclei (Fig 1b black arrowheads) varied from faint (+) to strong (+++). The large round nuclei of fetal gonocytes (Fig 1b asterisks) were stained intensely (+++/++++). In contrast, ER α immunostaining was confined to cells within the interstitium (Fig 1c) consistent with previous reports that these receptors are expressed in fetal and adult Leydig cells (Fisher *et al.* 1997). In the neonatal testis, Sertoli cell nuclei remained immunopositive (e.g. day 3, Fig 1d, black arrowheads) and on this day, and days 5 and 7 (not shown), nuclei of interstitial cells and large round germ cells, presumed to be gonocytes, were also immunopositive (asterisk, Fig 1d).

Immature and adult testis

Sertoli cell nuclei were immunopositive in all samples examined from immature (day 10 Fig 1e), day 14 (not shown) and

day 21 (Fig 1f) testes in which they formed a distinct, almost continuous layer of cells. On day 10 basal spermatogonia (Fig 1e, white arrowheads) were also stained. At day 21, the pattern of immunopositive cell nuclei was variable depending upon the complement of germ cells present in the tubule cross sections with prominent staining of basal spermatogonia and luminal pachytene spermatocytes (asterisk, Fig 1f) being observed. In the adult rat, ER β was localised to SC at all stages of the spermatogenic cycle (e.g. Fig 1g, h, black arrowheads) and expression was not reduced in testes from animals in which Leydig cells had been ablated 6 days previously (Fig 1i, j, k, black arrowheads).

Immunopositive staining was also detected in intermediate and B-type spermatogonia (+++, e.g. Fig 1j, white arrowheads) but was absent from leptotene, zygotene and early pachytene spermatocytes in stages XIV (Fig 1k, square bracket) and I. Immunopositive staining for ER β was present in the nuclei of pachytene spermatocytes between stages III and XII and was most intense at stages V to XI (e.g. asterisks in Fig 1h). A weak dispersed immunopositive signal was present in the cytoplasm of secondary spermatocytes during the second meiotic division (stage XIV, see Fig 1k, §) and some faint staining persisted in the nuclei and cytoplasm of round spermatids up to stage III.

Discussion

The detection of ER β protein in multiple testicular cell types using an anti-peptide antibody directed against the hinge domain of the receptor is consistent with detection of specific ER β mRNA in the testes of man and rat (Kuiper *et al.* 1997; Mosselman *et al.* 1996) but in conflict with the reported absence of ER β in the testis of wild type and ERKO male mice (Paech *et al.* 1997). It has been accepted for decades that testosterone exerts pervasive regulatory effects on the testis, and in particular on spermatogenesis (Sharpe 1994). The sites of expression of androgen receptors (AR; (Bremner *et al.* 1994) fit with this view, though the absence of AR from germ cells has led to the conclusion that testosterone regulates spermatogenesis indirectly via stage-specific effects on Sertoli cells (Sharpe 1994). This interpretation will now need to be reconsidered in view of the present demonstration of ER β in spermatogonia and in pachytene and later spermatocytes, and other findings showing the expression of aromatase in spermatocytes, spermatids and spermatozoa (Dorrington & Khan 1993; Janulis *et al.* 1996; Nitta *et al.* 1993). These developments raise the possibility that some of the effects of testosterone on spermatogenesis may occur directly on the germ cells after the local conversion of testosterone to oestradiol. Similarly in the fetal/neonatal testis, the demonstration of ER β in gonocytes coupled with the well-established ability of immature Sertoli cells to synthesise oestradiol (Dorrington & Khan 1993; Weniger 1993), suggest a role for locally-produced oestradiol in the early development of primordial germ cells. This view is reinforced by the demonstration that oestradiol is able to stimulate the proliferation of gonocytes isolated from the day 3 rat testis,

Figure 1. Immunolocalisation of ER β to multiple cell types in rat testes. All sections were lightly counterstained with haematoxylin. a) fetal day 20.5, inset pre-immune serum, small arrow indicates an immunopositive peritubular cell, L= clump of fetal Leydig cells, x40 b) higher power view of (a). Note that within the seminiferous cords, Sertoli cell (SC) nuclei are immunopositive (arrowheads) as are those of gonocytes (asterisks); c) fetal day 20.5 immunostained with antibody specific for ER α , note that immunopositive Leydig cells (L) are only present in the interstitium, x40; d) neonatal testis, day 3, x100, showing immunoexpression in SC nuclei (arrowheads), gonocytes (asterisk) and peritubular cells (arrow) e) immature testis, day 10, x40, showing immunoexpression in SC nuclei, spermatogonia (arrows) and Leydig cells (L), inset shows preimmune serum; f) pubertal day 21, nuclei of pachytene spermatocytes contain intense immunopositive staining (asterisk), whilst in the basal portion of the tubule immunopositive staining was present in both SC and spermatogonia, insert shows pre-immune serum. ER β were present in nuclei of all SC (arrowheads) and some pachytene spermatocytes (asterisks) and spermatogonia (short arrows) within the seminiferous epithelium of adult rats (g, h, i, j, k) in both the presence (control untreated, g,h) and absence of testosterone (i, j, k, EDS-treated, 6 days). Note that at stage XIV(k) SC nuclei are immunopositive (arrowheads), diffuse cytoplasmic staining was present in secondary spermatocytes (§) but pachytene spermatocytes (I) are not stained.

and effect which is blocked by an oestrogen receptor antagonist (Li *et al.* 1997).

The present findings show that ER β was expressed in a far wider range of testicular cell types than either ER α or AR. Receptors for ER α are confined to Leydig cells and cells of the excurrent ducts (Fisher *et al.* 1997). AR are abundant in peritubular cells during fetal and neonatal life (Majdic *et al.* 1995) but not expressed in either Leydig or Sertoli cells until the testis matures postnatally (Bremner *et al.* 1994). In the adult rat testis, AR are expressed in Sertoli cells in a stage-dependent manner and immunoexpression is lost after androgen withdrawal induced by Leydig cell ablation with EDS (Bremner *et al.* 1994). In contrast, in the present study there was no evidence for stage-dependent expression of ER β in adult Sertoli cells and treatment with EDS six days previously did not affect the pattern of expression of ER β in any cell type within the seminiferous epithelium although testosterone levels are undetectable after this treatment (Sharpe *et al.* 1990).

The available data suggest that ER β and ER α receptors have similar specificity for a wide range of ligands and bind oestradiol with similar affinity (Kuiper *et al.* 1997). The two ER subtypes can exist as either homodimers (ER α /ER α or ER β /ER β) or heterodimers (ER α /ER β) depending upon which types are expressed within the cell (Cowley *et al.* 1997; Pettersson *et al.* 1997). The binding affinity of ER α /ER α and ER α /ER β dimers for a consensus DNA oestrogen receptor response element (ERE) appears higher than that of ER β /ER β dimers (Cowley *et al.* 1997). In addition there is evidence suggesting that pattern of gene transcription induced by the ligand-activated receptor dimers may vary depending upon which form of ER dimer is formed. Paech *et al.* (1997) have reported that each of the homodimers showed the same transactivation profiles using a reporter gene linked to an ERE when activated with oestradiol, DES or several antiestrogens. In contrast in the same series of experiments, ER α /ER α or ER β /ER β homodimers complexed with oestradiol were shown to signal in opposite ways at an AP-1 site. In the ERKO mouse studies of oestrogen action on the uterus suggest that other pathways of oestrogen action, independent of the presence of either ER α or ER β , may exist (Das *et al.* 1997). These findings have important implications for oestrogen regulation of cell function in the testis as we have shown that there are cell types expressing both receptors α and β (Leydig cells) whereas the majority of cell types (Sertoli, peritubular myoid, gonocytes, spermatogonia and pachytene spermatocytes) express ER β alone and no cell type expresses ER α alone.

In conclusion, ER β is expressed in multiple cell types within the testis. Oestrogens capable of activating the receptor are present in testicular blood and can be produced locally from testosterone via the action of testicular aromatase. These findings are consistent with a role for oestrogens in modulating testicular gene expression and spermatogenesis.

Acknowledgements

We thank Julie Wilson, Sheila Macpherson, Ted Pinner and Jim Macdonald for their assistance. JSF was supported by a MRC studentship.

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