Andrologie 2003, 13, N° 1 43-50

Oestrogen receptors in the human and primate testis and reproductive tract

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ABSTRACT

The impact of oestrogens on the male reproductive system remains the subject of intensive research activity and debate. Oestrogen action is mediated via high affinity intracellular receptors expressed in target tissues. Two subtypes of oestrogen receptor known as ERα (NR3A1) and ERß (NR3A2) have been cloned and hERß variant isoforms identified. In target cells these receptors can exist as homo- or heterodimers. We have used immunohistochemistry to examine the patterns of expression of ERs in human and non-human primates as a first step in determining the cellular targets for oestrogen action in the male.

ERα was detected in the epithelial cells of efferent ductules (ED) occasionally in epithelial and stromal cells within the epididymis but was undetectable in human or primate testes. Using a polyclonal antibody raised against the hinge domain of ERß, immunopositive staining was detected in multiple cell types within the testis and in epithelial and stromal cell nuclei throughout the male reproductive system (ED, epididymis, vas deferens, seminal vesicles, prostate) and in the bladder. We have also used monoclonal antibodies that distinguish between wild type, full-length ERß (ERß1), and a splice variant isoform called ERßcx/ERß2 that does not bind oestrogens. ERß1 and ERß2 proteins were both detected in human testis and have distinct but overlapping patterns of expression. ERß1 was also detected in ED, epididymis and vas.

In conclusion, oestrogen receptors are widely expressed in the male urogenital system and with the exception of the ED there are more cells that express ERß than ERα. In the adult human the testicular cells most likely to be targets for oestrogens are round spermatids in which levels of expression of full-length wild type receptor (ERß1) are high.

Key words : *spermatogenesis, oestrogen receptor, ERß, spermatid*

I. INTRODUCTION

Oestrogen action is mediated via high affinity intracellular receptors expressed in target tissues. Following ligand binding the receptors undergo a conformational change, dimerize, bind regulator regions within genes, recruit co-factors and thereby regulate the transcription of target genes (for recent reviews see [20, 28]). Two oestrogen receptor cDNAs known as ERα (NR3A1) and ERß (NR3A2), encoded by genes located on different chromosomes [8] have been cloned from human and rodent tissues [14, 26, 31]. Like other members of the steroid receptor superfamily both receptors have a common arrangement of five structure-function domains, denoted A-F [3]. In 1998 a novel human ERß variant, named ERßcx, was identified in a human testis cDNA library [32] (accession AB006589). In separate experiments Moore et al [25] identified a number of mRNAs in human tissues, including testis, which encoded hERß isoforms including one identical to hERßcx that they named hERß2

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(reviewed in [43]). To avoid confusion, the original hERß protein identified as the homologue of the rat ERß will be referred to as hERß1 and the hERßcx/hERß2 splice variant will be referred to as hERß2/ßcx for the rest of this paper (Figure 1).

Within the adult testis testosterone is synthesised by the interstitial Leydig cells [35] and several studies have shown that the concentrations of testosterone within the testis are up to 100 fold higher than those in the general circulation [23]. In addition testosterone is locally converted to oestrogens by the aromatase P450 which is expressed in Leydig cells [34], haploid germ cells [4, 46] and spermatozoa [15].

II. MATERIALS AND METHODS

1. Tissues

Testicular tissues were obtained from men (n=7) undergoing surgical investigations : the men gave informed consent. Vas deferens was obtained from men undergoing vasectomy. Other human tissues were obtained from the Peterborough Hospitals NHS Trust tissue bank. Collection of tissues from captive bred common marmosets (*Callthrix jacchus*) and stump-tailed macaques (*Macaca arctoides*) has been described previously [41]. All tissues were fixed in Bouin and processed using standard methods.

2. Antibodies

Mouse monoclonal anti-human ERα was purchased from DAKO (Cambridge, UK, clone 1D5). ERß proteins were detected using three different antibodies and the locations of the peptides used to generate them are shown in Figure 1. Firstly, an affinity purified sheep polyclonal antiserum directed against hERß peptide P4 (hinge domain) was prepared and validated as described in [40] ; thereafter, two specific mouse monoclonal antibodies directed against ERß1 (wild type, peptide P7) and ERß2/ßcx (peptide P8) were prepared and validated as described in detail in [40, 42]. Specificity of the antibodies has been confirmed by Western blotting [40, 41, 42].

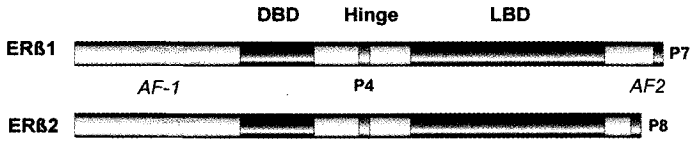

Figure 1 : Diagrammatic representation of human ERß1 (wild type) and human ERß2/ßcx (c-terminal) splice variant. The positions of the DNA (DBD), ligand (LBD) and hinge (H) domains are shown in relation to the peptides (P4, P7 and P8) used to raise specific antibodies.

3. Immunohistochemistry

Sections were subjected to heat induced antigen retrieval as described in [41], blocked for 30 min in normal rabbit serum (NRS: Diagnostics Scotland, Carluke,UK) diluted 1:4 in TBS containing 5% bovine serum albumin (NRS/TBS/BSA). Antibodies were diluted (ERα 1:50, ERß 1:800, ERß1 1:50, ERß2 1:50) in NRS/TBS/BSA and applied to the sections at 4°C overnight. Sections were washed in TBS and incubated with the appropriate biotinylated secondary antibodies, for anti-ERß, rabbit anti-sheep (Vector), and for anti- ERα rabbit anti-mouse, (DAKO, Cambridge, UK) both of which were diluted 1:500 in NRS/TBS/BSA. Following washes in TBS, sections were incubated with avidin-biotin-horseradish peroxidase linked complex (DAKO) according to the manufacturer's instructions. Bound antibody was visualised using 3,3'-diaminobenzidine tetrahydrochloride (DAKO). Sections were counterstained with haematoxylin and images captured using a Kodak DCS330 camera (Eastman Kodak), stored on a Macintosh PowerPC computer and assembled using Photoshop 6.0 (Adobe, Mountain View, CA).

III. RESULTS

1. Oestrogen receptor alpha

ERα positive cells were detected in the epithelial layer of the efferent ductules (ED) of all three species examined (Figure 2 a, macaque). In the rest of the reproductive tract most epithelial cells were immunonegative although occasional immunopositive cells were observed in marmoset and macaque epididymes (see [41]) and in the basal cell population of the human prostate. ERα positive cells were detected in the stromal compartment of the seminal vesicle (Figure 2 b), prostate and bladder (Table 1).

2. Oestrogen receptor beta

Using an antibody directed against the hinge domain of human ERß [40] immunopositive cell nuclei were detected in the testes and throughout the reproductive system of the male. For example ERß positive cells were present in both epithelium and stroma of the ED (Figure 2c), epididymes, vas deferens, seminal vesicle, prostate (Figure 2 d) and bladder. Results are summarised in Table 1. In the testis somatic (Sertoli, peritubular myoid, Leydig) cells as well as pre (e.g. spermatogonia,) and post-meiotic (e.g. spermatids) germ cells were all immunopositive.

3. Oestrogen receptor beta variants

Although multiple cell nuclei stained with the antibody against the hinge domain of ERß (see above) when monoclonal antibodies directed against the C-terminal portion of the full length ERß protein were applied to sections from

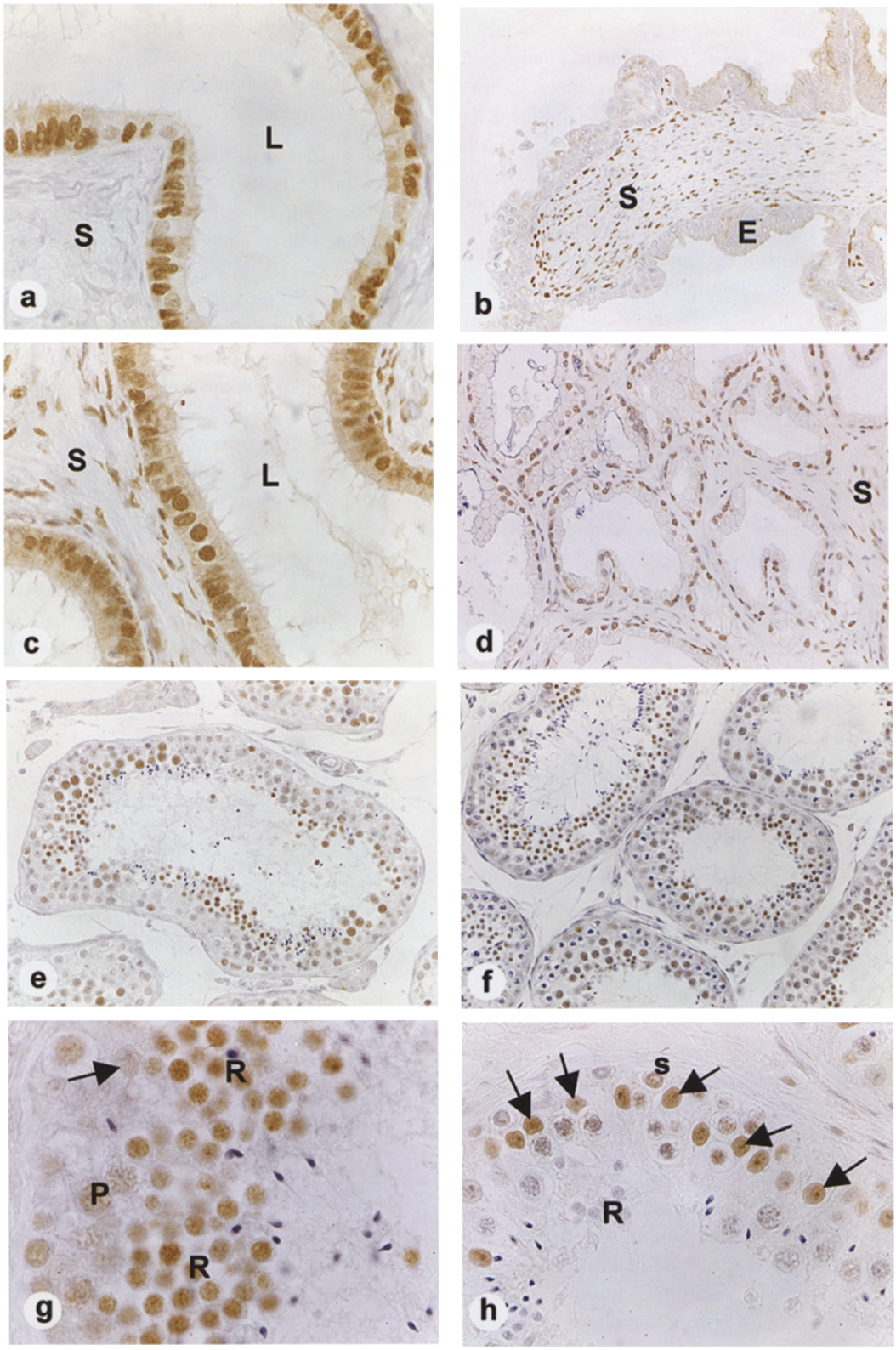

Figure 2 : Immunolocalisation of oestrogen receptors to tissues from the male. a) ERα *immunopositive staining of ED from macaque, note intense positive staining in the epithelial cells ; b) Marmoset seminal vesicle,* ERα *positive cells are found only in the stroma and not in the epithelium ; c) ERβ immunopositive staining of ED from macaque (parallel section to that shown in panel a) ; d) ERβ immunopositive cell nuclei in both the epithelium and stroma of the prostate of the marmoset ; e) Human testis stained with antibody specific for C-terminus of ERβ1 ; f) macaque testis stained with antibody specific for C-terminus of ERβ1 ; g) x100 magnification human testis stained with anti-ERβ1, note intense immunopositive reaction in round spermatids (R) but weak reaction in Sertoli cells (arrow) and pachytene spermatocytes (P) ; h) x100 magnification human testis stained with anti-ERβ2/βcx, note intense immunopositive staining of Sertoli cell nuclei (arrows) and spermatogonia (s).*

human (Figure 2 e) and primate (Figure 2 f, macaque) testes only a sub-set of the cells were immunopositive. The most intense immunopositive reaction was detected in the round spermatids (e.g. human Figure 2 g, labelled R) and immunopositive staining was barely detectable in the Sertoli cells. In contrast when antibody directed against the C-terminus of the ERß2 splice variant isoform was applied to the same sections the most intense immunopositive staining was located in Sertoli cell nuclei (Figure 2 h, arrows) and in a few germ cells located at the periphery of the seminiferous tubule which were tentatively identified as spermatogonia. A summary of the pattern of expression of ERs in the adult human testis is given in Table 2.

Differences between sequences at the C-terminus of the ERß2 variants in the macaque and marmoset and those of the human meant that the anti-ERß2 antibody did not stain tissues from these species (G.A Scobie and J E Sierens, unpublished observations). Preliminary studies using the monoclonal antibody directed against peptide P7 (C-terminus wild type protein) on sections of ED, epididymis and vas deferens from human and macaque revealed that most cells identified as ERß positive using the anti-P4 antibody were also positive with the anti-C terminal antibody suggesting that full length functional protein was expressed. Some cells within the epithelium and stroma of the human vas and epididymis were also ERß2 positive (not shown).

IV. DISCUSSION

The impact of oestrogens on male fertility has been investigated in a number of different ways, firstly as in the present study by defining the pattern of expression of oestrogen receptors, secondly by manipulating the hormonal environment for example by administration of oestrogens or anti-oestrogens and finally by targeted ablation of genes in mice. All three approaches have added to our understanding of the sites of action of oestrogens and have led to the general conclusion that male fertility can be affected by oestrogens as well as androgens [29, 30].

Some of the most convincing data for a role for oestrogens in germ cell development have come from studies in aromatase knockout (ArKO) mice. Although ArKO males are initially fertile, infertility is observed in adult animals from 4.5 months of age [9]. Analyses of the testes of these animals have shown that round spermatids undergo apoptosis, show disturbances in acrosome formation and fail to differentiate into mature elongate spermatids [36, 37]. Consistent with these data that suggest oestrogens may act as a germ cell survival factor, treatment of monkeys with an aromatase inhibitor also results in a reduction in the progression of round to elongate spermatids [44]. Furthermore in mice which develop immature seminiferous tubules without a full germ cell complement due to a deficiency in gonadotrophin levels (the hpg mouse model [5]), treatment of animals with low levels of oestradiol for 70 days resulted in a 5 fold increase in testis weight and qualitatively normal spermatogenesis [6].

We have shown that in both human and primate most epithelial cells in the efferent ductules (ED) express both nuclear ERα and ERß (present paper and [41], and similar findings have been reported for goats [13], dogs and cats [27], rats [10, 17] and mice [50]. The ED are an important site of fluid resorption within the male reproductive system [16, 18]. A role for oestrogens in modulating ED function has been highlighted by studies in rodents. For example in the ERα knockout mouse (ERKO [21, 22]) males become infertile due to excess accumulation of fluid within the lumens of the seminiferous tubules resulting in irreversible damage to the seminiferous epithelium [37]. Similarly, administration of an anti-oestrogen to rats has been shown to result in reduced fluid resorption within the ED resulting dilation of the ducts and an increase in testis weight [33]. Oestrogens have been shown to be essential for expression of a sodium transport protein by the ED and epithelial cell morphology [49]. It is notable that in the ERKO mice ERß expression within the ED can still be detected but is clearly not sufficient to maintain the normal functional competence of the epithelial cells.

Several studies have shown that most cells within the epithelia lining the epididymis and vas deferens express ERß [2, 27, 41, 50]. Expression of ERα is generally reported to be more abundant in stromal cells than in epithelial cells although there appear to be some differences between species. For example in the epididymes of the marmoset [41] and dog [27] few positive epithelial cells were detected whereas in the mouse [50] and cat [27] significant numbers of epithelial cells are immunopositive for ERα. In the rat neonatal exposure to high doses of the potent oestrogen diethylstilbestrol (DES) results in abnormalities in cell-specific and region-specific expression of ERα and suggested that oestrogens play a role in peripubertal development of the epididymis and vas [1]. Studies on mice have shown that the oestrogen sulphotransferase (EST) enzyme is also expressed in the epithelial cells of the epididymal corpus and cauda and in the luminal epithelium and smooth muscle cells of the vas deferens [45]. Studies in which expression of EST has been modulated (knockouts, hormonal manipulations) have suggested that the it plays a role in modulating oestrogen homeostasis and that disturbances in oestrogen levels in the luminal environment of the epididymis can have an adverse affect on motility of spermatozoa [45]. Several studies have shown that exposure to elevated oestrogens can alter gene expression in other parts of the male reproductive system. For example, Williams et al [48] reported that neonatal treatment with DES resulted in reduced expression of androgen receptors

Table 1 : Comparison between the patterns of expression of ERa and ERß in the male reproductive system of human and non-human primates.

Tissue	ERα		ERß (hinge)	
	Epithelium	Stroma	Epithelium	Stroma
Efferents ductules	++++	?	++	+
Caput epididymis	-/+	-	+++	++
Corpus epididymis	-/+	-	+++	+
Cauda epididymis	-/+	-	++	+
Vas déférens	-	-	++	++
Séminal vesicles	-	++	++	+
Prostate	-/+	+	++	+
Bladder	-	+	+++	+

Table 2 : Summary of cell specific patterns of expression of oestrogen receptors within the adult human testis.

Cell type	ERα	ERß1 (WT)	ERß2/ßcx
Sertoli cell	-	(+)	+++
Spermatogonia	-	(+)	+++
Spermatocyte	-		
PL	-	(+)	(+)
L	-	(+)	(+)
Z	-	(+)	(+)
P	-	+/++	+
D	-	(+)	+
2ndary	-	+++	-
Round Sptd	-	+++	-
Elongate Sptd	-	-	-
Leydig cell	-	(+)	(+)
PTM	-	+	++

and but induction of expression of progesterone receptors in the stroma of the seminal vesicles of rats at 18 days of age.

We, and others, have consistently failed to detect ERα mRNA or protein in samples from human [24, 41, 42], marmoset or macaque testes (unpublished observations). These findings are in marked contrast to the results obtained with other species including rodents [10, 50], dogs and cats [27] where ERα is expressed within Leydig cells and also within peritubular cells in some species.

In contrast ERß proteins have been reported to be expressed in multiple cell types including Sertoli cells, pre and post meiotic germ cells, Leydig cells and peritubular cells in rats [39, 47], mice [50], dogs and cats [27], primates [41] and human [24, 41] In addition mRNAs encoding variant isoforms of ERß, formed by alternative gene splicing, have been identified in cDNAs prepared from human testes [19, 25, 32, 43]. We have previously prepared antibodies specific for the full length wild type receptor and three of the splice variants and shown that the proteins they encode are expressed in the human testis in fetal life [12] and in adulthood [42] as well as in other tissues including the vas deferens [43].

To date the functional significance of the expression of human ERß splice variant proteins has been investigated using cells co-transfected with cDNA encoding the variant isoform and either full length hERα cDNA- or hERß cDNA-containing plasmids [19, 32]. In these reports the variant isoform (which did not bind ligand) was shown to act as a dominant negative regulator of reporter gene activation by ERα in the presence of oestradiol. The ERß2/ßcx variant protein which we have immunolocalised to Sertoli cells and spermatogonia in the testis (present paper and [42]) has also been immunolocalised to samples of normal and malignant breast [38] and prostate [11]. An association between increased expression of ERß2/ßcx and higher grade prostate cancers has been shown [11]. In the breast co-expression of ERα with ERß2/ßcx was associated with lowered expression of progesterone receptor in malignant cells [38]. In the human testis co-expression of ERß2 with ERα was not observed, and in some cells (e.g. Sertoli cells), ERß2/ßcx appears to be the only form of ER protein present. We are currently conducting further studies to determine the functional significance of these findings.

Using immunohistochemistry with a specific monoclonal antibody [42] we have detected expression of the full length ERß protein, i.e. the isoform which is capable of binding a range of oestrogenic ligands with high affinity and which can activate reporter gene expression *in vitro* in multiple cell types throughout the male reproductive system. In the adult testis of human, marmoset and macaque highest levels of expression were detected in round spermatids. Expression of ERß in these cells appears consistent with data from rodent and primate studies [36, 37, 44] which suggests that oestrogens are important for maturation of haploid germ cells as well as for fluid resorption in the ED.

V. CONCLUSIONS

A role for oestrogens in maintenance of normal male fertility is now well established. More cells within the male reproductive system express ERß than ERα. Highest levels of ERα were detected in the efferent ductules and expression of ERα in this tissue is essential for normal resorption of seminiferous tubule fluid. ERß protein was detected within epithelial and stromal cells throughout the male reproductive system. In the testes of primates and human both wild type, and ERß variant proteins are expressed. Based on the patterns of expression of ERß proteins within the seminiferous epithelium of the human we believe oestrogens may be important for development of round spermatids and some, but not all, spermatocytes.

ACKNOWLEDGEMENTS

The authors thank Sheila Macpherson, Keith Morris and Graeme Scobie for technical assistance and Drs D.S. Irvine and H.M. Fraser for providing tissue samples.

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