FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 42, No. 1, 2004
pp. 13-17

Immunolocalization of estrogen receptor β in the epididymis of mature and immature pigs

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Abstract: A growing body of evidence suggests a role of estrogens in the male reproduction *via* their specific estrogen receptors (ERα/ERβ). Estrogen receptor distribution along the genital tract tissues has been described in different species, but it is unknown in the pig. Therefore, the aim of the present study was to localize ERβ in the epididymis of mature and immature pigs (aged 2 and 18 months, respectively). Immunohistochemistry was carried out on paraffin-embedded tissues using a mouse anti-human monoclonal IgG against ERβ as the primary antibody, and a goat anti-mouse biotinylated IgG as the secondary antibody. Avidin-biotin-peroxidase complex was then applied followed by diaminobenzidine. In immature pigs, the epithelial cells from the caput, corpus and cauda epididymis showed no or very weak immunoreactivity for ERβ, whereas they were all strongly immmunoreactive in mature pigs. A various intensity of immunostaining from weak to strong in the smooth muscle cells as well as in the connective tissue cells were detected in the epididymis of both, young and adult pigs. This is the first report on the cellular localization of ERβ protein in porcine epidydimis. The present study demonstrated that (1) irrespectively of the epididymal region, the epithelial cells of caput, corpus and cauda epididymis of mature pigs revealed a strong immunoreactivity for ERβ, and (2) ERβ expression in the epididymal epithelium is regulated by puberty. Finally, although the biological activity of ERβ has not yet been established, the results of the present study suggest its involvement in estrogen modulation of pig epididymal function.

Key words: Estrogen receptor - Epididymis - Pig - Development - Male reproductive tract - Immunohistochemistry

Introduction

It is now accepted that estrogens play a physiological role in male reproduction *via* intracellular estrogen receptors (ERs) which mediate their genomic action in reproductive tissues [for review see, 2, 11, 24]. ERs are known in two forms, the classical ERα subtype and the novel ERβ subtype subsequently discovered in the rat, man and mouse [17, 21, 25, 30]. Estrogen binding throughout rat and mouse male reproductive tracts has been studied using autoradiography [12, 29]. More recently, a role of estrogens in the rat epididymis has been investigated using cultured epididymal epithelial cells [19, 32]. These cells have been shown to express aromatase mRNA and reported therefore as a source of estrogens [33].

Estrogen receptors were detected along the male genital tract of adult and/or immature animals, such as mouse [15, 34], rat [7, 12, 31], dog and cat [22], goat [8], rooster [18], monkey and man [5, 28] but their distribution in different regions was not uniform within and across the species. Furthermore, some of these studies did not discriminate between ERα and ERβ and, when the two ER subtypes were specifically detected, they often revealed a different topography in the multiple cell types of the genital organs. Therefore, the interpretation of the ERα/ERβ expression patterns in the male genital system is very complex and the biological significance of the two receptors has not yet been clarified. According to Hall and McDonnell [9], the role of ERβ in humans is to modulate ERα trancriptional activity.

Since the estrogen receptor distribution in reproductive organs of the pig is only scarcely known, the present study was addressed to investigate the localization of ERβ in epididymal tissues of mature and immature pigs by means of immunohistochemistry.

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Materials and methods

Animals. The investigation was carried out on 3 immature (aged 2 months) and 3 mature (aged 18 months) pigs (*Sus scrofa domestica*). Epididymal tissues were obtained after routine castration at local animal hospitals.

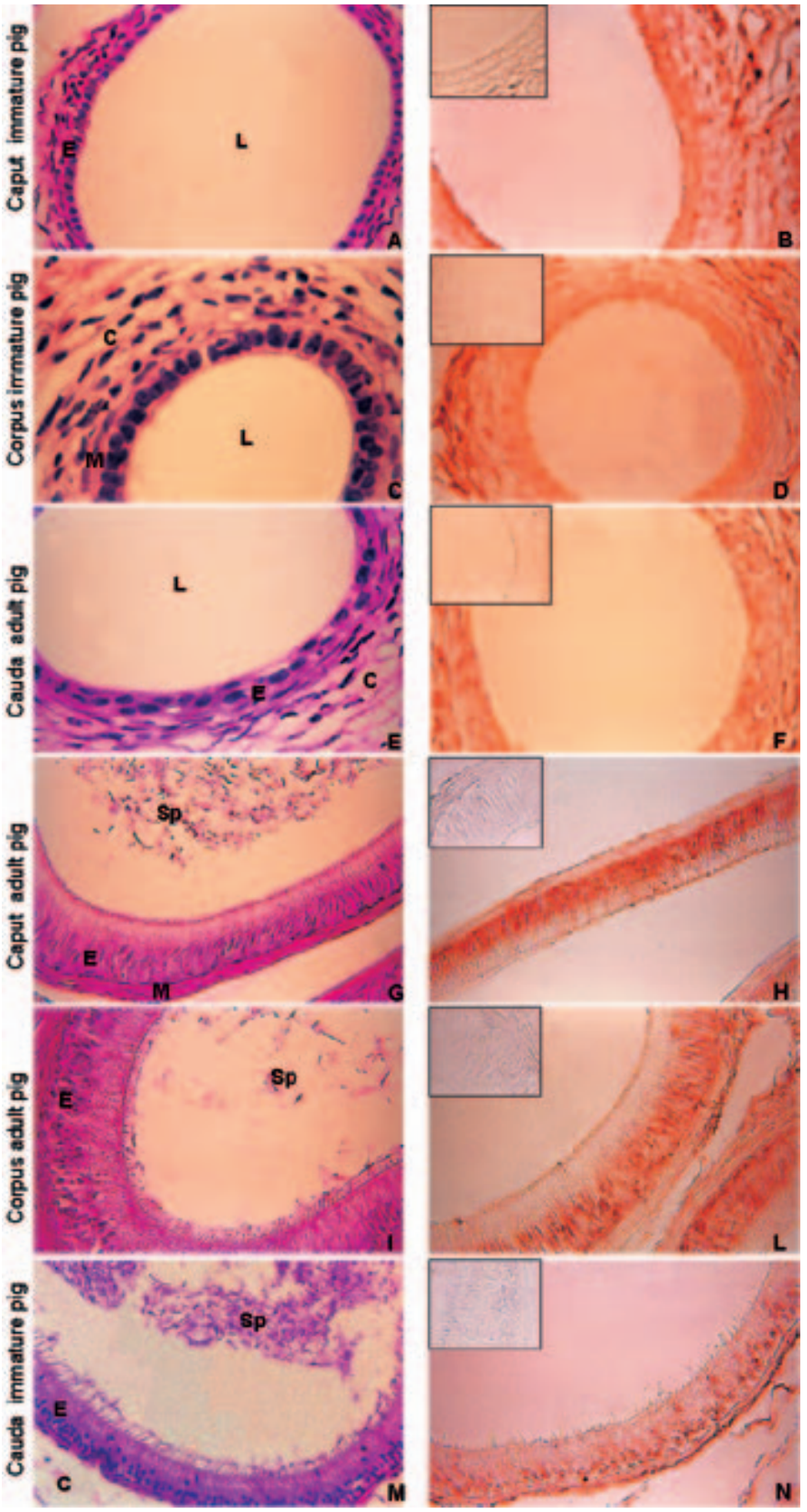

Fig. 1. Morphology (A, C, E, G, I, M) and ERβ immunodetection (B, D, F, H, L, N) of the caput, corpus and cauda epididymis of immature (A-F) and mature (G-N) pigs. Scale bars = 5 μm (A-F) and 12.5 μm (G-N). C - connective tissue cells; E - epithelial cells; L - lumen; M - smooth muscle cells; Sp - sperm cells. **A, C, E**: Morphology of the caput, corpus and cauda epididymis, respectively, of the immature pig. **G, I, M**: Morphology of the caput, corpus and cauda epididymis, respectively, of the mature pig. H+E staining. **B, D, F**: Immunolocalization of ERβ in epididymal sections of the caput, corpus and cauda, respectively, of immature pig. No or very weak ERβ immunostaining in the epithelial cells, moderate reactivity in smooth muscle cells and strong, positive reaction in connective tissue cells can be seen. Inserts represent the controls. **H, L, N**: Immunolocalization of ERβ in epididymal sections of the caput, corpus and cauda, respectively, of mature pig. Note strong ERβ immunostaining in the epithelial cells, weak reactivity in smooth muscle cells and strong, positive reaction in connective tissue cells. Inserts represent the controls.

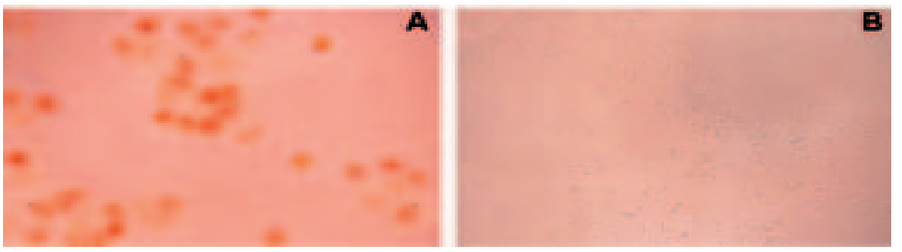

Fig. 2. ERβ immunostaining of HeLa cells. **A**: ERβ transfected cells. **B**: Immunonegative wild type cells. Scale bars = 12.5 μm.

Morphology and immunohistochemistry. Immediately after castration, the epididymis was dissected into the caput, corpus and cauda, three functional parts of the epididymis. For both, morphology and immunohistochemistry, the tissue was fixed in 4% neutral buffered formalin, embedded in paraffin and cut into 5 μm sections (6-7 serial sections for each sample). Morphological analysis was carried out by routine haematoxylin-eosin staining.

Immunohistochemical analysis was performed after standard heat-mediated antigen retrieval: sections were immersed in 10 mM citrate buffer (pH 6.0) and treated in a microwave oven (600 W) for 2×5 min [1]. Hydrogen peroxide (3% in distilled water) for 30 min was used to inhibit endogenous peroxidase activity and normal goat serum (10%) for 30 min was employed to block the non-specific binding sites. A mouse anti-human monoclonal IgG against ERβ 1 isoform (MCA1974, Serotec, Oxford, UK) was used as primary antibody (1:40) overnight at 4˚C, while a biotinylated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz CA,USA) served as secondary antibody (1:1000) for 1 h at room temperature. Avidin-biotin-horseradish peroxidase complex (Santa Cruz Biotechnology) amplification was then performed (30 min at RT) and the peroxidase activity was visualized with diaminobenzidine.

The same protocol was used for the ERβ immunocytochemical analysis of HeLa cells.

Cell culture and transfection. HeLa cells were maintained in DMEM (Sigma-Aldrich, Milan, Italy) without phenol red, supplemented with L-glutamine (2 mM), penicillin (100 U/ml) streptomycin (100 U/ml) and 10% fetal calf serum (FCS; Sigma-Aldrich, Milan, Italy). One day before transfection the cells were switched to the medium without serum. Transfection was performed for 6 h at 37˚C, in a humidified atmosphere of 5% of CO_2 in air, using the Fugene6 Reagent (Roche Diagnostics, Mannheim, Germany) with ERβ expression plasmid as recommended by the manufacturer.

Results

Microwave stabilization used at the begining of immunohistochemical procedure stopped the extraction of the protein from epididymal tissue, and unmasked the receptors.

Figure 1 shows representative cross sections of the caput, corpus and cauda epididymis in immature (A-F) and mature pigs (G-N). Typical morphology of these 3 epididymal regions of immature and mature pigs is shown in the micrographs A, C, E, and G, I, M, respectively. The positive ERβ immunoreactivity (B, D, F and H, L, N) was noted as nuclear staining and there was no nonspecific staining when sections were treated without the primary antibody (see inserts).

In immature pigs, epithelial cells of all the epididymal regions revealed no or very faint immunostaining of ERβ. More precisely, in the caput and cauda epididymis the epithelial cells expressed no immunoreaction for ERβ, whereas in the cells of the corpus epididymis very weak immunoreaction was observed (B, D, F). The intensity of immunostaining of stromal cells was variable, from strong to weak. In the caput and corpus epididymis, the smooth muscle cells were weakly reactive, while in the cauda epididymis they were moderately stained. Irrespectively of the region of epididymis, the connective tissue cells were strongly immunopositive (B, D, F).

In mature pigs, the intensity of ERβ immunostaining in the epithelial cells from the three epididymal regions was similar and strong throughout the whole tissue, as it was in the connective tissue cells (H, L, N), whereas the smooth muscle cells of the caput and cauda epididymis expressed weak intensity of immunostaining (H, N). The moderate intensity of immunostaining was only confined to the smooth muscle cells of the corpus epididymis (L).

The specificity of the ERβ antibody was verified with experiments on HeLa cells. The steroid receptor-negative HeLa cells, transfected with expression plasmid encoding ERβ, showed nuclear immunostaining which was not observed in the same cells transfected with the vector alone (Fig. 2).

Discussion

The present study has demonstrated for the first time the cellular ERβ expression in porcine epididymis, suggesting the genital duct as a target for estrogens also in this species. Despite of the extensive studies carried out in different animals [for review see 11], estrogen receptor distribution in porcine reproductive ducts was scantly known. In fact, to our knowledge, only one study revealed the ERα presence in efferent ductules, but not in the epididymis of the newborn piglets [23]. The efferent ductules have been reported as the major site for ERα expression across the species, supporting the hypothesis that estrogen regulates the fluid reabsorption in this

region by concentrating sperm cells as they enter the epididymis [10, 11].

Estrogen receptors β have been detected in ductus epididymis of adult mouse, rat, dog, cat and monkey [12, 22, 28, 34], although some data of ERβ distribution are conflicting. Our findings concerning the epithelium of the adult pig are consistent with these reports but all the epididymal regions of the pig revealed a strong epithelial ERβ expression, while a gradient-like staining intensity, from the caput to the cauda, was detected in other species [16].

ERβ expression has been reported in the epididymal epithelium of immature mice and rats [15, 27], however, a low ERβ reactivity was detected only in neonatal animals [27]. The results of the present study showed a different intensity and distribution of ERβ expression in immature and mature pigs. In fact, strong immunoreactivity for ERβ was observed in the epithelial cells of the caput, corpus and cauda epididymis only in the adult pig, while the ERβ reactivity in stromal cells was similar in pre- and post- pubertal animals. Therefore, the ERβ expression in the epididymal epithelial cells of pig appears to be regulated by puberty.

The findings of the present study confirm that ERβ is expressed in a species-specific manner in the epididymis and suggest that estrogens could modulate the epididymal function in the adult pig. Caput, corpus and cauda epididymis are involved in morphological and biochemical sperm maturation, in progression of sperm towards the vas deferens and in its storage. The strong, positive immunoreactivity for ERβ in the stromal cells of immature and mature epididymis observed in our study, could be due to possible interactions between these tissues. In fact, stroma can influence the epithelial differentiation and function through paracrine regulation [26, 34]. The function of ERs in stromal cells has also been studied by Mäkelä *et al.* [20] in the rat. The authors suppose that as in accessory glands, the stromal cells may modulate (*via* ERs) the function of epididymal epithelial cells. Recently, it has been reported that estrogens can influence epididymal contractility through the regulation of oxytocin receptors in the epithelial and smooth muscle cells of rabbit epididymis [6]. In spite of wider distribution of ERβ in genital tract in comparison to ERα [14], mice lacking ERα reveal morphological abnormality of the reproductive system and are infertile [4, 13], while mice lacking ERβ show normal genital tissues (with the exception of the elder prostate) and they are fertile [3, 16].

Taking into account data reported herein, the presence of ERβ in the epididymis could be a basis for future functional studies on the role of estrogens in porcine male reproductive system. However, this topic requires extensive investigations.

Acknowledgements: This study was supported by the Italian Ministry of University Education and Scientific and Technological Research.

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Accepted October 27, 2003