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Immunolocalization of estrogen and androgen receptors in the caput epididymidis of the fat sand rat (*Psammomys obesus*): Effects of seasonal variations, castration and efferent duct ligation

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ABSTRACT

The fat sand rat (*Psammomys obesus*) is a model to study seasonal reproductive cycle changes and several metabolic disorders. In order to show a possible involvement of estrogens in the male reproductive functions, the expression of estrogen receptors (ESR1 and ESR2) and androgen receptor (AR) were investigated in the caput epididymidis of fat sand rats during the breeding season, resting season, after castration, after castration followed by testosterone treatment, and after ligation of efferent ducts. In the breeding season, principal cells presented a strong immunostaining of AR in both nuclei and cytoplasm, a strong staining of ESR1, mainly in the apical zone, and a strong immunoexpression of ESR2, mainly in nuclei. In the resting season, a moderate immunostaining of AR in both cytoplasm and nuclei was observed. ESR1 staining showed a strong immunoreactivity in the nuclei. In contrast, the nuclei were negative for ESR2. After castration, a low and selective signal distribution was observed: the nuclei were moderately positive for AR and ESR2, and negative for ESR1. After castration and testosterone treatment, an androgen-dependence for AR and the restoration of ESR1 but not ESR2 immunoexpression were observed. After ligation of the efferent ducts, a considerable reduction of AR immunoreactivity was observed in contrast to ESR1 and ESR2, which gave a strong immunostaining signal. These results illustrate the complexity of the regulation of the androgen and estrogen receptor expression in the epididymis and argue for the coexistence of both androgenic and estrogenic pathways.

Introduction

Epididymal development and physiology are regulated by a complex interplay of hormones and testicular factors, among which testosterone is crucial for epididymal functions (Ezer and Robaire, 2002). However, some studies have provided compelling evidence for the role of estrogens, in regulating the functions of the efferent ducts and the epididymis (Hess, 2003; Shayu et al., 2005). Estrogen biosynthesis is catalyzed by a microsomal P450 enzyme complex, called aromatase, which is responsible for the irreversible transformation of androgens into estrogens. In the male reproductive tract of immature animals, estrogens are produced in Sertoli cells (Van der Molen et al., 1981), whereas in mature animals, they are present in germinal cells, in spermatozoa and in the Leydig cells (Payne et al., 1976; Levallet et al., 1998; Carreau et al., 2006, 2007). The epididymis and efferent ducts express moderate to high levels of estrogen receptors (Hess et al., 1997; Mowa and Iwanaga, 2001) and estradiol (E2) is one of the key hormones regulating their function (Hess et al., 2001a,b; Lee et al., 2001). The estrogens are of major importance for male fertility. The inactivation of estrogen receptor 1 (ESR1) in mice results in an abnormal epididymal phenotype (Eddy et al., 1996) and infertility (Hess et al., 1997) following defective fluid absorption in the efferent ducts. The estrogen receptor 2 (ESR2) knockout mice are fertile and have a normal epididymal phenotype (Krege et al., 1998). In several species, ESR2 is expressed

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in all epididymal regions (Yamashita, 2004), whereas the expression of ESR1 is localized in efferent ducts and in different segments of the epididymis, depending on the species (Hess, 2003). ESR2 was shown to be expressed in principal cells in mice (Zhou et al., 2002), stallions (Parlevliet et al., 2006) and boars (Pearl et al., 2007).

It has been demonstrated that estrogens play an important role in the absorption of luminal fluid and in pubertal development (Parlevliet et al., 2006). They also have other functions, including regulating the expression of lactoferrin, cystatin 12, and oxytocin receptor in rabbits (Yu and Chen, 1993; Filippi et al., 2002; Li et al., 2005) or of androgen receptors and ESR1 in rats (Oliveira et al., 2004).

Androgens control epididymal activity. Castration and efferent duct ligation result in the reduction of epididymal weight, cellular atrophy, tissue remodeling and changes in gene expression. A massive apoptosis in the epididymis following such manipulations was reported in Sprague-Dawley rats beginning 18 h after surgery in the initial segment and it ending on the seventh day in the distal epididymis (Fan and Robaire, 1998). Androgen receptors are present in principal cells of all epididymal regions in mice (Zhou et al., 2002), goats (Goyal et al., 1997), rats (Zhu et al., 2000), and stallions (Parlevliet et al., 2006). They are expressed in principal cells, basal cells and the smooth muscle cells in boars (Pearl et al., 2007). Androgens are essential for the maintenance of the principal cell morphology and prevention of apoptosis (Fan and Robaire, 1998; Ezer and Robaire, 2002). They regulate the expression of proteins involved in motility, storage and membrane maturation of spermatozoa (Briz et al., 1995; Syntin et al., 1999; Castellon and Huidobro, 1999). Androgens also control the expression of androgen receptors (Goyal et al., 1998; Zhu et al., 2000; Oliveira et al., 2004).

The fat sand rat (*Psammomys obesus*) is a diurnal rodent which lives in the North-West of the Algerian Sahara, near Wadis. It has a seasonal reproduction cycle with a breeding period from autumn through early spring and a resting phase from late spring through summer. This cycle has been described in both hormonal (Khammar, 1987) and cytophysiological terms (Gernigon et al., 1991; Gernigon, 1992; Menad, 2008). The aim of this study was to investigate the immunoexpression of estrogen and androgen receptors, as well as their seasonal and castration-induced variations in fat sand rats (*P. obesus*) as a model to study seasonal changes in the reproductive cycle and several metabolic disorders.

Material and methods

Animals and samples

Adult male fat sand rats (*P. obesus*) of average weight 145 g were trapped in the wild region of Béni Abbès (30°07′ N 2°10′ W) during the breeding ($n = 30$) or resting season ($n = 8$). Eight out of 30 animals caught during the breeding season and all the eight animals caught during the resting season (groups 1 and 2 respectively) were euthanized 48 h after capture, their caput epididymides were quickly excised, cleaned from the surrounding fat and weighed. Out of the remaining 22 animals caught during the breeding season, eight were castrated, eight castrated and treated with testosterone, and six subject to efferent duct ligation before euthanasia (groups 3, 4, and 5, respectively). All experiments were carried out in compliance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) following approval by the local Ethical Committee of the Houari Boumediene University of Sciences and Technology, Algeria.

Castration and hormonal replacement

Eight adult male fat sand rats (*P. obesus*) were bilaterally castrated (group 3). Surgical procedures were performed under sterile conditions. Animals were anesthetized by an intraperitoneal injection of ketamine hydrochloride (50 mg/kg, Ketalar, Pfizer, NY, USA) and xylazine hydrochloride (10 mg/kg, Rompun, Bayer, Toronto, Canada). The efferent ducts and the testicular vessels were ligated close to the testes, which were then removed. The epididymides were returned to the scrotum and the incisions closed with silk sutures. The animals were left to recover for 30 days, kept on a natural diet consisting of Chenopodiacae (low-calorie salt bush rich in salt and water) as described elsewhere (Daly and Daly, 1973), in conditions corresponding to their natural environment in terms of temperature and the photoperiod. After 30 days, they were euthanized. The capita epididymidis were subsequently removed, weighed and prepared for histological and immunohistochemical analyses.

A further group of eight animals (group 4) were castrated in the same manner. However, after 30 days and prior to euthanasia, they were submitted to daily intramuscular injections of testosterone oenantate (75 Ug per day dissolved in 40 μl olive oil) for 15 days as hormonal replacement.

Ligation of efferent ducts

Bilateral ligation of the efferent ducts for one month was carried out following castration on a group of six animals (group 5). After anesthesia, the testes and epididymides of rats were exposed through an incision of the anterior abdominal wall. Using a magnifying glass, a ligature was carefully placed around both right and left efferent ducts. The ligation was made simultaneously with castration and animals were maintained in the same conditions as those of groups 3 and 4 (castrated only or castrated and treated).

Histology

In all groups of animals, the epididymides were removed after euthanasia (in the morning), fixed in Bouin's solution, dehydrated in increasing concentrations of ethanol (70%, 95%, and 100%), cleared in toluene, and finally embedded in paraffin wax. Sections (5 μm thick) were cut with a Leitz microtome and mounted on histological slides or Superfrost® glass slides (Thermo Scientific, Menzel-Gläser, Brausschweig, Germany) for immunohistochemistry. All the organs were cut according to the sagittal plan to retain regionalization as previously described by Menad (2008). After hydration, the sections were stained with Masson's trichrome as described elsewhere (Martoja and Martoja, 1967; Gabe, 1968).

Morphometry and statistical analysis

A morphometric study of histological slides was carried out using a calibrated Zeiss microscope. The principal cell height was measured on 150 cells per batch using a 40× objective. Results were expressed as means ± standard measurement error (SEM). Comparisons were performed using a one-way variance analysis (Anova) followed by the Scheffe *post hoc* test. P values lower than 0.05 ($P < 0.05$) were considered as indicative of a significant difference. All calculations were performed using the Origin pro 7.5 software program (OriginLab Corp., Northampton, MA, USA).

Immunohistochemistry

The expression of estrogen receptors (ESR1 and ESR2) and androgen receptors (AR) was analyzed by immunohistochemistry. Sections were deparaffinized with cyclohexane and rehydrated with decreasing concentrations of ethanol. The slides were then washed in tap water for 10 min. For antigen retrieval, the slides were incubated at 95 °C in a 10 mM sodium citrate solution (H-3300, pH 6.0) for 45 min (for ESR1 and ESR2 analysis) or 30 min