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ORIGINAL ARTICLE

Testosterone influences the expression and distribution of the cation-dependent mannose-6-phosphate receptor in rat epididymis. Implications in the distribution of enzymes

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Summary

The mammalian epididymis plays a role in sperm maturation through its secretory activity. Among the proteins secreted by the epithelium, there are significant amounts of acid hydrolases. In most cell types, the normal distribution of lysosomal enzymes is mediated by mannose-6-phosphate receptors (MPRs). In this study, we analysed the expression and distribution of the cation-dependent MPR (CD-MPR) in epididymis from control, castrated or castrated rats with testosterone replacement. It was observed that expression of CD-MPR increased due to castration in all regions of the epididymis, which was reversed by injection of testosterone. We also measured the activity of α-mannosidase and observed that the castration tends to increase the retention of this enzyme in the tissue, which is reversed by the hormone replacement. In corpus, this resulted in a reduced secretion of the enzyme. Immunohistochemistry showed that CD-MPR has a supranuclear location (different from the cation-independent MPR), most likely in principal cells, and low reactivity in other cell types. The signal in castrated animals was more intense and tended to redistribute towards the apical cytoplasm. Thus, we concluded that expression and distribution of CD-MPR is affected by decrease of testosterone in rat epididymis, and this could change the distribution of lysosomal enzymes.

Introduction

It has been proposed that the secretory activity of mammalian epididymis contributes to sperm maturation (Turner *et al.*, 1995; Tulsiani *et al.*, 1998; Dacheux *et al.*, 2005; Tulsiani & Abou-Haila, 2011). Many of the proteins found in the epididymal fluid are acid hydrolases (Mayorga & Bertini, 1985; Gupta & Setty, 1995; Abou-Haila *et al.*, 1996; Tulsiani *et al.*, 1998; Belmonte *et al.*, 2002a,b). However, the presence of these enzymes in the fluid has not been well explained, as the epididymal environment is not optimal for the activity of acid hydrolases (Brown *et al.*, 1997; Turner, 2002). These enzymes are usually confined to the acidic environment of lysosomes, to exert their hydrolytic activity. During their synthesis and maturation, many lysosomal enzymes acquire mannose-6-phosphate residues to be recognised by specific receptors (mannose-6-phosphate receptors/MPRs) and to be transported selectively to lysosomes (Hille-Rehfeld, 1995; Dahms *et al.*, 2008). Two types of MPRs have been described so far, the cation-dependent (CD-MPR) and the cation-independent (CI-MPR) MPR, which co-exist in many cells and tissues (Hille-Rehfeld, 1995). At present, the coexistence of both receptors has not been well explained, although the fact that the CI-MPR can recognise other ligands that do not carry mannose-6-phosphate could ascribe additional functions (Dahms & Hancock, 2002; Olson *et al.*, 2002). In addition, evidence has shown that the CI-MPR participates in the re-uptake of extracellular ligands bearing mannose-6-phosphate residues, whereas the CD-MPR could be involved in exocytosis of these ligands (Chao *et al.*, 1990). From I-cell disease, characterised by impaired MPR pathway, the existence of alternative routes for the transport and secretion of hydrolytic enzymes has been predicted (Zhu & Conner, 1994; Dittmer *et al.*, 1999; Canuel *et al.*, 2008, 2009). In the case of the epididymis, our findings showed that cathepsin D (CatD) is secreted into the lumen and that

secretion of the enzyme (as the precursor procathepsin D, ProCatD) is significantly increased in cauda epididymis of castrated rats (Carvelli *et al.*, 2010). Based on our findings, and considering that the epididymis is a hormone-dependent organ (Robaire & Viger, 1995; Abou-Haila *et al.*, 1996; Ezer & Robaire, 2002; Robaire *et al.*, 2007; Robaire & Hamzeh, 2011), we thought that both the selective transport of acid hydrolases to lysosomes and secretion to the lumen might be mediated by CD-MPRs. Thus, it is possible that the expression and location of the receptor varies according to the hormonal levels. To test this hypothesis, we have studied in rats the expression and distribution of CD-MPR in the three epididymal regions, and attempted to correlate with distribution of an enzyme bearing mannose-6-phosphate (Belmonte *et al.*, 1998), in response to changes in testosterone levels.

Materials and methods

Reagents

The polyclonal anti-CD-MPR antibody raised in rabbits was kindly provided by Dr. Annette Hille-Rehfeld (Stuttgart, Germany). The mouse monoclonal anti-CI-MPR was purchased from DSHB (Iowa City, IA, USA). Chemiluminescent reagents were from Pierce Biotechnology Inc. (Rockford, IL, USA). The rabbit anti-actin antibody was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was from Dakocytoimation (Mississauga, Canada), and the HRP-conjugated anti-mouse antibody was from Sigma Chemical Co. The corresponding biotin-conjugated antibodies and peroxidase-conjugated avidin were also purchased from Sigma. The 4-methyl-umbelliferyl-α-D-mannopiranoside substrate was also from Sigma Chemical Co.

Castration and hormonal replacement

Twelve adult Sprague–Dawley male rats (90 days old and weighing 300–350 g), maintained under standard conditions (food and water *ad libitum* at 20–22 °C and cycles of 12 : 12 h light : dark) were divided into three groups: controls ($n = 4$), castrated ($n = 4$) and castrated with testosterone replacement ($n = 4$). The experimental animals (eight in total) were anesthetised by abdominal injection of ketamine hydrochloride (70 mg kg^{-1}) and xylazine (5 mg kg^{-1}), according to a protocol approved by the Committee for Animal Care of the Universidad Nacional de Cuyo, and they were castrated following the method of other authors (Mayorga & Bertini, 1985). The testes and epididymides were exposed by abdominal incision, and the testicular arteries were ligated without compromising the blood supply to the epididymides. Subsequently, the testes were removed, while the epididymides were placed back into the scrotum. After surgery, four of the castrated rats were injected intraperitoneally (at the time of surgery and 24 h after castration) with testosterone (1 mg kg^{-1} each injection in corn oil as vehicle), following the protocol of Fan & Robaire (1998). All the animals (castrated and castrated with testosterone replacement) were sacrificed at 48 h after castration by ether inhalation and decapitation, conformed to the policies and regulations of the Argentine National Institute of Health, and the National Research Council. The epididymides were removed and processed as detailed below. The controls were either intact animals ($n = 4$) or with abdominal incision without removal of testes ($n = 2$), because no differences were observed between both groups.

Processing of biological material

Epididymides of control and experimental animals (castrated or castrated with hormone replacement) were divided into three parts: caput, corpus and cauda, to be processed separately. The tissues were cut into small pieces with stainless steel blades. Samples were then suspended (1 : 3 w/v) in Hank's buffer at 32 °C for 30 min with gentle manual stirring. The minced tissues were left for 10 min at 4 °C for sedimentation, and the resulting supernatants were centrifuged to obtain the fluids (stored at −20 °C until use). Tissues were washed twice with Hank's buffer, weighed and suspended in (1 : 5 w/v) Buffer H (10 mM Tris-acetate, pH 7.2, containing 0.25 M sucrose, 0.25 M EDTA, 1 mM PMSF, 0.02% sodium azide and 5 mM glycerophosphate), and homogenised with a homogeniser glass/teflon. The homogenates were centrifuged at 800 ***g*** for 20 min at 4 °C, and the resulting post-nuclear supernatants were stored at −20 °C until use.

Electrophoresis (SDS-PAGE) and immunoblotting

All procedures were carried out according to Alberdi *et al.* (2005). Briefly, 45 μg of protein from each sample was solubilised and analysed on polyacrylamide gels (SDS-PAGE) according to the method of Laemmli (1970), and electrotransferred onto nitrocellulose membranes (0.2 μm pore; Pierce, Rockford, IL, USA), according to Burnette (1981). After blocking of non-specific binding sites with 5% delipidated milk, the membranes were incubated for 12 h at 4 °C with either anti-CD-MPR (1 : 1000) or anti-actin (1 : 1000) antibody, all diluted in PBS-T (10 mM NaH_2PO_4-Na_2HPO_4, pH 7.2, PBS, containing 0.05% (v/v) Tween 20). The membranes were then washed three times with PBS-T and incubated with the corresponding biotin-conjugated secondary

antibodies (1 : 5000) for 2 h. Finally, after three washes with PBS-T, the membranes were incubated with peroxidase-conjugated avidin (1 : 10 000) for 1 h, and the specific bands were detected by a chemiluminescence method (Pierce Biotechnology Inc.) according to manufacturers. Bands were quantified by densitometric scanning of the films, using an IMAGE J Program, and the values were normalised to the actin expression in each case.

Immunohistochemistry

The epididymides of control and experimental rats were removed and immediately fixed in Bouin for 48 h, then dehydrated and embedded in paraffin according to Hermo *et al.* (2007). Sections (5 μm) were cut and mounted on glass slides. After blocking with Peroxidase Block (Dako Cytomation), the sections were incubated for 12 h with anti-CD-MPR (1 : 200) or anti-CI-MPR (1 : 100) antibody diluted in dilution buffer from Dako-Cytomation (S0809). The anti-rabbit (K4010, DakoCytoimation kit) or HRP-conjugated anti-mouse (1 : 250) were used as secondary antibodies. Immunolocalisation of MPRs was performed using the Envision + peroxidase diaminobenzidine kit (K 4010, DakoCytoimation kit; 2007). Preimmune rabbit or mouse serum was used as control of antibody specificity. All digital images were taken with a Nikon Optiphot (Model Dn100, CCD colour camera; Garden City, NY, USA).

Other procedures

The activity of α-mannosidase (α-MAN) was measured fluorometrically from homogenates and fluids, using the corresponding 4-methyl-umbellyferyl substrate as described by Barret & Heath (1977). One unit of enzymatic activity corresponded to one nmol of substrate digested per hour incubation. Proteins were measured according to Lowry *et al.* (1951).

Statistics

The data obtained were subjected to Tukey's–Kramer multiple comparisons test, and the level of significance was set at $P < 0.05$.

Results

In this work, we studied the expression and distribution of the CD-MPR in the three regions of epididymis in rats subjected to hormonal changes; castration or castration followed by testosterone replacement. We noted that the CD-MPR tends to increase in the epididymal tissue due to castration. In corpus and cauda, the increase was significant, and reversed with hormone replacement (Fig. 1). Although the caput was less reactive to the hormonal changes, a tendency to increase with castration was observed (Fig. 1).

We have also attempted to study by immunohistochemistry the localisation of the CD-MPR in epididymal tissue, using the corresponding specific antibody. As shown in Fig. 2, reactivity to the CD-MPR in the three regions increased due to castration and was partially reversed by testosterone injection. These results were somehow consistent with the tendency observed by immunoblotting. We also noted a light redistribution of the CD-MPR due to castration; from a perinuclear location in the controls to a more dispersed signal in the

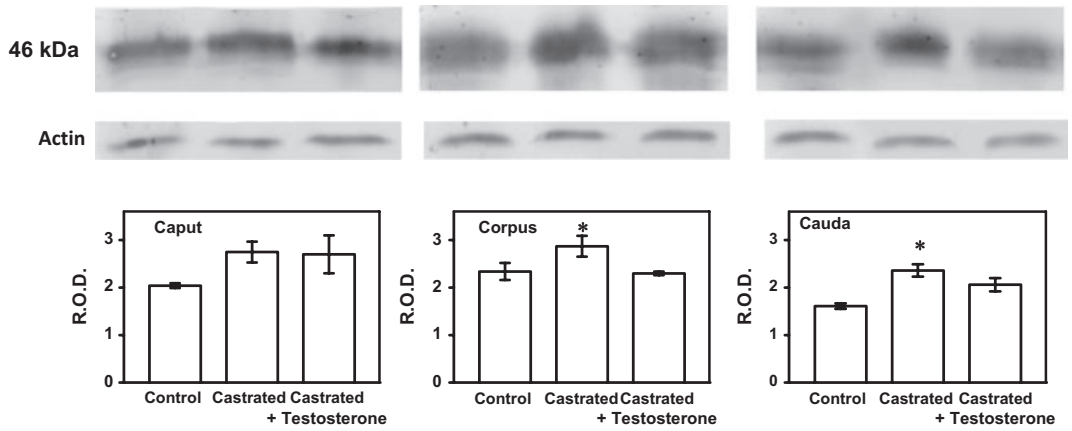

Fig. 1 Immunodetection of CD-MPR in tissue of rat epididymis from controls, castrated or castrated with hormone replacement. The three regions of the epididymis were processed separately as detailed in *Materials and methods*. The figure shows a representative immunoblot of each region and experimental condition and the quantification of the bands in each case. Values were normalised to expression of actin. Bars represent the relative optical density (R.O.D.) ± SD from four independent experiments (two epididymides per experiment). *Significantly different from the other two conditions ($P < 0.05$). CD, cation-dependent; MPR, mannose-6-phosphate receptors.

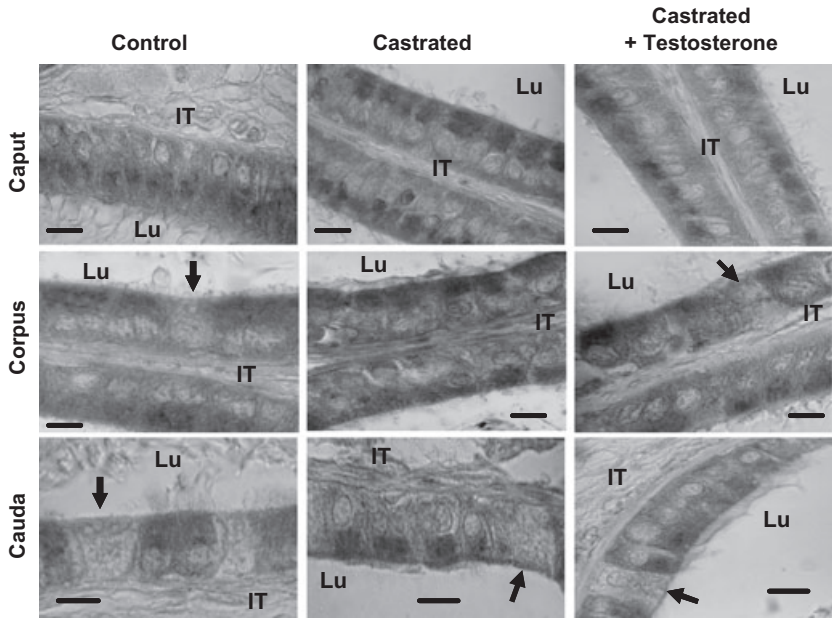

Fig. 2 Immunostaining of CD-MPR in caput, corpus or cauda of rat epididymis from controls, castrated or castrated with hormone replacement as indicated. The three regions of the epididymis were processed for immunohistochemistry as detailed in *Materials and methods*. Arrows indicate the clear cells. Scale bars = 10 μm. CD, cation-dependent; IT, intertubular space; Lu, lumen; MPR, mannose-6-phosphate receptors.

apical cytoplasm of castrated rats (Fig. 2). Moreover, while some cells (more likely principal cells) showed strong reactivity to the CD-MPR, other cell types ('clear cells', indicated with arrows) were poorly reactive, and there were no major changes in this cell type under the experimental conditions. It is important to note that the CI-MPR has a location different from that of the CD-MPR, as the CI-MPR showed a scattered distribution in the cytoplasm of the principal cells in controls, and with an apparent slight redistribution to the apical zone in the cauda of castrated rats (Fig. 3). This would indicate that both receptors fulfil different roles in the epididymis. Staining was negative when the samples were incubated with the respective secondary antibodies alone (not shown), confirming the specificity of the signal for the CD-MPR or CI-MPR. We also measured the activity of α-MAN, a lysosomal enzyme containing mannose-6-phosphate, to relate it to the behaviour of the CD-MPR. It was observed a tendency to retain the enzyme in tissues in response to castration, being this effect reversed by injection of testosterone. In the corpus, castration significantly affected the enzyme secretion, while in other regions, the effect was not so obvious (Fig. 4).

Discussion

For decades, the mammalian epididymis has attracted the attention of reproductive biologists due to its involvement in the storage and maturation of spermatozoa (Hermo & Robaire, 2002). However, it is still unclear the mechanism by which the epididymis confers to spermatozoa the ability to fertilise the oocyte. Currently, the most accepted concept is that this organ induces biochemical changes in gametes via the catalytic activity of acid hydrolases secreted by the epididymal epithelium (Turner *et al.*, 1995; Tulsiani *et al.*, 1998; Dacheux *et al.*, 2005; Tulsiani & Abou-Haila, 2011). The lysosomal enzymes are normally confined to lysosomes and are transported to this compartment by two MPRs; the cation-dependent and the cation-independent-MPR (CD-MPR and CI-MPR respectively; Chao *et al.*, 1990; Hille-

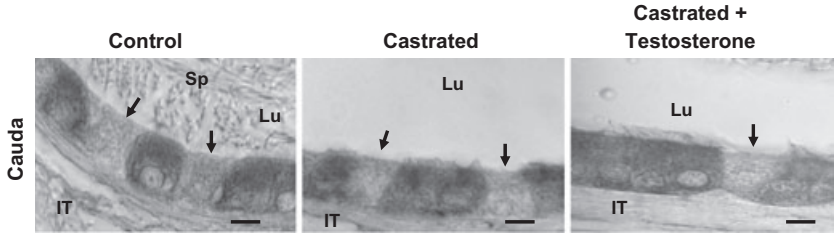

Fig. 3 Immunostaining of CI-MPR in cauda of rat epididymis from controls or castrated. Cauda epididymis was processed for immunohistochemistry as detailed in *Materials and methods*. Arrows indicate the clear cells. Scale bars = 10 μm. IT, intertubular space; Lu, lumen; MPR, mannose-6-phosphate receptors; Sp, spermatozoa.

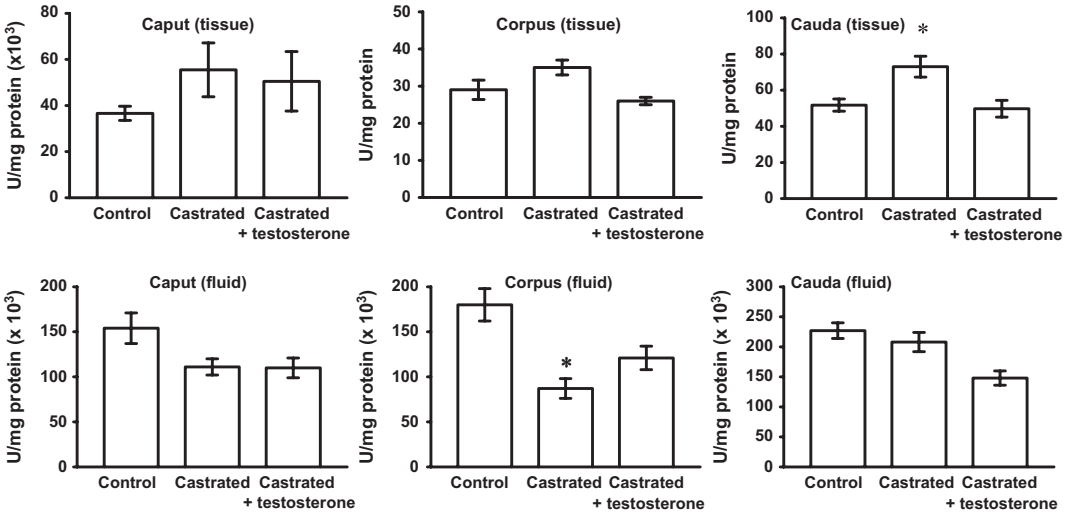

Fig. 4 Activity of α-mannosidase in the tissue and fluid of rat epididymis (caput, corpus or cauda) from controls, castrated or castrated with hormone replacement as indicated. Bars represent the means of specific activity ± SD from four independent experiments. *Significantly different from the other two conditions ($P < 0.05$).

Rehfeld, 1995). In this study, we observed that castration induces an increase in expression of CD-MPR in corpus and cauda of rat epididymis, and at lesser extent in caput. This effect was reversed by testosterone replacement, indicating that this steroid hormone may regulate the expression of CD-MPR in epididymis. If the functionality of these receptors can be regulated by hormones in some tissues has not been studied so far. It is well known that the epididymis is a hormone-dependent organ, because the withdrawal of testosterone induces changes in secretion and leads to involution of the organ (Robaire & Hermo, 1988; Robaire & Hamzeh, 2011). Moreover, castration has been used and long accepted as a model of androgen deprivation (Cheuk *et al.*, 2000; Ezer & Robaire, 2003). From these facts, we presume that this finding may have physiological relevance. From the differential response to castration between the three regions, we are tempted to speculate that this could be related to a specific role of the different epididymal regions on sperm maturation, and/or to differential response of epididymal regression to androgen deprivation, as it has been proposed by other authors (Robaire & Viger, 1995). By immunohistochemistry, it was observed that reactivity of CD-MPR increases in the three regions of epididymis of castrated rats and is reversed with hormonal replacement. This is, in some way, in line with changes in the expression of CD-MPRs observed by immunoblotting. Moreover, it was observed that castration induces a redistribution of the CD-MPR, from a perinuclear location (in controls) to a scattered distribution in the apical cytoplasm of castrated animals. The location of the CD-MPR does not overlap with that of the CI-MPR in controls, indicating that both receptors could play different roles in the epididymis. Although castration induces a slight increase in the expression of CI-MPR (Carvelli *et al.*, 2010) and also a slight redistribution to the apical area (Fig. 3), we cannot say that this receptor responds to hormonal changes in a way similar to the CD-MPR. Some epithelial cells, as 'clear cells', showed very low reactivity to both MPRs. If this is so, it is difficult to explain why they have very low levels of receptors, because this cell type is known to have abundant lysosomal apparatus and intense endocytic activity (Hermo & Robaire, 2002). We have also shown that α-MAN, an enzyme that is recognised by the MPRs (Belmonte *et al.*, 1998), is mostly retained by the epididymal tissue due to castration, in line with the increase in CD-MPR. The effect on the enzyme was similar to that of Belmonte *et al.* (2002a,b) in the cauda. In turn, this retention resulted in a decreased secretion of the enzyme in the corpus, but not in caput and cauda. The apparent discrepancy with the secretion of α-MAN observed by other authors in cauda (Belmonte *et al.*, 2002a,b; Carvelli *et al.*, 2010), could be explained by the differences in the method used to obtain the epididymal fluid. Regional differences observed in the secretion of α-MAN in response to hormonal changes, could be related to the existence of other ligands that compete with the enzyme in the interaction with CD-MPR. In conclusion, castration induces increased expression and relocation of CD-MPR in rat epididymis, and this may indicate that testosterone, directly or indirectly, regulates the trafficking of certain enzymes to lysosomes in rat epididymis. However, it should not be ruled out the possibility that the increase and redistribution of the CD-MPR due to castration is a compensatory response to other transport systems affected by the lack of testosterone (e.g. sortilin).

Some evidence has shown that the CD-MPR also participates in the exocytosis of enzymes in some cell types. This could suggest that the increase in the expression and the redistribution of the CD-MPR towards the apical area due to castration might also be responsible, in part, of the increased secretion of proCatD in epididymis, as it was previously observed (Carvelli *et al.*, 2010). Taken together, our results suggest that the lysosomal functionality in epididymis could be regulated by testosterone, mainly by controlling the expression and localisation of receptor proteins, such as the CD-MPR. This could be a starting point to explain the molecular basis of dysfunctions in the genital tract leading to male infertility.

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