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Research article

Aquaporin-1 and -9 are differentially regulated by oestrogen in the efferent ductule epithelium and initial segment of the epididymis

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Background information. Efferent ductules reabsorb more than 90% of the rete testis fluid, a process that involves ion transporters and AQP (aquaporin) water channels. Oestrogen has been shown to modulate the expression of the ion transporters involved in this activity, but reports of AQP regulation in the male tract have been confounding. To understand better the regulation of AQP1 and AQP9, we investigated their expression in rat efferent ductules and initial segment of the epididymis after treatment with the pure antioestrogen ICI 182,780 or bilateral efferent duct ligation, or castration, followed by hormone replacement.

Results. Results show that AQP9 is modulated by oestrogen in the efferent ductule epithelium, but not in the initial segment of the epididymis. DHT (5α-dihydrotestosterone) also modulated AQP9 in efferent ductules. AQP9 was down-regulated by the antioestrogen in efferent ductules on day 45 post-treatment, which occurred before the non-ciliated cells had shown significant loss of microvilli. DHT, but not oestradiol, modulated AQP9 expression in the initial segment of the epididymis. In contrast, testosterone, DHT, oestrogen or the antioestrogen did not alter AQP1 staining, indicating constitutive expression of AQP1 in the efferent ductule epithelium. AQP1 expression was induced in peritubular cells of efferent ductules and in the initial segment of the epididymis after castration and long-term treatment with the antioestrogen. Although peritubular AQP1 staining in efferent ductules was partially reversed by the androgens, it was not reversed after any treatment in the initial segment of the epididymis.

Conclusions. These results demonstrate that efferent ductules are unique in requiring both oestrogen and androgen to regulate an important mediator of fluid reabsorption, whereas the initial segment is dependent only on androgen stimulation.

Introduction

Efferent ductules are the region of the male reproductive tract responsible for reabsorption of more than 90% of the fluid coming from the testis (Clulow et al., 1994). This important function increases the concentration of sperm and facilitates their maturation and storage in the epididymis (Hess, 2002; Ilio and Hess, 1994). The reabsorption of fluid in the efferent ductules is iso-osmotic, inhibited by cAMP and sodium and is flow-dependent (Clulow et al., 1994, 1996; Hansen et al., 1999; Man et al., 2003), but independent of mineralocorticoids (Man et al., 1997). A complete mechanism to account for fluid reabsorption in the efferent ductules remains to be investigated, even though several important steps have been reported in recent years. Several ion transporters are present in the epithelial cell membrane, including Na^+/K^+-ATPase (Ilio and Hess, 1992, 1994), Na^+/H^+ exchanger-3 (Hansen et al., 1999),

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Abbreviations used: AQP, aquaporin; E2, 17-β-oestradiol-3-benzoate; ER, oestrogen receptor; DHT, 5α-dihydrotestosterone.

Cl^-/HCO_3^- exchanger down-regulated in adenoma (Lee et al., 2001) and chloride channel (cystic fibrosis transmembrane regulator) (Lee et al., 2001; Leung et al., 2001). AQP (aquaporin) water channels, including AQP1, AQP9 and AQP10 (Fisher et al., 1998; Elkjaer et al., 2000; Pastor-Soler et al., 2001, 2002; Zhou et al., 2001; Badran and Hermo, 2002; Hermo et al., 2004), are also found in the epithelium and may participate in the transcellular movement of water, in addition to passive diffusion of water by paracellular pathways (Ilio and Hess, 1992).

Genetic disruption of the ERα (oestrogen receptor α) [αERKO mouse (ERα knockout mouse)] or inhibition of the ERs by using antioestrogen ICI 182,780 impairs efferent ductule fluid reabsorption, evidenced by a significant increase in the luminal diameter of the ductules (Hess et al., 1997a, b, 2000; Lee et al., 2000; Oliveira et al., 2001, 2002). Since the efferent ductule contains the highest expression of ERα in the male tract (3.5 times greater than uterus) and oestrogen concentrations in the rete testis fluid approach 249 pg/ml in the rat (Free and Jaffe, 1979), it was hypothesized and later proven that oestrogen indeed does regulate fluid reabsorption in these tubules (Hess et al., 1997a). Others have also shown that oestrogen treatment or disruption of ERα function in efferent ductules alters the expression of several major ion transporters and of AQP1 (Fisher et al., 1998, 1999; Lee et al., 2000; Zhou et al., 2001; Oliveira et al., 2002).

AQP is a family of water channel proteins that allow the transcellular transport of water in a wide variety of tissues (Borgnia et al., 1999; Verkman, 2002; Agre and Kozono, 2003). AQP1 is required for efficient fluid reabsorption in the renal proximal tubules (Schnermann et al., 1998; Schrier et al., 2003). The high expression of AQP1 in the efferent ductule epithelium (Brown et al., 1993; Fisher et al., 1998) suggests a role for this water channel protein in fluid reabsorption. However, the AQP1 knockout mouse did not show evidence of impaired fluid reabsorption in efferent ductules (Zhou et al., 2001), which suggested that another isoform of AQP was present in this epithelium, or else transcellular water transport was not important in this leaky epithelium. The first explanation may be the correct one, as the AQP9 and AQP10 isoforms have now been detected in efferent ductule epithelium, revealing a new possible pathway for water and small non-charged solute transportation (Pastor-Soler et al., 2001; Badran and Hermo, 2002; Hermo et al., 2004).

Published reports dealing with hormonal regulation of AQP in the male have been controversial. According to Badran and Hermo (2002), neither AQP1 nor AQP9 is regulated by androgens in the rat efferent ductules and epididymis, but Pastor-Soler et al. (2001) reported an opposite result for AQP9 in the epididymis. Both of these studies used castration and testosterone replacement to assess the regulation of AQP; therefore the potential involvement of oestrogen cannot be ruled out. Both androgen receptor and ER are expressed abundantly in the efferent ductule epithelium. Thus the use of a non-aromatizable androgen, such as 5α-dihydrotestosterone (DHT), is recommended for identifying the effects of androgens without the interference of oestrogen synthesis from the testosterone.

To understand better the regulation of AQP1 and AQP9 in efferent ductules and to help resolve the existing conflicts in data, we investigated the expression of both the water channels in rat efferent ductules after bilateral ligation or castration, followed by hormone replacement. Testosterone and its metabolites DHT and oestradiol were used to alleviate potential indirect effects of testosterone alone that could occur through aromatization to oestrogen or 5α-reduction to DHT. The effects of a pure antioestrogen ICI 182,780 were also investigated because the compound inhibits efferent ductule fluid reabsorption, nearly identical with that seen in the αERKO mouse (Oliveira et al., 2001, 2002). The present results show that the expression of AQP9 in the efferent ductule epithelium of the rat, but not in the initial segment, is modulated by oestrogen. Concurrently, ICI 182,780 caused significant down-regulation of AQP9 in the efferent ductules, whereas AQP1 did not. DHT appears to influence the expression of AQP9 in both efferent ductules and initial segment of the epididymis. Androgens or oestrogens do not regulate AQP1.

Results

AQP1 and AQP9 expression in control rats

A summary of the results is found in Table 1. In control rats, both AQP1 and AQP9 were expressed in the efferent ductule epithelium, but not detected in the rete testis epithelium. In the efferent ductule

Table 1 | Comparison of the immunohistochemical staining for AQP1 and AQP9 in rete testis, efferent ductules and the initial segment of the epididymis of ligated, castrated, hormone-replaced, ICI 182,780-treated (as seen after 150 days of treatment) and control rats

NC, non-ciliated cells; C, ciliated cells; E, epithelium; PC, peritubular cells; Con, control; ICI, ICI 182,780-treated; Cas, bilateral castrated; T, testosterone propionate; —, negative; –, staining decreased to nearly undetectable; +, weak stain; +++, strong stain; ++/+, variable intensity of stain; –/+, intermittent stain; –/++, increased intermittent stain.

		Efferent ductules			Initial segment	
	Rete testis	NC	C	PC	E	PC
AQP1						
Con	—	+++	—	–/+	—	–/+
ICI	—	++/+	—	–/++	—	–/++
Sham-operated	—	+++	—	–/+	—	–/+
Ligated	—	+++	—	–/++	—	–/++
Castrated	—	+++	—	–/++	—	–/++
Cas + oil	—	+++	—	–/++	—	–/++
Cas + T (5 mg)	—	+++	—	–/+	—	–/++
Cas + DHT (5 mg)	—	+++	—	–/+	—	–/++
Cas + E2 (75 μg)	—	++	—	–/++	—	–/++
Cas + E2 (400 μg)	—	++	—	–/++	—	–/++
Cas + T + E2 (400 μg)	—	++	—	–/++	—	–/++
AQP9						
Con	—	+++	—	—	+++	—
ICI	—	–	—	—	+++	—
Sham-operated	—	+++	—	—	+++	—
Ligated	—	+	—	—	–	—
Castrated	—	+	—	—	–	—
Cas + oil	—	+	—	—	–	—
Cas + T (5 mg)	—	+	—	—	–	—
Cas + DHT (5 mg)	—	+++	—	—	+++	—
Cas + E2 (75 μg)	—	+++	—	—	–	—
Cas + E2 (400 μg)	—	+++	—	—	–	—
Cas + T + E2 (400 μg)	—	+++	—	—	–	—

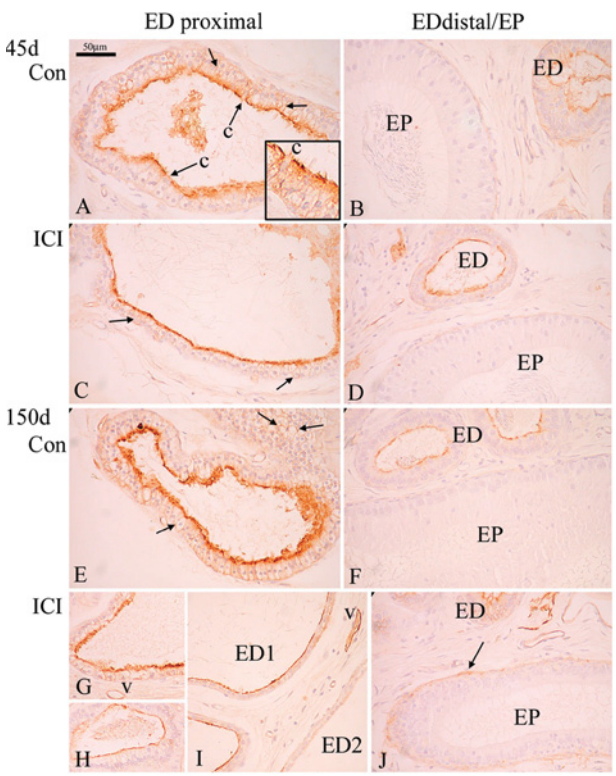
epithelium, non-ciliated cells were positive for AQP1 along the apical and basolateral membranes (Figures 1A, 1E and 3A). Ciliated cells were negative. The basolateral staining was better evidenced in tangential sections of the epithelium (Figure 1E). Sporadic stain for AQP1 was seen in the peritubular cells of the initial segment and, sometimes, an intermittent staining in the efferent ductules was also observed. Vascular endothelial cells were positive for AQP1. AQP9 was restricted to the non-ciliated cells in the efferent ductule epithelium, but only the apical membrane was positive (Figures 2A, 2E and 2I). The expression of AQP9 in the apical membrane of efferent ductule epithelium was relatively stronger than that for AQP1. The epithelium of the initial segment of the epididymis was immunonegative for AQP1 (Figures 1B, 1D, 1F and 1J), while being strongly positive for AQP9 along the microvillus border of the principal cells (Figures 2C, 2G and 2K).

AQP1 and AQP9 expression after ICI 182,780 treatment

After ICI 182,780 treatment, no distinguished changes in AQP1 expression were seen on days 7, 45 and 73 (Figure 1C); however, on days 100 and 150 post-treatment, some decrease in staining was observed in the efferent ductules (Figures 1G, 1H and 1I). The decrease in AQP1 was not homogeneous, with some areas presenting greater decrease in staining than others even in the same tissue section (Figure 1I). On day 150, a conspicuous AQP1 staining was detected in the peritubular tissue of the initial

Figure 1 | Effects of ICI 182,780 on the expression of AQP1 in rat efferent ductules (EDs) and the initial segment of the epididymis (EP)

In the control rats (**A**, **E**), AQP1 was expressed along the apical microvillus border of the non-ciliated cells in the ED epithelium. The basolateral plasma membranes were also positive (arrow), which is observed more clearly in transversal sections of the epithelium (between arrows in **E**). Ciliated cells ('c') were unstained (see inset to **A**). AQP1 was not detected in the epithelium of the initial segment of the EP in control rats (**B**, **F**). After 45 days of ICI treatment, no detectable changes in staining were found in the EDs (**C**) or epididymal (EP) epithelium (**D**). On days 100 and 150 after treatment, the intensity of staining was variable, with areas intensely stained (**G**), moderately stained (**H**) or the staining was barely detectable (ED2) in the ED epithelium (**I**). ED1 and ED2 represent difference in staining in the same section. v, blood vessel. (**J**) Treated rats on day 150 expressed AQP1 in the peritubular cells of the initial segment (EP).



segment and efferent ductules of treated animals (Figure 1J).

The effects of ICI 182,780 on AQP9 staining were not detected on day 7 post-treatment (Figure 2B); however, on day 45, AQP9 staining was significantly decreased in the efferent ductules (Figure 2F), but not in the initial segment (Figure 2H). On day 45, higher magnification of the non-ciliated cells revealed tall, compacted microvilli in control ductules (Figure 3A). In ICI 182,780-treated cells, the height of the epithelium was decreased significantly, but the microvilli were normal to slightly decreased in height, while their density appeared to be thinner (Figure 3B). On day 45, AQP9 staining was intense in controls (Figure 3C), whereas, in ICI 182,780-treated ductules, staining was greatly decreased even in areas containing nearly normal microvilli (Figure 3D). On days 100–150, AQP9 expression disappeared in the efferent ductules (Figure 2J), whereas no change was noted in AQP9 in the initial segment after treatment (Figure 2L).

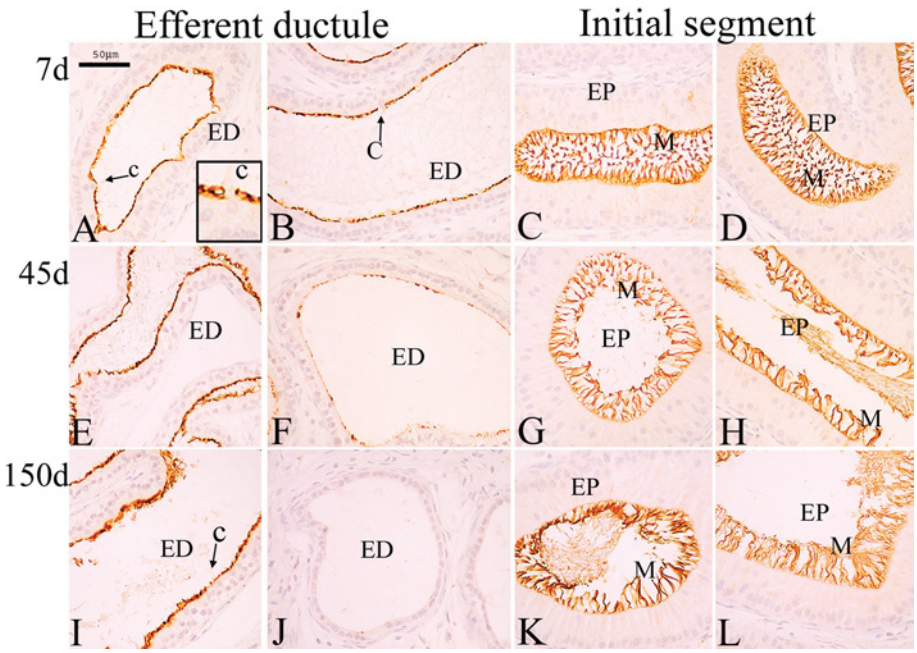
AQP1 expression after castration and hormone replacement

AQP1 staining in the efferent ductule epithelium was decreased slightly after castration or ligation of the efferent ductules (Figure 4B); however, there was an induction of AQP1 staining in peritubular tissue of the efferent ductules and initial segment of the epididymis (Figures 4B and 4H). In efferent ductules of castrated rats, the basolateral staining of AQP1 was decreased, specifically in the proximal area near the rete testis. After replacement with oestradiol alone or in combination with testosterone, there was a decrease in apical staining for AQP1 in efferent ductule epithelium, whereas the peritubular tissue remained similar to the castrated response (Figures 4C and 4F). Testosterone and DHT replacement caused a decrease in AQP1 staining along the efferent ductule peritubular region (Figures 4D and 4E), but no detectable changes were noted in the initial segment of peritubular tissue nor in endothelial cell staining after hormone replacement, compared with castrated animals (Figures 4G–4L).

AQP9 expression after castration and hormone replacement

AQP9 expression in the efferent ductule epithelium was decreased after castration or ligation (Figure 5B). Testosterone replenishment did not recover efferent ductule staining (Figure 5E); however, DHT, oestradiol alone or oestradiol + testosterone restored the efferent ductules staining to the control levels and appeared to have increased staining (Figures 5C, 5D

Figure 2 | Effects of ICI 182,780 on the expression of AQP9 in the rat ED and initial segment of the EP
In the control rats (**A**, **E**, **I**), AQP9 was expressed along the apical microvillus border of the non-ciliated cells in the ED epithelium. Ciliated cells ('c') were unstained (see inset to **A**). Intense expression of AQP9 was detected in the microvilli of epithelial cells in the initial segment of the EP in control rats (**C**, **G**, **K**). On day 7 of treatment, there was no effect on the expression of AQP9 in ED and initial segment (**B**, **D**). After 45 days of ICI treatment, there was a significant decrease in AQP9 expression in the ED (**F**), but not in the initial segment of epithelium (**H**). On day 150, the AQP9 staining basically disappeared in the ED epithelium (**J**), but the staining in the initial segment of epithelium was unchanged (**L**).



and 5F). In the initial segment, AQP9 staining decreased to nearly undetectable levels after castration or ligation (Figure 5H). DHT restored the initial segment staining to control levels (Figure 5J), but oestradiol or testosterone alone or in combination did not recover initial segment staining (Figures 5I, 5K and 5L).

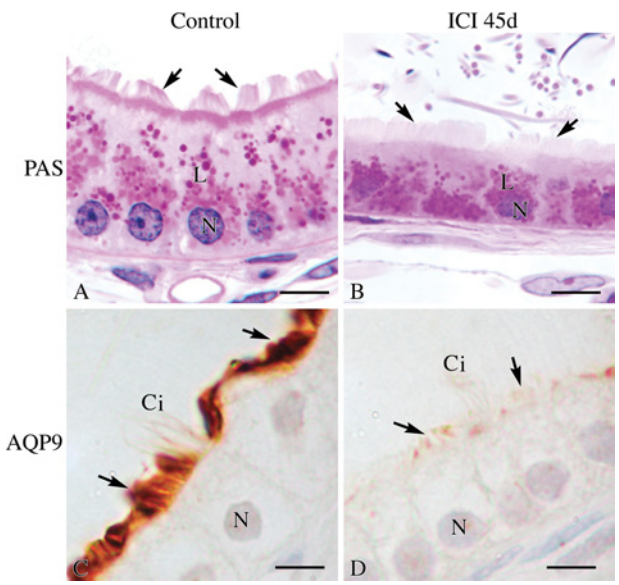
Discussion

The head of the epididymis, including efferent ductules and the initial segment of the epididymis in rodents, has a major function in the reabsorption of luminal fluids (Clulow et al., 1998). This region, particularly the efferent ductules, also expresses ERα more abundantly than any other region and shows the greatest sensitivity to ERα disruption (Hess et al., 1997a, 2002; Hess, 2003). Therefore we investigated the potential role of oestrogen in the regulation of two water channels, AQP1 and AQP9, in this region of the male reproductive tract. Both AQP1 and AQP9 were found within efferent ductule epithelium, but only AQP9 was expressed in the initial segment of the epididymis. A differential regulation of the water channels by oestrogen was noted. This is the first evidence that efferent ductules are unique in requiring both oestrogen and androgen to regulate an important mediator of fluid reabsorption, whereas the initial segment is dependent only on androgen stimulation.

The occurrence of AQP1 exclusively in the endothelium and efferent ductule epithelium, in contrast with the rete testis and epididymis, reveals the importance of this water channel for the concentrating capacity of the efferent ductules, where a large amount of testicular fluid requires rapid reabsorption (Clulow et al., 1998). In agreement with Badran and Hermo (2002), castration and ligation did not affect AQP1 expression on microvilli of the efferent ductule epithelium, indicating that expression of AQP1 on

Figure 3 | Higher magnification of EDs in control and ICI 182,780-treated rats showing (A, B) periodate–Schiff (PAS) and haematoxylin staining and (C, D) AQP9 staining

(**A**) In control EDs, the non-ciliated cells are columnar with basal nuclei (N), apical lysosomes (L) and a tall, dense brush border of microvilli (arrows). Scale bar, 10 μm. (**B**) In ICI 182,780-treated ductules, non-ciliated cells are shorter in height, with basal nuclei (N) surrounded by lysosomes (L). The microvillus border (arrows) is tall in this region, although their density appears to be less than that in controls. Scale bar, 10 μm. (**C**) AQP9 staining in control non-ciliated cells is intense along the microvillus border (arrows). Cilia (Ci), which protrude into the lumen, are unstained. Scale bar, 5 μm. (**D**) In ICI 182,780-treated non-ciliated cells, AQP9 staining is weak along the microvillus border (arrows) and even absent from some cells. Cilia (Ci) are seen in the lumen. Scale bar, 5 μm.



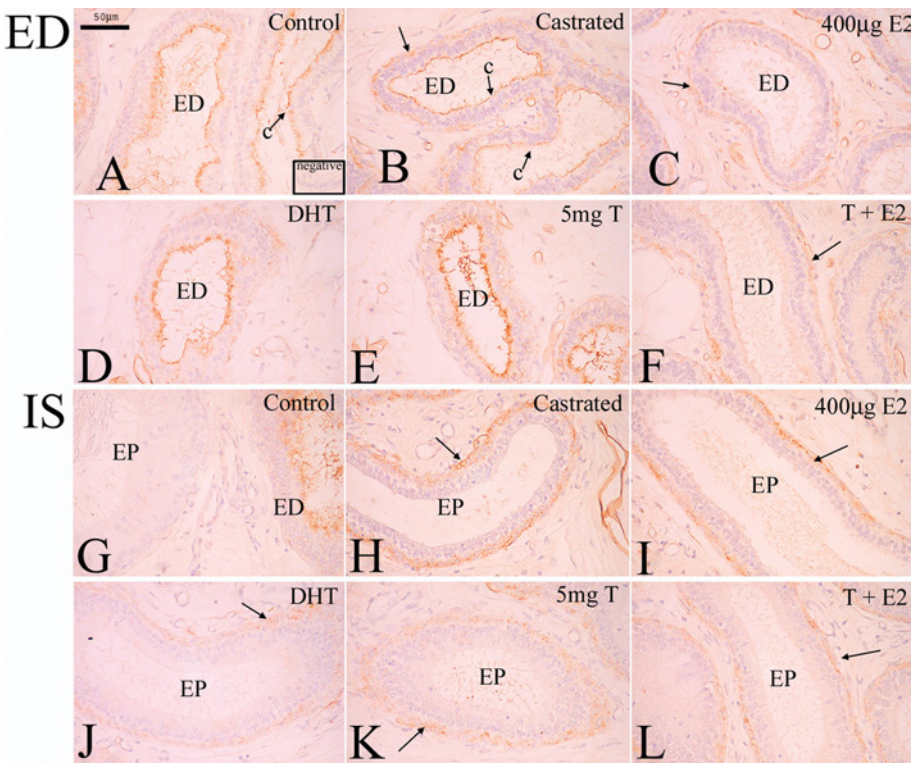
this membrane domain is not regulated by testicular factors. Similarly, expression of AQP1 in the efferent ductules was not affected by gonadotropin withdrawal (Fisher et al., 1998) or treatment with the antioestrogen ICI 182,780 (Zhou et al., 2001). On the other hand, DES treatment given neonatally (Fisher et al., 1998), as well as transgenic disruption of ERα (Zhou et al., 2001), resulted in decreased AQP1 protein, accompanied by luminal distension in efferent ductules and epithelial regression. In both cases, the decrease in AQP1 was attributed to a secondary effect resulting from changes in the epithelial morphology, more than direct effects of oestrogen on transcription (Fisher et al., 1998; Zhou et al., 2001). Taken together, these results are suggestive that epithelial AQP1 is probably constitutively expressed in the efferent ductules, as found in the proximal tubules of the kidney (Nielsen et al., 1993; Borgnia et al., 1999).

Although the epithelial expression of AQP1 in the efferent ductules was not affected by any treatment, it was surprising to find that castration and ligation, as well as long-term treatment with the pure antioestrogen ICI 182,780 (150 days), induced the expression of AQP1 in the peritubular tissue of the efferent ductules and initial segment. This result is coincident with a marked thickening of the efferent ductule peritubular layer after ICI 182,780 treatment (Oliveira et al., 2001), as well as exposures to DES and ethinyl oestradiol (Fisher et al., 1999; McKinnell et al., 2001). Treatment with ICI 182,780 induced complete testicular atrophy and regression of the efferent ductule epithelium (Oliveira et al., 2001, 2002), which indirectly would have decreased the luminal and transepithelial transport of fluid in the efferent ductules. It is possible that such a thickening of the peritubular region could be a compensatory response to the changes in efferent ductule morphology and physiology, which are common after ERα disruption, as the ducts respond to diminished water movement across the epithelium (Hess, 2003). Since androgens could partially reverse this overexpression of AQP1 in the peritubular cells of efferent ductules, it appears that androgens have an inhibitory effect on AQP1 expression in these cells, but not in epithelial cells.

AQP9 distribution included apical membranes of efferent ductule non-ciliated cells and microvilli of principal cells in the initial segment of the epididymis. The wider distribution of AQP9 contrasts with that of AQP1, which is restricted to efferent ductules. Also, in contrast with AQP1, castration and ligation promoted down-regulation of AQP9 in efferent ductules and initial segment of epithelium. DHT, but not testosterone, was capable of restoring AQP9 in both efferent ductules and the initial segment to control levels, showing that DHT is the principal androgenic mediator of AQP9 modulation. This finding explains why, in a recent study, testosterone could not regulate AQP9 in the efferent ductules and epididymis (Badran and Hermo, 2002). In contrast, another study showed that, in the cauda epididymis, AQP9 was regulated by testosterone (Pastor-Soler

Figure 4 | Regulation of AQP1 expression in the rat ED and initial segment of the EP

(**A**) In intact control animals, AQP1 was expressed in the apical border of the epithelial non-ciliated cells. Ciliated cells (c) were not stained for AQP1. Inset to (**A**) shows the negative control, which did not receive the primary antibody. (**B**) Castration did not affect the staining in the ED epithelium, but there was an induction of staining in the peritubular cells (arrow) of the ED. (**C**) The E2 (17-β-oestradiol-3-benzoate) replacement caused a decrease in the AQP1 staining in epithelial EDs, but the peritubular staining (arrow) persisted in the ED. Replacement with DHT (**D**) and testosterone (**E**) did not change the ED epithelial staining; however, there was a decrease in reactivity in the ED peritubular tissue (arrow) when compared with the castrated. (**F**) Combined E2 and testosterone caused a decrease in epithelial ED AQP1 staining, but peritubular staining was unchanged in the ED. (**G**) AQP1 was not detected in the epithelium of the initial segment of EP. (**H**) Castration induced positive AQP1 staining in the peritubular cells of the initial segment (arrow). (**I**) The peritubular AQP1 staining persisted after E2 replacement, but replacement with DHT (**J**) and testosterone (**K**) decreased the reactivity in the peritubular tissue (arrow). (**L**) Peritubular AQP1 staining was unchanged after combined E2 and testosterone treatment, when compared with the castrated rats. The results for bilateral ligated rats were similar to those of castrated, and sham-operated were similar to control rats, so they were not shown.

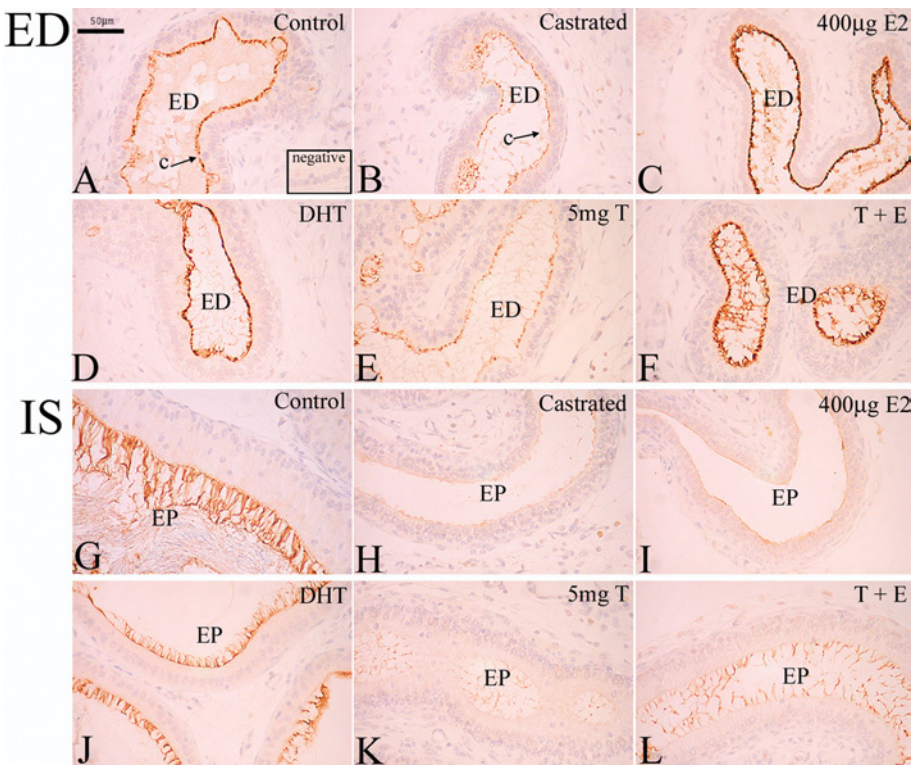


et al., 2002). The difference between these studies is probably due to the fact that the initial segment of the epididymis has the highest expression of 5α-reductase and it is the segment most sensitive to DHT, in comparison with the remaining regions of the epididymis (Roselli et al., 1991; Viger and Robaire, 1994).

Unexpectedly, oestradiol alone or in combination with testosterone also restored AQP9 expression to control levels; however, this response was restricted to the efferent ductules and did not occur in the initial segment of the epididymis. An oestrogen/ER role in the regulation of AQP9 was further confirmed by antioestrogen ICI 182,780 treatment, which induced a significant decrease in AQP9 expression. For AQP1, its decrease has been linked to the loss of apical microvilli (Fisher et al., 1998, 1999; Zhou et al., 2001). However, the down-regulation of AQP9 in rat efferent ductules was not detected until day 45 of treatment. Although the epithelium of ICI 182,780-treated ductules was decreased in height by nearly 50% and some areas also showed decrease in microvillus height, in most areas the microvilli remained

Figure 5 | Regulation of AQP9 expression in the rat ED and initial segment of the EP

(**A**) In intact control animals, AQP9 was expressed in the apical border of the epithelial non-ciliated cells. Ciliated cells (c) were not stained for AQP9. Inset: the negative control, which did not receive the primary antibody. (**B**) Castration caused a decrease in the AQP9 ED epithelial staining. The E2 (**C**) and DHT (**D**) replacements recovered the ED epithelial staining. (**E**) Testosterone alone did not recover the AQP9 expression, but (**F**) combined with E2 and testosterone treatment recovered epithelial ED AQP9 staining to control levels. (**G**) AQP9 was expressed in the microvilli of epithelial cells in the initial segment of the EP in control rats. (**H**) The AQP9 staining was decreased to barely detectable levels in the initial segment of epithelium after castration. The E2 (**I**), testosterone alone (**J**) or testosterone combined with E2 (**K**) was not capable of recovering the AQP9 staining in the initial segment. DHT (**J**) recovered the initial segment expression of AQP9. The results about bilateral ligation were similar to those of castrated rats and sham-operated were similar to control rats, so they were not shown.



significantly tall and even higher in some regions of treated ductules, compared with controls (Oliveira et al., 2002). Examination of the ductule epithelium with higher resolution demonstrated greatly decreased staining for AQP9 in the presence of well-defined microvilli. This ICI 182,780-induced decrease in AQP9 is coincident with a pronounced decrease in ERα expression, but not in androgen receptor expression (Oliveira et al., 2003), suggesting that AQP9 is regulated by oestrogen.

Altogether, these results confirm the large body of evidence that oestrogen and/or a functional ERα is essential for normal function of this reproductive tract segment, which is marked by an unusual capacity for fluid reabsorption (Clulow et al., 1998). Since the staining for AQP9 in efferent ductules is not completely lost after castration or ligation, maintenance of its expression is probably dependent on extra testicular factors. After castration, moderate levels of oestradiol are present in the serum (Sassa et al., 1994; Oliveira et al., 2004). Therefore it is possible that the oestrogen derived from aromatase activity in the peripheral tissues (Baird et al., 1973; de Jong et al., 1973) may be sufficient to provide a maintenance level of AQP9.

The relative contribution of AQP1 and AQP9 towards fluid reabsorption in the efferent ductules is unknown (Pastor-Soler et al., 2001). However, because

the AQP1 knockout mice failed to show disruption of fluid reabsorption in the efferent ductules (Zhou et al., 2001), it is reasonable to suggest that AQP9, or even AQP10 that has now been shown to be expressed in efferent ductules (Hermo et al., 2004), may be capable of compensating for the lack of an AQP1 channel. An investigation of the AQP9 and AQP10 knockout mice and the double or triple knockouts will be useful in testing this hypothesis. It is probably not a coincidence that AQP1, which is exclusively a water channel, does not appear to be regulated in the efferent ductule epithelium, whereas AQP9, which is permeable to both water and small uncharged solutes (Borgnia et al., 1999; Carbrey et al., 2003), is more closely regulated by both sex hormones, oestrogen and androgen. Such a regulation may be necessary in an organ where water reabsorption is largely secondary to reabsorption of electrolytes (Clulow et al., 1994; Ilio and Hess, 1994; Hansen et al., 1999).

In conclusion, these results show that there is differential expression and regulation of AQP1 and AQP9 in the efferent ductules and epididymis. It appears that both sex steroid-dependent and -independent pathways are responsible for regulating AQPs in the efferent ductules. AQP1 is not regulated by androgens or oestrogens in the male reproductive tract of rat, but appears to be constitutively expressed. In contrast, AQP9 is hormonally regulated in the head of the epididymis of the rat. DHT can influence the expression of AQP9 in both efferent ductules and initial segment of the epididymis. The expression of AQP9 in efferent ductule epithelium, but not in the initial segment, is modulated by oestrogen. Thus oestrogen does play a role in the movement of water in the efferent ductule epithelium.

Materials and methods

Animals

The present study was performed in 30 days old (for antioestrogen treatment) or 90 days old (for castration experiment) male Sprague–Dawley rats (Harlan Bioproducts, Indianapolis, IN, U.S.A.). The rats were allowed to adapt to the vivarium conditions for at least 7 days before treatment. They were maintained under constant conditions of light (12 h of light and 12 h of darkness) and temperature (22°C), with free access to water and diet (Teklad chow; Harlan Teklad, Madison, WI, U.S.A.). All experiments and surgical procedures were approved by the University of Illinois Division of Animal Resources and conducted in accordance with the Guide for the care and use of laboratory animals (National Research Council, 1996).

Antioestrogen treatment

Starting at 30 days of age, three rats were treated once a week with subcutaneous injections of ICI 182,780 (Faslodex, provided by AstraZeneca, Macclesfield, U.K.) at a dosage of 10 mg/animal in a volume of 0.2 ml of vehicle (Oliveira et al., 2001, 2002). The control group (three animals) received the same volume of castor oil. The animals were killed on days 7, 45, 73, 100 and 150 after the first treatment.

Surgical procedures

The adult rats (90 days old) were randomly divided into nine groups of three animals each, which were subjected to bilateral ligation, castration and hormone replacement as specified below. Three animals were not submitted to any treatment and served as intact experimental controls. For all other animals, surgical procedures were required. For this purpose, the rats were anaesthetized with intraperitoneal injection of sodium pentobarbital (0.08 ml/100 mg body weight) and the surgery was performed in aseptic conditions. The testis–epididymis was exposed through a mid-line ventral scrotal incision, in preparation for the ligation, castration or sham operation (Oliveira et al., 2004). For sham operation, the testes were exposed, manipulated and then reinserted intact into the scrotum. After surgery, the scrotal incision was closed by suture. Postoperative conditions of the animals were monitored daily.

Hormone replacement

Starting from the same day as the bilateral castration, groups of three animals were given subcutaneous injections once a day for 15 days with 5 mg of testosterone propionate (JT Baker Chemical, Phillipsburg, NJ, U.S.A.), 5 mg of DHT (Sigma, St. Louis, MO, U.S.A.), 75 or 400 μg of 17-β-oestradiol-3-benzoate (Sigma) and combined testosterone propionate (5 mg) + 17-β-oestradiol-3-benzoate (400 μg). Steroids were dissolved in corn oil (vehicle) and injected into a volume of 0.1 ml/day (Oliveira et al., 2004). The castration control group received the same volume of vehicle only.

Immunohistochemistry

Changes in the expression of AQP1 and AQP9 in the efferent ductules were investigated by immunohistochemistry in all experimental rats (3 rats/group). After 15 days of surgery and initiation of the hormone replacement or after each time period of ICI 182,780 treatment, the rats were anaesthetized (intraperitoneal sodium pentobarbital; 0.1 ml/100 g body weight) and perfused intracardially with 10% (v/v) neutral buffered formalin. After fixation, the efferent ductules were dissected from the testis and epididymis, embedded in paraffin, sectioned (5 μm) and mounted on electrostatically charged glass slides.

Tissue sections were dewaxed in xylene, rehydrated through a graded series of ethanol, washed with distilled water and PBS and then blocked for endogenous peroxidase by incubation with 0.6% H_2O_2 in methanol for 30 min. The slides were washed with PBS and then incubated for 30 min in 10% (v/v) normal goat serum. The sections were then incubated for 2 h at room temperature (22°C) with AQP1 or AQP9 polyclonal rabbit anti-rat primary antibody (Alpha Diagnostic International, San Antonio, TX, U.S.A.), diluted 1:1000. For negative controls, primary antibody was substituted for the PBS. After washing with PBS, the sections were exposed for 1 h to a biotinylated

secondary antibody, goat anti-rabbit (Dako, Carpinteria, CA, U.S.A.), used at 1:100 dilution. Labelling was visualized with an avidin–biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min, followed by immersion in 0.05% 3,3′-diaminobenzidine containing 0.01% H_2O_2 in 0.05 M Tris/HCl buffer (pH 7.6). The reaction was monitored microscopically and stopped by immersion in distilled water. Sections were counterstained with Mayer's haematoxylin and then dehydrated in ethanol, cleared in xylene and mounted.

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