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Actions of 5α-reductase inhibitors on the epididymis☆

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Abstract

Testosterone is converted to the more biologically active androgen, dihydrotestosterone (DHT), by steroid 5α-reductase. Two isozymes of 5α-reductase, types 1 and 2, are abundantly expressed in the epididymis. DHT is the androgen found in the nuclei of epididymal cells and is essential for the maturation of spermatozoa. Thus, one approach to block androgen action in the epididymis is to inhibit DHT formation. Several compounds have been reported to inhibit either one or both forms of 5α-reductase in many tissues. The first commercially available inhibitor of 5α-reductase, finasteride, has a predominant effect on the type 2 isozyme, while more recently developed agents, such as dutasteride, PNU157706 and FK143, act as dual inhibitors. We found that the treatment of adult rats with such agents results in pronounced effects on the expression of genes essential to the formation of the optimal luminal microenvironment that is required for proper sperm maturation. Furthermore, drug treatment caused a significant decrease in the percentage of progressively motile and morphologically normal spermatozoa in the cauda epididymides. Mating females to treated males resulted in fewer successful pregnancies and a higher rate of pre-implantation loss. Thus, there may be a role for dual 5α-reductase inhibitors as potential components of a male contraceptive.

Keywords: Dihydrotestosterone; Sperm motility; Sperm morphology; PNU157706; FK143; Male fertility; Rat

1. Epididymal function is dependent on dihydrotestosterone (DHT)

Testosterone acts directly on the androgen receptor in some tissues, such as muscle, needs to be aromatized to estradiol to mediate its action via the estrogen receptor in other tissues, such as some brain nuclei, and in others is converted to the 5α-reduced metabolite dihydrotestosterone (DHT) which binds to the androgen receptor with higher affinity than testosterone (Toth and Zakar, 1982; Blanchard and Robaire, 1997). In the epididymis, as in many other androgen responsive tissues, including the prostate, seminal vesicles, and skin, testosterone is converted to DHT by steroid 5α-reductase (EC 1.3.1.22), the rate-limiting enzyme in the pathway that leads from testosterone to its 5α-reduced metabolites (Fig. 1).

Some of the key findings that establish that androgen action in the epididymis is mediated by DHT are: (1) the active androgen present in epididymal cell nuclei after injection of radiolabelled testosterone is DHT (Tindall et al., 1972); (2) epididymal cells can synthesize 5α-reduced metabolites from testosterone in vitro (Gloyna and Wilson, 1969; Monsalve and Blaquier, 1977; Robaire et al., 1977b); (3) the results of micropuncture experiments confirm that, beyond the efferent ducts, the predominant androgens in epididymal luminal fluid are 5α-reduced metabolites of testosterone (Turner et al., 1984); and (4) the 5α-reduced metabolites of testosterone are more potent than testosterone in maintaining epididymal functions in vitro (Orgebin-Christ et al., 1976).

2. Steroid 5α-reductases in the epididymis

Although numerous studies, starting in the 1960's, established the activity, subcellular localization, and hormonal regulation of 5α-reductase enzyme activity in several tissues, the purification of the enzyme from any tissue, including the epididymis has proven to be elusive. Until 1992, the activity was believed to be due to a single protein; however, in that year a second isozyme was identified. The two isozymes were named according to the sequence in which their mRNAs were discovered. In the mouse, rat, dog, monkey, and man, both isozymes of 5α-reductase have been identified; they have different tissue distributions. Although several theories have been put forward (Mahendroo and Russell, 1999), little solid data exist to allow us to resolve the relative role of each isozyme. The consequences on

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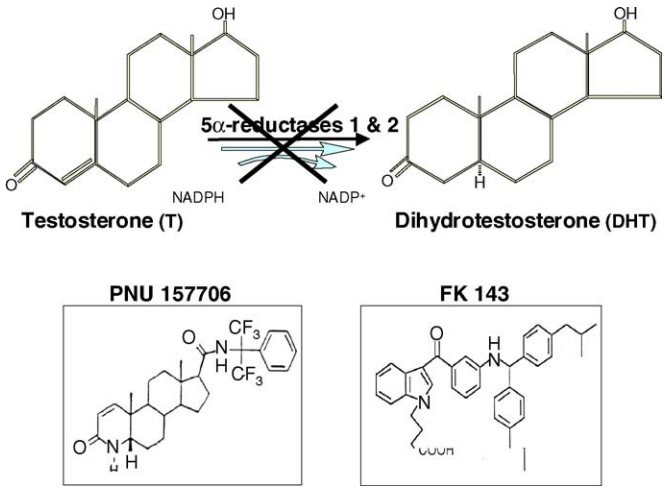

Fig. 1. Schematic representation of the conversion of testosterone to dihydrotestosterone by steroid 5α-reductases and of two compounds that act as dual inhibitors of the isozymes.

the female of a null mutation for 5α-reductase type 1 or the double null mutation for both isozymes have been well described (Mahendroo et al., 1996). Male null mutant mice are fertile; however, a detailed analysis of the consequences of deletion of 5α-reductase isozymes on spermatogenesis, spermatozoa in the epididymis, or on the epididymis itself has not been published. It appears that the mouse null mutation model does not accurately resemble the human condition of 5α-reductase deficiency (Mahendroo et al., 2001).

To understand the regulation of epididymal 5α-reductase isozymes, several approaches have been taken. These include studies designed to understand the genomic regulation (Seenundun and Robaire, 2005) of the two genes and their mRNA expression, as well as studies of the enzymatic activity in the tissue (reviewed in Robaire and Viger, 1995).

2.1. Regulation at the genomic level

The basic gene structure and chromosomal locations of both human and rat 5α-reductase genes have been reported (Jenkins et al., 1991; Labrie et al., 1992; Thigpen et al., 1992; Szpirer et al., 1997); however, until recently remarkably little was known about the 5′ upstream sequences or trans or cis acting factors for these genes. We have found that the proximal 5′ upstream region of type 1 rat 5α-reductase displays all the features of a CpG island and a bidirectional promoter activity (unpublished observations). We recently undertook the cloning and characterization of the 5′ upstream region of 5α-reductase type 2 genes in the rat to gain insight into its transcriptional regulation (Seenundun and Robaire, 2005). Sequential deletion analysis was done to map the 2243-bp cloned 5′ upstream region of this gene. The regulatory elements and the minimal promoter were mapped to the 485-bp region upstream of the start codon. Primer extension and 5′ RACE identified one transcriptional start site at 33-bp upstream of the start codon. Using electrophoretic mobility shift assays, a number of shifts were obtained when epididymal cell line (PC1) nuclear extracts were incubated with probes corresponding to selective regions of the upstream sequence. Supershift and mutational studies confirmed the binding of Sp1, and to a lesser extent Sp3, to the two potential Sp1 binding sites and the preference of these proteins to one binding site over the other. Thus, these results provide a basic framework for the further investigation of the genomic regulation of 5α-reductases in the rat epididymis.

2.2. Regulation at the mRNA level

The 5α-reductase type 1 mRNA is 2.5 kb in length and is most abundantly expressed in the initial segment of the epididymis, with concentrations three to seven-fold higher than in other segments (Viger and Robaire, 1991). Interestingly, the positional gradient in 5α-reductase type 1 mRNA expression in the epididymis is the same as that for nuclear 5α-reductase enzyme activity (Robaire et al., 1981). Endocrine and developmental regulation of the 5α-reductase type 1 mRNA in the epididymis reveal that: (1) orchidectomy results in a decrease in type 1 mRNA levels in all epididymal segments; (2) high dose exogenous testosterone maintains 5α-reductase type 1 mRNA at control steady state concentrations in all regions of the epididymis except the initial segment; (3) unilateral orchidectomy and efferent duct ligation cause a dramatic decrease on type 1 5α-reductase transcripts, selectively in the initial segment of the epididymis; and (4) the type 1 transcript is developmentally regulated (Viger and Robaire, 1991, 1992, 1996). Therefore, the primary regulator of 5α-reductase type 1 mRNA expression in the initial segment of the epididymis is a paracrine/lumicrine factor of testicular origin entering the epididymis via the efferent ducts, while 5α-reductase type 1 mRNA levels in the other epididymal regions appear to be controlled by circulating androgens.

The 5α-reductase type 2 mRNA transcript is 3.6 kb in length, is found in high concentrations in the epididymis (Jenkins et al., 1991; Viger and Robaire, 1996), and has a spatial distribution along the adult rat epididymis which resembles that of type 1 mRNA, except that it is highest in the caput epididymides, with similar concentrations in the initial segment, and does not decline as dramatically as type 1 in the more distal segments (Viger and Robaire, 1996). This observation was the first indication that although the 5α-reductase type 2 mRNA is present in great abundance relative to the type 1 mRNA in the epididymis, its regulation is clearly dissociated from that of the type 1 isoenzyme. While the 5α-reductase type 1 mRNA expression and enzyme activity are characterized by dramatic increases during postnatal development, just before the first appearance of spermatozoa in the epididymis (Viger and Robaire, 1991; Scheer and Robaire, 1980), 5α-reductase type 2 mRNA expression does not show any significant developmental changes; this is the case in all epididymal segments studied (Viger and Robaire, 1996).

2.3. Regulation of epididymal 5α-reductase protein concentrations

To study regulation at the protein level, rabbit polyclonal antibodies were obtained to a 24-mer peptide from the predicted protein sequence for rat 5α-reductase type 1. This peptide has a

high homology to the type 2 isozyme, thus providing an overall pattern of localization for both isozymes. In the adult rat, 5α-reductase protein expression is intensely immunolocalized in discrete lobules of the proximal initial segment of the epididymis (Viger and Robaire, 1994). A sharp decline in staining intensity occurs between the proximal initial segment and its adjacent region, followed by a progressive decrease in intensity beyond that point. In all epididymal regions, 5α-reductase immunoreactive protein is localized specifically in the epithelial principal cells and is uniquely associated with membranous cytoplasmic elements. Although no intranuclear staining is observed, very intense infranuclear staining is noted, specifically in the proximal initial segment. In the proximal caput epididymides, the immunoreactivity for 5α-reductase is restricted to an oval region above the nucleus, whereas in the other epididymal regions, weak staining is observed throughout the cytoplasm (Viger and Robaire, 1994; Thigpen and Russell, 1992). Thus, the intracellular localization of 5α-reductase changes as one moves down the epididymis. Savory et al. (1995) have reported the specific localization of 5α-reductase to the subcellular fraction containing outer nuclear membranes. We hypothesized that the infranuclear-localized form of 5α-reductase may be regulated by a paracrine/lumincrine factor of Sertoli cell origin (Viger and Robaire, 1994). Consistent with this suggestion, we found a remarkable decrease in 5α-reductase immunoreactivity selectively in the initial segment after efferent duct ligation, while immunoreactivity remained unaffected in the more distal segments of the epididymis (Robaire and Viger, 1995).

2.4. Regulation of epididymal 5α-reductase enzyme activity

5α-Reductase enzyme activity occurs in a striking positional gradient in the adult rat epididymis and is found in two subcellular fractions, the nuclear and microsomal fractions (Scheer and Robaire, 1983; Robaire et al., 1981). The activity associated with the nuclear fraction is highest in the initial segment and declines dramatically as one moves distally along the tissue. In fact, enzyme activity in the initial segment of the epididymis is higher than in any other reproductive tissue in the male. The hormonal regulation of epididymal 5α-reductase activity is complex. The enzyme activity found in the nuclear fraction is markedly decreased after bilateral orchidectomy and cannot be maintained by exogenous testosterone (Robaire et al., 1977b). Efferent duct ligation and unilateral orchidectomy both result in a dramatic decrease in epididymal 5α-reductase activity, especially in the proximal portion of the tissue (Robaire et al., 1977b; Robaire, 1979). Thus, we have proposed that nuclear fraction epididymal 5α-reductase activity is regulated in a paracrine/lumicrine manner by a substance directly entering the epididymis via the efferent ducts, and not via the general circulation (Robaire et al., 1981). This paracrine, or lumicrine (Hinton et al., 1998) mode of regulation has since been shown to be operative for a growing number of genes expressed selectively in the initial segment of the epididymis, including immobilin (Riuz-Bravo, 1988), proenkephalin (Garrett et al., 1990), cystatin-related epididymal spermatogenic (CRES) gene (Cornwall et al., 1992), and gamma-glutamyl transpeptidase (GGT) (Hinton et al., 1998). Several lines of evidence indicate that this regulatory factor(s) is of Sertoli cell origin. We have proposed that it may be androgen binding protein (ABP) (Robaire et al., 1981; Viger and Robaire, 1991), based on similarities in developmental profile (Hansson et al., 1975), hormonal control (Danzo et al., 1990), and the observation that ABP can be internalized in a receptor mediated mechanism by principal cells of the initial segment of the epididymis (Felden et al., 1992; Krupenko et al., 1994); however, there is also good evidence that other molecules, such as basic fibroblast growth factor, may serve as regulators of the initial segment of the epididymis (Hinton et al., 1998).

In marked contrast to the nuclear activity, microsomal 5α-reductase activity is found throughout the epididymis and is present at a lower level (Robaire et al., 1981). The developmental profile of epididymal microsomal 5α-reductase activity revealed that it increased coincidentally with serum androgens; this activity may be regulated by circulating androgens (Scheer and Robaire, 1983; Robaire et al., 1981).

3. Inhibitors of steroid 5α-reductases

Over the past 30 years, there has been a progression in the development of 5α-reductase inhibitors that reflects the quest for highly specific inhibitors of one or both isozymes that have minimal or no undesirable effects. As early as 1976, the ability of certain compounds and elements to inhibit (albeit non-specifically) 5α-reductase in the epididymis was demonstrated. Saksena et al. (1976) showed that the compound 3-oxo-4-androstene-17β-carboxylic acid inhibited the conversion of testosterone to DHT in the rat caput and cauda epididymides. Monsalve and Blaquier (1977) showed that the steroids progesterone and epitestosterone, as well as the elements Hg, Zn and Cu, were potent inhibitors of rat epididymal 5α-reductase.

In a series of studies, Rasmusson's group (Rasmusson et al., 1984; Liang et al., 1984) found that 4-aza-steroids were effective inhibitors of 5α-reductase activity in the prostate and a variety of other tissues. The lead compound (diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17β-carboxamide, 4-MA), a competitive inhibitor of the epididymal enzyme (Cooke and Robaire, 1986), ushered in a new era in potent inhibitors of this enzyme activity. The first report to identify an irreversible inhibitor of epididymal 5α-reductase activity described a family of 5,10-secosteroids that act as suicide substrates of the enzyme in vitro (Robaire et al., 1977a). However, these first generation compounds did not fulfill the requirements of selectively and specifically inhibiting 5α-reductase activity with no undesirable side effects. For example, 4-hydroxy-androstenedione also inhibited the aromatization of androgens to estrogens (Motta et al., 1986), while 4-MA also had affinity for the androgen receptor and inhibited 3β-hydroxysteroid dehydrogenase (Liang and Heiss, 1981; Cooke and Robaire, 1986; Perron and Belanger, 1994). In 1992, Zoppi et al. reported the differential effects of 4-MA and its derivatives on epididymal and prostate 5α-reductase activity (Zoppi et al., 1992).

Numerous 4-aza-steroid derivatives of 4-MA were subsequently developed and tested in order to achieve specific

inhibition of 5α-reductase with no or limited antagonism of the androgen receptor (Liang et al., 1984). These efforts led to the development of finasteride. In comparison to all previous compounds, finasteride is unquestionably the most well-studied 5α-reductase inhibitor, at both the basic and clinical research levels, with well over 1000 related publications. The structure and activity of finasteride were initially reported in 1986 by Rasmusson et al. and in 1992 finasteride became the first clinically available 5α-reductase inhibitor (Rasmusson et al., 1986; Gormley et al., 1992). In fact, until very recently, finasteride was the only commercially available 5α-reductase inhibitor (Gormley, 1995). However, the lack of specificity of finasteride for the type 1 isozyme has been proposed as the reason why this drug is only moderately effective in the treatment of benign prostatic hyperplasia (BPH) (Steers, 2001; Bartsch et al., 2002). Efforts to discover more therapeutically effective compounds, i.e. that decrease DHT levels to a greater extent, have led to the development of a novel class of dual 5α-reductase inhibitors (Foley and Kirby, 2003; di Salle et al., 1998; Frye et al., 1998; Hirosumi et al., 1995). Dutasteride (GI198745) is a dual 5α-reductase inhibitor that was selected for clinical development due to its remarkable potency. In early 2003, dutasteride (GI198745) became the first dual 5α-reductase inhibitor available for the treatment of BPH (Roehrborn et al., 2002). In comparison to finasteride, dutasteride and other dual compounds achieve almost total suppression of DHT levels (Foley and Kirby, 2003; di Salle et al., 1998; Roehrborn et al., 2002). Other inhibitors of both types 1 and 2 5α-reductase, such as PNU157706, or FK143, are under development (Fig. 1) (Zaccheo et al., 1998; Hirosumi et al., 1995).

4. Consequences of inhibiting steroid 5α-reductases

The major drive for the development of 5α-reductase inhibitors has been for the treatment of BPH, which is extremely prevalent in older males (>60% of men over 55 years old) (Rosen et al., 2004). More recently, 5α-reductase inhibitors have also been marketed for the treatment of male pattern baldness (Libecco and Bergfeld, 2004); basic and clinical research indicate potential roles for 5α-reductase inhibitors in the treatment of acne (Chen et al., 1996) and the early treatment or prevention of prostate cancer (Zaccheo et al., 1998; Andriole et al., 2004).

The consequences of inhibiting 5α-reductase activity on steroid concentrations, gene expression, epididymal epithelial cell function and sperm maturation have been studied only in a very limited manner. Cohen et al. (1981) showed that 5α-reductase inhibitors (3-oxo-4-androstene-17β-carboxylic acid and its methyl ester) could severely affect the fertilizing ability of spermatozoa from the cauda epididymides, while Turner and Futral (1992) showed that the intraluminal androgen balance was shifted dramatically by 5α-reductase inhibitors. Studies with finasteride showed that long-term treatment with a high dose of the drug resulted in a reversible loss of fertility, but this effect was ascribed to the action of the drug on the secretions of sex accessory tissues, reducing the formation of copulatory plugs, not on the epididymis, although effects on the histology of the epididymis were not reported and embryonic development was not allowed to progress beyond its very early phases (Cukierski et al., 1991; Wise et al., 1991).

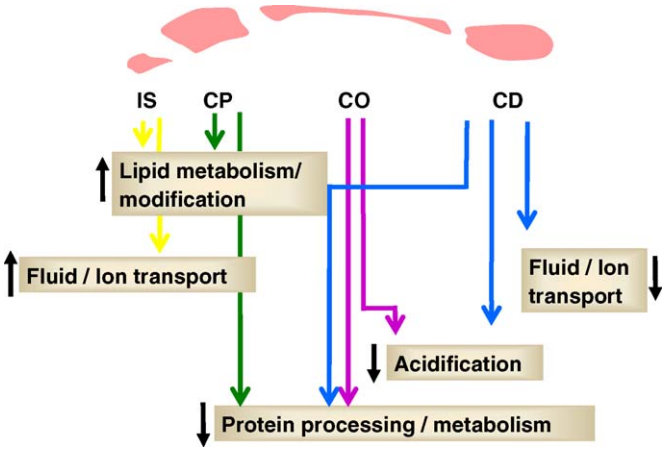

Fig. 2. Response of gene families along the rat epididymis to treatment for 28 days with the dual 5α-reductase inhibitor, PNU157706. IS, initial segment of the epididymis; CA, caput epididymides; CO, corpus epididymides; CD, cauda epididymides. Black arrows indicate the direction of change in gene expression; colored arrows indicate the segments where expression for a given gene family changes. Adapted from data in Henderson et al. (2004).

With the discovery that PNU157706 is a potent inhibitor of both isozymes of 5α-reductase, di Salle et al. (1998) showed, for the first time, that inhibition of 5α-reductases results in a reduction in epididymal weight. An investigation of the consequences of inhibiting both isozymes of 5α-reductase on gene expression profiling along the epididymis was undertaken in order to determine whether reduction of DHT production would have selective actions (Henderson et al., 2004). The effect of treatment with this drug on gene expression was dose-dependent and highly segment-specific; the initial segment responded uniquely in that a similar number of genes increased and decreased in expression, compared to the other segments where the majority of affected genes decreased in expression. Some of the more dramatically affected genes were involved in signal transduction as well as fatty acid and lipid metabolism, regulation of ion and fluid transport, luminal acidification, oxidative defense, and protein processing and degradation. These are essential processes, contributing to the formation of an optimal luminal microenvironment, as required for proper sperm maturation. The schematic in Fig. 2 illustrates the main changes after drug treatment along the epididymis in the gene families affected.

Quantitative RT-PCR was used to analyze the expression profiles of specific signaling genes in the epididymis and to assess their DHT-dependence by using two different dual 5α-reductase inhibitors (PNU157706 and FK143, Fig. 1); the two inhibitors had parallel effects. Specifically, in proximal regions, 5α-reductase 1, androgen receptor and TGF-β1 expression increased after treatment, while in distal segments expression of IGF-1, IGFBP-5, IGFBP-6, and FGF-10 decreased (Henderson and Robaire, submitted for publication). Together, these results provide novel insight into the DHT-dependent mechanisms that control epididymal functions and suggest the identity of potential candidate genes acting either upstream or downstream of DHT to regulate and/or mediate its actions in the epididymis.

The consequences of inhibiting both isoforms of 5α-reductase (types 1 and 2) on epididymal sperm maturation were examined by treating rats with PNU157706 and analyzing fertility as well as several key facets of sperm maturation. The motility of spermatozoa from the cauda epididymides of treated animals showed a significant decrease in both the percentage of motile and progressively motile sperm as well as altered motion parameters. The morphology of cauda epididymal spermatozoa was also adversely affected by the treatment; the most prominent effect was a markedly elevated proportion of sperm that retained their cytoplasmic droplet. Mating control females with treated males resulted in fewer successful pregnancies and a higher rate of pre-implantation loss. Surviving progeny were unaffected. The compromised sperm motility and morphology likely contribute to the subfertility of inhibitor-treated rats (Henderson and Robaire, 2005). These results indicate a role for dual 5α-reductase inhibitors in further studies of epididymal physiology and as a potential component of a male contraceptive.

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