Region-specific expression of androgen and growth factor pathway genes in the rat epididymis and the effects of dual 5 α -reductase inhibition

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Abstract

Dihydrotestosterone (DHT) is the primary androgen acting in the epididymis, the site of sperm maturation. Previously, we showed that the treatment of male rats with PNU157706, an inhibitor that acts on both isoforms of 5 α -reductase to prevent DHT formation, has effects on the expression of genes implicated in processes that create the optimal luminal microenvironment required for sperm maturation, and on sperm maturation itself. However, signaling pathways involved in regulating or mediating DHT actions in the epididymis remain largely unknown. The goals of this study were to determine the expression profiles of potential signaling systems in the epididymis and assess their DHT-dependence using two different dual 5 α -reductase inhibitors. Rats were untreated or gavaged with vehicle, 10 mg/kg per day PNU157706 or 32 mg/kg per day FK143 for 28 days and epididymal gene expression was analyzed. Gene array analysis

revealed analogous effects of FK143 on overall epididymal gene expression when compared with previous PNU157706 studies. Quantitative RT-PCR analysis of the expression of the 5 α -reductase isozymes, androgen receptor, and members of the IGF, FGF, TGF, and VEGF families revealed novel region-specific expression profiles in the epididymis that were differentially affected by 5 α -reductase inhibition; the two inhibitors had parallel effects. Specifically, in proximal regions, 5 α -reductase 1, androgen receptor, and TGF- β 1 expression increased after treatment, while in distal regions expression of IGF-1, IGFBP-5, IGFBP-6, and FGF-10 decreased. These results provide insight into epididymal signaling mechanisms and indicate potential candidates acting either upstream or downstream of DHT to regulate and/or mediate its actions in the epididymis.

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Introduction

The epididymis is a highly specialized tissue within the male excurrent duct system that functions in the transport, maturation, protection, and storage of spermatozoa (Orgebin-Crist 1967, Robaire & Hinton 2002). The four distinct regions of the epididymis that carry out the sperm-related functions are: the initial segment, caput, corpus and cauda epididymidis. Importantly, the acquisition of progressive motility and fertilizing ability (i.e. sperm maturation) requires the successive exposure of spermatozoa to the particular luminal environment created by different epididymal regions (Orgebin-Crist 1967, Robaire & Hinton 2002). The functional segmentation of the epididymis is reflected at the molecular level by complex region-specific gene expression profiles (Jervis & Robaire 2001, Cornwall *et al.* 2002). The unique patterns of gene expression along the duct contribute to the evolving repertoire of components that make up the highly specialized luminal fluid.

The structure and functions of the epididymis are highly dependent on androgens (Blaquier *et al.* 1972, Orgebin-Crist & Tichenor 1973). More specifically, several studies have clearly shown that the primary androgen acting in this tissue is dihydrotestosterone (DHT) (Gloyne & Wilson 1969, Tindall *et al.* 1972, Orgebin-Crist *et al.* 1976, Turner *et al.* 1984). 5 α -Reductase (EC 1.3.1.22) is the enzyme that catalyzes the conversion of testosterone to DHT. Both the isoforms of 5 α -reductase (type-1 and type-2) are present in the epididymis and are differentially expressed along the tubule (Viger & Robaire 1996).

Previously, using gene expression profiling, we have shown that the treatment of adult male rats with the dual 5 α -reductase inhibitor, PNU157706, has a highly region-specific effect on epididymal gene expression (Henderson *et al.* 2004). The affected genes are involved in fatty acid and lipid metabolism, regulation of ion and fluid transport, luminal acidification, oxidative defense, and protein

processing and degradation; these are essential processes that contribute to the formation of the optimal luminal microenvironment (Henderson *et al.* 2004).

Importantly, distinctive region-specific epididymal gene expression changes also occur during aging and in response to other experimental manipulations, such as orchidectomy, caloric restriction, and vitamin E treatments (Jervis & Robaire 2002, 2003, 2004, Ezer & Robaire 2003). These responses clearly demonstrate the differential regulation of this tissue, however, surprisingly, little is known about how this regulation is achieved. While DHT clearly has a prominent role in the epididymis, the involvement of other signaling molecules and pathways, for example, growth factor systems that have been recently characterized in other reproductive tissues (Culig *et al.* 1994, 1995, Gnani *et al.* 1997, Orio *et al.* 2002, Huang *et al.* 2004), remains to be deciphered. Therefore, the main objective of the present study was to examine the effects of 5 α -reductase inhibition on the expression of potential signaling systems in the epididymis, in order to elucidate possible mechanisms of DHT action in this tissue.

In the present study, gene arrays were used to analyze the effects of a second 5 α -reductase inhibitor, FK143, on gene expression; FK143 is a non-steroidal, non-competitive inhibitor of both the isozymes of 5 α -reductase (Hirosumi *et al.* 1995a,b). The cumulative array data from this study and our previous PNU157706 study (Henderson *et al.* 2004), as well as the present literature, were used as a guide in the selection of genes potentially involved in epididymal regulatory mechanisms for further characterization in the epididymis. The genes chosen include, both the isozymes of 5 α -reductase and the androgen receptor, which are directly involved in mediating androgen action in the epididymis, as well as those involved in different growth factor signaling systems. Interestingly, there is recent evidence supporting a role for various growth factors in epididymal function; for example, the overexpression of VEGF in the testis and epididymis of transgenic mice results in infertility (Korpelainen *et al.* 1998). In a different animal model, the GH-deficient dwarf (*dw/dw*) rat, the administration of insulin-like growth factor-I increases the motility and improves the morphology of immature spermatozoa (Vickers *et al.* 1999). Whether an epididymal mechanism contributes completely or in part to the effects of these growth factors in such animal models has not yet been determined, but it is certainly possible that these and other growth factor systems are involved in epididymal regulation.

The steady-state epididymal expression profiles of the selected genes were analyzed using the sensitive technique of quantitative real-time RT-PCR. We then assessed the effects of both 5 α -reductase inhibitors (PNU157706 and FK143) on the epididymal expression of these androgen and growth factor-signaling genes to determine whether they are dependent on DHT, and thus potentially acting as upstream (i.e. the 5 α -reductase isozymes) or downstream regulators of DHT-dependent gene expression and function.

Materials and Methods

Animals

Adult male Sprague–Dawley rats (325–350 g) were obtained from Charles River Canada (St Constant, PQ, Canada), maintained under controlled light (14 h light:10 h darkness) and temperature (22 °C), and provided with food and water *ad libitum*. The caput–corpus epididymides from 90-day-old animals that were previously stored at –80 °C were used for *in vitro* enzyme assays. For *in vivo* studies, adult male rats were left untreated (for determination of steady-state mRNA profiles, $n=5$) or were randomly divided into three treatment groups ($n=5$) and gavaged with 0.5 ml/kg of either the vehicle alone (control group) or 10 mg/kg PNU157706 or 32 mg/kg FK143 (inhibitor treatment groups) suspended in vehicle (0.5% methylcellulose solution (BDH, Montréal, QC, Canada) containing 0.4% Tween 80 (A&C American Chemicals Ltd, Montreal, QC, Canada)) for 28 consecutive days. Doses were selected based on reported effects on rat ventral prostate; the dosing regimen with 10 mg/kg per day PNU157706 or 32 mg/kg per day FK143 has been shown to decrease prostatic dihydrotestosterone levels by >90% (Hirosumi *et al.* 1995a,b, di Salle *et al.* 1998). Epididymides from untreated and treated rats were sectioned into initial segment, caput, corpus, and cauda epididymides and immediately frozen in liquid nitrogen. The tissues were stored at –80 °C until used for RNA extraction. The changes in reproductive tissue weights with treatment were consistent with what has been reported previously for both compounds (Hirosumi *et al.* 1995a,b, di Salle *et al.* 1998, Henderson *et al.* 2004). All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care (McGill Animal care Committee protocol no. 206).

In vitro 5 α -reductase assays

Unlabeled steroids were purchased from Steraloids, Inc., (Newport, RI, USA); (1,2,6,7-³H) testosterone (74.0 Ci/mmol) from Dupont/NEN (Boston, MA, USA); organic solvents from BDH; dimethylglutaric acid (DMG), NADPH, and dimethyl sulfoxide (DMSO) from Sigma and plastic-coated Whatman PE SIL G silica gel chromatography plates from Chromatographic Specialties (Montréal, QC, Canada). FK143 solutions were prepared in DMSO.

Frozen caput–corpus epididymides (only control, untreated tissues were used) were thawed, homogenized (Polytron) in DMG buffer (50 mM DMG, 5% glycerol, NaOH, and 0.5 mM NADPH) pH 6.5, and the nuclear and microsomal fractions were assayed for 5 α -reductase activity as previously described for PNU157706 (Henderson *et al.* 2004). Briefly, kinetic studies of 5 α -reductase activity were done at pH 6.5 with testosterone (nine concentrations ranging from 5 to 375 nM). To determine the effects of FK143 on 5 α -reductase activity, FK143 was

included at concentrations of 0, 3, 15, and 45 nM. A single incubation time of 1 h and an incubation volume 1 ml were used in kinetic studies. To obtain the apparent Michaelis constant ($K_{m(\text{app})}$) and apparent maximum velocity ($V_{\text{max}(\text{app})}$), the Wilkinson method (non-linear least squares analysis of a rectangular hyperbola) was used (Wilkinson 1961). Slope and intercept replots were obtained by the formula, $K_{m(\text{app})}/V_{\text{max}(\text{app})}$ vs $[I]$, and $1/V_{\text{max}(\text{app})}$ vs $[I]$ respectively. Linear regression analysis of the replots was employed to determine the $K_{i(\text{app})}$ values.

RNA extraction

From each sample, the total RNA was extracted using the RNeasy extraction kit with DNase1 treatment (Qiagen) according to the manufacturer's protocol, with the exception that the on-column DNase digestion was extended to 1 h. RNA concentration was assessed by optical density determination at 260 nm (Beckman DU7 spectrophotometer, Montréal, PQ, Canada). In addition to spectrophotometric reading, RNA quality was verified by conventional gel electrophoresis. Each sample consisted of a single epididymal region obtained from individual rats, i.e. no tissues were pooled.

cDNA arrays and hybridization

The effects of FK143 treatment on epididymal gene expression were analyzed with cDNA arrays as described previously (Henderson *et al.* 2004). Briefly, RNA samples were used to probe cDNA arrays (BD Biosciences Atlas Rat 1.2K array) according to the manufacturer's instructions. Four arrays per epididymal region per treatment group (control, FK143) were probed and referred as replicates ($n=4/\text{treatment per region}$). Arrays were exposed to phosphorimager plates (Molecular Dynamics, Sunnyvale, CA, USA) 24 h before scanning with a phosphorimager (Storm, Molecular Dynamics). Analysis of

array images with Atlas Image (Version 2.0, BD Biosciences) was done to quantify the intensity of each cDNA spot, which reflects the relative abundance of RNA in the sample. The raw data for each gene (intensity minus the background) were imported into GeneSpring 4.0.7 (Silicon Genetics, Redwood, CA, USA) for further analysis. For each replicate array in a given treatment group, a gene was considered detected if its intensity was above threshold, which is defined as two times the average background of that individual array. A gene was considered expressed if it was detected in at least three replicates in that group.

To minimize experimental variation and allow comparison of different treatment groups, data were normalized with the standard experiment-to-experiment normalization (GeneSpring). Specifically, the median level of expression on each array was defined as 1 and expression of each gene was normalized relative to 1. This value was averaged for all replicates in a group to generate what is referred to as the relative intensity for a given gene. A transcript was considered differentially expressed if the change in relative intensity between the control and the treatment group was twofold or greater (i.e. 50% decrease or 100% increase). The importance of replication for validation of gene-expression studies has been documented (Lee *et al.* 2000, Herwig *et al.* 2001).

Quantitative real-time RT-PCR

The expression of selected genes (Table 1) was analyzed by quantitative real-time RT-PCR. Analysis of the steady-state expression profiles for the selected genes along the epididymis was done using RNA samples extracted from the epididymides of untreated adult rats ($n=4-5$). Analysis of the effects of 5 α -reductase inhibition on the epididymal expression profiles of selected genes was done using RNA samples extracted from the epididymides of vehicle, PNU157706- and FK143-treated rats ($n=4-5$). As expected, the steady-state (untreated) and vehicle-treated expression profiles for all

Table 1 Details of primers used in quantitative real-time RT-PCR experiments

Gene	Accession no.	Forward primer	Reverse primer	Reference
5 α -R 1	NM_017070	CGTCCTGCTGGCTATGTTTC	GAAGGCCAAGACAAAGGTGA	NA
5 α -R 2	NM_022711	GGACCCTGATCCTGTGCTTA	ACACCACAAAGGAAGGCAAC	NA
AR	NM_012502	CTAGCGCGTGCCTTCCTTTACA	CCCACCTGCGGGAAGCT	Turner <i>et al.</i> (2003)
IGF-1	NM_178866	CACAGGCTATGGCTCCAGCAT	TCTCCAGCCTCCTCAGATCACA	Turner <i>et al.</i> (2003)
IGF-1R	NM_052807	CGCTGTGTGGACCGGATTT	GCTGGCACTCGCCATCGTG	Turner <i>et al.</i> (2003)
IGFBP-5	NM_012817	GTGACCGCAAAGGATTCTACAAGA	GGCAGCTTCATCCCATACTTGTC	Turner <i>et al.</i> (2003)
IGFBP-6	NM_013104	ACCTCGGCTGCCCTATAAGG	TCAGTCTGGAGCTGCTGCAGTAC	Turner <i>et al.</i> (2003)
FGF-10	NM_012951	GCTGCTGTTGCTGCTTCTTGTTG	GCTGCTGTTGCTGCTTCTTGTTG	Turner <i>et al.</i> (2003)
FGFR-2	XM_341940	TTCCCCAGATTACCTGGAGATAGC	TCGTGGTCTTCATTCCGGCAA	Turner <i>et al.</i> (2003)
TGF- β 1	NM_021578	GTTCTTCAATACGTCAGACATTCCG	CATTATCTTGTGTCACAAGAGC	Hanawa <i>et al.</i> (2002)
TGF- β 1R	NM_012775	AATGTTACGCCATGAAATATCCT	TACAACAGGTTCCATTTTCTTCA	Hanawa <i>et al.</i> (2002)
VEGF	NM_031836	CAGCTATTGCCGTCCAATTGA	CCAGGGCTTCATCATTGCA	Gomez <i>et al.</i> (2003)
VEGFR-2	NM_013062	TCAGAGACACTGAGCATGGAA	GTTTTCAGCTCTTCTGAGGCAA	Gomez <i>et al.</i> (2003)
Cyclophilin	NM_008907	ACACGCCATAATGGCACTGG	ATTTGCCATGGACAAGATGCC	Cupp <i>et al.</i> (1999)

NA, not applicable. Accession numbers are for the Genbank database.

the genes examined were indistinguishable. For simplicity, only the vehicle-treated profiles are shown, which are representative of the steady-state expression profiles, discussed in the Results section.

First-strand cDNA (cDNA) was synthesized from 1 μ g total RNA using SuperScript-II reverse transcriptase (Invitrogen-Life Technologies, Carlsbad, CA, USA) and oligo dT_(12–18) primers (Invitrogen-Life Technologies) according to the manufacturer's instructions, with the exception that only half the concentration of DTT was used (Lekanne Deprez *et al.* 2002). The reverse transcription (RT) reactions were then diluted in a ratio of 1:3 and 1 μ l aliquots of the diluted RT samples were used for subsequent quantitative real-time PCR analysis.

Quantitative real-time PCR was carried out on the LightCycler system (Roche) using the Quantitect SYBR green PCR kit (Qiagen) according to the manufacturer's protocol. The gene-specific primer sequences (Table 1) were obtained from the literature or designed using Primer3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/>). Briefly, each PCR contained 1 μ l cDNA (RT sample), 0.5 μ M gene-specific primers, and 10 μ l SYBR green master mix. The PCR cycling conditions were as follows: initial denaturation/enzyme activation for 15 min at 95 °C followed by 40–60 cycles of denaturation at 95 °C for 10 s, annealing at 57–60 °C for 5 s and elongation at 72 °C for 10 s. The production of a single PCR product was confirmed by melting-curve analysis and conventional gel electrophoresis.

The relative quantification of mRNA was done according to the standard curve method (refer to Applied Biosystems User Bulletin no. 2, reviewed in Bustin 2002). The same RT sample was used to assay each target gene and the reference gene cyclophilin in separate (LightCycler) PCR runs. Cyclophilin was chosen as the endogenous control because of its invariant expression throughout the epididymis and lack of response to androgen status (Palladino & Hinton 1994). A stock of standard curve cDNA was made as follows: total RNA was extracted from the four epididymal regions (initial segment, caput, corpus, and cauda epididymides) of an untreated adult rat, then equal amounts of RNA from each region were mixed together to create a standard 1 μ g/ μ l RNA solution. Subsequently, several 1 μ l aliquots of RNA standard were reverse-transcribed into cDNA as described previously and then pooled together to create a standard cDNA stock that was used in all PCR experiments. For each analysis, six serial dilutions of the standard cDNA were run as a calibration curve parallel to the samples. The levels of mRNA for each gene (target and reference genes) were determined by comparing each sample to the appropriate standard curve. All standards and samples were assayed in duplicate. Average values for the target gene RNA concentrations determined from each RT sample preparation were normalized to the average value for cyclophilin RNA concentration determined from the same RT sample preparation.

Statistical analysis

For the steady-state real-time PCR gene expression profiles, differences between epididymal regions were analyzed by ANOVA followed by Tukey's test. To analyze the differences in gene expression between the vehicle (control) and the inhibitor treatment groups, ANOVA followed by Dunnett's test was done. The level of significance was set at $P \leq 0.05$ for all analyses.

Results

Effect of FK143 on epididymal 5 α -reductase activity

The inhibitory effect of FK143 on 5 α -reductase was assessed *in vitro* by examining the effects of FK143 on 5 α -reductase activity of nuclear and microsomal epididymal fractions. The addition of FK143 to incubations at concentrations 0, 3, 15, and 45 nM inhibited epididymal 5 α -reductase as shown in Fig. 1A (nuclear fraction) and B (microsomal fraction). Kinetic analyses demonstrated that FK143 caused the $V_{\max(\text{app})}$ values to decrease (by about 75%) with no consistent effect on the $K_{\text{m}(\text{app})}$ values. Confirmation of these findings was achieved with slope ($K_{\text{m}(\text{app})}/V_{\max(\text{app})}$ vs [I]) and intercept ($1/V_{\max(\text{app})}$ vs [I]) replots, shown in Fig. 1C (nuclear) and D (microsomal); the slopes of the slope replots were negative or almost horizontal, whereas the slopes of the intercept replots were positive. The values for the inhibition constant ($K_{\text{i}(\text{app})}$) for FK143 determined from the slope and intercept replots were 56.6 and 26.5 for the nuclear 5 α -reductase fraction and 44.5 and 53.4 for the microsomal 5 α -reductase fraction respectively. Since, the $K_{\text{i}(\text{app})}$ values from both slope and intercept replots were almost identical, it was clear that the $K_{\text{m}(\text{app})}$ did not influence the $K_{\text{i}(\text{app})}$ values determined; these findings were consistent with FK143 being a non-competitive inhibitor of 5 α -reductase, as has been reported for other tissues (Hirosumi *et al.* 1995a,b).

Effects of FK143 on epididymal gene expression

Comparison of the gene array data from the present FK143 study to our previous study using PNU157706 (Henderson *et al.* 2004) revealed analogous effects of different inhibitors on epididymal gene expression; both the nature of the affected genes and the direction of expression changes (i.e. increased or decreased expression) were very similar. In the present study, the expression of a greater number of genes met our detection analysis criteria (i.e. were detectable above the set background expression threshold) therefore, a greater number of genes were analyzed. Novel changes in gene expression (twofold minimum) that were revealed in the present gene array study are reported in Table 2.

The comparison of the effects of two different inhibitors added greater power to our analyses such that we could identify genes with expression changes that were less than twofold, but consistently affected by treatment with both inhibitors.

Figure 1 Inhibition of rat nuclear (A) and microsomal (B) 5 α -reductase activity *in vitro* by different concentrations of FK143 (FK). For nuclear (C) and microsomal (D) 5 α -reductase, slope replots (squares) were obtained from $K_{m(app)}/V_{max(app)}$ vs (FK) and intercept replots (triangles) from $1/V_{max(app)}$ vs (FK).

Among these genes was 5 α -reductase type-2, which is of particular relevance to the present study as it is one of the isozymes that catalyzes the formation of DHT; the averaged response of this gene to 5 α -reductase inhibitor treatment (determined from the array studies) was 1.4-, 1.6-, and 1.8-fold decreased expression in the initial segment, caput, and corpus epididymides respectively. Also, among these genes were several insulin-like growth factor (IGF) family members. For example, following inhibitor treatment, the expression of IGF-binding protein-5 (IGFBP-5) decreased by an average of 1.1-, 1.4-, and 1.5-fold in the caput, corpus, and cauda epididymides respectively, and IGFBP-6 expression increased by an average of 1.1-fold in the initial segment and decreased by 1.4-fold in the corpus epididymidis. Lastly, the expression of IGF-I and IGF-I receptor (IGF-IR) decreased by an average of 1.4- and 1.6-fold respectively, in the cauda epididymidis following 5 α -reductase inhibitor treatment.

In accordance with the main objective of this study to identify potential signaling systems involved in epididymal regulation, we undertook to characterize the expression of the above-mentioned genes as well as other mediators of androgen action (5 α -reductase type-1, androgen receptor) and other growth factor family members (chosen based on the current literature) using more sensitive technique of quantitative real-time RT-PCR.

Expression of 5 α -reductases and the androgen receptor in the epididymis

We examined the steady-state expression levels of 5 α -reductase types-1 and 2 and the androgen receptor in the epididymis using real-time RT-PCR (Fig. 2, black bars). These three genes have been previously characterized in the epididymidis by northern blot analysis (Viger & Robaire 1995, 1996). The gene expression results obtained using these two techniques were analogous for all the three genes. 5 α -Reductase type-1 was expressed in the initial segment at levels approximately fourfold higher than the other regions of the epididymis. 5 α -Reductase type-2 was also highly expressed in the initial segment, peaked slightly in the caput epididymidis, and decreased in the corpus and cauda epididymides. Androgen receptor gene expression was relatively constant throughout the epididymis.

Dual 5 α -reductase inhibitor treatment had differential effects on the expression of 5 α -reductase types 1 and 2 and the androgen receptor in the epididymis (Fig. 2). Both compounds had similar effects on the different gene-expression profiles. 5 α -Reductase type-1 expression was uniquely affected in the initial segment where it increased maximally with FK143 treatment by nearly twofold. Androgen receptor gene expression also increased following

Table 2 Genes that showed a minimum twofold change in expression uniquely in one epididymal region following FK143 treatment

	Fold-change in expression			
	FK ^a	PNU ^b	Acc. number	Functional classification
Initial segment				
Glucose-regulated 78-kDa protein	↓2.7	↓1.4	M14050	Chaperones and heat shock proteins
Prohibitin	↑2.9	↑1.5	M61219	Intracellular adaptors and receptor-associated proteins
Neural visinin-like Ca ²⁺ -binding protein NVP-3	↑2.8	↑1.1	D13126	Calcium-binding proteins
Cytoplasmic β-actin	↑2.3	↑1.1	V01217	Cytoskeleton and motility proteins
Vasopressin V2 receptor	↑2.3	↑1.2	Z11932	Hormone/G protein-coupled receptors
Dual-specificity MAPKK2	↑2.2	↑1.1	D14592	Intracellular kinase network members
Casein kinase II	↑2.2	↑1.1	L15619	Intracellular kinase network members
Elongation factor 2	↑2.2	↑1.0	K03502	Translation factors
Aminopeptidase B	↑2.1	↑1.1	U61696	Metalloproteinases
Macrophage migration inhibitory factor	↑2.1	↑1.5	U62326	Growth factors, cytokines and chemokines
Cathepsin L	↑2.0	↑1.5	Y00697	Cysteine proteases
Cofilin	↑2.0	↑1.3	X62908	Other cytoskeleton and motility proteins
Caput epididymidis				
Gastric inhibitory polypeptide precursor	↓4.5	↓1.8	L08831	Other extracellular communication proteins
Sodium channel SCN2B	↓5.4	↓1.9	U37026	Voltage-gated ion channels
Potassium channel Kir6.2	↓4.2	↓1.8	X97041	Voltage-gated ion channels
C-type natriuretic peptide precursor	↓4.0	↓1.9	D90219	Hormones
Insulin receptor-related receptor-α	↓3.0	↓1.3	M90661	Intracellular kinase network members
rab12, ras related GTPase	↓2.9	↓1.5	M83676	G Proteins; other trafficking and targeting proteins
Gax, growth-arrest-specific protein	↓2.8	↓1.1	Z17223	Other transcription proteins; telomere-associated proteins
Cytochrome c oxidase, subunit IV	↓2.6	↓1.3	X14209	Energy metabolism
CD2, membrane glycoprotein	↓2.5	↓1.2	X05111	Cell-cell adhesion receptors; cell surface antigens
Vacuolar ATP synth. 16-kDa proteolipid subunit	↓2.3	↓1.4	D10874	ATPase transporters
erbB4, proto-oncogene	↓2.3	↓1.6	U52531	Growth factor/chemokine/protein kinase receptors
Creatine kinase	↓2.1	↓1.2	X59737	Energy/nucleotide metabolism
Apurinic/apyrimidinic endonuclease	↓2.1	↓1.3	D44495	DNA damage repair proteins and ligases
Corpus epididymidis				
Ca ⁺⁺ -dependent phospholipase A2 precursor	↓3.1	↓1.6	U03763	Phospholipases and phosphoinositol kinases
DNA-binding protein inhibitor ID3	↓2.4	↓1.9	D10864	Transcription activators and repressors
Monocarboxylate transporter MCT1	↓2.2	↓1.8	D63834	Symporters and antiporters
Pancreatic secretory trypsin inhibitor I precursor (PSTI-I)	↓2.1	↓1.8	M27882	Protease inhibitors
G1/S-specific cyclin D2	↓2.0	↓1.8	D16308	Cyclins; oncogenes and tumor suppressors
Ral A; GTP-binding protein	↑3.5	↑1.3	L19698	G Proteins
Angiotensin converting enzyme	↑3.1	↑1.1	U03734	Metalloproteinases
Tissue inhibitor of metalloproteinase-2	↑2.9	↑1.3	L31884	Protease inhibitors
Syntaxin 3	↑2.6	↑1.2	L20820	Trafficking and targeting proteins
14-3-3 protein theta	↑2.4	↑1.1	D17614	Kinase activators and inhibitors
GTP-binding protein; G-α-i2	↑2.4	↑1.0	M17528	G Proteins
Cauda epididymidis				
ATPase, transitional endoplasmic reticulum	↓2.6	↓1.4	U11760	Immune system proteins
Proteasome component C8	↓2.3	↓1.9	M58593	Proteasomal proteins
SYNAPTOTAGMIN XI	↓2.2	↓1.4	AF000423	Exocytosis/calcium-binding proteins
Heterogeneous nuclear ribonucleoprotein K	↓2.1	↓1.5	D17711	RNA processing, turnover and transport proteins
Plectin	↑2.6	↑1.3	X59601	Cytoskeleton and motility proteins
Tissue carboxypeptidase inhibitor	↑2.1	↑1.1	U40260	Protease inhibitors

Accession numbers (Acc. numbers) are for the Genbank database. Functional classifications are according to BD Bioscience (Clontech).

^aThe fold-change expression values following FK143 (FK) treatment were obtained in the present study.

^bThe fold-changes expression values following PNU157706 treatment (PNU) were obtained from a previous study (Henderson *et al.* 2004) and are included for comparison purposes.

Figure 2 Quantitative real-time RT-PCR analysis of 5 α -reductase type-1 (5 α R-1), 5 α -reductase type-2 (5 α R-2) and androgen receptor (AR) mRNAs in the initial segment (IS), caput (CA), corpus (CO), and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and represented as mean $\pm$ S.E.M. RNA extracted from epididymides of vehicle (black bars), PNU15776- (striped bars), and FK143- (white bars) treated rats ($n=5$ /group) was used to analyze the effect of 5 α -reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle- and inhibitor-treated groups ($P<0.05$).

treatment uniquely in the caput epididymidis. In contrast, the expression of 5 α -reductase type-2 remained essentially unaffected by treatment except for an approximate decrease of 66% in the corpus epididymidis. The corpus epididymidis was also the region where the greatest decrease in 5 α -reductase type-2 expression was detected using gene array technology.

Expression of IGF family members in the epididymis

We examined the steady-state expression of four IGF family members along the epididymis (Fig. 3, black bars). The expression of IGF-I was significantly lower in the caput epididymidis compared with the other regions, where it was expressed at similar levels. The expression of IGF-IR escalated longitudinally, increasing nearly threefold between

Figure 3 Quantitative real-time RT-PCR analysis of insulin-like growth factor-I (IGF-I), insulin-like growth factor-I receptor (IGF-IR), insulin-like growth factor-binding protein-5 (IGFBP-5), and -6 (IGFBP-6) mRNAs in the initial segment (IS), caput (CA), corpus (CO), and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and are represented as mean $\pm$ S.E.M. RNA extracted from epididymides of vehicle (black bars), PNU15776- (striped bars), and FK143- (white bars) treated rats ($n=5$ /group) was used to analyze the effect of 5 α -reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle- and inhibitor-treated groups ($P<0.05$).

the initial segment and the cauda epididymidis. The expression of IGFBP-5 was also highest in the cauda epididymidis, being on average threefold higher than the other segments. The relative expression values for IGFBP-6 suggest that it is more highly expressed in the epididymis compared with the other IGF family members. However, direct comparisons between different genes cannot be made conclusively, since different target gene primer pairs may not exhibit the same amplification efficiency in PCR experiments. Lastly, the expression profile of IGFBP-6 in the epididymis was very similar to that of IGF-I, such that it was significantly lower in the caput epididymidis compared with the remaining segments.

The effects of dual 5 α -reductase inhibition on the expression profiles of the four IGF family members are shown in Fig. 3. Again, treatment with two different dual inhibitors had similar effects on the expression profiles of all the IGF family members examined with FK143 having a more pronounced effect. Interestingly, the effects of 5 α -reductase inhibitor treatment were gene- and region-specific. The expression of all the four genes remained unaffected by treatment in the initial segment. In the more distal regions of the epididymis, treatment with either inhibitor caused a general decrease in the expression of most IGF family members examined. More specifically, the expression of IGF-I and IGFBP-5 was the most affected by treatment, maximally decreasing by 60 and 84% in the cauda epididymidis, respectively. Decreased expression was also observed for IGFBP-6, reaching significance in the corpus epididymidis. These changes in gene expression reflect what was found using gene array technology in the present study.

Expression of FGF family members in the epididymis

The steady-state expression profiles for the ligand, fibroblast growth factor-10 (FGF-10), and the receptor, fibroblast growth factor receptor-2 (FGFR-2) were examined in the epididymis (Fig. 4, black bars). The steady-state expression of FGF-10 was significantly lower in the caput epididymidis compared with all other regions. The steady-state expression of FGFR-2 was significantly higher in the cauda epididymidis compared with more proximal regions. The relative expression values for FGFR-2 suggest that it is quite highly expressed in the epididymis compared with the other genes analyzed.

The effects of PNU157706 and FK143 treatment on the epididymal expression of FGF-10 and FGFR-2 expression were analyzed (Fig. 4). FGF-10 gene expression was significantly affected by both the inhibitor treatments; it decreased maximally with FK143 treatment in the corpus and cauda epididymides approximately by 45 and 31% respectively. Inhibitor treatments did not result in a significant decrease in FGFR-2 expression.

Expression of TGF family members in the epididymis

The steady-state expression profiles for the ligand, transforming growth factor- β 1 (TGF- β 1), and the receptor, transforming

Figure 4 Quantitative real-time RT-PCR analysis of fibroblast growth factor-10 (FGF-10) and fibroblast growth factor receptor-2 (FGFR-2) mRNAs in the initial segment (IS), caput (CA), corpus (CO), and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and are represented as mean $\pm$ S.E.M. RNA extracted from epididymides of vehicle (black bars), PNU15776- (striped bars) and FK143- (white bars) treated rats ($n=5$ /group) was used to analyze the effect of 5 α -reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle- and inhibitor-treated groups ($P<0.05$).

growth factor- β 1 receptor (TGF- β 1R), were examined in the epididymis (Fig. 5, black bars). The expression of TGF- β 1 was relatively constant in the initial segment, caput, and corpus epididymides, and peaked in the cauda epididymidis nearly to double the levels in the proximal regions. In contrast, TGF- β 1R was expressed in the initial segment and corpus epididymidis at approximately twice the levels in the caput and cauda epididymides. The relative expression values for TGF- β 1R were the highest of all the genes examined.

The effects of dual 5 α -reductase inhibitor treatment on epididymal TGF- β 1 and TGF- β 1R expression were analyzed (Fig. 5). The only significant effects of 5 α -reductase inhibitor treatment were on the expression of TGF- β 1 in the caput epididymidis, which nearly doubled following treatment, and TGF- β 1R in the cauda epididymidis, which decreased.

Expression of VEGF family members in the epididymis

The steady-state expression profiles for the ligand, vascular endothelial growth factor (VEGF), and the receptor, vascular endothelial growth factor receptor-2 (VEGFR-2), were examined in the epididymis (Fig. 6, black bars). Both the

Figure 5 Quantitative real-time RT-PCR analysis of transforming growth factor- β 1 (TGF- β 1) and transforming growth factor- β 1 receptor (TGF- β 1R) mRNAs in the initial segment (IS), caput (CA), corpus (CO), and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and are represented as mean $\pm$ S.E.M. RNA extracted from epididymides of vehicle (black bars), PNU15776- (striped bars) and FK143- (white bars) treated rats ($n=5$ /group) was used to analyze the effect of 5 α -reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle- and inhibitor-treated groups ($P<0.05$).

genes were most highly expressed in the initial segment. VEGF expression was lowest in the caput epididymidis and increased slightly towards the cauda epididymidis. VEGFR-2 was expressed in the caput, corpus, and cauda epididymides at approximately 63–74% of the initial segment expression level.

The effects of dual 5 α -reductase inhibitor treatment on epididymal VEGF and VEGFR-2 expression were analyzed (Fig. 6). In contrast to all the other growth factors analyzed, there were no significant effects of treatment with either inhibitor in any region of the epididymis.

Discussion

Previously, we demonstrated that the dual 5 α -reductase inhibitor PNU157706 inhibited epididymal 5 α -reductase activity *in vitro* and had extensive effects on epididymal gene expression *in vivo* (Henderson *et al.* 2004). In the present study, we confirmed that a second compound, FK143, is a non-competitive inhibitor of nuclear and microsomal epididymal

Figure 6 Quantitative real-time RT-PCR analysis of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor-2 (VEGFR-2) mRNAs in the initial segment (IS), caput (CA), corpus (CO), and cauda (CD) epididymides. All gene expression values are expressed relative to the levels of cyclophilin mRNA and are represented as mean $\pm$ S.E.M. RNA extracted from epididymides of vehicle (black bars), PNU15776- (striped bars) and FK143- (white bars) treated rats ($n=5$ /group) was used to analyze the effect of 5 α -reductase inhibitor treatment on mRNA expression profiles. Asterisks (*) indicate significant differences between the vehicle- and inhibitor-treated groups ($P<0.05$).

5 α -reductase activity *in vitro*. Furthermore, it was found that treatment with FK143 elicited changes in epididymal gene expression that were analogous to those observed with PNU157706, as evidenced by gene array studies. These corroborating findings clearly indicate that the observed changes in gene expression are due to the common effect of both the compounds on the inhibition of DHT formation, as opposed to a mechanism different than 5 α -reductase inhibition.

Recently, we showed that the treatment of male rats with PNU157706 affects epididymal sperm maturation as evidenced by changes in epididymal sperm morphology, motility, and fertilizing ability (Henderson & Robaire 2005). It is highly likely that the effects of dual 5 α -reductase inhibition on epididymal sperm maturation are linked to the effects of inhibitor treatment on the expression of genes that can be functionally categorized according to important epididymal epithelial processes (i.e. processes that contribute to the formation of the epididymal luminal environment). What remains unclear is the identity of region-specific signaling/regulatory molecules and pathways involved in mediating the upstream and/or downstream effects of DHT on epididymal gene expression and function.

In the present study, we focused our gene expression analyses specifically on potential signaling molecules in the epididymis, including both 5 α -reductase isozymes and the androgen receptor, as well as several components of different growth factor systems. Moreover, we determined whether the expression of these genes was dependent on DHT by assessing the effects of dual 5 α -reductase inhibition. For the most part, these genes have not been extensively characterized in the epididymis with the exception of the 5 α -reductase isozymes and the androgen receptor. Thus, this is the first study demonstrating the differential distribution and regulation of many of these genes.

Expression of 5 α -reductases and the androgen receptor in the epididymis

The expression of both 5 α -reductase isozymes has been previously characterized in the epididymis (Viger & Robaire 1991, 1994, 1996). Moreover, the region-specific epididymal expression profiles of these genes show different patterns during development and aging, and respond differently to efferent duct ligation and bilateral orchidectomy (Viger & Robaire 1991, 1992, 1994, 1995, 1996). However, this is the first study to employ 5 α -reductase inhibitors to specifically examine the DHT regulation of the expression of these genes in the epididymis. Our results are consistent with the differential regulation of the 5 α -reductase isozymes in this tissue. Type-1 5 α -reductase expression was specifically increased in the initial segment following inhibitor treatment, suggesting a negative-feedback mechanism of regulation by DHT in this region. In contrast, the expression of 5 α -reductase type-2 was affected only in a limited manner by treatment in the corpus epididymidis. Interestingly, androgen status has been shown to regulate the expression of both 5 α -reductase isozymes differently in different tissues. For example, in the rat brain, type-1 5 α -reductase expression increases following castration, and androgen replacement with DHT reverses this increase to a larger extent than testosterone replacement (Torres & Ortega 2003a). In contrast, 5 α -reductase type-2 expression falls in the rat prefrontal cortex after castration and increases dramatically with testosterone replacement compared with DHT replacement (Torres & Ortega 2003a). In fact, the exogenous administration of testosterone, and to a lesser extent DHT, increased type-2 5 α -reductase expression even in intact animals (Torres & Ortega 2003a). Testosterone has also been shown to control the expression of 5 α -reductase type-2 in the rat prostate, while both testosterone and DHT positively regulate 5 α -reductase type-1 expression in the rat liver (Torres & Ortega 2003b, Torres *et al.* 2003). The differential regulation of both 5 α -reductase isozymes suggests that they play physiologically distinct roles in different tissues. Further studies are needed to fully characterize these distinct roles.

As expected, the present real-time PCR expression results were analogous to what has been previously described for the androgen receptor in the epididymis; androgen receptor gene

expression is relatively constant throughout the tissue with levels slightly higher in the caput epididymidis compared with those in the cauda epididymidis (Pujol & Bayard 1979, Tezon & Blaquier 1983, Viger & Robaire 1995). Studies have also shown that circulating androgens regulate the expression of the androgen receptor in the epididymis (Pujol & Bayard 1979, Tezon & Blaquier 1983); however, as with most of the genes previously examined in the epididymis, no differentiation was made between total androgens (i.e. testosterone plus DHT) and DHT alone. Our results suggest that androgen receptor expression is regulated by DHT specifically in the caput epididymidis.

Growth factor systems in the epididymis

Studies using prostate cell lines have provided evidence to support androgen receptor cross-talk with growth factor signaling pathways, resulting in both enhanced androgen responses and receptor ligand-independent activation and modulation of androgen receptor transactivation (Culig *et al.* 1994, 1995, Orio *et al.* 2002, Huang *et al.* 2004). Thus, it seems plausible that similar interactions occur in other reproductive tissues, including the epididymis; however, this remains to be examined. The characterization of recently established epididymal cell lines should facilitate further investigation into the interactions between androgen and growth factor signaling pathways in the epididymis (Kirchhoff *et al.* 2004, Tabuchi *et al.* 2005).

A physiological role for IGF-I in the regulation of epididymal functions was hypothesized over 10 years ago due to the varied immunohistochemical localization of IGF-I protein in the rat epididymis (Leheup & Grignon 1993). IGF-IR protein and mRNA have also been localized to the epididymis (Antich *et al.* 1995, Baker *et al.* 1996). Adult mice with a homozygous null mutation of the IGF-I gene are infertile dwarfs with reduced testosterone production, and consequently reduced spermatogenesis and reproductive organ size (Baker *et al.* 1996). Interestingly, the epididymal phenotype of IGF-I null mice is more severe in the distal regions of the epididymis with greater reductions in weight and decreased coiling. In the present study, the expression of IGF-I and IGF-IR was found to be highest in the distal epididymal regions. These results are consistent with a role for IGF signaling in the distal regions of the epididymis (corpus and cauda epididymides) and this conclusion is corroborated by results from the present study demonstrating the unique effects of 5 α -reductase inhibitor treatment on the expression of IGF-I and possibly IGF-IR, predominantly in the corpus and cauda epididymides.

The IGF network also includes numerous IGF-binding proteins that modulate the bioavailability and cellular functions of IGFs (Jones & Clemmons 1995). We analyzed the epididymal expression of two high-affinity binding proteins, IGFBP-5, and IGFBP-6. Again, these genes were more highly expressed in the distal regions of the epididymis and mainly affected by 5 α -reductase inhibitor treatment in these regions. IGFBP-5 expression was particularly affected by treatment, decreasing dramatically in the cauda epididymidis.

Various FGF receptors have been characterized exclusively in principal cells of the initial segment of the rat epididymis (Kirby *et al.* 2003). Our results also indicate a role for FGF signaling in the distal part of the epididymis, since the highest expression levels for FGF-10 and its receptor FGFR-2 were in the corpus and cauda epididymides. Furthermore, the effects of 5 α -reductase inhibitor treatment were most pronounced in these regions.

Differential expression profiles for TGF- β isoforms (TGF- β 1, TGF- β 2, and TGF- β 3) have previously been characterized in the rat epididymis by northern blot (Desai *et al.* 1998, Desai & Kondaiah 2000). In these studies, substantial TGF- β 1 expression was observed compared with other isoforms; TGF- β 1 expression was upregulated in the caput, corpus, and cauda epididymides following total androgen ablation by orchidectomy. In the present study, we also observed the upregulation of TGF- β 1 expression most predominantly in the caput epididymidis with no changes in the distal regions. Taken together, these data suggest that TGF- β 1 is uniquely regulated by different androgens in the epididymis; our results indicate that there is a DHT-dependent repression of TGF- β 1 in the proximal epididymis, while castration-induced increases in TGF- β 1 expression in the corpus and cauda epididymides, indicating a specific role for total androgens or testosterone in these regions. Roles for the TGF- β family have also been implicated in other DHT-dependent reproductive tissues, such as the seminal vesicle and ventral prostate (Tanji *et al.* 1994, Timme *et al.* 1994). TGF- β 1 in particular was also shown to be repressed by androgens in the regressing rat ventral prostate (Kyprianou & Isaacs 1989), similar to what was observed in the epididymis, further supporting a role for androgens in regulating the expression of this gene.

To the best of our knowledge, this is the first study demonstrating the differential distribution of any TGF- β receptor mRNA in the epididymis. The presence of both the ligand and the receptor strongly supports the TGF- β system in the epididymis; however, DHT only appears to regulate this system at the level of the ligand.

Vascular endothelial growth factor (VEGF) protein was shown to be localized in the rat epididymal epithelium in a region- and cell-specific pattern (Zhang *et al.* 2004); for example, clear cells in the caput, corpus, and cauda epididymides were immunoreactive for VEGF. In humans, VEGF protein has been localized to peritubular and basal cells of the epididymal duct and VEGFR-2 protein to vascular endothelial cells of the epididymis (Ergun *et al.* 1998). Interestingly, the overexpression of VEGF in the testis and epididymis of transgenic mice causes infertility (Korpelainen *et al.* 1998). These findings suggest a role for VEGF in the epididymis, likely as a paracrine regulator of the epididymal vasculature as opposed to the epididymal epithelium. In the present study, we confirmed the differential distribution of both the VEGF and VEGFR-2 mRNAs in the rat epididymis. Interestingly, unlike the other growth factor families examined, 5 α -reductase inhibitor treatment had no

significant effect on the expression of VEGF or VEGFR-2, reflecting a lack of DHT regulation of this growth factor system in the epididymis. This may be due to the predominant vascular localization as opposed to epithelial localization of this system in the epididymis, which is unique from other growth factor signaling systems.

It is of interest to note that the testis has been shown to produce and secrete many different growth factors, including components of the IGF, TGF, EGF, FGF, PDGF, and NGF systems (reviewed in Gnessi *et al.* 1997). It has been well established that the epididymis, in particular the initial segment, is dependent on the testicular input of factors, other than androgens, directly to the epididymal lumen (Robaire *et al.* 1981, Nicander *et al.* 1983, Turner & Riley 1999); depriving the epididymis of these testicular factors (i.e. via efferent duct ligation) has well-documented effects on gene expression in the initial segment (reviewed in Hinton *et al.* 1998). This type of paracrine regulatory mechanism has been coined 'lumicrine' regulation, since it occurs via a ductal system (Hinton *et al.* 1998). Thus, it is plausible that growth factors originating in the testis can act as 'lumicrine' regulators of the epididymis by acting on their corresponding receptors present in the epididymal epithelium. Similarly, it is also likely that the growth factors secreted in one region of the epididymis can act on the receptors located in the same or more distal regions of the epididymis representing autocrine or paracrine regulatory mechanisms.

In summary, the collective findings of the present study suggest that sex steroid and growth factor signaling become dysregulated following 5 α -reductase inhibition, leading to altered epididymal gene expression and function. Furthermore, the results of this study support the involvement of differential signaling mechanisms in regulating and/or mediating the actions of androgens (predominantly DHT) in the different regions of the epididymis. This regulatory diversity is likely to be important in controlling region-specific sperm-related functions.

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