Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway

Saveria Aquila1,2, Emilia Middea1,2, Stefania Catalano1, Stefania Marsico1,2, Marilena Lanzino1,2, Ivan Casaburi1, Ines Barone1,2, Rosalinda Bruno1, Silvia Zupo1,2 and Sebastiano Ando1,2,3

BACKGROUND: Results from mice lacking the androgen receptor (AR) showed that it is critical for the proper development and function of the testes. The aim of this study was to investigate whether a functional AR is present in human sperm. METHODS: The expression of AR and its effects on sperm were evaluated by RT-PCR, Western Blot, Immunocytochemistry, PI3Kinase and DNA laddering assays. RESULTS: We showed in human sperm that AR is located at the head region. Dihydrotestosterone (DHT), in a dose-dependent manner, leads to the rapid phosphorylation of the AR on tyrosine, serine and threonine residues and this effect was reduced by the AR antagonist hydroxyflutamide (OH-Flut). The effects of AR were evaluated on the phosphoinositide-3 kinase/protein kinase B (PI3K/AKT) pathway. Specifically, 0.1 and 1 nM DHT stimulated PI3K activity, whereas 10 nM DHT decreased PI3K activity and levels of p-AKT S473 and p-AKT T308, p-BCL2, and enhanced phosphatase and tensin homologue (PTEN) phosphorylation. In addition, 10 nM DHT was able to induce the cleavage of caspases 8, 9 and 3 and cause DNA laddering, and these effects were reversed either by casodex or OHFlut. By using wortmannin, a specific PI3K inhibitor, the cleavage of caspase 3 was reproduced, confirming that in sperm the PI3K/AKT pathway is involved in caspase activation. CONCLUSIONS: Human sperm express a functional AR that have the ability to modulate the PI3K/AKT pathway, on the basis of androgen concentration.

Keywords: androgen receptor; androgens; human sperm; male reproduction; PI3K/AKT

Introduction

A functional androgen receptor (AR) is required for male embryonic sexual differentiation, pubertal development and regulation of spermatogenesis in mammals. The role of AR during spermatogenesis has been the subject of intense interest for many years (Collins et al., 2003). Several findings have shown that AR function is required for the completion of meiosis and the transition of spermatocytes to haploid round spermatids (De Gendt et al., 2004). Studies of androgen withdrawal and disruption of AR activity, either by surgical, chemical or genetic means, have demonstrated that spermatogenesis rarely proceeds beyond meiosis. In all of these model systems, very few round and even fewer elongated spermatids are observed, as clearly demonstrated in a previous study (Yeh et al., 2002). However, the mechanisms by which androgens regulate male fertility are not fully understood and the sites of androgen action within the male reproductive system are not yet resolved.

Whereas few studies have raised the intriguing possibility that some germ cells may exhibit immunoreactive AR (Kimura et al., 1993; Vornberger et al., 1994), other reports point to Sertoli cells or Leydig cells or peritubular/myoid cells as the exclusive androgen target cells in the testis (Ruizeveld de Winter et al., 1991; Iwamura et al., 1994; Goyal et al., 1996; Suarez-Quian et al., 1999). Recently, the presence of the AR in human sperm was demonstrated by western blot and by immunofluorescence assay (Solakidi et al., 2005).

It is generally accepted that androgens bind to intracellular ARs resulting in mRNA and protein synthesis (McPhaul and Young, 2001). Nevertheless, rapid responses to androgens have been observed in different tissues, that cannot be explained by involvement of mRNA and protein synthesis (Peterziel et al., 1999; Castoria et al., 2004). These rapid, nongenomic effects are also seen for other steroid hormones (Cato et al., 2002) and their importance as a complementary route for cell regulation has recently become evident. Different nuclear receptors (Calogero et al., 2000; Aquila et al., 2004) have been found to be present in human spermatozoa, regulating cellular processes through nongenomic mechanisms. This may
represent an exclusive modality of action in spermatozoa since they are apparently transcriptionally inactive cells.

In addition to stimulating cell growth, androgens and/or AR play important roles in the promotion of cell apoptosis (Heisler et al., 1997; Olsen et al., 1998; Shetty et al., 2002; King et al., 2006). The term apoptosis defines programmed cell death, which is executed by the activation of caspases, a family of cytoplasmic cysteine proteases (Cohen, 1997) through two major pathways: the intrinsic and the extrinsic. The intrinsic pathway involves the cell sensing stress that triggers mitochondria-dependent processes, resulting in cytochrome c release and activation of caspase 9 (Olson and Kornbluth, 2001). The extrinsic pathway involves the final cleavage of caspase 8 (Schulz-Osthoff et al., 1998). Both caspases 8 and 9 can be directly regulated through protein phosphorylation from protein kinase B (AKT) (Cardone et al., 1998). AKT can be directly regulated through protein phosphorylation of multiple andrological pathologies such as impaired spermatogenesis, decreased sperm motility, increased levels of sperm DNA fragmentation, testicular torsion, varicocele and immunological infertility (Said et al., 2004). Further studies are needed to evaluate the full significance of caspases activation in spermatozoa. A direct link between AR and sperm survival has not been investigated previously.

In the present study, we have demonstrated the presence of a functional AR in sperm. It emerges from our data that low androgen concentrations stimulate PI3K activity, which is inhibited at higher levels. Additionally, in the latter circumstance increases in PTEN phosphorylation and cleavages of caspases 8, 9 and 3 were evident.

Materials and Methods

Chemicals

PMN Cell Isolation Medium was from BIOSPA (Milan, Italy). Total RNA Isolation System kit, enzymes, buffers, nucleotides 100 bp ladder used for RT-PCR were purchased from Promega (Milan, Italy). Moloney Murine Leukemia Virus (M-MLV) was from Gibco BRL. Life Technologies Italia (Milan, Italy). Oligonucleotide primers and TA Cloning kit were made by Invitrogen (Milan, Italy). Gel band purification kit was from Amersham Pharmacia Biotech (Buckinghamshire, UK). DMEM-F12 medium, BSA protein standard, laemnll sample buffer, prestrained molecular weight markers, percoll (colloidal PVP coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethyl sulfoxide, anti-rabbit IgG flourescein isothiocyanate (FITC) conjugated, Earle’s balanced salt solution, Hoechst 33342, steroids and all other chemicals were purchased from Sigma Chemical (Milan, Italy). RPMI 1640 medium was from Life Technologies, Inc. (Gaithersburg, MD) and DMEM (PRF-SFM) was from Eurobio (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100 and Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). ECL Plus western blotting detection system, Hybond ECL TM, [γ-32P]ATP and HEPES sodium salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Goat polyclonal actin antibody (1–19), monoclonal mouse anti-AR (AR 441) and anti-PIK3R1 antibodies, monoclonal anti-p-tyrrosine (PY99), normal mouse serum, peroxidase-coupled anti-rabbit and anti-goat and protein A/G-agarose plus were from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal mouse anti-p-SRC tyrosine kinase was from Oncogene (Milan, Italy). Polyclonal rabbit anti-p-serine, anti-p-threonine, anti-p- AKT2/AKT3 S473, anti-p-AKT1/AKT2/AKT3 T308, anti-p-BCL2, anti-p-PTEN, anti-caspases (8, 9 and 3) antibodies were from Cell Signaling (Milan, Italy). PY20 (Transduction Laboratories, Lexington, UK), anti-phospho-serine and anti-phospho-threonine Abs were from Zymed Laboratories (San Francisco, CA). Casodex (Cax) was from Astra Zeneca (Milan, Italy) and hydroxy-flutamide (OH-Flut) was from Schering (Milan, Italy). The specific caspases inhibitor Z-VAD-FMK (ZVF) was from R&D Systems (Milan, Italy). PCR 2.1 vector was from Promega (Milan, Italy). The specific caspases inhibitor Z-VAD-FMK (ZVF) was from R&D Systems (Milan, Italy).

Semen samples and spermatozoa preparations

Semen specimens from normozoospermic men were obtained after three days of sexual abstinence. The samples were ejaculated into sterile containers and left for at least 30 min in order to completely liquefy before being processed. Sperm from ejaculates with normal parameters of semen volume, sperm count, motility, vitality and morphology, according to the WHO Laboratory Manual (World Health Organization, 1999), were included in this study. In each experiment, three normal samples were pooled. Spermatozoa preparation was...
performed as previously described (Aquila et al., 2002). An independent observer, who observed several fields for each slide, inspected the cells. Percoll-purified sperm were washed with unsupplemented Earle’s medium and were incubated in the same medium (uncapacitating medium) for 30 min at 37°C and 5% CO₂, without (control) or with treatments (experimental). Some samples were incubated in capacitating medium (CAP) (Earle’s balanced salt solution medium supplemented with 600 mg BSA /100 ml and 200 mg sodium bicarbonate/100 ml). When the cells were treated with the inhibitors Cax, OH-Flut and ZVF, a pretreatment of 15 min was performed. The study was approved by the local medical Ethical Committees and all participants gave their informed consent.

**LNCaP cells culture**

LNCaP, human prostate adenocarcinoma cells, were grown in RPMI 1640 medium supplemented by 5% heat inactivated fetal bovine serum and Penicillin–Streptomycin 1%. Cultures were maintained at 37°C, 5% CO₂ and 100% humidity. In the experiments, steroids and growth factors were withdrawn from cells, and they were grown in phenol red-free DMEM containing 0.5% BSA and 2 mM l-glutamine for 24 h. LNCaP were treated for 30 min at 37°C and 5% CO₂, without (control) or with the indicated treatments (experimental).

**RNA isolation and RT–PCR**

Total RNA was isolated from human ejaculated spermatozoa and purified as previously described (Aquila et al., 2002). Contamination by leucocytes and germ cells in the sperm preparations was assessed by amplifying PTPRC and KIT transcripts, respectively. The applied PCR primers and the expected lengths of the resulting PCR products are shown in Table 1. AR primers were chosen to amplify the region of the DNA binding domain plus the hinge region of the receptor. PCR was carried for 40 cycles using the following parameters: 95°C/1 min, 55°C/1 min, 72°C/2 min for AR; 95°C/1 min, 52°C/1 min, 72°C/2 min for KIT; 95°C/1 min, 55°C/1 min, 72°C/2 min for PTPRC. For all PCR amplifications, negative (reverse transcription-PCR performed without M-MLV reverse transcriptase) and positive controls (LNCaP for AR, human testis for KIT and human leucocytes for PTPRC) were included.

**Table 1: Oligonucleotide sequences used for RT–PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’–3’)</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>5’-TGCCCATTGACTATTACTTCTCC-3’</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>5’-TGTCAGCAGCAACTACACC-3’</td>
<td></td>
</tr>
<tr>
<td>KIT</td>
<td>5’-AGTACATTGAGCATGAAACCTCG-3’</td>
<td>780</td>
</tr>
<tr>
<td></td>
<td>5’-GATTCCTGTCAGACATCGTCG-3’</td>
<td></td>
</tr>
<tr>
<td>PTPRC</td>
<td>5’-CAATAGCTACTTACCTCAAGAGCC-3’</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>5’-ATGCTTTATCGAGCAGTATAGT-3’</td>
<td></td>
</tr>
</tbody>
</table>

**Gel extraction and DNA sequence analysis**

The AR RT–PCR product was extracted from the agarose gel by using a gel band purification kit, and the purified DNAs were subcloned into PCR 2.1 vector and then sequenced.

**Western blot analysis of sperm proteins**

Sperm samples washed twice with Earle’s balanced salt solution (uncapacitating medium), were incubated for 30 min without or with the treatments indicated in the figures. During western blot analysis, sperm samples were processed as previously described (Aquila et al., 2002). The negative control was performed using a sperm lysate that was immunodepleted of AR (i.e. preincubation of lysates with anti-AR antibody for 1 h at room temperature and immunoprecipitated with protein A/G-agarose) (Aquila et al., 2004). As internal controls, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) of the first antibody and reprobed with anti-actin antibody. As a positive control, LNCaP (prostate cancer cell line) was used. The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program.

**Immunofluorescence assay**

Sperm cells, were rinsed three times with 0.5 mM Tris–HCl buffer (pH 7.5) and were fixed using absolute methanol for 7 min at −20°C. AR staining was carried out, after blocking with normal human serum (10%), using the monoclonal anti-human AR (1 μg/ml) as primary antibody and an anti-mouse IgG FITC conjugated (4 μg/ml) as secondary antibody. To stain DNA in living cells, Hoechst 33342 (Hoechst) was added at a final concentration of 10 μg/ml. The specificity of AR was tested by using normal mouse serum instead of the primary antibody; sperm cells incubated without the primary antibody were also used as negative controls. The cellular localization of AR and Hoechst was studied with a Bio-Rad MRC 1024 confocal microscope connected to a Zeiss Axiovert 135 M inverted microscope with 600×. The fluorophores were imaged separately to ensure no excitation/emission wavelength overlap, and a minimum of 200 spermatozoa per slide were scored.

**Immunoprecipitation of sperm proteins and LNCaP cells proteins**

Spermatozoa were washed in Earle’s balanced salt solution and centrifuged at 800g for 20 min. Sperm resuspended in the same uncapacitating medium and LNCaP cells were incubated without (control, UC) or in the presence of dihydrotestosterone (DHT) at increasing concentrations (0.1, 1, 10 and 100 nM) for 30 min. Other samples were pre-treated for 15 min with 10 μM OH-Flut. In order to evaluate the rapid effect of DHT on AR, spermatozoa were incubated in the unsupplemented Earle’s medium at 37°C and 5% CO₂ at different times (5 and 30 min and 1 h). To avoid non-specific binding, sperm lysates were incubated for 2 h with protein A/G-agarose beads at 4°C and centrifuged at 12 000g for 5 min. The supernatants (each containing 600 μg total protein) were then incubated overnight with 10 μl anti-AR and 500 μl HNTG (IP) buffer (50 mM HEPES, pH 7.4; 50 mM NaCl; 0.1% Triton X-100; 10% glycerol; 1 mM phenylmethylsulfonylfluoride; 10 μg/ml leupeptin; 10 μg/ml aprotinin and 2 μg/ml pepstatin). Immune complexes were recovered by incubation with protein A/G-agarose. The beads containing bound proteins were washed three times by centrifugation in immunoprecipitation buffer, then denaturated by boiling in Laemmli sample buffer and analysed by western blot to identify the coprecipitating effector proteins. Immunoprecipitation using normal mouse serum was used as negative control. Membranes were stripped of bound antibodies by incubation in glycine (0.2 M, pH 2.6) for 30 min at room temperature. Before reprobing with the different indicated antibodies, stripped membranes were washed extensively in Tris buffered saline with Tween 20 (TBS-T) and placed in blocking buffer (TBS-T containing 5% milk) overnight.

**Evaluation of sperm viability**

Viability was assessed by using Eosin Y method. Spermatozoa were washed in uncapacitating medium and centrifuged at 800g for 20 min. To test androgen effects on sperm viability, spermatozoa...
were incubated in unsupplemented Earle’s medium at 37°C and 5% CO₂ without (control, UC) or in the presence of DHT at increasing concentrations (0.1, 10 and 100 nM) or 10 nM testosterone (T) for 2 h. In a different set of experiments, sperm were incubated in unsupplemented Earle’s medium at 37°C and 5% CO₂ without (UC) or in the presence of 10 nM DHT or T at different times (0, 10 and 30 min, 2, 6 and 24 h). Some samples were pretreated with 15 min with 10 μM OH-Flut. 10 μl of Eosin Y [0.5% in phosphate-buffered saline (PBS)] were mixed with an equal volume of sperm sample on a microscope slide. The stained dead cells and live cells that excluded the dye, were scored among a total of 200 cells and by an independent observer. Further, viability was evaluated before and after pooling the samples.

**PI3K activity**
PI3K activity was performed as previously described (Aquila et al., 2004). The negative control was performed using a sperm lysate, where p110 catalyzing subunit of PI3K was previously removed by preincubation with the respective antibody (1 h at room temperature) and subsequently immunoprecipitated with protein A/G-agarose. The PI3K1 was precipitated from 500 μg of sperm lysates. The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4) and finally with 10 mM Tris, 100 mM NaCl and 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM MgCl₂, 50 μM ATP, 20 μCi [γ-³²P] ATP and 10 μg L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P₂) for 20 min at 37°C. Phospholipids were extracted with 200 μl CHCl₃/methanol. The labelled products of the kinase reaction, the PI phosphates, in the lower chloroform phase were spotted onto trans-1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid-treated silica gel 60 thin-layer chromatography plates state running solvent used for TLC. Radioactive spots were visualized by autoradiography.

**DNA laddering**
DNA laddering was determined by gel electrophoresis. Spermatozoa were washed in Earle’s balanced salt solution and centrifuged at 800g for 20 min, then were resuspended in the same uncapacitating medium and in different tubes containing no androgens (control, UC), T or DHT or estrogen or progesterone or wortmannin at the indicated concentrations for 30 min. Some samples were resuspended in CAP. Some samples were pretreated for 15 min with 10 μM Cax or 10 μM OH-Flut or ZVF alone or each combined with 10 nM DHT. After incubation cells were pelleted at 800g for 10 min. The samples were resuspended in 0.5 ml of extraction buffer (50 mM Tris-HCl [pH 8], 10 mM EDTA, 0.5% sodium dodecyl sulphate (SDS)) for 20 min in rotation at 4°C. DNA was extracted with phenol/chloroform for three times and once with chloroform. The aqueous phase was used to precipitate acids nucleic with 0.1 volumes or of 3 M sodium acetate and 2.5 volumes cold EtOH overnight at −20°C. The DNA pellet was resuspended in 15 μl of H₂O treated with RNase A for 30 min at 37°C. The absorbance of the DNA solution at 260 and 280 nm was determined by spectrophotometry. The extracted DNA (2 μg/lane) was subjected to electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide and then photographed.

**Statistical analysis**
The experiments for RT–PCR, immunofluorescence and immunoprecipitation assays were repeated on at least four independent occasions, and western blot analysis was performed in at least six independent experiments, PI3K activity and DNA laddering assay were performed in at least four independent experiments. The data obtained from viability (six replicate experiments using duplicate determinations) were presented as the mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) followed by Newman–Keuls testing to determine differences in means. P < 0.05 was considered as statistically significant.

**Results**

**AR mRNA and protein were detected in human sperm**
To determine whether mRNA for AR is present in human ejaculated spermatozoa, RNA isolated from percoll-purified sperm samples from normal men was subjected to reverse PCR. The nucleotide sequence of AR was deduced from the cDNA sequence of the human AR gene and our primers amplified a region from 1648 to 2055 bp corresponding to the DNA binding domain plus the hinge region of the AR. RT–PCR amplification of AR in human sperm revealed the expected PCR product size of 400 bp. This product was sequenced and found identical to the classical human AR. No detectable levels of mRNA coding either PTPRC, a specific marker of leukocytes, or KIT, a specific marker of germ cells, were found in the same semen samples (Fig. 1A), thus ruling out any potential contamination.

![Figure 1: AR expression in human ejaculated spermatozoa](https://academic.oup.com/humrep/article-abstract/22/10/2594/599660)

(A) reverse transcription-PCR analysis of human AR gene, KIT and PTPRC in percollated human ejaculated spermatozoa (S1), negative control (no M-MLV reverse transcriptase added) (-), positive control (LNCap, prostate cancer cell; T, human testis and L, human leucocytes), marker (M). Arrows indicate the expected size of the PCR products; (B) western blot of AR protein by using a monoclonal antibody raised against the epitope 299–316 of the AR from human origin: extracts of percoll sperm, were subjected to electrophoresis on 10% SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and probed with the above mentioned antibody. Expression of the receptors in three samples of ejaculated spermatozoa from normal men (S1, S2 and S3). LNCap cells were used as positive control. N, negative control performed as described in Materials and Methods. The experiments were repeated at least four times and the autoradiographs of the figure show the results of one representative experiment.
The presence of AR protein in human ejaculated spermatozoa was investigated by western blot using a monoclonal antibody raised against the epitope mapping at the 299–316 aa in the N-terminus of AR from human origin (Fig. 1B). The antibody revealed the presence in sperm of two protein bands with molecular weights of 110 and 85–87 kDa, the latter expressed to a greater extent.

**Immunolocalization of AR in human sperm**

Using an immunofluorescence technique, we identified a positive signal for AR in human spermatozoa (Fig. 2A). No immunoreaction was detected either by replacing the anti-AR antibody by normal mouse serum (Fig. 2D) or when the primary antibody was omitted (data not shown), demonstrating the immunostaining specificity. AR immunoreactivity was specifically compartmentalized at the sperm head (Fig. 2A), where the DNA is packaged, as it can be seen in Fig. 2B in which the DNA is stained by Hoechst. Fig. 2C shows the merged images of Fig. 2A and B.

![Figure 2: Immunolocalization of AR in human ejaculated spermatozoa](https://academic.oup.com/humrep/article-abstract/22/10/2594/599660/figure2)

Spermatozoa were extensively washed and incubated in the unsupplemented Earle’s medium for 30 min at 37°C and 5% CO2. Spermatozoa were then fixed and analyzed by immunostaining as detailed in Materials and Methods. (A) AR localization in sperm; (B) staining with Hoechst of spermatozoa nuclei; (C) overlapping images of A and B; (D) sperm cells incubated replacing the anti-AR antibody by normal mouse IgG were utilized as negative control. The pictures shown are representative examples of experiments that were performed at least four times with reproducible results.

**AR is phosphorylated in human sperm**

It was reported that the function of AR is strongly correlated with the phosphorylation status (Wang et al., 1999), which is rapidly enhanced upon androgen exposure when it is able to activate signal transduction pathways. AR immunoprecipitates were blotted with three different antibodies: anti-p-tyrosine, anti-p-threonine and anti-p-serine. As shown in Fig. 3A, two major AR antibody reactive proteins corresponding to the 85–87 and 110 kDa were observed. To determine if the changes in phosphorylation status of AR under androgen treatments may occur in ejaculated sperm, these were exposed for 30 min to varying concentrations of DHT (0.1–10 nM). We observed that the AR phosphorylation was enhanced in a dose related manner (Fig. 3A) and was significantly reduced by OH-Flut, an AR antagonist. To investigate if the enhanced phosphorylation status may represent an early event, we performed a time course study revealing that AR phosphorylations occurred rapidly as they were observed from 0 to 15 min and then dropped significantly after 1 h (Fig. 3B). Moreover, all three phospho-antibodies demonstrated a prevalence for phosphorylation of the 110 kDa isoform. Furthermore, we repeated the experiments with the LNCaP cells to see whether they show similar results. As evidenced in Fig. 3C, in LNCaP cells, the major phosphorylation event appears to affect serine residues to a higher extent.

**Androgens effect on sperm viability**

To evaluate sperm viability under androgen treatment, we performed different sets of experiments. Sperm were incubated in the presence of 10 nM T or 10 nM DHT at the indicated times (Fig. 4A). Other samples were incubated in uncapacitating medium for 2 h in the absence or presence of different T or DHT concentrations (0.1–100 nM). As shown in Fig. 4B, the majority of cells remained viable in the control at 2 h. Cell viability significantly decreased with 10 and 100 nM T or DHT. Interestingly, the effect of androgen was reversed by using OH-Flut, addressing an AR mediated effect. It should be mentioned that the 100 nM androgen concentration is much higher than that commonly found circulating in vivo in man, while about 3 nM is detected in the seminal plasma of normal subjects (Luboshitzky et al., 2002).

**Androgen action on PI3K activity, p-AKT, p-BCL2 and p-PTEN is mediated by AR**

As shown in Fig. 5A low androgen concentration (0.1 and 1 nM) induced PI3K activity, while it was reduced by using 10 and 100 nM DHT. Both 10 nM T and to a greater extent 10 nM DHT treatments decreased PI3K activity (Fig. 5A). The 10 nM DHT effect was reversed in the presence of OH-Flut. Concomitantly, we observed a reduction on the levels of the downstream p-AKT S473 and p-AKT T308 (Fig. 5B and C) as well as p-BCL2 (Fig. 5D), a known anti-apoptotic protein (Ito et al., 1997). Specifically, DHT but not T had a significant inhibitory effect on p-AKT S473 and p-AKT T308 levels. Further, 10 nM of T or DHT significantly increased the phosphorylation of PTEN, a specific inhibitor of PI3K (Fig. 5E). All the above mentioned effects were reversed by Cax and OH-Flut, indicating that the effects of androgens are mediated by the classic AR in sperm. Recently, it was found that estradiol (E2) enhances sperm survival signalling (Aquila et al., 2004). Therefore, we aimed to evaluate whether, in sperm, a functional interaction exists between androgen and estrogen on PI3K activity. In sperm samples incubated with 100 nM E2 combined with increasing DHT...
Figure 3: AR is phosphorylated in human sperm

AR phosphorylation was determined by immunoprecipitation using an AR specific antibody. The immunoprecipitates were blotted with three different antibody: anti-p-tyrosine (pTyrAR), anti-p-threonine (pThrAR) and anti-p-serine (pSerAR). (A) sperm were incubated without (control, UC) or in the presence of DHT at increasing concentrations (0.1, 1 and 10 nM) for 30 min. Some samples were pretreated for 15 min with 10 μM OH-Flut. Sperm lysates (600 μg) were immunoprecipitated using anti-AR and then blotted with specific antibodies raised to anti-p-tyrosine, anti-p-serine, anti-p-threonine, anti-AR. Immunoprecipitation by using normal mouse serum was used as negative control (N). The autoradiographs presented are representative examples of experiments that were performed at least four times with repetitive results. Molecular weight markers are indicated on the right of the blot. The histograms indicated on the right of each blot are the quantitative representation after densitometry of data (mean ± SD) of four independent experiments. *P < 0.05, **P < 0.01 DHT-treated versus untreated cells. (B) time course of sperm incubated without (control, UC) or in the presence of 10 nM DHT. The autoradiographs presented are representative examples of experiments that were performed at least four times with repetitive results. Molecular weight markers are indicated on the right of the blot. The histograms indicated on the right of each blot are the quantitative representation after densitometry of data (mean ± SD) of four independent experiments. *P < 0.05, **P < 0.01 DHT-treated versus untreated cells. (C) LNCaP cells were incubated without (control, C) or in the presence of DHT at increasing concentrations (0.1, 1 and 10 nM) for 30 min. Some samples were pretreated for 15 min with 10 μM OH-Flut. The autoradiographs presented are representative examples of experiments that were performed at least four times with repetitive results. Molecular weight markers are indicated on the right of the blot. The histograms indicated on the right of each blot are the quantitative representation after densitometry of data (mean ± SD) of four independent experiments. *P < 0.05, **P < 0.01 DHT-treated versus untreated cells.
samples, and the values represent the mean. Experiments were repeated at least six independent times with duplicate samples, and the values represent the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005 versus control concentrations, the E₂-induced PI3K activity progressively decreased (Fig. 5F).

Androgens induce AR, PIK3R1 and phospho-src tyrosine kinase coimmunoprecipitation in human sperm

It was reported that a triple complex between AR, PIK3R1 and SRC tyrosine kinase is required for androgen-stimulated PI3K/AKT activation (Castoria et al., 2003; Sun et al., 2003), therefore we investigated whether it also occurs in sperm. At the 0.1 nM DHT concentration, phospho-SRC tyrosine kinase coimmunoprecipitated with the two proteins immunodetected by the C-19 anti-AR antibody, migrating at 110 and 85–87 kDa. Remarkably, no association of phospho-SRC tyrosine kinase with AR occurred at the 100 nM DHT concentration. Fig. 6 shows immunocomplexes blotted with anti-AR (Fig. 6A) or phospho-SRC tyrosine kinase (Fig. 6B) or anti-PIK3R1 (Fig. 6C) antibodies. The possibility that androgen treatment could modify the AR level was excluded since in the immunoprecipitated proteins the same amount of AR was detected.

Since the coimmunoprecipitation of phospho-SRC tyrosine kinase and PI3-kinase with AR decreased as androgens concentration increased, we may suppose that this is the mechanism through which high DHT concentration reduced PI3k activity.

Androgens effects on caspasess are mediated by AR

On the basis of the abovementioned results we sought to evaluate androgen action on the caspases family (Paasch et al., 2004), since these proteins are involved in cell death. Particularly, caspase 3 which is the main effector of both caspasess 8 and 9, executes the final disassembly of the cell by cleaving a variety of cell structure proteins and generating DNA strand breaks. Our study revealed activation of the caspases 8, 9 and 3 upon 30 min of 10 nM T or 10 nM DHT treatments. The DHT effect was reversed by both AR antagonists, 10 μM Cax or 10 μM OH-Flut (Fig. 7A). Notably, the effect on caspasess was specific for androgen as it was not observed with E₂ or progesterone treatments. Particularly, progesterone treatment was performed because of the similarity in structure between progesterone receptor and AR. The cleavage of caspase 3 was increased by androgens in a dose-dependent manner (Fig. 7C). In the presence of wortmannin, a specific inhibitor of PI3K activity, the cleavage of caspase 3 was also observed, addressing a regulatory role of PI3K in caspase activation in sperm. Furthermore, in order to demonstrate a specific effect on caspase activation, an additional control experiment was included showing that activation of caspases by androgens can be inhibited with a specific caspases inhibitor such as ZVK. All these data were confirmed by DNA laddering assay (Fig. 7B and D).

Discussion

Androgens and AR have been shown to play critical roles in testis function (Collins et al., 2003). AR has been detected in Sertoli, Leydig, peritubular myoid and spermatid cells (round and elongated) (Kimura et al., 1993; Vornberger et al., 1994; Suarez-Quian et al., 1999). The currently prevailing view is that sperm does not contain AR and this stems from previous studies reporting that no AR immunostaining of germ cells was observed both in rat and in human testis (Suarez-Quian et al., 1999). However, several studies reported that in spermatozoa, the binding capacity of androgens was greater than that of estrogens or progesterone (Hyne and Boettcher, 1977; Cheng et al., 1981), and recently AR was shown to be present in sperm by western blot and immunofluorescence assays (Solakidi et al., 2005).

In this study, we have demonstrated the presence of AR in human sperm at different levels: mRNA expression, protein expression and immunolocalization. By RT–PCR, we amplified a gene region corresponding to the DNA binding domain plus the hinge region of the human AR. This product was sequenced and found to be identical to the classical human AR. As it concerns the presence of mRNAs in mammalian ejaculated spermatozoa, originally it was hypothesized that these transcripts were carried over from earlier stages of...
Figure 5: Androgens action on PI3K activity, p-AKT, p-BCL2 and p-PTEN is mediated by AR.
Washed pooled sperm from normal samples were incubated in the unsupplemented Earle's medium at 37°C and 5% CO2, in the absence (UC) or in the presence of 10 nM T or in the presence of DHT at increasing concentrations (0.1, 1, 10 and 100 nM) for 30 min. 500 µg of sperm lysates were used for PI3K activity in sperm incubated at the indicated DHT concentrations in the absence or in the presence of 10 µM OH-Flut (A). The autoradiograph presented is representative example of experiments that were performed at least four times with repetitive results. The histograms indicated on the bottom of the figure are the quantitative representation after densitometry of data (mean ± SD) of four independent experiments. **P < 0.01 T- and DHT-treated versus untreated cells, ††P < 0.01 10 and 100 nM DHT versus 0.1 and 1 nM DHT, **P < 0.01 and †P < 0.05 10 µM OH-Flut plus DHT-treated versus DHT-treated cells. 50 µg of sperm lysates were used for western blot analysis of p-AKT S473 (B) and...
forms, are expressed in a variety of fetal and adult (male and female) human tissues and especially in reproductive tissues (Wilson and McPhaul, 1994). The B form migrates with an apparent mass of 110 kDa and constitutes >80% of the immunoreactive receptor in most cell types. The A form of the AR migrates with an apparent mass of 87 kDa. It was identified as an NH2-terminally (from 1 to 187 aa) truncated protein of AR-B and it was first described in human genital skin fibroblasts. The detection of two distinct forms of the AR raised a number of issues. AR-A is expressed at low levels in many androgen-responsive tissues; however, it appears to have functions similar to those of the full-length AR-B isoform. Functional activities of cDNAs containing the two isoforms were assessed using cotransfection assays that employed two models of androgen-responsive genes (MMTV-luciferase and PRE2-tk-luciferase) in response to mibolerone, a potent androgen agonist, in three different cell lines (Gao and McPhaul, 1998). These studies demonstrated subtle differences in the activities of the A and B isoforms, which depended on the promoter and cell context. Additional studies failed to reveal any major differences in the responses of the AR-A and AR-B isoforms to a variety of androgen agonists and antagonists, suggesting that the previously reported functional defect of the AR-A is due principally to its level of expression. When assays of AR function are performed under conditions in which levels of expression of the two isoforms are equivalent, the AR-A and AR-B possess similar functional activities (Gao and McPhaul, 1998). The ratio of AR-B:AR-A may vary among tissues and at different stages of development. However, it is unknown whether these isoforms have divergent biologic signal transduction capacities in humans, therefore we cannot predict what is the physiological correlate of a low AR-B:AR-A ratio as observed in sperm. By immunohistochemical assays, we have demonstrated that AR protein is detectable in the sperm head. Solakidi et al. (2005) reported AR prevalently localized in the midpiece region and the labelling pattern was similar to that of ERα. The apparent discrepancy between the latter finding and ours may be due to the different methods used to process samples.

An increasing body of evidence suggests that androgens and other steroid hormones can exert rapid, nongenomic effects (Peterziel et al., 1999; Cato et al., 2002). Different nuclear receptors such as progesterone receptor (Calogero et al., 2000), estrogen receptors α and estrogen receptor β (Aquila et al., 2004) were found to be present in human ejaculated spermatozoa, regulating cellular processes through nongenomic mechanisms. All these findings strengthen the importance of the nuclear receptors in nongenomic signalling (Cato et al., 2002) which may represent their exclusive modality of action.

**Figure 6:** AR, PIK3R1 and phospho-SRC tyrosine kinase coimmunoprecipitate in human sperm

Washed spermatozoa from normal samples were incubated in the unsupplemented Earle’s medium for 30 min at 37°C and 5% CO2, without (UC) or in the presence of DHT at increasing concentrations (0.1, 1, 10 and 100 nM). 600 µg of sperm lysates were immunoprecipitated using anti-AR antibody and then blotted with specific antibodies raised to AR (A), p-SRC tyrosine kinase (B) and PIK3R1 (C). LnCap lysates were used as positive control (lane 1); Immunoprecipitation by using normal mouse serum was used as negative control (N). The autoradiographs presented are representative examples of experiments that were performed at least four times with repetitive results. Molecular weight markers are indicated on the left of the blot. *P < 0.05, **P < 0.01 DHT-treated versus untreated cells.
Figure 7: Androgens effects on caspases are mediated by AR

(A) washed pooled sperm from normal samples were incubated in the unsupplemented Earle’s medium at 37°C and 5% CO2 (UC) in the presence of 10 nM T or 10 nM DHT or 100 nM E for 30 min. Some samples were washed with the unsupplemented Earle’s medium and incubated in capacitating medium (CAP). Some samples were treated with Cax or Flut or ZVF each alone or combined with 10 nM DHT. The sperm were lysed and subjected to western blot analysis. 70 μg of sperm lysates were used for western blot analysis of caspase 8, caspase 9 and caspase 3. (B) DNA laddering was performed in sperm treated as indicated. (C) effect of increasing DHT concentrations (0.1–100 nM), 100 nM PRG and 10 μM wortmannin (W) on caspase 3 cleavage. The experiments were repeated at least six times and the autoradiographs of the figure show the results of one representative experiment. (D) DNA laddering was performed in sperm treated as indicated.
in spermatozoa since they are apparently transcriptionally inactive. Here, we have demonstrated that in human ejaculated sperm, short exposure to androgens produces an increase in AR phosphorylation in a dose-dependent manner, while the antagonist OH-Flut significantly reduces this effect. Furthermore, we observed the most prominent phosphorylation on the 110 kDa band which is the less expressed isoform in sperm. It is known that the function of nuclear receptors is strongly correlated with their phosphorylation status rather than the level of total receptor proteins. The 110 kDa isoform exhibits a major length of the N-terminal domain which is an important effector of the cell signalling (Wilson and McPhaul, 1994,1996) This may explain why the phosphorylated status of the 110 kDa appears much more pronounced than the smaller isoform. From these findings it emerges that in sperm the 110 kDa is the most involved isoform in mediating AR trafficking signals. Furthermore, in sperm, the phosphorylation of the less expressed isoform appears to occur, upon androgen binding, in tyrosine, threonine and to a greater extent with respect to the basal values, on serine aminoacidic residues. Therefore, we repeated the experiments with the LNCaP cells to see whether they show a similar result. In LNCaP cells, the major phosphorylation event appears to affect serine residues. In any case, we should take into account that even in uncapacitated sperm, the autocrine effect of a pool of cytokines, insulin-like growth factors, as we previously demonstrated (Aquila et al., 2005a,b) may per sé influence the phosphorylation status of AR in addition to that determined by its natural ligand.

On the basis of our data androgens are able to modulate sperm survival depending on their concentration. To investigate the molecular mechanism involved in these effects we evaluated their action on the PI3K/AKT pathway, since it represents the main cell survival pathway and it was identified in sperm (Aquila et al., 2004). The 0.1 and 1 nM androgens induced PI3K activity, which was reduced by higher concentrations (10 and 100 nM). The 10 nM DHT was able to reduce the PI3K downstream signalling, while phosphorylation of PTEN, a proapoptotic marker which inhibits the PI3K pathway, was enhanced. To gain further insight into the mechanism involved in the PI3K/AKT modulation by AR, we investigated the association between AR/PIK3R1/p-SRC tyrosine kinase since it was reported depending on androgen concentration in somatic cells (Castoria et al., 2003; Sun et al., 2003). In our study, high androgen concentrations (10 and 100 nM) produce a detachment of SRC tyrosine kinase from the PIK3R1/AR complex, confirming that the triple complex is needed for the PI3K pathway activation. Furthermore, wortmannin, a specific PI3K/AKT inhibitor, induced caspase 3 cleavage in sperm, showing that the PI3K/AKT pathway is involved in the modulation of the caspases activity. The sperm death under high androgens (10 nM T, 10 and 100 nM DHT) was confirmed both by DNA laddering and cleavage of caspases 8, 9 and 3. In addition, increasing androgen concentrations were able to counteract the E2-induced PI3K activity previously documented (Aquila et al., 2004).

It is well established that in men intratesticular T levels are ~800 nM (Coviello et al., 2004), whereas they are ranging from 16 to 20 nM in serum (Luboshitzky et al., 2002; Coviello et al., 2004). The androgenic milieu in seminal plasma is dependent on circulating androgen levels and no longer intratesticular levels (Kuwahara, 1976; Andò et al., 1983). The biologically active amount of T, represented by its free fraction, in the genital tract is mostly converted in DHT by 5 alpha-reductase which is particularly expressed in the epithidymis and in the adnexal glands (Steers, 2001). A careful evaluation of the total androgenic milieu in seminal plasma, prevalently represented by the two most important androgens T and DHT, reveals the presence of about 1 nM of T and 2 nM of DHT and their ratio is about T/DHT 0.61 (Andò et al., 1983). Therefore, the seminal androgenic milieu, prevalently represented by the total molar concentration of T plus DHT corresponds to ~3 nM. In our study, the effects induced by 10 nM DHT were opposite to those induced by the lower doses and the same opposite pattern of androgen effects on PI3K pathway was previously documented in other cell type (Castoria et al., 2003).

In conclusion, the importance of androgen in the completion of male gamete maturation during the epididymal transit has been proved by the presence of AR in epididymal tissue (Zhou et al., 2002). Now on the basis of our results we may speculate how the importance of androgen in the sperm maturation process goes beyond the length of their life in seminiferous tubules, and continues when they became transcriptionally silent, through the AR non-genomic signalling. Even though to date, we cannot establish the physiopathologic correlates of these findings, we observed that an excess of androgens in the local hormonal milieu inhibits PI3K activity and negatively interferes with sperm survival. Further work will be required to more fully elucidate the role that AR plays in this aspect of male fertility.

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