Towards a physiological role for cytochrome P450 aromatase in ejaculated human sperm

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BACKGROUND: Advances in the definition of the function and the mechanism of estrogen action in different tissues have come from human and animal models of estrogen insufficiency. Recently we have demonstrated that aromatase is present and biologically active in human ejaculated sperm, suggesting that autonomous estradiol sperm production may influence sperm functions. In the present study we investigate a possible physiological role for enzymatically active P450 aromatase in human ejaculated sperm.

METHODS AND RESULTS: To confirm the presence of mRNA coding for P450 aromatase, total RNA isolated from human sperm underwent RT-PCR and then Southern blot analysis. In non-capacitating medium, we observed that only estradiol and aromatizable steroids were able to increase sperm motility/migration; concomitantly they enhanced protein tyrosine phosphorylation and increased p-44/42 extracellular signal-regulated kinase activity. When we tested acrosin activity, it emerged that estradiol and aromatizable androgens were also able to induce the acrosome reaction evaluated by two different cytological staining techniques (triple-stain and fluorescein isothiocyanate-\textit{Pisum sativum} agglutinin). All these events were enhanced by the 2$'\text{-}$O$'$-dibutyryladenosine-3$'$,5$'$-cyclic monophosphate and inhibited in the presence of the specific aromatase inhibitor, letrozole.

CONCLUSIONS: From this study, it appears that a link exists between the locally produced estradiol (from ejaculated sperm), sperm capacitation and the acrosome reaction. The induction of both events by aromatizable androgens in the absence of exogenous mediators suggests that estrogen biosynthesis in ejaculated sperm is a process that may influence the intrinsic sperm fertilizing capability.

Key words: acrosome reaction/aromatizable androgens/cytochrome P450 aromatase/ejaculated sperm/estrogen

Introduction

The generation of knockout mice for estrogen receptor (ER) $\alpha$ and $\beta$ as well as for the cytochrome P450 aromatase (P450arom) genes has provided evidence for a significant function of estrogens in male reproduction addressing their crucial role in maintaining normal spermatogenesis (Eddy et al., 1996; Hess et al., 1997; Cooke et al., 1998; Krege et al., 1998; Lee et al., 2000).

ER$\alpha$ knockout (ER$\alpha$KO) mice, from the onset of puberty, were infertile and showed a dysmorphismogenesis of the seminiferous tubules. This alteration resulted in a decreased spermatogenesis and inactive sperm, due to the accumulation of the luminal fluid and a lack of fluid reabsorption in the efferent ductules consequent to an altered ion transport and fluid movement (Lee et al., 2000). ER$\beta$ knockout (ER$\beta$KO) mice were fertile, while the reproductive phenotype of the double ER$\alpha$$\beta$KO was similar to that of ER$\alpha$KO mice. These data indicate that estrogen action through ER$\alpha$ is needed for normal male mouse reproduction. A P450arom knockout (ArKO) mouse was initially fertile, but displayed a progressive long term deterioration of spermatogenesis comprising in abnormalities in post-meiotic early cells. These alterations are evidenced by a decrease of $\sim$40% in round and elongated spermatids associated with an increase in apoptosis (Fisher et al., 1998; Robertson et al., 1999). Sperm maturation was substantially reduced or, in some cases, completely absent in the cauda epididymis. Taking into account that the above-mentioned spermatogenetic anomalies appear from 4–5 months of age, the progression of this phenotype may be intrinsic to the mechanism of action of estrogens in the seminiferous epithelium of adults.

A surprising finding in ArKO mice was that round spermatids, which did not undergo apoptosis early in spermiogenesis, had acrosomal dysgenesis (Robertson et al., 1999). The abnormal acrosome development in ArKO mice suggests that acrosome biogenesis could be an estrogen-dependent process. Another ArKO mouse lacking exons 1 and 2 of the aromatase gene (Honda et al., 1998) showed that at the age of 10–18 weeks sperm were present in the epididymis, but they were infertile. Moreover, ArKO mice show a 50%
decrease in ICSI fertilization ability, indicating that aromatase deficiency also affects the normal acquisition of sperm fertilizing potential (Luconi et al., 2002).

The importance of local production of the estrogens in the human testis was also shown in two men with homozygous inactivating mutation in the ER alpha gene (Smith et al., 1994) or in the P450arom gene (Carani et al., 1997). The patient lacking a functional ER alpha gene had normal genitalia and sperm density, but sperm motility was severely decreased. In the second male, the mutation in the aromatase gene resulted in infertility with a decreased sperm count and 100% immotile sperm.

In human testis it has been demonstrated that estradiol acts as a survival factor for round spermatids, which in absence of local production of estrogen undergo apoptosis, failing to differentiate into elongated spermatids (Pentikainen et al., 2000). These data suggest an important role for estrogen in sperm differentiation as well as in sperm fertilizing capability.

Several studies carried out in different species immunolocalized a biologically active P450arom in pachytene spermatocytes, round spermatids, elongated spermatids, flagella of late spermatids, and sperm within the epididymis (Nitta et al., 1993; Tsubota et al., 1993; Hess et al., 1995; Kwon et al., 1995; Carreau et al., 1998; Levallet et al., 1998). Recently in the human, P450arom was associated with the cytoplasm surrounding elongated spermatids (Turner et al., 2002) and with ejaculated sperm (Aquila et al., 2002).

In the present study we investigated a physiological role for estradiol produced by the human ejaculated sperm.

Materials and methods

**Chemicals**

PMN Cell Isolation Medium was from Biopsa (Milan, Italy). Total RNA iso-lation System kit, enzymes, buffers, and DNA 100 bp ladder used for RT–PCR were purchased from Promega (Italy); Moloney murine leukemia virus (M-MLV) was from Gibco BRL–Life Technologies Italia (Italy). Oligonucleotide primers were made by Invitrogen (Italy). Dulbecco’s modified Eagle’s medium–Ham’s F-12, 2’-O-dibutyryladenosine-3’,5’-cyclic monophosphate [(Bu)2cAMP], bovine serum albumin (BSA) protein standard, activated charcoal, Laemmli sample buffer, prestained molecular weight markers, Percoll (colloidal polyvinylpyrrolidone-coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, testosterone (17β-hydroxy-4-androsten-3-one), estradiol [3α,5α,10-12,17β-diol] (E2), 4-androstene-3,17-dione (Δ4), 5α-androstane-17α-ol-3-one (DHT), 5α,17β-dihydroxy-7,17-dimethylestr-4-en-3-one (mibolerone), dimethyldisulphoxide (DMSO), benzamidine, Nα-benzoyl- DL-arginine P-nitroanilide (BAPNA), Fibronecin, Earle’s balanced salt solution and all other chemicals were purchased from Sigma Chemical (Italy). Eosin Y was from Farmitalia Carlo Erba (Italy). Letrozole was provided by Novartis (Italy). Acrylamide bisacrylamide was from Laebek Eurobio (Italy). Triton X-100, HEPES Sodium Salt ECL Plus Western blotting detection system and Hybond™ ECL™ were from Amersham Pharmacia Biotech (UK). Mouse anti-phosphotyrosine monoclonal antibody (PY-99), peroxidase-coupled anti-rabbit and anti-mouse IgG were from Santa Cruz Biotechnology (Germany). Rabbit p-44/42 extracellular signal-regulated kinase (ERK1/2) was from Cell Signaling (Italy). Bradford protein assay was performed using a kit from Bio-Rad Laboratories, Inc. (Italy). The chamber well polycarbonate membranes were provided by Corning Costar Corporation (Italy). The fluorescent probe fluorescein isothiocyanate (FITC)-labelled *Pisum sativum* agglutinin (PSA), Trypan blue, Bismark brown Y and Rose Bengal were procured from Sigma. Nylon membranes were provided by Roche Diagnostics Corporation (USA).

**Semen samples**

Ejaculates were collected from normozoospermic men otherwise undergoing semen analysis for infertility, by masturbation after 3 days of sexual abstinence. Samples were allowed to completely liquefy for 30–60 min. Semen analysis (semen volume, sperm count, motility, vitality and morphological evaluation) was performed within 1.5 h after ejaculate collection and according to the World Health Organization (1999). Samples with sperm concentration <20 × 10⁶/ml and/or the presence of leukocytes and/or immature germ cells at a concentration of >2 × 10⁹ cells/ml were not included in the study. Ejaculates with abnormal viscosity were also excluded.

**Sperm purification**

After liquefaction, normal semen samples were pooled and subjected to centrifugation (800 g) on a discontinuous Percoll density gradient (80:40% v:v) (World Health Organization, 1999). The 80% Percoll fraction was examined using an optical microscope equipped with a ×100 oil objective to ensure that a pure sample of sperm was obtained. An independent observer, who observed several fields for each slide, inspected the cells. The pellet was then washed twice with BSA-free Ham’s F-10 medium.

Sperm were immediately used for RNA isolation or protein extraction. The purity of sperm preparations was determined by microscopic examination and by the use of the Percoll gradient. Additionally, the purity of our sperm preparation was confirmed using RT–PCR to detect Myelo Pox, a marker employed to examine granulocyte contamination (Aquila et al., 2002). RT–PCR of Myelo Pox in the sperm samples was not detectable, providing evidence that sperm preparations were not contaminated.

**Evaluation of sperm viability**

Measurement of acrosome reaction is indicative of occurred capacitation and acrosomal exocytosis. Since the release of acrosomal enzymes may also be a consequence of cell death, sperm viability was measured to evaluate some possible effects of the exposure to different treatments.

Viability was estimated using eosin Y exclusion. Ten microlitres of eosin Y (0.5% in PBS) were mixed with an equal volume of sperm sample on a microscope slide. The stained dead and living cells that excluded the dye, were scored among a total of 100 cells and by a blinded observer.

**RNA isolation, RT–PCR and Southern blot analysis**

Total RNA was isolated from human ejaculated sperm using Total RNA Isolation System kit as described by the manufacturer. The purity and quantity of the RNA was assessed spectrophotometrically before carrying out the analytical procedures. A total of 3 μl of each mRNA were visualized on a 0.9% formaldehyde-agarose gel to assess the RNA integrity.

RNA was amplified by RT–PCR. Briefly, 5 μl of total RNA was reverse-transcribed by 200 IU M-MLV reverse transcriptase in a reaction volume of 20 μl (0.4 μg oligo-d-T, 0.5 mmol/l deoxy-NTP and 24 IU Rnasin) for 30 min at 37°C, followed by heat denaturation for 5 min at 95°C. PCR amplification of complementary DNA (cDNA) employed 2 IU of Taq DNA polymerase and a sense primer specific for exon 7 (5’-CTGGAGAATAATGTAGGACTT-3’) and an antisense primer specific for exon 10 (5’-GATCATTTCCACGATTTT-3’) of...
P450arom. These pairs of primers were able to amplify 659 bp of human P450arom, including the helical aromatic and haem-binding region. The temperature program used was 94°C for 1 min, 60°C for 1 min and 72°C for 2 min, for 35 cycles. For all amplifications negative controls were included that consisted of water added instead of sample to test for contamination with extraneous DNA. As a positive control for each reaction, P450arom was amplified from a plasmid containing human CYP19 (pcDNA3.1-CYP19), the gene encoding P450arom (Simpson et al., 1997). Standard DNA markers (100 bp DNA ladder) were also run to determine the size of amplified products. To prove the coincidence of amplified products with the selected region of aromatase gene, Southern blot analysis was performed. Amplified DNA obtained by RT-PCR was subjected to electrophoresis and blotted on Nylon membrane, positively charged. DNA was transferred with the use of a capillary method and lasted for 16 h. Then DNA was covalently linked to the nylon membrane by exposure to UV light (254 nm) at 1.5 J/cm². The prehybridization was carried out in a blocking solution for 2 h at 55°C. A total of 1–2 ng of RNA probe (S-TTCTAAGGCTTTGCGCATGAC-3' located in Exon IX) was labelled with [γ-32P]ATP using polynucleotide kinase and was added to a second solution identical to the prehybridization solution. The hybridization was carried out at 65°C for 6 h. The membrane was exposed to Kodak XAR-2 film with intensifying screens overnight at −80°C.

Assessment of sperm-hyperactivated motility using a chemotaxis assay
We identified hyperactivated sperm using a chemotaxis assay since sperm acquire their chemotactic responsiveness as a part of capacitation process and lose it when the capacitated state is terminated (Cohen-Dayag et al., 1995).

Changes in the motility of sperm were assessed by their ability to migrate through a polycarbonate membrane containing 8 μm pores. The lower wells of the 24-well chamber were filled with a swim-up preparation of human sperm (1×10⁹). Sperm were prepared in unsupplemented Earle’s balanced salt solution (non-capacitating medium) in the presence (experimental) or absence (control) of different amounts of the following steroids: E2 (100 nmol/l, 1 μmol/l), Δ4 (100 nmol/l, 1 μmol/l), testosterone (100 nmol/l, 1 μmol/l), DHT (100 nmol/l, 1 μmol/l). In addition, Δ4 treatments were tested in the presence of 10 μmol/l of letrozole and/or with 1 mmol/l of (Bu)2cAMP. Other samples were treated with letrozole or (Bu)2cAMP alone. Treated sperm suspensions were incubated for 1 h at 37°C, 5% CO2 and then centrifuged for 5 min at 5000 g. The pellet was resuspended in lysis buffer [62.5 mmol/l Tris–HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 1 mmol/l Na3VO4, 1 mmol/l phenylmethylsulphonyl fluoride]. The lysates were shaken for 15 min protein concentration was estimated using Bradford’s assay (Bradford, 1976). After protein measurement the sperm extracts, each containing the same protein amount (15 μg), were adjusted with lysis buffer to the same volume and diluted in an equal volume of reducing 2×standard Laemmlidi SDS buffer. Samples were boiled for 5 min and electrophoresed on 10% SDS–polyacrylamide gels. After SDS–polyacrylamide gel electrophoresis (PAGE), proteins were transferred to nitrocellulose membranes. Nitrocellulose filters with transferred proteins were blocked overnight in TTBS (0.1% Tween-20, 20 mmol/l Tris and 150 mmol/l NaCl) containing 5% BSA, then washed three times for 15 min each in TTBS. Mouse anti-phosphotyrosine monoclonal antibody (PY-99) and rabbit anti-p-ERK 44/42 were diluted in 5% BSA–TTBS, and then incubated with the blotted membranes. The antigen–antibody complexes were detected by incubation (1 h) of the membranes with peroxidase-coupled anti-mouse IgG and peroxidase-coupled anti-rabbit IgG respectively and visualized using the ECL Plus Western blotting detection system.

Acrosin activity assay
Acrosin activity was assessed by the method of Kennedy et al. (1989). Pergcoll-purified sperm were washed in Earle’s balanced salt solution medium supplemented with CaCl2 (266 mg/100 ml), BSA (600 mg/100 ml), sodium pyruvate (3 mg/100 ml), sodium lactate (360 μl/100 ml) and sodium bicarbonate (200 mg/100 ml), centrifuged at 800 g for 20 min. Sperm were resuspended (final concentration of 1–10×10⁶ sperm/ml) in the same capacitating medium and in different tubes containing no steroid (control) or different amounts of the following steroids (experimental): E2 (100 nmol/l, 1 μmol/l), testosterone (100 nmol/l, 1 μmol/l), Δ4 (100 nmol/l, 1 μmol/l), DHT (100 nmol/l, 1 μmol/l) and mibolerone (100 nmol/l, 1 μmol/l). Some samples were pretreated for 30 min with 1 μmol/l of (Bu)2cAMP or with 10 μmol/l of letrozole. Then 1 ml of substrate–detergent mixture (23 mmol/l BAPNA in DMSO and 0.01% Triton X-100 in 0.055 mol/l NaCl, 0.055 mol/l HEPES at pH 8.0 respectively) for 3 h at room temperature was added. The effect induced by Δ4 (1 μmol/l), with or without the letrozole, was focused on sperm resuspended in unsupplemented Earle’s medium and subsequently incubated with 1 ml of substrate (23 mmol/l BAPNA in DMSO) without the detergent mixture which destroys acrosomal membrane. Aliquots (50 μl) were removed at 0 and 3 h, and the percentages of viable cells were determined. No significant differences were detected between the
control and experimental conditions. After incubation, 0.5 mol/l benzamidine was added (0.1 ml) to each of the tubes and then centrifuged at 1000 g for 30 min. The supernatants were collected and the acrosin activity measured spectrophotometrically at 410 nm. In this assay, the total acrosin activity is defined as the amount of the active (non-zymogen) acrosin associated with sperm plus the amount of active acrosin that is obtained by proacrosin activable.

Quantification of acrosin activity
One international unit (IU) of acrosin activity was defined as the amount of enzyme that hydrolysed 1 µmol/l BAPNA/min at room temperature. To obtain whole numbers, the acrosin activity was expressed as µIU/106 sperm. The activity was calculated as the difference in optical density at 410 nm between the mean of the test assays and the control, multiplied by 106 and divided by the product of the total number of sperm and a correction factor, i.e:

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\text{µIU acrosin/10}^6 \text{ sperm } = \frac{[(\text{mean OD}_{\text{test}}) - \text{OD}_{\text{control}}] \times 10^6}{1485 \times \text{total number of sperm} (\times 10^6)}
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Assessment of acrosomal status in human ejaculated sperm
AR was scored using two methods: the FITC–PSA, which binds to intra-acrosomal contents (Mendoza et al., 1992); and the triple-stain technique of Talbot and Chacon (1981), which discriminates between dead and live sperm which have intact acrosomes or have already undergone the acrosome reaction. Aliquots (50 µl) were removed before and at the end of treatments, the percentages of viable cells were determined and no significant differences were detected between the control and experimental conditions.

Processing of ejaculated sperm for the AR scoring
Percoll-puriﬁed sperm were washed with 0.5 mol/l Tris–HCl buffer pH 7.5 and were incubated in the unsupplemented Earle’s medium for 1 h at 37°C and 5% CO2, without (control) or with (experimental) Δ4 (1 µmol/l) or DHT (1 µmol/l). In addition, Δ4 treatment was tested in the presence of letrozole (10 µmol/l).

FITC–PSA staining
At the end of incubation, sperm cells were washed three times with 0.5 mol/l Tris–HCl buffer, pH 7.5 and were allowed to settle onto slides. Smears, dried in air, were dipped in absolute methanol for 15 min and left at room temperature. The smears were then incubated with a solution of FITC–PSA in Tris–HCl buffer (50 µg/ml) in a humid chamber at room temperature. After 30 min, the slides were washed with distilled water and then they were examined immediately after drying in an epifluorescent microscope (Olympus B202). A minimum of 200 live sperm were examined for each treatment and the following patterns were scored: (i) selective staining of the whole acrosome (unreacted cell); (ii) no staining at all, or staining limited to the equatorial segment (reacted cells). Staining was assessed according to a published scoring system (Mendoza et al., 1992).

Talbot and Cachon triple-stain technique
Sperm cells were diluted with 2% Trypan Blue (1/1) and were incubated at 37°C for 15 min and centrifuged at room temperature for 10 min at 600 g. The sperm pellet was resuspended in deionized water and again centrifuged. This procedure was continued until the supernatant was clear. The sperm were then ﬁxed for 60 min in 2.5% glutaraldehyde. After glutaraldehyde removal, sperm cells were smeared on glass slides and air-dried. The slides were then stained with 0.8% Bismark Brown Y, at 40°C for 5 min and then washed for several seconds. Slides were stained with 0.8% Rose Bengal, at 24°C for 30 min, washed in water, dehydrated in an alcohol series and cleared in xylene. A minimum of 200 sperm were examined for each treatment, scoring living sperm displaying two staining patterns: (i) light brown post-acrosomal regions and pink acrosomes (unreacted sperm); (ii) light brown post-acrosomal regions and white acrosomes (reacted sperm).

Statistical analysis
The experiments for Southern and Western blot were repeated on at least three independent occasions. The data obtained from replicate experiments of acrosin activity (six replicate experiments), motility/migration assay (six replicate experiments), staining assay (six replicate experiments) are presented as mean ± SD. The differences between experimental groups were analysed by one-way analysis of variance (Sheffé et al., 1959) and the signiﬁcant differences between groups, when appropriate, were established using Student’s t-test. In all cases, the results were considered signiﬁcant if P < 0.05. Error bars denote SD.

Results
Presence of P450arom mRNA in human ejaculated sperm
To give further conﬁrmation of our previous data, concerning the presence of mRNA encoding P450arom in human ejaculated sperm, total RNA isolated from puriﬁed samples was subjected to RT–PCR with a speciﬁc P450arom set of primers and then subjected to Southern blot analysis (Figure 1). Hybridized band of cDNA ampliﬁed from human sperm (S) revealed the same mobility as that ampliﬁed from a plasmid containing the full coding region of P450arom (+), which was used as a positive control.

Sperm motility/migration is enhanced by estradiol and aromatizable substrates
When we incubated sperm in simple deﬁned medium containing only metabolic energy substrates (non-capacitating medium) with or without the various steroids tested, we observed that only E2, testosterone and Δ4 were able to produce the enhancement, in a dose related manner, of sperm motility/migration as result of capacitation occurring in the chemotactic assay (Figure 2A).

The increase induced by Δ4 was completely abrogated in the presence of an inhibitor of aromatase such as letrozole (Figure 2B). On the basis of our previous ﬁndings demonstrating that (Bu)2cAMP ampliﬁes aromatase activity, we wondered if the well-documented effect of cAMP on sperm motility and capacitation (White et al., 1989) was further ampliﬁed concomitantly with aromatization. We noticed that upon combined exposure to (Bu)2cAMP and Δ4, sperm motility/migration was signiﬁcantly enhanced with respect the samples treated with (Bu)2cAMP alone. This potentiating effect was reversed by letrozole (Figure 2C).

Similar results upon Δ4 were obtained when we scored at the microscope observation, the percentage of hyperactivated sperm, characterized by pronounced flagellar movements, marked lateral excursion of the sperm head and a non-linear trajectory (Ho et al., 2001) (data not shown). No signiﬁcant difference was observed between treated and untreated samples when we scored the percentage of viable cells.
Estradiol and aromatizable substrates are able to increase sperm protein tyrosine phosphorylation and to activate ERK1/2

During capacitation, human sperm undergo tyrosine phosphorylation of a characteristic set of proteins (Naz, 1996). Figure 3 shows SDS–PAGE patterns of proteins extracted from human sperm suspensions with or without (control) the various steroids tested, in non-capacitating medium. Mouse anti-phosphotyrosine monoclonal antibody (PY-99) specifically recognized protein bands predominantly belonging to four molecular regions as largely documented (Naz, 1996; Visconti et al., 1998). The intensity of the protein tyrosine phosphorylation increased in the samples treated with E₂, D₄ and testosterone, while in DHT-treated samples was similar to untreated. It is noteworthy that the different treatments mainly affected the 95–97 kDa tyrosine-phosphorylated proteins whose intensity increased under E₂, Δ₄ and testosterone treatment, while the up-regulatory effect of the Δ₄ was inhibited by letrozole.

Figure 1 Southern blot analysis of P450 aromatase (P450arom) in ejaculated sperm of normal man. Total RNA was isolated from pooled sperm of normal men, amplified by RT–PCR and subjected to Southern blot analysis. Aromatase-specific fragment was detected by hybridization of the membrane with specific oligonucleotide for exon IX (see Materials and methods) of the human P450arom gene. Lane 1: sperm (S). Lane 2: vector containing the coding region of human P450arom used as the positive control (+). Lane 3: negative control (no cDNA added). Molecular weight marker is shown on the left (in base pairs). The autoradiography presented in the figure is a representative example of experiments that were performed a minimum of three times with similar results.

Figure 2 Effect of estradiol (E₂) and different sex steroids (aromatizable and non-aromatizable) on sperm motility/migration in non-capacitating medium. (A) The effect of E₂ (100 nmol/l, 1 μmol/l), 4-androstene-3,17-dione (Δ₄) (100 nmol/l, 1 μmol/l), testosterone (100 nmol/l, 1 μmol/l) and 5α-androstan-17α-ol-3-one (DHT) (100 nmol/l, 1 μmol/l) on the motility/migration of human sperm through an 8 μm pore membrane of Boyden chambers (see Materials and methods). ***P < 0.0001 with respect to control (C); **P < 0.001 with respect to control; *P < 0.05 with respect to control. (B) The effect of different concentrations of Δ₄ with or without 10 μmol/l letrozole (L) on the motility/migration of human sperm. ***P < 0.0001 with respect to control; **P < 0.001 with respect to control; *P < 0.05 with respect to control; *P < 0.05 with respect to Δ₄ (100 nmol/l); *P < 0.01 with respect to Δ₄ (1 μmol/l). (C) The effect of 2'-O-dibutyryladenosine-3',5'-cyclic monophosphate [(Bu)₂cAMP] (1 mmol/l) and/or Δ₄ (1 μmol/l) with or without 10 μmol/l letrozole on the motility/migration of human sperm. ***P < 0.0001 with respect to control; **P < 0.001 with respect to control; *P < 0.05 with respect to control; *P < 0.05 with respect to Δ₄ alone; *P < 0.05 with respect [(Bu)₂cAMP]; **P < 0.05 with respect to Δ₄ alone; ***P < 0.001 with respect [(Bu)₂cAMP + Δ₄]; ***P < 0.001 with respect [(Bu)₂cAMP + Δ₄]. The results represent the mean ± SD of at least six independent experiments.
The densitometric evaluation of the double 95–97 kDa band, revealed that the tyrosine phosphorylation signal increased significantly upon E₂, Δ₄ and testosterone whereas it was reversed in the presence of letrozole.

A similar modulatory pattern induced by E₂, Δ₄ and testosterone in the expression of ERK1/2 phosphoproteins has been observed (Figure 4). Moreover we observed a synergistic effect of (Bu₂)₂cAMP plus Δ₄ on both tyrosine phosphorylation and ERK1/2 activation.

Acrosin activity is enhanced by estradiol and aromatizable substrates

Acrosin activity was used to evaluate the induction of the AR; moreover, the AR has been a useful endpoint to determine whether the cells are fully capacitated.

The effect of various steroids on acrosin activity was tested in the capacitating medium and in simple defined medium containing only metabolic energy substrates.

In the presence of capacitating medium E₂, Δ₄ and testosterone at two different concentrations (100 nmol/l and 1 μmol/l) were able to enhance acrosin production in a dose-related manner, while no substantial changes were observed
with DHT (100 nmol/l, 1 μmol/l) and mibolerone (100 nmol/l, 1 μmol/l) (Figure 5A). In the presence of Δ4 plus letrozole, the pattern of acrosin response overlap that of control (Figure 5B).

We evaluated whether the enhancement exerted by Δ4 on acrosin activity could also be affected by the addition of (Bu)2cAMP. As indicated in Figure 5C, the effect of (Bu)2cAMP on the Δ4-enhanced acrosome reaction was specifically related to an increase of aromatase activity because this effect was also abolished by letrozole.

We then asked if aromatizable androgens per se were able to induce AR without exogenous mediators and we focused our attention on the effect of Δ4 in sperm incubated in non-capacitating medium. We observed a significant increase of

Figure 5. Effect of estradiol (E2) and different sex steroids (aromatizable and non-aromatizable) on acrosin activity in capacitating medium. (A) The effect of E2 (100 nmol/l, 1 μmol/l), 4-androstene-3,17-dione (Δ4) (100 nmol/l, 1 μmol/l), testosterone (T) (100 nmol/l, 1 μmol/l), 7α,17β-17-hydroxy-7,17-dimethylestr-4-en-3-one (mibolerone, MIB) (100 nmol/l, 1 μmol/l) and 5α-androstan-17α-ol-3-one (DHT) (100 nmol/l, 1 μmol/l) on the acrosin activity. ***P < 0.005 with respect to control (C); **P < 0.01 with respect to the control; *P < 0.05 with respect to Δ4 alone. (B) The effect of different concentrations of Δ4 with or without letrozole (L) on the acrosin activity. ***P < 0.0001 with respect to control; **P < 0.005 with respect to control; *P < 0.01 with respect to Δ4 alone. (C) The effect of different doses of Δ4 on acrosin activity in the presence or without 2'-O-dibutyryladenosine-3',5'-cyclic monophosphate [(Bu)2cAMP] (1 mmol/l). The combined effect of Δ4 plus (Bu)2cAMP was also evaluated with or without 10 μmol/l letrozole. ***P < 0.005 with respect to control; **P < 0.01 with respect to the control; *P < 0.05 with respect to Δ4 alone; **P < 0.05 with respect to (Bu)2cAMP + Δ4. The results depicted represent the mean ± SD of at least six independent experiments.

Figure 6. Effect of 4-androstene-3,17-dione (Δ4) on acrosin activity in capacitating and non-capacitating medium. The results depicted in the figure summarized the effect of Δ4 (1 μmol/l) with or without 10 μmol/l letrozole (L) both in capacitating (grey) and in non-capacitating (black) medium. *P < 0.01 with respect to control (C) in capacitating medium; +P < 0.05 Δ4 treatment in non-capacitating medium with respect to control in capacitating medium. The results depicted represent the mean ± SD of at least six independent experiments.
acrosin production in the sample treated with Δ4 with respect to the control in capacitating medium, and it was completely reversed by letrozole (Figure 6). No significant differences were observed between treated and untreated samples when we scored the percentage of viable cells.

**Acrosome reaction induced by aromatizable substrates is evidenced through two different cytological staining techniques**

The FITC–PSA staining revealed a significant difference between the treatments. A highly significant increase in reacted sperm cells was seen in sperm incubated with 1 μmol/l Δ4 with respect to the basal samples. The incidence of reacted sperm in samples treated with 1 μmol/l of Δ4 plus 10 μmol/l of letrozole or with DHT was similar to the control (Figure 7A).

Similar results were obtained using the Talbot and Chacon triple stain (Figure 7B). Each slide was examined by two independent observers and 200 sperm were counted by each observer. For every sample, the viability of 200 sperm was determined, and no significant changes were observed between untreated and treated samples.

**Discussion**

For many years the expression of P450arom in the human male genital tract has been limited to prostate (Matzkin et al., 1992) and Leydig cells (Inkster et al., 1995); only recently has it been observed in elongated spermatids (Turner et al., 2002) and in ejaculated sperm (Aquila et al., 2002). In this study, we confirm that P450arom mRNA in ejaculated sperm bear the functional domains of the codified protein, and we have investigated a physiological relevance for P450arom in human ejaculated sperm.

The possible functional role of this enzyme in the male gamete has been focused on its possible link to the processes of capacitation and acrosome reaction. The ejaculated sperm cannot immediately bind to an oocyte and undergo the acrosome reaction. They need a series of biochemical and functional modifications collectively referred to as capacitation (Yanagimachi et al., 1994; Visconti and Kopf, 1998), through which they acquire hyperactivated motility and become acrosomally responsive. The effects of estradiol on human sperm have been associated with an enhanced motility, oocyte penetration, longevity, oxygen intake, lactate production and metabolization of several exogenous substrates (Idaomar et al., 1989). In addition, estradiol leads to a rapid increase of intracellular cAMP, calcium concentrations, and to an enhanced tyrosine phosphorylation of proteins in sperm (Luconi et al., 1999; Baldi et al., 2000). It is noteworthy that these effects of estradiol in sperm recall the events that characterize capacitation.

The mechanism of the induction of sperm capacitation remains enigmatic and many paracrine or endocrine candidates have been proposed (Wu et al., 2001). The activation of extracellular signal-regulated kinases during capacitation suggests that the process requires extracellular signals like those that occur following residence in the female tract (e.g. estradiol and/or environmental conditions). However, an intriguing aspect of the process is that it occurs spontaneously in vitro by the removal of sperm from seminal plasma and the incubation in simple media (Visconti et al., 1995). In these circumstances, the pattern of protein tyrosine phosphorylation occurs in a time-dependent fashion in the absence of an external stimulus during capacitation (Visconti et al., 1995). These observations suggest that the initiation and the completion of the capacitated state may be regulated by processes intrinsic to the sperm cell itself.

Towards this aim, we first focused our investigation on sperm motility/migration concomitantly with the tyrosine phosphoprotein status, reliable parameters of capacitation status. Our results provide evidence that E2 and aromatizable substrates were able to enhance sperm motility/migration in non-capacitating medium and that this effect was reversed by letrozole. Afterwards we observed that E2 and aromatizable steroids were able to enhance tyrosine phosphoprotein status of sperm, as shown by the 95–97 kDa bands, which are reported to be expressive during capacitation (Naz, 1996). Since several of the effectors involved in the signal transduction pathways that produce AR begin to be tuned during capacitation, we performed acrosin activity assessment in human sperm treated...
with E₂, aromatizable and non-aromatizable steroids, in non-capacitating and capacitating conditions. The low amount of acrosin produced in non-capacitating medium fits with the evidence that in the absence of any specific stimuli only a small percentage of human sperm can undergo a spontaneous AR. For instance, it has been suggested that under these circumstances self-aggregation of the sperm receptor for ZP, after removal of seminal plasma, may account for this spontaneous acrosome reaction (Baldi et al., 2000). In non-capacitating conditions, acrosin production is elevated upon exposure to aromatizable steroids and specifically linked to the process of aromatization since it was reversed by letrozole. No substantial changes were observed in the presence of DHT and mibolerone. These findings correlated well with both staining techniques utilized to demonstrate that the number of acrosome reacted sperm increase significantly in the presence of aromatizable androgens in non-capacitating medium. It is noteworthy that Δ₄ enhances sperm acrosin production to a greater extent than the capacitating medium alone. Moreover, we observed a synergistic effect of (Bu)₂cAMP and Δ₄ on both sperm motility/migration and acrosin activity which was abrogated by letrozole, strengthening a role of aromatization process in inducing both events.

The synergistic effect of (Bu)₂cAMP and Δ₄ may be due to an enhanced Δ₄ aromatization upon the nucleotide exposure, as we previously demonstrated (Aquila et al., 2002). The non-genomic, cAMP-mediated, action of estradiol has been demonstrated in previous studies (Sabewral et al., 2002; and references therein). Likewise, we may explain why the up-regulatory effect of Δ₄ on both ERK1/2 and tyrosine phosphorylation appears to be potentiated by (Bu)₂cAMP.

Given the coincident location of some cAMP/PKA pathway effectors (dynein and PKA anchoring proteins), ERα (Durkee et al., 1998; Baldi et al., 2000) and aromatase in the tail of sperm (Aquila et al., 2002), it is reasonable to postulate how estradiol produced by sperm may activate, via its own receptor, cAMP cascade signalling which somehow recapitulates the events that characterize the capacitation process.

Our results indicate how aromatization in sperm induces per se the activation of multiple pathways involved both in capacitation and in AR. From all these findings it may be hypothesized that autonomous estrogen production by human ejaculated sperm, through a paracrine mechanism or an autocrine short loop, may act to trigger the events occurring in capacitation and then in AR, since the two processes are sequentially and functionally linked. The physiological role of enzymatically active P450arom in human ejaculated sperm raises the potential use of aromatizable androgens as a substrate in seminal plasma (Purvis et al., 1975; Andò et al., 1983; Bujan et al., 1993) as well as in female genital fluid (Chew et al., 1989) priming, together with environmental conditions, the events of sperm capacitation. Further support for this assumption is raised by the recent finding that estradiol stimulates capacitation in non-capacitating medium (Adeoya-Osiguwa et al., 2003).

In conclusion, our data address aromatization as an important process that may influence the intrinsic fertilizing capability of human ejaculated sperm.

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