UNIVERSITÀ DELLA CALABRIA

Facoltà di Farmacia e Scienze della Nutrizione e della Salute

DIPARTIMENTO FARMACO –BIOLOGICO (MED/04 PATOLOGIA GENERALE)

Dottorato di Ricerca in "Biochimica Cellulare ed Attività dei Farmaci in Oncologia" (XXIII ciclo)

Estrogens modulate the expression of the deleted gene homolog phosphatase and tensin on chromosome 10 (PTEN) in human seminoma cells TCam2

Docente Tutor

Ch.mo Prof. Sebastiano Andò

Coordinatore

Prof. Diego Sisci

Dottoranda

Carmela Guido

Carmela Guido

Anno Accademico 2010 – 2011

UNIVERSITÀ DELLA CALABRIA

Facoltà di Farmacia e Scienze della Nutrizione e della Salute
DIPARTIMENTO FARMACO –BIOLOGICO (MED/04 PATOLOGIA GENERALE)

Dottorato di Ricerca in "Biochimica Cellulare ed Attività dei Farmaci in Oncologia" (XXIII ciclo)

Estrogens modulate the expression of the deleted gene homolog phosphatase and tensin on chromosome 10 (PTEN) in human seminoma cells TCam2

Docente Tutor
Ch.mo Prof. Sebastiano Andò
Coordinatore
Prof. Diego Sisci

Dottoranda
Carmela Guido

Anno Accademico 2010 – 2011

INDEX

Scientific Publications

- *Estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) influence human sperm metabolism and interact with cytoskeletal proteins: evidence on the pathophysiology of varicocele-associated male infertility." (revision 1 on Journal of Cellular Physiology)*

- *Conventional progesterone receptors (PR) B and PRA are expressed in human spermatozoa and may be involved in the pathophysiology of varicocoele: a role for progesterone in metabolism. Accepted 08.08.2010 on Int J Androl. 2010*

- *Rimonabant (SR141716) induces metabolism and acquisition of fertilizing ability in human sperm. Br J Pharmacol. 2010 159(4):831-41*

- *Human sperm anatomy: ultrastructural localization of the cannabinoid1 receptor and a potential role of anandamide in sperm survival and acrosome reaction. Anat Rec (Hoboken). 2010 293(2):298-309.*

- *Human male gamete endocrinology: 1alpha, 25-dihydroxyvitamin D3 (1,25(OH)2D3) regulates different aspects of human sperm biology and metabolism. Reprod Biol Endocrinol. 2009 30;7:140*[*]*These authors contributed equally to this work*

- *Insulin and IR-beta in pig spermatozoa: a role of the hormone in the acquisition of fertilizing ability. Int J Androl. 2010 33(3):554-62.*

- *A new role of anandamide in human sperm: focus on metabolism. J Cell Physiol. 2009 221(1):147-53.*

- *Leptin and its receptor are expressed in the testis and in the epididymis of young and adult pigs. Anat Rec (Hoboken). 2009 292(5):736-45.*

- *Human sperm anatomy: ultrastructural localization of 1alpha,25-dihydroxyvitamin D receptor and its possible role in the human male gamete. J Anat. 2008 213(5):555-64.*

- *Leptin and leptin receptor in pig spermatozoa: evidence of their involvement in sperm capacitation and survival. Reproduction. 2008 136(1):23-32.*

SUMMARY

Testicular germ cell tumors of adults and adolescents (TGCTs) are the most common tumor in male. TGCTs can be classified into two main histological subtypes, seminoma (SE) and nonseminoma (NS). Here the focus is on SE, by using the TCam-2 cell lines, containing typical features of human seminoma and originated from a primary testicular seminoma of a 35-year-old patient. Testicular cancer research continues to investigate and study therapies aimed to induce cell death in tumor cells. Estrogens (E2) are regulators of testicular physiology and function. *PTEN*, a factor involved in the mechanisms that induce apoptosis, appears to be essential for the differentiation of testicular germ cell tumor. The aim of this study was to investigate a potential functional crosstalk between E2 and *PTEN* in human seminoma cells, to improve our understanding in the biology of testicular tumor and in the regulation of the *PTEN* gene. First, as the presence of the main mediators of E2 action, estrogen receptor ERα and ERβ, in our cell type was never reported, we evidenced that the TCAM2 cells express the ERβ and not the classical ERα. Upon increasing E2, by western blot and RT-PCR we observed an increase of *PTEN* both at protein and mRNA levels.

To evidence if this effect occurs at genomic level, we used a series of constructs expressing either full length or different deleted regions of the *PTEN* promoter gene. Using these mutants, by in vitro trasfection assays, we identified the sequences required for the modulation of *PTEN* gene by E2/ER. Besides, based on the informations concerning the structure of human *PTEN* promoter gene we functionally characterized the *PTEN* promoter gene region responsive to E2, evidencing that it contains many Sp1 transcription factor sites.

The second specific aim of this research was to study the molecular interactions among the transcription factors that are involved in *PTEN* gene transactivation by E2/ER. By EMSA assay and the ChIp assay we evidenced that the binding of E2/ER at the promoter gene region of *PTEN* identified occurs through Sp1. Furthermore, by ChIp assay we investigated the expression of RNA pol II, evidencing an increase in the E2-induced *PTEN* transcriptional activity. Besides, overexpressing the dominant negative of ERβ we confirmed that the regulation of *PTEN* by E2 occurs through the ERβ in human seminoma cells.

PTEN is the main regulator of the PI3K/AKT pathway that regulates different cell activities including the cell survival. To test our hypothesis that the biological significance of the link among E2/ER/*PTEN* in our cells is an induction of the cell death, by trypan blue assay we revealed that this was the case, since TCAM2 cells treated with increasing E2 showed a significantly reduction of cell viability. E2 10 nM in combination with ICI reverted the effect indicating the ERβ involvement as our cells do not express the classical ERα. It was also interesting to show that the effect is potentiated by using a specific agonist of ERβ, the diarylpropionitrile (DPN).

To gain more insight in the biology of testicular tumors by western blotting, we first studied the effect of increasing E2 concentrations on the PI3K/AKT pathway to evaluate if the cell death occurs through the apoptosis. Our data evidenced that E2 was able to decrease AKT expression, concomitantly the FHKR and BAD increased suggesting an apoptotic role of the hormone through the ERβ. However, a faint increase of caspase-9 and PARP-1 cleavages was obtained and DNA fragmentation did not occur presenting instead a smear upon E2 10nM. These results led us to consider other types of cell death.

Since PI3K/AKT pathway is shared with the molecular mechanisms that regulate another type of cell death, ie, autophagy, we wanted to test the influence of E2 on different proteins that are mainly involved in the process of autophagy, such as PI3KIII, Beclin-1, AMBRA and UVRAG. Our results evidenced that E2 increased all these proteins and the effect was abolished by ICI addressing that E2/ER can induce autophagy in our cell type.

The biological counterpart of the autophagy in our context was confirmed by the monodansylcadaverine (MDC) assay and the transmission electron microscopy (TEM).

Our study suggesting a tumor suppressor role for the ERβ in human seminoma, indicates that the mechanisms through which E2 induces cell death in TCAM2 occur mainly through autophagy and not by apoptosis. Concluding, these data support estrogen-dependency of human testicular seminoma and candidate the ERβ-ligands for a therapeutic use in the treatment of this pathological condition.

INTRODUCTION

The most abundant malignancies among male population between the ages of 17 and 45 years are germ cell tumors (GCTs) and its incidence has increased 3-4 fold in the last 50 years. They comprise a heterogeneous group of neoplasms in terms of their histology, marker expression, and age of manifestation. Testicular germ cell tumors of adolescents and adults (TGCTs) can be classified into seminomatous (SE) and nonseminomatous tumors (NSE). Within the testis, three types of GCTs can be diagnosed: type I (teratomas and yolk-sac tumors of neonates and infants); type II (seminomas and nonseminomas); type III (spermatocytic seminomas).

Testicular cancer research continues to modify current therapies aimed to induce cancer cell death and one important cell survival signalling pathway is mediated by phosphoinositide 3-OH kinase (PI3K) and its downstream target AKT that is a serine/threonine protein kinase also known as PKB. A key negative regulator of PI3K/AKT pathway is the tumor suppressor gene phosphatase and tensin homolog deleted on chromosome ten (*PTEN*), (Downes et al., 2007; Stiles et al., 2004), encoding a protein with phospholipid and protein phosphatase activity (Maehama et al., 2001; Simpson and Parsons 2001; Waite and Eng 2002).

PTEN negatively regulates the PI3K (Leslie et al., 2002) through the dephosphorylation in position D3 of phosphatidylinositol 3,4,5-triphosphate (PIP3) and then generating inactive PIP2. PIP3 regulates PDK1, a kinase that in turn phosphorylates and activates AKT (FIG. 1).

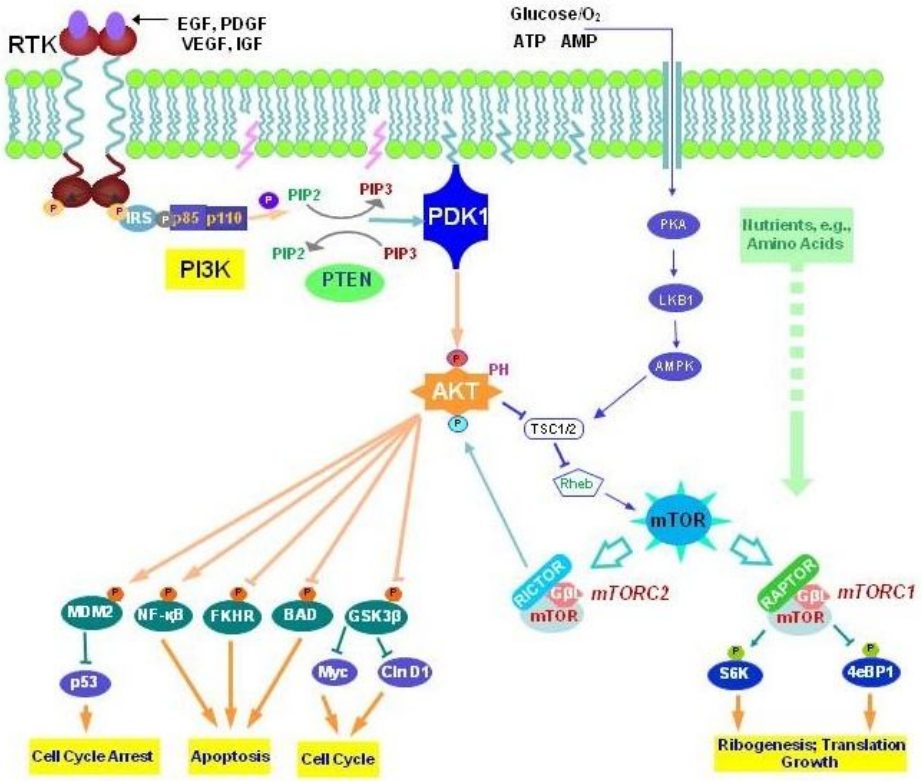

FIG.1: The PI3K/AKT/*PTEN* pathway

Loss of *PTEN* function in embryonic stem cells and human cancer cell lines results therefore in PIP3 accumulation and the activation of its downstream signalling molecule, AKT/PKB. Subsequently, activation of the PI3K/AKT pathway by the loss of *PTEN* stimulates various biological functions, such as cell cycle progression, cell survival and cell migration The tumor suppressor gene *PTEN*, is frequently mutated in human cancers, including brain, breast, endometrial, prostate, and kidney tumors (Cantley and Neel 1999; Simpson and Parsons 2001) and it is abundantly expressed in germ cells whereas it was virtually absent from 56% of seminomas as well as from 86% of embryonal carcinomas, leading to an uncontrolled stimulation of growth and survival signals.

PTEN appears to be essential for germ cell differentiation and an important factor in testicular germ cell tumor formation and primordial germ cells (PGCs), which are the embryonic precursors of gametes (Kimura et al.,

2003). Increased mitotic levels, higher percentages of apoptotic cells, and teratoma formation were observed *in vivo* for *PTEN* mutant male gonads. Despite extensive characterization of *PTEN* mutations in human cancers and relatively good understanding of the molecular roles of *PTEN* in the control of cellular processes, little is known about the modes of *PTEN* gene regulation. Recently, scientists have paid more attention to the regulation of PTEN expression and it was reported that the transcription of *PTEN* could be regulated by p53 and Sp1 (Stambolic et al., 2001; Han et al., 2003). Salvesen *et al*., found that *PTEN* promoter methylation was relatively frequent in endometrial carcinoma (Salvesen et al, 2001). Till now, the regulation of *PTEN* expression is still unclear especially in testicular tumor cells.

SE resembles primordial germ cells or early gonocytes, the cells from which all TGCTs are thought to be derived. The most widely accepted model of TGCTs development proposes an initial tumorigenic event in utero and the development of a precursor lesion known as intratubular germ cell neoplasia undifferentiated (ITGCNU), also known as carcinoma in situ (Skakkebaek, 1972). This is followed by a period of dormancy until after puberty when TGCTs emerge and this suggests that the TGCTs development is hormone dependant.

In addition to the role of androgens, recent studies have demonstrated the importance of estrogen in influencing the male reproductive function (Hess et al., 1997, Sharpe, 1998). It is well-known capacity of the testis to convert androgens into estrogens by the enzyme P450 aromatase. The localization of this enzyme and the role of estrogen in the testis were the subject of considerable interest during the last decade (Carreau et al., 1998). The testicular expression of this enzyme has been demonstrated in several species both in somatic (Sertoli cells and Leydig cells) and in germ cells. It was shown that estrogens are able to stimulate proliferation of rat neonatal

gonocytes *in vitro*, to induce spermatogenesis in the hypogonadal mouse (Li et al., 1997, Ebling et al., 2000). Interestingly, 17ß-estradiol (E2) appears to be a potent germ cell survival factor in the human testis since is able to prevent apoptosis of human adult postmeiotic germ cells cultivated in preserved seminiferous tubules (low concentrations of 17ß-estradiol, 10^{-9} and 10^{-10} mol/L, effectively inhibited male germ cell apoptosis). Conversely, it was shown that E2 is able to inhibit human embryonal carcinoma cell proliferation *in vitro* through an estrogen receptor (ER)β-dependent mechanism suggesting that ERβ acts on germ cells as a tumor suppressor (Roger et al., 2005) according to the observations made in ERβ knockout mice by Delbes *et al.* on neonatal gonocytes (Delbes et al., 2004). In human testis, gonocytes (Gaskell et al., 2003) and most adult germ cells (Mäkinen et al., 2001) express mainly ERβ. However, the precise role of estrogens/ERs (Dupont et al., 2000) and the underlying mechanism(s) in the control and in the biology of testicular tumors remain to be determined.

PI3K/AKT pathway is shared with the molecular mechanisms that regulate another type of cell death. Recent reports show that some previously known oncogenes and tumor suppressor genes impinge upon autophagic pathways in addition to their other well-known functions. Thus, some steps in the malignant transformation could result from the deregulation of these autophagy-related aspects in their mode of action. The *PTEN* tumor suppressor gene, class I PI 3-kinase and AKT oncogenes fall into this category. The tumor suppressor *PTEN*, has been recently shown to promote autophagy in HT-29 colon cancer cells (Arico et al., 2001), where *PTEN* activity blocked the AKT survival pathway and induced autophagy. This type of programmed cell death, the autophagy, is becoming an important area in cancer research. Autophagy is a physiologic process that is involved in degradation and in the turnover of cytoplasmic organelles. However,

progressive autophagy can be cytotoxic and it can substitute, under certain settings, the apoptosis in induction of cell death.

The process of autophagy has been extensively reviewed elsewhere. Briefly, a double membrane vesicle forms in the cytosol that encapsulates whole organelles and bulk cytoplasm. This autophagosome then fuses with the lysosome where the contents are degraded and recycled (FIG. 2).

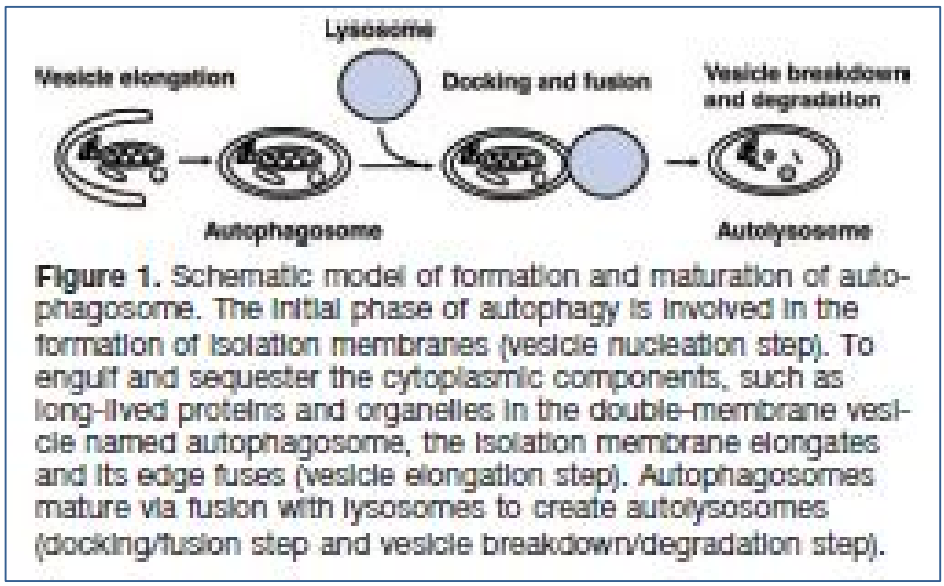

Figure 1. Schematic model of formation and maturation of autophagosome. The initial phase of autophagy is involved in the formation of isolation membranes (vesicle nucleation step). To engulf and sequester the cytoplasmic components, such as long-lived proteins and organelles in the double-membrane vesicle named autophagosome, the isolation membrane elongates and its edge fuses (vesicle elongation step). Autophagosomes mature via fusion with lysosomes to create autolysosomes (docking/fusion step and vesicle breakdown/degradation step).

FIG. 2: Schematic model of formation and maturation of autophagosome

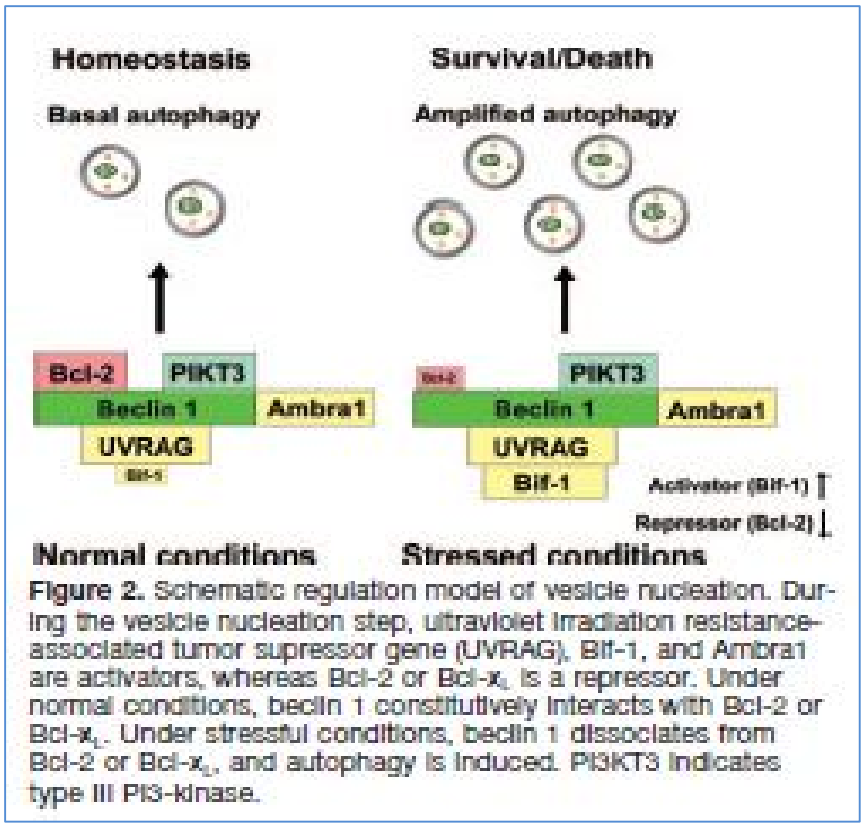

Figure 2. Schematic regulation model of vesicle nucleation. During the vesicle nucleation step, ultraviolet irradiation resistance-associated tumor supressor gene (UVRAG), Bif-1, and Ambra1 are activators, whereas Bcl-2 or Bcl-x_L is a repressor. Under normal conditions, beclin 1 constitutively interacts with Bcl-2 or Bcl-x_L. Under stressful conditions, beclin 1 dissociates from Bcl-2 or Bcl-x_L, and autophagy is induced. PI3KT3 indicates type III PI3-kinase.

FIG. 3: Schematic regulation of vesicle nucleation.

Moreover, loss of autophagy is correlated with tumorigenesis and several inducers of autophagy are tumor-suppressor genes.

Autophagy induction and autophagosome maturation are greatly altered in cancer tissue. The tumor microenvironment is commonly poor in nutrients and oxygen, favouring autophagy induction (Mathew et al., 2007). However, human cancers frequently display inactivating mutations in pro-autophagy genes (e.g. *PTEN*, p53, TSC1, TSC2, Beclin 1, UVRAG, Bif-1) and the activation of anti-autophagy genes (e.g. PI3KCI, AKT, Ras, Bcl-2), suggesting that autophagy functions as an important barrier for cellular transformation (Kroemer and Jaattela 2005, Mathew et al., 2007, Gozuacik D and Kimchi A 2004) (FIG. 3 and 4). However, the relation of autophagy to cancer development is complex and multifaceted, depending on the genetic composition of the cell as well as on the extra-cellular stresses.

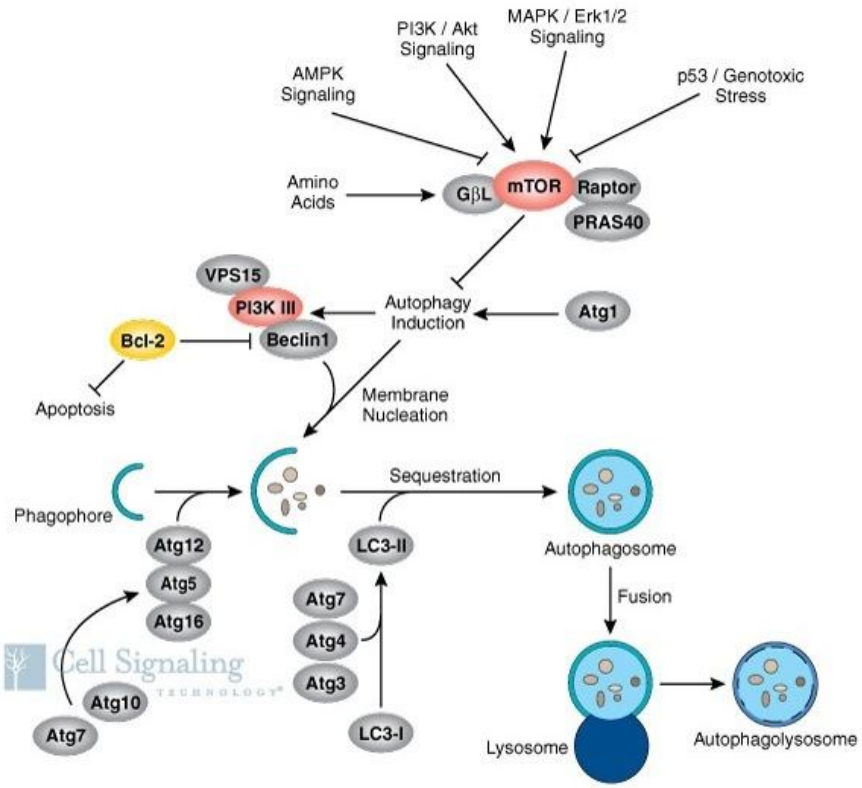

FIG 4: Autophagy signalling

Many lines of evidence also describe a role for autophagy in antagonizing cell survival and promoting cell death, classified as autophagic, or type II, cell death. This discrepancy is partially resolved by the presumption that extensive activation of autophagy might lead to the disintegration of major cellular systems, such that ‘‘the point of no return’’ is crossed and recovery of the cell is unattainable.

In this project we will focalize on SE, by using the TCam-2 cell lines, containing typical features of human seminoma (Eckert et al., 2008). As a functional crosstalk between the ER and *PTEN* was never reported in the literature, we will test increasing E2 concentrations to evaluate eventual effects on the *PTEN* expression as well as the biological significance of this potential link. Three different concentration of E2 will be tested as well as the combination of E2 with ICI 182,780 (ICI) to evaluate the more efficacious dose able to induce the eventual cell death and if the effect is

ER-mediated in human seminoma cell line. Diarylpropionitrile (DPN), specific agonist of ERβ, will be also used.

Results of this study will begin to determine the regulatory mechanisms involved in the progression of the testicular tumors, expanding our existing knowledge concerning the mechanisms which govern estrogen action and the regulation of the *PTEN* gene.

MATERIALS AND METHODS

Mizuno and coworkers reported isolation and characterization of a cell line named TCAM-2 (Mizuno et al., 1993). This cell line originated from a primary lesion of a left testicular seminoma (typical pure type seminoma) of an 35 aged male patient and was generated initially by in vitro culture, and also propagated as xenografts in SCID mice. Using a multidisciplinary approach, it was concluded that TCAM-2 is representative for seminoma (de Jong J. et al., 2008).

Cell cultures - Human TCAM-2 seminoma cell line (a gift from Dr. Leendert H. J. Looijenga Department of Pathology, Erasmus MC-University Medical Center Rotterdam, Josephine Nefkens Institute, TCAM-2 cells were obtained from Sohei Kitazawa (Division of Molecular Pathology, Kobe University, Japan) were grown in RPMI plus 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 200 mM glutamine, at 37 °C in a humidified cell culture incubator with 5% carbon dioxide.

Chemicals - Estradiol (oestra-1,3,5,(10)-triene-3,17β-diol) (E2), the Sp1 specific inhibitor mithramycin A and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Eosin Y was from Farmitalia Carlo Erba. 2,3-bis (4-hydroxyphenyl)-propionitrile - Diarylpropionitrile (DPN) and ICI 182,780 (ICI) were purchased from Tocris chemical (Bristol, UK). Monoclonal mouse antibodies (Abs) to human ER α (F-10), to human Beclin1, to human AMBRA, to human UVRAG, to human PI3KCIII, to human RIP1, rabbit polyclonal Abs to human ER α (D-20), ER β (H-150), β-actin (AC-15), AKT, FKHR, Poly ADP-ribose polymerase-1(PARP-1), normal mouse and rabbit IgG, peroxidase-coupled anti-rabbit and anti-mouse IgG were from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-caspase 9 Ab was from Cell Signaling (Milan, Italy).Salmon sperm

DNA/protein A agarose (UBI, DBA Srl, Milan - Italy) Fugene 6 reagent was from Roche Diagnostics (Mannheim, Germany). ECL system (Amersham Pharmacia, Buckinghamshire, UK). Total RNA Isolation System kit, enzymes, buffers, nucleotides, and 100-bp ladder used for RT-PCR were purchased from Promega Corp. (Milan, Italy). TRIZOL reagent (Invitrogen) Bradford protein assay was performed using a kit from Bio-Rad Laboratories, Inc. (Milan, Italy). E2 and ICI were dissolved in ethanol (0.02% final concentration) and used as solvent controls did not induce any positive result in all in vitro assays (data not shown).

Plasmids **-** The firefly luciferase reporter plasmid containing the full-length of the *PTEN* promoter region [pGL3-2768 (-2 927/-160)] and the different deletion constructs of *PTEN* [pGL3-612 (-1 389/-778), pGL3-341 (-1 118/-778), pGL3-139 (-916/-778)] were kind gifts from Prof. Xi-Liang Zha, (Shanghai Medical College, Fudan University, Shanghai). The ERβ antisense plasmid (AS/ERβ) contains, in reverse orientation, a 1170 bp fragment of the coding sequence of the human ERβ cloned in pIRESpuro2 vector (Clontech). The wild type human ERα expression vector (HEGO) consists of the full-length ERα cDNA fused with the SV40 early promoter and expressed in the pSG5 vector (HeG0, Tora et al.1989). The Renilla reniformis luciferase expression vector used was pRL-Tk (Promega, USA).

Immunoblotting – TCAM2 cells were grown in 10 cm dishes to 70-80 % confluence and exposed to treatments as indicated in 5% dextran coated charcoal (DCC). Cells were then harvested in cold phosphate-buffered saline (PBS) and resuspended in lysis buffer containing 20 mM HEPES pH 8, 0.1mM EDTA, 5mM $MgCl_2$, 0.5M NaCl, 20 % glycerol, 1 % NP-40, inhibitors (0.1mM Na_3VO_4, 1 % PMSF, 20 mg/ml aprotinin). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA USA). A 70µg portion of protein lysates was used for Western Blotting (WB), resolved on a 12 % SDS-polyacrylamide

gel, transferred to a nitrocellulose membrane and probed with the indicated Abs. As internal control, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 minutes at room temperature) of the first antibody and reprobed with anti-β-actin Ab.

The antigen-antibody complex was detected by incubation of the membranes for 1 hour at room temperature with the appropriated secondary antibodies peroxidase-coupled and revealed using the enhanced chemiluminescence system. Blots were then exposed to film. The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assay

TCAM2 cells were grown in 10 cm dishes to 70-80 % confluence and exposed to treatments for 24 hours in 5% DCC. Total cellular RNA was extracted using TRIZOL reagent as suggested by the manufacturer. The purity and integrity were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures. The evaluation of gene expression was performed by RT-PCR method as previously described. For *PTEN* and the internal control gene 36B4, the primers were: 5'CCACCACAGCTAGAACTTATC *-3′* (*PTEN* forward) and 5'ATCTGCACGCTCTATACTGC3′ (*PTEN* reverse), 5’-CTC AAC ATC TCC CCC TTC TC-3’ (36B4 forward) and 5’-CAA ATC CCA TAT CCT CGT CC-3’ (36B4 reverse) to yield respectively products of 700 bp with 25 cycles and 408 bp with 12 cycles. The results obtained as optical density arbitrary values were transformed to percentage of the control (percent control) taking the samples from untreated cells as 100 %.

Transient transfection assay

TCAM2 cells were transferred into 24-well plates with 500 µl of regular growth medium/well the day before transfection. The medium was replaced with 5% dextran coated charcoal (DCC) on the day of transfection, which

was performed using Fugene 6 reagent as recommended by the manufacturer with a mixture containing 0.5 µg of promoter-luciferase reporter plasmids. After 24 hours transfection, treatments were added in 5% DCC as indicated and cells were incubated for further 24 hours. In a set of experimets a pre-treatment for 2 hours was performed in the presence of mithramycin and ICI. Empty vectors were used to ensure that DNA concentrations were constant in each transfection and pRL-Tk (25 ng/well) was used to normalize the transfection efficiency. Firefly and Renilla luciferase activities were measured using the Dual Luciferase Kit (Promega). The firefly luciferase values of each sample were normalized by Renilla luciferase activity and data were reported as Relative Light Units (RLU) values. All the luciferase assays were carried out at least in triplicate, and the experiments were repeated thrice.

Chromatin immunoprecipitation (ChIP) and Re-ChIP assays

For ChIP assay, TCAM2 cells were grown in 10 cm dishes to 50-60 % confluence, shifted to 5% DCC for 24 hours and then treated with 10 nM E2 for 1 hour or pre-incubated with mithramycin for 1 hour where required. Thereafter, cells were washed twice with PBS and crosslinked with 1 % formaldehyde at 37 °C for 10 minutes. Next, cells were washed twice with PBS at 4 °C, collected and resuspended in 200 µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1) and left on ice for 10 minutes. Then, cells were sonicated four times for 10 seconds at 30 % of maximal power (Sonics, Vibra Cell 500 W) and collected by centrifugation at 4 °C for 10 minutes at 14,000 rpm. The supernatants were diluted in 1.3 ml of IP buffer (0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80 µl of sonicated salmon sperm DNA/protein A agarose for 1 hour at 4 °C. The precleared chromatin was immunoprecipitated with anti-ER Ab and re-immunoprecipitated with anti-RNA Pol II Ab (Santa Cruz Biotechnology).

At this point, 60 µl salmon sperm DNA/protein A agarose were added and precipitation was further continued for 2 hours at 4 °C. After pelleting, precipitates were washed sequentially for 5 minutes with the following buffers: Wash A (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), Wash B (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1 % NP-40, 1 % sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immunocomplexes were eluted with elution buffer (1 % SDS, 0.1 M $NaHCO_3$). The eluates were reverse crosslinked by heating at 65 °C and digested with proteinase K (0.5 mg/ml) at 45 °C for 1 hour. DNA was obtained by phenol/chloroform/isoamyl alcohol extraction. 2 µl of 10 mg/ml yeast tRNA (Sigma) were added to each sample and DNA was precipitated with 70 % ethanol for 24 hours at –20 °C, and then washed with 95 % ethanol and resuspended in 20 µl of TE buffer. A 5 µl volume of each sample was used for PCR amplification with the following primers flanking a sequence of *PTEN* promoter: 5'- CAC GCT CGG CTG AGA GCT TTC AT -3' (forward) and 5'- CTT CCC CCA AAT CTG TGT CCT CA – 3'(reverse) corresponding to the -1398 to -1111 region (Gene Bank, accession number: AF067844). The PCR conditions were 45 seconds at 94 °C, 40 seconds at 58 °C, and 90 seconds at 72 °C. The amplification products obtained in 30 cycles were analysed in a 2 % agarose gel and visualized by ethidium bromide staining. The negative control was provided by PCR amplification without DNA sample. Samples pretreated with mytramicin or trasfected with DN/ERb were also used as control.

Electrophoretic Mobility Shift Assay (EMSA)

TCAM2 cells plated into 10 cm dishes were grown to 70-80 % confluence shifted to SFM for 24 hours and then treated with increasing concentrations of E2 (1 nM, 10 nM, and 100 nM) or with 100nM ICI or with 100nM ICI

combined with 10 nM E2, for 6 hours. Thereafter, cells were scraped into 1.5 ml of cold PBS. Cells were pelleted for 10 seconds and resuspended in 400 µl cold buffer A (10 mM HEPES-KOH pH 7.9 at 4 °C, 1.5mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) by flicking the tube. The cells were allowed to swell on ice for 10 minutes and then vortexed for 10 seconds. Samples were centrifuged for 10 seconds and the supernatant fraction discarded. The pellet was resuspended in 50 µl of cold Buffer B (20 mM HEPES-KOH pH 7.9, 25 % glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) and incubated in ice for 20 minutes for high-salt extraction. Cellular debris were removed by centrifugation for 2 minutes at 4 °C and the supernatant fraction (containing DNA binding proteins) was stored at –70 °C. The probe was generated by annealing single stranded oligonucleotides and labeled with ^{32}P-ATP (Amersham Pharmacia) and T4 polynucleotide kinase (Promega) and then purified using Sephadex G50 spin columns (Amersham Pharmacia). The DNA sequence, containing multiple Sp1sites, obtained from the native *PTEN* promoter gene used as probe or as cold competitor is the following: F 5'- GAG AGG TGG GGC GCT GCA AGG GAG CCG GA and R 5'-TCC GGC TCC CTT GCA GCG CCC CAC CTC TC. The protein binding reactions were carried out in 20 µl of buffer 20 mM Hepes pH 8, 1 mM EDTA, 50 mM KCl, 10 mM DTT, 10% glicerol, 1mg/ml BSA, 50 µg/ml poly dI/dC with 50,000 cpm of labeled probe, 5 µg of TCAM2 nuclear protein and 5 µg of poly (dI-dC). The mixtures were incubated at room temperature for 20 minutes in the presence or absence of unlabeled competitors oligonucleotides. For the experiments involving the anti-ERβ, anti-ERα and anti-Sp1 Abs (Santa Cruz Biotechnology), the reaction mixture was incubated with Ab at 4 °C for 30 minutes before addition of labeled probe. The entire reaction mixture was electrophoresed through a 6 % polyacrylamide gel in 0 .25 X Tris

borate-EDTA for 3 hours at 150 V. Gel was dried and subjected to autoradiography at –70 °C.

Cell viability assay

TCAM2 were seeded (100 000 cells/well) in a complete RPMI medium in six-well plates and become attached to the bottom of the well overnight. On the second day, the medium was changed and shifted for 24 h with 5% DCC. At the end of this time, the cells were treated with increasing concentrations of E2 (1 nM, 10 nM, and 100 nM) or with 100 nM ICI or with 100 nM ICI combined with 10 nM E2, and incubated for 24 h in the incubator at 37 °C. At the end of this period the cells were harvested by trypsinization and counted in a hemocytometer using trypan blue exclusion assay (0.2% trypan blue). The results were expressed as percentage of the controls, determined by standardizing untreated cells to 100%.

DNA Fragmentation assay

DNA fragmentation was determined by gel electrophoresis. TCAM2 cells were grown in 10-cm dishes to 80% confluence and treated with with increasing concentrations of E2 (1 nM, 10 nM, and 100 nM) or with 100 nM ICI or with 100 nM ICI combined with 10 nM E2. After 24 h cells were collected and washed with PBS and pelletted at 1800 rpm for 5 min. The samples were resuspended in 0.5 ml of extraction buffer (50 mM Tris-HCl, pH 8; 10 mM EDTA, 0.5% SDS) for 20 min in rotation at 4 C. DNA was extracted with phenol-chloroform three times and once with chloroform. The aqueous phase was used to precipitate acids nucleic with 0.1 vol 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 H_2O 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 (40 µg/lane)

was subjected to electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide and then photographed.

Monodansylcadaverine (MDC) Staining

The MDC is an autofluorescence substance able to selectively mark the autophagosomes. A 10 mM stock solution of MDC was prepared in PBS. Following treatment with with increasing concentrations of E2 (1 nM, 10 nM, and 100 nM) or with 100nM ICI or with 100nM ICI combined with 10 nM E2, for 24 h, cells were stained with MDC at a final concentration of 10 µM for 10 min at 37 °C, and fixed using 3% paraformaldehyde solution in PBS for 30min. Cells were washed and then examined by fluorescence microscopy (Leica AF2006).

Trasmission Electron Microscopy (TEM)

The transmission electron microscopy (TEM) allows the study of ultrastructural details of a biological sample. For routine transmission electron microscopy, cells were fixed in 3% glutaraldehyde solution in 0.1 M phosphate buffer (pH. 7.4) for 2 h at 4 °C. Then the samples were postfixed in osmium tetroxide (3%) for 2 h, dehydrated in graded acetone, and embedded in Araldite (Fluka, Buchs, Switzerland). Ultrathin sections were prepared using a diamond knife, collected on copper grids and contrasted using both lead citrate and uranyl acetate. The grids were examined in a “Zeiss EM 10” electron microscope.

STATISTICAL ANALYSIS

The data obtained (six replicate experiments using duplicate determinations) are presented as the mean±SEM. The differences in mean values were calculated using ANOVA with Newman–Keuls *post hoc* test. Values of $P<0.05$ were taken to show a significant difference between means.

RESULTS

ERs expression in human seminoma cells

Although an extensive characterization of the TCam-2 cell line to prove that it is representative for human seminoma was performed, the presence of the ERs was never reported. As it concerns the classical ERα, the protein was not detectable in our cells compared with MCF-7, a human breast cancer cell line used as positive control, neither by using an anti-ERα Ab raised against the carboxy-terminal part of ERα (Fig. 5A), nor with an Ab recognizing the amino-terminal part of ERα Fig 5B. On the contrary, an intense band was detected by Western blotting at 60 kDa (Fig. 5C) corresponding to the molecular mass of the ERβ long form.

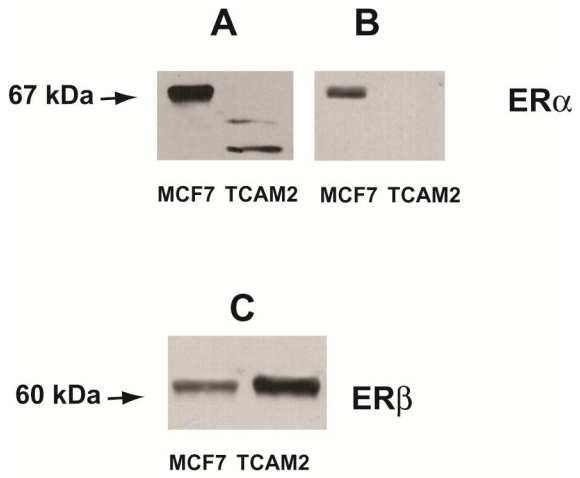

FIG.5:Immunoblots of estrogen receptors (ERα, ERβ) from protein extracts of TCam2

Western blot analysis of proteins showed expression of the estrogen receptors (ERs). Extracts of Tcam2 protein were subjected to electrophoresis on 12% SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and probed with mouse Ab to human ERα (A), rabbit polyclonal Abs to human ERα (B) and ERβ (C). MCF-7 extracts were used as controls. The number on the left corresponds to molecular masses (kilodaltons) of the marker proteins. The experiments were repeated at least six times, and the autoradiographs of the figure show the results of one representative experiment.

PTEN protein and mRNA increased in response to E2

The precise role of estrogens/ERs and the underlying mechanism(s) in the control and in the biology of testicular tumors remain to be determined (Dupont et al. 2000). Despite a relatively good understanding of the molecular roles of *PTEN* in the control of cellular processes, little is known about modes of *PTEN* gene regulation. In order to evaluate a potential functional cross-talk between E2/ERs and *PTEN* we studied the effect of increasing concentrations of E2 on *PTEN* expression. Interestingly, E2 upregulated the *PTEN* protein expression in a dose-dependent manner from 1 nM to 10 nM, while 100 nM did not give significantly effects (Fig.6 A).

Next, we investigated the mRNA expression of *PTEN*, and it was induced by E2 in a similar pattern as it was obtained for the protein (Fig. 6 B). ICI failed to cause an increase in *PTEN* protein and mRNA levels, suggesting a direct involvement of the ER in mediating this effect.

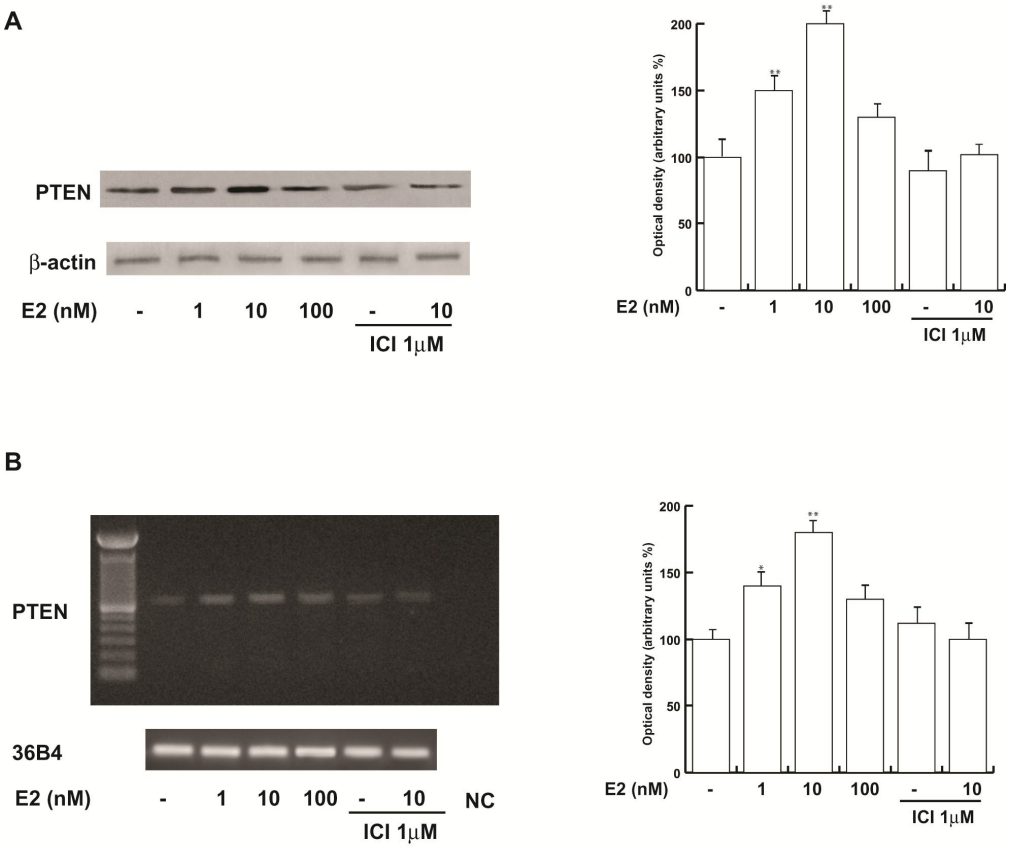

FIG. 6: E2 up-regulates *PTEN* protein and mRNA expression in TCAm2 cells.

(A) Immunoblots of *PTEN* from TCam2 cells treated for 24 h with vehicle (-), increasing E2 (1,10,100 nM) concentrations, 1μM ICI alone or in combination with 10 nM E2. b-actin was used as loading control. The side panel shows the quantitative representation of data (mean ± S.E.M.) of three independent experiments including that of A. (B) Semiquantitative RT-PCR evaluation of *PTEN* mRNA expression. TCam2 cells were treated as in A. 36B4 mRNA levels were determined as control. The side panel shows the quantitative representation of data (mean ± SEM) of six independent experiments including that of B after densitometry and correction for 36B4 expression. *P<0.05 E2-treated vs untreated cells; **P<0.01 E2-treated vs. untreated cells

E2 Transactivates the *PTEN* Promoter

To investigate whether one of the mechanisms involved in the ER-mediated increase of *PTEN* expression could be a direct modulation of the transcriptional activity of its promoter, transient trasfection assays were performed in TCAM2 cells. Luciferase reporter constructs containing a series of deletion mutants of the human *PTEN* promoter gene were used to more precisely define the effect of E2 on *PTEN* transcription. A construct contains the fragment of the 5'-flanking region from human *PTEN* gene, corresponding to the region spanning from bases -2927/-160 bp (pG13-2768, full length). Further deletions of the 5'-flanking region and a deletion of the 5'-untranslated region from *PTEN* gene were present in the following constructs: -1389/-778 (pG13-612), -1118/-778 (pG13-341), -916/-778 (pG3-139) (Fig. 7A).

The results shown in Fig. 7B indicated that transcription from the construct pG13-2768 was detectable, particularly it was enhanced by 1 nM E2 while it decreased upon 10 nM and 100 nM E2. The plasmid pGL3-2768 (-2 927/-160), which contained full-length promoter, appeared to have lower activity than that of pGL3-612 (-1 389/-778), suggesting the presence of potential negative regulatory regions located within the sequence spanning from -777/-160. In fact, by using the pG13-612 construct, the promoter activity increased upon E2 treatment in a dose-dependent manner, while this effect was abrogated by ICI, indicating that *PTEN* promoter activation by E2 requires the presence of a functional ER (Fig. 7B). E2 failed to enhance the transcriptional activity of the *PTEN* promoter when cells were transfected with pG13-341 and pG3-139 constructs, suggesting that the segment located between -1398 and –1118 relative to the translation initiation site is important for E2-responsiveness.

By analyzing this DNA sequence to search potential binding sites for transcription factors nucleotide sequences, we observed that the

aforementiond region contains several DNA motifs known to bind the transcription factor Sp1. Mithramycin is a drug able to bind to GC boxes and then to inhibit Sp1 binding selectively blocking mRNA synthesis from genes that contain functional

recognition sites both in vitro and in vivo (Blume et al., 1991). As shown in Fig. 7C, in TCAM2 cells transfected with pG13-612 construct and treated with mithramycin, the *PTEN* transactivation upon 10 nM E2 was prevented. Altogether these data strongly suggest that Sp1 plays an important role in the transcriptional regulation of *PTEN* by E2/ER.

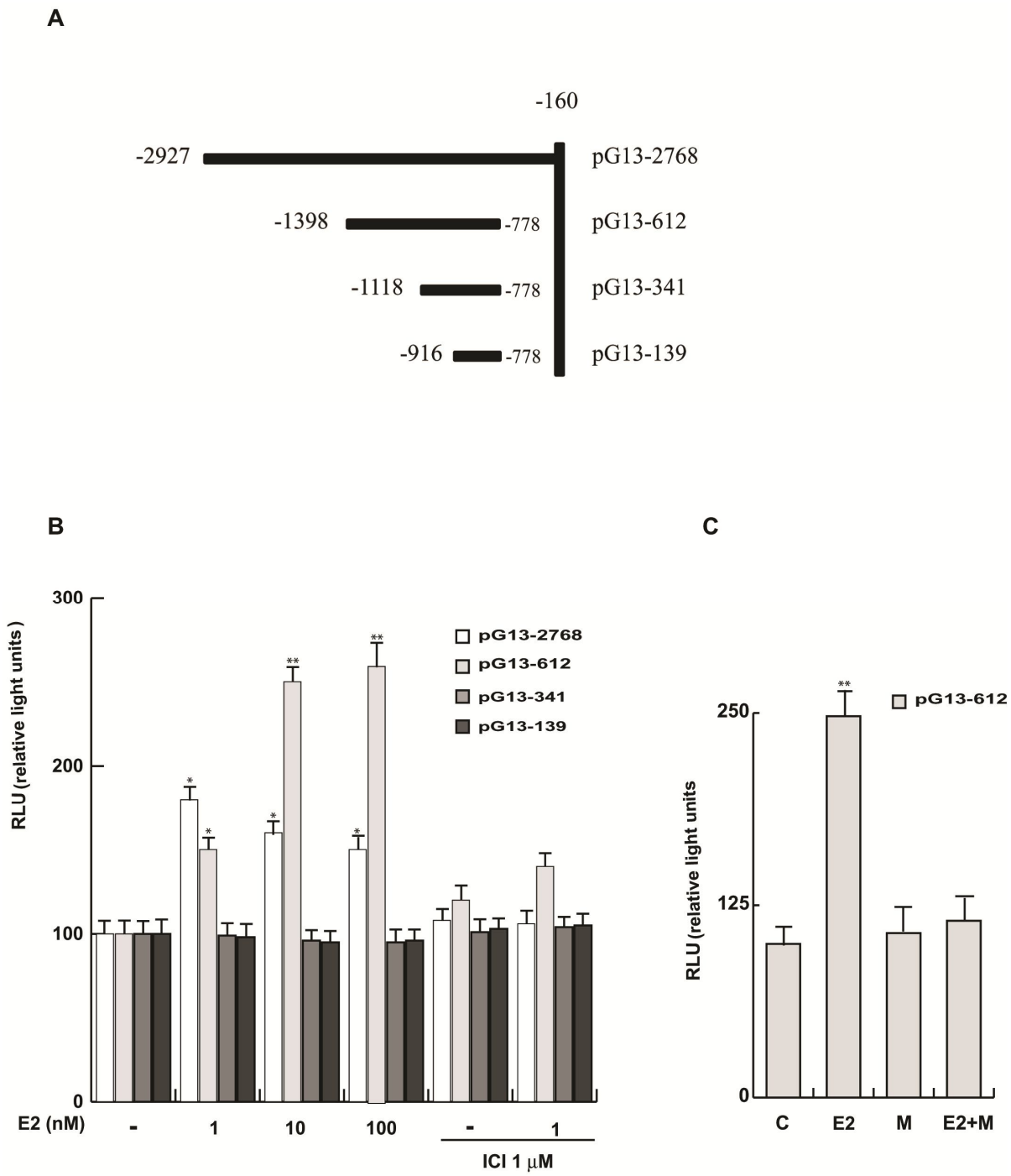

FIG. 7: E2 transactivates *PTEN* promoter gene in TCam2 cells.
Schematic map of the *PTEN* promoter fragments used in this study. (B) TCam2 cells were treated for 24 h with vehicle (-), increasing E2concentrations, 1μM ICI alone or in combination with 10 nM E2. (C)TCam2 cells were treated for 24 h with vehicle (-), 10 nM E2, 100 nM mithramycin (M) alone or in combination with 10 nM E2. The luciferase activities were normalized to the Renilla luciferase as internal transfection control and data were reported as RLU values. Columns are mean ± SEM of six independent experiments performed for six times. *P<0.05 E2-treated vs untreated cells, **P<0.01 E2-treated vs untreated cells. RLU, Relative Light Units.

Functional interaction of ERβ with *PTEN* by chromatin immuno-precipitation (ChIP) assay

The interaction of ERβ with the *PTEN* gene promoter was further investigated by ChIP assay. TCam2 chromatin was immunoprecipitated with the anti-ERβ Ab and then reprecipitated with the anti-RNA-Pol II or anti-Sp1 Abs. PCR was used to determine the recruitment of ERβ to the PTEN region containing the Sp1 sites. The results indicated that ERβ was weakly constitutively bound to the *PTEN* promoter in untreated cells and this recruitment was increased upon E2 treatment (Fig.8). In addition, by Re-ChIP assays an increased association of Sp1 was obtained. Particularly the augmented RNA-Pol II recruitment indicated that a positive regulation of *PTEN* transcriptional activity was induced by E2 (Fig.10). The physical interaction of ERβ and Sp1 proteins to our sequence was strengthened by the absence of amplification in samples trasfected with the DN/ERβ or treated with mytramicin (Fig.8). The negative control was performed by PCR amplification without DNA sample.

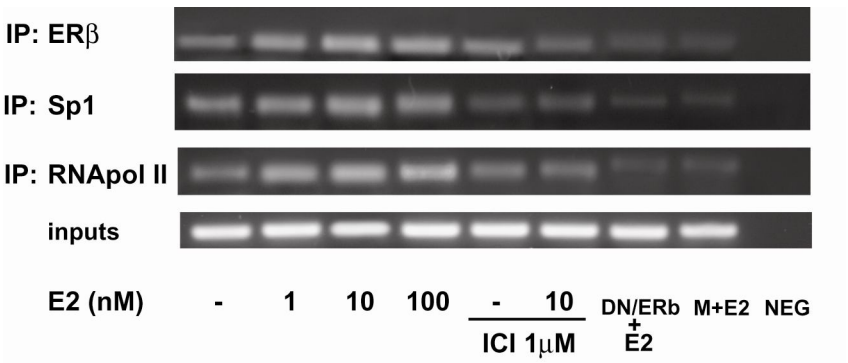

FIG. 8: **Functional interaction between ERβ and *PTEN* promoter.**
TCam2 cells were treated as indicated, then cross-linked with formaldehyde and lysed. The soluble chromatin was immunoprecipitated with the anti-ERβ Ab, (Re-ChIP with the anti-RNA Pol II and anti-Sp1 Abs). The *PTEN* promoter sequence containing Sp1 was detected by PCR with specific primers, as described in Materials and Methods. To control input DNA, *PTEN* promoter was amplified from 30 μl of initial preparations of soluble chromatin (before immunoprecipitations). M: mithramycin. NEG is a negative control provided by PCR amplification without DNA sample.

ERβ interacts with *PTEN* promoter gene through Sp1 site in EMSA

To gain further insight into the mechanism involved in the *PTEN* transactivation induced by E2, we performed EMSA using a synthetic oligodeoxyribonucleotide which contains the E2-responsive region of the *PTEN* promoter identified and containing the Sp1 binding motifs. We tested if E2-action on the above identified *PTEN* promoter region occurs through the interactions of ERβ and /or Sp1.

In nuclear extracts from TCam2 cells we observed the formation of a specific complex (Fig 9, lane 1) which was increased upon 10 nM E2 treatment (lane 3). The binding was abrogated by 100 fold molar excess of unlabelled probe (lane 2), demonstrating the specifity of the DNA-binding complex.

To confirm that *PTEN* transcription induced by E2 in TCam2 cells is regulated by Sp1, nuclear extracts were incubated with mithramycin and in the reaction mixture strongly reduced the intensity of the bands (Fig. 9 lane 4). The involvement of ERβ and Sp1 in the DNA-binding complexes was confirmed by using the specific anti-ERβ (lane 5) and anti-Sp1 Abs (lane 6), since both induced supershift and immunodepletion of the bands. By using the anti-ERα Ab no difference with respect the non treated sample was observed (lane 7). The ERβ and the Sp1 synthesized in vitro (lanes 8 and 9 respectively) demonstrated that the binding to our probe occurs through the Sp1. In control samples (IgG),normal mouse or rabbit IgG, respectively, was used instead of the primary Abs as control of Ab specificity. Lane 11 containing probe alone did not show any complex

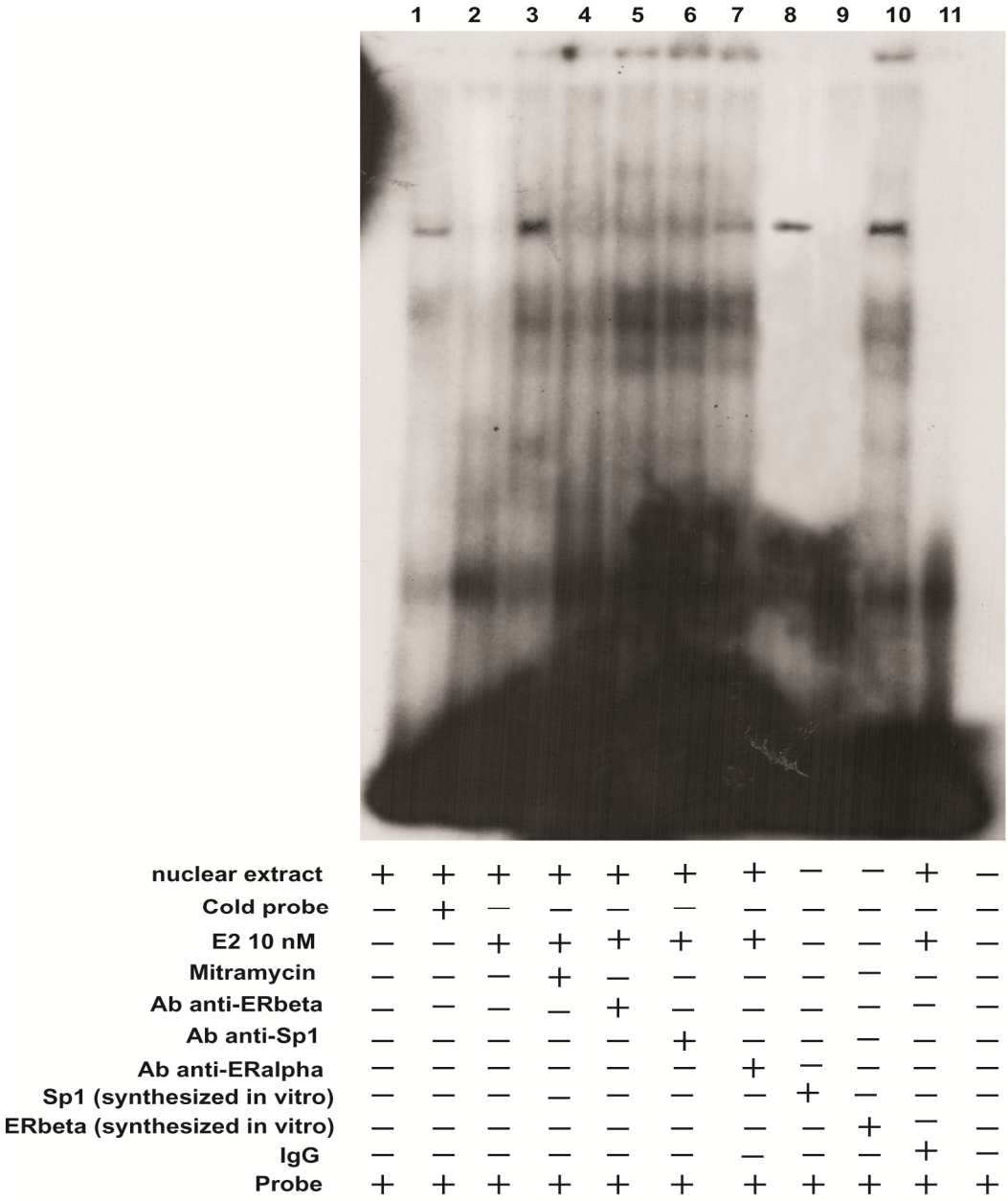

FIG. 9: ERβ binds through the Sp1 within the *PTEN* promoter region in EMSA. Nuclear extracts from TCam2 cells (lane 1) were incubated with a labeled probe or not labeled used as cold competitor containing bases, obtained from the native *PTEN* promoter gene from: F 5'- GAG AGG TGG GGC GCT GCA AGG GAG CCG GA and R 5'-TCC GGC TCC CTT GCA GCG CCC CAC CTC TC and subjected to electrophoresis in a 6% polyacrylamide gel. In lane 3, nuclear extracts from *PTEN* were treated with 10 nM E2. Competition experiments were performed adding as competitor a 100-fold molar excess of unlabeled *PTEN* probe (lane 2) or 100 nM mithramycin (lane 4). Anti-ERβ and anti-Sp1 Abs were incubated with nuclear extracts from TCam2 cells treated with 10 nM E2 (lanes 5 and 6, respectively). In lanes 8 and 9 the ERβ and the Sp1 synthesized in vitro respectively, were incubated with the probe alone. In control samples (IgG),normal mouse or rabbit IgG, respectively, was used instead of the primary Abs as control of Ab specificity. Lane 11 contains probe alone.

E2 induces cell death in TCAM2 cells

PTEN negatively regulates cell growth and survival in different cell types. Therefore, we studied if the functional significance of the E2/ERβ/*PTEN* link may consist in the modulation of TCAM2 cell survival by trypan blue exclusion assay. Our cells exposed to increasing E2 exhibited a dose–responsive decrease in cell viability after 24 hours. Lower E2 concentration did not induce significant changes, while 10 nM and 100 nM E2 produced a significant decrease (Fig. 10). Cells treated with increasing concentration of a specific ERβ agonist, DPN evidenced an earlier decrease of the cell viability from 1 nM DPN.

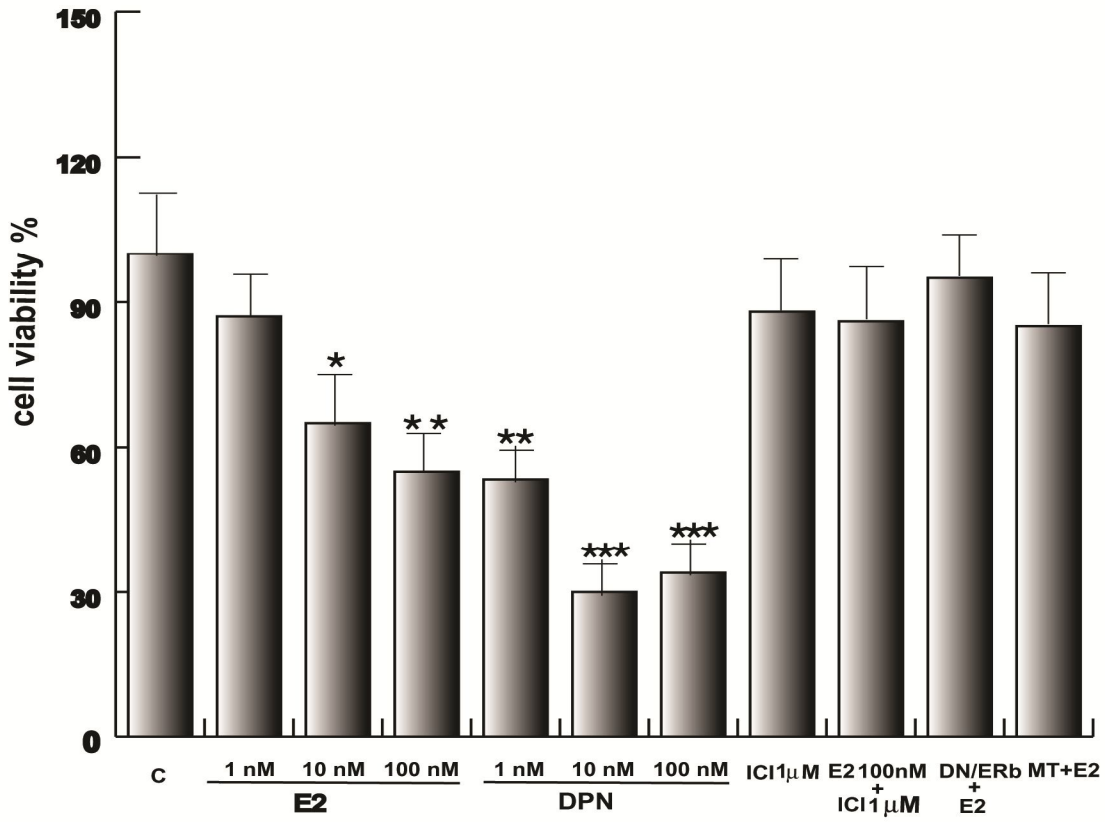

FIG. 10: E2 induces cell death in TCAM2 cells and DPN potentiates the effect. The viability of TCam2 cells decreased significantly at 10 and 100 nM of E2. The effect results higher with increasing concentration of DPN. Treatment of cells with ICI alone or in combination with E2 as like as DN/ERβ trasfected cells and mytramicin in combination with E2 does not affect the cell viability. Columns are mean ± SEM of six independent experiments. *P<0.05 E2-treated vs untreated cells, **P=0.01 E2-treated vs untreated cells. ***P<0.01 E2-treated vs untreated cells.

ERβ is Required for E2-Mediated effects in TCAM2 cells

The *PTEN* protein dephosphorylates position D3 of phosphatidylinositol 3,4,5-triphosphate (PIP3) and generates inactive PIP2. PIP3 is a direct product of PI3K that regulates and activates AKT, and then *PTEN* is a key negative regulator of the AKT activity. Therefore, we first studied whether E2 stimulation of TCAM2 has some effect on AKT expression.

As shown in Fig. 11 the AKT content diminished particularly at 10 nM E2, while the combination of ICI and E2 restored AKT levels, addressing an ER-mediated effect. In this manuscript we demonstrated that TCAM2 cells express ERβ and not the classical ERα, however to sustain that the E2 action in this context is mediated by ERβ we transfected the cells with DN/ERβ and then treated for 24 hours as previously indicated. As expected in the absence of ERβ no difference in the AKT content was observed (Fig. 11) among the different treatments. On the contrary, in the presence of ectopic classic ERα (cells trasfected with HEGO), a dose-dependent increase in the Akt content was obtained upon E2.

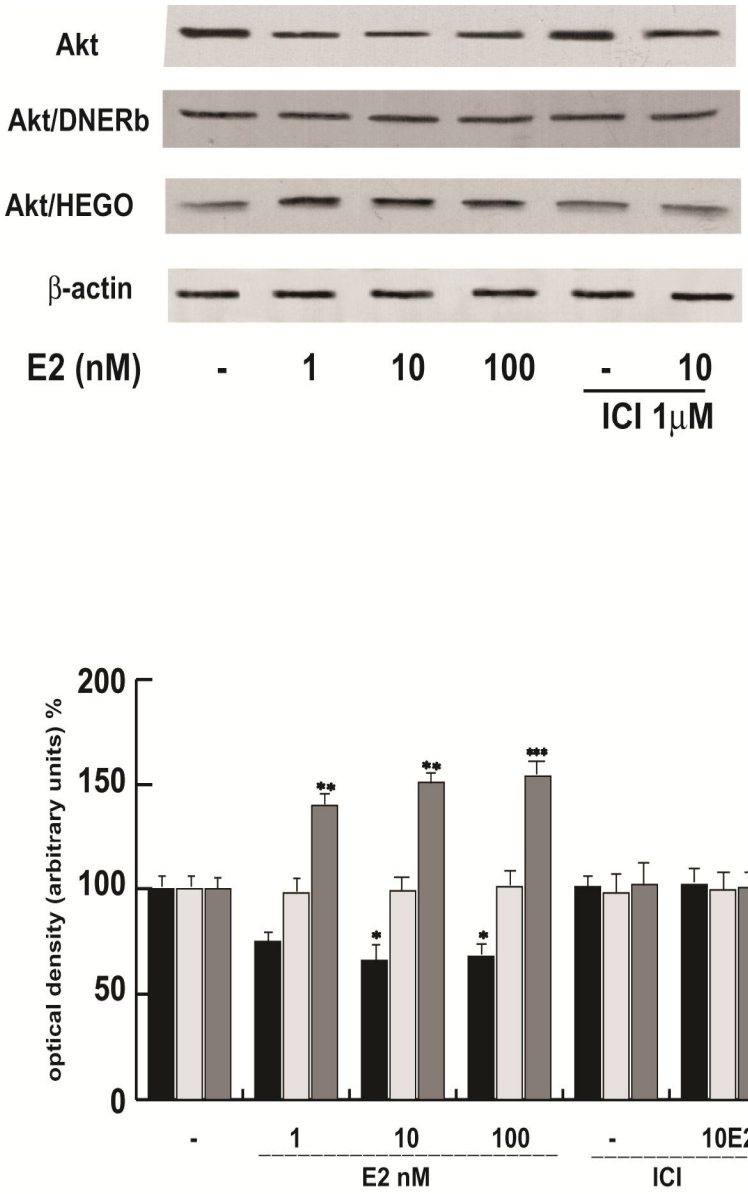

FIG. 11: E2 effects on AKT

Immunoblots of AKT from TCam2 cells treated for 24 h with vehicle (-), increasing E2 (1,10,100 nM) concentrations, 1μM ICI alone or in combination with 10 nM E2. β-actin was used as loading control. AKT not revealed difference in TCam 2 cells transfected with DN/ERβ while results increased in cells transfected with pSG5-HEGO encoding ERα. The lower panel shows the quantitative representation of data (mean ± S.E.M.) of six independent experiments . Columns are mean ± SEM of six independent experiments. *P<0.05 E2-treated vs untreated cells, **P=0.01 E2-treated vs untreated cells. ***P<0.01 E2-treated vs untreated cells.

E2 effects on some markers related to the apoptosis.

PTEN acts as a tumor suppressor by antagonizing PI3K, which activates the AKT that in turn activates proliferative and antiapoptotic signaling pathways. A number of downstream targets of AKT have been described, including the proapoptotic proteins BAD and FKHR. The amount of these two proteins increased upon E2 treatment and the effect was abrogated by ICI (Fig. 12A). These data may indicate that E2 induces apoptosis in TCAM2 cells, therefore we evaluated the effects of the steroid on different markers of the apoptosis such as caspase9, PARP1 and DNA fragmentation. During apoptosis, caspase-7 and caspase-3 cleave PARP1 into two fragments: p85 and p24, inactivating the protein (Tewari et al., 1995; Germain et al., 1999). PARP1 cleavage aims at preventing the activation of PARP1 by the ensuing DNA fragmentation and thereby preserving cellular energy for certain ATP sensitive steps of apoptosis.

It appears from the panel A and B in the Fig. 12 C that faint cleavages occurred both for caspase9 and PARP1 upon E2 treatments. DNA degradation is considered a diagnostic hallmark of cells undergoing apoptosis. Unfortunately, no DNA fragmentation was obtained under E2 treatment in TCam2 cells instead a DNA smear occurred. The smear was not observed when we treated the cells with ICI or mytramicin.

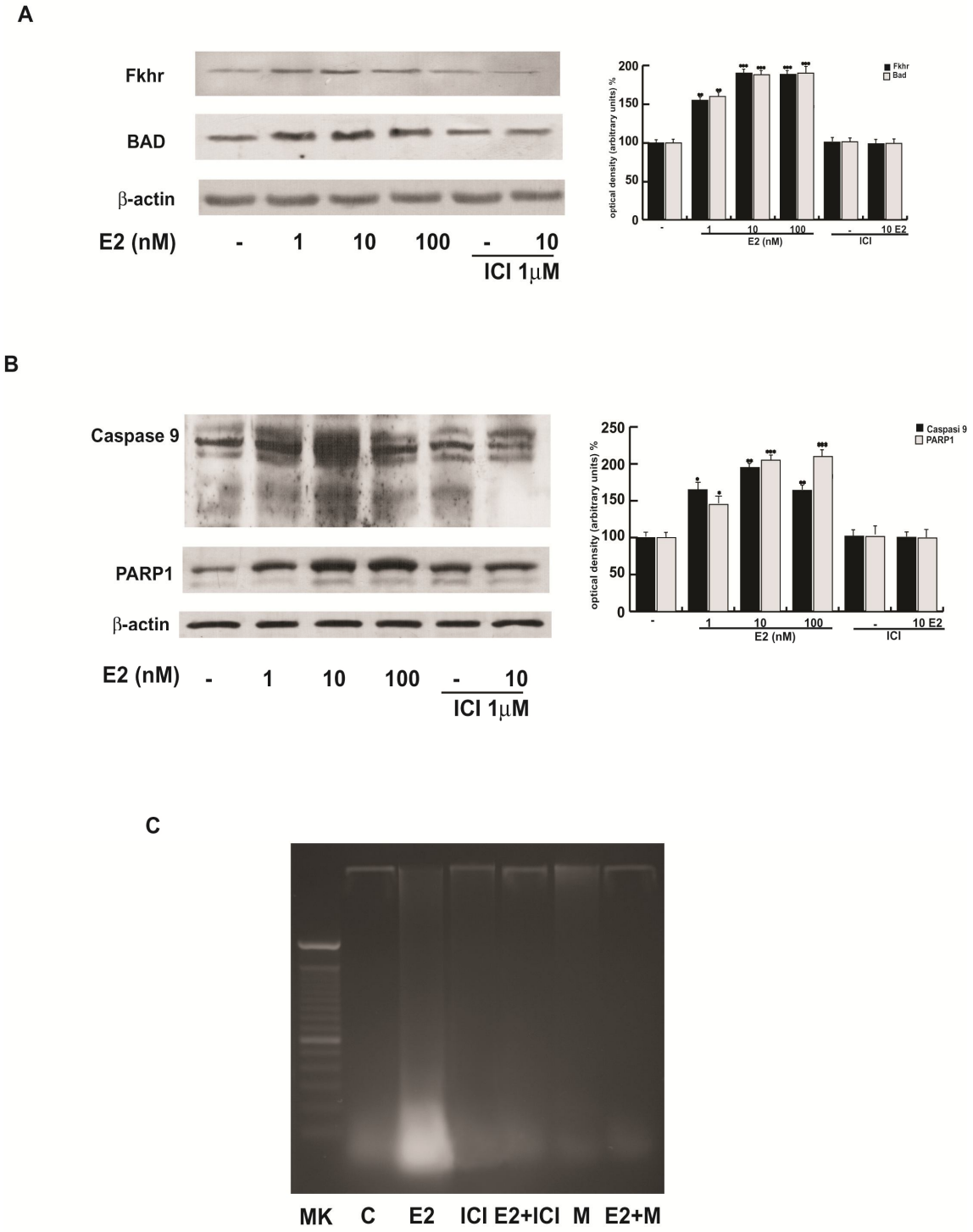

FIG. 12: E2 did not induces apoptosis in TCAM2 cells.
TCam2 cells were treated for 24 h as indicated. Fkhr and BAD (A) protein expression (evaluated by WB) in TCam2 cells increased upon increasing concentration of E2. b-actin was used as loading control. Caspase 9 and PARP 1 and their faint cleavage product are evidenced upon E2 (B).One of three similar experiments is presented. β-actin was used as loading control on the same stripped blot. The side panels show the quantitative representation of data (mean ± SEM) of six independent experiments. Columns are mean ± SEM of six independent experiments (C) DNA laddering was performed in TCAM2 cells treated for 24h as indicated.. *P<0.05 E2-treated vs untreated cells, **P<0.01 E2-treated vs untreated cells.

However to investigate if the selective ligand of ERβ was able to enhance cell death in human seminoma cells, we used increasing DPN. In Fig. 13 it is possible to note that DPN potentiated the effect of E2 both on AKT and PTEN expression, this also occurs in the panel B with casapse 9 and PARP1.

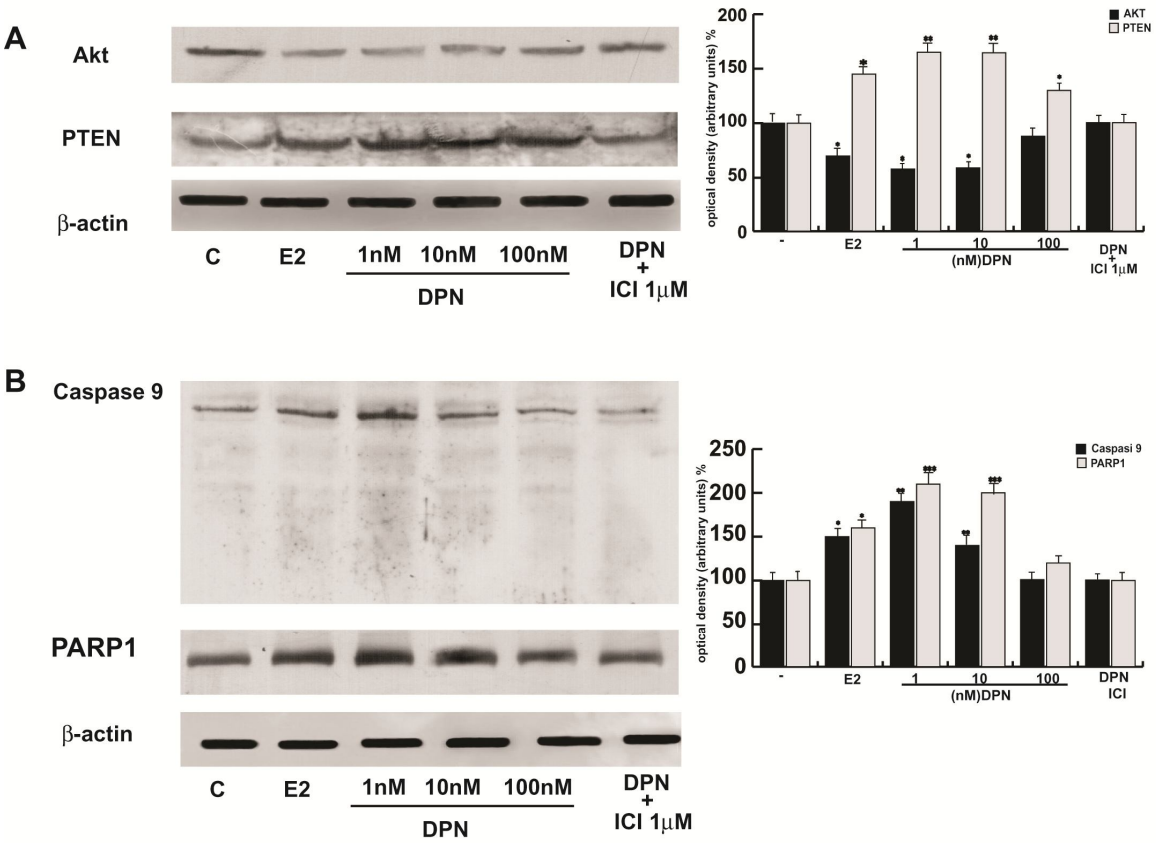

FIG. 13: DPN potentiates the effect of E2 in human seminoma cells
By Western Blot on protein extracts from TCam2 cells as previously described and treated as indicated, selective ERβ agonist DPN potentiate the effect of E2 both on AKT and *PTEN* expression (A), this also occurs in the panel B with caspase9 and PARP1 (B). To verify equal loading, the membranes were stripped and probed with an antibody against β-actin. The side panels show the quantitative representation of data (mean ± SEM) of six independent experiments. Columns are mean ± SEM of six independent experiments. *P<0.05 E2-treated vs untreated cells, **P=0.01 E2-treated vs untreated cells. ***P<0.01 E2-treated vs untreated cells,

E2 induces autophagy in TCAM2 cells

In order to define the type of cell death in our context and as the autophagy converge also at the PI3K/AKT signalling we examined if E2/ERβ induced autophagy in TCAM2 cells. First, we evaluated the occurrence of this other programmed cell death at molecular level, by analyzing important marker such as PI3KIII, Beclin1, UVRAG and AMBRA that have a prevalent role in the first phases of the autophagic process. TCAM 2 were treated for 24 hs with increasing concentration of E2 (1, 10, 100 nM) and with 1 μM ICI alone or in combination with 10nM E2. All the proteins involved in the autophagic pathway increase upon E2. As control we treated cells trasfected with DN/ERβ evidencing that the effect did not occur (Fig. 14).

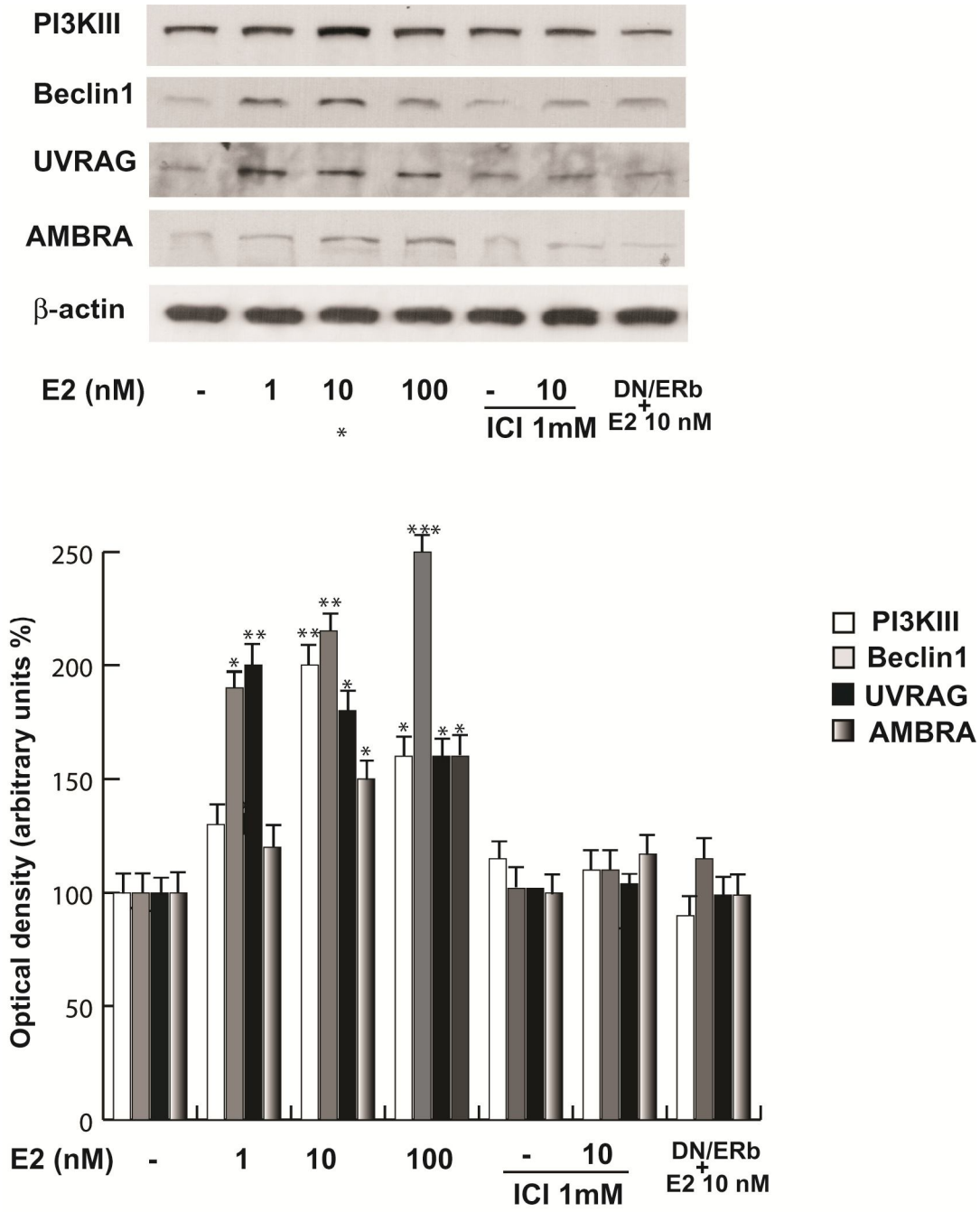

FIG. 14: E2 induces some hallmarkers proteins of autophagy

Western blot analysis of PI3KIII, Beclin1, UVRAG and AMBRA from TCam2 cells treated for 24 h with vehicle s (-), increasing E2 (1,10,100 nM) concentrations, 1μM ICI alone or in combination with 10 nM E2 and cells trasfected with DN/ERβ. b-actin was used as loading control. The lower panel shows the quantitative representation of data (mean ± SEM) of six independent experiments. Columns are mean ± SEM of six independent experiments. *P<0.05 E2-treated vs untreated cells, **P=0.01 E2-treated vs untreated cells. ***P<0.01 E2-treated vs untreated cells,

An increased effect by DPN with respect to E2 was also tested on authophagy (Fig. 15).

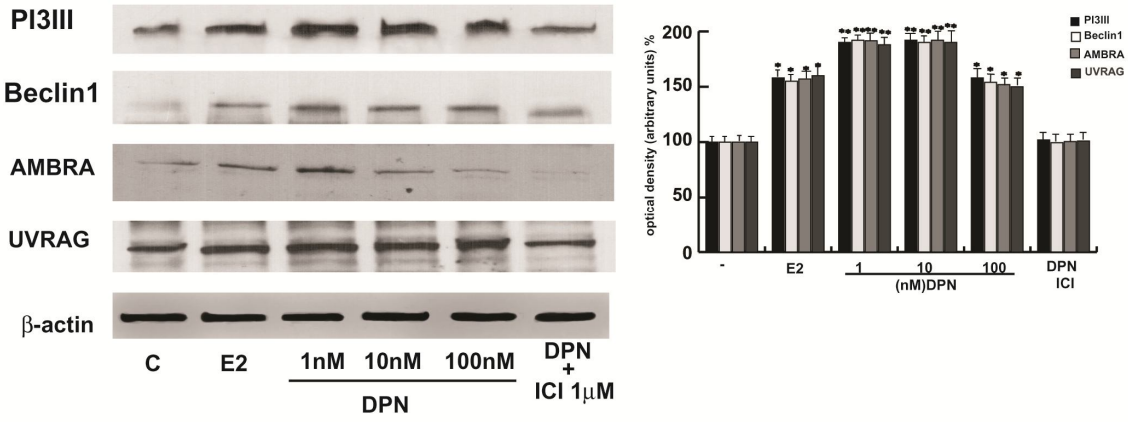

FIG. 15: DPN potentiates the effect of E2 on autophagy in human seminoma cells
By Western Blot on protein extracts from TCam2 cells as previously described and treated as indicated, selective ERβ agonist DPN potentiate the effect of E2. To verify equal loading, the membranes were stripped and probed with an antibody against β-actin. The side panels show the quantitative representation of data (mean ± SEM) of six independent experiments. Columns are mean ± SEM of six independent experiments. *P<0.05 E2-treated vs untreated cells, **P=0.01 E2-treated vs untreated cells. ***P<0.01 E2-treated vs untreated cells,

To confirm if autophagy occurred in TCam2 cells, we monitored autophagic vesicle formation using biochemical (MDC) and ultrastructural methods (TEM) (Klionsky et al. 2007). It has been reported that MDC is an autofluorescent compound and has been proposed as a tracer for autophagic vacuoles (Biederbick et al. 1995). When the cells were viewed under a fluorescence microscope, MDC-labeled autophagic vacuoles appeared as distinct dot like structures distributing in cytoplasm or in perinuclear. As such, we studied the incorporation of MDC in TCam2 cells using fluorescence microscopy. As shown in Fig.18, TCAM2 cells were treated for 24 hs, with 10 nM E2, 1μM ICI alone or in combination with 10 nM E2, with the specific agonist for ERβ 10 nM DPN, 100 nM mytramicin alone or combined with 10 nM E2 or trasfected with DN/ERβ and then treated with 10 nM E2. The fluorescent density MDC-labeled particles of

TCam2 were higher in the cells treated with 10 nM E2 and with DPN, while fewer autophagic vacuoles were observed in cells treated with ICI alone or combined with E2, mytramicin and in that trasfected with DN/ERβ.

By TEM, ultrastructural analysis revealed TCAM2 cells with all the organelles, plasmatic membrane and nucleus well preserved (Fig. 16, Control). Cell treated with 10 nM E2 showed the majority of the cells with autophagic bodies (lower panels, autophagy).

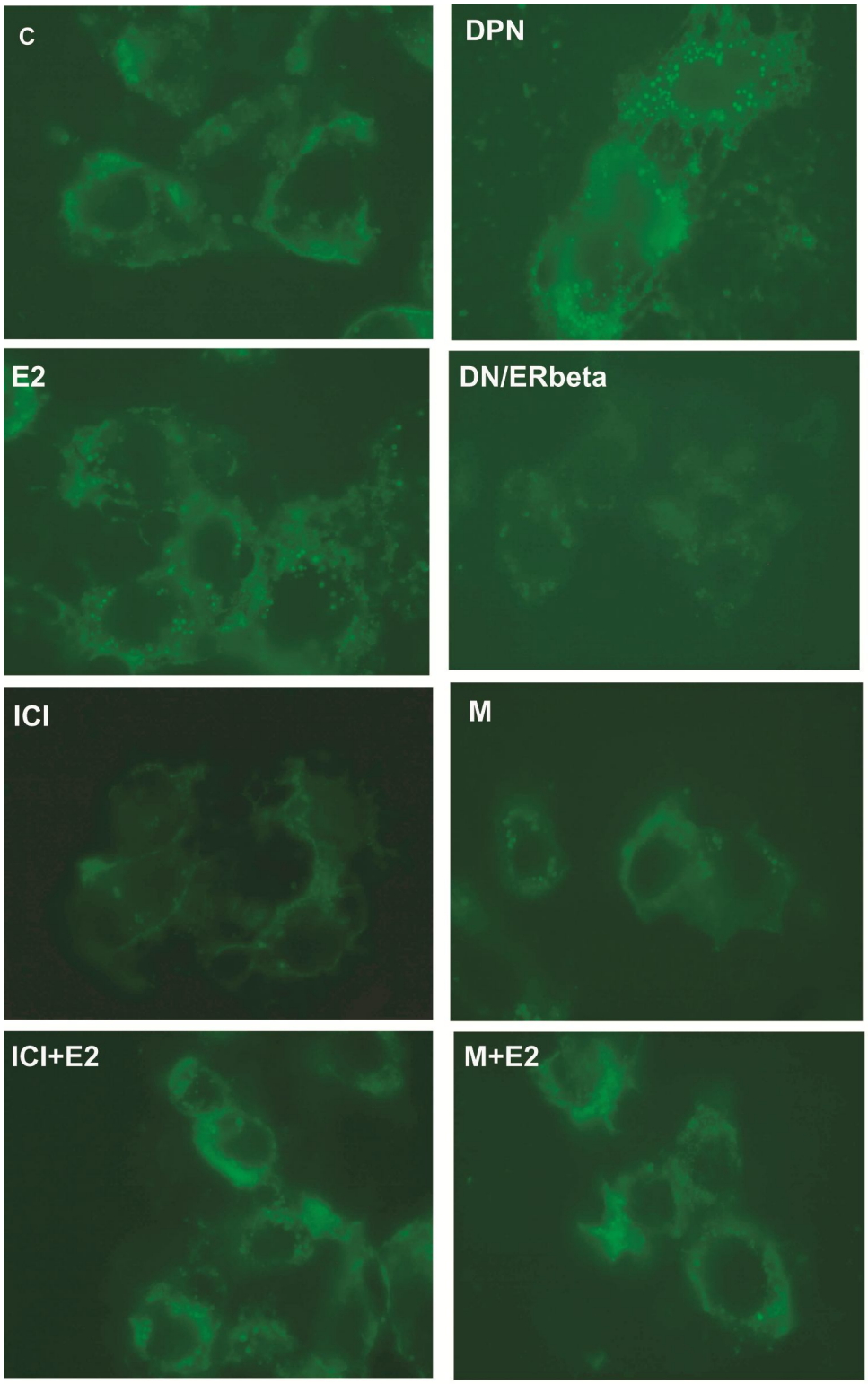

FIG. 16: Monodansylcadaverine-labeled vacuoles in TCAM2 cells.

Autophagic vacuoles were labeled with 0.05 mmol/L monodansylcadaverine (MDC) in phosphate-buffered saline (PBS) at 37°C for 10 min. The fluorescent density and the MDC-labeled particles in TCam2 cells were higher upon E2 and DPN treatments than in control group. The number of MDC-labeled particles in TCam2 cells was lower in ICI or mytramicin alone or combined with E2 and in DN/ERb trasfected cells (× 400 magnifications).

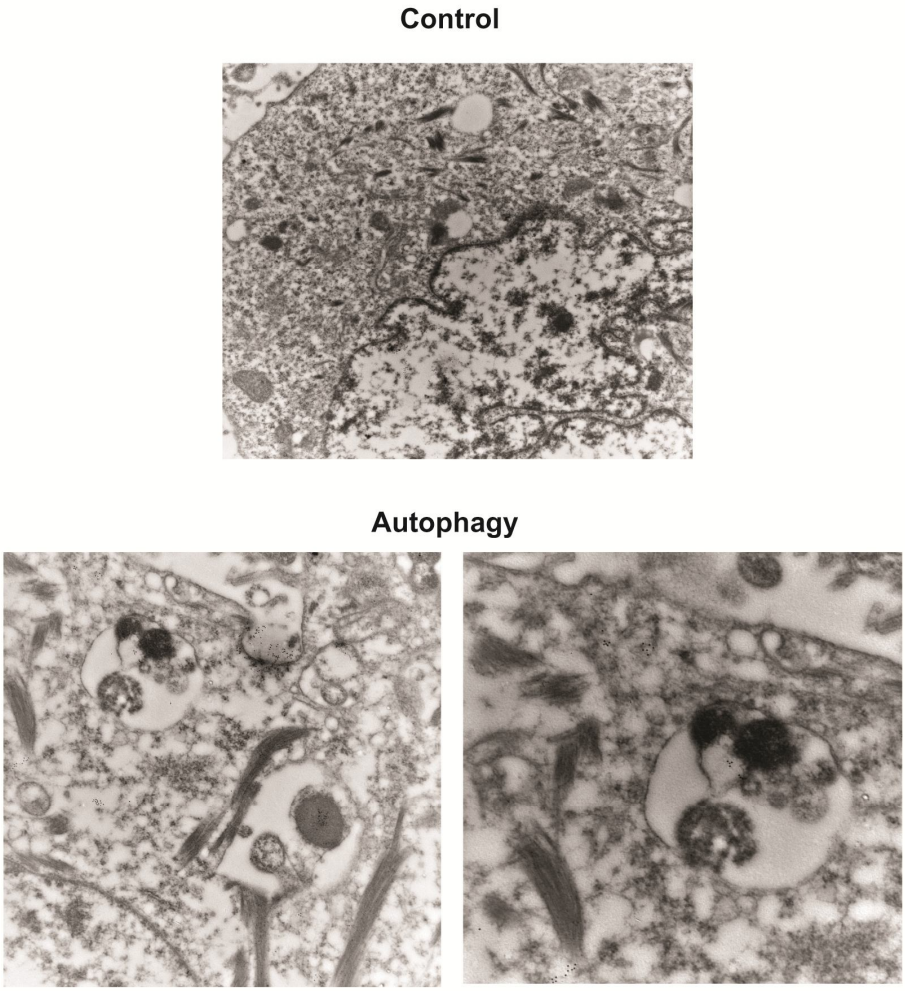

FIG. 16: E2 induces autophagy in TCAM2 cells

Transmission electron microscopy observation (Magnification ×10,000) demonstrates autophagy following E2 treatment. TCam2 cells were treated with E2 for 24h and then were processed for electron microscopy as described in Materials and Methods. Morphologic findings characteristic of each type of cell death are enlarged to highlight the findings. Lower panels: Autophagosomes (white arrowheads) and autolysosomes (black arrowheads) can be seen in autophagic cells present in E2-treated cells, compared with control.

DISCUSSION

Most of the all tumors malignancies (60%) diagnosed in men between 17 and 45 years of age are germ cell tumors (GCT). GCT arise from carcinoma in situ cells, which are thought to originate from a transformed fetal germ cell, the gonocyte. In last ten years the incidence of testicular GCT (TGCT) has increased annually by 3%–6% in the Caucasian population (Oosterhuis and Looijenga 2005). The half of the Type II TGCTs manifests as pure seminoma, that represents the most frequent subtypes of GCT together with embryonal carcinoma. Up to date, the nature of the molecular pathways involved in seminoma pathobiology remains elusive. Given the importance of both E2 and *PTEN* in the regulation of the testicular function, we investigated a potential functional cross talk between these two factors in human seminoma, by using the TCAM-2 cell line that contain typical features of seminoma.

Although, a multidisciplinary approach was undertaken to characterize the TCAM-2 cells, the presence of the ERs was never reported. The analysis of Western blotting showed strong expression of the ERβ long isoform at 60 kDa, whereas two different antibodies used in the evaluation of ERα did not show any signal at 67 kDa that is the molecular weight reported for the classical ERα, clearly expressed in the MCF7 breast cancer cells used as positive control. A recent study of the JKT-1 cells, embryonic carcinoma cells and testicular non-seminoma as further evidenced by the literature (Roger et al., 2005), shows the total absence of ERα. However, JKT-1, considered by the authors cells of seminoma, through characterization, appeared devoid of genes commonly found in seminoma and therefore do not constitute a suitable cellular model for studying the tumorogenesis in seminoma. Moreover, in human testis, gonocytes (fetal germ cells which differentiate into spermatogonia after birth) (Gaskell *et al.* 2003) and most

adult germ cells (Mäkinen *et al.* 2001) express mainly the ERβ. The presence of ERβ in TCAM2 suggests an estrogen-dependence (or sensitivity to estrogen) and testicular cancer supports a possible involvement of endogenous estrogens and / or environmental carcinogenesis in the testis. It has been reported that estrogen affects all cell types of the male genital tract and that may affect the molecular level the normal development of the testes. However, the molecular mechanisms through which they act have not been defined.

PTEN, which is a lipid phosphatase, as well as being an important factor involved in the mechanisms that induce apoptosis, seems to be involved in the differentiation of testicular germ cell tumor.

In order to evaluate a possible link between estrogen and *PTEN*, never reported in the literature, we tested the possible action of three different concentrations of estrogen on the expression of *PTEN*. Our results show that E2 is able to increase the expression of *PTEN*, both at protein and mRNA levels. This action appears to be mediated by estrogen receptor since the effect is reversible by ICI, specific antagonist of both ERs isoforms. Our data showed for the first time that E2 and its receptor are able to modulate the expression of *PTEN*, and this is likely to occur through a genomic action. To confirm this hypothesis we performed transient transfection experiments on TCAM2 cells using a series of constructs conjugated with luciferase containing the promoter of *PTEN*. The trasfections were performed with plasmid constructs containing progressively deleted regions in the 5'flanking region of the *PTEN* promoter to assess what the region is responsible for the modulator effects by E2. Interesting responsiveness to E2 depends on the plasmid construct used. Our results showed that luciferase activity increased with the construct PG13-2768 using 1 nM E2, and decreased with E2 10 nM and 100 nM. The plasmid pGL3-2768 (-2927/-160), which contained the full-

length promoter, seemed to have a lower activity than that obtained with the plasmid pGL3-612 (-1389/-778), suggesting the presence of potential negative regulatory regions within the sequence that goes from -777/-160. In fact, using the construct PG13-612, the promoter activity increased upon treatment with E2 in a dose-dependent, this effect was also abrogated by ICI, indicating that activation of *PTEN* promoter regulated by E2 requires the presence a functional ER. The E2 does not induce the transcriptional activity of the *PTEN* promoter in cells transfected with PG13-PG3 341-139, thus suggesting that the segment is located between -1398 and -1118 from the transcription start site is involved in the induction *PTEN*-mediated transcriptional activity from the ER.

By analyzing the DNA sequence spanning from -1398 and -1118 we observed that it contained various DNA motifs known to bind the transcription factors Sp1. Several studies indicate that many mammalian gene types are controlled by Sp1, including genes for structural proteins, metabolic enzymes, cell cycle regulators, transcription factors, growth factors, surface receptors, and others (Black et al., 2003). Recently, it was suggest that Sp1 is a possible transcription factor that regulates the constitutive expression of *PTEN* (Han et al, 2003). These findings are in agreement with our results since in TCAM2 cells transfected with the construct PG13-612 and treated with mithramycin in combination with 10 nm E2 the ER-mediated transactivation of *PTEN* promoter was abolished.

Altogether, these data suggest that the E2 most likely bind to the ERβ through the mediation of other transcription factors such as Sp1. To confirm our hypothesis and to go inside the molecular mechanism through which the functional cross talk occurs, we investigated the physical interaction of these factors at the promoter level. The physiological relevance of ERβ and Sp1 within the *PTEN* promoter in vivo is pointed out by ChIP analysis showing that ERβ and Sp1 occupancy of the Sp1

containing promoter region is concomitant with an increase in RNA Pol II recruitment, consistent with the increased PTEN transcriptional activity. ChIP assay also indicates that both ERβ and Sp1 are required for a full E2-dependent induction of the PTEN promoter activity. To better define the E2/ERβ action on the PTEN promoter we did EMSA experiments using a [32P]-labeled sequence identified to be E2-responsive in the PTEN promoter gene. Nuclear extracts of TCAM2 showed a single band in untreated cells which increased upon 10 nM E2, while decreased in the presence of mytramicin. The presence of anti-ERβ or of anti-Sp1 antibodies, supershifted and reduced the signal intensity. These observations prompted us to evaluate the binding of ERβ or Sp1 translated *in vitro* proteins to the [32P]-labeled PTEN promoter sequence considered. The band was generated by Sp1 and not by ERβ. Taken together, our data show that ERβ binds to the *PTEN* sequence through Sp1, and that estrogen-regulated PTEN gene expression appears to be dependent on the coordinated interactions of the Sp1-ERβ to the identified region.

In various cell types has been demonstrated that *PTEN* is deputy to the regulation of proliferation and cell death. To explore the biological significance of this E2/ERβ/PTEN functional link we performed exclusion of trypan blue test showing that E2 effectively reduced TCAM2 cell survival and significantly at 10 nM and 100 nM concentrations. The combined treatment with ICI partially reversed the effect induced by 100 nM E2. Interestingly, by using the DPN a selective agonist for ERβ, the TCAM2 cell viability strongly decreased from 1 nM concentration.

The *PTEN* gene encodes a lipid phosphatase that negatively regulates the PI3K/AKT pathway, classically involved in cell survival (Haas-Kogan et al., 1998; Stambolic et al., 1998). To investigate if the regulation of *PTEN*

by E2/ERβ is involved in TCAM2 cell death, we evaluated the expression of AKT in cells of seminoma treated with increasing concentrations of E2. Our results confirm a negative action of E2 on the AKT expression and this effect was reverted by ICI. In this research we demonstrated that TCAM2 cells express ERβ and not ERα and ICI is a pure antagonist for both ERs and that in our cells E2 effects are mediated by ERβ. In fact, in TCAM2 cells trasfected with DN/ERβ no changes were observed in the AKT expression, confirming the role of ERβ in mediating cell death in human seminoma cells. Overexpression of ERα induces the AKT content, supporting the hypothesis that the presence of ERα is associated with the proliferative effects of estrogens, whereas the bulk of current evidence implies that ERβ has growth-suppressive activities in various types of cancer cells (Paruthiyil S et al, 2004; Strom A et al., 2004).

The PI3K/AKT pathway generally induces cell death through apoptosis. Apoptosis involves a sequence of specific morphological changes in the dying cell: condensation of the cytoplasm and nuclear chromatin, followed by breakage of cells into membrane bound apoptotic bodies containing a variety of cytoplasmic organelles and nuclear fragments, which are then engulfed by neighboring cells and macrophages. Then to expand on the molecular mechanism(s) responsible of the E2-dependent seminoma cell death we evaluated the expression of two proapoptotic proteins such as FKHR and BAX and as a biological counterpart we evaluated caspase 9 and PARP1 expression as well as DNA fragmentation assay. While both FKHR and BAX expression increased, less clear are the cleavages of caspase9 and PARP1. The PARP1 is an ubiquitous enzyme in eukaryotic cells, particularly abundant in the testis. PARP1, responsible for the integrity of DNA, is readily activated when a mild damage happens then is cleaved and thus inactivated by caspases, and the cell goes into apoptosis.

During apoptosis, caspases cleave PARP-1 into two fragments: p89 and p24 (Tewari et al., 1995; Germain et al., 1999). Cleavage fragments contribute to the suppression of PARP1 activity aimed to preserve cellular energy for certain ATP sensitive steps of apoptosis. If the damage is excessive PARP overstraining, resulting in the depletion of reserves of NAD and ATP. The consumption of ATP prevents the removal of the cell to apoptosis, the performance of which requires considerable energy consumption, therefore, cell death occurs by necrosis.

From our data, the apoptotic response to E2 was not really clear, as also DNA fragmentation was not obtained and this led us to investigate an other kind of cell death observed in our experimental conditions. Two major morphologies of cell death have been described: apoptosis (type I) and cell death associated with autophagy (type II). Therefore as the same PI3K/AKT pathway is shared with the molecular mechanisms that regulate another type of cell death, ie, autophagy, we wanted to test the influence of E2 on certain proteins involved in the early events of autophagy, such as PI3KIII, Beclin-1 AMBRA and UVRAG.

Autophagy is a process that consists in the lysosomal degradation of macromolecules, organelles and membranes and plays a key role in the normal control of cell growth and reductions in energy intake. Although considered primarily a homeostatic response, the presence of features in autophagic dying cells it implies a function in the process of dying, since excessive autophagic activity may destroy portions of cytosol and organelles and determine the collapse of cellular functions. Beclin-1 is a protein that is encoded by the gene in humans BECN1 and is also known as autophagy-related gene (ATG) 6, belongs to the class III PI3K complex and participates in the very early stages of autophagosome formation inducing fusion of autophagic vesicles. This complex has a role in the formation and size of the pre-membrane autophagosome inducing the

recruitment of cytosolic proteins. Recently, it was evidenced that UVRAG is able to bind Beclin 1 by acting as a positive regulator of Beclin 1-dependent autophagic program, thus facilitating the interaction of Beclin 1 with PI3KIII. AMBRA 1also bind to Beclin 1 upon autophagic stimuli. Our results suggest that there is an increase upon E2 of all these proteins involved in the autophagic pathway and therefore t estrogen can induce autophagy in our cell type. In addition to highlighting the biological correlates of autophagy we performed two different methodological approaches: the assay with the MDC and observation by TEM of the autophagosomes. The assay clearly shows that during MDC in cells transfected with DN/ERβ or mytramicin autophagy does not occur. Interestingly, the TEM allows the visualization of a large number of double membranes enveloping the various cellular organelles and autophagosomes.

.

Many lines of evidence now indicate that common death stimuli, previously attributed to the induction of apoptosis solely, trigger autophagy as well. Another layer of complexity stems from the finding that there exists a cross talk between autophagy and apoptosis. Moreover, these cross talks varies from positive to negative feedbacks in different scenarios. Under some circumstances, autophagy contributes to apoptosis and consequently augments caspase-dependent cell death. On the other hand, under certain settings autophagy is activated only when apoptosis is blocked, indicative of a negative feedback between the two processes and or that the apotosis machinery is defective.

Therefore we propose that apoptosis is the primary response of TCAM2 in those subpopulations which are most sensitive and ready to trigger apoptosis. More resistant cells trigger autophagy as a defense mechanism,

supplying indispensable amino acids and energy to maintain cellular homeostasis and survival (Lemasters et al. 1998).

The autophagic effects of E2 are mediated by a link among ERβ/*PTEN*, leading to inhibition of the PKB/AKT-dependent survival pathway. Up to now, the effects of estrogen on autophagy was never investigated. However, we still do not know if this effect is directly or indirectly mediated by other factors. It cannot be excluded that our cell have a defective apoptotic pathway.

From our research it also emerges that DPN potentiate the E2-induced effects and then selective ERβ agonists could provide a promising new strategy could be taken into account in setting therapies for testicular cancer disease. On the other hand, a combination of therapies based on autophagy mediators could also offer a more effective approach in the therapy of human seminoma.

The reported finding evidenced that a functional cross talk between E2/ERβ and *PTEN* occurs in inducing cell death in TCAM2 cells. We discovered that ERβ is a regulator of *PTEN* gene and it may function as a tumor suppressor expanding our knowledge on data concerning the regulation of *PTEN* gene and the biology of seminoma.

BIBLIOGRAFIA

Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P, Ogier-Denis E. (2001) The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *Biol Chem*. ;276(38):35243-6.

Behrens D, Gill JH, Fichtner I. (2007) Loss of tumourigenicity of stably ERbeta-transfected MCF-7 breast cancer cells. Mol Cell Endocrinol. ;274(1-2):19-29.

Biederbick A, Kern HF, Elsässer HP. (1995) Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *Eur J Cell Biol*. 66(1):3-14.

Black AR, Black JD, Azizkhan-Clifford J. (2003) Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer. *J Cell Physiol* 188:143–160.

Blume SW, Snyder RC, Ray R, Thomas S, Koller CA, Miller DM. (1991) Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo. *J Clin Invest*.;88(5):1613-21.

Cantley LC, Neel BG. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A*.; 13;96:4240-5.

Carreau S, Bilinska B, Levallet J. (1998) Male germ cells. A new source of estrogens in the mammalian testis A*nn Endocrinol* .;59(2):79-92.

de Jong J, Stoop H, Gillis AJ, Hersmus R, van Gurp RJ, van de Geijn GJ, van Drunen E, Beverloo HB, Schneider DT, Sherlock JK, Baeten J, Kitazawa S, van Zoelen EJ, van Roozendaal K, Oosterhuis JW, Looijenga LH. (2008) Further characterization of the first seminoma cell line TCam-2. *Genes Chromosomes Cancer*.;47(3):185-96.

Delbes G, Levacher C, Pairault C, Racine C, Duquesne C, Krust A, Habert R. (2004) ERbeta-mediated inhibition of male germ cell line development in mice by endogenous estrogens during perinatal life. *Endocrinology;* 145:3395–3403.

Downes CP, Ross S, Maccario H, Perera N, Davidson L, Leslie NR. (2007) Stimulation of PI 3-kinase signaling via inhibition of the tumor suppressor phosphatase, PTEN. *Adv Enzyme Regul*.;47:184-94.

Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. (2000) Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development;* 127:4277-4291.

Ebling FJ, Brooks AN, Cronin AS, Ford H, Kerr JB. (2000) Estrogenic induction of spermatogenesis in the hypogonadal mouse. *Endocrinology* 141:2861–2869.

Eckert D, Nettersheim D, Heukamp LC, Kitazawa S, Biermann K, Schorle H. (2008) TCam-2 but not JKT-1 cells resemble seminoma in cell culture. *Cell Tissue Res*.; 331(2):529-38.

Gaskell TL, Robinson LL, Groome NP, Anderson RA, Saunders PT. (2003) Differential expression of two estrogen receptor beta isoforms in the human fetal testis during the second trimester of pregnancy. *J Clin Endocrinol Metab*.; 88(1):424-32.

Germain M, Affar EB, D'Amours D, Dixit VM, Salvesen GS, Poirier GG. (1999) Cleavage of automodified poly(ADP-ribose) polymerase during apoptosis. Evidence for involvement of caspase-7. *J Biol Chem.;* 1;274(40):28379-84.

Golstein P, Aubry L, Levraud JP. (2003) Cell-death alternative model organisms: why and which? *Nat Rev Mol Cell Biol.* ;4(10):798-807.

Gozuacik D & Kimchi A. (2004) Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* 23, 2891–2906.

Haas-Kogan D, Shalev N, Wong M, Mills G, Yount G, Stokoe D. (1998) Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. *Curr Biol.* 22;8(21):1195-8.

Han B, Dong Z, Liu Y, Chen Q, Hashimoto K, Zhang JT. (2003) Regulation of constitutive expression of mouse PTEN by the5'-untranslated region. *Oncogene*; 22: 5325-5337.

Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, Lubahn DB. (1997) A role for oestrogens in the male reproductive system. *Nature*.; 390(6659):509-12.

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J. (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell*. ;135(7):1311-23.

Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. (1993) Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res*.;53(17):3976-85.

Kimura T, Suzuki A, Fujita Y, Yomogida K, Lomeli H, Asada N, Ikeuchi M, Nagy A, Mak TW, Nakano T (2003) Conditional loss of PTEN leads to testicular teratoma and enhances embryonic germ cell production. *Development.*;130(8):1691-700.

Klionsky DJ et al., (2008) Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy.*;4(2):151-75.

Kroemer G & Jaattela M. (2005) Lysosomes and autophagy in cell death control. *Nat Rev Cancer*; 5, 886–897.

Leslie NR, Downes CP. (2002) PTEN: The down side of PI 3-kinase signaling. *Cell Signa*l.; 14:285-95.

Li H, Papadopoulos V, Vidic B, Dym M, CultyM. (1997) Regulation of rat testis gonocyte proliferation by platelet-derived growth factor and estradiol: identification of signaling mechanisms involved. *Endocrinology;* 138:1289–1298.

Maehama T, Dixon JE. (1999) PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends Cell Biol.;*9(4):125-8.

Maehama T, Taylor GS, Dixon JE. (2001) PTEN and myotubularin:novel phosphoinositide phosphatases. *Annu Rev Biochem*; 70: 247-279.

Mäkinen S, Mäkelä S, Weihua Z, Warner M, Rosenlund B, Salmi S, Hovatta O, Gustafsson JA. (2001) Localization of oestrogen receptors alpha and beta in human testis. *Mol Hum Reprod.;* 7(6):497-503

Mathew R, Karantza-Wadsworth V , White E. (2007) Role of autophagy in cancer. *Nat Rev Cancer* 7, 961–967.

Mizuno Y, Gotoh A, Kamidono S, Kitazawa S. (1993) Establishment and characterization of a new human testicular germ cell tumor cell line (TCam-2) *Nippon Hinyokika Gakkai Zasshi*.;84(7):1211-8.

Moe-Behrens GH, Klinger FG, Eskild W, Grotmol T, Haugen TB, De Felici M. (2003) Akt/PTEN signaling mediates estrogen-dependent proliferation of primordial germ cells in vitro. Mol Endocrinol.;17(12):2630-8.

Oosterhuis JW, Looijenga LH. (2005) Testicular germ-cell tumours in a broader perspective. *Nat Rev Cancer*.;5(3):210-22.

Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. (2004) Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res.* 1;64(1):423-8.

Pinton G, Brunelli E, Murer B, Puntoni R, Puntoni M, Fennell DA, Gaudino G, Mutti L, Moro L. (2009) Estrogen receptor-beta affects the prognosis of human malignant mesothelioma. *Cancer Res.* 1;69(11):4598-604.

Roger C, Lambard S, Bouskine A, Mograbi B, Chevallier D, Nebout M,Pointis G, Carreau S, Fenichel P. (2005) Estrogen-induced growth inhibition of human seminoma cells expressing estrogen receptor beta and aromatase. *J Mol Endocrinol* ;35:191–199.

Salvesen HB, MacDonald N, Ryan A, Jacobs IJ, Lynch ED, Akslen LA, Das S. (2001) PTEN methylation is associated with advanced stage and microsatellite instability in endometrial carcinoma. *Int J Cancer*.; 91(1):22-6.

Sharpe RM. 1998 The roles of oestrogen in the male. Trends Endocrinol Metab. ;9(9):371-7.

Simpson L, Parsons R. (2001) PTEN: life as a tumor suppressor. *Exp Cell Res*; 264: 29-41.

Skakkebaek NE (1972) Possible carcinoma-in-situ of the testis. *Lancet* ; 2(7776):516-7.

Speirs V, Malone C, Walton DS, Kerin MJ, Atkin SL. (1999a) Increased expression of estrogen receptor beta mRNA in tamoxifen-resistant breast cancer patients. *Cancer Res*. 1;59(21):5421-4.

Speirs V, Parkes AT, Kerin MJ, Walton DS, Carleton PJ, Fox JN, Atkin SL. (1999b) Coexpression of estrogen receptor alpha and beta: poor prognostic factors in human breast cancer? *Cancer Res*. 1;59(3):525-8.

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. (1998) Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell.* 2;95(1):29-39.

Stambolic V, MacPherson D, Sas D, Lin Y, Snow B, Jang Y,Benchimol S, Mak TW. (2001) Regulation of PTEN transcription byp53. *Mol Cell*; 8: 317-325.

Stiles B, Groszer M, Wang S, Jiao J, Wu H. (2004) PTEN less means more. *Dev Biol.* ;273(2):175-84.

Ström A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. (2004) Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci U S A.*;101(6):1566-71.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS. (1995) VMYama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell.* ;81(5):801-9.

Tora, L., Mullick, A., Metger, D., Ponglikitmongkol, M., Park, I., and Chambon, P. (1989) The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties *EMBO J.;* 8: 1981-1986.

Waite KA, Eng C. (2002) Protean PTEN: form and function. *Am J Hum Genet*; 70: 829-844.

Wang H, Yu SW, Koh DW, Lew J, Coombs C, Bowers W, Federoff HJ, Poirier GG, Dawson TM, Dawson VL. (2004) Apoptosis-inducing

factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. *J Neurosci.*;24(48):10963-73.

Williams C, Edvardsson K, Lewandowski SA, Ström A, Gustafsson JA. (2008). A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. *Oncogene*. 7;27(7):1019-32.

International Journal of Andrology
international journal of andrology ISSN 0105-6263

ORIGINAL ARTICLE

Conventional progesterone receptors (PR) B and PRA are expressed in human spermatozoa and may be involved in the pathophysiology of varicocoele: a role for progesterone in metabolism

F. De Amicis,*†[1] C. Guido,*†[1] T. Perrotta,‡ P. Avena,*† S. Panza,*† S. Andò*§[2] and S. Aquila*†[2]

*Centro Sanitario, †Department of Pharmaco-Biology, ‡Department of Ecology, and §Department of Cellular Biology, University of Calabria, Arcavacata di Rende (CS), Italy

Keywords:
human ejaculated spermatozoa, male reproduction, PR

Correspondence:
Saveria Aquila, Centro Sanitario, University of Calabria, Arcavacata di Rende (CS) 87036, Italy. E-mail: aquisav@libero.it

[1]These authors contributed equally to the study
[2]These authors are joint senior authors

Received 7 May 2010; revised 20 July 2010; accepted 8 August 2010

doi:10.1111/j.1365-2605.2010.01111.x

Summary

The physiological roles of intracellular progesterone (PRG) receptors (PRs) have been studied intensively in female mammals, while their functions in male are scarce. Conventional PRs were evidenced in our study by Western blotting, concomitantly in healthy spermatozoa and in oligoasthenoteratozoospermic samples without and with varicocoele. Transmission electron microscopy revealed the presence of the PRs on the membrane as well as in the nucleus, mitochondria and flagellum. A reduced expression of the PRs was observed only in varicocoele spermatozoa. Responses to PRG treatment on cholesterol efflux, tyrosine phosphorylation, src and Akt activities, acrosin activity and acrosome reaction in varicocoele spermatozoa were reduced or absent. To further investigate PRG significance in human male gamete, we focused its action on lipid and glucose metabolism. The evaluation of the triglycerides content, lipase and acyl-CoA dehydrogenase activities suggests that PRG through the PRs exerts a lipolytic effect on human spermatozoa. An increase in glucose-6-phosphate dehydrogenase activity was also obtained, evidencing a role for PRG on glucose metabolism. In 'varicocoele' spermatozoa, the PRG did not induce energy consumption. The action of PRs on sperm metabolism is a novel finding that renews the importance of PRG in male fertility. Our results showed that varicocoele may lead to male factor infertility by a mechanism involving a decreased PR expression in human spermatozoa that evidences a detrimental effect on spermatozoa at the molecular level, going beyond the abnormal sperm morphology described to date.

Introduction

The enigma of the varicocoele has always attracted the researchers' attention as attested by the substantial body of literature on the topic. Varicocoeles, defined as abnormally dilated scrotal veins, are present in 15% of the normal male population and in approximately 40% of men presenting infertility [World Health Organization (WHO) 1992; Koksal *et al.*, 2007]. Although it is widely accepted that varicocoele is the most common cause of male infertility (Romeo & Santoro, 2009), scientific support for this contention is almost lacking. The preponderance of experimental data from clinical and animal models demonstrates an adverse effect of varicocoeles on spermatogenesis, as venous reflux and testicular temperature elevation cause impaired spermatogenesis (Zorgniotti & Macleod, 1973; Gorelick & Goldstein, 1993). Studies using light microscopy have shown in ejaculated spermatozoa from men with varicocoele an increased number of elongated tapered sperm heads (Portuondo *et al.*, 1983), although the pathophysiology of the spermatogenesis underlying the relationship between sperm cell quality and varicocoele is still poorly understood.

It is well known that progesterone (PRG) is an essential regulator of several female reproductive events such as ovulation, regulation of the menstrual cycle, implanta-

tion and maintenance of pregnancy (Rothchild, 1983; Graham & Clarke, 1997). In contrast to the established unequivocal roles of PRG in female reproductive physiology, there are limited data on the role of PRG in male reproductive events. The actions of PRG are generally mediated via conventional intracellular PRG receptors (PRs) that belong to the superfamily of transcription factors (Horwitz & Alexander, 1983; Kastner *et al.*, 1990) expressed in a variety of female foetal and adult tissues (Jensen, 1996; Lau *et al.*, 1996). Gene targeting strategies showed pleiotropic reproductive abnormalities in PR null female mice (Lydon *et al.*, 1995; Conneely & Lydon, 2000). Deletion of both PR isoforms, PRB and the N terminally truncated PRA renders female mice infertile with ovarian deficits that prevent ovulation (Pinter *et al.*, 1996; Conneely *et al.*, 2001). Far less is known of the reproductive consequences of PR disruption or blockade in male animals. Although the reproductive phenotype of PR knockout male mice has not been reported in detail, mice null for steroid receptor coactivator-1 (SRC-1; an intracellular PR coactivator) show reduced testicular growth and fertility compared with their wild-type littermates (Xu *et al.*, 1998).

Ejaculated mammalian spermatozoa are highly differentiated attractive cells showing intriguing features: extreme polarization of cellular architecture and functions; a little amount of cytoplasm; they seem to be transcriptionally inactive; they go through two different physiological conditions comprising a steady state in the male genital tract and a functional maturation, known as capacitation, in the female genital tract (Yanagimachi, 1994; Rathi *et al.*, 2001; Suarez, 2008). Capacitation is a multifaceted process occurring in the female genital tract by which spermatozoa acquires the ability to fertilize an oocyte. PRG has been shown to activate several signalling pathways involved in the regulation of sperm functions. It was reported that PRG induces hyperactive motility and acrosome reaction (AR) of mammalian spermatozoa during the transit along the female reproductive tract (Kay *et al.*, 1994; Gadkar-Sable *et al.*, 2005). Cheng *et al.* (1998a,b) were the first to report the existence of a sperm plasma membrane PR in stallion spermatozoa. Later, a non-genomic plasma membrane PR (Contreras & Llanos, 2001) was found in the acrosomal region (Wu *et al.*, 2005, 2006). Specific PRG sperm-binding sites are located on the plasma membrane of the spermatozoon (Blackmore *et al.*, 1994). Binding studies also revealed the presence of two classes of PRs in the human spermatozoon: one class has an elevated affinity constant (nanomolar) and it is specific for PRG, whereas the other class has an affinity constant in the micromolar range. The blocking of these surface receptors inhibits PRG-induced AR (Sabeur *et al.*, 1996; Cheng *et al.*, 1998b). Besides, PRG has been shown to activate several signalling pathways, such as generation of cAMP, increase of intracellular calcium (Ca^{2+}), promotion of tyrosine phosphorylation of proteins, activation of phospholipases and many others, all involved in the regulation of human spermatozoa physiology (Thomas & Meizel, 1989; Blackmore *et al.*, 1990). However, the mechanism/s through which PRG exerts its effects in this context and the role of PRG in male reproductive events are still complex to define.

In this study, we investigated the expression of the classical PRs and their ultrastructural location in human spermatozoa and interestingly, 'varicocoele' spermatozoa showed a reduced expression of the receptors. The functional role of PRG/PRs was tested on capacitation, Akt and p60c-src (src) activities and AR. To define further the significance of PRG in human male gamete, we evaluated its action on lipid and glucose metabolism as it has never been investigated.

Materials and methods

Chemicals

Percoll (colloidal PVP-coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethyl sulphoxide (DMSO), Earle's balanced salt solution, PRG, fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and all other chemicals were purchased from Sigma Chemical (Milan, Italy). The anti-progestin RU486 {RU38486, mifepristone, 17(-hydroxy-11(8-[4-(dimethylamino)phenyl]-17a-propynylestra-4,9-dien-3-one} binds with high affinity to the intracellular PR receptor in most vertebrate species (Schatz *et al.*, 2003). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100 and Eosin Y were from Farmitalia Carlo Erba (Milan, Italy). ECL Plus Western blotting detection system, Hybond ECL, Hepes Sodium Salt were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Colloidal gold-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody (Ab) was from Sigma-Aldrich. Rabbit polyclonal anti-human PR (C-19) Ab, anti-phosphotyrosine Ab (PY99), goat polyclonal actin Ab (1-19), peroxidase-coupled anti-mouse, anti-rabbit and anti-goat IgG secondary Abs were from Santa Cruz Biotechnology (Heidelberg, Germany). Cholesterol-oxidase (CHOD)–peroxidase (POD) enzymatic colorimetric kit, triglycerides assay kit, lipase activity kit, glucose-6-phosphate dehydrogenase (G6PDH) activity assay kit and insulin radioimmunoassay (RIA) kit were from Inter-Medical (Biogemina Italia Srl, Catania, Italy). PRG and RU486 were dissolved in ethanol (0.02% final concentration in culture) and used, as solvent controls did not induce any positive result in all in vitro assays (data not shown).

Semen samples and spermatozoa preparations

Human semen sample was collected, according to the WHO-recommended procedure, by masturbation from healthy volunteer donors of proven fertility. Spermatozoa preparations were performed as described previously (Aquila *et al.*, 2005a). Briefly, semen samples with normal parameters of volume, sperm count, motility, morphology and vitality, according to the WHO's (1992) *Laboratory Manual*, were included in this study. Varicocoele samples of patients who consulted us for fertility investigation were also included in our study. Reflux of blood in the pampiniform plexus was determined by palpation employing the Valsalva manoeuvre. Physical examination is the reference standard to diagnose varicocoeles in subfertile men. Additional radiological imaging is not necessary to diagnose subclinical varicocoele because only a varicocoele detected by physical examination should be considered potentially significant (Pryor & Howards, 1987). Varicocoele samples used in this study were from oligoasthenoteratozoospermic (OAT) patients with diagnosed varicocoele of grade III (visible without palpation) on the left testis and their ejaculates were found to have total sperm count of 17×10^6 sperm cells per ejaculate, percentage of motility a + b of 30%, percentage of normally formed features of 30% and viability percentage of 67%. Samples of OAT patients without varicocoele, but with similar semen characteristics with respect to those with varicocoele, were considered as control in our study to isolate a specific effect of varicocoele. Mean ± SD age of men with normal semen parameters, OAT samples without and with varicocoele was 30.8 ± 4.5, 31.4 ± 3.8 and 29.7 ± 4.1 years, respectively. Testicular volume, measured with an ellipsoid Prader orchidometer comparing the sizes of both testes, was 15.8 ± 2.5 mL right testis (Rt) and 15.4 ± 2.5 mL left testis (Lt) in men with normal semen parameters; 15.2 ± 3.5 mL Rt and 15.0 ± 2.2 mL Lt in OAT patients without varicocoele; 14.9 ± 3.5 mL Rt and 14.3 ± 3.5 mL Lt in varicocoele patients. Despite statistically significant differences in the hormones of some varicocoele patients that were reported by many investigators (Andò *et al.*, 1984), the actual values were within normal limits (Al-Ali *et al.*, 2010). In our study, all subjects were also evaluated by reproductive plasma hormone determinations, including follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone. Mean plasma levels for FSH were 6.6 ± 0.5 mlU/mL in men with normal semen parameters, 6.9 ± 0.7 mlU/mL in OAT patients without varicocoele and 7.2 ± 0.5 mlU/mL in varicocoele patients. Mean plasma levels for LH were 10.2 ± 0.4 mlU/mL in men with normal semen parameters, 10.0 ± 0.7 in OAT patients without varicocoele and 10.8 ± 0.5 mlU/mL in varicocoele patients. Mean plasma levels for testosterone were 487 ± 19.9 ng/100 mL in men with normal semen parameters, 478 ± 10.9 ng/100 mL in OAT patients without varicocoele and 446 ± 16.9 ng/100 mL in varicocoele patients. There have also been some controversial studies on this latter issue (Mohammed & Chinegwundoh, 2009).

The study was approved by the local medical-ethical committee and all participants gave their informed consent.

Processing and treatments of ejaculated spermatozoa

For each experiment (many times repeated as reported in the 'Statistical analysis' section), three normozoospermic samples or four OAT or four varicocoele samples were pooled. In fact, after liquefaction, semen samples were first pooled and then subjected to centrifugation (800 ***g***) on a discontinuous Percoll density gradient (80% : 40% v : v; Aquila *et al.*, 2002). The 80% Percoll fraction was examined using an optical microscope equipped with a 100× oil objective to ensure that a pure sample containing only spermatozoa was obtained. These spermatozoa had a motility of about 65% (grades a + b, WHO, 1999) and a viability of 80% for both normal or pathological samples. An independent observer inspected several fields for each slide. Particularly, the same number for both normal and pathological samples of Percoll-purified spermatozoa was washed with unsupplemented Earle's medium (uncapacitating medium) and the samples were incubated for 30 min at 37 °C and 5% CO_2, without (control, NC) or with the following treatments: increasing PRG concentrations (3, 30 and 60 μM) or with RU486 (10 μM) alone or combined with 30 μM PRG. When the cells were treated with RU486, a pre-treatment of 15 min was performed. It deserves to be mentioned that in humans, PRG is present in low concentrations in blood, but it can be extraordinarily high in periovulatory follicular fluid (up to 20 μg/mL; Saaranen *et al.*, 1993); therefore, high levels of PRG may become available to spermatozoa at the time of fertilization.

Immunogold labelling for PR

Spermatozoa fixed overnight in 4% paraformaldehyde were washed in phosphate-buffered saline (PBS) to remove excess fixative, dehydrated in graded alcohol, infiltrated in LR white resin, polymerized in a vacuum oven at 45 °C for 48 h, while 60 nm ultra-thin sections were cut and placed on coated nickel grids for post-embedding immunogold labelling with the rabbit polyclonal Ab to human PR. Potential non-specific labelling was blocked by incubating the sections in PBS containing 5% normal goat serum, 5% bovine serum albumin and

0.1% cold water fish gelatin at room temperature for 1 h. Sections were then incubated overnight at 4 °C with rabbit polyclonal PR Ab at a dilution of 1 : 500 in PBS buffer. They were then incubated in 10-nm colloidal gold-conjugated anti-mouse or anti-rabbit IgG secondary Abs at 1 : 50 dilution for 2 h at room temperature. The sections were then subsequently washed in PBS, later fixed in gluteraldehyde, counterstained in uranyl acetate and lead acetate and examined under a Zeiss EM 900 (Zeiss, Oberkochen, Germany) transmission electron microscope (TEM). To assess the specificity of the immunolabelling, negative controls were carried out in corresponding sections of spermatozoa that were labelled with colloidal gold-conjugated secondary Ab with normal rabbit serum instead of primary Ab.

Western blot analysis of sperm proteins

Percoll-purified sperm samples, washed twice with uncapacitating medium, were incubated as mentioned before and then centrifuged for 5 min at 5000 ***g***. The pellet was resuspended in lysis buffer as previously described (Aquila *et al.*, 2002). An equal amount of protein (80 μg) was boiled for 5 min, separated on a 11% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and probed with an appropriate dilution of the indicated primary Ab. The binding of the secondary Ab was observed with the ECL Plus Western blotting detection system, according to the manufacturer's instructions. As internal control, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) and re-probed with anti β-actin Ab or with total Akt or src. The protein bands were quantified by scanning densitometry (Imaging Densitometer GS-700; Bio-Rad, Hercules, CA, USA). Western blot analysis was performed in at least four independent experiments and more representative results are shown.

Measurement of cholesterol in the sperm culture medium

Cholesterol was measured in duplicate by a CHOD–POD enzymatic colorimetric method according to the manufacturer's instructions in the incubation medium from human spermatozoa, as described previously (Aquila *et al.*, 2006, 2009). Percoll-purified sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) or in capacitating medium for 30 min at 37 °C and 5% CO_2. Some samples were incubated in the presence of increasing PRG concentrations (3–60 μM). Other samples were incubated in the presence of 10 μM RU486 alone or combined with 30 μM PRG. At the end of sperm incubation, the culture media were recovered by centrifugation, lyophilized and subsequently dissolved in 1 mL of buffer reaction. The samples were incubated for 10 min at room temperature and then the cholesterol content was measured spectrophotometrically at 505 nm. Cholesterol standard used was 200 mg/dL. The limit of sensitivity for the assay was 0.05 mg/dL. Inter- and intra-assay variations were 0.04% and 0.03%, respectively. Cholesterol results are presented as mg per 10×10^6 number of spermatozoa.

Acrosin activity assay

Acrosin activity was assessed by the method of Kennedy *et al.* (1989) and as described previously (Aquila *et al.*, 2003). Spermatozoa were washed in Earle's medium and centrifuged at 800 ***g*** for 20 min, then resuspended in different tubes (final concentration of 10×10^6 sperm/mL) in the presence and absence of treatments. One millilitre of substrate–detergent mixture (23 mmol/L of BAPNA in DMSO and 0.01% Triton X-100 in 0.055 mol/L of NaCl, 0.055 mol/L of HEPES at pH 8.0, respectively) was added for 3 h at room temperature. Aliquots (20 μL) were removed at 0 and 3 h and the percentage of viable cells was determined for each treatment. After incubation, a final concentration of 0.5 mol/L of benzamidine was added to each tube and then centrifuged at 1000 ***g*** for 30 min. Supernatants were collected and the acrosin activity was measured using a spectrophotometer at 410 nm. In this assay, the total acrosin activity was defined as the amount of active (non-zymogen) acrosin associated with spermatozoa plus the amount of active acrosin that is obtained by proacrosin activable. The acrosin activity was expressed as μIU/10^6 spermatozoa. Quantification of acrosin activity was performed as described previously (Aquila *et al.*, 2003).

Acrosome reaction

The evaluation of AR was performed by utilizing FITC-PNA. At the end of incubation, sperm cells were washed thrice with 0.5 mmol/L of 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 samples were then incubated with a solution of FITC-PNA in a humid chamber at room temperature. After 30 min, the slides were washed with PBS to remove the excess label. Scoring was completed within 24 h of staining that was assessed according to a published scoring system (Mendoza *et al.*, 1992). A minimum of 200 live spermatozoa were examined for each treatment and they were classified into two main categories based on the FITC-PNA staining as follows: (i) acrosome-reacted cells with uniform green FITC-PNA

fluorescence of the acrosome cap; (ii) acrosome-intact cells without any fluorescence. Values were expressed as percentage of acrosome-reacted cells.

Triglycerides assay

Triglycerides were measured in duplicate by a GPO-POD enzymatic colorimetric method according to the manufacturer's instructions in sperm lysates and as described previously (Aquila *et al.*, 2006). Percoll-purified sperm samples, washed twice by centrifugation with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37 °C and 5% CO_2. Other samples were incubated in the presence of the indicated treatments. At the end of sperm incubation, 10 μL of the lysate was added to 1 mL of the buffer reaction and incubated for 10 min at room temperature. Then, the triglycerides content was measured at 505 nm using a spectrophotometer. Data are presented as μg/10^6 spermatozoa.

Lipase activity assay

Lipase activity was evaluated by the method of Panteghini *et al.* (2001) based on the use of 1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester (DGGR) as substrate; 50 μg of sperm extracts was loaded into individual cuvettes containing buffer for spectrophotometric determination. DGGR is cleaved by lipase, resulting in an unstable dicarbonic acid ester, which is spontaneously hydrolysed to yield glutaric acid and methylresorufin, a bluish-purple chromophore with peak absorption at 580 nm. The absorbance of samples was read every 20 s for 1.5 min. The rate of methylresorufin formation is directly proportional to the lipase activity in the sample. Analysis of total imprecision gave a coefficient of variation between 0.02% and 0.032%. The estimated reference interval was 6–38 U/L (μmol/min/mg protein). The enzymatic activity was determined with three control media: one without the substrate, another without the co-enzyme (colipase) and the third without either substrate or co-enzyme (data not shown).

Assay of acyl-CoA dehydrogenase activity

Acyl-CoA dehydrogenases are a class of enzymes, which function to catalyse the initial step in each cycle of fatty acid β-oxidation in the mitochondria of cells. Assay of acyl-CoA dehydrogenase was performed on spermatozoa, using a modification of the method described by Lehman *et al.* (1990). In brief, after protein lysis, 70 μg of sperm proteins was added to the buffer containing 20 mM Mops, 0.5 mM EDTA and 100 μM FAD^+ at pH 7.2. Reduction of FAD^+ to FADH was read at 340 nm upon addition of octanoyl-CoA (100 μM) every 20 s for 1.5 min. Data are expressed as nmol/min/mg protein. The enzymatic activity was determined with three control media: one without octanoyl-CoA as substrate, another without the coenzyme (FAD^+) and the third without either substrate or coenzyme (data not shown).

Assay of the G6PDH activity

The conversion of $NADP^+$ to NADPH, catalysed by G6PDH, was measured by the increase in absorbance at 340 nm (Aquila *et al.*, 2009). Spermatozoa samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37 °C and 5% CO_2. Other samples were incubated in the presence of the indicated treatments. After incubation, 50 μL of sperm extracts was loaded into individual cuvettes containing buffer (100 mM triethanolamine, 100 mM $MgCl_2$, 10 mg/mL glucose-6-phosphate, 10 mg/mL $NADP^+$, pH 7.6) for spectrophotometric determination. The absorbance of samples was read at 340 nm every 20 s for 1.5 min. Data are expressed as nmol/min/10^6 spermatozoa. The enzymatic activity was determined with three control media: one without glucose-6-phosphate as substrate, another without the coenzyme ($NADP^+$) and the third without either substrate or coenzyme (data not shown).

Statistical analysis

The experiments for TEM and AR were performed in at least three independent experiments. The experiments for the Western blotting analysis were performed in at least eight independent experiments. The data obtained from cholesterol assay, acrosin activity, triglycerides assay, lipase activity, acyl-CoA dehydrogenase activity, G6PDH activity (10 replicate experiments using duplicate determinations) were presented as the mean ± SEM. The differences in mean values were calculated using analysis of variance (ANOVA) with a significance level of $p \leq 0.05$.

Results

Western blotting analysis of PRs

The presence of PR protein in human ejaculated spermatozoa was investigated by Western blot (Fig. 1A) using a rabbit polyclonal Ab raised against the c-terminal region of the human PR. Two immunoreactive bands, corresponding to the molecular mass values of 116 and 94 kDa were observed. As positive controls, T47D and MCF7 breast cancer cells, which expressed both isoforms as previously reported (Sartorius *et al.*, 1994), were used. Interestingly, it appears that varicocoele samples exhibit a

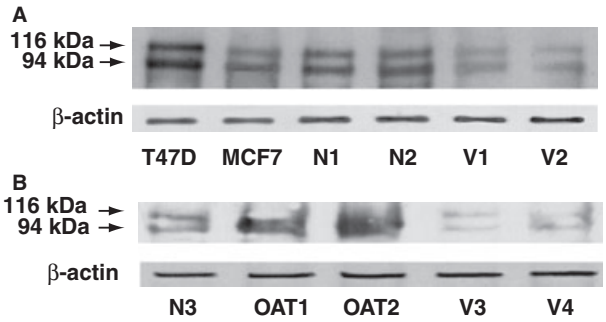

Figure 1 Western blot analysis of human sperm proteins showed expression of the conventional progesterone receptors (PRs). Extracts of pooled purified ejaculated spermatozoa were subjected to electrophoresis on 11% sodium dodecyl sulphate (SDS)-polyacrylamide gels, blotted onto nitrocellulose membranes and probed with rabbit polyclonal antibody to human PR. (A) N1 and N2 expression of PRs in two samples of ejaculated spermatozoa from normal men. V1 and V2 expression of PRs in two samples of ejaculated spermatozoa from varicocoele men. T47D and MCF-7 extracts were used as controls. (B) N3, expression of PRs in samples of ejaculated spermatozoa from normal men (lane 1). OAT1 and OAT2 expression of PRs in samples of ejaculated spermatozoa from oligoasthenoteratozoospermic patients. V3 and V4 expression of PRs in two samples of ejaculated sperm from varicocoele men. The number on the left corresponds to molecular masses (kilodaltons) of the marker proteins. The experiments were repeated at least eight times, and the autoradiographs of the figure show the results of one representative experiment.

reduced expression of PRs. When we compared PR expression among normal, OAT and varicocoele spermatozoa, only the latter showed a drastic decrement addressing a role for PRs in varicocoele pathophysiology (Fig. 1B).

Immunogold localization of PRs in human spermatozoa

Ultrastructural analysis of spermatozoa by TEM revealed that it is immunoreactive to the conventional PRs (Fig. 2). Interestingly, the label decorated mostly the head (at both the membrane and nucleus levels; Fig. 2, part a). The midpiece with the mitochondria also showed an appreciable presence of gold particles (Fig. 2, part d). PR expression was progressively reduced from the principal piece of the flagellum up to the end piece (Fig. 2, part g). Intriguingly, receptors were present not only on the sperm membrane but also as components of the flagellum, between the ribs of the fibrous sheath, outer dense fibres and axoneme. Simultaneous negative control experiments with the normal rabbit serum did not show any label in the corresponding regions (Fig. 2, parts c, f and i). In 'varicocoele' spermatozoa, a strong reduction in the gold particles was detected in the head (Fig. 3, parts a and b), along the midpiece and the tail of all the spermatozoa observed (Fig. 3, parts e and h), in agreement with the Western blotting data. Negative control experiments with the normal rabbit serum did not show signal in the corresponding sperm regions (Fig. 3, parts c, f and i).

PRG induces cholesterol efflux and protein tyrosine phosphorylation in human spermatozoa

To further investigate the PRG significance in male fertility, we evaluated its effects on two hallmarks of capacitation process, sperm membrane cholesterol efflux and protein tyrosine phosphorylation, considering both normozoospermic and varicocoele samples. Capacitation encompasses different features and sperm membrane cholesterol efflux contributes to one signalling mechanism that controls the process (Visconti *et al.*, 1995; Aquila *et al.*, 2006). Cholesterol efflux initiates signalling events leading to tyrosine phosphorylation of sperm proteins (Visconti *et al.*, 1995). Our results showed a dose-dependent increase in the cholesterol efflux upon PRG treatments (Fig. 4A). This effect was attenuated by using the PR antagonist RU486. In varicocoele samples, a slight, not significant, dose-dependent increase upon PRG was observed.

We then tested the effects of hormone on protein tyrosine phosphorylations; the treatments under our experimental conditions particularly affected the 95 ± 97 kDa tyrosine-phosphorylated proteins as previously reported (Aquila *et al.*, 2003). The densitometric evaluation of the double 95 ± 97 kDa band revealed a significant increase in the tyrosine phosphorylation in normal samples from 3 μM to 60 μM concentrations (Fig. 4B, and panel on the right side), whereas in varicocoele spermatozoa, although to a lesser extent, an increment from 30 μM to 60 μM PRG was obtained (Fig. 4C, and panel on the right side). In both cases, the RU486 abolished the PRG-induced effects. A significant increase in both serine and threonine phosphorylations upon increasing PRG was obtained as reported in supplementary data (Fig. S1).

PRG activates src and Akt in human spermatozoa

The mechanisms involved in the control of sperm functions are not well known yet; strong evidence indicates that they are associated with or controlled by different signal transduction elements. It appears that the tyrosine kinase src is a key player in the signal transduction cascade involved in the regulation of tyrosine phosphorylation occurring during sperm capacitation. To evaluate the impact of PRG on src activity, we used an Ab that specifically recognizes the active form of this kinase, by targeting an activating tyrosine phosphorylation at position 139. The results of this study clearly showed that PRG significantly induced src activity in a dose-dependent

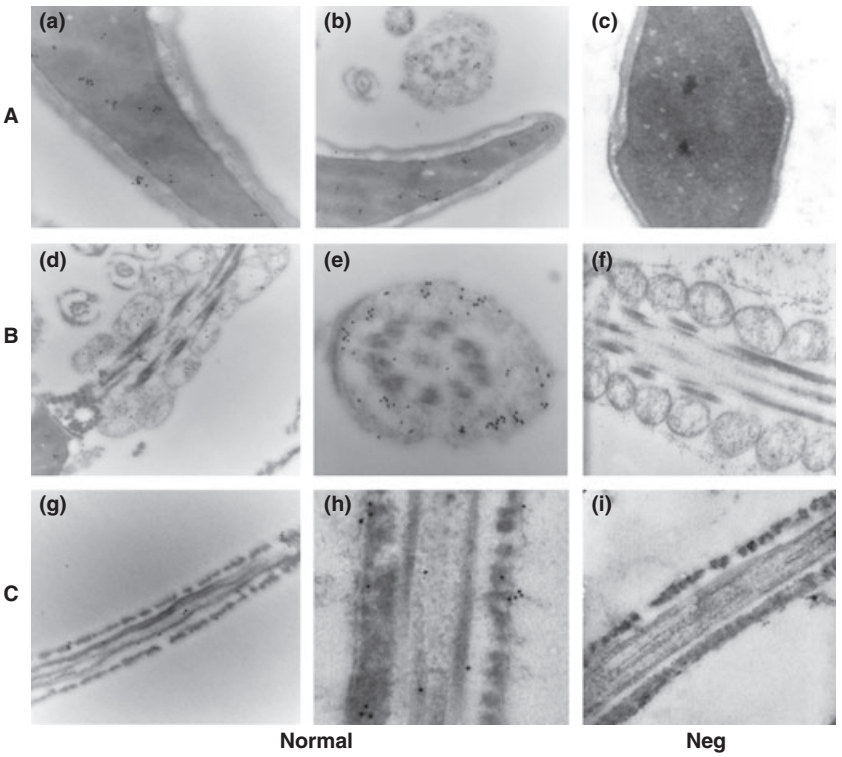

Figure 2 Immunoelectron localization of progesterone receptors (PRs) in spermatozoa of normozoospermic patients. Spermatozoa were collected and prepared as described in 'Materials and methods'. Micrographs of sections from ejaculated spermatozoa of normozoospermic patients were probed with rabbit polyclonal antibody (Ab) to human PR: panels a, b, d, e, g and h, original magnification, a, ×80 000; b, ×50 000; d, ×40 000; e, ×63 000; g, ×50 000; h, ×83 000. Panels c, f and i are the negative controls (Neg) carried out in corresponding sections of spermatozoa that were labelled with colloidal gold-conjugated secondary Ab with normal rabbit serum instead of primary Ab, original magnification, c, ×63 000; f, ×50 000; i, ×63 000. In all cases, a secondary anti-rabbit antibody conjugated to 10-nm colloidal gold particles was used for labelling. (A) Longitudinal sections through the head; (B) longitudinal and cross-sections of the midpiece of the flagellum; (C) longitudinal sections of the principal piece of the flagellum. Representative of three similar experiments.

manner and the combination with RU486 lightly attenuated the PRG-induced effects (Fig. 5A, upper panel). These data suggested that PRG is capable of inducing capacitation via mechanisms that lie upstream of src activation and that it does not act through the PRs alone. In varicocoele spermatozoa, no significant effects were observed (Fig. 5A, lower panel) upon PRG treatment.

In somatic cells, downstream signalling proteins potentially involved in mediating PRG activity include Akt, a key kinase involved in the metabolism and survival of the cells and also identified in spermatozoa (Aquila *et al.*, 2004, 2007). Increasing doses of the PRG resulted in an induction of the Akt phosphorylation on both Ser473 and Thr308 residues, indicating a full activation of the kinase (Fig. 5B, upper panel). The PRG-induced actions were not completely reversed by RU486. Similar to the src activity, the varicocoele spermatozoa did not show being responsive to PRG action on Akt phosphorylations.

Acrosin activity and AR are lower in varicocoele spermatozoa

PRG, which is secreted by cumulus cells, has been indicated as a physiological stimulus or co-stimulus for initiating the AR in spermatozoa. To give a biological evidence of this observation and with the aim to strengthen our data at the molecular level, we examined the role of PRG/PRs on acrosin activity in both healthy and varicocoele spermatozoa. The enzymatic activity highly increased upon PRG in normal samples (Fig. 6A), whereas a weak enhancement was obtained in pathological spermatozoa. The RU486 did not completely reverse the PRG-induced effect, suggesting that factors other than PRs are involved in mediating PRG action in this sperm activity. It deserves to be mentioned that in humans, PRG is present in low concentrations in blood, but it can be extraordinarily high in periovulatory follicular fluid (up to 20 μg/mL; Saaranen *et al.*, 1993); therefore, high levels of PRG may become available to spermatozoa at the time of fertilization.

Moreover, the AR increased in a dose-dependent manner upon PRG (Fig. 6B). In varicocoele samples, the percentage of reacted spermatozoa significantly increased from 30 μM to 60 μM PRG, albeit by a lower extent with respect to normal spermatozoa.

PRG influences both lipid and glucose metabolism in human spermatozoa

A role of PRG in lipid metabolism was reported in brown adipose tissue (Monjo *et al.*, 2003; Caprio *et al.*, 2008); however, in spermatozoa, a similar action of the hormone was never tested. We first investigated triglyceride intracellular content upon increasing PRG levels. As shown in

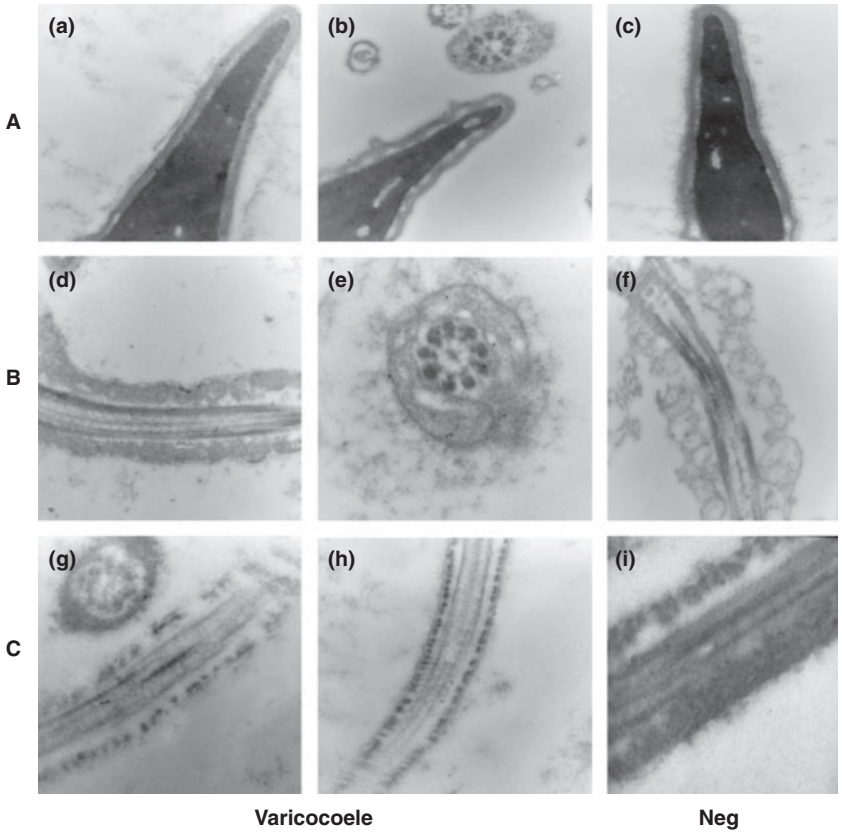

Figure 3 Immunoelectron microscopic localization of progesterone receptors (PRs) in varicocoele spermatozoa. Spermatozoa were collected and prepared as described in 'Materials and methods'. Micrographs of sections from ejaculated spermatozoa of varicocoele patients probed with rabbit polyclonal antibody (Ab) to human PR: panels a, b, d, e, g and h, original magnification, a, ×50 000; b, ×40 000; d, ×40 000; e, ×63 000; g, ×62 000; h, ×50 000. Panels c, f and i are the negative controls (Neg) carried out in the corresponding sections of spermatozoa that were labelled with colloidal gold-conjugated secondary Ab with normal rabbit serum instead of the primary Ab, original magnification, c, ×40 000; f, ×40 000; i, ×70 000. In all cases, a secondary anti-rabbit antibody conjugated to 10-nm colloidal gold particles was used for labelling. (A) Longitudinal sections through the head; (B) longitudinal and cross-sections of the midpiece of the flagellum; (C) longitudinal sections of the principal piece of the flagellum. Representative of three similar experiments.

Fig. 7A, PRG was able to decrease significantly the triglycerides, and 30 μM PRG plus 10 μM RU reversed the effect. These data suggest that PRG may induce a lipolytic effect in human spermatozoa. On the contrary, in varicocoele, a decrease in triglycerides content was not obtained. Therefore, to investigate the mechanism through which this hormone may influence sperm lipid metabolism, we evaluated its action on lipase and acyl-CoA dehydrogenase activities. Interestingly, lipase activity was enhanced by PRG in a dose-dependent manner and 30 μM PRG combined with 10 μM RU reduced the PRG-induced action (Fig. 7B). Strong activation of the acyl-CoA dehydrogenase activity was concomitantly obtained, indicating also a role for PRG/PRs in the β-oxidation of the fatty acids in human spermatozoa (Fig. 7C). The combination of 10 μM RU plus 30 μM PRG reversed the PRG-induced effect. In varicocoele samples, the hormone did not increase the enzymatic activities.

To gain a greater insight into sperm energy management, we evaluated the PRG action on glucose metabolism. The effect of glucose on the fertilizing ability of spermatozoa appears to be mediated by its metabolism through the pentose phosphate pathway (PPP); therefore, we investigated whether PRG was able to modulate the G6PDH activity, the key rate-limiting enzyme in the PPP. As shown in Fig. 7D, PRG greatly and unequivocally induced the enzymatic activity; 30 μM PRG combined with 10 μM RU reversed PRG action. Similar to the other enzymatic activities, the varicocoele spermatozoa did not seem to be responsive to PRG.

Discussion

Varicocoele is one of the most common causes of male infertility; however, the mechanisms through which it negatively affects male reproduction are not still fully clarified. Similarly, despite the demonstration that responsiveness to PRG is related to fertilization, the role of this female hormone in male reproduction is yet to be defined. Besides, levels of PR expression are only slightly lower in males than in females (Gadkar-Sable *et al.*, 2005), and their distributions throughout target tissues such as the brain and pituitary gland are similar in the two genders (Shannon *et al.*, 1982; Scarpin *et al.*, 2009). The presence of both ligand and receptor strongly suggests a physiological role for PRG and PRs in male reproductive physiology and/or behaviour; yet, few studies have directly assessed this possibility. The present study was designed to identify and localize clearly the conventional PR isoforms in human 'healthy' spermatozoa and to examine a possible molecular

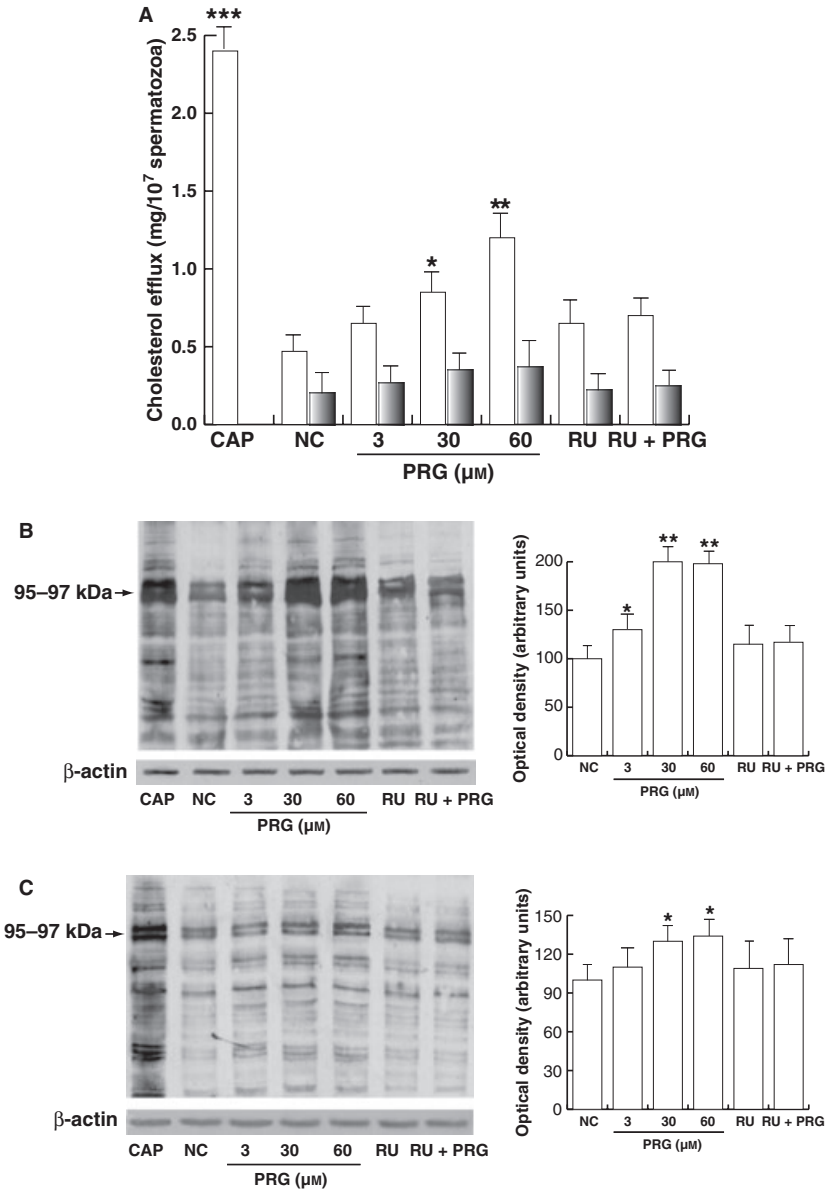

Figure 4 Effects of progesterone on capacitation. Purified spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or in the presence of indicated treatments. (A) Cholesterol in culture medium from human ejaculated spermatozoa was measured by enzymatic colorimetric assay. Columns are mean ± SEM of 10 independent experiments performed in duplicate. Data are expressed in mg/10^7 spermatozoa. *$p \leq 0.05$ vs. control ; **$p < 0.02$ vs. control : □, normal; ■, varicocoele. (B) Exactly 70 μg of sperm lysates was used for Western blot analysis of protein tyrosine phosphorylations in normal samples. Actin was used as loading control. On the right, quantitative representation after densitometry of the double 95 ± 97-kDa band, representative of protein tyrosine phosphorylations in human spermatozoa. (C) Protein tyrosine phosphorylation in varicocoele samples. On the right, quantitative representation after densitometry of the double 95 ± 97-kDa band. The autoradiographs presented are representative examples of experiments that were performed at least eight times with repetitive results. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control; ***$p < 0.001$ vs. control.

difference in 'varicocoele' spermatozoa to highlight the pathophysiology of this condition. By assessing a more profound approach on the role of PRG in sperm physiology, we evaluated different events of capacitation and we tested for the first time its action in the modulation of lipid and glucose metabolism.

In spermatozoa, a large number of studies have attempted to define the molecular mechanisms underlying PRG action as well as to identify the receptors mediating its effects. Despite many efforts, the identity of the sperm receptor for the hormone remains uncertain and contradictory. Most of the studies on the PR subtypes in spermatozoa have supported the original hypothesis that they function as specific membrane receptors through which progestins induce rapid, non-genomic responses in target cells (Thomas *et al.*, 2009). In fact, there is universal agreement that the effects of PRG on spermatozoa occur only via membrane-bound PRs and this reflects the accepted description that spermatozoa are highly differentiated, specialized cells of minimal cytoplasm and compacted nucleus that is transcriptionally inactive. However, in the recent years, a new picture of this cell is emerging: it expresses various receptor types, including nuclear receptors (Travis & Kopf, 2002; Aquila *et al.*, 2005a,b, 2007), and it also produces their ligands, suggesting that through an autocrine short loop, it may modulate its

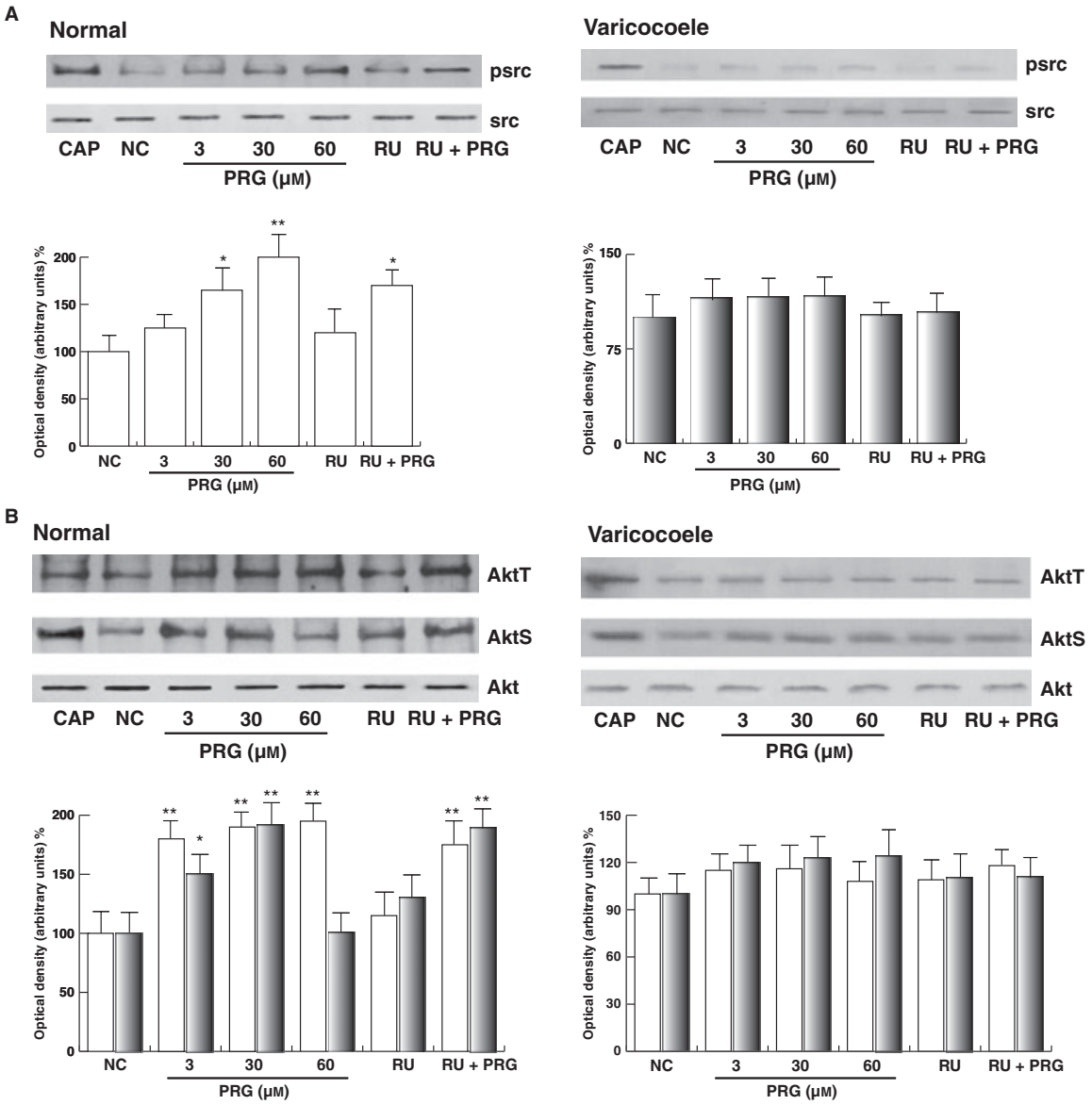

Figure 5 Progesterone (PRG) increases src, AktS and AktT phosphorylations. Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or in the presence of increasing PRG concentrations (3, 30 and 60 μm). Some samples were treated with 10 μm RU486 (RU) alone or combined with 30 μm PRG. Exactly 70 μg of sperm lysates were used for Western blot analysis of src (A), AktS and AktT (B) phosphorylations. (A) Upper panel represents p-src evaluated in normal samples, while the lower panel in varicocoele samples. Total src was used as loading control. (B) Upper panel represents AktS and AktT phosphorylations evaluated in normal samples, while the lower panel in varicocoele samples. Total Akt was used as loading control. On the right, quantitative representation after densitometry. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control: □, normal; ■, varicocoele.

own functions independently by the systemic regulation. Intriguingly, it has been demonstrated that spermatozoa is able to translate de novo by mitochondrial-type ribosomes (Gur & Breitbart, 2006). Despite this, while questions linger, different intriguing avenues remain to be extended on the biology of this cell. However, it is well established that post-translational modifications are the major means by which spermatozoa acquire full functionality (Ross *et al.*, 1990; Baker *et al.*, 2004).

It has been reported that the membrane fraction analysis shows the existence of PRG-binding proteins (Luconi *et al.*, 1998) and that spermatozoa lack genomic PRs (Castilla *et al.*, 1995). Several attempts have been made to identify the sperm membrane PR protein (Buddhikot

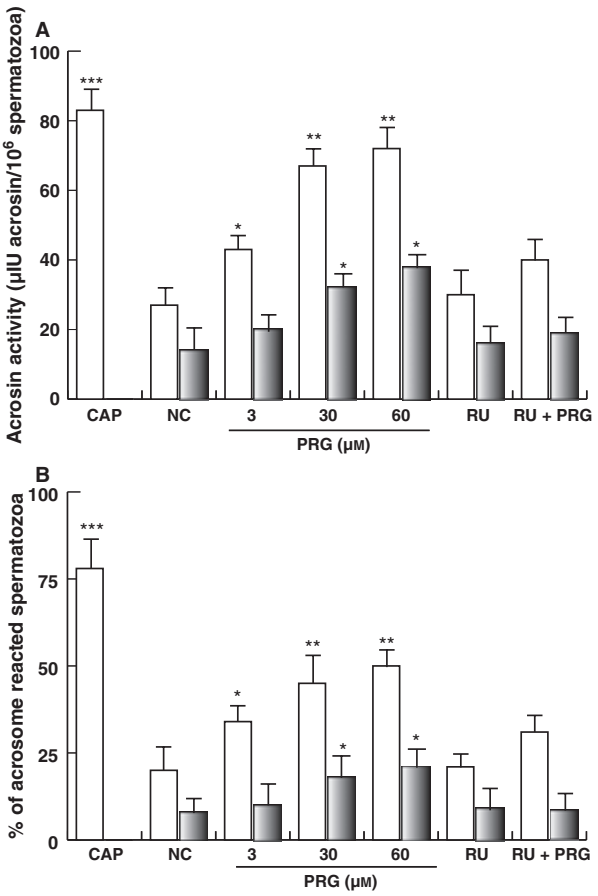

Figure 6 Progesterone (PRG) induces acrosin activity and acrosome reaction in human spermatozoa. Washed spermatozoa were incubated in unsupplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or in the presence of PRG (from 3 μm to 60 μm) and 1 μm RU486 alone or in combination with 30 μm PRG. (A) Acrosin activity was determined as described in 'Materials and methods'. Columns represent mean ± SEM of 10 independent experiments each performed in duplicate. $*p < 0.05$ and $**p < 0.01$ vs. control: CAP, capacitated spermatozoa; □, normal; ■, varicocoele. (B) Acrosome reaction was determined as described in 'Materials and methods' and the values are expressed as percentage of acrosome-reacted cells. Columns represent mean ± SEM of three independent experiments each performed in duplicate. $*p < 0.05$ and $**p < 0.02$ vs. control; $***p < 0.001$ vs. control; CAP, capacitated spermatozoa; □, normal; ■, varicocoele.

et al., 1999; Luconi *et al.*, 2002; Zhu *et al.*, 2003; Thomas *et al.*, 2004) and specific PRs have been discovered in the plasma membrane of human spermatozoa by Western blotting analysis and ligand blot analysis (Luconi *et al.*, 1998). The classic PRs are not seen with antibodies to the DNA-binding domain (DBD) or the amino-terminal domains. Using antibodies directed against the C-terminal region of the conventional PR, proteins of 5l–57 kDa in sperm lysate have been revealed by Western blot analysis (Blackmore & Lattanzio, 1991; Sabeur *et al.*, 1996; Luconi *et al.*, 1998), a 52-kDa antigen has been found on the spermatozoa head (Thomas *et al.*, 2004); the same Ab detected four bands of molecular masses of 28, 54, 57 and 66 kDa in human spermatozoa preparations (Luconi *et al.*, 1998). The presence of a 55-kDa protein in testicular and sperm lysates using another Ab that recognized the epitopes encompassing the DBD and the hormone binding domain (HBD) regions of conventional PR was also reported (Shah *et al.*, 2005a).

In our study, an Ab produced against the C-terminal region of the human PR revealed the expression of the conventional PRB and PRA. Classically, PRB (116 kD) and PRA (94 kD) have been considered nuclear receptors controlling gene transcription. PRA is a truncated version of B, lacking the first 164 amino acids. It is now clear that there are two main kinds of cellular effects mediated by nuclear receptors. One involves the alteration in gene expression (De Amicis *et al.*, 2009) and the other is associated with a rapid onset of cellular effects. This latter model of action appears to be the more appropriate modality of PRG in spermatozoa as it happens too quickly because of gene regulation. The apparent contradiction among previous studies and our study probably originates from the real presence of different types of PRs in the spermatozoa and/or from different methods of sperm processing.

Intriguingly, the expression of conventional PRs was evidenced concomitantly in semen samples from healthy volunteer donors of proven fertility, semen samples of OAT patients with or without varicocoele, but both with similar semen parameters. A decreased expression of PRs seems to be related only to varicocoele as it distinguishes healthy and OAT spermatozoa from those with varicocoele, suggesting that the stress caused by this pathology impairs spermatogenesis bringing a reduced PR expression. It was reported that glucocorticoid receptor is rapidly degraded in heat-shocked cells (Vedeckis *et al.*, 1989); therefore, it may also be possible that the increased testicular temperature in varicocoele subjects determines this effect.

An nPR-like form A of 55 kDa has been localized to the posterior head and the acrosome region of spermatozoa (Blackmore & Lattanzio, 1991); this observation is consistent with three other studies: the first reporting that only 30% of spermatozoa have detectable PRs on their heads (Sabeur *et al.*, 1996; Benoff, 1998), the second localizing PRs at the equatorial region of the sperm head (Luconi *et al.*, 1998) and the third in the mid-head region (Buddhikot *et al.*, 1999). In the present study, TEM with immunogold analysis improved understanding of the human spermatozoa anatomical regions containing the PRs and confirmed the abated expression of the receptors in the varicocoele spermatozoa. In fact, in 'healthy' spermatozoa, numerous gold particles decorated the head

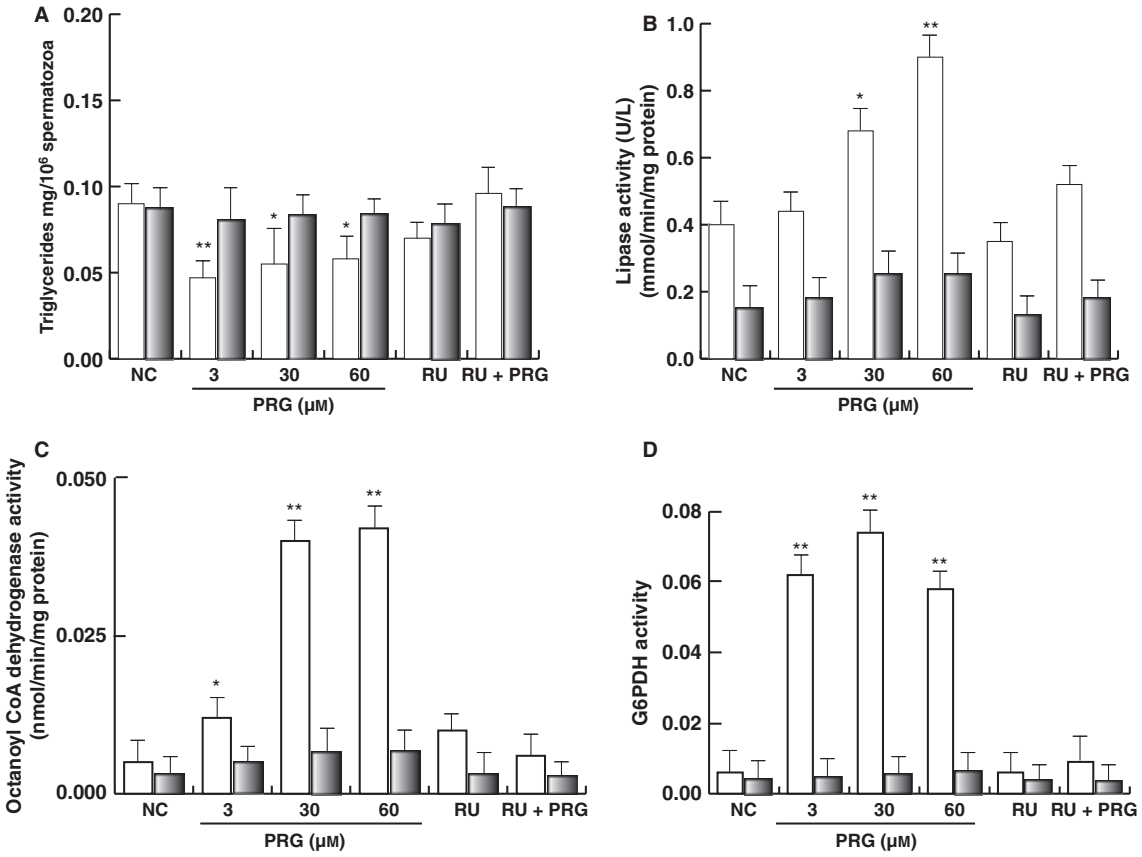

Figure 7 Effects of progesterone (PRG) on lipid and glucose metabolism in human spermatozoa. Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or in the presence of increasing PRG concentrations (3, 30 and 60 μm). Some samples were treated with 10 μm RU486 (RU) alone or combined with 30 μm PRG. (A) Triglycerides assay was performed as reported in 'Materials and methods'. Columns represent mean ± SEM. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control. (B) Lipase activity was performed as reported in 'Materials and methods'. Columns represent mean ± SEM. *$p < 0.05$ vs. control; **$p < 0.01$ vs. control. (C) Octanoyl-CoA dehydrogenase activity was performed as reported in 'Materials and methods'. Columns represent mean ± SEM. *$p \leq 0.05$ vs. control; **$p < 0.001$ vs. control. (D) G6PDH activity was performed as reported in 'Materials and methods'. Columns represent mean ± SEM. **$p < 0.001$ vs. control: □, normal; ■, varicocoele.

both at the membrane and at the nucleus. Interestingly, PR expression was progressively reduced from the principal piece of the flagellum up to the end piece. Particularly, the midpiece comprising mitochondria contains PRs. Therefore, the receptors were present not only on the sperm membrane but also inside the body spermatozoa as component of the nucleus and the flagellum, between the mitochondria, the ribs of the fibrous sheath, outer dense fibres and axoneme. The high polarization in the structure and function of spermatozoa requires a compartmentalization of metabolic and signalling pathways in the regions where they are needed. Altogether, based on these observations, the PRs could be implicated in different sperm activities according to their specific cellular localization. Interestingly, in 'varicocoele' spermatozoa, a reduction in the PR expression was noticed, confirming Western blotting data and suggesting that the PRs could be involved in the diminished functionality of the male gamete under this pathological condition.

The presence of PRs in all the spermatozoa body supports earlier evidence that the responses to progestins in mammals involve different signalling pathways many of which result in the capacitation and the AR (Kay *et al.*, 1994; Cheng *et al.*, 1998a; Shah *et al.*, 2005b). During its life, spermatozoa goes through two different physiological conditions: uncapacitated condition during which spermatozoa remain in a resting state accumulating and/or economizing energy substrates, which are going to be successively spent, and the capacitated condition, which allows spermatozoa to achieve the final competence to fertilize the oocyte. Capacitation implies striking changes in all the sperm activities and many of these were tested upon different PRG concentrations (Harper *et al.*, 2004). In this study, we evidenced PRG action on known hall-

marks of capacitation by evaluating its effects on cholesterol efflux and tyrosine phosphorylations as well as on two key kinases such as src and Akt. During capacitation, an increase in the cholesterol efflux and tyrosine phosphorylation of sperm proteins occurs. From our results, it could be observed that PRG was able to induce these activities considered priming events of the process, therefore confirming a role of the hormone in capacitation.

Following capacitation-associated cholesterol efflux and its consequent increase in membrane fluidity, spermatozoa show a rise in cyclic AMP, which precedes an upregulation of sperm motion parameters and tyrosine phosphorylation (Luconi *et al.*, 2005). It was speculated that the sperm dysfunction associated with varicocoele may be related to an alteration in plasma membrane dynamic, which causes tyrosine phosphorylation insufficiency and the consequent alteration in sperm function (Buffone *et al.*, 2006). Therefore, our data reporting a decreased cholesterol efflux, which in turn decreases sperm membrane fluidity and reduces protein tyrosine phosphorylation, are consistent with the study of Buffone *et al.* (2006) in considering these hallmarks of capacitation as two pathophysiological defects associated with grade II/III infertile varicocoele. Interestingly, in addition in our research, we demonstrated that the mechanism through which this effect occurs may be related to a diminished expression of the PRs in varicocoele.

In somatic cells, activation of src is an extranuclear function of the PR (Boonyaratanakornkit *et al.*, 2007) and PRG potentiates IP3-mediated calcium signalling through Akt/PKB (Koulen *et al.*, 2008). Therefore, these two kinases are involved in the PRG signalling and in spermatozoa src was found to play an important role in capacitation (Pujianto *et al.*, 2010), whereas Akt in survival (Aquila *et al.*, 2004, 2007). From our data, given the activation of src and Akt by PRG, it may be supposed that the hormone concurs in these activities during spermatozoa life. The negligible or absence of action of PRG on src and Akt activities in varicocoele samples may be explained by the reduced expression of the PRs.

When we performed the acrosin activity and AR to give biological evidence of the data obtained at the molecular level, a reduced enzymatic activity and percentage of reacted spermatozoa, both in the basal levels and in PRG-treated varicocoele sperm were observed. Few reports in the literature showed the relationship between acrosin activity and varicocoele. Although the acrosin activity previously described in varicocoele was measured by a different method, it was significantly lower in the varicocoele group than in the normal samples (El Mulla *et al.*, 1995), in agreement with our data. Besides, the RU486 did not completely reverse the PRG-induced effect in normal spermatozoa, suggesting that different factors other than PRs are involved in mediating PRG action in this sperm activity.

The sperm energy management is an intriguing issue and it appears that this cell is able to regulate its own metabolism independently by the systemic regulation. A role of PRG in lipid metabolism was observed in somatic cells (Correia *et al.*, 2007; Wada *et al.*, 2010); however, this action was not investigated in spermatozoa cells. From our data, it could be observed that PRG was able to reduce the triglycerides content, whereas it induced lipase and acyl-CoA dehydrogenase activities, suggesting a lipolytic effect on human spermatozoa metabolism. During capacitation, energy demand increases and capacitated spermatozoa shows an increased metabolism and overall energy expenditure, and therefore it may be assumed of the possibility of PRG co-working with other factors to stimulate such enzymatic activities providing additional metabolic fuel to sustain capacitation process. Nature has endowed spermatozoa with striking cellular peculiarities given its essential role in the propagation of life, but with a single, irreversible chance to fertilize an egg; therefore, its metabolism needs to be fine-tuned and probably independent of the systemic regulation. Interestingly, PRG strongly increased G6PDH activity and this is in agreement with its reported insulinotropic effect (Landau & Poulos, 1971; Beck, 1977). Previous data from our laboratory lead us to speculate that insulin might be considered an endogenous factor involved in the autocrine induction of the capacitation (Aquila *et al.*, 2005a) and that the insulin secretion by spermatozoa may provide an autocrine regulation of glucose metabolism as proved on G6PDH activity. The induced G6PDH activity, as we found in this study, confirms the importance of PRG/PRs in human spermatozoa functional maturation.

Our results of PRG action on metabolism in varicocoele spermatozoa renew the role of PR expression in the human male gamete, also indicating that a metabolic dysfunction is present in these cells.

To strengthen the idea that the PRG effects are 'extranuclear' and 'non-genomic' and happen through a membrane receptor in spermatozoa, there is general consensus that the effects of the hormone are not counteracted by classical PRG antagonists such as mifepristone, RU486 (Schatz *et al.*, 2003). From our data, it emerges that RU486 was not able to abolish all the PRG-induced effects tested, and this may be imputable to different mechanisms in the hormone action and/or that different proteins responding to PRG exist. Particularly, the PRG-induced action was reversed by RU486 on metabolic studies, suggesting that in this context, the conventional PRs mediate PRG action in spermatozoa.

In conclusion, varicocoele affects testicular function in a variety of ways, in spermatogenesis, in semen quality, in

sperm functions and in morphology. From our data, it emerges that this pathology may induce damage in the gamete at molecular level, opening a new chapter in the already multifactorial pathophysiology of the varicocoele and complicating this issue. By the time of ovulation, PRG is almost everywhere in the egg microenvironment affecting ability of the spermatozoa to fertilize. Therefore, the reduced expression of PRs in varicocoele spermatozoa, as we evidenced, may negatively affect different sperm activities. Undoubtedly, there is a need for more molecular and genetic studies to clarify the pathophysiology of this condition.

Acknowledgements

The authors offer their special thanks to Dr. Vincenzo Cunsolo (Biogemina Italia Srl, Catania, Italy) for technical and scientific assistance. They also thank Perrotta Enrico for the excellent technical assistance and Serena and Maria Clelia Gervasi for the English language review of the manuscript. This work was supported by MIUR Ex 60% – 2010.

References

Al-Ali BM, Marszalek M, Shamloul R, Pummer K & Trummer H. (2010) Clinical parameters and semen analysis in 716 Austrian patients with varicocoele. *Urology* 75, 1069–1073.

Andò S, Giacchetto C, Colpi G, Beraldi E, Panno ML, Lombardi A & Sposato G. (1984) Physiopathologic aspects of Leydig cell function in varicocoele patients. *J Androl* 5, 163–170.

Aquila S, Sisci D, Gentile M, Middea E, Siciliano L & Andò S. (2002) Human ejaculated spermatozoa contain active P450 aromatase. *J Clin Endocrinol Metab* 87, 3385–3390.

Aquila S, Sisci D, Gentile M, Carpino A, Middea E, Catalano S, Rago V & Andò S. (2003) Towards a physiological role for cytochrome P450 aromatase in ejaculated human sperm. *Hum Reprod* 18, 1650–1659.

Aquila S, Sisci D, Gentile M, Middea E, Catalano S, Carpino A, Rago V & Andò S. (2004) Estrogen receptor (ER) alpha and ER beta are both expressed in human ejaculated spermatozoa: evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway. *J Clin Endocrinol Metab* 89, 1443–1455.

Aquila S, Gentile M, Middea E, Catalano S & Andò S. (2005a) Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* 146, 552–557.

Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V & Andò S. (2005b) Leptin secretion by human ejaculated spermatozoa. *J Clin Endocrinol Metab* 90, 4753–4761.

Aquila S, Bonofiglio D, Gentile M, Middea E, Gabriele S, Belmonte M, Catalano S, Pellegrino M & Ando S. (2006) Peroxisome proliferator-activated receptor (PPAR) gamma is expressed by human spermatozoa: its potential role on the sperm physiology. *J Cell Physiol* 20, 977–986.

Aquila S, Middea E, Catalano S, Marsico S, Lanzino M, Casaburi I, Barone I, Bruno R, Zupo S & Andò S. (2007) Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway. *Hum Reprod* 22, 2594–2605.

Aquila S, Guido C, Laezza C, Santoro A, Pezzi V, Panza S, Andò S & Bifulco M. (2009) A new role of anandamide in human sperm: focus on metabolism. *J Cell Physiol* 221, 147–153.

Baker MA, Hetherington L, Ecroyd H, Roman SD & Aitken RJ. (2004) Analysis of the mechanism by which calcium negatively regulates the tyrosine phosphorylation cascade associated with sperm capacitation. *J Cell Sci* 117, 211–222.

Beck P. (1977) Effect of progestins on glucose and lipid metabolism. *Ann N Y Acad Sci* 286, 434–445.

Benoff S. (1998) Modelling human sperm–egg interactions *in vitro*: signal transduction pathways regulating the acrosome reaction. *Mol Hum Reprod* 4, 453–471.

Blackmore PF & Lattanzio FA. (1991) Cell surface localization of a novel non-genomic progesterone receptor on the head of human sperm. *Biochem Biophys Res Commun* 181, 331–336.

Blackmore PF, Beebe SJ, Danforth DR & Alexander N. (1990) Progesterone and 17α-hydroxyprogesterone: novel stimulators of calcium influx in human sperm. *J Biol Chem* 265, 1376–1380.

Blackmore PF, Im WB & Bleasdale JE. (1994) The cell surface progesterone receptor which simulates influx in human sperm is unlike the A ring reduced steroid site on the $GABA_A$ receptor/chloride channel. *Mol Cell Endocrinol* 104, 237–243.

Boonyaratanakornkit V, McGowan E, Sherman L, Mancini M, Cheskis B J & Edwards Dean P. (2007) The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. *Mol Endocrinol* 21, 359–375.

Buddhikot M, Falkenstein E, Wehling M & Meizel S. (1999) Recognition of a human sperm surface protein involved in the progesterone-initiated acrosome reaction by antisera against an endomembrane progesterone binding protein from porcine liver. *Mol Cell Endocrinol* 158, 187–193.

Buffone MG, Brugo-Olmedo S, Calamera JC, Verstraeten SV, Urrutia F, Grippo L, Corbetta JP & Doncel GF. (2006) Decreased protein tyrosine phosphorylation and membrane fluidity in spermatozoa from infertile men with varicocoele. *Mol Reprod Dev* 73, 1591–1599.

Caprio M, Zennaro MC, Fève B, Mammi C, Fabbri A & Rosano G. (2008) Potential role of progestogens in the control of adipose tissue and salt sensitivity via interaction with the mineralocorticoid receptor. *Climateric* 11, 258–266.

Castilla JA, Gil T, Molina J, Hortas ML, Rodriguez F, Torres-Muñoz J, Vergara F & Herruzo AJ. (1995) Undetectable expression of genomic progesterone receptor in human spermatozoa. *Hum Reprod* 10, 1757–1760.

Cheng FP, Fazeli AR, Voorhout WF, Tremoleda JL, Bevers MM & Colenbrander B. (1998a) Progesterone in mare follicular fluid induces the acrosome reaction in stallion spermatozoa and enhances *in vitro* binding to the zona pellucida. *Int J Androl* 21, 57–66.

Cheng FP, Gadella BM, Voorhout WF, Fazeli A, Bevers MM & Colenbrander B. (1998b) Progesterone-induced acrosome reaction in stallion spermatozoa is mediated by a plasma membrane progesterone receptor. *Biol Reprod* 59, 733–742.

Conneely OM & Lydon JP. (2000) Progesterone receptors in reproduction: functional impact of the A and B isoforms. *Steroids* 65, 571–577.

Conneely OM, Mulac-Jericevic B, Lydon JP & De Mayo FJ. (2001) Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. *Mol Cell Endocrinol* 179, 97–103.

Contreras HR & Llanos MN. (2001) Detection of progesterone receptors in human spermatozoa and their correlation with morphological and functional properties. *Int J Androl* 4, 246–252.

Correia JN, Conner SJ & Kirkman-Brown JC. (2007) Non-genomic steroid actions in human spermatozoa. ''Persistent tickling from a laden environment''. *Semin Reprod Med* 25, 208–219.

De Amicis F, Zupo S, Panno ML, Malivindi R, Giordano F, Barone I, Mauro L, Fuqua SA & Andò S. (2009) Progesterone receptor B recruits a repressor complex to a half-PRE site of the estrogen receptor alpha gene promoter. *Mol Endocrinol* 23, 454–465.

El Mulla KF, Köhn FM, El Beheiry AH & Schill WB. (1995) The effect of smoking and varicocoele on human sperm acrosin activity and acrosome reaction. *Hum Reprod* 10, 3190–3194.

Gadkar-Sable S, Shah C, Rosario G, Sachdeva G & Puri C. (2005) Progesterone receptors: various forms and functions in reproductive tissues. *Front Biosci* 10, 2118–2130.

Gorelick JI & Goldstein M. (1993) Loss of fertility in men with varicocoele. *Fertil Steril* 59, 613–616.

Graham JD & Clarke CL. (1997) Physiological action of progesterone in target tissues. *Endocr Rev* 18, 502–519.

Gur Y & Breitbart H. (2006) Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. *Genes Dev* 20, 411–416.

Harper CV, Barratt CL & Publicover RSJ. (2004) Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. *J Biol Chem* 279, 46315–46325.

Horwitz KB & Alexander PS. (1983) *In situ* photolinked nuclear progesterone receptors of human breast cancer cells: subunit molecular weights after transformation and translocation. *Endocrinology* 113, 2195–2201.

Jensen EV. (1996) Steroid hormones, receptors, and antagonists. *Ann N Y Acad Sci* 784, 1–17.

Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H & Chambon P. (1990) Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J* 9, 1603–1610.

Kay VJ, Coutts JR & Robertson L. (1994) Effects of pentoxifylline and progesterone on human sperm capacitation and acrosome reaction. *Hum Reprod* 9, 2318–2313.

Kennedy WP, Kamisky JM, Van der Ven HH, Jeyendran RS, Reid DS, Blackwell J, Biefeld P & Zaneveld LJD. (1989) A simple, classical assay to evaluate the acrosin activity of human spermatozoa. *J Androl* 10, 221–231.

Koksal IT, Ishak Y, Usta M, Danisman A, Guntekin E, Bassorgun IC & Ciftcioglu A. (2007) Varicocoele-induced testicular dysfunction may be associated with disruption of blood-testis barrier. *Arch Androl* 53, 43–48.

Koulen P, Madry C, Duncan RS, Hwang JY, Nixon E, McClung N, Gregg EV & Singh M. (2008) Progesterone potentiates IP(3)-mediated calcium signaling through Akt/PKB. *Cell Physiol Biochem* 21, 161–172.

Landau RL & Poulos JT. (1971) The metabolic influence of progestins. *Adv Metab Disord* 5, 119–147.

Lau TM, Witjaksono J & Rogers PA. (1996) Progesterone receptor in Norplant endometrium. *Hum Reprod* 2, 90–94.

Lehman TC, Hale DE, Bhala A & Thorpe C. (1990) An acyl-coenzyme A dehydrogenase assay utilizing the ferricenium ion. *Anal Biochem* 186, 280–284.

Luconi M, Bonaccorsi L, Maggi M, Pecchioli P, Krausz C, Forti G & Baldi E. (1998) Identification and characterization of functional nongenomic progesterone receptors on human sperm membrane. *J Clin Endocrinol Metab* 83, 877–885.

Luconi M, Bonaccorsi L, Bini L, Liberatori S, Pallini V, Forti G & Baldi E. (2002) Characterization of membrane nongenomic receptors for progesterone in human spermatozoa. *Steroids* 67, 505–509.

Luconi M, Porazzi I, Ferruzzi P, Marchiani S, Forti G & Baldi E. (2005) Tyrosine phosphorylation of the A kinase anchoring protein 3 (AKAP3) and soluble adenylate cyclase are involved in the increase of human sperm motility by bicarbonate. *Biol Reprod* 72, 22–32.

Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr, Shyamala G, Conneely OM & O'Malley BW. (1995) Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* 9, 2266–2278.

Mendoza C, Carreras A, Moos J & Tesarik J. (1992) Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin. *J Reprod Fertil* 95, 755–763.

Mohammed A & Chinegwundoh F. (2009) Testicular varicocoele: an overview. *Urol Int* 82, 373–379.

Monjo M, Rodríguez AM, Palou A & Roca P. (2003) Direct effects of testosterone, 17 beta-estradiol, and progesterone on adrenergic regulation in cultured brown adipocytes: potential mechanism for gender-dependent thermogenesis. *Endocrinology* 144, 4923–4930.

Osheroff JE, Visconti PE, Valenzuela JP, Travis AJ, Alvarez J & Kopf GS. (1999) Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. *Mol Hum Reprod* 5, 1017–1026.

Panteghini M, Bonora R & Pagani F. (2001) Measurement of pancreatic lipase activity in serum by a kinetic colorimetric assay using a new chromogenic substrate. *Ann Clin Biochem* 38, 365–370.

Pinter JH, Deep C & Park-Sarge OK. (1996) Progesterone receptors: expression and regulation in the mammalian ovary. *Clin Obstet Gynecol* 39, 424–435.

Portuondo JA, Calabozo M & Echanojauregui AD. (1983) Morphology of spermatozoa in infertile men with and without varicocoele. *J Androl* 4, 312–315.

Pryor JL & Howards SS. (1987) Varicocele. *Urol Clin N Am* 14, 499–513.

Pujianto DA, Curry BJ & Aitken RJ. (2010) Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. *Endocrinology* 151, 1269–1279.

Rathi R, Colenbrander B, Bevers MM & Gadella BM. (2001) Evaluation of *in vitro* capacitation of stallion spermatozoa. *Biol Reprod* 65, 462–470.

Romeo C & Santoro GJ. (2009) Varicocele and infertility: why a prevention? *J Endocrinol Invest* 32, 559–561.

Ross P, Kan FW, Antaki P, Vigneault N, Chapdelaine A & Roberts KD. (1990) Protein synthesis and secretion in the human epididymis and immunoreactivity with sperm antibodies. *Mol Reprod Dev* 26, 12–23.

Rothchild I. (1983) Role of progesterone in initiating and maintaining pregnancy. In: Progesterone and Progestins (eds Bardin CW, Milgrom E & Mauvais-Jarvis P), pp. 219–229. Raven Press, New York.

Saaranen MJ, Calvo L, Dennison L, Banks S, Bustillo M, Dorfmann AD, Goldstein M, Thorsell L, Schulman JD & Sherins RJ. (1993) Acrosome reaction inducing activity in follicular fluid correlates

with progesterone concentration but not with oocyte maturity or fertilizability. *Hum Reprod* 8, 1448–1454.

Sabeur K, Edwards DP & Meizel S. (1996) Human sperm plasma membrane progesterone receptor(s) and the acrosome reaction. *Biol Reprod* 54, 993–1001.

Sartorius CA, Groshong SD, Miller LA, Powell RL, Tung L, Takimoto GS & Horwitz KB. (1994) New T47D breast cancer cell lines for the independent study of progesterone B- and A-receptors: only anti-progestin-occupied B-receptors are switched to transcriptional agonists by cAMP. *Cancer Res* 54, 3868–3877.

Scarpin KM, Graham JD, Mote PA & Clarke CL. (2009) Progesterone action in human tissues: regulation by progesterone receptor (PR) isoform expression, nuclear positioning and coregulator expression. *Nucl Recept Signal* 7, e009.

Schatz F, Krikun G, Caze R, Rahman M & Lockwood CJ. (2003) Progestin-regulated expression of tissue factor in decidual cells: implications in endometrial hemostasis, menstruation and angiogenesis. *Steroids* 68, 849–860.

Shah C, Modi D, Sachdeva G, Gadkar S, D'Souza S & Puri C. (2005a) N-terminal region of progesterone receptor B isoform in human spermatozoa. *Int J Androl* 28, 360–371.

Shah C, Modi D, Sachdeva G, Gadkar S & Puri C. (2005b) Coexistence of intracellular and membrane-bound progesterone receptors in human testis. *J Clin Endocrinol Metab* 90, 474–483.

Shannon JM, Cunha GR, Taguchi O, Vanderslice KD & Gould SF. (1982) Autoradiographic localization of steroid binding in human tissue labeled *in vitro*. *J Histochem Cytochem* 30, 1059–1065.

Suarez SS. (2008) Regulation of sperm storage and movement in the mammalian oviduct. *Int J Dev Biol* 52, 455–462.

Thomas P & Meizel S. (1989) Phosphatidylinositol 4,5-biphosphate hydrolysis in human stimulated with follicular fluid or progesterone is dependent upon Ca^{2+} influx. *Biochem J* 264, 539–546.

Thomas P, Pang Y, Zhu Y, Detweiler C & Doughty K. (2004) Multiple rapid progestin actions and progestin membrane receptor subtypes in fish. *Steroids* 69, 567–573.

Thomas P, Tubbs C & Garry VF. (2009) Progestin functions in vertebrate gametes mediated by membrane progestin receptors (mPRs): identification of mPRalpha on human sperm and its association with sperm motility. *Steroids* 74, 614–621.

Travis AJ & Kopf GS. (2002) The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *J Clin Invest* 110, 731–736.

Vedeckis WV, Ali M & Allen HR. (1989) Regulation of glucocorticoid receptor protein and mRNA levels. *Cancer Res* 49, 2295s–2302s.

Visconti PE, Baley JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS. (1995) Capacitation in mouse spermatozoa I. Correlation between the capacitation state and protein phosphorylation. *Development* 121, 1129–1137.

Wada T, Hori S, Sugiyama M, Fujisawa E, Nakano T, Tsuneki H, Nagira K, Saito S & Sasaoka T. (2010) Progesterone inhibits glucose uptake by affecting diverse steps of insulin signaling in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 298, E881–E888.

World Health Organization. (1992) The influence of varicocoele on parameters of fertility in a large group of men presenting to infertility clinics. *Fertil Steril* 57, 1289–1293.

Wu JT, Tsai PS, Lee SL & Cheng FP. (2005) Characterisation of the progesterone receptor on canine spermatozoa. *Reprod Fertil Dev* 17, 733–741.

Wu JT, Chiang KC & Cheng FP. (2006) Expression of progesterone receptor(s) during capacitation and incidence of acrosome reaction induced by progesterone and zona proteins in boar spermatozoa. *Anim Reprod Sci* 93, 34–45.

Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai MJ & O'Malley BW. (1998) Partial hormone resistance in mice with disruption of the steroidal receptor coactivator-1 (SRC-1) gene. *Science* 279, 1922–1925.

Yanagimachi R. (1994) Fertility of mammalian spermatozoa: its development and relativity. *Zygote* 2, 371–382.

Zhu Y, Bond J & Thomas P. (2003) Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc Natl Acad Sci USA* 100, 2237–2242.

Zorgniotti AW & Macleod J. (1973) Studies in temperature, human semen quality, and varicocoele. *Fertil Steril* 24, 854–863.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Progesterone induces phosphorylation of the proteins on serine and threonine residues in human spermatozoa.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 159, 831–841

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

Rimonabant (SR141716) induces metabolism and acquisition of fertilizing ability in human sperm

S Aquila[1,2], C Guido[1,2], A Santoro[3], P Gazzerro[3], C Laezza[4], MF Baffa[1], S Andò[1,2,5] and M Bifulco[3]

[1]*Department of Pharmaco-Biology,* [2]*Centro Sanitario, University of Calabria,* [3]*Department of Pharmaceutical Sciences, University of Salerno, Via Ponte don Melillo, 84084 Fisciano, Salerno,* [4]*IEOS-CNR Naples and* [5]*Department of Cellular Biology, University of Calabria 87036 Arcavacata di Rende, Cosenza, Italy*

Background and purpose: The endocannabinoid system and the cannabinoid CB_1 receptor have been identified in human sperm, and it is well known that endocannabinoids have pronounced adverse effects on male and female reproduction. In order to elucidate further the pathophysiological role of the endocannabinoid system in male fertility, we investigated the activity of the CB_1 receptor antagonist rimonabant (SR141716) on the fertilizing ability of human sperm.
Experimental approach: We evaluated *in vitro* the effects of rimonabant on motility, survival, capacitation, acrosin activity and metabolism of human sperm. Particularly, capacitation was studied by using three different approaches: intracellular free Ca^{2+} content assay, cholesterol efflux assay and protein tyrosine phosphorylation analysis.
Key results: Rimonabant significantly increased sperm motility and viability through the induction of pAkt and pBcl2, key proteins of cell survival and metabolism, and it induced acrosome reaction and capacitation as well. Rimonabant reduced the triglyceride content of sperm, while enhancing lipase and acyl-CoA dehydrogenase activities, implying an overall lipolytic action in these cells. Rimonabant also affected sperm glucose metabolism by decreasing phosphorylation of glycogen synthase kinase 3 and increasing glucose-6-phosphate dehydrogenase activity, suggesting a role in inducing sperm energy expenditure. Intriguingly, agonism at the CB_1 receptor, with an anandamide analogue or a selective inhibitor of fatty acid amide hydrolase, produced opposing effects on human sperm functions.
Conclusions and implications: Our data suggest that blockade of the CB_1 receptor by rimonabant induces the acquisition of fertilizing ability and stimulates energy expenditure in human sperm.
British Journal of Pharmacology (2010) **159,** 831–841; doi:10.1111/j.1476-5381.2009.00570.x; published online 8 January 2010

Keywords: rimonabant; CB_1 receptor; human sperm; male fertility; reproduction; endocannabinoids

Abbreviations: AEA, anandamide, *N*-arachidonoylethanolamine; CB receptor, cannabinoid receptor; DGGR, 1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester; EC, endocannabinoid; FAAH, fatty acid amide hydrolase; G6PDH, glucose-6-phosphate dehydrogenase; GSK3, glycogen synthase kinase 3; MF-AEA, 2-methylarachidonyl-2′-fluoro-ethylamide; PPP, pentose phosphate pathway; SR141716, rimonabant (*N*-(piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4dichlorophenyl)-4-methyl-pyrazole-3-carboxamide); THC, delta-9-tetrahydrocannabinol; URB, URB597 (3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate); ZP, zona pellucida

Introduction

The endogenous cannabinoid (EC) system, comprising the most abundant cannabinoid type-1 (CB_1) and the more restricted CB_2 receptors, their endogenous ligands (endocannabinoids) and the enzymes catalyzing their biosynthesis and degradation, is an almost ubiquitous signalling system involved in the control of several physiological functions from energy homeostasis to movement, memory and pain (Elphick and Ergetova, 2001; Bifulco *et al.*, 2007; Bellocchio *et al.*, 2008). During the last years, evidence has accumulated for an important role of the EC system in both male and female fertility (Wang *et al.*, 2006). The CB_1 receptor is expressed in human testis (Gerard *et al.*, 1991), and sea urchin and human sperm cells possess a functional EC system (Schuel *et al.*, 1987; 1994; Wang *et al.*, 2006). In human sperm, the CB_1 receptor subtype is present in the membranes of the head and middle piece, and is also localized on the mitochondria (Rossato *et al.*, 2005; Aquila *et al.*, 2009a). Moreover, the CB_1 agonist anandamide (AEA) inhibited sperm

Correspondence: Professor Maurizio Bifulco, Department of Pharmaceutical Science, University of Salerno, Via Ponte don Melillo, 84084 Fisciano, Salerno, Italy. E-mail: mbifulco@unisa.it; alternate E-mail: aquisav@libero.it
Received 25 June 2009; revised 9 September 2009; accepted 30 September 2009

motility and viability (Rossato *et al*., 2005; Aquila *et al*., 2009a,b). These observations together with the finding that agonism at CB_1 receptors, either by the endogenous agonist AEA or by the natural CB_1 agonist delta-9-tetrahydrocannabinol (THC) has negative effects on human reproduction, suggest that targeting the CB_1 receptor or controlling the endogenous tone of endocannabinoids may represent a therapeutic tool in reproductive pathological situations involving an imbalance of the EC system (Maccarrone and Finazzi-Agrò, 2004; Bifulco *et al*., 2007; Maccarrone, 2008). So far, there are very few data on the effects of CB_1 receptor blockade on male fertility, whereas there is a large body of the literature focused on the interaction and possible regulation of reproductive processes by endocannabinoids (Wang *et al*., 2006). The only available data concern the ability of the highly selective CB_1 antagonist SR141716 (rimonabant) to counteract AEA-mediated inhibition of human sperm viability and motility, when administered prior to AEA (Rossato *et al*., 2005). Similar results were reported in the frog *Rana esculenta* by Cobellis *et al*. (2006) who found that micromolar concentrations of rimonabant alone were able to induce a slight, but significant, increase of viable and motile spermatozoa. Rimonabant also induced penile erection in male rats when injected into the paraventricular nucleus of male rat hypothalamus (Melis *et al*., 2006; Succu *et al*., 2006). This effect was associated with an increase of extracellular glutamic acid leading to the activation of NO synthase in oxytocinergic neurons mediating penile erection (Succu *et al*., 2006).

In order to increase our knowledge of the pathophysiological role of the EC system in male fertility, and to analyse the activity of rimonabant on human sperm functions, we investigated the effects of rimonabant on sperm viability and motility, and focused on different aspects of the capacitation process, acrosome reaction and metabolism. Human spermatozoa do not possess the ability to fertilize an oocyte immediately after ejaculation, but they acquire this ability after some time in contact with the female reproductive tract. This time-dependent acquisition of fertilizing ability is known as capacitation (Yanagimachi, 1994), and includes acquisition of hyperactivated motility (Suarez, 2008) increase in both intracellular Ca^{2+} concentration and protein phosphorylation (Visconti *et al*., 2002; Jha *et al*., 2003), and efflux of cholesterol from sperm (Travis and Kopf, 2002). Capacitation enables the sperm to bind to the zona pellucida (ZP) and undergo the acrosome reaction, a process by which powerful hydrolyzing enzymes present in the acrosome are released into its surroundings. Acrosome reaction serves at least two functions: first, to facilitate the penetration of the ZP by the and, subsequently, to aid in the oocyte–sperm fusion process (Yanagimachi, 1994). Hence, by analyzing the key biochemical changes of capacitation and acrosome reaction, we could assess the influence of rimonabant on male fertilizing potential. In addition, as there is a close link between energy balance and reproduction (Chehab, 2000; Altarejos *et al*., 2008), and we recently reported that sperm cells are able to modulate their own metabolism independently of systemic regulation by expressing and secreting both insulin and leptin (Andò and Aquila, 2005; Aquila *et al*., 2005; 2006), we evaluated the action of rimonabant on lipid and glucose metabolism in human sperm.

Methods

Semen samples and spermatozoa preparations

Human semen was collected, according to the World Health Organization (WHO) recommendations, by masturbation from healthy volunteer donors of proven fertility undergoing semen analysis in our laboratory. Spermatozoa preparations were performed as previously described (Aquila *et al*., 2006). Briefly, sperm samples 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. Each sperm sample was obtained by pooling the ejaculates of three different normozoospermic healthy donors. In our experience, this was necessary to obtain enough cells to perform all the tests (Aquila *et al*., 2005; 2009a,b). In addition, each assay was performed at least three times using three different sperm samples. Washed pooled sperm samples were subjected to the indicated treatments, and incubated for 30 min at 37°C and 5% CO_2. Then, the samples were centrifuged and the pellet containing sperm was lysed to perform Western blots, triglyceride assay, Ca^{2+} assay, acyl-CoA dehydrogenase assay, glucose-6-phosphate dehydrogenase (G6PDH) activity and lipase activity. Prior to the centrifugation, several aliquots were used to measure sperm motility and viability. The study was approved by the local medical ethical committees, and all participants gave their informed consent.

Processing of ejaculated sperm

After liquefaction, the 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 at 1000× magnification to ensure that a pure sample of sperm was obtained. An independent observer, who observed several fields for each slide, checked the cells. Percoll-purified sperm was washed with uncapacitating medium (Earle's balanced salt solution medium without supplementation with BSA, sodium bicarbonate or Ca^{2+}), and incubated for 30 min at 37°C and 5% CO_2, without (control, NC) or with the indicated concentrations of rimonabant. When combined treatments were performed, the cells were pretreated for 15 min with the CB_1 receptor antagonist rimonabant (1 μM), and then 2-methylarachidonyl-2′-fluoroethylamide (MF-AEA) at the indicated concentrations or URB597 (0.1 μM) was added.

Evaluation of sperm motility and viability

Sperm motility was assessed by means of light microscopy examining aliquots of each sperm. An independent observer scored at least 200 cells. Sperm motility was expressed as percentage of total motile sperm.

Viability was assessed by red eosin exclusion test using eosin Y. Sperm vitality was assessed by means of light microscopy examining an aliquot of each sperm sample in the absence (NC) or in the presence of increasing concentrations of rimonabant, and then incubated for 30 min. An

independent observer scored 200 cells for stain uptake (dead cells) or exclusion (live cells), and sperm viability was expressed as percentage of total live sperm.

Evaluation of Ca^{2+} in sperm
Intracellular Ca^{2+} concentration has been estimated spectrophotometrically with the indicator arsenazo III (Thomson and Wishart, 1989) using sperm lysates according to our previous study (Aquila *et al.*, 2009b). At a neutral pH, the Ca^{2+} forms with arsenazo III a complex, the colour intensity of which is directly proportional to the concentration of Ca^{2+} in the sample. Normal sperm samples were treated as mentioned earlier. Ca^{2+} content was measured at 600 nm. The Ca^{2+} standard used was 2.5 mM (100 mg·L^{-1}). Inter- and intra-assay variations were 0.24 and 0.37% respectively. Ca^{2+} results are presented as μM per 10^7 spermatozoa.

Measurement of cholesterol in the sperm culture medium
Cholesterol was measured in duplicate by a cholesterol oxidase–peroxidase (CHOD–POD) enzymatic colorimetric method according to the manufacturer's instructions in the incubation medium from human spermatozoa. Each sperm sample, washed twice with uncapacitating medium, was incubated in the same medium (control) in the presence or in the absence of testing compounds for 30 min at 37°C and 5% CO_2. At the end of the sperm incubation, culture media were recovered by centrifugation, lyophilized and subsequently dissolved in 1 mL of reaction buffer. The samples were incubated for 10 min at room temperature, then the cholesterol content was measured spectrophotometrically at 505 nm. The cholesterol standard used was 2 g·L^{-1}. The limit of sensitivity for the assay was 0.005 mg·L^{-1}. Inter- and intra-assay variations were 0.04 and 0.03% respectively. Cholesterol results are shown as mg per 10^7 spermatozoa.

Acrosin activity assay
Acrosin activity was assessed by the method of Kennedy *et al.* (1989) and as previously described (Aquila *et al.*, 2003). Sperm cells were washed in Earle's medium and centrifuged at 800× *g* for 20 min, and then were resuspended in different tubes (final concentration of 10^7 spermatozoa mL^{-1}) in the presence and absence of treatments. One millilitre of substrate–detergent mixture (23 mM BAPNA in DMSO and 0.01% Triton X-100 in 0.055 M NaCl, 0.055 M HEPES at pH 8.0, respectively) was added for 3 h at room temperature. Aliquots (20 μL) were removed at 0 and 3 h, and the percentage of viable cells was determined for each treatment. After incubation, benzamidine 0.5 M final concentration was added to each tube, and then centrifuged at 1000× *g* for 30 min. Supernatants were collected, and the acrosin activity was measured by using a spectrophotometer at 410 nm. In this assay, the total acrosin activity was defined as the amount of the active (non-zymogen) acrosin associated with sperm plus the amount of active acrosin that is obtained by pro-acrosin activable. The acrosin activity was expressed as μIU/10^6 spermatozoa. Quantification of acrosin activity was performed as previously described (Aquila *et al.*, 2003).

Western blot analysis of sperm proteins
Each sperm sample, washed twice with an uncapacitating medium, was incubated and treated as mentioned earlier and then centrifuged for 5 min at 5000× *g*. The pellet was resuspended in lysis buffer as previously described (Aquila *et al.*, 2002). An equal amount of protein (80 μg) was boiled for 5 min, separated on a 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and probed with an appropriate dilution of the indicated primary antibody. The binding of the secondary antibody was revealed with the ECL Plus Western blotting detection system, according to the manufacturer's instructions. As internal control, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) and reprobed with anti-β-actin antibody or with antibody to total Akt, Bcl2 or glycogen synthase kinase 3 (GSK3). The protein bands were quantified by scanning densitometry (Imaging Densitometer GS-700, Bio-Rad, Hercules, CA, USA). Western blot analysis was performed in at least three independent experiments, and more representative results are shown.

Triglyceride assay
Triglycerides were measured in duplicate by a glycerol-3-phosphate oxidase–POD enzymatic colorimetric method according to the manufacturer's instructions and as previously described (Aquila *et al.*, 2006). Sperm samples, washed twice with an uncapacitating medium, were incubated in the same medium (control) for 30 min at 37°C and 5% CO_2 in the presence or in the absence of the testing compounds. At the end of the incubation, 10 μL of sperm lysate was added to 1 mL of reaction buffer, and incubated for 10 min at room temperature. Triglyceride content was measured at 505 nm by using a spectrophotometer. Data are presented as μg/10^6 spermatozoa.

Lipase activity assay
Lipase activity was evaluated by the method of Panteghini *et al.* (2001) based on the use of 1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester (DGGR) as substrate (Aquila *et al.*, 2009b). Then, 50 μg of sperm extracts, treated as described earlier, was loaded into individual cuvettes containing buffer for spectrophotometric determination. DGGR was cleaved by lipase, resulting in an unstable dicarbonic acid ester which was spontaneously hydrolysed to yield glutaric acid and methylresorufin, a bluish purple chromophore with peak absorption at 580 nm. The absorbance of the samples was read every 20 s for 1.5 min. The rate of methylresorufin formation is directly proportional to the lipase activity in the sample. Analysis of total imprecision gave a coefficient of variation of between 0.01 and 0.03%. The estimated reference interval was 6–38 U·L^{-1} (μmol·min^{-1}·mg^{-1} protein). The enzymatic activity was determined with three control media: one without the substrate, one without the coenzyme (colipase) and the third without either substrate or coenzyme (data not shown).

Assay of acyl-CoA dehydrogenase activity
Assay of acyl-CoA dehydrogenase was performed on sperms, using a modification of the method described by Lehman

et al. (1990) (Aquila *et al*., 2006). In brief, after protein lysis, 70 μg of sperm proteins was added to the buffer containing 20 mM Mops, 0.5 mM EDTA and 100 μM FAD^+ at pH 7.2. Reduction of FAD^+ to FADH was read at 340 nm upon the addition of octanoyl-CoA (100 μM) every 20 s for 1.5 min. Data are expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$ protein. The enzymatic activity was determined with three control media: one without octanoyl-CoA as substrate, one without the coenzyme (FAD^+) and the third without either substrate or coenzyme (data not shown).

G6PDH activity

The G6PDH activity was performed as previously described (Aquila *et al*., 2005). Briefly, sperm samples, washed twice with an uncapacitating medium, were treated as mentioned earlier and incubated in the same medium for 30 min at 37°C and 5% CO_2. After incubation, 50 μL of sperm extracts was loaded into individual cuvettes containing buffer (100 mM triethanolamine, 100 mM $MgCl_2$, 10 $mg \cdot mL^{-1}$ glucose-6-phosphate, 10 $mg \cdot mL^{-1}$ $NADP^+$, pH 7.6) for spectrophotometric determination. The conversion of $NADP^+$ to NADPH, catalysed by G6PDH, was measured by the increase of absorbance at 340 nm every 20 s for 1.5 min. Data are expressed in $nmol \cdot min^{-1}/10^6$ spermatozoa. The enzymatic activity was determined with three control media: one without glucose-6-phosphate as substrate, one without the coenzyme ($NADP^+$) and the third without either substrate or coenzyme (data not shown).

Statistical analysis

The data obtained from Western blots, Ca^{2+} assay, cholesterol efflux assay, triglyceride assay, G6PDH activity, acyl-CoA dehydrogenase activity, lipase activity, acrosin activity, viability and motility (six replicate experiments using duplicate determinations) are presented as the mean ± SEM. The differences in mean values were calculated using ANOVA with Newman–Keuls *post hoc* test. Values of $P < 0.05$ were taken to show a significant difference between means.

Materials

BSA protein standard, Laemmli sample buffer, pre-stained molecular weight markers, Percoll (colloidal PVP-coated silica for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, Earle's balanced salt solution (uncapacitating medium) and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100 and eosin Y were from Farmitalia Carlo Erba (Milan, Italy). ECL Plus Western blotting detection system, Hybond ECL and HEPES sodium salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Triglyceride assay kit, cholesterol assay kit, lipase activity kit, calcium (Ca^{2+}) assay kit, G6PDH activity assay and CHOD–POD enzymatic colorimetric kit were from Inter-Medical (Biogemina Sas, Catania, Italy). Goat polyclonal β-actin antibody (Ab); polyclonal rabbit anti-phosphoAkt (S473); and total Akt Abs, POD-coupled anti-mouse, anti-rabbit and anti-goat IgG secondary Abs were from Santa Cruz Biotechnology (Heidelberg, Germany). Polyclonal anti-phosphoBcl2 (S70), anti-total Bcl2 Abs, anti-phosphoGSK3-beta (S9) and total GSK3 were from Cell Signaling (Milan, Italy). The CB_1 receptor antagonist rimonabant was kindly provided by Sanofi-Aventis (Montpellier, France). The MF-AEA was purchased from Sigma Chemical, and the fatty acid amide hydrolase (FAAH) inhibitor, 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate (URB597) was from Alexis Biochemicals (Firenze, Italy).

The nomenclature of cannabinoid receptors, their agonists and antagonists and endocannabinoid-metabolizing enzymes follows Alexander *et al*. (2008).

Results

CB_1 receptor blockade by rimonabant influences sperm motility and viability

Flagellar sperm motility was determined in increasing concentrations of rimonabant from 1 nM to 1 μM. Our data indicated that sperm motility was increased by rimonabant at 1 and 10 nM (Figure 1A), but unchanged at higher concentrations (100 nM or 1 μM). Similar results were obtained when we analysed the effects of the same concentrations of rimonabant on sperm viability, even though 10 nM appears to be more efficacious compared with 1 nM of rimonabant (Figure 1B). Notably, the CB_1 receptor agonist, MF-AEA, at 0.1 μM caused opposing effects either on sperm motility or viability. URB597 (URB, 0.1 μM), which increases the level of endogenous AEA by inhibiting the activity of its degrading enzyme, FAAH (Piomelli *et al*., 2006), also reduced the motility and viability of sperm. In particular, MF-AEA plus rimonabant or URB plus rimonabant resulted in the strengthening of the rimonabant-induced effects.

In order to provide further insights on the molecular action of rimonabant, we also evaluated the phosphorylation levels of key proteins controlling cell survival such as Akt and Bcl2. Our findings indicate (Figure 2) that rimonabant alone induced the phosphorylation of both Akt and Bcl2, whereas MF-AEA and URB reduced this effect.

Effects of rimonabant on human sperm capacitation

To investigate the possible action of rimonabant on sperm capacitation, we studied the variation of intracellular free Ca^{2+} content, cholesterol efflux and tyrosine protein phosphorylation after treatment with increasing concentrations of rimonabant in the presence and absence of MF-AEA and URB. Results showed that rimonabant, up to 100 nM, produced a concentration-dependent increase in the intracellular free Ca^{2+} (Figure 3A). Increased cholesterol efflux (Figure 3B) was also observed upon treatment with rimonabant, even though the effect was not concentration dependent, and 10 nM was the most effective concentration. In addition, we observed an enhancement in protein tyrosine phosphorylation at 1 and 10 nM rimonabant (Figure 3C and D). All the effects induced by rimonabant were opposite to those of MF-AEA and URB (both used at concentrations of 0.1 μM) and 1 μM rimonabant fully reversed the agonist-mediated decrease of

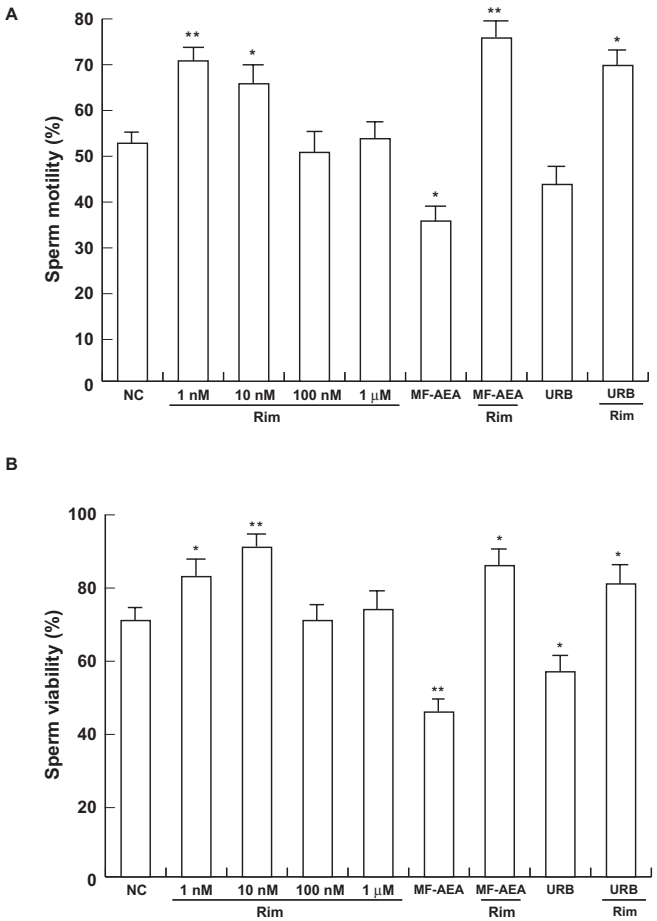

Figure 1 Rimonabant positively affects sperm motility and viability. Spermatozoa were incubated in unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing concentrations of rimonabant, MF-AEA (0.1 μM) and URB (0.1 μM) alone or in combination with 1 μM rimonabant. Sperm motility (A) and viability (B) were assessed as reported in Methods. Histograms represent mean ± SEM of three independent experiments, each in duplicate. *$P < 0.05$ and **$P < 0.02$ versus control.

cholesterol efflux and protein tyrosine phosphorylation. Rimonabant also reversed the effect of the agonist on free Ca^{2+} content.

Rimonabant induces acrosin activity

A sperm was treated with increasing concentrations of rimonabant (1, 10 and 100 nM, and 1 μM), and incubated under uncapacitating conditions (see Methods for details) to evaluate the activity of acrosin, the major enzyme contained in the acrosome. A significant increase was produced by rimonabant, and 10 nM was the most effective concentration (Figure 4). Intriguingly, in this assay, the effect of the treatment with the CB_1 receptor agonist was not different to that of rimonabant alone and rimonabant 1 μM plus 10 nM MF-AEA (i.e. the concentration at which we have already observed an effect of MF-AEA on the acrosome reaction) (Aquila *et al.*, 2009a). Similarly, URB (0.1 μM) alone or plus rimonabant (1 μM) all increase acrosin activity.

Rimonabant-induced effects on sperm lipid metabolism

In order to evaluate whether rimonabant had the ability to modulate sperm lipid metabolism, we first investigated its action on the intracellular content of triglycerides. Our data indicated that rimonabant decreased the triglyceride content in a concentration-independent manner (Figure 5A). Moreover, treatment with rimonabant induced an increase of lipase activity (Figure 5B) and the β-oxidation of fatty acids, as assayed by the octanoyl-CoA dehydrogenase activity (Figure 5C), within the same concentration range. MF-AEA and URB attenuated the effect of rimonabant alone (Figure 5) exerting *per se* a lipogenic action on human sperm cells.

Rimonabant-induced effects on sperm glucose metabolism

To further investigate the potential effects of rimonabant on human sperm metabolism, we chose to evaluate expression of phosphorylated GSK3 and G6PDH activity. Rimonabant treatment was able to significantly induce G6PDH activity at 1 and 10 nM (Figure 6A). Enhancement of endogenous AEA by URB or treatment with the agonist MF-AEA at the same concentrations as used to study lipid metabolism induced no change in G6PDH activity. These effects were reversed by pretreatment with rimonabant. In terms of GSK3 (Figure 6B), at 1 nM rimonabant reduced GSK3 phosphorylation, whereas MF-AEA and URB induced the opposite effect. Also in this case, the treatment with both rimonabant and CB_1 receptor agonist attenuated the effect of the agonist alone. Altogether, these data suggest that rimonabant increased glucose expenditure in human sperm.

Discussion

The EC system is involved in mammalian reproduction, but the significance of (endo)cannabinoid signalling and the pharmacological implications of CB receptor modulation in spermatogenesis, fertilization and embryonic implantation and growth remain still largely unknown (Wang *et al.*, 2006). An autonomous EC system has been found in sperm (Schuel *et al.*, 1994; Rossato *et al.*, 2005). Several reports have demonstrated CB_1 receptor-mediated inhibitory effects of endocannabinoids on mammalian sperm functions (Maccarrone *et al.*, 2005; Rossato *et al.*, 2005; Aquila *et al.*, 2009a,b), supporting the idea that the physiological role of the EC system in sperm is to maintain a quiescent, uncapacitated condition before interacting with the egg (Rossato, 2008). In the present study, we clearly showed that rimonabant was able to increase human sperm motility and viability, and, at molecular level, it appears that Akt and Bcl2 proteins are involved. These enzymes control key pro-survival pathways, and the phosphatidylinositol 3′-kinase/Akt signalling pathway has also been shown to be a positive regulator of cell metabolism (Maddika *et al.*, 2007). On the contrary, AEA reduced sperm motility and viability, and rimonabant completely reversed this action. We obtained similar effects by using the highly selective FAAH inhibitor URB597, suggesting that the blockade of CB_1 receptor is involved in the observed effects. The present findings, together with our already reported evidence that the CB_2 antagonist SR144528 and the vanilloid receptor

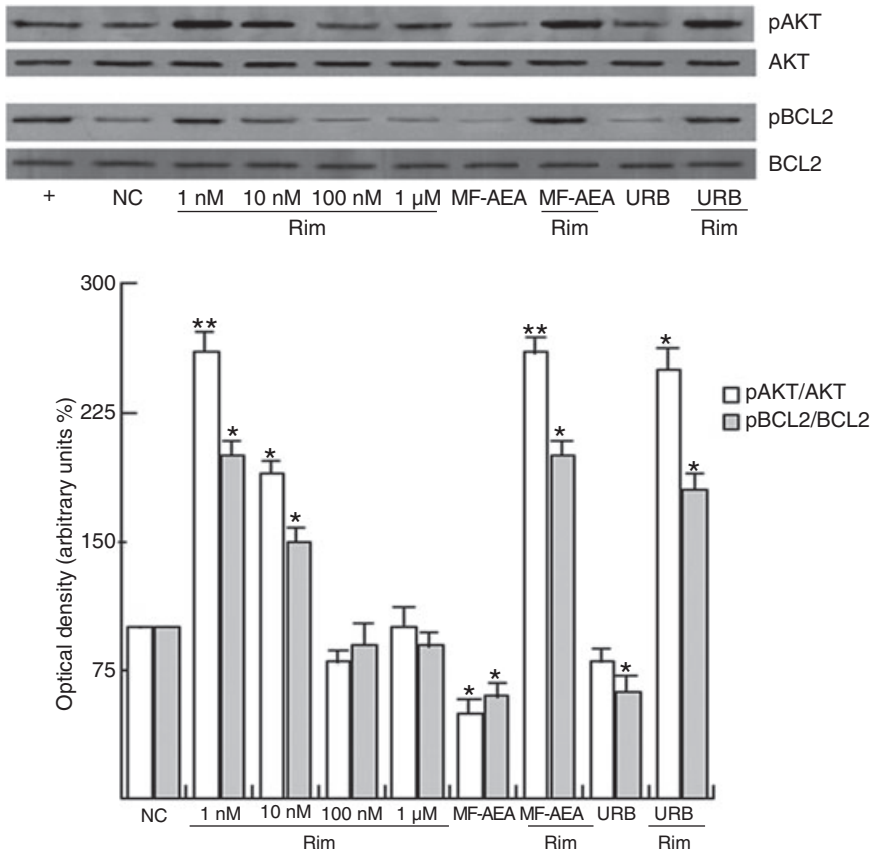

Figure 2 Rimonabant induces Akt and Bcl2 phosphorylation in sperm cells. Washed spermatozoa were incubated in uncapacitating medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing concentrations of rimonabant, MF-AEA (0.1 μM) and URB (0.1 μM) alone or in combination with 1 μM rimonabant. Capacitating medium (+) was used as a positive control. Representative Western blots of phosphoAkt (pAKT) and phosphoBcl2 (pBCL2) are shown. Densitometric analysis (mean ± SEM) of four independent experiments are reported below as pAkt/Akt and pBcl2/Bcl2 relative intensity. *$P < 0.05$ and **$P < 0.02$ versus control.

antagonist capsazepine were unable to counteract AEA-mediated action on sperm survival and Akt phosphorylation (Aquila *et al.*, 2009a), also support a CB_1 receptor-mediated action as the underlying mechanism. Our results are also in agreement with previous data showing that rimonabant induced Akt activity and glucose uptake in skeletal muscle cells in a CB_1-dependent manner (Esposito *et al.*, 2008). Moreover, this evidence is corroborated by Ricci *et al.* (2007) who reported that sperm from CB_1 receptor knockout mice showed a strong increase of motility in the head of epididymus, compared to wild-type mice.

During life, sperm exists in two different physiological conditions: a quiescent state in the seminal plasma and a capacitated state upon ejaculation and movement through the female reproductive tract. Capacitation involves numerous physiological changes including destabilization of the plasma membrane, cholesterol efflux, alterations of intracellular ion concentrations and protein phosphorylation (Yanagimachi, 1994). In order to gain further insight into the effects of rimonabant on sperm fertilization, we tested rimonabant's effects on Ca^{2+} concentration, cholesterol efflux and protein tyrosine phosphorylation, which are hallmarks of the capacitation status (Visconti *et al.*, 2002; Jha *et al.*, 2003; Suarez, 2008). Our results demonstrated a concentration-dependent increase of Ca^{2+} content, whereas cholesterol efflux was significantly induced until 1 μM in a concentration-independent manner. Protein tyrosine phosphorylation was also induced. These findings indicate a stimulation of the adenylate cyclase/cAMP/PKA signalling, which plays an important role not only in capacitation (Travis and Kopf, 2002; Jha *et al.*, 2003), but also in the acrosome reaction (Salicioni *et al.*, 2007). Moreover, our data demonstrating an increase in acrosin activity after treatment of sperm with rimonabant are in line with this finding and with previous observations showing that this pathway is inhibited by endocannabinoids in a CB_1 receptor-dependent manner (Maccarrone *et al.*, 2005; Aquila *et al.*, 2009a). Interestingly, AEA did not decrease acrosin activity, and in accord with our previous findings, we found that acrosin was stimulated by the addition of 10 nM MF-AEA, which corresponds to the physiological concentration in mid-cycle oviductal fluid (Schuel *et al.*, 2002). Furthermore, treatment with both MF-AEA and rimonabant potentiated, rather than reversed, the effect of MF-AEA alone. This finding is not surprising because synergic effects of MF-AEA and rimonabant have already been reported (Malfitano *et al.*, 2008). On the other hand, we also demonstrated that treatment with MF-AEA plus rimonabant increased free Ca^{2+} content in human sperm, even though MF-AEA alone induced similar effects (Aquila *et al.*, 2009b).

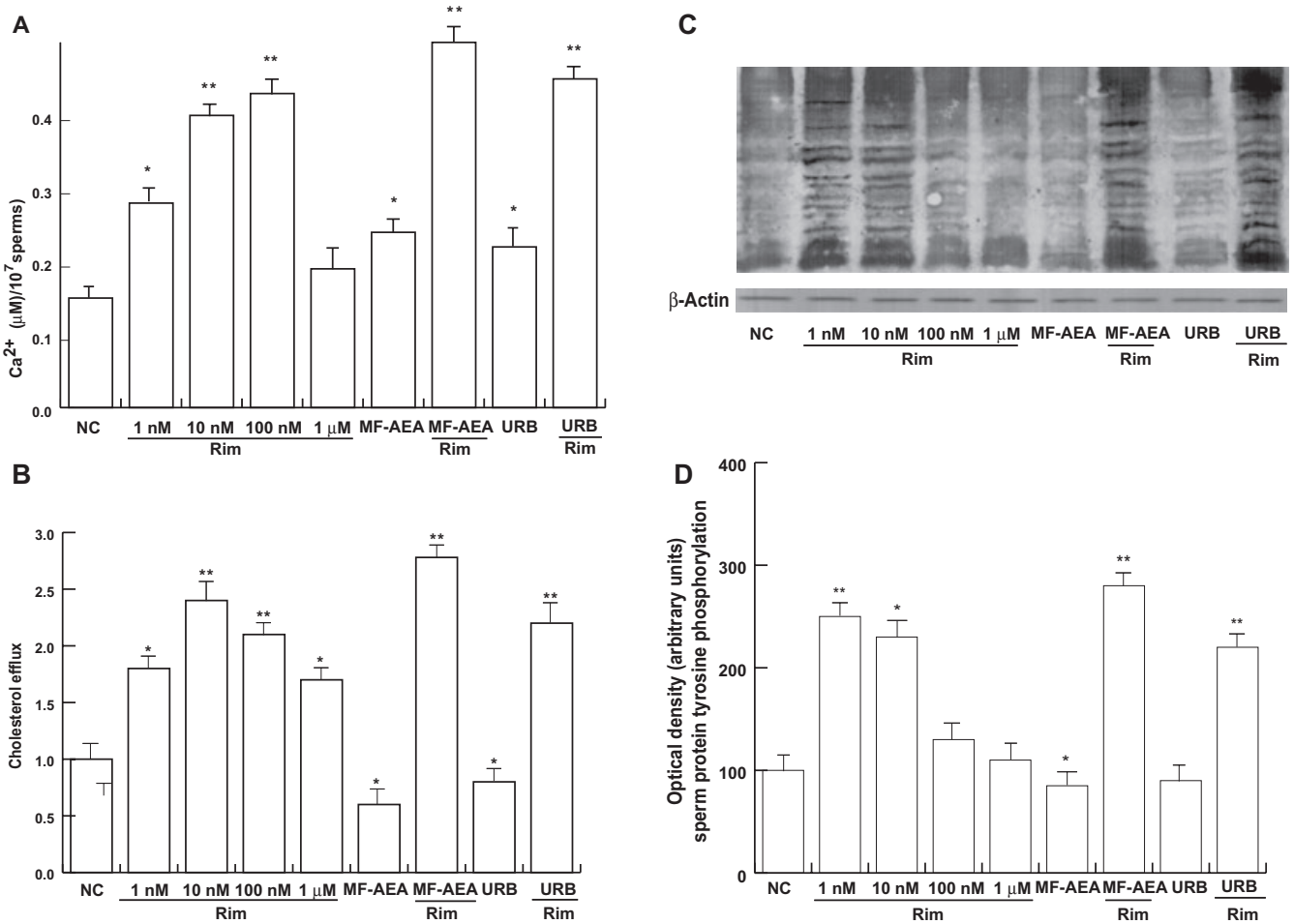

Figure 3 Rimonabant increases free intracellular Ca^{2+} cholesterol efflux and protein tyrosine phoshorylation. Sperm cells were incubated in unsupplemented Earle's medium (uncapacitating medium) for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing concentrations of rimonabant, MF-AEA and URB (0.1 μM, respectively) alone or in combination with 1 μM rimonabant. Free intracellular Ca^{2+} (A) and cholesterol efflux (B) were measured, and values shown represent mean ± SEM. *$P < 0.05$ and **$P < 0.02$ versus control. (C) Sperm lysates were used for Western blot analysis performed to determine protein tyrosine phosphorylation. Actin was used as a loading control. (D) Quantitative representation after densitometric evaluation of the 95 kDa band/actin. Autoradiograph presented is a representative example of independent experiments performed four times. *$P < 0.05$ versus control, **$P < 0.02$ versus control.

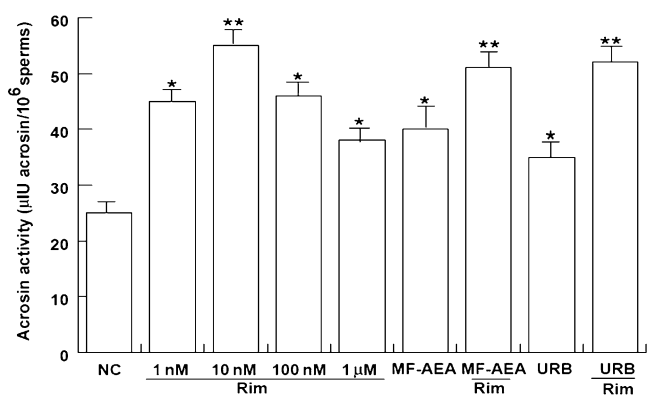

Figure 4 Rimonabant increases acrosin activity in human sperm. Washed spermatozoa were incubated in unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of rimonabant (from 1 nM to 1 μM), MF-AEA (10 nM) and URB (0.1 μM) alone or in combination with 1 μM rimonabant. Acrosin activity was determined, and data shown represent mean ± SEM of three independent experiments each in duplicate. *$P < 0.05$ and **$P < 0.02$ versus control.

The interaction between energy balance and reproduction is subject of intensive investigations, and ATP generation is an important process in human sperm especially after the initiation of capacitation (Yanagimachi, 1994; Miki, 2007). Capacitated sperm displays an increased metabolic rate and overall energy expenditure, presumably to effect the changes in sperm signalling and function during capacitation. It appears that each event characterizing male fertilization requires different substrates and activates different metabolic pathways. For example, the acrosome reaction requires lactate or pyruvate for ATP production by oxidative phosphorylation, while successful gamete fusion requires glucose to produce NADPH through the pentose phosphate pathway (PPP) (Urner and Sakkas, 1999a; Miki, 2007). From this point of view, we investigated whether the capacitated status and increased motility induced by rimonabant would also involve specific changes in lipid and glucose metabolism. We found that the compound induced human sperm energy expenditure by stimulating lipase and octanoyl-CoA dehydrogenase activities concomitantly with reducing the level of triglycerides. As for

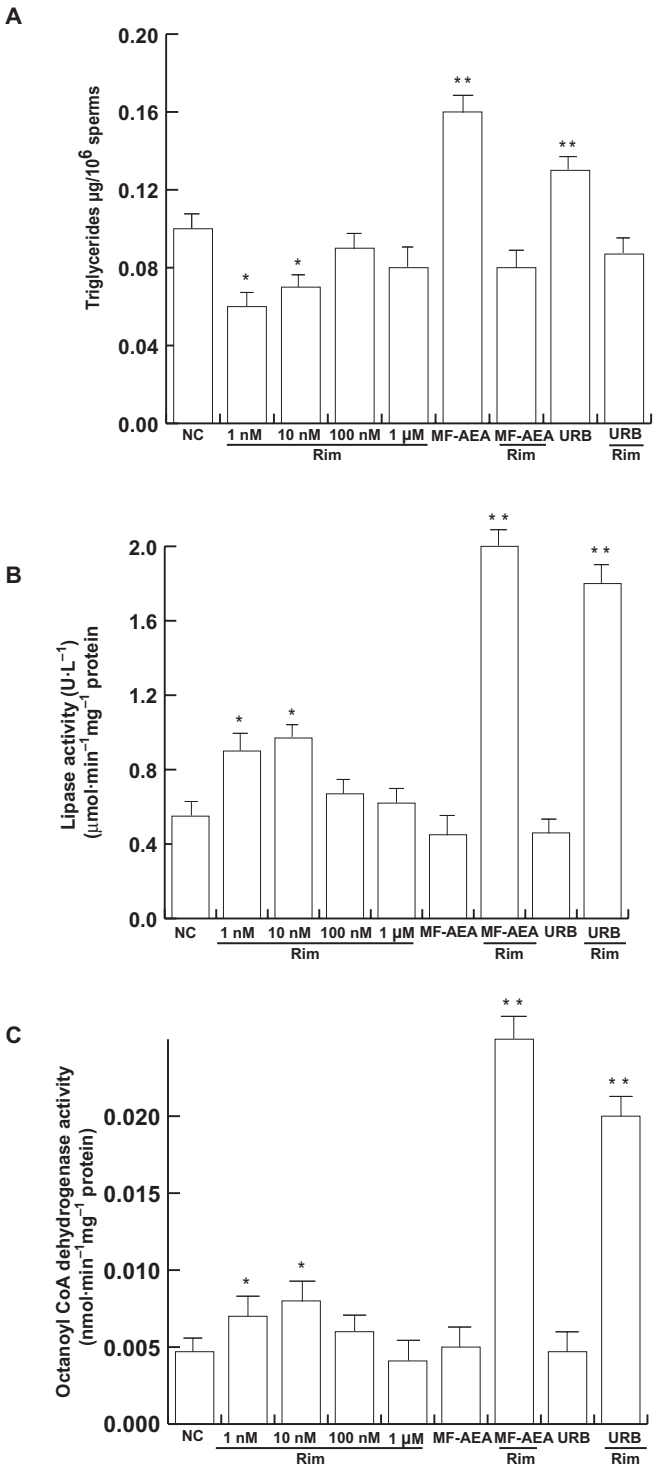

Figure 5 Rimonabant increases lipid metabolism in human sperm. Washed spermatozoa were incubated in uncapacitating medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of rimonabant (from 1 nM to 1 µM), MF-AEA (0.1 µM) and URB (0.1 µM) alone or in combination with 1 µM rimonabant. Triglyceride content (A), lipase activity (B) and octanoyl-CoA dehydrogenase activity (C) were determined, and data shown represent mean ± SEM of four independent experiments each in duplicate. *$P < 0.05$ and **$P < 0.02$ versus control.

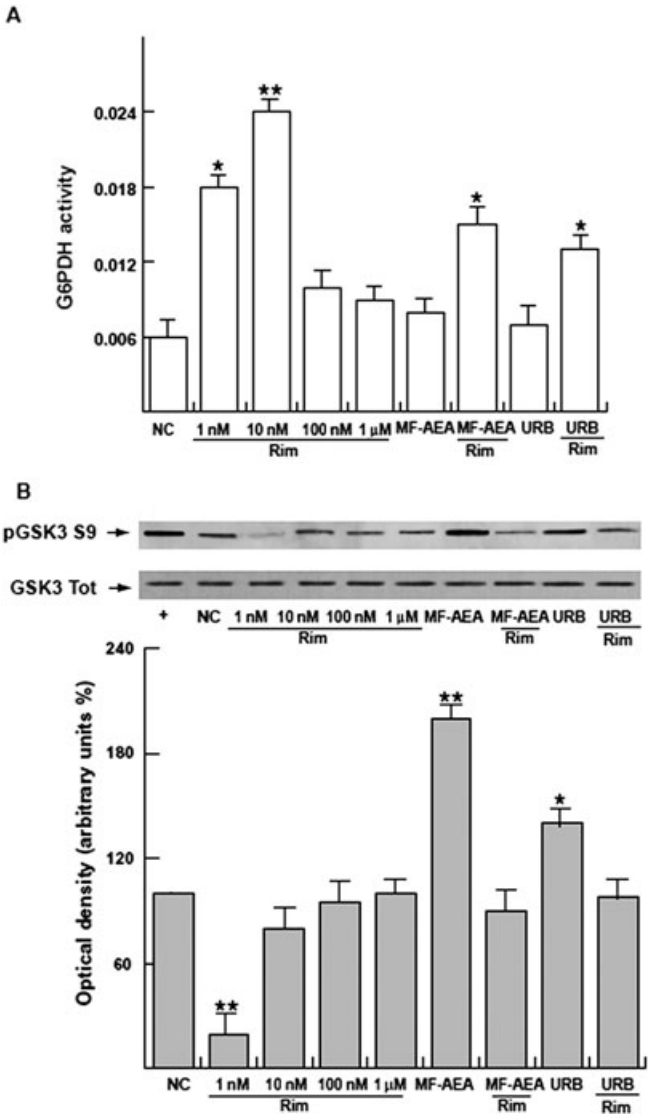

Figure 6 Rimonabant increases glucose metabolism in human sperm. Sperm samples, washed twice with an uncapacitating medium, were incubated in the same medium (NC) for 30 min at 37°C and 5% CO_2, and treated with or without rimonabant in the presence or absence of MF-AEA and URB (0.1 µM respectively). (A) The conversion of $NADP^+$ to NADPH catalysed by G6PDH was measured by the increase of absorbance at 340 nm every 20 s for 1.5 min. Data are expressed in nmol·min^{-1}/10^6 spermatozoa, and represent mean ± SEM. *$P < 0.05$ and **$P < 0.02$ versus control. (B) Western blot and quantitative representation after densitometric evaluation of the ratio of phosphorylated GSK3/total GSK3. *$P < 0.05$ versus control, **$P < 0.02$ versus control. Autoradiograph presented is representative of three independent experiments. +, MCF7 tumour breast cancer cells used as positive control.

glucose metabolism, we observed an enhanced G6PDH activity and a reduced phosphorylation of GSK3 at 1 and 10 nM rimonabant concentrations, also addressing to an induction of energy consumption. GSK3 is known to be implicated in the storage of glucose to form glycogen in mammalian sperm (Ballester *et al*., 2000; Andò and Aquila, 2005; Aquila *et al*., 2005). Particularly, in uncapacitated sperm, GSK3 is tightly blocked and phosphorylated, whereas during capacitation there is a de-phosphorylation and then an activation of the

enzyme. G6PDH, the rate-limiting enzyme in the PPP that catalyses the oxidation of glucose 6-phosphate and regulates the production of NADPH by controlling glucose metabolism (Urner and Sakkas, 1999b) is crucial for sperm-fertilizing ability (Urner and Sakkas, 1999a; Travis *et al.*, 2001; Urner *et al.*, 2001). Metabolic fluxes between glycolysis and the PPP are also particularly relevant for maintaining the mitochondrial transmembrane potential, and balancing cellular requirements for energy and the production of reactive oxygen intermediates (Perl, 2007), and it has been reported that cannabinoid receptor agonists inhibit mitochondrial membrane potential and ATP production in rat testicular tissue (see Rossato, 2008). Our findings demonstrating that nanomolar concentrations of rimonabant favour G6PDH activity are in line with the observation that AEA, unlike rimonabant, is able to affect human sperm metabolism by exerting a lipogenic effect and favouring the accumulation of energy substrates (Aquila *et al.*, 2009b). Moreover, the effect of the enhancement of endogenous AEA by URB-mediated action and the treatment with MF-AEA at the concentration used to study lipid metabolism, supported the hypothesis that rimonabant, through action at CB_1 receptors, induced glucose metabolism in sperm.

It is well known that the EC system contributes to the physiological regulation of energy balance, and rimonabant reduces food intake inducing favourable changes in lipid and glucose metabolism in obese patients, as well as in animal models (see Bifulco *et al.*, 2007; 2009). Moreover, endocannabinoids are regulated negatively by leptin and *ob*/*ob* and *db*/*db* mice, characterized by an impairment of leptin signalling are obese, infertile and fail to undergo normal sexual maturation (Chehab, 2000; Di Marzo *et al.*, 2001). In *ob/ob* mice, exogenous leptin restored fertility (Chehab *et al.*, 1996), and it has been demonstrated that leptin controls AEA degradation, but not its binding to the CB_1 receptor. Rimonabant induced a significant decrease in food intake and body weight loss in *ob/ob* mice, but failed to improve female fertility when administered alone (Maccarrone *et al.*, 2004). These results, together with ours, suggest that CB_1 receptor modulation might be important in human fertility, and rimonabant might be used to develop special media to improve *in vitro* fertilization.

In addition, rimonabant, by acting as a CB_1 antagonist, was able, at 0.1 μM, to reverse the negative effects of AEA on boar spermatozoa (Maccarrone *et al.*, 2005), a finding in agreement with those we have obtained in human spermatozoa. In the majority of the tests we performed, rimonabant was active at concentrations in the range of the highest affinity for the CB_1 receptor (1–20 nM) (Rinaldi-Carmona *et al.*, 1994; Pertwee, 2005), and had opposite effects compared to those produced by the exogenous and endogenous AEA. Besides, the tested compound attenuated the effect of the agonist even though an enhancement of the antagonist-induced effect can be observed in the presence of both CB_1 receptor agonist and antagonist. Moreover, above 100 nM, we did not observe any significant effects of rimonabant, suggesting that starting from this concentration rimonabant could display neutral antagonism, while, in combination with MF-AEA, it behaves as an inverse agonist. Rimonabant has been shown to act as a neutral antagonist, competitive antagonist and inverse agonist (Hurst *et al.*, 2005; Pertwee, 2005). As an inverse agonist, rimonabant produces effects in some CB_1 receptor containing bioassay systems that are opposite in direction from those produced by agonists for these receptors. It was proposed that inverse agonism at the CB_1 receptor may be explained in terms of a three-state model in which the receptor can switch between two conformational states, a ground or inactive R state and an active R* state, which are in equilibrium with each other (Leff, 1995). An agonist has higher affinity for R*, and agonist binding is thought to shift the equilibrium towards R*, resulting in G protein activation with an increase in GDP/GTP exchange. An inverse agonist has higher affinity for R and its binding shifts the equilibrium towards R, resulting in a decrease in the activation of the signalling pathway. The binding of a neutral/null antagonist is thought not to alter the equilibrium between R and R*, because the neutral antagonist has equal affinity for both states. It is likely that the efficacy for the production of inverse cannabimimetic effects will be governed by the degree of endocannabinoid release at CB_1 receptors (Aquila *et al.*, 2009b).

Finally, we have to point out that in our experimental conditions, rimonabant is able to antagonize endocannabinoid-mediated inhibition of human sperm functions, in agreement with those obtained earlier in boar sperm by Maccarrone *et al.* (2005), in frog sperm by Cobellis *et al.* (2006) and human sperm by Rossato *et al.* (2005).

In conclusion, in the present study, we demonstrate for the first time that rimonabant is able to improve fertilization by the human male gamete, and stimulates energy expenditure in sperm.

Acknowledgements

This work was supported by Sanofi-Aventis (M.B.), MURST ex 60% (2007), and the Associazione Educazione e Ricerca Medica Salernitana. Our special thanks to Dr Vincenzo Cunsolo (Biogemina Italia Srl, Catania, Italy) for technical and scientific assistance. We also thank Serena and Maria Clelia Gervasi for the English language review of the manuscript.

Conflict of interest

Authors declare that there is no conflict of interest to disclose.

References

Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edn. *Br J Pharmacol* **153**: S1–S209.

Altarejos JY, Goebel N, Conkright MD, Inoue H, Xie J, Arias CM *et al.* (2008). The Creb1 coactivator Crtc1 is required foe energy balance and fertility. *Nat Med* **14**: 1112–1117.

Andò S, Aquila S (2005). Arguments raised by the recent discovery that insulin and leptin are expressed in and secreted by human ejaculated spermatozoa. *Mol Cell Endocrinol* **245**: 1–6.

Aquila S, Sisci D, Gentile M, Middea E, Siciliano L, Andò S (2002). Human ejaculated spermatozoa contain active P450 aromatase. *J Clin Endocrinol Metab* **87**: 3385–3390.

Aquila S, Sisci D, Gentile M, Carpino A, Middea E, Catalano S *et al*. (2003). Towards a physiological role for cytocrome P450 aromatase in ejaculated human sperm. *Hum Reprod* **18**: 1650–1659.

Aquila S, Gentile M, Middea E, Catalano S, Andò S (2005). Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* **146**: 552–557.

Aquila S, Bonofiglio D, Gentile M, Middea E, Gabriele S, Belmonte M *et al*. (2006). Peroxisome proliferator-activated receptor (PPAR) gamma is expressed by human spermatozoa: its potential role on the sperm physiology. *J Cell Physiol* **209**: 977–986.

Aquila S, Guido C, Perrotta I, Santoro A, Laezza C, Bifulco M *et al*. (2009a). Human sperm anatomy: ultrastructural localization of the cannabinoid1 receptor (CB1-R) and a potential role of anandamide in sperm survival and acrosome reaction. *Anat Rec* (in press).

Aquila S, Guido C, Laezza C, Santoro A, Pezzi V, Panza S *et al*. (2009b). A new role of anandamide in human sperm: focus on metabolism. *J Cell Physiol* **221**: 147–153.

Ballester J, Fernandez-Novell JM, Rutllant J, Garcia-Rocha M, Jesus Palomo M, Mogas T *et al*. (2000). Evidence for a functional glycogen metabolism in mature mammalian spermatozoa. *Mol Reprod Dev* **56**: 207–219.

Bellocchio L, Cervino C, Pasquali R, Pagotto U (2008). The endocannabinoid system and energy metabolism. *J Neuroendocrinol* **20**: 850–857.

Bifulco M, Grimaldi C, Gazzerro P, Pisanti S, Santoro A (2007). Rimonabant: just an antiobesity drug? Current evidence on its pleiotropic effects. *Mol Pharmacol* **71**: 1445–1456.

Bifulco M, Santoro A, Laezza C, Malfitano AM (2009). Cannabinoid receptor CB1 antagonists state of the art and challenges. *Vitam Horm* **81**: 159–189.

Chehab FF (2000). Leptin as a regulator of adipose mass and reproduction. *Trends Pharmacol Sci* **21**: 309–313.

Chehab FF, Lim ME, Lu R (1996). Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet* **12**: 318–320.

Cobellis G, Cacciola G, Scarpa D, Meccariello R, Chianese R, Franzoni MF *et al*. (2006). Endocannabinoid system in frog and rodent testis: type-1 cannabinoid receptor and fatty acid amide hydrolase activity in male germ cells. *Biol Reprod* **75**: 82–89.

Di Marzo V, Goparaju SK, Wang L, Liu J, Bátkai S, Járai Z *et al*. (2001). Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* **410**: 822–825.

Elphick MR, Ergetova M (2001). The neurobiology and evolution of cannabinoid signalling. *Philos Trans R Soc Lond B* **356**: 381–408.

Esposito I, Proto MC, Gazzerro P, Laezza C, Miele C, Alberobello AT *et al*. (2008). The cannabinoid CB1 receptor antagonist rimonabant stimulates 2-deoxyglucose uptake in skeletal muscle cells by regulating the expression of phosphatidylinositol-3-kinase. *Mol Pharmacol* **74**: 1678–1686

Gerard CM, Mollerau C, Vassart G, Parmentier M (1991). Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem J* **279**: 129–134.

Hurst DP, Lynch DL, Barnett-Norris J, Hyatt SM, Seltzman HH, Zhong M *et al*. (2005). *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A) interaction with LYS 3.28(192) is crucial for its inverse agonism at the cannabinoid CB1 receptor. *Mol Pharmacol* **62**: 1274–1287.

Jha KN, Kameshwari DB, Shivaji S (2003). Role of signaling pathways in regulating the capacitation of mammalian spermatozoa. *Cell Mol Biol* **49**: 329–340.

Kennedy WP, Kamisky JM, Van der Ven HH, Jeyendran RS, Reid DS, Blackwell J *et al*. (1989). A simple, classical assay to evaluate the acrosin activity of human spermatozoa. *J Androl* **10**: 221–231.

Leff P (1995). The two-state model of receptor activation. *Trends Pharmacol Sci* **16**: 89–97.

Lehman TC, Hale DE, Bhala A, Thorpe C (1990). An acyl-coenzyme A dehydrogenase assay utilizing the ferricenium ion. *Annal Biochem* **186**: 280–284.

Maccarrone M (2008). CB2 receptor in reproduction. *Br J Pharmacol* **153**: 189–198.

Maccarrone M, Finazzi-Agrò A (2004). Anandamide hydrolase: a guardian angel of human reproduction? *Trends Pharmacol Sci* **25**: 353–357.

Maccarrone M, Fride E, Bisogno T, Bari M, Cascio MG, Battista N *et al*. (2004). Up-regulation of the endocannabinoid system in the uterus of leptin knockout (*ob/ob*) mice and implications for fertility. *Mol Hum Reprod* **11**: 21–28.

Maccarrone M, Barboni B, Bernabò N, Gasperi V, Pistilli MG, Fezza F *et al*. (2005). Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction. *J Cell Sci* **118**: 4393–4404.

Maddika S, Ande SR, Panigrahi S, Paranjothy T, Weglarczyk K, Zuse A *et al*. (2007). Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. *Drug Resist Updat* **10**: 13–29.

Malfitano AM, Laezza C, Pisanti S, Gazzerro P, Bifulco M (2008). Rimonabant (SR141716) exerts anti-proliferative and immunomodulatory effects in human peripheral blood mononuclear cells. *Br J Pharmacol* **153**: 1003–1010.

Melis MR, Succu S, Mascia MS, Sanna F, Melis T, Castelli MP *et al*. (2006). The cannabinoid receptor antagonist SR-141716A induces penile erection in male rats: involvement of paraventricular glutamic acid and nitric oxide. *Neuropharmacology* **50**: 219–228.

Miki K (2007). Energy metabolism and sperm functions. *Soc Reprod Fertil Suppl* **65**: 309–325.

Panteghini M, Bonora R, Pagani F (2001). Measurement of pancreatic lipase activity in serum by a kinetic colorimetric assay using a new chromogenic substrate. *Ann Clin Biochem* **38**: 365–370.

Perl A (2007). The pathogenesis of transaldolase deficiency. *IUBMB Life* **59**: 365–373.

Pertwee RG (2005). Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. *Life Sci* **76**: 1307–1324.

Piomelli D, Tarzia G, Duranti A, Tontini A, Mor M, Compton TR *et al*. (2006). Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). *CNS Drug Rev* **12**: 21–38.

Ricci G, Cacciola G, Altucci L, Meccariello R, Pierantoni R, Fasano S *et al*. (2007). Endocannabinoid control of sperm motility: the role of epididymus. *Gen Comp Endocrinol* **153**: 320–322.

Rinaldi-Carmona M, Barth F, Healume M, Shire D, Calandra B, Congy C *et al*. (1994). SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350**: 240–244.

Rossato M (2008). Endocannabinoids, sperm functions and energy metabolism. *Mol Cell Endocrinol* **286S**: S31–S35.

Rossato M, Ion Popa F, Ferigo M, Clari G, Foresta C (2005). Human sperm express cannabinoid receptor CB1, the activation of which inhibits motility, acrosome reaction, and mitochondrial function. *J Clin Endocrinol Metab* **90**: 984–991.

Salicioni AM, Platt MD, Wertheimer EV, Arcelay E, Allaire A, Sosnik J *et al*. (2007). Signalling pathways involved in sperm capacitation. *Soc Reprod Fertil* (Suppl 65): 245–259.

Schuel H, Schuel R, Zimmerman AM, Zimmerman S (1987). Cannabinoids reduce fertility of sea urchin sperm. *Biochem Cell Biol* **65**: 130–136.

Schuel H, Goldstein E, Mechoulam R, Zimmerman AM, Zimmerman S (1994). Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. *Proc Natl Acad Sci USA* **91**: 7678–7682.

Schuel H, Burkman LJ, Lippes J, Crickard K, Mahony MC, Giuffrida A *et al*. (2002). Evidence that Anandamide-signaling regulates human sperm functions required for fertilization. *Mol Reprod Dev* **63**: 376–387.

Suarez SS (2008). Control of hyperactivation in sperm. *Hum Reprod Update* **14**: 647–657.

Succu S, Mascia MS, Sanna F, Melis T, Argiolas A, Melis MR (2006). The cannabinoid CB1 receptor antagonist SR141716 induces penile erection by increasing extra-cellular glutamic acid in the paraventricular nucleus of male rats. *Behav Brain Res* **169**: 274–281.

Thomson MF, Wishart GJ (1989). Elucidation of the mechanism responsible for the temperature-dependent reversible inactivation of the motility of fowl spermatozoa. *Br Poult Sci* **30**: 687–692.

Travis AJ, Kopf GS (2002). The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *J Clin Invest* **110**: 731–736.

Travis AJ, Jorgez CJ, Merdiushev T, Jones BH, Dess DM, Diaz-Cueto L *et al*. (2001). Functional relationships between capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa. *J Biol Chem* **276**: 7630–7636.

Urner F, Sakkas D (1999a). A possible role for the pentose phosphate pathway of spermatozoa in gamete fusion in the mouse. *Biol Reprod* **60**: 733–739.

Urner F, Sakkas D (1999b). Characterization of glycolysis and pentose phosphate pathway during sperm entry into the mouse oocyte. *Biol Reprod* **60**: 973–978.

Urner F, Leppens-Luisier G, Sakkas D (2001). Protein tyrosine phosphorylation in sperm during gamete interaction in the mouse: the influence of glucose. *Biol Reprod* **64**: 1350–1357.

Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB (2002). Novel signaling pathways involved in sperm acquisition of fertilizing capacity. *J Reprod Immunol* **53**: 133–150.

Wang H, Dey SK, Maccarrone M (2006). Jekill and Hyde: two faces of cannabinoid signaling in male and female fertility. *Endocr Rev* **27**: 427–448.

World Health Organization (1999). *Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interactions*, 4th edn. Cambridge University Press: Cambridge.

Yanagimachi R (1994). Mammalian fertilization. In: Knobil E, Neill JD (eds). *The Physiology of Reproduction*. Raven Press Ltd: New York, pp. 189–317.

Human Sperm Anatomy: Ultrastructural Localization of the Cannabinoid1 Receptor and a Potential Role of Anandamide in Sperm Survival and Acrosome Reaction

SAVERIA AQUILA,[1,2] CARMELA GUIDO,[1,2] ANTONIETTA SANTORO,[3] IDA PERROTTA,[4] CHIARA LAEZZA,[5] MAURIZIO BIFULCO,[3]* AND ANDÒ SEBASTIANO[6,7]

[1]Department of Pharmaco-Biology, University of Calabria, Arcavacata di Rende, Cosenza, Italy
[2]Centro Sanitario, University of Calabria, Arcavacata di Rende, Cosenza, Italy
[3]Department of Pharmaceutical Sciences, University of Salerno, Fisciano, Salerno, Italy
[4]Department of Ecology, University of Calabria, Arcavacata di Rende, Cosenza, Italy
[5]IEOS-CNR, Napoli, Italy
[6]Department of Cellular Biology, University of Calabria, Arcavacata di Rende, Cosenza, Italy
[7]Faculty of Pharmacy, University of Calabria, Arcavacata di Rende, Cosenza, Italy

ABSTRACT

Recently, the endocannabinoid (EC) system and the presence of CB1 receptor (CB1-R), have been identified in human sperm. However, the effects of EC receptor ligands such as anandamide (N-arachidonoylethanolamine) and the role of EC system in male fertility is still largely unexplored. In the present study, we investigated the ultrastructural compartmentalization of CB1-R and analyzed the effects of its stimulation by using a stable analog of anandamide, 2-methylarachidonyl-2′-fluoro-ethylamide (MET-F-AEA). We focused particularly on sperm survival and acrosin activity. The study of human sperm anatomy by transmission electron microscopy with immunogold analysis revealed the location of the CB1-R prevalently in the sperm membranes of the head and interestingly on the mitochondria. The effect of different concentrations of MET-F-AEA from 100 nM to 1 μM evidenced a significant decrease of sperm survival. Interestingly, we analyzed this negative effect at molecular level, testing the EC action on different known sperm survival targets. MET-F-AEA-treatment decreased both pBCL2 and pAkt, two prosurvival proteins, and increased pPTEN expression which is the main regulator of the PI3K/Akt pathway. Moreover, a biphasic effect was observed with increasing MET-F-AEA concentrations on the acrosin activity. The blockage of the CB1-R by using its selective antagonist SR141716 (rimonabant) induced an opposite action on sperm survival supporting a role for this receptor in the biology of the male gamete. Anat Rec, 293:298–309, 2010. © 2009 Wiley-Liss, Inc.

Key words: CB1-R; anandamide; sperm; male reproduction; SR141716

Grant sponsors: Associazione Educazione e Ricerca Medica Salernitana, ERMES and by PRIN-MIUR and Ex 60%-2007.

Aquila Saveria and Guido Carmela equally contributed to this work.

*Correspondence to: Maurizio Bifulco, Dipartimento di Scienze Farmaceutiche, Universitá di Salerno, Via Ponte don Melillo, Fisciano, Salerno 84084, Italy. E-mail: maubiful@unina.it or maubiful@unisa.it or aquisav@libero.it

Received 1 July 2008; Accepted 16 June 2009

DOI 10.1002/ar.21042

Published online 24 November 2009 in Wiley InterScience (www.interscience.wiley.com).

During the past years, a great deal of data have been accumulated demonstrating that cannabinoids/endocannabinoids (ECs) control several physiological functions including reproductive system functions (Di Marzo et al., 2004). ECs affect secretion of pituitary gonadotrophic hormones and gonadal steroids, spermatogenesis, ovulation, implantation of blastocysts into the uterine endometrium, and fetal growth; (Powell and Fuller, 1983; Maykut, 1985; Smith and Asch, 1987; Murphy et al., 1994; Schuel et al., 1999; Maccarrone et al., 2000; Paria and Dey, 2000; Mani et al., 2001; Paria et al., 2001).

The well-known effects of exogenous cannabinoids are prevalently established acting as ligands of cannabinoid receptors (CB-Rs) (Park et al., 2004) whereas Anandamide (N-arachidonoyl-ethanolamine, AEA) is the most intensively studied endogenous agonist for the CB-Rs (Devane et al., 1992). To date, two different CB-Rs subtypes have been identified and cloned, the brain-type CB1-R and the spleen-type CB2-R (Devane et al., 1988; Matsuda et al., 1990; Galiegue et al., 1995). Both CB1-R and CB2-R are widely distributed in many other tissues including uterus and testis (Schuel et al., 2002a). Recently, it has been demonstrated that rat testis is able to synthesize AEA (Sugiura et al., 1996) and this compound has been detected also in human seminal plasma (Schuel et al., 2002a). The presence of CB1-Rs in Leydig cells and their involvement in testosterone secretion have been demonstrated in mice, whereas mouse Sertoli cells have been shown to possess CB2-Rs (Wenger et al., 2001).

Evidence for the presence of functional cannabinoid receptors in sperm was first obtained with sea urchin sperm (Chang et al., 1993; Schuel et al., 1994), and subsequently in human sperm (Schuel et al., 2002b). Particularly, it has been reported that human sperm express the CB1-R, located in the head and middle piece sperm (Rossato et al., 2005). However, while Rossato et al. (2005) failed to detect CB2-R in human sperm using Western blots, Maccarrone et al. (2005) detected low levels of CB2-R in boar sperm with Western blots and radioligand binding procedures. Sperm are also equipped with the typical machinery regulated by endocannabinoids, since recently, the EC system was discovered in boar spermatozoa, supporting a physiological role of AEA in controlling male fertility (Maccarrone et al., 2005). Experimental data have accumulated confirming that ECs negatively influence the motility of sperm in different mammalian species. It was shown that AEA reduces human sperm motility by reducing mitochondrial functions (Rossato et al., 2005). In fact, it was reported that ECs may interfere with mitochondrial electron transport and decrease both mitochondrial activity via depletion of NADH and mitochondrial permeability transition (Sarafian et al., 2003). In addition, in sperm CB1-R activation determines the inhibition of capacitation and acrosome reaction (Schuel et al., 1994; Schuel et al., 2002a; Maccarrone et al., 2005; Rossato et al., 2005; Cobellis et al., 2006; Aquila et al., 2009). Sperm motility, together with capacitation and acrosome reaction, are all energy consuming processes (Miki, 2007; Ruiz-Pesini et al., 2007), therefore, the aforementioned inhibitory effects of ECs on mitochondrial functions might be well correlated. Altogether, these findings have led to the suggestion that the EC network plays a role in the male fertility (Maccarrone et al., 2003b), however, the molecular mechanisms through which CB1-R stimulation acts in this context needs to be further elucidated.

The signaling pathways through which ECs induce apoptosis is under active investigation and may vary based on cell type (Dobrosi et al., 2008; Turco et al., 2008). While apoptosis in somatic cells and in (testicular) spermatocytes and spermatids *in vivo* is well established, the presence and significance of apoptosis in ejaculated human sperm is still unresolved (Oehninger et al., 2003). Human spermatozoa have been documented to display features of several apoptosis signal transduction pathways such as the externalization of phosphatidylserine, disruption of the transmembrane mitochondrial potential, and activation of caspases (Glander and Schaller, 1999; Oehninger et al., 2003; Paasch et al., 2004c). Interestingly, a strong correlation of apoptosis markers with sperm parameters exists, denoting that it might affect the sperm fertilization potential (Paasch et al., 2004a,b).

Recently, in human sperm, we have explored one of the main pathway involved in cell survival, the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway (Aquila et al., 2004, 2007). In our studies, we have evidenced a regulation of human sperm survival through the PI3K/Akt pathway by evaluating some representative proteins downstream to the pathway, such as Akt (Cantley, 2002) and Bcl-2 (Ito et al., 1997) phosphorylation, which is required for their anti-apoptosis function, together with PTEN, an upstream regulator of the same pathway (Wu et al., 1998).

In the aim to investigate human sperm anatomy at molecular level, in the present finding, we assessed for the first time the ultrastructural compartmentalization of the CB1-R by means of transmission electron microscopy (TEM). Besides, we evaluated the effects of 2-methylarachidonyl-2′-fluoro-ethylamide (MET-F-AEA) in human sperm focusing on survival and acrosin activity. Since AEA binds to CB-Rs and to the vanilloid receptor (TRPV1), discovered in sperm (Schuel and Burkman, 2005), the specific targets of AEA have been investigated by using the CB1-R antagonist SR141716 (SR1), the CB2-R antagonist SR144528 (SR2), and capsazapine (CZ), which is a TRPV1 antagonist. Interestingly, the study of known sperm survival targets under different MET-F-AEA concentrations led us to provide insight in the molecular mechanisms through which the cannabinoids exert their negative effects on human reproduction.

MATERIALS AND METHODS

Chemicals

Percoll (colloidal PVP coated silica for cell separation), Sodium bicarbonate, Sodium lactate, Sodium pyruvate, Dimethyl Sulfoxide (DMSO), Earle's balanced salt solution (uncapacitating medium), and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). ECL Plus Western blotting detection system, Hybond^{TM} ECL^{TM}, Hepes Sodium Salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Goat polyclonal actin antibody (Ab), polyclonal rabbit anti-CB2-R Ab, anti-phospho-Akt S473 Ab, peroxidase-coupled anti-rabbit, and anti-goat

TABLE 1. Mean of the semen data from all the sample used (N = 18)

Semen parameters	Mean ± SD
Volume (mL)	4.17 ± 0.2
Sperm concentration (10^6/mL)	80 ± 2
Motility (%)	45 ± 1
Morphology (%)	50 ± 1.2

IgG secondary Abs were from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal anti-CB1-R Ab and CB1-R peptide were from abcam (Milan, Italy). Polyclonal anti-phospho-PTEN and anti-phospho-BCL2 Abs were from Cell Signaling (Milan, Italy).

Drugs

2-Methylarachidonyl-2′-fluoro-ethylamide, referred in all the text and in figures as MET-F-AEA, dissolved in ethanol (EtOH), was purchased from Sigma Chemical (Milan, Italy). Selective CB1-R antagonist SR141716 (Rimonabant, SR1) (McPartland et al., 2007), and CB2-R antagonist, SR144528 (SR2) (McPartland et al., 2007), both dissolved in dimethylsulfoxide (DMSO), were kindly provided by Sanofi-Aventis (Montpellier, France). Capsazapine (CZ) a vanilloid receptor (TRPV1) antagonist (Liu and Simon, 1997), dissolved in DMSO, was from Alexis Biochemicals (Milan, Italy). DMSO (0.01% final concentration in culture) and EtOH (0.02% final concentration in culture) used as solvent controls did not induce any positive result in all *in vitro* assays.

Semen Samples and Spermatozoa Preparations

Human semen was collected, according to the World Health Organization (WHO) recommended procedure, by masturbation from healthy volunteer donors of proven fertility undergoing semen analysis in our laboratory (Table 1). Spermatozoa preparations were performed as previously described (Aquila et al., 2005). Briefly, semen samples with normal parameters of volume, sperm count, motility, vitality, and morphology, according to the World Health Organization (WHO) Laboratory Manual (WHO, 1999), were included in this study. Each experiment conducted in our study was performed on sperm cells processed by pooling the ejaculates of three normozoospermic samples. Washed in Earle's balanced salt solution (uncapacitating medium), by centrifugation, sperm were subjected to the indicated treatments and incubated at 37°C and 5% CO_2. Before centrifugation, several aliquots were used to perform sperm viability. The study has been approved by the local medical-ethical committees and all participants gave their informed consent.

Processing of Ejaculated Sperm

After liquefaction, normal semen samples were centrifuged (800*g*), pooled and subjected on a discontinuous Percoll density gradient (80:40% v:v) (WHO, 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. Percoll-purified sperm were washed with unsupplemented Earle's medium (uncapacitating medium) and were incubated for 30 min at 37°C and 5% CO_2, without (control) or with treatments (experi-mental).

The treatments were the following: increasing MET-F-AEA concentrations (10 nM, 100 nM, and 1 μM); SR1 (1 μM); SR2 (1 μM); CZ (1 μM). When the cells were treated with a receptor antagonist [SR1 (1 μM), SR2 (1 μM), or CZ (1 μM)] each combined with MET-F-AEA, a pre-treatment of 15 min was performed with the antagonist. DMSO (0.01% final concentration in culture) and EtOH (0.02% final concentration in culture) used as solvent controls did not induce any different result with respect to the control in all *in vitro* assays. We have chosen the dose of 10 nM MET-F-AEA to mimic the AEA concentrations observed in human seminal plasma (12.3 nM) and in mid-cycle oviductal fluid (10.5 nM) (Schuel et al., 2002a), while 100 nM and 1 μM MET-F-AEA are supraphysiological levels.

Immunogold Labeling for CB1-R

Immunogold labeling for CB1-R was performed as previously reported (Aquila et al., 2008). Briefly, sperms fixed overnight in 4% paraformaldehyde were washed in phosphate buffered saline (PBS) to remove excess fixative, dehydrated in graded alcohol, infiltrated in LR white resin, polymerized in a vacuum oven at 45°C for 48 hr and 60 nm ultrathin sections were cut and placed on coated nickel grids for post-embedding immunogold labeling for anti-CB1-R Ab. Potential non specific labeling was blocked by incubating the sections in PBS containing 5% normal goat serum, 5% bovine serum albumin, and 0.1% cold water fish gelatine at room temperature for 1 hr. Sections were then incubated overnight at 4°C with polyclonal rabbit anti-CB1-R primary Ab at a dilution of 1:500 in PBS buffer. Incubated in 10 nm colloidal gold conjugated goat anti-rabbit IgG secondary Ab at 1:50 dilution for 2 hr at room temperature. The sections were then subsequently washed in PBS, further fixed in gluteraldehyde, counterstained in uranyl acetate and lead acetate, and examined under a Zeiss EM 900 transmission electron microscope. To assess the specificity of the immunolabeling, a negative control was carried out in those sections of sperm that were labeled with colloidal gold conjugated secondary Ab without the primary Ab.

Western Blot Analysis of Sperm Proteins

Sperm samples, washed by centrifugation twice with Earle's medium, were incubated without (NC) or with the indicated treatments, and then, centrifuged for 5 min at 5,000*g*. The pellet was resuspended in lysis buffer as previously described (Aquila et al., 2002). Equal amounts of protein (80 μg) were boiled for 5 min, separated by 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and probed with an appropriate dilution of the indicated Abs. The bound of the secondary antibody was revealed with the ECL Plus Western blotting detection system according to the manufacturer's instructions. As internal control, all the membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) of the first Ab and reprobed with anti-β actin Ab.

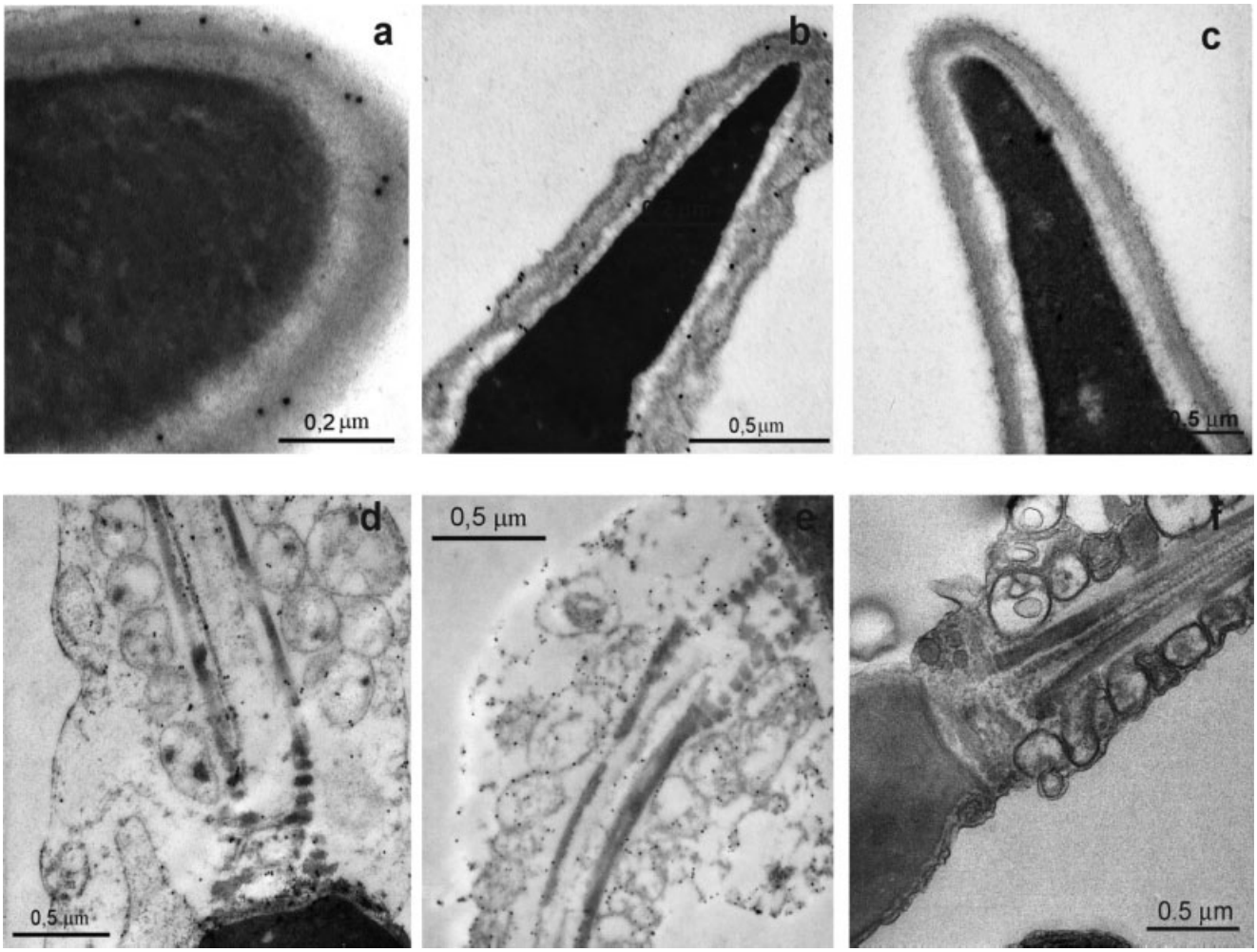

Fig. 1. Subcellular localization of CB1-R in human ejaculated spermatozoa by immunoelectron microscopy. Sperms from normozoospermic subjects were processed as reported in Materials and Methods section. (**a**,**b**) Region of the head, ×85,000 and ×50,000 respectively, scale bars = 0.2 μm and 0.5 μm, respectively; (**d**,**e**) Regions of the neck and midpiece, ×30,000, scale bars = 0.5 μm; (**c**,**f**) Electron micrograph of control sections incubated without the primary Ab, where the sperm are totally without reaction product, ×85,000 and ×30,000, respectively; scale bars = 0.5 μm and 0.5 μm, respectively.

Evaluation of Sperm Viability

Viability was performed by red-eosin exclusion test using Eosin Y (WHO, 1999). Sperm vitality was assessed by means of light microscopy examining an aliquot of each sperm sample in absence (NC) or in the presence of MET-F-AEA increasing concentrations (10 nM, 100 nM, and 1 μM), in addition 100 nM MET-F-AEA were combined with 1 μM SR1 or 1 μM SR2 or 1 μM CZ, and then, incubated for 30 min. An independent observer scored 200 cells for stain uptake (dead cells) or exclusion (live cells), and sperm viability was expressed as the percentage of total live sperm.

Acrosin Activity Assay

Acrosin activity was assessed by the method of Kennedy et al. (1989) and as previously described (Aquila et al., 2003). Sperm were washed in Earle's medium and centrifuged at 800*g* for 20 min, then were resuspended (final concentration of 10×10^6 sperm/mL) in different tubes containing no treatment (control) or MET-F-AEA increasing concentrations (10 nM, 100 nM, and 1 μM). In addition, 10 nM MET-F-AEA were combined with 1 μM SR1 or 1 μM SR2 or 1 μM CZ. Some samples were also treated with each antagonist alone; 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 hr at room temperature was added. Aliquots (20 μL) were removed at 0 and 3 hr and the percentages of viable cells were determined. After incubation, 0.5 mol/L benzamidine was added (0.1 mL) to each of the tubes, and then, centrifuged at 1,000*g* for 30 min. The supernatants were collected and the acrosin activity measured with the spectrophotometer 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. The acrosin activity was expressed as μIU/10^6 sperms. Quantification of acrosin activity was performed as previously described (Aquila et al., 2003).

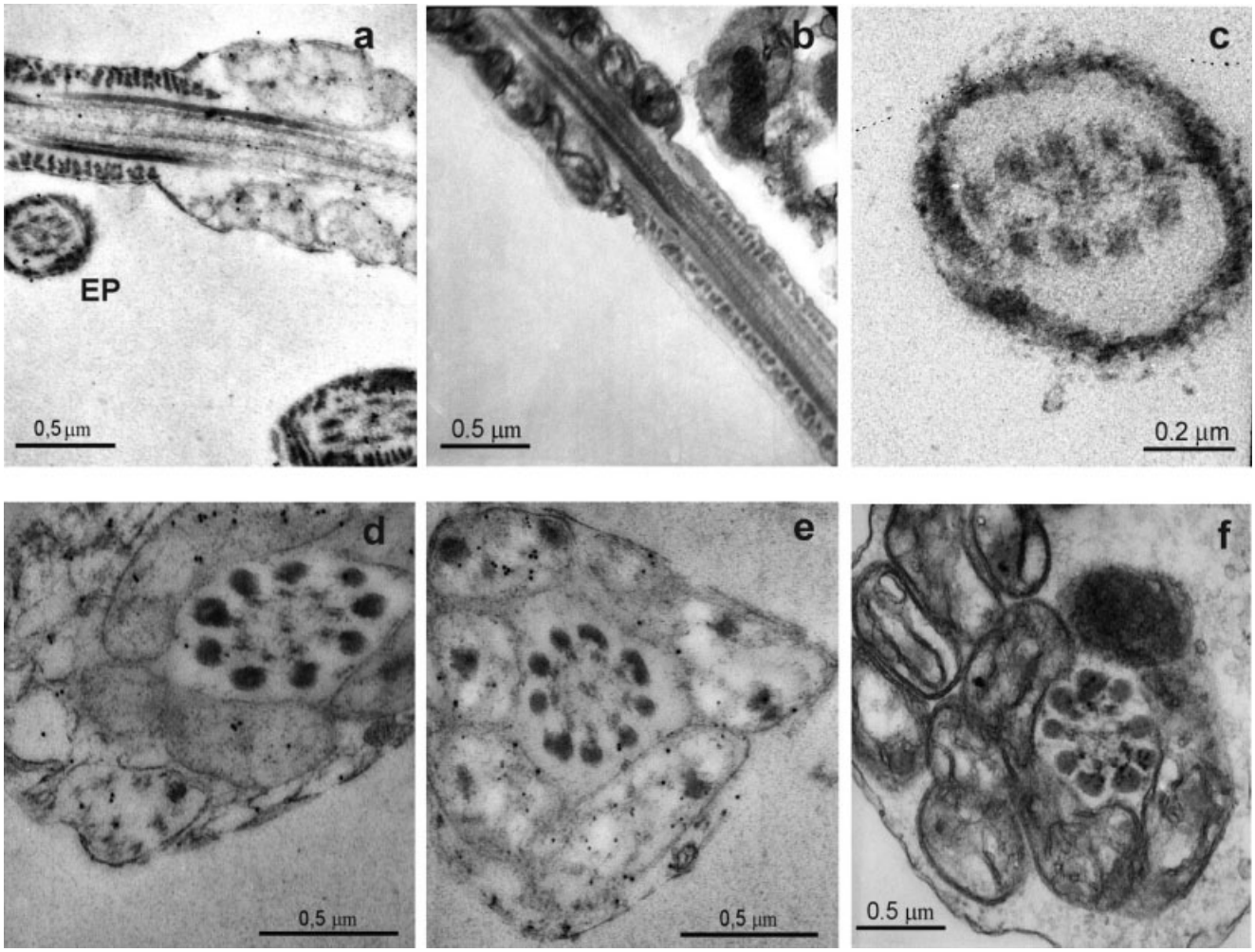

Fig. 2. CB1-R location in the midpiece and along the tail in human spermatozoa. Electron micrographs of sperms allowed to react with Ab directed against CB1-R. (**a**) Electron micrograph of a sagital section of the midpiece and the tail showing the transition between the middle piece with its mitochondria and the principal piece, probed with anti-CB1-R Ab; EP, transverse section of the end-piece; ×30,000, scale bar = 0.5 μm. (**d**,**e**) Transverse section of the midpiece probed with anti-CB1-R Ab, ×50,000, scale bars = 0.5 μm. (**b,c**,**f**) In the electron micrograph of control sections incubated without the primary Ab, no immunoreaction was present: (b) ×30,000, scale bar = 0.5 μm; (c) ×20,000, scale bar = 0.2 μm; (f) ×50,000, scale bar = 0.5 μm.

Statistical Analysis

The experiments for Immunogold ultrastructural and Western blot analysis were performed in at least four independent experiments. The data obtained from viability and acrosin activity (six replicate experiments using duplicate determinations) were presented as the mean ± S.E.M. The differences in mean values were calculated using analysis of variance (ANOVA) with a significance level of $P \leq 0.05$.

RESULTS

Immunogold Localization of CB1-R

Human sperm is immunoreactive to CB1-R. Particularly, the membranes of the head, the neck (segmented columns), and the mitochondria in midpiece of human sperm are decorated with gold particles (Figs. 1, 2). The head in Fig. 1a,b shows label on the sperm membranes, whereas in negative control (Fig. 1c) there is no label in sperm incubated without the primary Ab. The segmented columns and the midpiece demonstrated immunopositivity to CB1-R (Fig. 1d,e), whereas the corresponding regions in the sperm incubated without the primary Ab are devoid of any label (Fig. 1f). Particularly, in the midpiece, the gold particles are prevalently present on the mitochondria (Fig. 2a,d,e) and the corresponding negative controls without the primary Ab (Fig. 2b,f) are free of any label. Reduced label is seen through the flagellum till the end-piece (Figs. 1d, 2a) and the negative controls, sperm incubated without the primary Ab (Fig. 2b,c) showed no immunogold labeling in corresponding regions. The spermatozoa were observed both in transverse and sagital sections through the end-piece.

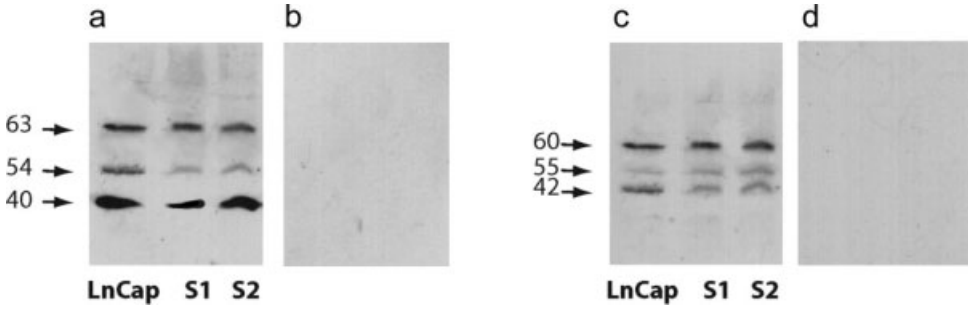

Fig. 3. CB1-R and CB2-R are both expressed by human sperm. Western blot analysis of CB1-R and CB2-R proteins from human sperm. Sperm lysates were subjected to electrophoresis on 10% SDS-Polyacrylamide gels, blotted onto nitrocellulose membranes and probed with the indicated antibodies. Expression of the CB1-R (**a**) and CB2-R (**c**) receptors in two samples of ejaculated spermatozoa from normal men (S1, S2). LNCap cells were used as positive control (+). Specificity control experiments incubating the anti-CB1 Ab with the immunizing protein (**b**) or processing immunoblots without primary antibody anti-CB2-R (**d**) show no immunoreactivity. The experiments were repeated at least four times and the autoradiographs of the figure show the results of one representative experiment.

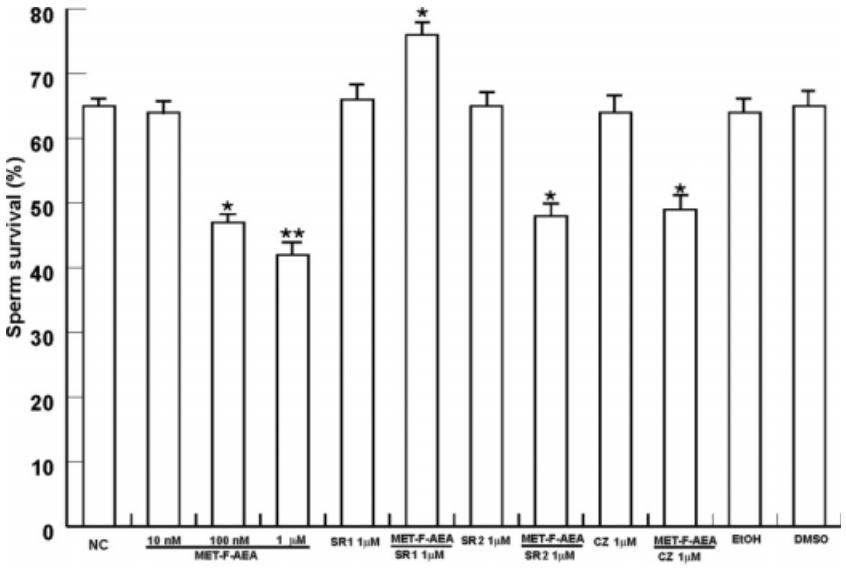

Fig. 4. Effect of MET-F-AEA on sperm viability. Viability was assessed by using Eosin Y as described in Materials and Methods. Washed sperm were incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2 without (NC) or in the presence of the indicated MET-F-AEA concentrations. Some samples were pre-treated for 15 min with 1 μM SR1 or 1 μM SR2 or 1 μM CZ, and then, treated with 100 nM MET-F-AEA. Some samples were treated with 1 μM SR1 or 1 μM SR2 or 1 μM CZ each alone. EtOH and DMSO samples were used as solvent controls. The values represent the Mean ± SEM. *P = 0.05, **P = 0.01 versus control.

Human Sperm Expresses CB1-R and CB2-R

In the aim to evaluate the specific AEA target and since the data reported on the presence of CB1-R and CB2-R in sperm are not in agreement (Maccarrone et al., 2005; Rossato et al., 2005), we performed Western blot analysis to clarify which type of cannabinoid receptor is expressed in the sperm. Interestingly, our anti-CB1-R antibody recognized three bands at about 63, 54, and 40 kDa (Fig. 3a) as it was previously reported (Matsuda et al., 1990; Song and Howlett, 1995; Porcella et al., 2000). Three bands at about 60, 55, and 42 kDa (Fig. 3c) were also detected for the CB2-R protein in a pattern similar to that reported for spleen, brainstem, and cerebellum (Van Sickle et al., 2005). Processing immunoblots by pre-absorbing with antigenic peptide (Fig. 3b) or without primary antibody (Fig. 3d) abolished the identified bands. LnCap, a prostate cancer cell line, was used as positive control both for CB1-R and CB2-R (Sarfaraz et al., 2005).

MET-F-AEA Decreases Human Sperm Survival

A functional assessment of the sperm biological activity under increasing MET-F-AEA was performed by analyzing sperm survival. Sperm viability was not affected by 10 nM MET-F-AEA, whereas it significantly decreased at 100 nM and 1 μM MET-F-AEA (Fig. 4). Intriguingly, the treatment with 1 μM SR1 completely reverted the effect of MET-F-AEA alone but also induced a significant increase on sperm survival; 1 μM SR2 plus 100 nM MET-F-AEA and 1 μM CZ combined with 100 nM MET-F-AEA partially reverted the effect of 1 μM MET-F-AEA alone; 1 μM SR1 alone as well as 1 μM SR2 or 1 μM CZ used as antagonist controls, did not induce any significant effect on

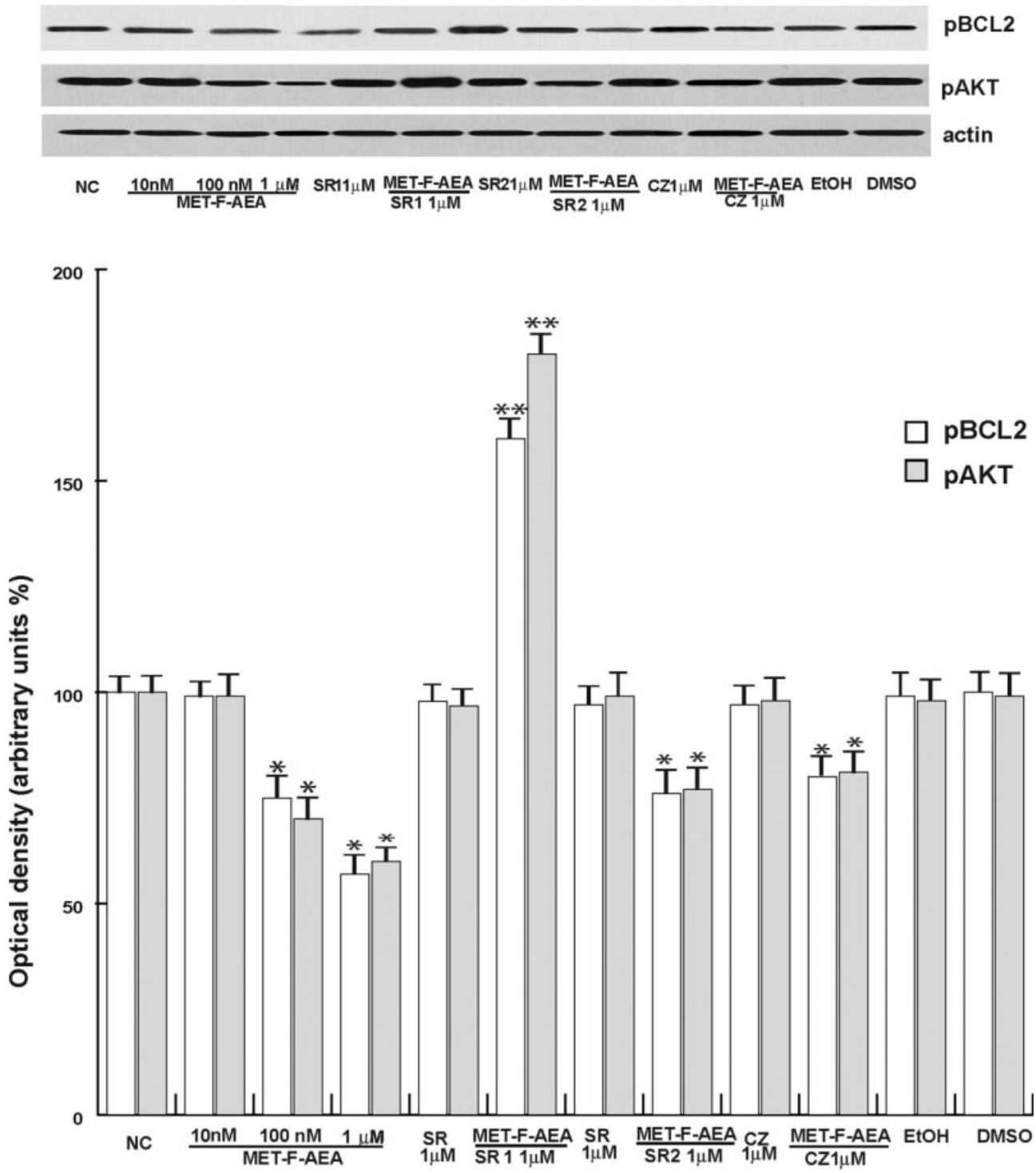

Fig. 5. MET-F-AEA action on p-AKT and p-BCL2. Washed sperm from normal samples were incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2, in the absence (NC) or in the presence of the indicated MET-F-AEA concentrations for 30 min. Some samples were pre-treated for 15 min with 1 μM SR1 or 1 μM SR2 or 1 μM CZ, and then, treated with 100 nM MET-F-AEA. Other samples were treated with 1 μM SR1 or 1 μM SR2 or 1 μM CZ each alone. EtOH and DMSO samples were used as solvent controls; 80 μg of sperm lysates were used. Actin was used as loading control. The autoradiographs presented are representative examples 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 ± SEM) of four independent experiments. *$P = 0.05$, **$P = 0.01$ versus control.

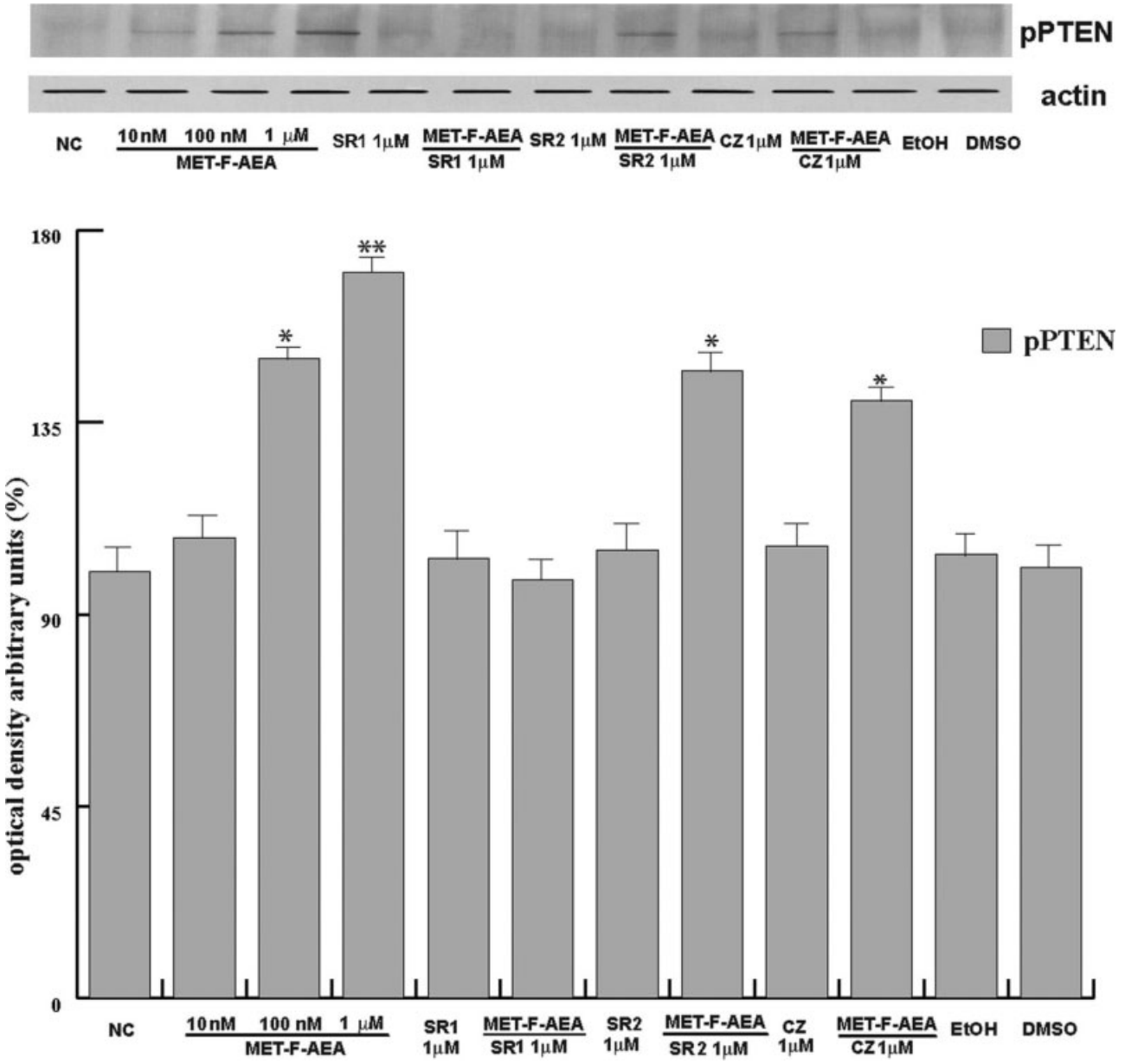

Fig. 6. MET-F-AEA modulates the PI3K/Akt pathway through pPTEN. Washed sperm from normal samples were incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2, in the absence (NC) or in the presence of the indicated MET-F-AEA levels for 30 min. Some samples were pre-treated for 15 min with 1 μM SR1 or 1 μM SR2 or 1 μM CZ, and then, treated with 100 nM MET-F-AEA. Other samples were treated with 1 μM SR1 or 1 μM SR2 or 1 μM CZ each alone. EtOH and DMSO samples were used as solvent controls. Eighty microgram of sperm lysates were used. Actin was used as loading control. 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 ± SEM) of four independent experiments. *$P = 0.05$, **$P = 0.01$ versus control.

sperm viability compared to the untreated sample. The solvent controls, DMSO and EtOH, did not induce any different result with respect to the control.

MET-F-AEA Effects on pBCL2, pAkt, and pPTEN

To analyze at molecular level, the negative effect of MET-F-AEA on sperm viability, we tested its action on different known sperm survival targets. As shown in Fig. 5, 100 nM and 1 μM AEA decreased both pBCL2 and pAkt, whereas at the same concentrations an increase of pPTEN expression was observed (Fig. 6). In the presence of 100 nM MET-F-AEA plus 1 μM SR1 both pAkt and pBCL2 increased, whereas the PTEN phosphorylation disappeared; 1 μM SR2 plus 100 nM MET-F-AEA as well as 1 μM CZ plus 100 nM MET-F-AEA produced similar results to that obtained with 100 nM

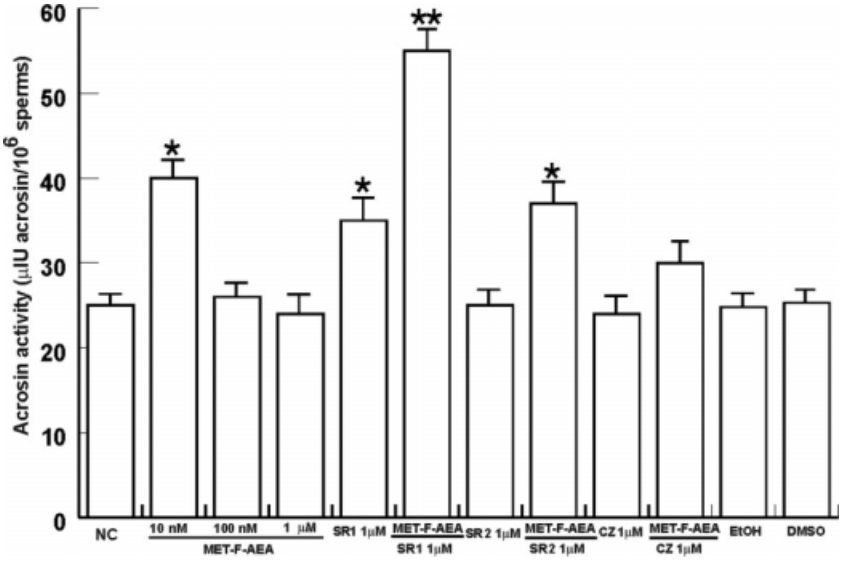

Fig. 7. MET-F-AEA effects on acrosin activity. Washed sperm from normal samples were incubated in the unsupplemented Earle's medium at 37°C and 5% CO_2, in the absence (NC) or in the presence of the indicated MET-F-AEA levels for 30 min. Some samples were pretreated for 15 min with 1 μM SR1 or 1 μM SR2 or 1 μM CZ, and then, treated with 10 nM MET-F-AEA. Other samples were treated with 1 μM SR1 or 1 μM SR2 or 1 μM CZ each alone. EtOH and DMSO samples were used as solvent controls. Acrosin activity was assessed as reported in Materials and methods. Columns are Mean ± SEM of six independent experiments performed in duplicate. *$P < 0.05$ versus control; **$P < 0.01$ versus control.

MET-F-AEA alone; 1 μM SR1 alone, 1 μM SR2 or 1 μM CZ alone were not significantly different with respect to the control. Also, in this case, the combined action of MET-F-AEA and SR1 induced a significant increase of pBCL2 and pAKT that were higher than that of the compounds alone. The solvent controls, DMSO and EtOH did not induce any different result with respect to the control. Moreover, no differences in protein expression levels were observed by analyzing total BCL2, AKT, and PTEN (data not shown).

MET-F-AEA Regulates Acrosin Activity

The acrosin is one of the most representative enzyme involved in the acrosome reaction (Cui et al., 2000; Buffone et al., 2008). Washed sperm were treated as aforementioned and incubated under uncapacitating conditions (Fig. 7; see Materials and Methods). A significant increase was observed with 10 nM MET-F-AEA, whereas higher MET-F-AEA levels did not induce the enzymatic activity; 10 nM MET-F-AEA combined with 1 μM SR1 significantly increased the acrosin activity; 1 μM SR2 plus 10 nM MET-F-AEA and 1 μM CZ plus 10 nM MET-F-AEA have a similar behavior to that observed by using 10 nM MET-F-AEA alone; 1 μM SR1 alone induced acrosin activity, whereas 1 μM SR2 as well as 1 μM CZ were not significantly different with respect to the untreated sample. The solvent controls, DMSO and EtOH did not induce any different result with respect to the control.

DISCUSSION

Among the AEA biological activities, the regulation of mammalian fertility has been attracted growing interest. Particularly, different sperm actions were reported to be regulated by the ECs, however, their effects on human male gamete are not completely characterized. In our finding, to study the human sperm anatomy at molecular level we evidenced for the first time the ultrastructural compartmentalization of CB1-R by using TEM. Moreover, to further elucidate the potential role of EC system in human male fertility, the MET-F-AEA action was studied on sperm survival and acrosin activity. In addition, we also evaluated the molecular mechanisms through which the MET-F-AEA negative effect occurs on survival.

Mammalian spermatozoa are highly differentiated cells that display extreme polarization of architecture and function. Briefly, the mature sperm cell has three highly specialized regions: the sperm head, involved in sperm oocyte interaction; the midpiece with mitochondria, involved in energy production; the flagellum, involved in motility. In our finding, the CB1-R was compartmentalized at the membranes of the sperm head and in the midpiece, prevalently on the mitochondria, while reduced label is seen through the tail. In regard to the cell polarization, it was hypothesized that sperm possess compartmentalized metabolic and signaling pathways in the regions where they are needed. Altogether these observations and the evidence of the AEA synthesis in sperm (Maccarrone et al., 2005), suggest that through an autocrine short loop, AEA and its own receptor may modulate sperm functional maturation and metabolism. Previous finding, by immunofluorescence assay evidenced the CB1-R location in the head and in the midpiece (Rossato et al., 2005). Besides, it is at our knowledge the first time that the CB1-R was evidenced at the mitochondria level, and this may be in agreement with the reported CB1-R modulation of mitochondrial functionality both in sperm (Rossato et al., 2005) and in other cellular type (Athanasiou et al., 2007).

While Rossato et al. (2005) failed to detect CB2-R in human sperm using Western blots, Maccarrone et al. (2005) did detect low levels of CB2-R in boar sperm with Western blots and radioligand binding procedures. With this respect, we evidenced by Western blots that human sperm contains both CB1-R and CB2-R. Specifically, our antibodies detected three variants for each receptor with the about 63 kDa band corresponding to the expected molecular weight of CB1-R according to other authors (Matsuda et al., 1990; Song and Howlett, 1995; Porcella et al., 2000). Our results are consistent with these and other observations (Shire et al., 1995; Ryberg et al., 2005). In our cellular type it is difficult to perform other molecular analysis to clarify whether this pattern of CB1-R expression is linked to different splicing variants and/or they are the consequence of different post transductional modifications. Concomitantly, our results on CB2-R expression revealed the presence of three species of expressed CB2-R proteins at about 42, 55, and 60 kDa according to the previous data reporting three different immunoreactive bands in rat spleen and brainstem (Matias et al., 2002; Van Sickle et al., 2005). The apparent discrepancy between our expression analysis of the CB2 receptor and that of Rossato et al. (2005) could be due to the heterogeneity of human semen, and thus, to different expression levels of this protein among human sperm samples. However, the presence of a functional CB2 receptor in human spermatozoa has been recently demonstrated by Agirregoitia et al. (2009) by analyzing semen from 50 normozoospermic, healthy human donors.

Successively, we evaluated the effects of different MET-F-AEA concentrations on sperm survival, observing that at 100 nM and 1 μM, MET-F-AEA significantly decrease sperm viability. Recently, sperm viability showed was not significantly influenced by AEA concentrations up to 1.0 μM, whereas at higher doses, sperm survival decreased (Rossato et al., 2005). Our data showing that lower concentrations of MET-F-AEA are able to reduce cell viability, might indicate that MET-F-AEA is more toxic to human sperm than AEA. The AEA action on the survival of different cellular models has been shown to occur through the activation of different receptors, which in turn trigger different signal transduction pathways, depending on the cellular type (Maccarrone et al., 2003a and references therein). It has been shown that cannabinoids are able to modulate, through CB1-R, the PI3K/Akt pathway, which serves as a pivotal antiapoptotic signal (Gómez del Pulgar et al., 2000). Transductional pathways regulated by ECs have not been studied until now in the male gamete, and then, we explored the MET-F-AEA effect on human sperm viability by evaluating the PI3K/Akt pathway which induces different cellular activities such as motility, metabolism and survival (Parsons, 2004). At higher MET-F-AEA concentrations, we observed a reduction in the Akt and BCL2 phosphorylations that was concomitantly with an increase of the pPTEN, suggesting a negative modulation of this pathway by AEA in the human male gamete. The treatments of sperm with MET-F-AEA combined with SR1 or with SR2 or with CZ indicated that the AEA effect on sperm survival and PI3/K were dependent on its interaction with the CB1-R. All these observations are in agreement with our already reported data demonstrating that MET-F-AEA is also able to induce human sperm metabolism by increasing lipogenesis and favoring the accumulation of energy substrates (Aquila et al., 2009).

Successful sperm maturation depends on sequential steps both in male and female reproductive tracts. After ejaculation, to fertilize an egg the male gamete must undergo to capacitation process, that is a prerequisite for fertilization of mammalian spermatozoa. Capacitation is an extratesticular maturational process that *in vivo* occurs in the female reproductive tract and confers to the sperm the ability to undergo to the acrosome reaction (Yanagimachi, 1994; Visconti et al., 1995). Spermatozoa must experience the "acrosome reaction" to release the enzymes needed to penetrate the ova vestments and to be able to fuse with the ovum's plasma membrane (Fraser, 1998). It was reported that in sperm CB1-R activation determines an inhibition of capacitation and acrosome reaction (Schuel et al., 1994; Schuel et al., 2002a; Maccarrone et al., 2005; Rossato et al., 2005; Cobellis et al., 2006). In our finding, through the evaluation of acrosin activity, the main enzyme expressed in the acrosome (Cui et al., 2000; Buffone et al., 2008), we evidenced that it is stimulated by the addition of 10 nM Met-F-AEA which corresponds to the physiological concentration in midcycle oviductal fluid (Schuel et al., 2002a). Furthermore, the treatment with both MET-F-AEA and SR1, potentiate, rather than reverting the effect of MET-F-AEA alone. This finding is not surprising since synergic effects of MET-F-AEA and SR141716 have been already reported in the ability of these compounds to inhibit the proliferation of stimulated peripheral blood mononuclear cells (Malfitano et al., 2008). On the other hand, we also demonstrated that the treatment of MET-F-AEA with SR141716 increased Ca^{2+} content in human sperm even though Met-F-AEA alone induced similar effects (Aquila et al., 2009). These findings together with our results obtained treating sperm cells with both CB2-R and TRPV1 receptor inhibitors support the hypothesis that the AEA action on sperm survival and acrosin activity could be mediated by CB1-R. In our experiments, it appears that the SR1 could display a neutral antagonism as well as an inverse agonism. SR1 has been shown to act as neutral antagonist, competitive antagonist, and inverse agonist (Hurst et al., 2002; Pertwee, 2005; Aquila et al., 2009). It is likely that the efficacy for the production of inverse cannabimimetic effects will be governed by the degree of ongoing endocannabinoid release onto CB1 receptors. Therefore, we could also hypothesize that SR1, by acting as an inverse agonist is able to enhance MET-F-AEA effects as already proposed in other experimental system by our group (Esposito et al., 2008; Santoro et al., 2009).

Finally, as sperm leave seminal plasma during their transit in the female reproductive tract, they are deprived of decapacitating factors and exposed to female genital tract fluids that contain AEA (Schuel et al., 2002a). Our results, showing that AEA and its receptor CB1-R have the ability to modulate sperm survival and acquisition of fertilizing ability, also imply that suprapysiological levels of the EC within female reproductive tracts could impair human reproduction. Intriguingly, our data showing direct biological effects of the CB1-R stimulation by its agonist AEA in human sperm evidenced for the first time the precise human sperm anatomic regions target of the CB1-R and revealed, at least

in part, the molecular mechanisms involved in the AEA modulation of human sperm survival.

ACKNOWLEDGEMENTS

The authors thank Dr. Vincenzo Cunsulo for the technical and scientific assistance (Biogemina Italia Srl, Catania, Italy). They also thank Perrotta Enrico for the excellent technical assistance, Serena and Maria Clelia Gervasi for the English review.

LITERATURE CITED

Agirregoitia E, Carracedo A, Subirán N, Valdivia A, Agirregoitia N, Peralta L, Velasco G, Irazusta J. The CB(2) cannabinoid receptor regulates human sperm cell motility. Fertil Steril, in press.

Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V, Andò S. 2005. Leptin secretion by human ejaculated spermatozoa. J Clin Endocrinol Metab 90:4753–4761.

Aquila S, Guido C, Laezza C, Santoro A, Pezzi V, Panza S, Andò S, Bifulco M. 2009. A new role of anandamide in human sperm: focus on metabolism. J Cell Physiol 221:147–153.

Aquila S, Guido C, Perrotta I, Tripepi S, Nastro A, Andò S. 2008. Human sperm anatomy: ultrastructural localization of 1alpha, 25-dihydroxyvitamin D receptor and its possible role in the human male gamete. J Anat 213:555–564.

Aquila S, Middea E, Catalano S, Marsico S, Lanzino M, Casaburi I, Barone I, Bruno R, Zupo S, Andò S. 2007. Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway. Hum Reprod 22:2594–2605.

Aquila S, Sisci D, Gentile M, Carpino A, Middea E, Catalano S, Rago V, Ando S. 2003. Towards a physiological role for cytochrome P450 aromatase in ejaculated human sperm. Hum Reprod 18: 1650–1659.

Aquila S, Sisci D, Gentile M, Middea E, Catalano S, Carpino A, Rago V, Andò S. 2004. Estrogen receptor (ER)alpha and ER beta are both expressed in human ejaculated spermatozoa: evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway. J Clin Endocrinol Metab 89:1443–1451.

Aquila S, Sisci D, Gentile M, Middea E, Siciliano L, Andò S. 2002. Human ejaculated spermatozoa contain active P450 aromatase. J Clin Endocrinol Metab 87:3385–3390.

Athanasiou A, Clarke AB, Turner AE, Kumaran NM, Vakilpour S, Smith PA, Bagiokou D, Bradshaw TD, Westwell AD, Fang L, Lobo DN, Constantinescu CS, Calabrese V, Loesch A, Alexander SP, Clothier RH, Kendall DA, Bates TE. 2007. Cannabinoid receptor agonists are mitochondrial inhibitors: a unified hypothesis of how cannabinoids modulate mitochondrial function and induce cell death. Biochem Biophys Res Commun 364:131–137.

Buffone MG, Foster JA, Gerton GL. 2008. The role of the acrosomal matrix in fertilization. Int J Dev Biol 52:511–522.

Cantley LC. 2002. Phosphoinositide 3-kinase pathway. Science 296:1655–1657.

Chang MC, Berkery D, Schuel R, Laychock SG, Zimmerman AM, Zimmerman S, Schuel H. 1993. Evidence for a cannabinoid receptor in sea urchin sperm and its role in blockade of the acrosome reaction. Mol Reprod Dev 36:507–516.

Cobellis G, Cacciola G, Scarpa D, Meccariello R, Chianese R, Franzoni MF, Mackie K, Pierantoni R, Fasano S. 2006. Endocannabinoid system in frog and rodent testis: type-1 cannabinoid receptor and fatty acid amide hydrolase activity in male germ cells. Biol Reprod 75:82–89.

Cui YH, Zhao RL, Wang Q, Zhang ZY. 2000. Determination of sperm acrosin activity for evaluation of male fertility. Asian J Androl 2:229–232.

Devane WA, Dysarz FA, III, Johnson MR, Melvin LS, Howlett AC. 1988. Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 34:605–613.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R. 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258:1946–1949.

Di Marzo V, Bifulco M, De Petrocellis L. 2004. The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Discov 3:771–784.

Dobrosi N, Tóth BI, Nagy G, Dózsa A, Géczy T, Nagy L, Zouboulis CC, Paus R, Kovács L, Bíró T. 2008. Endocannabinoids enhance lipid synthesis and apoptosis of human sebocytes via cannabinoid receptor-2-mediated signaling. FASEB J 22:3685–3695.

Esposito I, Proto MC, Gazzerro P, Laezza C, Miele C, Alberobello AT, D'Esposito V, Beguinot F, Formisano P, Bifulco M. 2008. The cannabinoid CB1 receptor antagonist rimonabant stimulates 2-deoxyglucose uptake in skeletal muscle cells by regulating the expression of phosphatidylinositol-3-kinase. Mol Pharmacol 74: 1678–1686.

Fraser LR. 1998. Sperm capacitation and the acrosome reaction. Hum Reprod 1:9–19.

Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P. 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte populations. Eur J Biochem 232:54–61.

Glander HJ, Schaller J. 1999. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. Mol Hum Reprod 5:109–115.

Gómez del Pulgar T, Velasco G, Guzman M. 2000. The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. Biochem J 347:369–373.

Hurst DP, Lynch DL, Barnett-Norris J, Hyatt SM, Seltzman HH, Zhong M, Song ZH, Nie J, Lewis D, Reggio PH. 2002. N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A) interaction with LYS 3.28(192) is crucial for its inverse agonism at the cannabinoid CB1 receptor. Mol Pharmacol 62:1274–1287.

Ito T, Deng X, Carr BK, May WS. 1997. Bcl-2 phosphorylation required for anti-apoptosis function. J Biol Chem 272:11671–11673.

Kennedy WP, Kaminski JM, Van der Ven HH, Jeyendran RS, Reid DS, Blackwell J, Bielfeld P, Zaneveld LJ. 1989. A simple, clinical assay to evaluate the acrosin activity of human spermatozoa. J Androl 10:221–231.

Liu L, Simon SA. 1997. Capsazepine, a vanilloid receptor antagonist, inhibits nicotinic acetylcholine receptors in rat trigeminal ganglia. Neurosci Lett 228:29–32.

Maccarrone M, Barboni B, Paradisi A, Bernabò N, Gasperi V, Pistilli MG, Fezza F, Lucidi P, Mattioli M. 2005. Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction. J Cell Sci 118:4393–4404.

Maccarrone M, Cecconi S, Rossi G, Battista N, Pauselli R, Finazzi-Agrò A. 2003b. Anandamide activity and degradation are regulated by early postnatal aging and follicle-stimulating hormone in mouse Sertoli cells. Endocrinology 144:20–28.

Maccarrone M, De Felici M, Bari M, Klinger F, Siracusa G, Finazzi-Agrò A. 2000. Down-regulation of anandamide hydrolase in mouse uterus by sex hormones. Eur J Biochem 267:2991–2997.

Maccarrone M, Finazzi-Agro A. 2003a. The endocannabinoid system, anandamide and the regulation of mammalian cell apoptosis. Cell Death Differ 10:946–955.

Malfitano AM, Proto MC, Bifulco M. 2008. Cannabinoids in the management of spasticity associated with multiple sclerosis. Neuropsychiatr Dis Treat 4:847–853.

Mani SK, Mitchell A, O'Malley BW. 2001. Progesterone receptor and dopamine receptors are required in D9-tetrahydrocannabinol modulation of sexual receptivity in female rats. Proc Natl Acad Sci USA 98:1249–1254.

Matias I, Pochard P, Orlando P, Salzet M, Pestel J, Di Marzo V. 2002. Presence and regulation of the endocannabinoid system in human dendritic cells. Eur J Biochem 269:3771–3778.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564.

Maykut MO. 1985. Health consequences of acute and chronic marihuana use. Prog Neuropyschopharmacol Biol Psychiatry 9: 209–238.

McPartland JM, Glass M, Pertwee RG. 2007. Meta-analysis of cannabinoid ligand binding affinity and receptor distribution: interspecies differences. Br J Pharmacol 152:583–593.

Miki K. 2007. Energy metabolism and sperm function. Soc Reprod Fertil Suppl 65:309–325.

Murphy LL, Cher J, Steger RW, Bartke A. 1994. Effects of Delta-9-tetrahydrocannabinol on copulatory behavior and neuroendocrine responses of male rats to female conspecifics. Pharm Biochem Behav 48:1011–1017.

Oehninger S, Morshedi M, Weng SL, Taylor S, Duran H, Beebe S. 2003. Presence and significance of somatic cell apoptosis markers in human ejaculated spermatozoa. Reprod Biomed Online 7:469–476.

Paasch U, Grunewald S, Agarwal A, Glander H-J. 2004a. The activation pattern of caspases in human spermatozoa. Fertil Steril 81:802–809.

Paasch U, Grunewald S, Dathe S, Glander HJ. 2004c. Mitochondria of human Spermatozoa are preferentially susceptible to apoptosis. Ann N Y Acad Sci 1030:403–409.

Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha E, Thomas AJ, Jr, Glander HJ, Agarwal A. 2004b. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. Biol Reprod 71:1828–1837.

Paria BC, Dey SH. 2000. Ligand-receptor signaling with endocannabinoids in preimplantation embryo development and implantation. Chem Phys Lipids 108:211–220.

Paria BC, Song H, Wang X, Schmid PC, Krebsbach RJ, Schmid HHO, Bonner TL, Zimmer A, Dey SK. 2001. Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. J Biol Chem 276:20523–20528.

Park B, McPartland JM, Glass M. 2004. Cannabis, cannabinoids and reproduction. Prostaglandins Leukot Essent Fatty Acids 70: 189–197.

Parsons R. 2004. Human cancer, PTEN and the PI-3 kinase pathway. Semin Cell Dev Biol 15:171–176.

Pertwee RG. 2005. Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. Life Sci 76:1307–1324.

Porcella A, Maxia C, Gessa GL, Pani L. 2000. The human eye expresses high levels of CB1 cannabinoid receptor mRNA and protein. Eur J Neurosci 12:1123–1127.

Powell DJ, Fuller RW. 1983. Marijuana and sex: strange bedpartners. J Psychoactive Drugs 15:269–280.

Rossato M, Ion Popa F, Ferigo M, Clari G, Foresta C. 2005. Human sperm express cannabinoid receptor Cb1, the activation of which inhibits motility, acrosome reaction, and mitochondrial function. J Clin Endocrinol Metab 90:984–991.

Ruiz-Pesini E, Díez-Sánchez C, López-Pérez MJ, Enríquez JA. 2007. The role of the mitochondrion in sperm function: is there a place for oxidative phosphorylation or is this a purely glycolytic process? Curr Top Dev Biol 77:3–19.

Ryberg E, Vu HK, Larsson N, Groblewski T, Hjorth S, Elebring T, Sjögren S, Greasley PJ. 2005. Identification and characterisation of a novel splice variant of the human CB1 receptor. FEBS Lett 579:259–264.

Santoro A, Pisanti S, Grimaldi C, Izzo AA, Borrelli F, Proto MC, Malfitano AM, Gazzerro P, Laezza C, Bifulco M. 2009. Rimonabant inhibits human colon cancer cell growth and reduces the formation of precancerous lesions in the mouse colon. Int J Cancer 125:996–1003.

Sarafian TA, Kouyoumjian S, Khoshaghideh F, Tashkin DP, Roth MD. 2003. Delta 9-tetrahydrocannabinol disrupts mitochondrial function and cell energetics. Am J Physiol Lung Cell Mol Physiol 284:298–306.

Sarfaraz S, Afaq F, Adhami VM, Mukhtar H. 2005. Cannabinoid receptor as a novel target for the treatment of prostate cancer. Cancer Res 65:1635–1641.

Schuel H, Burkman LJ. 2005. A tale of two cells: endocannabinoid-signaling regulates functions of neurons and sperm. Biol Reprod 73:1078–1086.

Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D, Giuffrida A. 2002a. N-Acylethanolamines in human reproductive fluids. Chem Phys Lipids 121:211–227.

Schuel H, Burkman LJ, Lippes J, Crickard K, Mahony MC, Giuffrida A, Picone RP, Makriyannis A. 2002b. Evidence that anandamide-signaling regulates human sperm functions required for fertilization. Mol Reprod Dev 63:376–387.

Schuel H, Chang MC, Burkman LJ, Picone RP, Makriannis A, Zimmerman AM, Zimmerman S. 1999. Cannabinoid receptors in sperm. In: Nahas G, Sutin KM, Agurell S, editors. Marihuana and medicine. Totowa, NJ: Humana Press. p 335–345.

Schuel H, Goldstein E, Mechoulam R, ZImmerman AM, Zimmerman S. 1994. Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. Cell Biol 91:7678–7682.

Shire D, Carillon C, Kaghad M, Calandra B, Rinaldi-Carmona M, Le Fur G, Caput D, Ferrara P. 1995. An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. J Biol Chem Feb 270:3726–3731.

Smith CG, Asch RH. 1987. Drug abuse and reproduction. Fertil Steril 48:355–373.

Song C, Howlett AC. 1995. Rat brain cannabinoid receptors are N-linked glycosylated proteins. Life Sci 56:1983–1989.

Sugiura T, Kondo S, Sukagawa A, Tonegawa T, Nakane S, Yamashita A, Waku K. 1996. Enzymatic synthesis of anandamide, an endogenous cannabinoid receptor ligand, through N-acylphosphatidylethanolamine pathway in testis: involvement of Ca(2+)-dependent transacylase and phosphodiesterase activities. Biochem Biophys Res Commun 218:113–117.

Turco MY, Matsukawa K, Czernik M, Gasperi V, Battista N, Della Salda L, Scapolo PA, Loi P, Maccarrone M, Ptak G. 2008. High levels of anandamide, an endogenous cannabinoid, block the growth of sheep preimplantation embryos by inducing apoptosis and reversible arrest of cell proliferation. Hum Reprod 23:2331–2338.

Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD, Sharkey KA. 2005. Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science 310:329–332.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. 1995. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 121:1129–1137.

Wenger T, Ledent C, Csernus V, Gerendai I. 2001. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. Biochem Biophys Res Commun 284:363–368.

World Health Organization. 1999. Laboratory manual for the examination of human semen and sperm-cervical mucus interactions. 4th ed. Cambridge, UK: Cambridge University Press.

Wu X, Senechal K, Neshat MS, Whang YE, Sawyers CL. 1998. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci USA 95:15587–15591.

Yanagimachi R. 1994. Mammalian fertilization. Physiol Reprod 189–317.

Reproductive Biology and Endocrinology

BioMed Central

Research

Human male gamete endocrinology: 1alpha, 25-dihydroxyvitamin D3 (1,25(OH)2D3) regulates different aspects of human sperm biology and metabolism

Saveria Aquila*†[1,2], Carmela Guido†[1,2], Emilia Middea[1,2], Ida Perrotta[3], Rosalinda Bruno[1,2], Michele Pellegrino[3] and Sebastiano Andò[2,3,4]

Address: [1]Dept Pharmaco-Biology, University of Calabria 87036 Arcavacata di Rende (Cosenza), Italy, [2]Centro Sanitario, University of Calabria 87036 Arcavacata di Rende (Cosenza), Italy, [3]Dept Cellular Biology, University of Calabria 87036 Arcavacata di Rende (Cosenza), Italy and [4]Faculty of Pharmacy, University of Calabria 87036 Arcavacata di Rende (Cosenza), Italy

Email: Saveria Aquila* - aquisav@libero.it; Carmela Guido - guidosisters@libero.it; Emilia Middea - emiliamiddea@virgilio.it; Ida Perrotta - pida@libero.it; Rosalinda Bruno - ros.bru@yahoo.it; Michele Pellegrino - mipelle@libero.it; Sebastiano Andò - sebastianoando@unical.it

* Corresponding author †Equal contributors

Published: 30 November 2009

Reproductive Biology and Endocrinology 2009, **7**:140 doi:10.1186/1477-7827-7-140

Received: 4 September 2009
Accepted: 30 November 2009

This article is available from: http://www.rbej.com/content/7/1/140

Abstract

Background: A wider biological role of 1alpha,25-Dihydroxyvitamin D3 (1,25(OH)2D3), the active metabolite of vitamin D3, in tissues not primarily related to mineral metabolism was suggested. Recently, we evidenced the ultrastructural localization the 1,25(OH)2D3 receptor in the human sperm. However, the 1,25(OH)2D3 action in human male reproduction has not yet been clarified.

Methods and Results: By RT-PCR, Western blot and Immunofluorescence techniques, we demonstrated that human sperm expresses the 1,25(OH)2D3 receptor (VDR). Besides, 25(OH)D3-1 alpha-hydroxylase, evidenced by Western blot analysis, indicated that in sperm 1,25(OH)2D3 is locally produced, highlighting the potential for autocrine-paracrine responses. 1,25(OH)2D3 through VDR, increased intracellular Ca2+ levels, motility and acrosin activity revealing an unexpected significance of this hormone in the acquisition of fertilizing ability. In sperm, 1,25(OH)2D3 through VDR, reduces triglycerides content concomitantly to the increase of lipase activity. Rapid responses stimulated by 1,25(OH)2D3 have been observed on Akt, MAPK and GSK3 implying that this secosteroid is involved in different sperm signalling pathways.

Conclusion: Our data extended the role of 1,25(OH)2D3 beyond its conventional physiological actions, paving the way for novel therapeutic opportunities in the treatment of the male reproduction disorders.

Background

1 alpha,25-dihydroxyvitamin D_3 (1,25(OH)2D3), the active metabolite of vitamin D_3, is formed after hydroxylation by the rate-limiting enzyme 25(OH)D_3-1 alpha-hydroxylase (1 alpha-hydroxylase), abundantly expressed in the renal proximal tubule [1]. 1,25(OH)2D3 is a secosteroid whose actions are mediated by binding to its cognate nuclear receptor, the vitamin D receptor (VDR), a

member of the nuclear hormone receptor superfamily [2]. The VDR exerts 1,25(OH)2D3-dependent responses in the nucleus as a ligand-activated transcription factor [3]. In addition to these relatively slow genomic effects, 1,25(OH)2D3 generates rapid responses including Ca^{2+} uptake from intestine [4,5], augmentation of insulin secretion from pancreatic β-cells [6,7], growth and differentiation of vascular smooth muscle cells [8] and keratinocytes [9]. The initial signal is amplified by production of second messengers including inositol triphosphate and diacylglycerol in the plasma membrane by phospholipase C, phosphoinositol 3-kinase [8], production of cAMP [10], and activation of the MAPK pathway [11]. Vitamin D regulates Ca^{2+} homeostasis and VDR expression is not limited to organs involved in Ca^{2+} regulation, suggesting that 1,25(OH)2D3 may perform different functions in a tissue specific manner.

Previous studies suggested that 1,25(OH)2D3 has some role in reproductive functions. The VDR is widely distributed in male and female reproductive tissues [12], implying a 1,25(OH)2D3 action in these organs. In humans, VDR was observed in the testis, in the prostate and in spermatozoa [12-14]. Particularly, our group by immunogold analysis showed that VDR was localised uniformly in the sperm nucleus, although some particles also decorated the neck of the sperm [15]. Vitamin D deficiency and vitamin D receptor null mutant mice showed gonadal insufficiencies [16,17]. Uterine hypoplasia and impaired folliculogenesis were observed in the female, decreased sperm count and motility with histological abnormality of the testis were observed in the male. However, the role of 1,25(OH)2D3 in the testis is unclear. Different nuclear receptors [18,19] were found to be present in human ejaculated spermatozoa, regulating cellular processes through their nongenomic mechanisms. Sperm is a useful cellular type to study these effects since they are transcriptional inactive cells [20]. Indeed, sperm functionalities need to be rapidly activated to accommodate dynamic changes in the surrounding milieu. The significance of 1,25(OH)2D3/VDR in male fertility is not yet been fully investigated. The current finding evaluated the potential role of 1,25(OH)2D3/VDR system in human sperm physiology by studying its effect on Ca^{2+} levels, motility, acrosin activity, glucose and lipid metabolism. Furthermore, rapid 1,25(OH)2D3 responses were evaluated on different signalling transductional pathways identified in sperm.

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 - Life Technologies Italia (Milan, Italy). Oligonucleotide primers were made by Invitrogen (Milan, Italy). BSA protein standard, Laemmli sample buffer, prestained molecular weight markers, Percoll (colloidal PVP coated silica for cell separation), Sodium bicarbonate, Sodium lactate, Sodium pyruvate, Dimethyl Sulfoxide (DMSO), Earle's balanced salt solution (uncapacitating medium), 1alpha,25-Dihydroxyvitamin D_3 (1,25(OH)2D3) and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, fetal calf serum (FCS) was from Invitrogen (Milan, Italy), Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). ECL Plus Western blotting detection system, Hybond™ECL™, Hepes Sodium Salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Triglycerides assay kit, lipase activity kit, calcium (Ca^{2+}) assay kit, Glucose-6-phosphate dehydrogenase (G6PDH) activity assay kit were from Inter-Medical (Biogemina Italia Srl, Catania, Italy). Goat polyclonal actin Ab (1-19), monoclonal mouse anti-VDR (D-6) Ab, rabbit anti-p-Akt1/Akt2/Akt3 S473 Ab, peroxidase-coupled anti-rabbit and anti-goat IgG secondary Abs, anti-rabbit IgG FITC conjugated, Protein A/G-agarose plus were from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit anti-p-extracellular signal-regulated kinase (ERK 1/2) and anti-p-GSK3 Abs were from Cell Signalling (Milan, Italy). Mouse anti-1α-hydroxylase Ab was from the Binding Site Ltd (Birmingham, UK).

Semen collection, sperm processing and experimental treatments

Human semen was collected, according to the World Health Organization (WHO) recommended procedure by masturbation from healthy volunteer donors of proven fertility undergoing semen analysis in our laboratory. Spermatozoa preparations were performed as previously described [21]. Briefly, sperm samples with normal parameters of semen volume, sperm count, motility, vitality and morphology, according to the WHO Laboratory Manual [22], were included in this study. Further, the results of routine semen analysis on subjects included in the study are reported in the Table 1. Each sperm sample was obtained by pooling the ejaculates of three different normozoospermic healthy donors. After liquefaction, normal semen samples were pooled and subjected to cen-

Table 1: Mean of the semen parameters from all the sample used (n. 21)

Semen Parameters	Mean ± SD
Volume (mL)	3.54 ± 0.3
Sperm concentration (10^6/mL)	75.2 ± 2.4
Motility (%)	47.2 ± 1.82
Morfology (%)	45 ± 1.2

trifugation (600 *g*) on a discontinuous Percoll density gradient (95:40% v:v) [23]. The 95% 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 examined several fields for each slide, inspected the cells. Percoll-purified sperm were washed with unsupplemented Earle's balanced salt solution medium (uncapacitating medium) and were incubated in the same medium for 30 min at 37°C and 5% CO_2, without (control) or with increasing concentrations of 1,25(OH)2D3 (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with anti-VDR Ab combined with 0.1 nM 1,25(OH)2D3. When the cells were treated with the anti-VDR Ab, a pre-treatment of 15 min was performed. Other samples were incubated in capacitating medium (Earle's balanced salt solution medium supplemented with 266 mg/100 ml $CaCl_2$, 600 mg/100 ml BSA, 3 mg/100 ml sodium pyruvate, 360 μl/100 ml sodium lactate, and 200 mg/100 ml sodium bicarbonate).

After the incubation time, the samples were centrifuged and the pellet containing sperm was lysed to perform RT-PCR, western blots, triglycerides assay, Ca^{2+} assay, acyl-CoA dehydrogenase assay, glucose6-phosphate dehydrogenase (G6PDH) activity, lipase activity. Prior the centrifugation several aliquots were used to perform sperm motility, viability and acrosin activity. To evaluate the expression of the VDR in samples with severe oligoasthenozoospermia (subjects with a sperm count less than 10 × 10^6/ml and motility less than 20%) the western blotting analysis was performed by pooling three different samples with purified sperms. The study was approved by the local medical-ethical committees and all participants gave their informed consent.

RNA isolation, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from human ejaculated spermatozoa purified as abovementioned. Before RT-PCR, RNA was incubated with ribonuclease-free deoxyribonuclease (Dnase) I in single-strength reaction buffer at 37°C for 15 min. This was followed by heat inactivation of Dnase I at 65°C for 10 min. Two micrograms of Dnase-treated RNA samples were reverse transcribed by 200 IU M-MLV reverse transcriptase in a reaction volume of 20 μl (0.4 μg oligo-dT, 0.5 mM 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) was performed with 2 U of Taq DNA polymerase, 50 pmol primer pair in 10 mM Tris-HCL (pH 9.0) containing 0.1% Triton X-100, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.25 mM each dNTP. Potential contamination by leucocytes and germ cells in our sperm cells preparations was assessed by amplifying CD45 and c-kit transcripts respectively. The applied PCR primers and the expected lengths of the resulting PCR products are shown in Table 2. For all PCR amplifications (40 cycles), negative (reverse transcription-PCR performed without M-MLV reverse transcriptase) and positive controls were included: MCF7 breast cancer cells for VDR [24], human germ cells for c-Kit and human leucocytes for CD45. Cycling conditions were: 95°C/1 min, 55°C/1 min, 72°C/2 min for VDR; 95°C/1 min, 52°C/1 min, 72°C/2 min for c-kit; 95°C/1 min, 55°C/1 min, 72°C/2 min for CD45. The PCR-amplified products were subjected to electrophoresis in 2% agarose gels stained with ethidium bromide and visualised under UV transillumination.

Gel extraction and DNA sequence analysis

The VDR RT-PCR product was extracted from the agarose gel by using a gel band purification kit, the purified DNA was subcloned into PCR 2.1 vector and then sequenced by MWG AG Biotech (Ebersberg, Germany).

Evaluation of sperm motility

Sperm motility was assessed by means of light microscopy examining an aliquot of each sperm sample in absence (NC, control) or in the presence of increasing concentrations of 1,25(OH)2D3 (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with anti-VDR Ab combined with 0.1 nM 1,25(OH)2D3 and incubated for 30 min under uncapacitating conditions (experimental). Sperm motility was expressed as percentage of total motile sperm.

Western blot analysis of sperm proteins

Percoll-purified sperm, washed twice with Earle's balanced salt solution, were incubated without (NC) or with the treatments indicated in the legend of each figure, and then centrifuged for 5 min at 5000 × g. Besides, some samples were washed and incubated in capacitating medium.

Table 2: Oligonucleotide sequences used for RT-PCR

Gene	Sequence (5' - 3')	Size of PCR product (bp)
VDR	5' - CTCCCCCTGCCAGTGCCTTACCTC - 3' 5' - CCCCGCTCTCCCTTCCCACACT - 3'	299
KIT	5' - AGTACATGGACATGAAACCTGG - 3' 5' - GATTCTGCTCAGACATCGTCG - 3'	780
PTPRC	5' - CAATAGCTACTACTCCATCTAAGCCA - 3' 5' - ATGTCTTATCAGGAGCAGTACATG - 3'	230

The pellet was resuspended in lysis buffer as previously described [23]. Equal amounts of protein (80 μg) were boiled for 5 min, separated by 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and probed with an appropriate dilution of the indicated Abs. The bound of the secondary antibody was revealed with the ECL Plus Western blotting detection system according to the manufacturer's instructions. As internal control, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) of the first Ab and reprobed with anti-β actin.

Immunofluorescence assay

Sperm cells, recovered from Percoll gradient, were rinsed three times with 0.5 mM Tris-HCl buffer, pH 7.5 and fixed with absolute methanol for 7 min at -20°C. VDR staining was carried out, after blocking with normal horse serum (10%), using a monoclonal anti-human VDR as primary Ab (1 μg/ml) and an anti-mouse IgG FITC conjugated (4 μg/ml) as secondary Ab. Sperm cells incubated without the primary Ab or with normal mouse serum instead of the primary Ab were utilized as the negative controls. The slides were examined under a fluorescence microscope (Olympus BX41, Milan Italy), and a minimum of 200 spermatozoa per slide were scored.

Evaluation of Ca^{2+} in sperm lysate

Ca^{2+} was determined according to the manufacturer instructions [25] and as previously described [26]. At a neutral pH, the Ca^{2+} forms with arsenazo III a complex, the colour intensity of which is directly proportional to the concentration of Ca^{2+} in the sample. Percoll-purified sperm, washed twice with Earle's balanced salt solution, were incubated in the absence (NC, control) or in the presence of increasing concentrations of 1,25(OH)2D3 (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with anti-VDR Ab combined with 0.1 nM 1,25(OH)2D3 for 30 min in a Ca^{2+} serum free medium (uncapacitating medium). 1 ml of the reagent, 4-morpholinoethanesulfonic acid (MES) at pH 6.5 (100 mM) and Arsenazo III (200 μM), was added to the sperm lysate, mixed and incubated for 5 minutes at +15-25°C. The optical density was measured with the spectrophotometer at 600 nm. Ca^{2+} content Ca^{2+} standard used was 2.5 mM (100 mg/l). Inter- and intra-assay variation were 0.24% and 0.37%. Ca^{2+} results are presented as μM per 10×10^6 number of spermatozoa.

Acrosin activity assay

Acrosin activity was assessed by the method of Kennedy *et al.* [27] and as previously described [28]. Sperm were washed in Earle's medium and centrifuged at 800 *g* for 20 min, then were resuspended (final concentration of 10×10^6 sperm/ml) in different tubes containing no treatment (NC, control) or sperm were treated with increasing concentrations of 1,25(OH)2D3 (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with anti-VDR Ab combined with 0.1 nM 1,25(OH)2D3 and incubated for 30 min under uncapacitating conditions (experimental). 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) was added and incubated for 3 hours at room temperature. Aliquots (20 μl) were removed at 0 and 3 hours and the percentages of viable cells were determined. 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 with the spectrophotometer 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. The acrosin activity was expressed as μIU/10^6 sperms. Quantification of acrosin activity was performed as previously described [28].

Triglycerides Assay

Triglycerides were measured in duplicate by a GPO-POD enzymatic colorimetric method according to manufacturer's instructions in sperm lysates and as previously described [29]. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) or in capacitating medium for 30 min at 37°C and 5% CO_2. Other samples were incubated in the presence of increasing concentrations of 1,25(OH)2D3 (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with anti-VDR Ab combined with 0.1 nM 1,25(OH)2D3, for 30 min under uncapacitating conditions.

At the end of the sperm incubation 50 μg of sperm extracts were added to the 1 ml of buffer reaction and incubated for 10 min at room temperature. Then the triglycerides content was measured with the spectrophotometer at 505 nm. Data are presented as μg/10^6 sperms.

Assay of acyl-CoA dehydrogenase activity

Assay of acyl-CoA dehydrogenase was performed on sperm, using a modification of the method described by Lehman *et al.* [30]. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37°C and 5% CO_2. Other samples were incubated in the presence of increasing concentrations of 1,25(OH)2D3 (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with anti-VDR Ab combined with 0.1 nM 1,25(OH)2D3, for 30 min under uncapacitating conditions. In brief, after lysis, 70 μg of sperm protein was added to buffer containing 20 mM Mops, 0.5 mM EDTA, and 100 μM FAD^+ at pH 7.2. Reduction of FAD^+ to FADH was read at 340 nm upon addition of octanoyl-CoA (100 μM) every 20 seconds for 1.5 min. Data are expressed as nmol/min/mg protein. The enzymatic activity was deter-

mined with three control media: one without octanoyl-CoA as substrate, one without the coenzyme (FAD^+), and the third without either substrate or coenzyme (data not shown).

Lipase activity assay

Lipase activity was evaluated, by the method of Panteghini [31] based on the use of 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester (DGGR) as substrate. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37°C and 5% CO_2. Other samples were incubated in the presence of increasing concentrations of 1,25(OH)2D3 (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with anti-VDR Ab combined with 0.1 nM 1,25(OH)2D3, for 30 min under uncapacitating conditions. 50 μg of sperm extracts were loaded into individual cuvettes containing buffer for determination with the spectrophotometer. DGGR is cleaved by lipase, resulting in an unstable dicarbonic acid ester which is spontaneously hydrolysed to yield glutaric acid and methylresorufin, a bluish-purple chromophore with peak absorption at 580 nm. The absorbance of samples was read every 20 seconds for 1.5 min. The rate of methylresorufin formation is directly proportional to the lipase activity in the sample. Analysis of total imprecision gave a coefficient of variation of between 0.01% and 0.03%. The estimated reference interval was 6-38 U/L (μmol/min/mg protein). The enzymatic activity was determined with three control media: one without the substrate, one without the co-enzyme (colipase) and the third without either substrate or co-enzyme (data not shown).

G6PDH activity

The conversion of $NADP^+$ to NADPH, catalyzed by G6PDH, was measured by the increase of absorbance at 340 nm. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37°C and 5% CO_2. Other samples were incubated in the presence of sperm treated with increasing concentrations of 1,25(OH)2D3 (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with anti-VDR Ab combined with 0.1 nM 1,25(OH)2D3 and incubated for 30 min under uncapacitating conditions. After incubation, 50 μg of sperm extracts were loaded into individual cuvettes containing buffer (100 mM triethanolamine, 100 mM $MgCl_2$, 10 mg/ml glucose-6-phosphate, 10 mg/ml $NADP^+$, pH 7.6) for determination with the spectrophotometer. The absorbance of samples was read at 340 nm every 20 seconds for 1.5 min. Data are expressed in nmol/min/10^6 sperms. The enzymatic activity was determined with three control media: one without glucose-6-phosphate as substrate, one without the coenzyme ($NADP^+$), and the third without either substrate or coenzyme (data not shown).

Statistical analysis

The experiments for RT-PCR and immunofluorescence assays were repeated on at least three independent occasions, whereas Western blot analysis was performed in at least seven independent experiments. The data obtained from triglycerides Assay, G6PDH activity, acyl-CoA dehydrogenase activity, Ca^{2+} assay and lipase activity (seven replicate experiments using duplicate determinations), motility (five replicate experiments using duplicate determinations), acrosin activity (seven replicate experiments using duplicate determinations) were presented as the mean ± SD. The differences in mean values were calculated using analysis of variance (ANOVA) with a significance level of $P \leq 0.05$.

Results

VDR expression in human sperm

To determine whether mRNA for VDR is present in human ejaculated spermatozoa, RNA isolated from percoll-separated sperm of normal men was subjected to reverse PCR. The primer sequences were based on the human VDR gene sequence and the RT-PCR amplification revealed the expected PCR product size of 299 bp of the coding region of the human VDR cDNA (Fig 1A). This product was sequenced and found to be corresponding to the classical human VDR (data not shown). No detectable levels of mRNA coding either CD45, a specific marker of human leucocytes, or c-kit, a specific marker of human germ cells, were found in the same semen samples (Fig 1A), ruling out any potential contamination. In addition, the RT-PCR products were not a result of any DNA contamination as the RNA samples were subjected to DNAse treatment before RT-PCR.

The presence of VDR protein in human ejaculated sperm was investigated by Western blot (Fig 1B) using a mouse monoclonal Ab raised against amino acids 344-424 of VDR (D-6) of human origin, purchased by Santa Cruz Biotechnology. Two immunoreactive bands, corresponding to the molecular mass values of 52 and 57 kDa were observed. Two different cellular types were used as positive controls: MCF7, breast cancer cells, that showed two bands, one of 48 kDa as previously reported [32] and another one at 52 kDa; LNCap, prostate cancer cells, that showed one band at 52/54 [33]. Interestingly, as shown in the panel C, it appears that pathologic samples exhibit a reduced expression of VDR, particularly of the 52 kDa band.

A local Vitamin D Metabolism occurs in human sperm

The 1 alpha-hydroxylase is a member of the cytochrome P450 superfamily and it is a key enzyme of vitamin D metabolism. In order to investigate whether in sperm a local Vitamin D metabolism exists, we did perform a west-

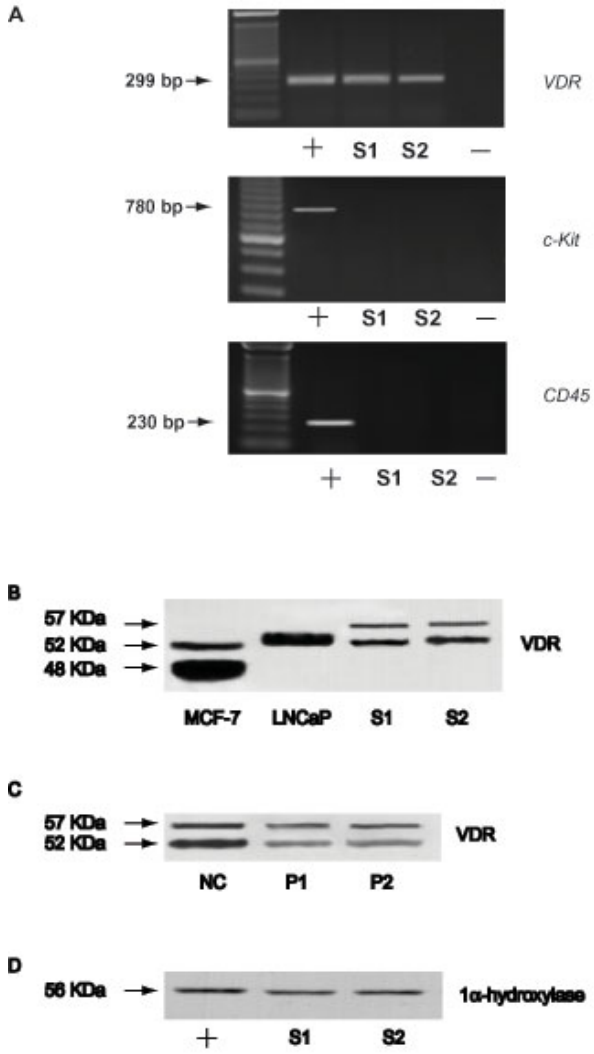

Figure 1

VDR expression in human ejaculated spermatozoa. **A**: Reverse transcription-PCR analysis of human VDR, CD45 and c-Kit genes in percolled human spermatozoa (S1 and S2), negative control (no M-MLV reverse transcriptase added) (-), positive controls (MCF7 breast cancer cells for VDR, human germ cells for c-Kit and human leucocytes for CD45) (+), marker (lane M). Arrows indicated the expected size of the PCR products. **B**: Western blot of VDR protein in human sperm, expression in two samples of ejaculated spermatozoa from normal men (S1, S2). MCF-7 and LNCaP extracts were used as positive controls. **C**: VDR expression in severe oligoastenozoospermic patients. NC = Normal uncapacitated sample; P1, P2 = pathologic samples. **D**: Western blot of 1alpha--hydroxylase protein in human sperm, expression in two samples of ejaculated spermatozoa from normal men (S1, S2). MCF-7 extracts was used as positive control (+). The experiments were repeated at least four times for RT-PCR, seven times for Western blot and the autoradiographs of the figure show the results of one representative experiment.

ern blot by using an anti-1alpha-hydroxylase Ab. As shown in Fig. 1D one band, corresponding to the molecular mass value of 56 kDa was observed like in somatic cells [34].

VDR localization in human sperm

Using a immunofluorescence technique and the same anti-VDR Ab used for western blot, we obtained a positive signal for VDR in human spermatozoa. The immunoreactivity was predominantly compartmentalized in the sperm head (Fig 2A) and a weak staining was also observed in the midpiece. No fluorescent signal was obtained when primary Ab was omitted (Fig 2B) or when the normal mouse IgG was used instead of the primary Ab (Fig. 2C), thus further confirming the specificity of the antibody binding.

1,25(OH)2D3 regulates intracellular Ca^{2+} content in human sperm

1,25(OH)2D3 plays not only a pivotal role in systemic Ca^{2+} homeostasis but also in the intracellular Ca^{2+} homeostasis of various tissues [35]. Recently it was demonstrated that internal sperm Ca^{2+} stores provide sufficient Ca^{2+} for the induction of hyperactived motility [36]. It is important to point out that serum human 1,25(OH)2D3 levels between 37.5 and 150 pM (15 - 60 pg/ml) can be regarded as physiological concentrations [37] whereas 1 nM and 10 nM are supraphysiological levels. Our results showed that 1,25(OH)2D3 from 0.01 nM to 1 nM is able to increase intracellular Ca^{2+}, however not in a dose dependent manner and 10 nM 1,25(OH)2D3 didn't show a significant increase (Fig. 3); the combination of anti-VDR Ab with 0.1 nM 1,25(OH)2D3 reduced this action. These data may address an important role of 1,25(OH)2D3/VDR in sperm Ca^{2+} regulation.

1,25(OH)2D3 influences sperm motility and acrosin activity

As it was never investigated, a functional assessment of the sperm under 1,25(OH)2D3 was performed to evaluate motility and acrosin activity. Sperm motility was enhanced upon 0.01 nM and 0.1 nM 1,25(OH)2D3, while the combination of the anti-VDR Ab with 0.1 nM 1,25(OH)2D3 reduced this effect. 1 nM and 10 nM 1,25(OH)2D3 appear to be ineffective (Fig. 4A). Besides, we evaluated whether 1,25(OH)2D3 is able to influence the sperm extratesticular maturation by evidencing its potential action on acrosin activity.

A significant a dose-dependent effect from 0.01, 0.1 and 1 nM 1,25(OH)2D3 on increased acrosin activity was observed (Fig. 4B). The 10 nM 1,25(OH)2D3 didn't show a further increase with respect to the 1 nM concentration. The process was significantly reduced by using the anti-

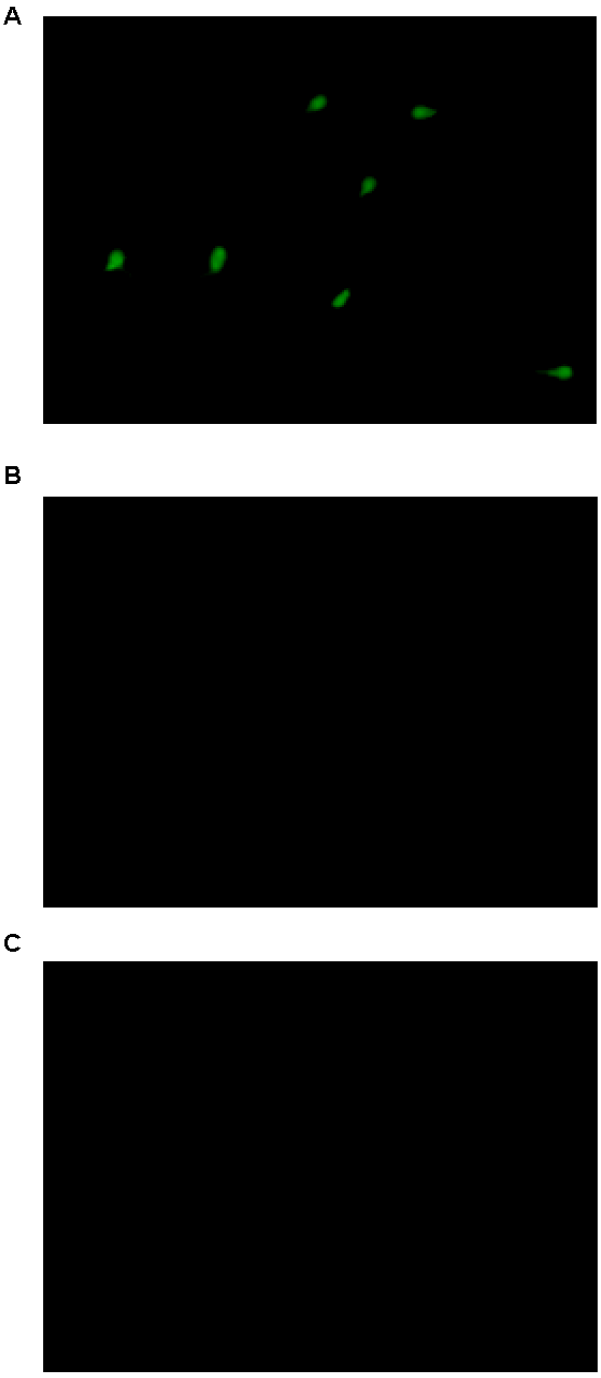

Figure 2
Immunofluorescence localization of VDR in human ejaculated spermatozoa. **A**: VDR Immunolocalization; **B**: Sperm cells incubated without the primary Ab were utilized as negative control. **C**: Sperm cells incubated replacing the anti-VDR Ab by normal rabbit serum were utilized as negative control. The pictures shown are representative examples of experiments that were performed at least three times with reproducible results.

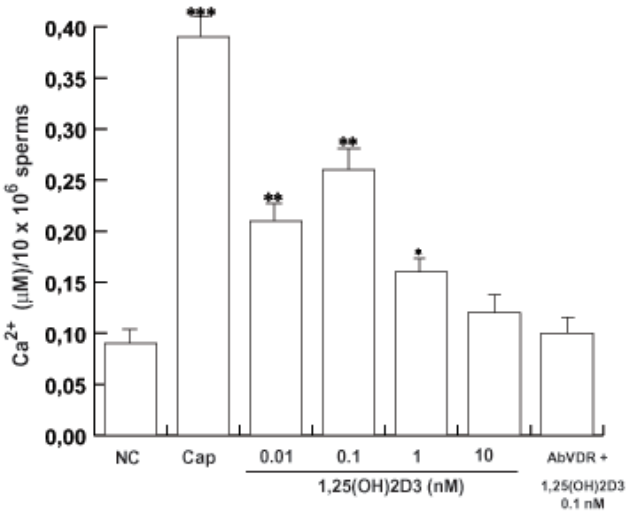

Figure 3
1,25(OH)2D3 increases intracellular Ca^{2+}. Percoll-purified sperm washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing 1,25(OH)2D3 concentrations (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with 0.1 nM 1,25(OH)2D3 combined with anti-VDR Ab (AbVDR). Other samples were incubated in capacitating medium (Cap). Intracellular calcium was measured as reported in Materials and Methods. The calcium assay presented are representative examples of experiments that were performed at least seven times with repetitive results. Columns represent mean ± S.D. **P* < 0.05 versus control, ***P* < 0.02, ****P* < 0.01 versus control.

VDR Ab combined with 0.1 nM 1,25(OH)2D3 suggesting an involvement of the receptor in the acrosome reaction.

1,25(OH)2D3 affects human sperm metabolism

During sperm extratesticular maturation an overall increase in sperm metabolism occurs. However, the mechanisms that govern this event are still poorly understood. In order to give further insight on this aspect of sperm physiology we studied a potential role of VDR in lipid and glucose sperm metabolism, by evaluating the intracellular levels of triglycerides, lipase activity, acyl-CoA dehydrogenase activity and G6PDH activity. 1,25(OH)2D3 stimulation induced a significant dose-dependent decrease from 0.01, 0.1 and 1 nM 1,25(OH)2D3, addressing a lipolytic effect, while the 10 nM 1,25(OH)2D3 was ineffective. The anti-VDR Ab reversed the 0.1 nM 1,25(OH)2D3 effect addressing a VDR-dependent event (Table 3). The lipase activity was enhanced from 0.01 nM to 1 nM 1,25(OH)2D3, however not in a dose dependent manner. 10 nM 1,25(OH)2D3 appears to be ineffective and the combination of anti-VDR Ab with 0.1 nM 1,25(OH)2D3 indicates a VDR-mediated effect. No differences between treated and control samples were observed both in the

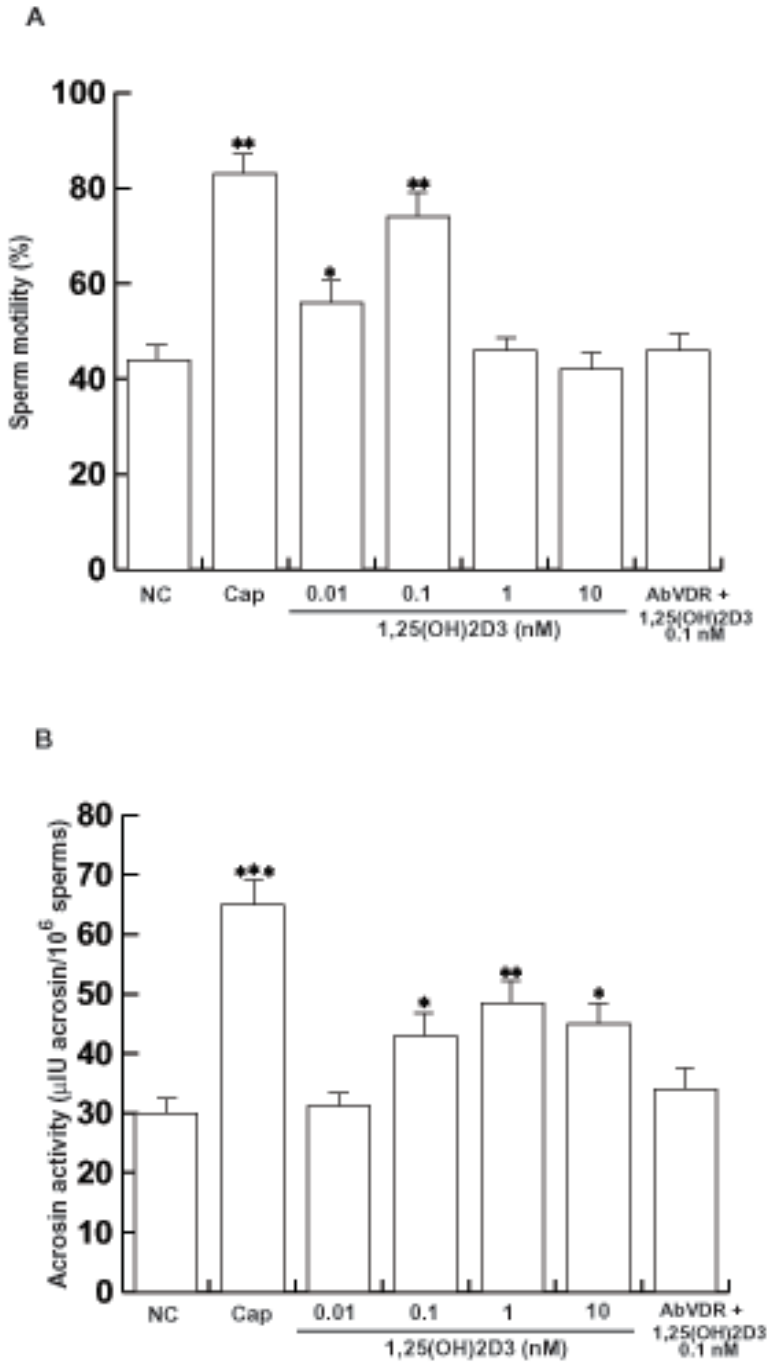

Figure 4

1,25(OH)2D3 effects on motility and acrosin activity are VDR-mediated. Percoll-purified sperm washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing 1,25(OH)2D3 concentrations (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with 0.1 nM 1,25(OH)2D3 combined with anti-VDR Ab (AbVDR). Other samples were incubated in capacitating medium (Cap). Sperm motility and acrosin activity were assessed as reported in *Materials and methods*. The sperm motility presented are representative examples of experiments that were performed at least five times with repetitive results while acrosin activity were performed at least seven times with repetitive results. Columns are mean ± S.D. Data are expressed as % for motility and as μIU acrosin/10^6 sperms for acrosin activity. *$P < 0.05$ versus control; ** $P < 0.02$, ***$P < 0.01$ versus control.

acyl-CoA dehydrogenase and in the G6PDH activities upon increasing 1,25(OH)2D3.

1,25(OH)2D3 activates ERK1/2, Akt and GSK3 in human sperm

The mechanisms involved in the control of sperm functions are not yet well known; strong evidences indicate that they are associated with or controlled by different signal transduction elements. Therefore, we aimed to investigate 1,25(OH)2D3 rapid action on different kinases identified in sperm, such as the ERK1/2, AKT and GSK3, by evaluating their phosphorylations. Increasing doses of the secosteroid from 0.01 nM to 0.1 nM resulted in significant induction of the ERK1/2, AKT and GSK3 phosphorylations (Fig. 5A), while the 1 nM and 10 nM concentrations appear to be ineffective. Particularly, the 1 nM 1,25(OH)2D3 increased only the AKT phosphorylation. Anti-VDR Ab abolished 0.1 nM 1,25(OH)2D3-induced effect, demonstrating that in sperm 1,25(OH)2D3 is able to activate different signalling pathways through VDR and therefore it might contribute to different sperm biological functions.

To investigate if the phosphorylations of the abovementioned kinases, induced by 0.1 nM 1,25(OH)2D3 may represent an early event, we performed a time course study (0, 5, 10, 30 and 60 min). This experiment revealed that the ERK1/2, AKT and GSK3 phosphorylations occurred rapidly as they were observed from 5 min, increased at 10 min and were sustained until 30 min, then dropped significantly after 1 hour (Fig. 5B).

Discussion

1,25(OH)2D3 is a key regulator of Ca^{2+} homeostasis through binding to specific receptor-VDR [38]. The archetypal target organs of this hormone include bone, intestines and kidney. Indeed, diverse array of tissues that do not participate in mineral and bone metabolism possess specific VDR and sequentially respond to 1,25(OH)2D3, including testis [13]. However, the role of 1,25(OH)2D3 in the physiology of genitourinary organs, is still principally unknown. In our study we aimed to investigate 1,25(OH)2D3 functional role in sperm physiology and the molecular mechanisms through which this secosteroid may affect human male reproduction, discovering new 1,25(OH)2D3 actions.

First, we identified VDR in sperm at different levels: mRNA presence, protein expression and its localization. Previous reports demonstrating the size of the VDR by gel electrophoresis, showed that it varies depending on the species studied with a molecular weight ranging from 50 to 60 kDa. Human testis showed a protein of 57 and another one of 52 kDa molecular weight compared with 57 and 37 kDa in the rat testis [39]. Human prostate evidenced protein of 52 kDa compared to rat ventral (57 and 37 kDa) and dorsal prostate (52 and 26 kDa). Our data on VDR isoforms are super imposable to that found in human testis. Recently, in human sperm, VDR was reported at 50 kDa size [14], and this discrepancy with our data may be due to different methodological approaches and/or to the antibody used. It appears that a lower amount of VDR is expressed in pathologic samples that have severe oligoastenozoospermia. The VDR localization by immunofluorescence assay confirmed the data observed in our previous study, showing by immunogold analysis that VDR was localised in the sperm nucleus, although some particles also decorated the neck [15]. In somatic cells, the receptor-hormone complex becomes localized to the nucleus and then interacts with the 1,25(OH)2D3-responsive element, modifying transcription of the target genes. In addition to their classic genomic action, nuclear receptors regulate cellular processes through a non-genomic mechanism [40]. It is generally accepted that the sperm nucleus is transcriptional inactive due to the highly condensed architecture of its chromatin. In this study, we investigated the rapid effects of the VDR as we have also observed previously for other nuclear receptors in human sperm [18,29,41]. Indeed, this mode of action seems to be particularly appropriate

Table 3: Effects of 1,25(OH)2D3 on sperm metabolism

	Triglycerides mg/10^6 sperms	Lipase activity (U/L)mmol/ min/ mg protein	Acyl CoA dehydrogenase activity nmol/min/mg protein	G6PDH mmol/min/10^6 sperms
NC	0,08 ± 0.0013	0,5 ± 0.02	0,004 ± 0.0001	0,005 ± 0.0001
1,25(OH)2D3 0.01 nM	0,05 ± 0.001*	1,3 ± 0.04*	0,0039 ± 0.0001	0,0048 ± 0.0002
1,25(OH)2D3 0.1 nM	0,025 ± 0.0029**	1,8 ± 0.03**	0,0041 ± 0.0002	0,0047 ± 0.0002
1,25(OH)2D3 1 nM	0,015 ± 0.0032**	0,8 ± 0.032*	0,0040 ± 0.0003	0,0051 ± 0.00018
1,25(OH)2D3 10 nM	0,07 ± 0.002	0,6 ± 0.031	0,0038 ± 0.0001	0,0046 ± 0.0001
Ab VDR + 0.1 nM 1,25(OH)2D3	0,077 ± 0.0019	0,4 ± 0.041	0,0038 ± 0.0002	0,0049 ± 0.0002
CAP	0.06 ± 0.002	2,2 ± 0,045**	0,01 ± 0,0005**	0.01 ± 0,0003*

Data are presented as mean ± S.D. of seven independent experiments performed in duplicate.
*$P < 0.05$ versus control; ** $P < 0.01$ versus control (NC). CAP = capacitated sperms.

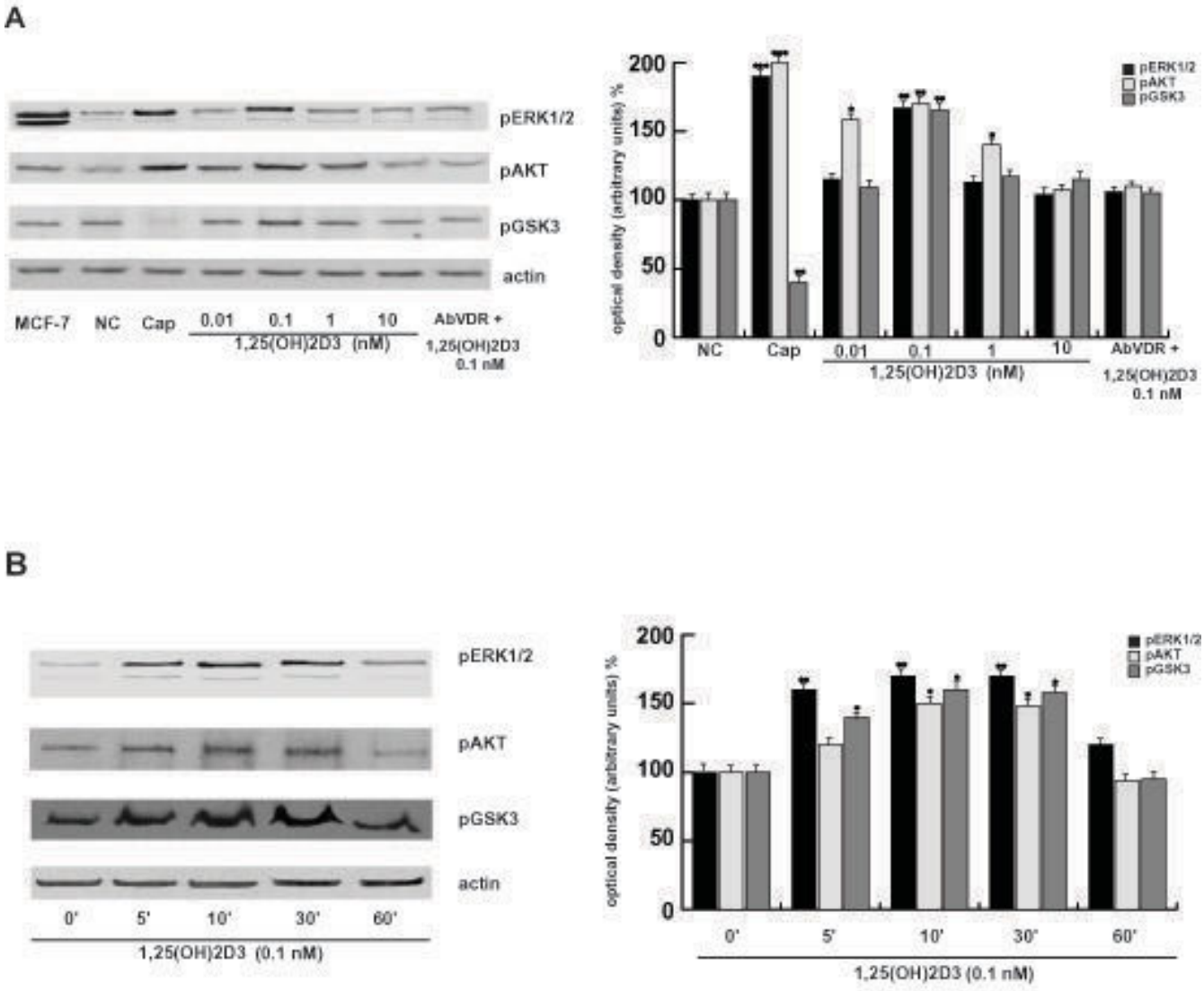

Figure 5

1,25(OH)2D3 induces pERK1/2, AKT and GSK3 phosphorylations in human sperm through VDR. **A**: Percoll-purified sperm washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing 1,25(OH)2D3 concentrations (0.01 nM, 0.1 nM, 1 nM and 10 nM) or with 0.1 nM 1,25(OH)2D3 combined with anti-VDR Ab (AbVDR). Other samples were incubated in capacitating medium (Cap). **B**: Time course study (0, 5, 10, 30 and 60 min) of ERK1/2, AKT and GSK3 phosphorylations treated with 0.1 nM 1,25(OH)2D3. Actin was used as loading control. On the side are reported the densitometric evaluations normalised against actin levels. The autoradiographs presented are representative examples of experiments that were performed at least seven times with repetitive results. **P* < 0.05 versus control, ***P* < 0.01, ****P* < 0.002 versus control.

in the male gamete because sperm functions need to be activated rapidly to accommodate dynamic changes in the surrounding milieu. Importantly, our study also evidenced that 1,25(OH)2D3 is a locally produced hormone as the male gamete expresses the 1α, hydroxylase. The presence of this protein in sperm and the VDR raises the possibility of an autocrine short loop.

Sperm physiology depends on nongenomic signals amongst which Ca2+ have an important role [42,43], however, its regulation is poorly understood. Autonomous 1,25(OH)2D3 production together the presence of VDR led us to hypothesize an autocrine regulation of Ca2+ in sperm, on the basis of this classical 1,25(OH)2D3 role at systemic level. There is considerable evidences that Ca^{2+} stores exist in mammalian spermatozoa and recently it was demonstrated that they play an important role in triggering hyperactivated motility. In fact, internal Ca^{2+} stores could provide sufficient Ca^{2+} for the induction of hyperactivation, afterwards the Ca^{2+} influx is required to

maintain intracellular Ca^{2+} levels sufficient to maximize and sustain this process that led to the acrosome reaction [36]. From our data it emerges that 1,25(OH)2D3, through VDR, is able to increase intracellular Ca^{2+} levels, addressing a role for the receptor in the induction of hyperactivated motility, that in turn triggers or contributes to the sperm changes associated to capacitation and acrosome reaction. The mechanisms controlling the interaction between energy balance and reproduction are the subject of intensive investigations. Capacitated sperm display an increased metabolism and overall energy expenditure, however, the signalling pathways associated with the change in sperm metabolism energy are poorly understood. In this study, we observed in sperm that 1,25(OH)2D3, through VDR, reduces triglycerides content concomitantly to the increase of the lipase activity. The rate-limiting step in the metabolism of neutral lipids in adipose tissue lies at the level of the lipase, which catalyzes the hydrolysis of triglyceride [44], high lipase activity was previously demonstrated in the spermatozoa [45]. However, the endogenous substrate used for energy metabolism by spermatozoa is species specific and to what degree triglycerides supply the energy demands of mammalian spermatozoa is not clear. Particularly, from our study it appears that 1,25(OH)2D3 doesn't affect FFA β-oxidation or G6PDH activities and this may be explained by the fact that the increased lipase activity is regulated by Ca^{2+} [46] and therefore, this effect might be imputable to 1,25(OH)2D3 calcemic action.

Intriguingly, it appears that 1,25(OH)2D3 differently influences lipid metabolism in adipocytes and in sperm. In human adipocytes 1,25(OH)2D3 stimulates Ca^{2+} influx, promotes lipogenesis and inhibits lipolysis via a rapid nongenomic action [47]. It was demonstrated that the suppression of 1,25(OH)2D3 by high dietary Ca^{2+} stimulates lipolysis, inhibits lipogenesis [48] and thereby shifts the partitioning of dietary energy from energy storage to energy expenditure. Similarly, this 'anti-obesity' effect of Ca^{2+} may occur in sperm during capacitation when Ca^{2+} influx strongly increases. Therefore, it seems that, in spite of adipose tissue, 1,25(OH)2D3 has a lipolytic effect in sperm, however an intricate cooperation of endocrine and autocrine/paracrine factors may exist in this cell leading to a fine regulation of the energy needed to the different physiological conditions. The early increase in sperm intracellular free Ca^{2+} induced by 1,25(OH)2D3 might be involved in switching the metabolism from lipogenesis to lipolysis. Indeed, it may be speculated that 1,25(OH)2D3 increases the intracellular Ca^{2+} mobilization, stimulating the induction of capacitation that requires energy. Therefore the lipid metabolism increases to meet the energetic demands during the process by reducing energy storage and increasing energy expenditure. Besides, sperm lipid metabolism might be more sensitive to Ca^{2+} variations given the importance of this signalling in sperm. Infact it is important to point out that intracellular Ca^{2+} is approximately 50-100 nM in uncapacitated sperm, 200-1000 nM in hyperactivated sperm [49] and it may increase to approximately 10 μM during the acrosome reaction [50].

A wide array of rapid responses stimulated by 1,25(OH)2D3 have been reported [51]. ERK1/2, AKT and GSK3 have been demonstrated to be involved in different sperm activities [18,21]. In this study 1,25(OH)2D3 rapidly induces all of these pathways indicating that VDR is involved in various sperm functions and thus corroborating the unexpected physiological significance of the hormone in the human male gamete.

The hormone concentration can be crucial in determining the type of cell responsiveness. It was observed in different cell systems that the hormone level regulates the association between different receptors and signalling effectors suggesting that assembly or disassembly of different modules are involved in the effects triggered by low and high hormone concentrations [52,53]. In our study, the response to 1,25(OH)2D3 appears to be biphasic with a stimulatory effect at lower concentrations, and becoming inhibitory or ineffective at the higher levels. The outcome of signalling activation depending on differences in ligand level was also recently demonstrated in human sperm [15,18,21,29,41] and a possible explanation relies in the down regulation of the receptors at elevated hormone concentration [54]. The observation that different hormone levels trigger different responses in sperm cells is a remarkable example of the pronounced flexibility of this cell in the responsiveness to steroids.

Concluding, sperm local source of 1,25(OH)2D3 may participate with different actions in the sperm functional maturation. The data provided by the current experiments clearly establish a molecular role of the VDR in sperm physiology and it may be considered as a novel modulator of sperm fertilizing ability. VDR is present in the seminiferous tubules, in spermatogonia [12] and in spermatozoa [14,15]. In addition to sex steroid hormones, the classic regulators of reproduction, vitamin D also modulates reproductive processes in the human female. After sperm are deposited into the vagina via ejaculation, they must travel through the cervical mucus into the uterus and then into the fallopian tube before they can meet with the egg. The 1 alpha-hydroxylase is expressed in cervical and uterine tissues [55,56]. Vaginal epithelium [57], cervical, endometrial cells [58] and fallopian epithelial cells express VDR [59,12] implicating a physiological role of the 1,25(OH)2D3 in this context. It was reported that the amount of 1,25(OH)2D3 present in follicular fluid is significantly lower than in the concurrent serum [60]. These

observations may also support our results concerning the biphasic effect of 1,25(OH)2D3 doses, since lower hormone levels induce the majority of the sperm activities evaluated, while higher concentrations appear to be ineffective. However, the physiological significance and the specific role of 1,25(OH)2D3/VDR in both gametes need to be further investigated.

Taken together, our results extended the role of 1,25(OH)2D3 beyond its conventional physiological actions, enhanced our knowledge on human sperm at molecular level and our understanding of the vitamin D signaling pathway, paving the way for novel therapeutic opportunities in the treatment of the male fertility disorders. The modulation of the VDR might also provide a mechanism by which environmental or dietary vitamin D can influence sperm fertilizing ability and therefore male reproduction.

Abbreviations

(1,25(OH)2D3): 1alpha,25-Dihydroxyvitamin D_3; (VDR): 1,25(OH)2D3 receptor; (1α-hydroxylase): 25(OH)D_3-1alpha-hydroxylase; (G6PDH): Glucose-6-phosphate dehydrogenase; (DMSO): Dimethyl Sulfoxide; (M-MLV): Moloney Murine Leukemia Virus; (ERK 1/2): Rabbit anti-p-extracellular signal-regulated kinase; (WHO): World Health Organization; (Dnase): ribonuclease-free deoxyribonuclease; (cDNA): complementary DNA; (DGGR): 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester; (Ab): Antibody.

Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Authors' contributions

AS, author responsible for conception, design, coordinating the experiments, the analysis and interpretation of data as well as of drafting manuscript, revising it critically and final approval of the version. GC, the author responsible for sperm isolation, hormonal treatments, protein extraction and participating in the analysis and interpretation of data. ME, the author responsible for substantial contributions to conception and design of the research, performing acrosin activity and RT-PCR assays and participating in the analysis interpretation of data and for helping to draft the manuscript. PI, the author responsible for performing the immunofluorescence experiments. BR, the author responsible for performing the assays testing metabolic sperm functions. PM, the author responsible for performing western blot analysis. AS*, the author participating in the design and coordination of the study. All authors read and approved the final manuscript.

Footnotes

Contract grant sponsor: This work was supported by MURST and Ex 60% -2009.

Acknowledgements

Our special thank to D. Sturino (Faculty of Pharmacy, University of Calabria - Italy) for the English review of the manuscript, to Dr. Cunsulo Vincenzo (Biogemina Italia Srl, Catania - Italy) and to Perrotta Enrico for the technical and scientific assistance.

References

1. Zehnder D, Bland R, Walker EA, Bradwell AR, Howie AJ, Hewison M, Stewart PM: **Expression of 25-hydroxyvitamin D_3-1alpha-hydroxylase in the human kidney.** *J Am Soc Nephrol* 1999, **10:**2465-2473.
2. Sunn KL, Cock TA, Crofts LA, Eisman JA, Gardiner EM: **Novel N-Terminal Variant of Human VDR.** *Mol Endocrinol* 2001, **15:**1599-1609.
3. Jurutka PW, Whitfield GK, Hsieh JC, Thompson PD, Haussler CA, Haussler MR: **Molecular Nature of the Vitamin D Receptor and its Role in Regulation of Gene Expression.** *End & Metab Disorders* 2001, **2:**203-216.
4. Nemere I, Yoshimoto Y, Norman AW: **Calcium transport in perfused duodena from normal chicks: enhancement within 14 minutes of exposure to 1α,25-dihydroxyvitamin D_3.** *Endocrinology* 1984, **115:**1476-1483.
5. Norman AW, Okamura WH, Hammond MW, Bishop JE, Dormanen MC, Bouillon R, Van Baelen H, Ridall AL, Daane E, Khoury R: **Comparison of 6-*s*-*cis* and 6-*s*-*trans* locked analogs of 1,25(OH)$_2$-vitamin D_3 indicates that the 6-*s*-*cis* conformation is preferred for rapid nongenomic biological responses and that neither 6-s-*cis* nor 6-s-*trans* locked analogs are preferred for genomic biological responses.** *Mol Endocrinol* 1997, **11:**1518-1531.
6. Kadowaki S, Norman AW: **Time-course study of insulin secretion after 1α,25-dihydroxyvitamin D_3 administration.** *Endocrinology* 1985, **117:**1765-1771.
7. Kajikawa M, Ishida H, Fujimoto S, Mukai E, Nishimura M, Fujita J, Tsuura Y, Okamoto Y, Norman AW, Seino Y: **An insulinotropic effect of vitamin D analog with increasing intracellular Ca^{2+} concentration in pancreatic β-cells through nongenomic signal transduction.** *Endocrinology* 1999, **140:**4706-4712.
8. Rebsamen MC, Sun J, Norman AW, Liao JK: **1α,25-Dihydroxyvitamin D_3 induces vascular smooth muscle cell migration via activation of phosphatidylinositol 3-kinase.** *Circ Res* 2002, **91:**17-24.
9. Gniadecki R: **Activation of Raf-mitogen-activated protein kinase signaling pathway by 1α,25-dihydroxyvitamin D_3 in normal human keratinocytes.** *J Invest Dermatol* 1996, **106:**1212-1217.
10. Massheimer V, Boland R, De Boland AR: **Rapid 1,25(OH)$_2$-vitamin D_3 stimulation of calcium uptake by rat intestinal cells involves a dihydropyridine-sensitive cAMP-dependent pathway.** *Cell Signal* 1994, **3:**299-304.
11. Buitrago C, Gonzalez PV, Russo DB, Boland R: **Activation of RAF-1 through RAS and PKC mediates 1,25(OH)2D3 regulation of the map kinase pathway in muscle cells.** *J Biol Chem* 2002, **278:**2199-2205.
12. Johnson JA, Grande JP, Roche PC, Kumar R: **Immunohistochemical detection and distribution of the 1,25-dihydroxyvitamin D3 receptor in rat reproductive tissues.** *Histochem Cell Biol* 1995, **105:**7-15.
13. Habib FK, Maddy SQ, Gelly KJ: **Characterisation of receptors for 1,25-dihydroxyvitamin D_3 in the human testis.** *Steroid Biochem* 1990, **35:**195-199.
14. Corbett ST, Hill O, Nangia AK: **Vitamin D receptor found in human sperm.** *Urology* 2006, **68:**1345-1349.
15. Aquila S, Guido C, Perrotta I, Tripepi S, Nastro A, Andò S: **Human sperm anatomy: ultrastructural localization of 1alpha,25-dihydroxyvitamin D receptor and its possible role in the human male gamete.** *J Anat* 2008, **213:**555-564.

16. Kwiecinksi GG, Petrie GI, DeLuca HF: **1,25-Dihydroxyvitamin D3 restores fertility of vitamin D-deficient female rats.** *Am J Physil* 1989, **256:**483-487.
17. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S: **Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia, and growth retardation after weaning.** *Nat Genet* 1997, **16:**391-396.
18. Aquila S, Sisci D, Gentile M, Middea E, Catalano S, Carpino A, Rago V, Andò S: **Estrogen receptor (ER)alpha and ER beta are both expressed in human ejaculated spermatozoa: evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway.** *J Clin Endocrinol Metab* 2004, **89:**1443-1451.
19. El-Hefnawy T, Manna PR, Luconi M, Baldi E, Slotte PJ: **Huhtaniemi: Progesterone Action in a Murine Leydig Tumor Cell Line (mLTC-1), Possibly through a Nonclassical Receptor Type.** *Endocrinology* 2000, **141:**247-255.
20. Grunewald S, Paasch U, Glander HJ, Anderegg U: **Mature human spermatozoa do not transcribe novel RNA.** *Andrologia* 2005, **37:**69-71.
21. Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V, Andò S: **Leptin Secretion by Human Ejaculated Spermatozoa.** *J Clin Endocrinol Metab* 2005, **90:**4753-4761.
22. World Health Organization: **Laboratory manual for the examination of human semen and sperm-cervical mucus interactions.** 4th edition. Cambridge UK Cambridge University Press; 1999.
23. Aquila S, Sisci D, Gentile M, Middea E, Siciliano L, Andò S: **Human Ejaculated Spermatozoa Contain Active P450 Aromatase.** *J Clin Endocrinol Metab* 2002, **87:**3385-3390.
24. Diesing D, Cordes T, Fischer D, Diedrich K, Friedrich M: **Vitamin D--metabolism in the human breast cancer cell line MCF-7.** *Anticancer Res* 2006, **26:**2755-2759.
25. Ovsiannikov AI, Kon'kov SA, Gurkalo VK: **Measurement of calcium content in the blood and other biological fluids by Arsenazo III complexone.** *Klin Lab Diagn* 1999, **1:**16-18.
26. Aquila S, Guido C, Laezza C, Santoro A, Pezzi V, Panza S, Andò S, Bifulco M: **A new role of anandamide in human sperm: focus on metabolism.** *J Cell Physiol* 2009, **221:**147-153.
27. Kennedy WP, Kaminski JM, Ven HH Van der, Jeyendran RS, Reid DS, Blackwell J, Bielfeld P, Zaneveld LJ: **A simple, clinical assay to evaluate the acrosin activity of human spermatozoa.** *J Androl* 1989, **10:**221-231.
28. Aquila S, Sisci D, Gentile M, Carpino A, Middea E, Catalano S, Rago V, Andò S: **Towards a physiological role for cytochrome P450 aromatase in ejaculated human sperm.** *Hum Reprod* 2003, **18:**1650-1659.
29. Aquila S, Bonofiglio D, Gentile M, Middea E, Gabriele S, Belmonte M, Catalano S, Pellegrino M, Andò S: **Peroxisome proliferator-activated receptor (PPAR)gamma is expressed by human spermatozoa: its potential role on the sperm physiology.** *J Cell Physiol* 2006, **209:**977-986.
30. Lehman TC, Hale DE, Bhala A, Thorpe C: **An acyl-coenzyme A dehydrogenase assay utilizing the ferricenium ion.** *Anal Biochem* 1990, **186:**280-284.
31. Panteghini M, Bonora R, Pagani F: **Measurement of pancreatic lipase activity in serum by a kinetic colorimetric assay using a new chromogenic substrate.** *Ann Clin Biochem* 2001, **38:**365-370.
32. Mork Hansen C, Rohde L, Madsen MW, Hansen D, Colston KW, Pirianov G, Holm PK, Binderup L: **MCF-7/VD^R A new vitamin D resistant cell line.** *J Cell Biochem* 2001, **82:**3.
33. Leman ES, Arlotti JA, Dhir R, Getzenberg RH: **Vitamin D and androgen regulation of prostatic growth.** *J Cell Biochem* 2003, **90:**138-147.
34. Lou YR, Laaksi I, Syvala H, Blauer M, Tammela TLJ, Ylikomi T, Tuohimaa P: **25-Hydroxyvitamin D3 is an active hormone in human primary prostatic stromal cells.** *Faseb J* 2004, **18:**332-334.
35. Banerjee P, Chatterjee M: **Antiproliferative role of vitamin D and its analogs--a brief overview.** *Mol Cell Biochem* 2003, **253:**247-254.
36. Kong LJ, Yang YY, Wang GL: **CatSper and sperm hyperactivation.** *Zhonghua Nan Ke Xue* 2007, **13:**164-167.
37. Peacock M: **Vitamin D receptor gene alleles and osteoporosis: a contrasting view.** *J Bone Miner Res* 1995, **10:**1294-1297.
38. Reichel H, Koeffler HP, Norman AW: **The role of the vitamin D endocrinesystem in health and disease.** *N Engl J Med* 1989, **320:**980-991.
39. Nangia AK, Butcher JL, Konety BR, Vietmeie BN, Getzenberg RH: **Association of vitamin D receptors with the nuclear matrix of human and rat genitourinary tissues.** *Steroid Biochem* 1998, **66:**241-246.
40. Norman AW, Mizwicki MT, Norman DP: **Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model.** *Nat Rev Drug Discov* 2004, **3:**27-41.
41. Aquila S, Middea E, Catalano S, Marsico S, Lanzino M, Casaburi I, Barone I, Bruno R, Zupo S, Andò S: **Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway.** *Hum Reprod* 2007, **22:**2594-2605.
42. Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CM: **A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels.** *Biol Reprod* 1998, **59:**12-16.
43. Suarez SS, Ho HC: **Hyperactivation of mammalian sperm.** *Cell Mol Biol* 2003, **49:**351-356.
44. Steinberg D: **Prostaglandins as adrenergic antagonists.** *Ann N Y Acad Sci* 1967, **139:**897-909.
45. Mita M, Yasumasu I, Nakamura M: **Energy metabolism of spermatozoa of the sand dollar Clypeaster japonicus: the endogenous substrate and ultrastructural correlates.** *J Biochem* 1994, **116:**108-113.
46. Zhang L, Lookene A, Wu G, Olivecrona G: **Calcium triggers folding of lipoprotein lipase into active dimers.** *J Biol Chem* 2005, **280:**42580-42591.
47. Shi H, Norman AW, Okamura WH, Sen A, Zemel MB: **1alpha,25-Dihydroxyvitamin D3 modulates human adipocyte metabolism via nongenomic action.** *FASEB J* 2001, **15:**2751-2753.
48. Schrager S: **Dietary Calcium Intake and Obesity.** *J Am Board Fam Practice* 2005, **18:**205-210.
49. Suarez SS, Dai X: **Intracellular calcium reaches different levels of elevation in hyperactivated and acrosome-reacted hamster sperm.** *Mol Reprod Dev* 1995, **42:**325-333.
50. Dube C, Tardif S, LeClerc P, Bailey JL: **The importance of calcium in the appearance of p32, a boar sperm tyrosine phosphoprotein, during in vitro capacitation.** *J Androl* 2003, **24:**727-733.
51. Norman AW: **Minireview: vitamin D receptor: new assignments for an already busy receptor.** *Endocrinology* 2006, **147:**5542-5548.
52. Marshall CJ: **Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation.** *Cell* 1995, **80:**179-185.
53. Castoria G, Lombardi M, Barone MV, Bilancio A, Di Domenico M, Bottero D, Vitale F, Migliaccio A, Auricchio F: **Androgen-stimulated DNA synthesis and cytoskeletal changes in fibroblasts by a nontranscriptional receptor action.** *J Cell Biol* 2003, **161:**547-556.
54. Uotani S, Bjørbaek C, Tornøe J, Flier J: **Functional properties of leptin receptor isoforms: internalization and degradation of leptin and ligand-induced receptor downregulation.** *Diabetes* 1999, **48:**279-286.
55. Friedrich M, Villena-Heinsen C, Axt-Fliedner R, Meyberg R, Tilgen W, Schmidt W, Reichrath J: **Analysis of 25-hydroxyvitamin D3-1alpha-hydroxylase in cervical tissue.** *Anticancer Res* 2002, **22:**183-186.
56. Evans KN, Bulmer JN, Kilby MD, Hewison M: **Vitamin D and placental-decidual function.** *J Soc Gynecol Investig* 2004, **11:**263-271.
57. Becker S, Cordes T, Diesing D, Diedrich K, Friedrich M: **Expression of 25 hydroxyvitamin D3-1alpha-hydroxylase in human endometrial tissue.** *J Steroid Biochem Mol Biol* 2007, **103:**771-775.
58. Yildirim BN, Abban G, Rota S, Jetten A: **Immunohistochemical Detection and Distribution Cornifin and Vitamin D Receptor Expression in Vaginal Epithelium Treated With Vitamin. D3.** *J Turkish-German Gynecol Assoc* 2007, **8:**33-37.
59. Pérez-López FR: **Sunlight, the vitamin D endocrine system, and their relationships with gynaecologic cancer.** *Maturitas* 2008, **59:**101-113.
60. Teilmann SC, Clement CA, Thorup J, Byskov AG, Christensen ST: **Expression and localization of the progesterone receptor in mouse and human reproductive organs.** *J Endocrinol* 2006, **191:**525-535.

International Journal of Andrology
international journal of andrology ISSN 0105-6263

ORIGINAL ARTICLE

Insulin and IR-β in pig spermatozoa: a role of the hormone in the acquisition of fertilizing ability

A. Carpino,* V. Rago,* C. Guido,†‡ I. Casaburi†‡ and S. Aquila†‡

*Department of Cell Biology, †Department of Pharmaco-Biology (Faculty of Pharmacy), and ‡Centro Sanitario, University of Calabria, Arcavacata di Rende (Cosenza), Italy

Keywords:
fertility, insulin, insulin receptor β, pig spermatozoa, reproduction

Correspondence:
Carpino Amalia, Dipartimento di Biologia Cellulare, Università della Calabria, 87030 Arcavacata di Rende, Cosenza, Italy.
E-mail: am_carpino@yahoo.it

Received 29 January 2009; revised 11 March 2009, 1 April 2009; accepted 9 April 2009

doi:10.1111/j.1365-2605.2009.00971.x

Summary

Recent studies have revealed that insulin, the main regulator of the glucose homeostasis in somatic cells, is expressed in human spermatozoa which are also able to secrete it. This study investigated the expression of insulin and insulin receptor β in pig spermatozoa, at immunohistochemical protein and mRNA level. The immunofluorescence assay revealed that insulin and its receptor were co-localized in the sperm midpiece, while insulin was also detected in the acrosomal region. Western blot evidenced a 36 kDa band for insulin and a 95 kDa band for insulin receptor, such as reported in somatic cells. In addition, both insulin and insulin receptor transcripts were detected in pig spermatozoa. Interestingly, a possible biological role of the hormone was evidenced during pig sperm capacitation and acrosome reaction. In fact, the results showed that insulin (0.01 and 0.1 nM) can induce both the activities. A possible autocrine short loop of insulin in pig spermatozoa was suggested by the evaluation of the hormone secretion in both uncapacitated and capacitated spermatozoa. Furthermore, spontaneous sperm capacitation and acrosome reaction were stimulated by glucose and inhibited by the blockage of insuline release (nifedipine). In conclusion, this work has firstly demonstrated the expression of insulin and of its receptor, as well as the insulin secretion by pig spermatozoa, thereby suggesting an unexpected significance of the hormone in the acquisition of the male gamete fertilizing ability.

Introduction

In adult mammals, insulin (Ins) is produced in highly specialized cells (β-cells) of the endocrine pancreas. In young animals, Ins is also secreted by the thymus (Throsby *et al.*, 1998) and, additionally, extra-pancreatic Ins gene expression is detectable in the yolk sac (Philippe, 1991) and transiently in brain tissue (Alpert *et al.*, 1988) during embryonic development of rodents.

Insulin has been shown to exhibit pleiotropic effects involving mitogenic and/or metabolic events and its effects are tissue/development dependent. Ins may transduce its signal through a variety of pathways (White & Kahn, 1994); the two major pathways, described to date, employ Ins receptors (IRs) as the primary targets, signalling via mitogen-activated protein kinases and phosphoinositol-3 kinase. IRs exist in two isoforms (Seino *et al.*, 1989): the A type (IR-A) and the B type (IR-B) but the functional significance of these IR isoforms remains still unclear.

In somatic cells, the IR is a heterotetrameric transmembrane protein composed of two α- and two β-subunits linked by disulphide bonds (Kahn & White, 1988). The α-subunits are totally extracellular while the β-subunits are anchored in the plasma membrane. The binding of Ins to the α-subunits activates the intrinsic tyrosine-specific kinase of the intracellular β-subunits to phosphorylate tyrosine residues and also to phosphorylate intracellular substrates. These events are necessary for the signal transduction to elicit various Ins actions.

In farm animals, similarly as in humans, reproduction is affected by changes in the level of energetic balance. Therefore, Ins may play a role in the regulation of gonadal function. In addition to gonadotropins, reproductive function is affected by the metabolic factors (Adashi *et al.*, 1985), which act on hypothalamo-pituitary axis as

well as on the gonads by directly altering gametogenesis. Different studies suggested a positive effect of Ins in female fertility, particularly on pre-ovulatory follicular development of swine (Cox *et al.*, 1987; Britt *et al.*, 1988; Matamoros *et al.*, 1990, 1991), while the significance of Ins in male fertility is not defined yet.

In men, it was reported that Ins promotes spermatogonial differentiation into primary spermatocytes by binding to the IGF-I receptor (Nakayama *et al.*, 1999). Further, in men affected by Ins-dependent diabetes, with malfunction of Ins signalling, spermatozoa have severe structural defects, significantly lower motility and concentration as well as abnormal morphology (Baccetti *et al.*, 2002; Ramalho-Santos *et al.*, 2008) and, lower ability to penetrate hamster eggs (Shrivastav *et al.*, 1989). Furthermore, increased levels of nuclear DNA fragmentation, mitochondrial DNA deletions and oxidative DNA modification were reported in spermatozoa from men with type 1 diabetes (Agbaje *et al.*, 2007, 2008).

Recently, studies from our laboratory discovered that human ejaculated spermatozoa express and secrete Ins (Aquila *et al.*, 2005), opening a new field of study in reproductive biology. Aim of this study was to investigate the expression of Ins and of its receptor (IR β) in ejaculated spermatozoa of the pig. A possible biological role of the hormone on the functional property of the male gamete has been also explored.

Materials and methods

Chemicals

BSA protein standard, Laemmli sample buffer, prestained molecular weight markers, Percoll (colloidal PVP-coated silica for cell separation), sodium bicarbonate, dimethyl sulphoxide, Earle's balanced salt solution, porcine Ins, propidium iodide (PI), fluorescein isothiocyanate-labelled peanut (Arachis hypogaea) agglutinin (FITC–PNA), D-glucose, nifedipine and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, ECL Plus Western blotting detection system and Hybond^{TM} ECL^{TM} were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Polyclonal rabbit anti-Ins (H-86), peroxidase-coupled anti-rabbit, peroxidase-coupled anti-mouse, FITC-conjugated anti-rabbit IgG and Texas Red-conjugated anti-mouse IgG antibodies (Abs) were from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal mouse anti-IRβ (C18C4) was from Stressgene (Bologna, Italy). Cholesterol-oxidase (CHOD)-peroxidase (POD) enzymatic colorimetric kit was from Inter-Medical (Biogemina Italia Srl, Catania, Italy). The Ins RIA kit was purchased from Diagnostic Systems Laboratories ICN (Biogemina Italia Srl, Catania, Italy). Total RNA Isolation System kit, enzymes, buffers and nucleotides 100 bp ladder used for RT-PCR were purchased from Promega Corp. (Milan, Italy). Moloney murine leukaemia virus (M-MLV) was from Gibco-Life Technologies Italia. Oligonucleotide primers were purchased from Invitrogen Life Technologies, Inc., (Milan, Italy).

Animals and semen samples

The investigation has been conducted on semen from eight fertile male pigs (*Sus scrofa domestica*, Large White) kept at 'Swine Artificial Insemination Centre' (Rende, Cosenza, Italy). The animals were 20 to 30 month-old and their weights were from 260 to 330 kg. Individual fresh ejaculates were collected using the gloved hand method and filtered immediately by Universal Semen bags (Minitub, Tiefenbech, Germany). Semen was transported within half an hour to the laboratory. It was diluted 1 : 10 with TBS buffer and centrifuged on a discontinuous Percoll density gradient (72%/90%) to remove bacteria and debris (Kuster *et al.*, 2004).

Evaluation of sperm viability

Viability of pig spermatozoa was assessed using the DNA-specific fluorochrome propidium iodide (PI). Sperm suspension (1×10^6 mL) was exposed to PI (12 μM) for 5 min at room temperature. Then spermatozoa were fixed by adding 1 μL of 12.5% (w/v) paraformaldehyde in 0.5 M Tris (pH 7.4) and the slides were immediately examined under an epifluorescence microscope (Olympus BX41, Olympus Italia Srl, Segrate, Milano, Italy) observing a minimum of 200 spermatozoa per slide (100× objective).

Sample treatments

Percoll-purified spermatozoa were washed with unsupplemented Earle's medium (uncapacitating medium) and were incubated for 30 min at 39 °C and 5% CO_2, without (NC : uncapacitated) or with increasing concentration of Ins (0.01, 0.1 and 1 nM). These doses were chosen on the basis of the physiological Ins plasma levels reported in the pig (McNeel *et al.*, 2000). The cells were also pre-treated (15 min) with the anti-IRβ Ab before the addition of 0.1 nM Ins. A further control was performed incubating pig spermatozoa in Earle's balanced salt solution medium supplemented with 600 mg BSA/100 mL and 200 mg sodium bicarbonate/100 mL (capacitating medium)(Cap). No adverse effects among the different treatments have been observed on pig sperm viability.

Immunofluorescence assay

Following Percoll separation, sperm cells were rinsed three times with 0.5 mM Tris-HCl buffer (pH 7.5); then 10 μL of cell suspension was added to 250 μL drop of warm (37 °C) TBS (0.05M Tris- buffered normal saline, pH 7.6) and allowed to settle onto slides in a humid chamber. The overlying solution was carefully pipetted off and replaced by absolute methanol for 7 min at −20 °C. After methanol removal, sperm cells were washed in TBS, containing 0.1% Triton X-100 and were treated for immunofluorescence.

Insulin and IRβ immunostainings were carried out, after blocking with normal goat serum (10%), using anti-Ins (1 : 100) and anti-IRβ (1 : 100) as primary antibodies, followed by anti-rabbit IgG FITC conjugated and anti-mouse IgG Texas Red conjugated antibodies (1 : 200) respectively. Sperm cells incubated without the primary antibodies were utilized as negative controls. The slides were immediately examined under an epifluorescence microscope (Olympus BX41) observing a minimum of 200 spermatozoa per slide (100× objective). The experiments were repeated three times for each sample.

Western blot analysis of sperm proteins

Western blot (WB) analysis was used to identify Ins and IRβ in pig spermatozoa. Sperm samples were washed twice with unsupplemented Earle's medium (uncapacitating medium) and then centrifuged for 5 min at 5000 ***g***. The pellets were resuspended in lysis buffer as previously described (Kuster *et al.*, 2004). Equal amounts of proteins (80 μg) were boiled for 5 min, separated by 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and probed with a dilution of 1 : 500 for the anti-Ins Ab or with a dilution of 1 : 1000 for the anti-IRβ Ab. The binding of the secondary antibody was revealed with the ECL Plus WB detection system according to the manufacturer's instructions. The negative control was performed using a sperm lysate that was immunodepleted of Ins or IRβ (i.e. pre-incubate lysate with anti-Ins Ab or anti-IRβ Ab for 1:00 h at room temperature and immunoprecipitate with Protein A/G-agarose). β-actin served as a control for equal loading. The experiments were repeated at least four times for each sample.

RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from pig spermatozoa purified as previously described (Aquila *et al.*, 2002). Before RT-PCR, RNA was incubated with ribonuclease-free deoxyribonuclease (DNAse) I in single-strength reaction buffer at 37 °C for 15 min. This was followed by heat inactivation of DNAse I at 65 °C for 10 min. Two micrograms of DNAse-treated RNA samples were reverse transcribed by 200 IU M-MLV reverse transcriptase in a reaction volume of 20 μL (0.4 μg oligo-dT, 0.5 mM 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) was performed with 2 U of Taq DNA polymerase, 50 pmol primer pair for both Lep and Ob-R in 10 mM Tris-HCL (pH 9.0) containing 0.1% Triton X-100, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.25 mM each dNTP. The applied PCR primers and the expected lengths of the resulting PCR products are the following: forward, 5′-GCC TTT GTG AAC CAA CAC CTG-3′; reverse, 5′-GTT GCA GTA GTT CTC CAG CTG-3′. For Ins with a product size of 261 bp; 5′-TTTCCATGTTCTGTGTAC-GTGCG-3′ and 5′-AGTCTCCGTGAGCCATCAATTCA-3′ for IR with a product size of 215 bp. Cycling conditions were: 95 °C/1 min, 62 °C/1 min, 72 °C/2 min for Ins; 95 °C/1 min, 65 °C/1 min, 72 °C/2 min for IR. For all PCR amplifications, negative controls (reverse transcription-PCR performed without M-MLV reverse transcriptase) were performed. The PCR-amplified products were subjected to electrophoresis in 2% agarose gels stained with ethidium bromide and visualized under UV transillumination.

Measurement of Ins secreted by pig spermatozoa

A competitive radioimmunoassay was applied to measure Ins in the pig sperm culture medium. Sperm cultures were incubated under uncapacitating (unsupplemented Earle's medium alone) or capacitating conditions (Earle's balanced salt solution medium supplemented with 600 mg BSA/100 mL and 200 mg sodium bicarbonate /100 mL) for 30 min in a 39 °C water bath at a final concentration of 10×10^6 sperm/500 μL. Each group was treated with 1, 4.5 and 8.3 mM glucose. At the end of the sperm incubations, the culture media were recovered by centrifugation. Ins concentrations were determined in duplicate using an Ins RIA kit according to manufacturer's instructions. Ins standards ranged from 0–300 μIU/mL. The limit of sensitivity for the assay was 0.01 μIU/mL. Inter- and intra- assay variations were 5.8% and 4.7% respectively. Ins results are presented as the original concentrations of the supernatants and are expressed as μIU/ml. The experiments were repeated at least six times for each sample.

Measurement of the cholesterol efflux in the sperm culture medium

Cholesterol was measured (in duplicate) in the incubation medium from pig spermatozoa by a CHOD–POD

enzymatic colorimetric method according to manufacturer's instructions as previously reported (Aquila *et al.*, 2008). Sperm samples (10×10^6) were washed twice with uncapacitating medium and incubated in the presence of 0.01, 0.1 and 1 nM Ins concentrations for 30 min at 39 °C and 5% CO_2. Control samples (NC) were incubated only in the same medium, while other samples were incubated with anti-IRβ Ab combined with 0.1 nM Ins. A further control has been carried out by using pig spermatozoa incubated in capacitating medium (Cap). At the same time, spermatozoa were also incubated with 4.5 nM glucose and 25 μM nifedipine. At the end of the sperm incubation, the culture media were recovered by centrifugation, lyophilized and subsequently dissolved in 1 mL of working reagent. The samples were incubated for 10 min at room temperature, then the cholesterol content was measured with the spectrophotometer at 505 nm. The cholesterol standard used was 200 mg/dL. The limit of sensitivity for the assay was 0.05 mg/dL. Inter and intra assay variations were 0.71% and 0.57% respectively. The experiments were repeated at least six times for each sample.

Acrosome reaction

Spermatozoa incubated with 0.01, 0.1 and 1 nM Ins concentrations, spermatozoa incubated with anti-IRβ Ab combined with 0.1 nM Ins and spermatozoa incubated only with buffer (NC) were re-suspended in unsupplemented Earle's medium (5×10^6 sperm/mL), placed in a conical tube and cultured for 2 h in an atmosphere of 5% CO_2 in air at 39 °C. A further control has been carried out incubating sperm samples in capacitating medium (Cap). At the same time, spermatozoa were also incubated with 4.5 nM glucose and 25 μM nifedipine. Then acrosomal status was monitored using the acrosome-specific fluorochrome fluorescein isothiocyanate-labelled peanut (Arachis hypogaea) agglutinin (FITC–PNA) in conjunction with DNA-specific fluorochrome propidium iodide (PI) as a viability test (Funahashi, 2002). Briefly, sperm suspension (1×10^6 mL) was exposed to FITC–PNA (10 μg/mL) and propidium iodide (12 μM) for 5 min at 39 °C and then fixed by adding 1 mL of 12.5% (w/v) paraformaldehyde on 0.5 mol Tris/L (pH 7.4). The slides were immediately examined with an epifluorescence microscope (Olympus BX41) with a multiple fluorescence filter (U-DM-DA/FI/TX2) observing a minimum of 200 spermatozoa per slide (100× objective). Acrosomal status was assessed according to the staining patterns.

Staining patterns

Spermatozoa with a nuclear red PI staining were considered as dead cells while sperm cells without PI staining were considered as live cells. Live spermatozoa were classified into two main categories on the basis of the FITC–PNA staining as follows: (i) acrosome-reacted cells with uniform green FITC–PNA fluorescence of acrosome cap (ii) acrosome-intact cells without any fluorescence. Values were expressed as percentage. Three replicate experiments were performed for each semen sample.

Statistical analysis

Data, presented as mean ± SEM, were evaluated using the one-way ANOVA. The differences in mean values were calculated at a significance level of $p \leq 0.05$.

Results

Immunolocalization of Ins and IRβ in pig spermatozoa

A brilliant green light revealed the Ins presence in the acrosome and midpiece of pig sperm cells (Fig. 1a). An intense red fluorescence showed IRβ confined in the sperm midpiece while the other cellular regions were unlabelled (Fig. 1b). In addition, no fluorescent signal was obtained when primary antibodies (anti-Ins Ab or anti- IRβ Ab) were omitted, thus confirming the specificity of the antibody binding.

Ins and IRβ expression in pig spermatozoa

Our anti-Ins antibody revealed one immunoreactive band at 36 kDa in different lysates from pig sperm samples

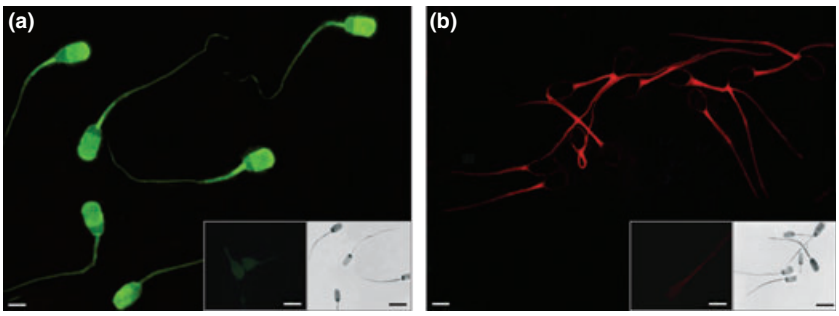

Figure 1 Representative immunofluorescence labelling of insulin and IRβ in pig spermatozoa: (a) A green brilliant light showed insulin in the acrosoma and in the midpiece of the spermatozoa. (b) A red intense fluorescence localized IRβ only in the sperm midpiece. *Inserts*: negative controls and transmitted light pictures of sperm cells. *Scale bars*: 5 μM, insert: 12.5 μM.

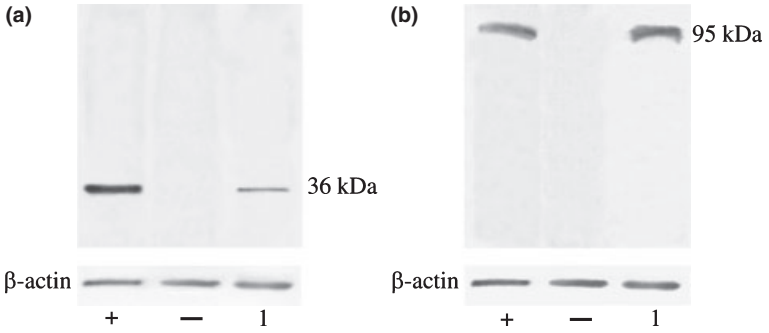

Figure 2 Insulin and insulin receptor expression in pig spermatozoa. (a): Western blot of insulin protein. Human spermatozoa (*lane* +), representative pig spermatozoa (*lane* 1), negative control (*lane* -). (b): Immunoblot of insulin receptor in pig spermatozoa. MCF7 cells (*lane* +), representative pig spermatozoa (*lane* 1), negative control (*lane* -).

(Fig. 2a), at the same mobility of the human sperm extract used as positive control, while no bands were observed in the negative control. The 36 kDa protein probably corresponds to the Ins hexameric form.

Western blot of pig sperm extracts evidenced one band at 95 kDa (Fig. 2b), such as in the MCF7 (breast cancer cell line) used as positive control. The band was not detected in the negative control.

Pig spermatozoa express mRNA for Ins and IR

To determine whether mRNA for Ins is present in pig spermatozoa, RNA isolated from percoll-purified spermatozoa was subjected to reverse PCR. The primer sequences were based on the pig gene sequence and the RT-PCR amplification revealed the expected PCR product size of 261 bp (Fig. 3a). To evaluate mRNA for IR in pig spermatozoa, RNA isolated from percoll-purified spermatozoa was subjected to reverse PCR. The primer sequences were based on the IR pig gene sequence and the RT-PCR amplification revealed the expected PCR product size of 215 bp (Fig. 3b). These products were sequenced and found identical to the pig gene sequences considered.

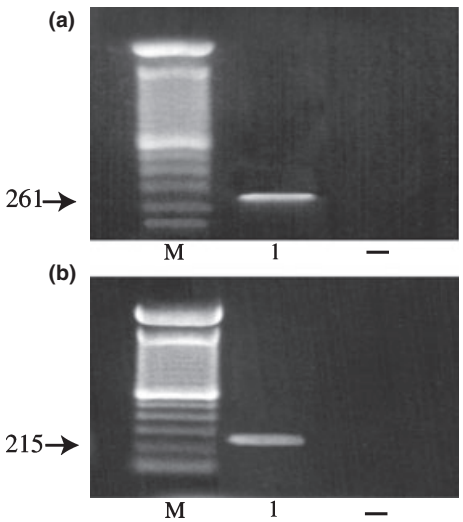

Figure 3 RT-PCR analysis of insulin (a) and insulin receptor (b) in one representative pig sperm sample (lane 1), negative control (lane-), and markers (lane M). The *arrow* indicates the expected size of the PCR product.

Measurement of Ins secretion by pig spermatozoa

Ins secretion from 10×10^6 spermatozoa incubated in uncapacitating medium was significantly lower than one measured from spermatozoa incubated under capacitating conditions ($p < 0.05$) (Fig. 4). Furthermore, a dose-response curve for Ins secretion in response to glucose stimulation was recorded for both uncapacitated and capacitated spermatozoa. As shown in Fig. 4, glucose significantly stimulated Ins secretion in a dose-dependent way.

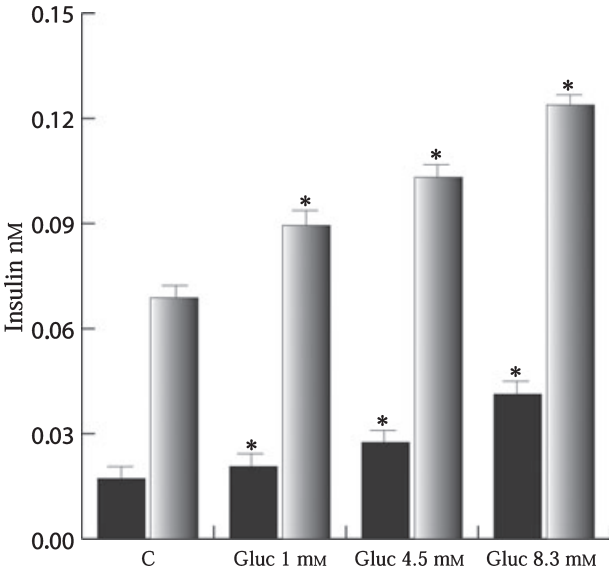

Figure 4 Effects of glucose on insulin secretion from pig ejaculated spermatozoa in uncapacitating (■) or capacitating (□) conditions. Control spermatozoa (C). Each group was treated with 1, 4.5 and 8.3 mM glucose (Gluc). The results are given as mean ± SEM. *, $p < 0.05$ vs. control. Statistical difference between capacitated and uncapacitated spermatozoa within the same treatment group was $p < 0.01$.

Ins modulates pig sperm capacitation influencing cholesterol efflux through its receptor

Sperm membrane cholesterol efflux represents one of the early events associated with capacitation in mammalian spermatozoa. Our results showed a significant increase in cholesterol efflux upon 0.01 and 0.1 nM Ins treatment in uncapacitated spermatozoa which reached the levels of capacitated spermatzoa (Fig. 5). Furthermore, the 1 nM Ins was ineffective and the anti-IRβ Ab was able to abolish the 0.1 nM Ins effect (Fig. 5). At the same time, 4.5 nM glucose significantly increased the cholesterol efflux from uncapacitated spermatozoa while 25 μM nifedipine was able to decrease it.

Ins affects pig spermatozoa acrosome reaction

Acrosome reaction is the end-point of fully capacitated spermatozoa. Fig. 6 shows a representative fluorescence pattern of pig spermatozoa, stained with FITC–PNA + PI for the assessment of acrosome status and sperm viability, after Ins treatment (A1) and in control samples (A).

A similar incidence of dead spermatzoa (PI positive cells) was observed in spermatozoa incubated in uncapacitating medium with 0.01, 0.1 and 1 nM Ins (16 ± 4%) and in control (15 ± 4%) as well as in capacitated spermatozoa (14 ± 5%).

Furthermore, in uncapacitaing conditions, a higher percentage of acrosome-reacted cells (FITC–PNA positive cells) was detected in spermatozoa incubated with both 0.01 nM Ins (16 ± 4%) and 0.1 nM Ins (21 ± 5%) with respect to the control spermatozoa (NC) (10 ± 3%), so reaching the value of capacitated control spermatozoa (Cap) (20 ± 5%) (Fig. 6b). Conversely, the percentage of acrosome-reacted cells which was detected in 1 nM Ins spermatozoa (11 ± 2%) and in spermatozoa incubated with Anti-IRβ Ab/0.1 nM Ins (9 ± 2%) was similar to that of the control spermatozoa (NC) (Fig. 6b). At the same time, 4.5 nM glucose significantly increased acrosome reaction extent (18 ± 4%) of uncapacitated spermatozoa while 25 μM nifedipine was able to decrease it (5 ± 2%).

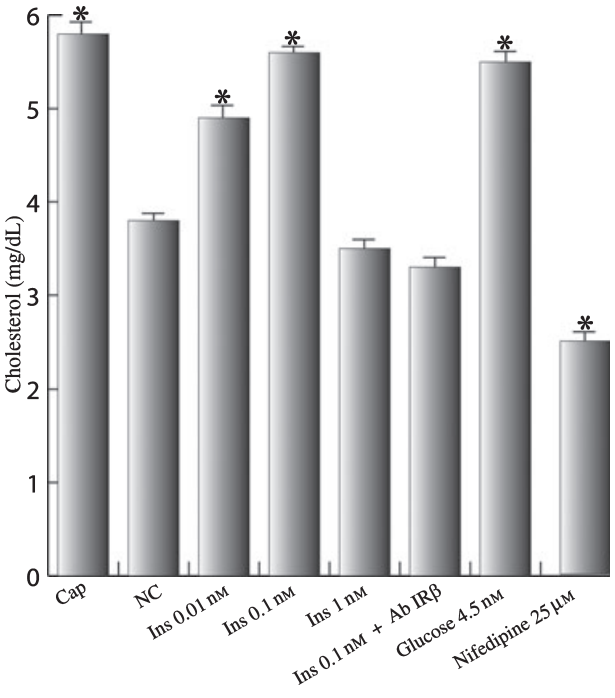

Figure 5 Insulin affects cholesterol efflux from pig spermatozoa. Capacitated spermatozoa as control(Cap). Uncapacitated spermatozoa were incubated in the absence (NC: uncapacitated control spermatozoa) or in the presence of insulin (0.01, 0.1 and 1 nM) as well as in presence of anti IRβ antibody + 0.1 nM insulin. Furthermore, 4.5 nM glucose increased the cholesterol efflux from uncapacitated spermatozoa while 25 μM nifedipine was able to decrease it. Results are presented as cholesterol amount (mg/dl) in culture medium from 10×10^6 sperm and are given as mean ± SEM. *, $p < 0.05$ vs. control (NC).

Discussion

Insulin and its signalling systems are implicated in both central and peripheral mechanisms governing the ingestion, distribution, metabolism and storage of nutrients in organisms ranging from worms to humans. New data have also shown that Ins signalling is a determinant of lifespan and reproductive function, broadening the importance of Ins to diverse areas of physiology and biomedicine.

Reproductive function is affected by metabolic factors in addition to gonadotropins (Adashi *et al.*, 1985). Particularly, involvement of Ins in ovarian follicular function in swine was suggested by increased ovulation rate (Cox *et al.*, 1987) and reduced follicular atresia (Matamoros *et al.*, 1990, 1991) when exogenous Ins was given during the pre-ovulatory period in cyclic gilts. Furthermore, exogenous Ins restored follicular growth in nutritionally anoestrous females (Britt *et al.*, 1988) and Ins increased the developmental potential of porcine oocytes and embryos (Lee *et al.*, 2005). However, the Ins effect on male reproduction is scarcely known.

Recently, we have discovered that human ejaculated spermatozoa express Ins and they have the autonomous capability to manage their energy status according to the metabolic needs, independently of the systemic regulation (Aquila *et al.*, 2005).

In this study, we have demonstrated the presence of Ins and IRβ in pig spermatozoa at different levels: mRNA expression, protein expression and immunolocalization,

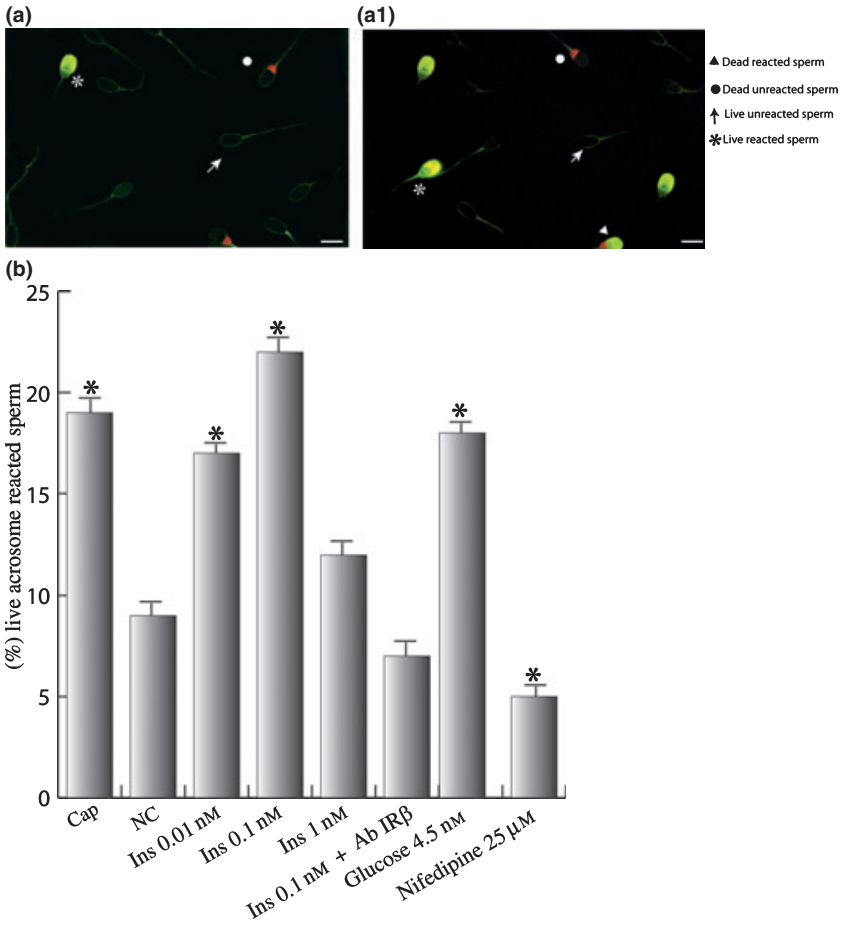

Figure 6 Effect of insulin on acrosome reaction of pig spermatozoa. Representative fluorescence pattern of pig spermatozoa incubated in absence of insulin (a) and in presence of 0.1 nM of insulin (a1) *Scale bars*: 5 μM. (b) Uncapacitated spermatozoa were incubated in the absence (NC) or in the presence of insulin (0.01, 0.1 and 1 nM) as well as in presence of anti IRβ antibody + 0.1 nM insulin. Capacitated spermatozoa as control (Cap). Furthermore, 4.5 nM glucose increased the acrosome reaction from uncapacitated spermatozoa while 25 μM nifedipine was able to decrease it. Values are mean percentage ± SD. *$p < 0.05$ vs. control (NC).

thereby evidencing for the first time IRβ in the male gamete. Using WB analysis, we detected the two proteins at molecular level at the same molecular weight as they were reported in somatic cells. Particularly, Ins was evidenced at 36 kDa, similarly as in β-pancreatic cells (Coore *et al.*, 1969) and in human spermatozoa (Aquila *et al.*, 2005), very probably corresponding to the Ins hexameric form of storage granules. In addition, we also detected Ins and IRβ mRNAs in pig spermatozoa. The presence of different mRNAs has been previously reported in mammalian ejaculated spermatozoa; however, the significance of mRNA in these cells is currently under investigation (Miller, 2000; Ostermeier *et al.*, 2002; Andò & Aquila, 2005; Aquila *et al.*, 2005, 2008). Current dogma holds that spermatozoa deliver to the oocyte the paternal haploid genome, activation signals and the sperm centrosome. However, new data suggest that spermatozoa also provide a unique set of paternal mRNAs. With respect to the role(s) of sperm RNA, it has been suggested that it is not residual to spermatogenesis. Possible functions include de-novo translational replacement of degraded proteins, repackaging of chromatin, post-fertilization (delivering some essential RNAs to the oocyte) (Miller & Ostermeier, 2006).

The immunofluorescence assay localized Ins in the acrosome and in the midpiece of all the spermatozoa observed, while IRβ was compartmentalized only at the midpiece level, with an overlap of the two proteins in the midpiece.

Mammalian spermatozoa are highly differentiated cells that display extreme polarization of cellular architecture and function. The mature sperm cell has three highly specialized regions: the sperm head, involved in sperm oocyte interaction; the midpiece with mitochondria involved in energy management; the flagellum, involved in motility. With regard to this polarization of function, it was hypothesized that spermatozoa possess compartmentalized metabolic and signalling pathways in the regions where they are necessary. Additionally, the specific functionality of spermatozoa from a single mammalian species may be strongly related to its metabolic characteristics. In fact, parameters like the life-span inside the female genital tract (ranging from the long life-span of the human to the short survival time of the boar)

(Zinamam *et al.*, 1989; Gordon, 2004) are strongly linked to the energy-management mechanisms. On the basis of these observations, we can hypothesize that Ins localization may imply its role in the acquisition of fertilizing ability and in metabolism of pig spermatozoa. Besides, the co-localization of the two proteins in the sperm midpiece intriguingly suggests that Ins, through an autocrine short loop, acts on its own receptor to induce sperm energy regulation.

Interestingly, physiological role of Ins and IRβ was studied on both capacitation and acrosome reaction. To fertilize the oocyte, mammalian spermatozoa must undergo a maturation process in the female uterus and oviduct, including the capacitation process and the acrosome reaction. Capacitation, which involves a number of biochemical and functional sperm changes, is mediated by the modification of the ion channels in the sperm plasmalemma, which triggers a cholesterol efflux (Travis & Kopf, 2002). Our results evidenced that Ins has the ability to modulate, through its receptor, this important process. Particularly, a biphasic effect of different Ins concentration was observed, as low Ins induced the cholesterol efflux, while high Ins seemed to be ineffective. A similar effect was detected in human and pig spermatozoa after leptin treatment and it has been explained by a receptor down-regulation under high doses of the hormone (Aquila *et al.*, 2008). Capacitation prepares the spermatozoon to a process of specialized exocytosis known as acrosome reaction (Bedford & Cross, 1999), which enables it to penetrate the zona pellucida of the ovum. Our results demonstrated that Ins has the ability to modulate the acrosome reaction in a biphasic fashion, such as observed for capacitation. Interestingly, when uncapacitated spermatozoa are stimulated by Ins (0.1 nM), they reach capacitation and acrosome reaction extents similar to the spontaneous capacitated spermatozoa. The enhancement of acrosome reaction, in response to Ins, has been recently evidenced also in human spermatozoa (Lampiao & Du Plessis, 2008).

The potential for an autocrine Ins loop in pig spermatozoa is supported by the co-expression of Ins and IRβ in the midpiece, as well as by the modular effect of the hormone through its own receptor on both capacitation and acrosome reaction. With the aim to better address this issue, we evidenced that pig spermatozoa are able to secrete Ins as it was demonstrated in human spermatozoa (Aquila *et al.*, 2005). In addition, our results revealed that Ins secretion from pig spermatozoa is also responsive to glucose, the main secretagogue of Ins in pancreatic β-cells (Aspinwall *et al.*, 1999), showing a similar behaviour between pig and human spermatozoa. Further findings support our hypothesis; in fact, glucose has stimulated spontaneous capacitation and acrosome reaction of pig spermatozoa, whereas the blockage of insulin release, by nifedipine, has inhibited both the functional properties, as previously demonstrated in human spermatozoa (Aquila *et al.*, 2005).

Conclusions

The present investigation has identified, for the first time, insulin and insulin receptor in ejaculated pig spermatozoa. In addition, the ability of Ins to modulate the acquisition of fertilizing ability in the male gamete has been evidenced, perhaps beyond the classical role of the hormone in the energy homeostasis regulation. Therefore, this study evidences an important role of Ins and IR in pig sperm biology, even if their physiological action on the male gamete is just at the beginning of the knowledge.

Acknowledgements

The authors thank Dr Rocco Panza, chief of the Swine Artificial Insemination Centre, APA, Cosenza (Italy), who provided animals for sample collections. The authors also express their special thanks to Dr. Vincenzo Cunsulo (Biogemina Italia Srl, Catania – Italy) and to prof. Antonietta Martire for the English reviewing of this manuscript.

Footnotes: This work was supported by MURST and Ex 60% -2007.

References

Adashi, E. Y., Resnick, C. E., D'Ercole, J., Svoboda, M. E. & Van Wyk, J. J. (1985) Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocrine Review* 6, 400–420.

Agbaje, I. M., Rogers, D. A., Mc Vicar, C. M., McClure, N., Atkinson, A. B., Mallidis, C. & Lewis, S. E. M. (2007) Insulin dependant diabetes mellitus: implications for male reproductive function. *Human Reproduction* 22, 1871–1877.

Agbaje, I. M., Mc Vicar, C. M., Schock, B. C., McClure, N., Atkinson, A. B., Rogers, D. & Lewis, S. E. (2008) Increased concentration of the oxidative DNA adduct 7,8-dihydro-8-oxo-2-deoxyguanosine in the germ-line of men with type 1 diabetes. *Reproductive Biomedicine Online* 16, 401–409.

Alpert, S., Hanahan, D. & Teitelman, G. (1988) Hybrid insulin genes reveal a dev life-span inside the female genital tract elopmental lineage for pancreatic endocrine cells and imply a relationship with neurons. *Cell* 53, 295–308.

Andò, S. & Aquila, S. (2005) Arguments raised by the recent discovery that insulin and leptin are expressed in and secreted by human ejaculated spermatozoa. *Molecular and Cellular Endocrinology* 245, 1–6.

Aquila, S., Sisci, D., Gentile, M. E., Middea, E., Siciliano, L. & Andò, S. (2002) Human ejaculated spermatozoa contain active P450 aromatase. *Journal of Clinical Endocrinology and Metabolism* 87, 3385–3390.

Aquila, S., Gentile, M. E., Middea, E., Catalano, S. & Andò, S. (2005) Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* 146, 552–557.

Aquila, S., Rago, V., Guido, C., Zupo, S., Casaburi, I. & Carpino, A. (2008) Leptin and leptin receptor in pig spermatozoa: evidence of their involvement in sperm capacitation and survival. *Reproduction* 136, 23–32.

Aspinwall, C. A., Lakey, J. R. & Kennedy, R. T. (1999) Insulin-stimulated insulin secretion in single pancreatic β cells. *Journal of Biological Chemistry* 274, 6360–6365.

Baccetti, B., La Marca, A., Piomboni, P., Capitani, S., Bruni, E., Petraglia, F. & De Leo, V. (2002) Insulin-dependent diabetes in men is associated with hypothalamo-pituitary derangement and with impairment in semen quality. *Human Reproduction* 17, 2673–2677.

Bedford, M. J. & Cross, N. L. (1999) Sperm Capacitation. In: Encyclopedia of Reproduction, Vol 4 (eds E. Knobil & J. D. Neill), pp. 597–602. Academic, San Diego.

Britt, J. H., Armstrong, J. D. & Cox, N. M. (1988) Metabolic Interfaces Between Nutrition and Reproduction. In: Proceeding of 11th International Congress of Animal Reproduction, A.I., Dublin. pp. 117–125.

Coore, H. G., Hellman, B., Pihl, E. & Taljedal, I. B. (1969) Physicochemical characteristics of insulin secretion granules. *Biochemical Journal* 111, 107–113.

Cox, N. M., Stuart, M. J., Althen, T. G., Bennett, W. A. & Miller, H. W. (1987) Enhancement of ovulation rate in gilts by increasing dietary energy and administering insulin during follicular growth. *Journal of Animal Science* 64, 507–513.

Funahashi, H. (2002) Induction of capacitation and the acrosome reaction of boar spermatozoa by L-arginine and nitric oxide synthesis associated with the anion transport system. *Reproduction* 124, 857–864.

Gordon, I. (2004) Artifical Insemination. In: Reproductive technologies in farm animals, chapter 2. pp. 49–75. CABI Publishing, Oxford, UK.

Kahn, C. R. & White, M. F. (1988) The insulin receptor and the molecular mechanism of insulin action. *Journal of Clinical Investigation* 82, 1151–1156.

Kuster, C. E., Hess, R. A. & Althouse, G. C. (2004) Immunofluorescence reveals ubiquitination of retained distal cytoplasmic droplet on ejaculated porcine spermatozoa. *Journal of Andrology* 25, 340–347.

Lampiao, F. & Du Plessis, S. S. (2008) Insulin and leptin enhance human sperm motility, acrosome reaction and nitric oxide production. *Asian Journal of Andrology* 10, 799–807.

Lee, M. S., Kang, S. K., Lee, B. C. & Hwang, W. S. (2005) The beneficial effects of insulin and metformin on in vitro developmental potential of porcine oocytes and embryos. *Biology of Reproduction* 73, 1264–1268.

Matamoros, I. A., Cox, N. M. & Moore, A. B. (1990) Exogenous insulin and additional energy affect follicular distribution, follicular steroid concentration, and granulosa cell human chorionic gonadotropin binding in swine. *Biology of Reproduction* 43, 1–7.

Matamoros, I. A., Cox, N. M. & Moore, A. B. (1991) Effects of exogenous insulin and body condition on metabolic hormones and gonadotropin-induced follicular development in prepubertal gilts. *Journal of Animal Science* 69, 2081–2091.

McNeel, R. L., Ding, S. T., Smith, E. O. & Mersmann, H. J. (2000) Effect of feed restriction on adipose tissue transcript concentrations in genetically lean and obese pigs. *Journal of Animal Science* 78, 934–942.

Miller, D. (2000) Analysis and significance of messenger RNA in human ejaculated spermatozoa. *Molecular and Reproductive Development* 56, 259–264.

Miller, D. & Ostermeier, G. C. (2006) Towards a better understanding of RNA carriage by ejaculate spermatozoa. *Human Reproduction Update* 12, 757–767.

Nakayama, Y., Yamamoto, T. & Abe, S. I. (1999) IGF-I, IGF-II and insulin promote differentiation of spermatogonia to primary spermatocytes in organ culture of newt testes. *International Journal of Developmental Biology* 43, 343–347.

Ostermeier, G. C., Dix, D. J., Miller, D., Khatri, P. & Krawetz, S. A. (2002) Spermatozoal RNA profiles of normal fertile men. *Lancet* 360, 772–777.

Philippe, J. (1991) Structure and pancreatic expression of the insulin and glucagon genes. *Endocrine Review* 12, 1–20.

Ramalho-Santos, J., Amaral, S. & Oliveira, P. J. (2008) Diabetes and the impairment of reproductive function: possible role of mitochondria and reactive oxygen species. *Current Diabetes Reviews* 4, 46–54.

Seino, S., Seino, M., Nishi, S. & Bell, G. I. (1989) Structure of the human insulin receptor gene and characterization of its promoter. *Proceedings of the National Academy of Sciences of the United States of America* 86, 114–118.

Shrivastav, P., Swann, J., Jeremy, J. Y., Thompson, C., Shaw, R. W. & Dandona, P. (1989) Sperm function and structure and seminal plasma prostanoid concentrations in men with IDDM. *Diabetes Care* 12, 742–744.

Throsby, M., Homo-Delarche, F., Chevenne, D., Goya, R., Dardenne, M. & Pleau, J. M. (1998) Pancreatic hormone expression in the murine thymus: localization in dendritic cells and macrophages. *Endocrinology* 139, 2399–2406.

Travis, A. J. & Kopf, G. S. (2002) The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *Journal of Clinical Investigation*, 110, 731–736.

White, M. F. & Kahn, C. R. (1994) The insulin signaling system. *Journal of Biological Chemistry* 269, 1–4.

Zinamam, M., Dobronis, E. Z., Morales, P., Brazil, C., Kiel, M., Cross, N. L., Hanson, F. W. & Overstreet, J. W. (1989) The physiology of sperm recovered from the human cervix: acrosomal status and response to inducers of the acrosome reaction. *Biology of Reproduction* 41, 790–797.

Journal of Cellular Physiology

A New Role of Anandamide in Human Sperm: Focus on Metabolism

SAVERIA AQUILA,[1,2] CARMELA GUIDO,[1,2] CHIARA LAEZZA,[3] ANTONIETTA SANTORO,[4] VINCENZO PEZZI,[1,5] SALVATORE PANZA,[1,2] SEBASTIANO ANDÒ,[1,2,5] AND MAURIZIO BIFULCO[4]*

[1]*Department of Pharmaco-Biology, University of Calabria, Arcavacata di Rende, Cosenza, Italy*

[2]*Centro Sanitario, University of Calabria, Arcavacata di Rende, Cosenza, Italy*

[3]*Institute of Endocrinology and Experimental Oncology, CNR Napoli, Italy*

[4]*Department of Pharmaceutical Sciences, University of Salerno, Fisciano, Salerno, Italy*

[5]*Faculty of Pharmacy, University of Calabria, Arcavacata di Rende, Cosenza, Italy*

The endocannabinoid system and the presence of CB1 receptor (CB1-R) target of the anandamide were identified in human sperm, however the anandamide action in this context needs to be further elucidated. At this purpose we analyzed the effects of anandamide on human sperm capacitation and motility. Afterwards, we focused on lipid and glucose sperm metabolism and also investigated the interrelationship between anandamide and insulin secretion by sperm. By intracellular free Ca^{2+} content assay and proteins tyrosine phosphorylation, we evidenced that anandamide did not induce capacitation process and a negative effect was obtained on sperm motility. The blockage of CB1-R by the specific antagonist SR141716 increased both capacitation and sperm motility suggesting an involvement of the CB1-R in the acquisition of sperm fertilizing activity. The evaluation of the triglycerides content, lipase and acyl-CoA dehydrogenase activities, suggest that anandamide exerts a lipogenetic effect on human sperm lipid metabolism. Concerning the glucose metabolism, anandamide increases GSK3 phosphorylation indicating that it is involved in the accumulation of energy substrates. G6PDH activity was not affected by anandamide. Interestingly, AEA is involved in insulin secretion by sperm. As insulin had been demonstrated to be an autocrine factor that triggers capacitation, the endocannabinoid might be inserted in the signaling cascade that induces this process. Altogether these findings highlight a pivotal involvement of the CB1-R in the control of sperm energy homeostasis and propose a new site of action for endocannabinoids in the control of energy metabolism.

J. Cell. Physiol. 221: 147–153, 2009.

Cannabinoids are the main constituents of the marijuana plant (*Cannabis sativa*), and it is well known that delta-9-tetrahydrocannabinol, the primary psychoactive substance in marijuana, has marked adverse effects on male and female reproductive systems (Habayeb et al., 2002; Wang et al., 2006). Beside cannabinoids, a family of unsaturated fatty acid derivatives, have been identified and are known as endocannabinoids (ECs); the main ones are anandamide (*N*-arachidonoylethanolamine, AEA), 2-arachidonoylglycerol (2-AG), and 2-arachidonoylglycerylether (noladin ether) (Hanus et al., 1993; Sugiura et al., 1995; Di Marzo et al., 1998, 2004). Cannabinoids and ECs exert their effects through the activation of specific cannabinoid receptors (CB-Rs), the brain-type CB1-R and the spleen-type CB2-R (Devane et al., 1988; Matsuda et al., 1990; Galiegue et al., 1995). CB-Rs are linked to inhibitory and stimulatory guanine nucleotide binding proteins (G_i and G_s proteins) and are widely distributed in many other tissues including placenta, uterus and testis (Schuel et al., 2002; Wang et al., 2006). They regulate several signal-transduction pathways in cells, by modulating ionic currents, activating focal adhesion kinase and mitogen-activated protein kinase (Mackie and Hille, 1992; Bouaboula et al., 1996; Glass and Felder, 1997).

Studies demonstrating the expression of functional CB-Rs in sea urchin sperm, provided the first evidence that cannabinoids could directly affect fertilization (Schuel et al., 1987; Wang et al., 2006). In sea urchin, it has been shown that sperm synthesizes AEA (Bisogno et al., 1997), AEA binds to CB-Rs and reduces fertilizing capacity of sperm (Schuel et al., 1994; Schuel and Burkman, 2005). Human seminal plasma contains nanomolar concentrations of AEA and human sperm expresses CB1-R (Wang et al., 2006). It has been shown that AEA reduces human sperm motility by reducing mitochondrial activity (Rossato et al., 2005). In addition, AEA inhibits acrosome reaction and its effects are prevented by the CB1 antagonist SR141716 (rimonabant, SR) (Rossato et al., 2005; Bifulco et al. 2007). Altogether, these findings suggest that the control of endogenous tone of ECs and its interaction with the CB-Rs are checkpoints in reproduction (Wang et al., 2006). To date the effects of ECs and the role of EC system in male fertility are still largely unexplored.

Abbreviations: 2-AG, 2-arachidonoyl glycerol; AEA, anandamide, *N*-arachidonoylethanolamine; CB-R, cannabinoid receptor; EC, endocannabinoid, Met-F-AEA, 2-methylarachidonyl-2′-fluoro-ethylamide; SR, SR141716, *N*-(piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4dichlorophenyl)-4-methyl-pyrazole-3-carboxamide; PPP, pentose phasphate pathway.

Contract grant sponsor: MURST (Ex 60%, 2008).
Contract grant sponsor: Associazione Educazione e Ricerca Medica Salernitana (ERMES).

*Correspondence to: Maurizio Bifulco, Via Ponte don Melillo, 84084 Fisciano, Salerno, Italy. E-mail: maubiful@unisa.it

Received 2 April 2009; Accepted 21 April 2009

Published online in Wiley InterScience (www.interscience.wiley.com.), 2 June 2009.
DOI: 10.1002/jcp.21837

In the present study we evaluated the effects of a stable analogue of AEA, Met-F-anandamide (2-methylarachidonyl-2′-fluoro-ethylamide, Met-F-AEA) on different aspects of human sperm biology, such as capacitation and motility. Sperm capacitation, a complex and not well-elucidated series of physiological changes, induces an increase of metabolism and energy expenditure, however the mechanisms through which these events occur are poorly understood (Andò and Aquila, 2005). Interestingly, we focused on lipid and glucose sperm metabolism since it was never investigated, in order to shed light on the possible pathophysiological role of the EC system in male fertility and to correlate the energy metabolism profile of human sperm with EC-induced events. With this respect we also investigated the interrelationship between AEA and insulin secretion by sperm.

Materials and Methods

Chemicals

BSA protein standard, Laemmli sample buffer, prestained molecular weight markers, Percoll (colloidal PVP coated silica for cell separation), Sodium bicarbonate, Sodium lactate, Sodium pyruvate, Earle's balanced salt solution (uncapacitating medium), stable analogue of anandamide, 2-methylarachidonyl-2′-fluoro-ethylamide (Met-F-AEA) and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, Eosin Y was from Farmitalia Carlo Erba (Milan, Italy). ECL Plus Western blotting detection system, Hybond^{TM} ECL^{TM}, Hepes Sodium Salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Triglycerides assay kit, lipase activity kit, calcium (Ca^{2+}) assay kit, Glucose-6-phosphate dehydrogenase (G6PDH) activity assay and insulin RIA kit were from Inter-Medical (Biogemina Italia Srl, Catania, Italy). Goat polyclonal actin antibody (Ab), monoclonal mouse anti-p-Tyr Ab, rabbit anti-insulin Ab, peroxidase-coupled anti-mouse, anti-rabbit and anti-goat IgG secondary Abs were from Santa Cruz Biotechnology (Heidelberg, Germany). CB1-R antagonist SR141716 (rimonabant, SR) was kindly provided by Sanofi-Aventis (Montpellier, France).

Semen samples and spermatozoa preparations

Human semen was collected, according to the World Health Organization (WHO) recommended procedure by masturbation from semen samples from healthy volunteer donors of proven fertility undergoing semen analysis in our laboratory. Spermatozoa preparations were performed as previously described (Aquila et al., 2006). Briefly, sperm samples 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. For each experiment three normal samples were pooled. Washed pooled sperm were subjected to the indicated treatments and incubated for 30 min (min) at 37°C and 5% CO_2. Then, samples were centrifuged and the upper phase was used to determinate the insulin levels, while the pellet containing sperm was lysed to perform western blots, triglycerides assay, Ca^{2+} assay, acyl-CoA dehydrogenase assay, G6PDH activity, lipase activity. Prior the centrifugation several aliquots were used to perform sperm motility. The study was approved by the local medical-ethical committees and all participants gave their informed consent. MET-F-AEA was dissolved in ethanol (EtOH), while SR in dimethylsulfoxide (DMSO). EtOH (0.02% final concentration in culture) and DMSO (0.1% final concentration in culture) used as solvent controls did not induce any positive result in all in vitro assays.

Processing of ejaculated sperm

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. Percoll-purified sperm was washed with unsupplemented Earle's medium (uncapacitating medium, Earle's balanced salt solution medium without supplementation with BSA sodium bicarbonate or calcium) and were incubated for 30 min at 37°C and 5% CO_2, without (control, NC) or with increasing MET-F-AEA (10 nM, 100 nM, and 1 μM). When the cells were treated with the CB1-R antagonist SR (1 μM), a pre-treatment of 15 min was performed, and then 100 nM MET-F-AEA were added.

Evaluation of Ca^{2+} in sperm

Intracellular Ca^{2+} concentration has been estimated spectrophotometrically with the indicator Arsenazo III using disrupted spermatozoa (Thomson and Wishart, 1989), according to the manufacturer instructions. At a neutral pH, the Ca^{2+} forms with arsenazo III a complex, the color intensity of which is directly proportional to the concentration of Ca^{2+} in the sample. Ca^{2+} content was measured at 600 nm. The Ca^{2+} standard used was 2.5 mM (100 mg/L). Inter- and intra-assay variation were 0.24% and 0.37%. Ca^{2+} results are presented as μM per 10×10^6 number of spermatozoa.

Western blot analysis of sperm proteins

Sperm samples, washed twice with Earle's balanced salt solution (uncapacitating medium), were incubated in the presence and absence of the test substances and then centrifuged for 5 min at 5,000*g*. The pellet was resuspended in lysis buffer as previously described (Aquila et al., 2002). An equal amount of protein (80 μg) were boiled for 5 min, separated on a 10% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and probed with an appropriate dilution of the indicated primary antibody. The bound of the secondary antibody was revealed with the ECL Plus Western blotting detection system according to the manufacturer's instructions. As internal control, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) and reprobed with anti-β actin antibody. The protein bands were quantified by scanning densitometry (Imaging Densitometer GS-700 Bio-Rad, Milan, Italy).

Evaluation of sperm motility

Sperm motility was assessed by means of light microscopy examining aliquots of each sperm sample in absence (NC) or in the presence of increasing concentrations of MET-F-AEA (10 nM, 100 nM, and 1 μM). Some samples were treated with 1 μM SR alone or combined with 100 nM MET-F-AEA. A blinded observer scored at least 200 cells. Sperm motility was expressed as percentage of total motile sperm.

Triglycerides assay

Triglycerides were measured in duplicate by a GPO-POD enzymatic colorimetric method according to manufacturer's instructions in sperm lysates and as previously described (Aquila et al., 2006). Sperm samples, washed twice by centrifugation with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37°C and 5% CO_2. Other samples were incubated in the presence of the indicated treatments. At the end of the sperm incubation 10 μl of lysate were added to the 1 ml of buffer reaction and incubated for 10 min at room temperature. Then the triglycerides content was measured at 505 nm by using a spectrophotometer. Data are presented as μg/10^6 sperms.

Assay of acyl-CoA dehydrogenase activity

Assay of acyl-CoA dehydrogenase was performed on sperm, using a modification of the method described by Lehman et al. (1990). In brief, after protein lysis, 70 μg of sperm proteins were added to the buffer containing 20 mM Mops, 0.5 mM EDTA, and 100 μM FAD^+ at pH 7.2. Reduction of FAD^+ to FADH was read at 340 nm upon addition of octanoyl-CoA (100 μM) every 20 sec for 1.5 min. Data are expressed as nmol/min/mg protein. The enzymatic activity was determined with three control media: one without octanoyl-CoA as substrate, one without the coenzyme (FAD^+), and the third without either substrate or coenzyme (data not shown).

Lipase activity assay

Lipase activity was evaluated, by the method of Panteghini (Panteghini et al., 2001) based on the use of 1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester (DGGR) as substrate. Fifty micrograms of sperm extracts were loaded into individual cuvettes containing buffer for spectrophotometric determination. DGGR is cleaved by lipase, resulting in an unstable dicarbonic acid ester which is spontaneously hydrolyzed to yield glutaric acid and methylresorufin, a bluish-purple chromophore with peak absorption at 580 nm. The absorbance of samples was read every 20 sec for 1.5 min. The rate of methylresorufin formation is directly proportional to the lipase activity in the sample. Analysis of total imprecision gave a coefficient of variation of between 0.01% and 0.03%. The estimated reference interval was 6-38 U/L (μmol/min/mg protein). The enzymatic activity was determined with three control media: one without the substrate, one without the co-enzyme (colipase) and the third without either substrate or co-enzyme (data not shown).

G6PDH activity

The conversion of $NADP^+$ to NADPH, catalyzed by G6PDH, was measured by the increase of absorbance at 340 nm. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37°C and 5% CO_2. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) or in capacitating medium for 30 min at 37°C and 5% CO_2. Other samples were incubated in the presence of the indicated treatments. After incubation, 50 μl of sperm extracts were loaded into individual cuvettes containing buffer (100 mM triethanolamine, 100 mM $MgCl_2$, 10 mg/ml glucose-6-phosphate, 10 mg/ml $NADP^+$, pH 7.6) for spectrophotometric determination. The absorbance of samples was read at 340 nm every 20 sec for 1.5 min. Data are expressed in nmol/min/10^6 sperms. The enzymatic activity was determined with three control media: one without glucose-6-phosphate as substrate, one without the coenzyme ($NADP^+$), and the third without either substrate or coenzyme (data not shown).

Measurement of insulin secreted by human spermatozoa

A competitive RIA was applied to measure insulin in the sperm culture medium. Spermatozoa were washed twice with unsupplemented Earle's medium and incubated in the same medium for 30 min at 37°C in 5% CO_2. A final concentration of 10×10^6 sperm/500 ml was used. Sperm were treated with 10 nM, 100 nM, and 1 μM MET-F-AEA. Some samples treated with 1 μM SR alone or combined with 100 nM MET-F-AEA.

At the end of the sperm incubations, the culture media were recovered by centrifugation. Human insulin concentrations were determined in duplicate using an insulin RIA kit according to manufacturer's instructions. Insulin standards ranged from 0 to 300 μIU/ml. The limit of sensitivity for the assay was 0.01 μIU/ml. Inter- and intraassay variations were 4.4% and 5.1%, respectively.

Insulin results are presented as the original concentrations of the supernatants and are expressed as micro international units per milliliter.

Statistical analysis

The experiments for Western blot analysis were performed in at least four independent experiments. The data obtained from Ca^{2+} assay, Triglycerides Assay, G6PDH activity, acyl-CoA dehydrogenase activity, lipase activity, insulin assay, acrosin activity and motility (six replicate experiments using duplicate determinations), were presented as the mean ± SEM. The differences in mean values were calculated using analysis of variance (ANOVA) with a significance level of $P \leq 0.05$.

Results

MET-F-AEA effects on intracellular free Ca^{2+} content and on tyrosine phosphorylation of the proteins in human sperm

A first functional assessment of the sperm biological activities under increasing MET-F-AEA concentrations was performed on the capacitation process. To evaluate whether AEA was able to influence the sperm extratesticular maturation, we studied its potential action on intracellular free Ca^{2+} content and proteins tyrosine phosphorylation. Recently, it was demonstrated that internal sperm Ca^{2+} stores provide sufficient Ca^{2+} for the induction of hyperactivated motility (Kong et al., 2007), which is indicative of the capacitation status. It appears that 100 nM MET-F-AEA were able to produce an increase in the intracellular free Ca^{2+} while 10 nM and 1 μM did not (Fig. 1A). Surprisingly, the combined treatment of MET-F-AEA with 1 μM of the CB1-R antagonist SR, significantly increased the Ca^{2+} content.

Human sperm capacitation is correlated with an augment in intracellular cAMP levels that in turn induces an increase in tyrosine phosphorylation of a variety of substrates, while the AEA generally inhibits adenylate cyclase activity (Bifulco and Di Marzo, 2002). In order to assess this issue, the status of the proteins phosphorylation was investigated in human sperm after the treatment with increasing MET-F-AEA. A significant decrease in the sperm proteins phosphorylation (Fig. 1B) was observed by using different MET-F-AEA doses, while the treatment with 100 nM MET-F-AEA plus 1 μM SR induced a significant increase of tyrosine phosphorylated proteins.

MET-F-AEA has the ability to modulate human sperm motility

Motility is a critical sperm biological function that at the time of fertilization allows or at least facilitates passage of the sperm through the zona pellucida. A non-motile or abnormally motile sperm is not going to fertilize. Hence, assessing the fraction of a sperm population that is motile is perhaps the most widely used measure of semen quality. In our experiments, sperm motility decreased from 10 to 100 nM MET-F-AEA in a dose-dependent manner (Fig. 2), while it was enhanced by using 1 μM SR plus 100 nM MET-F-AEA.

MET-F-AEA effects on the triglycerides content in human sperm

During sperm extra-testicular maturation an overall increase in sperm metabolism occurs, however, the mechanisms that govern this event are still poorly understood. MET-F-AEA in somatic cells is an important lipid metabolism regulator (Wang et al., 2006) and in sperm its role in this issue was never evaluated. We first investigated triglycerides intracellular content. As shown in Figure 3, MET-F-AEA was able to significantly increase the triglycerides content and the

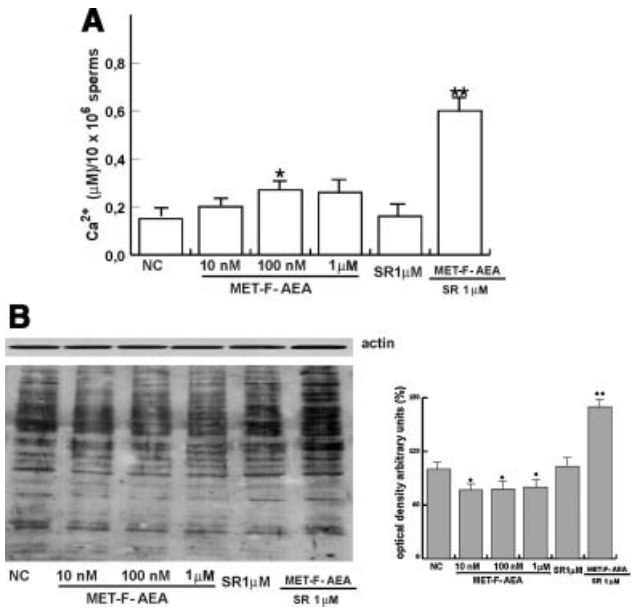

Fig. 1. MET-F-AEA influences free intracellular Ca^{2+} and protein tyrosine phosphorylations in sperm. Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing MET-F-AEA concentrations (10 nM, 100 nM, and 1 μM). Some samples were treated with 1 μM SR alone or combined with 100 nM MET-F-AEA. A: Free intracellular calcium was measured as reported in Materials and Methods Section. Columns represent mean ± SEM $^{*}P < 0.05$ versus control, $^{**}P < 0.02$ versus control. B: Eighty micrograms of sperm lysates were used for Western blot analysis performed to determine protein tyrosine phosphorylations. Actin was used as a loading control. On the right, quantitative representation after densitometry evaluation of the 95-kDa band. Autoradiograph presented is a representative example of experiments that were performed at least four times with repetitive results. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control.

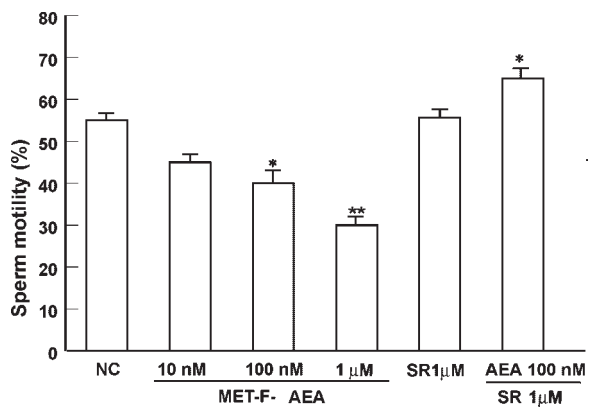

Fig. 2. MET-F-AEA effects on sperm motility. Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing MET-F-AEA concentrations (10 nM, 100 nM, and 1 μM). Some samples were treated with 1 μM SR alone or combined with 100 nM MET-F-AEA. Sperm motility was assessed as reported in Materials and Methods Section. Columns represent mean ± SEM. Data are expressed as % of sperm motility $^{*}P < 0.05$ versus control; $^{**}P < 0.02$ versus control.

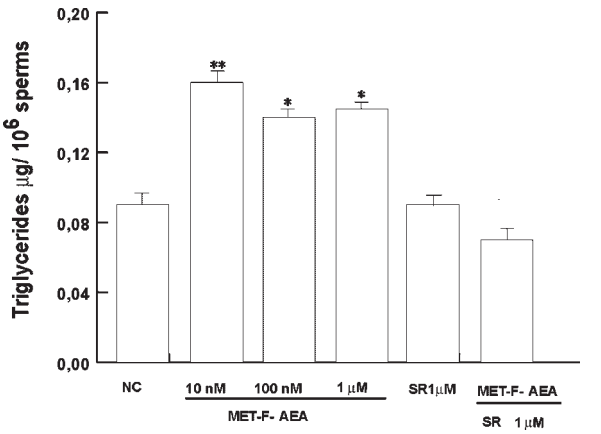

Fig. 3. MET-F-AEA effects on the triglycerides content in human sperm. Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing MET-F-AEA concentrations (10 nM, 100 nM, and 1 μM). Some samples were treated with 1 μM SR alone or combined with 100 nM MET-F-AEA. Triglycerides content was measured as reported in Materials and Methods Section. Columns represent mean ± SEM. $^{*}P < 0.05$ versus control, $^{**}P < 0.02$ versus control.

combination with SR was able to attenuate the effect of MET-F-AEA.

MET-F-AEA effects on acyl-CoA dehydrogenase and lipase activities

To further investigate the role of AEA in sperm lipid metabolism, we evaluated both acyl-CoA dehydrogenase and lipase activities. It appears that MET-F-AEA treatment alone did not produce effects on lipase activity (Fig. 4A) as well as on β-oxidation of the fatty acids (Fig. 4B). Interestingly, the combined treatment of 100 nM MET-F-AEA plus 1 μM SR increased both fatty acids-β oxidation and lipase activity.

MET-F-AEA effects on sperm glucose metabolism

It was recently demonstrated that human sperm express the GSK-3 and that the enzymatic activity is higher in uncapacitated than in capacitated sperm (Aquila et al., 2005a). In the present study, MET-F-AEA induced a dose-dependent increase in the GSK3 phosphorylation (Fig. 5A).

To gain insight into the MET-F-AEA regulation of sperm glucose metabolism we evaluated the G6PDH activity upon increasing MET-F-AEA concentrations. G6PDH, the rate-limiting enzyme in the pentose phosphate pathway (PPP), has been shown to be crucial in the acquisition of fertilizing capability as well as to mediate gamete fusion (Aquila et al., 2005b). From our results it emerges that MET-F-AEA was unable to significantly induce G6PDH activity (Fig. 5B).

MET-F-AEA effects on insulin secretion by sperm

In mouse pancreatic β-cells, cannabinoids inhibit insulin secretion via CB1-R (Nakata and Yada, 2008). In our recent study, we had shown that insulin is expressed in and secreted from human ejaculated spermatozoa, leading us to suppose an autocrine regulation of glucose metabolism according to the sperm energetic needs independently of the systemic insulin (Aquila et al., 2005b). In our cellular type, MET-F-AEA induced a very weak dose-dependent increase in insulin secretion reaching a maximal level of 0.6 μIU/ml of the hormone

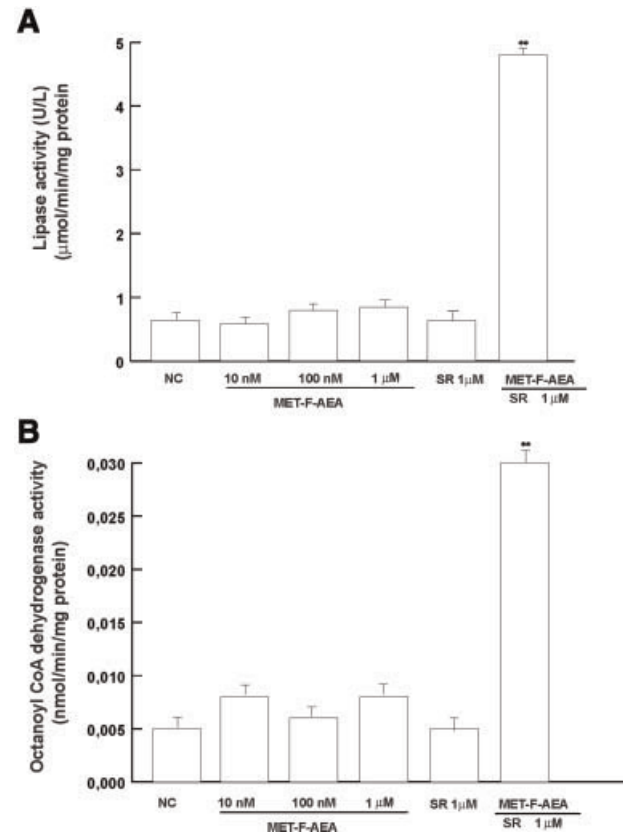

Fig. 4. MET-F-AEA effects on acyl-CoA dehydrogenase and lipase activities. Washed spermatozoa were incubated in the unsupplemented Earle's medium for 30 min at 37°C and 5% CO_2, in the absence (NC) or in the presence of increasing MET-F-AEA concentrations (10 nM, 100 nM, and 1 μM). Some samples were treated with 1 μM SR alone or combined with 100 nM MET-F-AEA. A: Lipase activity and (B) octanoyl-CoA dehydrogenase activity were performed as reported in Materials and Methods Section. Columns represent mean ± SEM. $^{**}P < 0.01$ versus control.

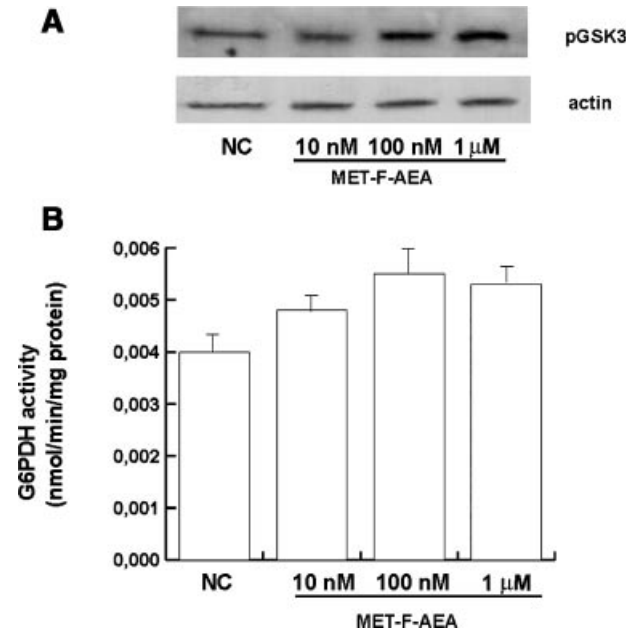

Fig. 5. MET-F-AEA effects on sperm glucose metabolism. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control) for 30 min at 37°C and 5% CO_2. A: Western blotting evaluating GSK3 phosphorylation. Actin was used as a loading control. Autoradiograph presented is a representative example of experiments that were performed at least four times with repetitive results. B: G6PDH activity was performed as reported in Materials and Methods Section. Columns represent mean ± SEM. $^{***}P < 0.01$ versus control.

concentration (Fig. 6A). These results indicate that in sperm, similarly to the endocrine pancreatic cells, CB1-R is involved in modulating insulin secretion. We also performed insulin western blot on sperm lysates to evidence that the increase in insulin secretion is related to a decrease in insulin sperm content (Fig. 6B).

Discussion

The EC system first emerged as a major neuromodulatory system in the brain, however, it has also been shown to play an important role in various peripheral organs, including testis. Recently, EC system was identified in boar sperm and CB1-R was demonstrated in human sperm (Rossato et al., 2005), suggesting a possible physiological role of the AEA in controlling male fertility. In our study, we evidenced the AEA role in different aspects of sperm biology, such as capacitation and motility. Particularly, we focused our researches on lipid and glucose metabolism and we also evaluated AEA action on insulin secretion by sperm.

The physiological changes that confer to the sperm the ability to fertilize a metaphase II-arrested egg are collectively called "capacitation." This extratesticular maturation is a complex process and a functional change that involves different sperm activities. During capacitation, spermatozoa become responsive to the stimuli that induce the acrosome reaction and prepare the male gamete to the penetration of the egg investments prior fertilization. Many gaps exist in our knowledge on the capacitation at molecular levels. It appears that the process involves modifications of intracellular Ca^{2+}, lipid remodeling in sperm plasma membrane as well as changes in proteins phosphorylation. Therefore, we first aimed to evaluate AEA action in some events that characterize the capacitation process, such as intracellular Ca^{2+} levels and tyrosine phosphorylation of sperm proteins.

Ca^{2+} signaling in sperm is critical for fertilization and recently it was demonstrated that internal Ca^{2+} stores provide sufficient Ca^{2+} for the induction of motility hyperactivation, whereas Ca^{2+} influx is required to maintain intracellular Ca^{2+} levels to sustain hyperactivation (Florman et al., 1998; Suarez and Ho, 2003). From our results it emerges that MET-F-AEA is able to slightly increase free intracellular Ca^{2+} levels and the inactivation of the CB1-R significantly enhanced this effect suggesting that the CB1-R status is implicated in the regulation of intracellular free Ca^{2+} and then in the induction of hyperactivated motility. Our data are not in agreement with previous studies concerning the AEA effects on Ca^{2+} levels in sperm (Rossato et al., 2005), however we may to take into account that different method and medium were used.

It has been demonstrated that proteins tyrosine phosphorylation is tightly associated to the initial stage the capacitation process (Visconti et al., 1995a,b). In somatic cells a major CB1-R-dependent signaling pathway involves the down-regulation of adenylate cyclase activity (Bifulco and Di Marzo, 2002). In our study, MET-F-AEA decreases sperm proteins tyrosine phosphorylation, while this effect was prevented by the CB1-R antagonist SR, leading to the

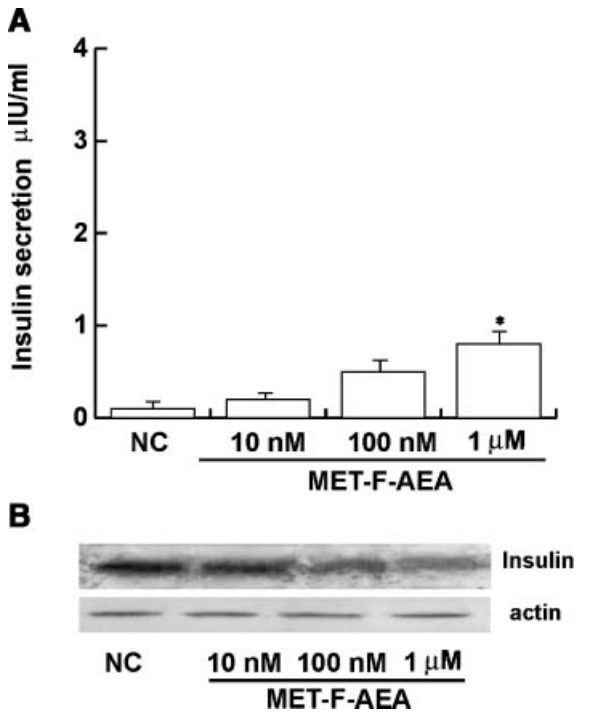

Fig. 6. MET-F-AEA effects on insulin secretion by sperm. Spermatozoa were washed twice with unsupplemented Earle's medium and incubated in the same medium for 30 min at 37 C in 5%CO_2. A final concentration of 10×10^6 sperm/500 ml was used. Sperm were treated with 10 nM, 100 nM, and 1 μM MET-F-AEA. At the end of the sperm incubations, the culture media were recovered by centrifugation. A: Human insulin concentrations were determined in duplicate using an insulin RIA kit according to manufacturer's instructions. Columns represent mean ± SEM, $^*P < 0.05$ versus control, $^{***}P < 0.01$ versus control. B: Western blotting to evaluate insulin inside sperm after the indicated treatment. Actin was used as a loading control. Autoradiograph presented is a representative example of experiments that were performed at least four times with repetitive results.

suggestion that this receptor is involved in the modulation of an important early event of the capacitation process. These results are in keeping with previous data demonstrating that the inhibitory effect of AEA on capacitation depends on its ability to reduce intracellular levels of cAMP (Bifulco and Di Marzo, 2002).

Sperm cell features comprise a high polarization of structure and functions, including motility that is a crucial requisite for the male gamete to reach the oocyte. In our study we observed that AEA induces a dose-dependent reduction of sperm motility and that SR was able to significantly enhance this effect addressing an important role of CB1-R in this sperm function. Our results confirm those of previous studies reporting that AEA negatively affects human sperm motility (Rossato et al., 2005). However, it deserves to be mentioned that different concentrations were used in our finding, in fact we have chosen the nanomalar doses of 10 nM to mimic the AEA levels observed in human seminal plasma (12.3 nM) and in mid-cycle oviductal fluid (10.5 nM) (Schuel et al., 2002), while 100 nM and 1 μM are supraphysiological levels. Particularly, we would like to point out that the spermatozoa used in our experiments are washed from seminal plasma and then they are deprived of the decapacitating factors, reproducing in some manner the sperm physiological conditions as they have in the female genital tract.

The mechanisms controlling the interaction between energy balance and reproduction are the subject of intensive investigations. Capacitated sperm display an increased metabolic rate and overall energy expenditure, presumably to affect the changes in sperm signaling and function during capacitation process. The EC system has been recognized as a new crucial player in energy balance control. In general the net effect at diverse sites in the brain and throughout the body is anabolic, facilitating increased energy intake, decreased energy expenditure and increased accumulation of body fat. An increase of the EC tone has been reported in the hypothalamus of obese animals and the administration of ECs has been shown to increase food intake and to promote weight gain (Després, 2007). In white adipocytes, CB1-R activation stimulates lipogenesis, while in contrast, CB1-R antagonists in vitro and in vivo reduce the expression of enzymes involved in lipogenesis (Pagotto et al., 2005). Similarly, MET-F-AEA seems to exert a lipogenetic effect on human sperm lipid metabolism, since the augment in the triglycerides content is compatible with the behavior of both lipase and acyl-CoA dehydrogenase activities. Our data clearly evidenced that the blockage of CB1-R induces an increase in the energy expenditure, corroborating the idea that the receptor activation in sperm is related to the uncapacitated status.

In the majority of the experiments performed, the 1 μM SR alone displayed a neutral antagonism, while in combination with MET-F-AEA it behaves as inverse agonist. SR has been shown to act as neutral antagonism, competitive antagonist and inverse agonist in host cells transfected with exogenous CB1 receptor, as well as in biological preparations endogenously expressing CB1 (Hurst et al., 2005; Pertwee, 2005). As inverse agonist, SR produces effects in some CB1 containing bioassay systems that are opposite in direction from those produced by agonists for these receptors. It was proposed that inverse agonism at the CB1 receptor may be explained in terms of a three-state model in which the receptor can switch between two receptor conformational states, a ground or inactive R state and an active R^* state, which are in equilibrium with each other (Leff, 1995). An agonist has higher affinity for R^* and agonist binding is thought to shift the equilibrium toward R^*, resulting in G protein activation with an increase in GDP/GTP exchange. An inverse agonist has higher affinity for R and its binding shifts the equilibrium toward R, resulting in a decrease in the activation of the signaling pathway. The binding of a neutral/null antagonist is thought not to alter the equilibrium between R and R^* because the neutral antagonist has equal affinity for both states. It is likely that the efficacy for the production of inverse cannabimimetic effects will be governed by the degree of ongoing endocannabinoid release onto CB1 receptors.

In our previous study, we demonstrated that sperm express and secrete insulin, the classical hormone involved in the body energy homeostasis (Aquila et al., 2005b). Moreover in uncapacitated sperm, insulin increased GSK-3 S9 phosphorylation, while during capacitation the kinase is not phosphorylated. These results suggested that the hormone modulates sperm energetic substrates availability on the basis of sperm energy needs (Ballester et al., 2000). We observed that under MET-F-AEA, GSK3 phosphorylation increases indicating that the endocannabinoid in uncapacitated sperm is involved in the accumulation of energy substrates, which would be spent during capacitation and thus mirroring the insulin behavior in this context. In fact, in uncapacitated sperm the GSK3 is tightly blocked, whereas during capacitation there is an activation of the enzyme. Intriguingly, glycogen deposits and GSK3 enzyme are present in the head and midpiece of spermatozoa where CB1-R is also localized and this support a physiological relevance of our finding. Although glycolysis is important for sperm functions this metabolic pathway does not appear to be responsible for successful gamete fusion (Urner and Sakkas,

1996; Travis et al., 2001). Instead, the beneficial effect of glucose on the acquisition of fertilizing ability as well as on gamete fusion is mediated by glucose metabolism through the PPP (Urner and Sakkas, 1999a; Urner et al., 2001) where G6PDH is the key rate limiting enzyme that regulates the production of NADPH (Urner and Sakkas, 1999b). Accordingly, with the above-mentioned results on GSK3, MET-F-AEA was unable to significantly increase the G6PDH activity.

In our previous study we have showed that sperm express and secrete insulin and we have demonstrated that a great difference in insulin secretion between incapacitated and capacitated sperm exists, therefore relating the hormone concentration to the different gamete physiological conditions. In mouse pancreatic islets it was shown that cannabinoids inhibit insulin secretion via CB1-R (Nakata and Yada, 2008). From the present work it emerges that MET-F-AEA induce an increase in insulin secretion, although the concentrations obtained are comprised in the typical range showed by sperm during uncapacitated status (0.1–0.73 μIU/ml). Insulin secretion is significantly higher in sperm during capacitation (4–12 μIU/ml), Our previous data lead us to speculate that insulin might be considered an endogenous factor involved in the autocrine induction of the capacitation, here we found that MET-F-AEA is able to modulate insulin secretion, therefore the EC could be considered a regulator of the capacitation process. On the other hand, the presence of AEA at higher concentration in seminal plasma prevents premature capacitation activating CB1-R, instead in the female genital tract spermatozoa are exposed to a progressively reduced concentration of AEA (Schuel et al., 2002) and sperm capacitation might occur as a consequence of reduced action of AEA on CB1-R. These observations, together with our findings, raise the possibility that defective AEA-signaling may likewise impair sperm acquisition of fertilizing ability and thus male fertility.

Apart from classical hormones like leptin and insulin, the AEA ability to modulate both lipid and glucose metabolism highlights also a pivotal involvement of this EC in the control of sperm energy homeostasis. Concluding, the present finding discovered a new site of action for ECs in the control of energy metabolism.

Acknowledgments

Our special thanks to Dr. Vincenzo Cunsolo (Biogemina SAS, Catania, Italy) for the technical and scientific assistance. We also thank Serena and Maria Clelia Gervasi for the English language review of the manuscript. This work was supported by MURST - Ex 60% -2008, and Associazione Educazione e Ricerca Medica Salernitana (ERMES).

Literature Cited

Andò S, Aquila S. 2005. Arguments raised by the recent discovery that insulin and leptin are expressed in and secreted by human ejaculated spermatozoa. Mol Cell Endocrinol 245:1–6.
Aquila S, Sisci D, Gentile M, Middea E, Siciliano L, Andò S. 2002. Human ejaculated spermatozoa contain active P450 aromatase. J Clin Endocrinol Metab 87:3385–3390.
Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V, Andò S. 2005a. Leptin Secretion by Human Ejaculated Spermatozoa. J Clin Endocrinol Metab 90:4753–4761.
Aquila S, Gentile M, Middea E, Catalano S, Andò S. 2005b. Autocrine regulation of insulin secretion in human ejaculated spermatozoa. Endocrinology 146:552–557.
Aquila S, Bonofiglio D, Gentile M, Middea E, Gabriele S, Belmonte M, Catalano S, Pellegrino M, Ando S. 2006. Peroxisome proliferator-activated receptor (PPAR)gamma is expressed by human spermatozoa: Its potential role on the sperm physiology. J Cell Physiol 209:77–986.
Ballester J, Fernandez-Novell JM, Rutllant J, Garcia-Rocha M, Jesus Palomo M, Mogas T, Pena A, Rigau T, Guinovart JJ, Rodriguez-Gil JE. 2000. Evidence for a functional glycogen metabolism in mature mammalian spermatozoa. Mol Reprod Dev 56:207–219.
Bifulco M, Di Marzo V. 2002. Targeting the endocannabinoid system in cancer therapy: A call for further research. Nat Med 8:547–550.
Bifulco M, Grimaldi C, Gazzerro P, Pisanti S, Santoro A. 2007. "Rimonabant: Just an antiobesity drug? Current evidence on its pleiotropic effects". Mol Pharmacol 71:1445–1456.
Bisogno T, Ventriglia M, Milone A, Mosca M, Cimino G, Di Marzo V. 1997. Occurrence and metabolism of anandamide and related acyl-ethanolamides in ovaries of the sea urchin Paracentrotus lividus. Biochim Biophys Acta 1345:338–348.
Bouaboula M, Poinot-Chazel C, Marchand J, Canat X, Bourrie B, Rinaldi-Carmona M, Calandra B, Le Fur G, Casellas P. 1996. Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. Eur J Biochem 237:704–711.
Després JP. 2007. The endocannabinoid system: A new target for the regulation of energy balance and metabolism. Crit Pathw Cardiol 6:46–50.
Devane WA, Dysarz FA, Johnson MR, Melvin LS, Howlett AC. 1988. Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 34:605–613.
Di Marzo V, Melck D, Bisogno T, De Petrocellis L. 1998. Endocannabinoids: Endogenous cannabinoid receptor ligands with neuromodulatory actions. Trends Neurosci 21:521–528.
Di Marzo V, Bifulco M, De Petrocellis L. 2004. The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Discov 3:771–784.
Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CM. 1998. A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: A tale of two channels. Biol Reprod 59:12.
Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G, Casellas P. 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte populations. Eur J Biochem 232:54–61.
Glass M, Felder CC. 1997. Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: Evidence for a Gs linkage to the CB1 receptor. J Neurosci 17:5327–5333.
Habayeb OMH, Bell SC, Konje JC. 2002. Endogenous cannabinoids: Metabolism and their role in reproduction. Life Sci 70:1963–1977.
Hanus L, Gopher A, Almong S, Mechoulam R. 1993. Two neu unsaturated fatty acid ethanolamides in brain that bind to the cannabinoid receptor. J Med Chem 36:3032–3034.
Hurst DP, Lynch DL, Barnett-Norris J, Hyatt SM, Seltzman HH, Zhong M, Song Z, Nie J, Lewis D, Reggio PH. 2005. N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A) interaction with LYS 3.28(192) is crucial for its inverse agonism at the cannabinoid CB1 receptor. Mol Pharmacol 62:1274–1287.
Kong LJ, Yang YY, Wang GL. 2007. CatSper and sperm hyperactivation. Zhonghua Nan Ke Xue 13:164–167.
Leff P. 1995. The two-state model of receptor activation. Trends Pharmacol Sci 16:89–97.
Lehman TC, Hale DE, Bhala A, Thorpe C. 1990. An acyl-coenzyme A dehydrogenase assay utilizing the ferricenium ion. Anal Biochem 186:280–284.
Mackie K, Hille B. 1992. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proc Natl Acad Sci USA 89:3825–3829.
Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564.
Nakata M, Yada T. 2008. Cannabinoids inhibit insulin secretion and cytosolic Ca2+ oscillation in islet beta-cells via CB1 receptors. Regul Pept 14:49–53.
Pagotto U, Vicennati V, Pasquali R. 2005. The endocannabinoid system and the treatment of obesity. Ann Med 37:270–275.
Panteghini M, Bonora R, Pagani F. 2001. Measurement of pancreatic lipase activity in serum by a kinetic colorimetric assay using a new chromogenic substrate. Ann Clin Biochem 38:365–370.
Pertwee RG. 2005. Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. Life Sci 76:1307–1324.
Rossato M, Ion Popa F, Ferigo M, Clari G, Foresta C. 2005. Human sperm express cannabinoid receptor Cb1, the activation of which inhibits motility, acrosome reaction, and mitochondrial function. J Clin Endocrinol Metab 90:984–991.
Schuel H, Burkman LJ. 2005. A tale of two cells: Endocannabinoid-signaling regulates functions of neurons and sperm. Biol Reprod 73:1078–1086.
Schuel H, Schuel R, Zimmerman AM, Zimmerman S. 1987. Cannabinoids reduce fertility of sea urchin sperm. Biochem Cell Biol 65:130–136.
Schuel H, Goldstein E, Mechoulam R, Zimmerman AM, Zimmerman S. 1994. Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. Proc Natl Acad Sci USA 91:7678–7682.
Schuel H, Burkman LJ, Lippes J, Crickard K, Mahony MC, Giuffrida A, Picone RP, Makriyannis A. 2002. Evidence that Anandamide-signaling regulates human sperm functions required for fertilization. Mol Reprod Dev 63:376–387.
Suarez SS, Ho HC. 2003. Hyperactivation of mammalian sperm. Cell Mol Biol 49:351–356.
Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K. 1995. 2-Arachidonoyl-glycerol: A possible endogenous cannabinoid receptor ligand in brain. Biochem Biophys Res Commun 215:88–97.
Thomson MF, Wishart GJ. 1989. Elucidation of the mechanism responsible for the temperature-dependent reversible inactivation of the motility of fowl spermatozoa. Br Poult Sci 30:687–692.
Travis AJ, Jorgez CJ, Merdiushev T, Jones BH, Dess DM, Diaz-Cueto L, Storey BT, Kopf GS, Moss SB. 2001. Functional relationships between capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa. J Biol Chem 276:7630–7636.
Urner F, Sakkas D. 1996. Glucose is not essential for the occurrence of sperm binding and zona pellucida-induced acrosome reaction in the mouse. Int J Androl 19:91–96.
Urner F, Sakkas D. 1999a. Characterization of glycolysis and pentose phosphate pathway activity during sperm entry into the mouse oocyte. Biol Reprod 60:973–978.
Urner F, Sakkas D. 1999b. A possible role for the pentose phosphate pathway of spermatozoa in gamete fusion in the mouse. Biol Reprod 60:733–739.
Urner F, Leppens-Luisier G, Sakkas D. 2001. Protein tyrosine phosphorylation in sperm during gamete interaction in the mouse: The influence of glucose. Biol Reprod 64:1350–1357.
Visconti PE, Baley JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. 1995a. Capacitation in mouse spermatozoa I. Correlation between the capacitation state and protein phosphorylation. Development 121:1129–1137.
Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS. 1995b. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121:1139–1150.
Wang H, Dey SK, Maccarrone M. 2006. Jekill and Hyde: Two faces of cannabinoid signaling in male and female fertility. Endo Rev 27:427–448.
World Health Organization. 1999. Laboratory manual for the examination of human semen and sperm-cervical mucus interactions. 4th edition. Cambridge, UK: Cambridge University Press.

Leptin and Its Receptor Are Expressed in the Testis and in the Epididymis of Young and Adult Pigs

VITTORIA RAGO,[1] SAVERIA AQUILA,[2] CARMELA GUIDO,[2] AND AMALIA CARPINO[1]*

[1]Department of Cell Biology, Faculty of Pharmacy, University of Calabria, 87036 Arcavacata di Rende, Cosenza, Italy

[2]Department of Pharmaco-Biology, Faculty of Pharmacy, University of Calabria, 87036 Arcavacata di Rende, Cosenza, Italy

ABSTRACT

Recent studies indicated that leptin, a 16 kDa hormone, is a regulatory signal in human and rodent male reproduction. This work was designed to investigate the expression of leptin and its receptor in testes and epididymides from immature and mature pigs. Immunolocalization revealed that leptin and its receptor were confined only in the interstitial compartment of immature testes, whereas both proteins were detected in Leydig cells and within seminiferous tubules of mature gonads. The immunostaining of epididymal tissues showed that leptin was absent in the epithelial cells of immature pigs but it was present in all the three regions of mature epididymides, although with a minor signal in the cauda. Conversely, leptin receptor was observed in all the epithelial cells of both immature and mature epididymides. Western blot analysis of tissue extracts detected a 16 kDa band for leptin and five/six isoforms, ranging from 120 to 40 kDa, for leptin receptor. In conclusion, this work has identified, for the first time, leptin and leptin receptor in the testis and in the epididymis of the pig showing a differential cell-type expression pattern of the two proteins in young and adult animals. Therefore, our findings suggest a possible involvement of leptin in endocrine or autocrine/paracrine control of porcine male reproductive structures. Anat Rec, 292:736–745, 2009.

Key words: epididymis; leptin; leptin receptor; pig; testis

Leptin (Lep) is a 16 KDa hormone which plays a key role in the regulation of energy homeostasis via the control on food intake, energy expenditure, and metabolic rate (Kamohara et al., 1997; Rossetti et al., 1997; Bouloumie et al., 1998). Furthermore, leptin, reflecting the nutrition state, can act as a metabolic signal to the neurendocrine reproductive system which is sensitive to the energy stores. In fact, in different species, a regulatory role of Lep in the female and male reproductive processes has been reported through the control of the hypothalamic-pituitary-gonadal axis activity (Barb, 1999; Foster and Nagatani, 1999; Ahima and Flier, 2000; Amstalden et al., 2000; Budak et al., 2006).

Grant sponsor: Ministero dell'Università e della Ricerca Scientifica e Tecnologica.

*Correspondence to: Amalia Carpino, Dipartimento di Biologia Cellulare, Università degli Studi della Calabria, 87030 Arcavacata di Rende, Cosenza, Italy. Fax: +39 0984 492911.
E-mail: am_carpino@yahoo.it

Received 29 July 2008; Accepted 22 December 2008

DOI 10.1002/ar.20880

Published online 20 March 2009 in Wiley InterScience (www.interscience.wiley.com).

The effects of Lep are mediated by its specific receptor (Ob-R) in target tissues. The Ob-R, a single membrane-spanning glycoprotein, belongs to the class I cytokine receptor superfamily. Several leptin receptor isoforms have been detected in target tissues, particularly a long form (Ob-Rb) and truncated short isoforms (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Rf, and Ob-Re) (Takaya et al., 1996; Lollmann et al., 1997; Murakami et al., 1997; Bjorbaek et al., 1998; Dieterich and Lehnert, 1998). The full length isoform Ob-Rb contains intracellular motifs required for activation of the JAK/STAT (Jasus kinases/signal transducers and activators of transcription) pathway and it is considered to be a fully functional receptor (Thomas, 2004). The MAPK (mitogen activated protein kinase) patway can also be stimulated by Ob-Rb and Ob-Ra, although to lesser extent by the latter (Fruhbeck, 2006). In addition, Ob-Ra is thought to mediate leptin transport across anatomical barriers, such as the blood–brain barrier (Kastin et al., 1999) and the placenta (Smith and Waddell, 2002). Both Ob-Rb and Ob-Ra mediate also leptin internalization and its lysosomal degradation (Uotani et al., 1999) Ob-Re, which lacks the transmembrane and intracellular domain, serves as soluble receptor and represents the leptin bioavailability, being the hormone binding protein in plasma (Zastrow et al., 2003). However, the different target tissues have revealed variable expression levels of the Ob-R isoforms (Cioffi et al., 1996; Tartaglia, 1997; Glasow et al., 1998; Breidert et al., 1999; Margetic et al., 2002).

In both male and female rodents, it has been shown that leptin has direct effects on fertility since the hormone is able to reverse the infertility of *ob*/*ob* mice, lacking of the leptin gene (Cunningham et al., 1999). Compelling evidences support the role of leptin in the physiology of female reproductive system in a paracrine and/or endocrine fashion (Casanueva and Dieguez, 1999; Ahima and Flier, 2000; Wauters et al., 2000; Moschos et al., 2002). In contrast, leptin involvement in the control of male reproduction is still not well defined.

Some studies demonstrated the Lep regulation of male reproduction via the central nervous system (Tena-Sempere and Barreiro, 2002), but recently a peripheral direct action of the hormone on target reproductive structures has been hypothesized. In fact, Lep and/or Ob-R were detected in rodent testes (Caprio et al., 2003; El-Hefnawy et al., 2000; Herrid et al., 2008), in human testes and seminal plasma (Glander et al., 2002; Soyupek et al., 2005; Ishikawa et al., 2007), in human sperm (Aquila et al., 2005), in boar sperm (De Ambrogi et al., 2007), and in pig sperm (Aquila et al., 2008). Furthermore, a recent paper reported the effects of leptin treatment, alone or in combination with dietary energy restriction, on different reproductive parameters of male rats (Sirotkin et al., 2008).

In the pig, the hormone has been identified as a metabolic signal to the hypothalamus and pituitary gland modulating the GnRH/LH secretory axis (Barb et al., 2005). However, our recent work detected leptin and Ob-R in pig sperm showing a role of the hormone on the male gamete biology (Aquila et al., 2008). Therefore, aim of this work was to provide some evidence of leptin involvement in the control of male reproductive structures by investigating Lep and Ob-R in testicular and epididymal cells of immature and mature pigs.

MATERIALS AND METHODS

Chemicals

BSA (bovine serum albumin) protein standard, Laemmli sample buffer, prestained molecular weight marker, Haematoxylin, Eosin, Bradford reagent, and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, ECL Plus Western blotting detection system, Hybond™ ECL™, were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). Polyclonal rabbit anti-leptin (A-20), polyclonal rabbit anti-Ob-R mapping within the Ob-R internal domain (H-300), peroxidase-coupled anti-rabbit, β-actin antibodies (Abs), Ob, and OB-R blocking peptides (sc842P and sc1834P) were from Santa Cruz Biotechnology (Heidelberg, Germany). Biotinylated goat anti-rabbit IgG, avidin-biotin-horseradish peroxidase (ABC) complex, and diaminobenzidine (DAB) were from Vector Laboratories (Santa Cruz, CA). Total RNA Isolation System Kit, enzymes, buffers, nucleotides 100 bp ladder used for RT-PCR were purchased from Promega. Moloney murine leukemia virus (M-MLV) was from Gibco-Life Technologies Italia. Oligonucleotide primers were made by Invitrogen.

Animals

The investigation has been conducted on testicular and epididymal tissues from five fertile adult pigs and five infertile young animals (*Sus scrofa domestica*, Large White) kept at "Swine Artificial Insemination Centre" (Rende, Cosenza, Italy). Immature pigs were 2–3 month old and their weights were from 25 to 35 kg, whereas mature animals were 18–24 month-old and their weights were from 280 to 320 kg. Testes and epididymides were removed during routine castrations at the local animal hospital. All surgical procedures followed approved guidelines for the ethical treatment of animals. Immediately after castration, the epididymides were carefully dissected in caput, corpus, and cauda.

Morphological analysis was carried out by standard haematoxylin-eosin staining.

Immunohistochemistry

Removed tissues were immediately fixed in neutral buffered formalin (4%), dehydrated in a series of ethanol concentrations, and paraffin-embedded. Then the (5 μm) sections were cut (8–9 serial sections, randomly selected, for each sample), mounted on polylysine-precoated slides, deparaffinized, and dehydrated. Immunochemistry was performed after heat-mediated antigen retrieval (sections microwaved in a 0.01 M citrate buffer solution, pH 6, for 18 min). Hydrogen peroxide (3% in distilled water for 30 min) was used to inhibit endogenous peroxidase activity. Normal goat serum (10% for 30 min) was used to block nonspecific binding sites. Anti-leptin (1:100) and anti-Ob-R (1:100) were used as primary Abs (overnight at 4°C), whereas biotinylated goat anti-rabbit IgG was utilized as secondary antibody (1 hr at RT). ABC complex amplification was then performed (30 min at RT) and the peroxidase reaction was developed with DAB. All the sections were counterstained with haematoxilyn. The primary antibodies were replaced by normal

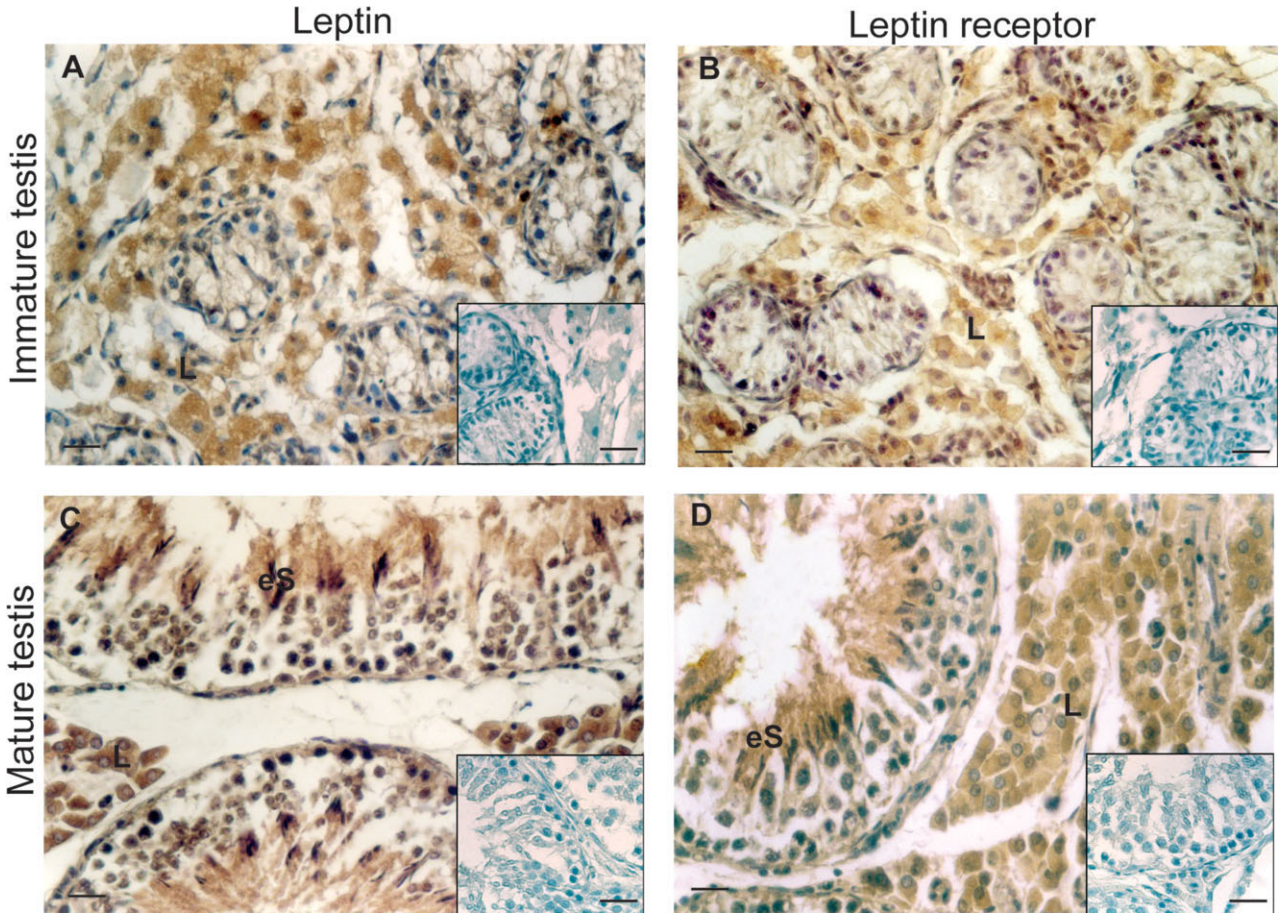

Fig. 1. Representative leptin and leptin receptor immunostainings of immature and mature pig testes: (**A**) leptin immunoreactivity in the immature testis. (**B**) Leptin receptor immunostaining in the immature pig. (**C**) Leptin immunoreactivity in the mature testis. (**D**) Leptin receptor immunodetection in the mature animal. Inserts: immunonegative absorption controls. L, Leydig cells; eS, elongating spermatids. Scale bars: 12.5 μm.

rabbit serum in the ordinary controls (data not shown). In addition, absorption controls were assessed by using primary antibodies preabsorbed with an excess of Ob and Ob-R blocking peptides for 48 hr at 4°C.

Western Blot Analysis

Frozen testicular and epididymal tissues from immature and mature pigs were homogenized (GLAS-COL, Terre Haute) and lysed in buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM EDTA, 25% glycerol,1 mM 1,4-dithiothreitol, 0.5 mM, Na_3VO_4, 0.2% Nonidet P-40, and a mixture of protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonylfluoride, pepstatin). Lysates were quantified using Bradford protein assay reagent and equal amounts of proteins (40 μg) were resolved on a 10% sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in glycine buffer (0.02 mM Tris, 0.2 mM glycine, 1% SDS). Proteins were then transferred to a nitrocellulose membrane and probed overnight at 4°C with antibodies against Lep, Ob-R, and β-actin (loading control). Finally, the bound of the secondary antibody was revealed with the ECL Plus WB detection system according to the manufacturer's instructions.

Pig adipocyte and pig sperm extracts were used as positive controls for leptin and Ob-R, respectively. The negative controls were performed using tissue lysates previously immunodepleted of leptin or Ob-R (i.e., lysates preincubated with anti-leptin Ab or anti-Ob-R Ab for 1 hr at room temperature and immunoprecipitated with Protein A/G-agarose).

To further validate the results for Ob-R, as negative control, the nonimmune rabbit serum replaced the primary antibody at the same dilution ratio (1:1,000).

The experiments were repeated at least four times for each sample.

RNA isolation, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR analysis of epidydimal and testicular tissue extracts has been performed to confirm Ob and Ob-R expression in young and adult pigs. Concerning Ob-R, the authors have performed the RT-PCR by using

TABLE 1. Leptin and Ob-R immunostaning[a] in testis and epididymis of young and adult pigs

		Leptin		Ob-R	
		young	adult	young	adult
Testis	Leydig cell	+++	+++	+++	+++
	Peritubular myoid cell	–	–	–	–
	Sertoli cell	–	–	–	–
	Spermatogonia	–	–	–	–
	Spermatocytes	/	–	/	+/–
	Elongating spermatids	/	+++	/	+++
Epididymis	Caput				
	Epithelial cells	–	+++	++	+++
	Stromal cells	++	++	++	++
	Corpus				
	Epithelial cells	–	+++	++	+++
	Stromal cells	++	++	++	++
	Cauda				
	Epithelial cells	–	+++	++	+++
	Stromal cells	++	++	++	++

[a]Staining intensity scores as follows: – negative; +/– very weak; ++ moderate; +++ strong; / cell type absence.

primers designed on the basis of the published sequence of the transmembrane leptin receptor (Gene bank, accession number AF090422). Different Ob-R isoforms of Sus Scrofa have not been cloned yet. The only complete sequences published are relative to Leptin receptor mRNA (Long form) and to Leptin transmembrane receptor mRNA (included in the first one). Therefore, we amplified the last sequence that is potentially present in all the Ob-R isoforms.

Testes and epididymides, removed from immature and mature pigs, were dissected and immediately frozen in liquid nitrogen. Subsequently, total RNA extraction was performed by tissue homogenization followed by guanidinium/chloroform purification, according to Freije (Freije et al., 1997).

Before RT-PCR, RNA was incubated with ribonuclease-free deoxyribonuclease (Dnase) I in single-strength reaction buffer at 37°C for 15 min. This was followed by heat inactivation of Dnase I at 65°C for 10 min. Purity and integrity of RNA extracted was checked spectroscopically and by gel electrophoresis before use. Ten micrograms of total RNA was reverse transcribed using 200 IU M-MLV reverse transcriptase in a reaction volume of 20 μL (0.4 μg oligo-dT, 0.5 mM deoxy-NTP, and 24 IU Rnasin) for 30 min at 37°C, followed by heat denaturation for 5 min at 95°C and stored at −20°C. PCR amplification of complementary DNA (cDNA) was performed with 2 U of Taq DNA polymerase, 50 pmol primer pair for both Lep and Ob-R in 10 mM Tris-HCL (pH 9.0) containing 0.1% Triton X-100, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.25 mM each dNTP. The applied PCR primers and the expected lengths of the resulting PCR products are the following: 5′ ATTCCTGGCTTGGCCC 3′ and 5′ AAGGCAGACTGGTGAGGATCTGTT 3′ for Lep with a product size of 248 bp; 5′ ACTTCCTCTTG CCTGCTGGAATCT 3′ and 5′ GACACAGGCACATGG-CATTCACAA 3′ for Ob-R with a product size of 460 bp. Cycling conditions were: 95°C/1 min, 55°C/1 min, 72°C/2 min for Lep; 95°C/1 min, 52°C/1 min, 72°C/2 min for Ob-R. For all PCR primer amplifications, negative RT-PCR was performed without M-MLV reverse transcriptase. The PCR-amplified products were subjected to electrophoresis in 2% agarose gels stained with ethidium bromide and visualized under UV transillumination.

RESULTS

Leptin and Ob-R Immunolocalization

Testis. Immature testes showed a strong immunostaining of leptin and Ob-R in the cytoplasm of interstitial cells, whereas no immunoreactivity was observed in myoid peritubular cells and inside the small closed seminiferous tubules (Fig. 1A,B) (Table 1). Conversely, in the mature gonads both the hormone and its receptor were strongly evidenced in the Leydig cell clusters and, inside the active seminiferous tubules, mainly in elongating spermatids (Fig. 1C,D) (Table 1). No immunoreactivity was observed in the negative (data not shown) and absorption controls (inserts).

Epididymis. The epithelial cells of the immature genital duct were immunonegative for leptin (Fig. 2A,C,E), whereas a strong cytoplasmic immunostaining was observed in the caput and corpus of mature epididymis together with a moderate immunoreactivity in the cauda (Fig. 2B,D,F) (Table 1).

A moderate Ob-R signal was evidenced in the epithelial cells of caput, corpus, and cauda of young pigs (Fig. 3A,C,E), whereas the immunoreactivity was strong in the three regions of adult epididymides (Fig. 3B,D,F) (Table 1). No Ob-R immunostaining was observed in the negative (data not shown) and absorption controls (inserts).

In addition, connective tissue of all epididymal samples showed a moderate leptin and Ob-R immunoreactivity (Fig. 3).

WB Analysis

Leptin. One immunoreactive band was observed at 16 kDa for leptin in the lysates from immature and mature testes (Fig. 4A,B, lane 1) showing the same mobility of the adipocyte extract used as positive control (Fig. 4A,B, lane C+).

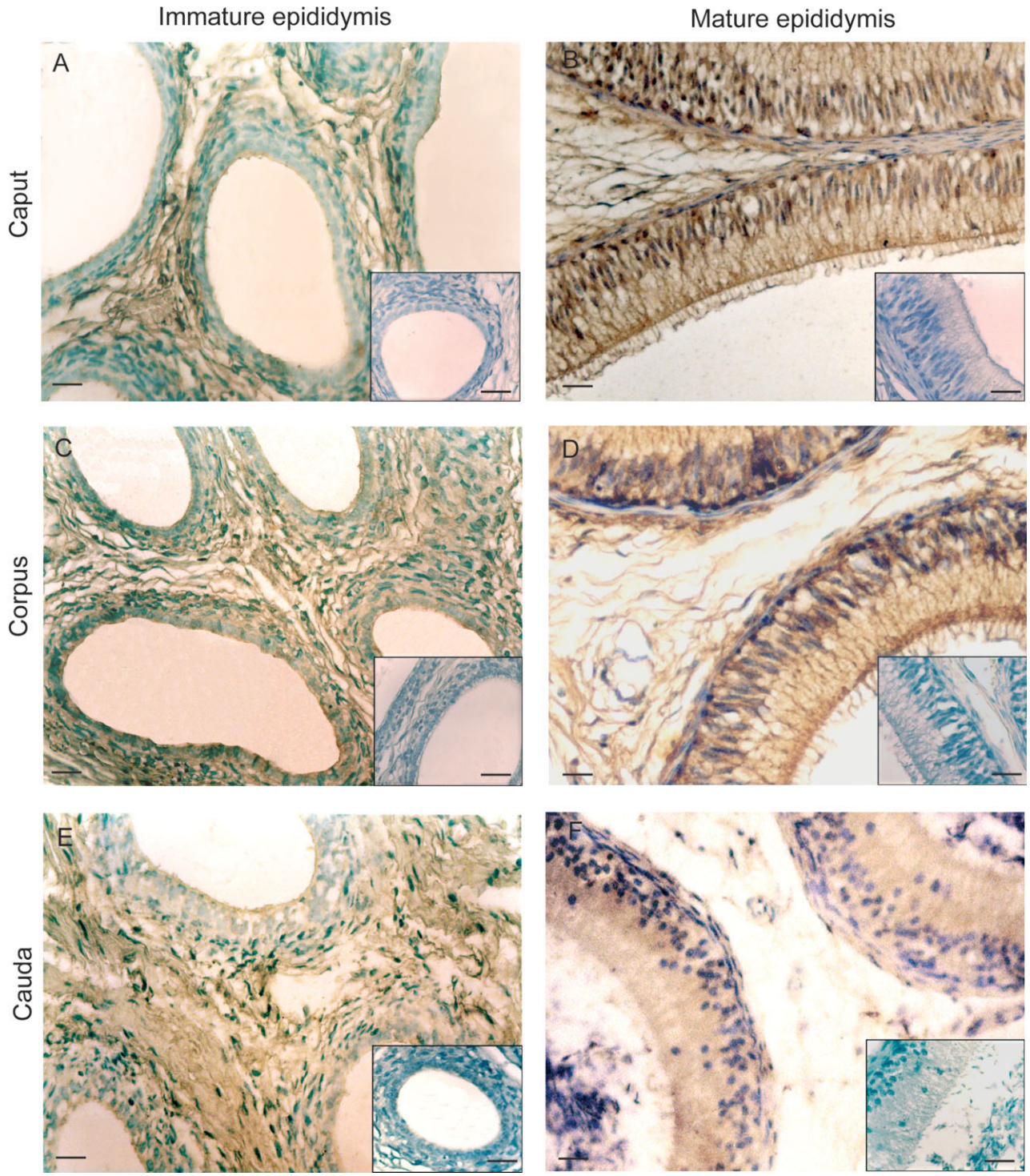

Fig. 2. Representative leptin immunodetection of caput, corpus, and cauda epididymis in immature (**A, C, E**) and mature (**B, D, F**) pigs. Inserts: immunonegative absorption controls. Scale bars: 12.5 μm.

A very thin16 kDa band was observed in the extracts from the three regions of the immature epididymides, probably corresponding to the positive immunostaining of the connective tissues (Fig. 4A, lanes: 2, 3, 4). Conversely, a high expression of leptin was detected with a 16 kDa band in the lysates from caput, corpus, and cauda of the mature ducts (Fig. 4B, lanes: 2, 3, 4). No band has been detected in the negative controls (data not shown).

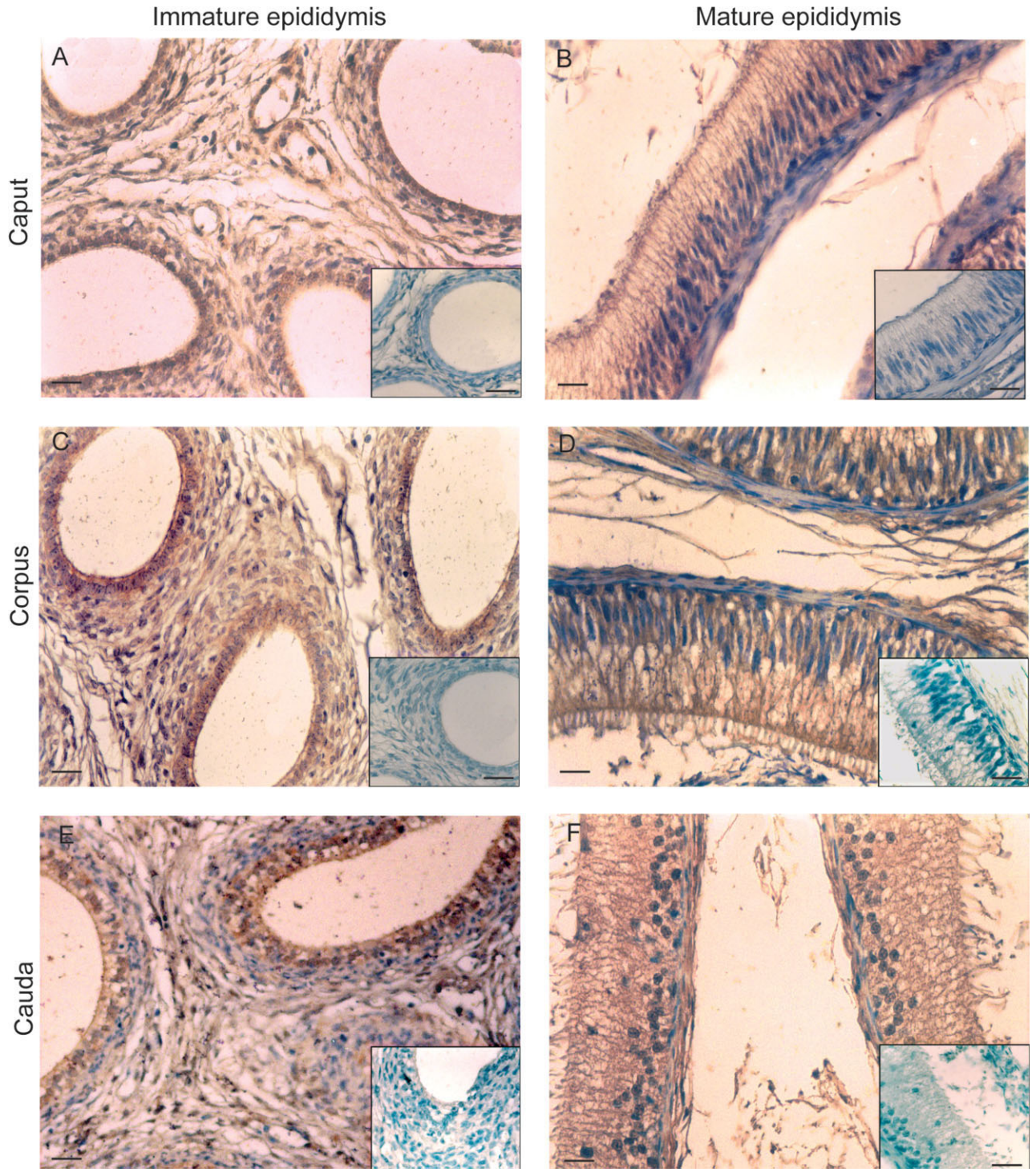

Fig. 3. Representative leptin receptor immunoreactivity of caput, corpus, and cauda epididymis in immature (**A, C, E**) and mature (**B, D, F**) pigs. Inserts: immunonegative absorption controls. Scale bars: 12.5 μm.

Ob-R. In agreement with the specificity of the antibody used in this study, WB analysis evidenced multiple immunoreactive bands corresponding to long and short Ob-R isoforms. Six different immunoreactive Ob-R isoforms (120, 90, 80, 65, 60, and 40 kDa) were detected in testicular extracts from both mature and immature pigs (Fig. 5A,B, lane 1) such as in the lysate of ejaculated pig sperm used as positive control (Fig. 5A,B, lane C+). The same bands were observed in epididymal extracts from the mature pigs (Fig. 5B, lanes: 2, 3, 4), whereas the

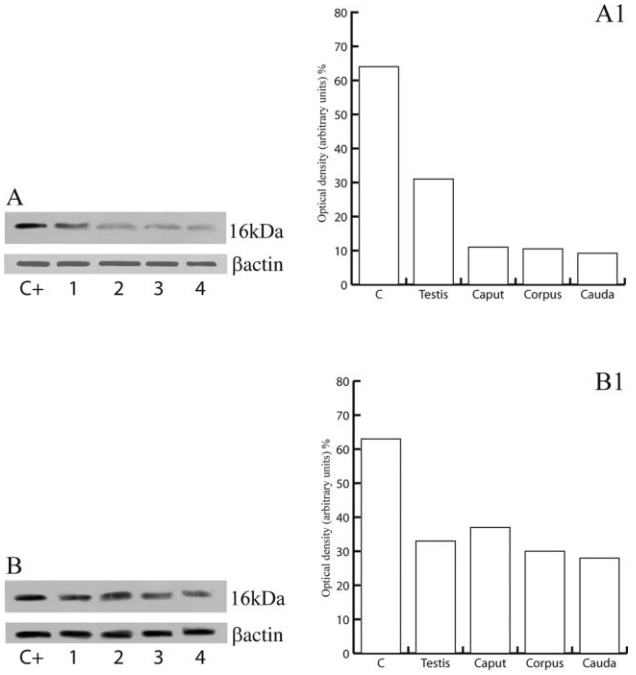

Fig. 4. Western blot results of leptin in testicular and epididymal lysates from representative immature (**A**) and mature (**B**) pigs. lane C+: adipocyte cells used as positive control; lane 1: testis; lane 2: caput; lane 3: corpus: lane 4: cauda. β-actin served as loading control. Numbers on the right-hand side correspond to molecular weights of detected proteins. (**A1**) and (**B1**): Band intensities were evaluated in terms of arbritrary densitometric units. Values are as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

40 kDa band was absent in the lysates from the immature ducts (Fig. 5A, lanes: 2, 3, 4).

The immunoreactive bands were not detected by non-immune rabbit serum indicating that these proteins are specific for Ob-R in pig (data not shown).

RT-PCR Analysis

Expression of leptin and Ob-R mRNA was examined in testicular and epididymal tissues from immature and mature pigs. RNA isolated from testicular and epididymal tissues was subjected to reverse PCR. Concerning leptin, the primer sequences were based on the leptin pig gene sequence and the RT-PCR amplification revealed the expected PCR product size of 280 bp (Fig. 6).

Concerning Ob-R, the primer sequences were based on the OB-R pig gene sequence of the transmembrane region and the RT-PCR amplification revealed the expected PCR product size of 460 bp (Fig. 7).

DISCUSSION

Leptin represents a metabolic signal affecting central regulation of reproduction in the pig. In fact, Lep is detected by the central nervous system and, by means of the neuroendocrine axis, regulates hypothalamic gonadotropin-releasing hormone (GnRH) release and subsequent luteinizing hormone (LH) secretion (Barb et al., 2005). However, our recent work demonstrated a role of leptin in pig sperm acquisition of fertilizing ability (Aquila et al., 2008) suggesting a possible peripheral effect of the hormone in porcine male reproduction.

This study has identified leptin and its receptor in testis and epididymis of young and adult pigs demonstrating the presence of the two proteins at different levels: mRNA expression, protein expression, and immunolocalization.

Immunohistochemical analysis has shown a differential cell-type expression of leptin and leptin receptor in immature and mature pig testes. In fact, the hormone and its receptor have been detected in Leydig cells of young and adult animals, whereas the two proteins were identified only inside seminiferous tubules of mature gonads. The expression of leptin and Ob-R in both immature and mature pig Leydig cells suggests that the hormone may be directly involved in regulation of Leydig cell development and in steroidogenesis by paracrine and/or autocrine mechanisms. Previous studies could support this hypothesis, in fact, interstitial tissue is reduced more than 50% in the leptin deficient *ob*/*ob* mice but leptin treatment induced the Leydig cell regeneration (Hellman, 1965; Mounzih et al., 1997). Furthermore, a direct receptor-mediated action of leptin on androgen production has been demonstrated in cultured adult rat Leydig cells (Caprio et al., 1999).

Our study has shown leptin and Ob-R inside seminiferous tubules of mature testes, mainly in elongating spermatids, but not in seminiferous tubules of immature gonads. These findings suggest a role of leptin in the control of pig sperm cell differentiation. This hypothesis agrees to previous investigations in rodents and humans. In fact, the *ob*/*ob* mouse testes are characterized by hollow seminiferous tubules with aberrant multinucleated spermatids and few spermatozoa, but leptin treatment can restore the spermatogenetic arrest (Jones and Ainsworth-Harrisson, 1957; Mounzih et al., 1997). Furthermore, leptin has been suggested to regulate the proliferation and differentiation of mouse testicular germ cells by the activation of STAT3 (El-Hefnawy et al., 2000) and, in human testis, the dysfunction of spermatogenesis appears to be associated with an altered leptin and leptin receptor expression (Ishikawa et al., 2007; Chen et al., 2008).

In comparison to previous studies on mammals, our data reveal a species-specificity of leptin and Ob-R expression in pig testes. In fact, in the rat, leptin was expressed in seminiferous tubules and in the interstitium of the adult gonads (Chen et al., 2008), whereas Ob-R was confined only in the Leydig cells of mature testes (Caprio et al., 2003), but it was absent in immature gonads. In contrast, in the mouse, leptin and its receptor were detected exclusively in germ cells of immature and mature testes (El-Hefnawy et al., 2000; Herrid et al., 2008). Furthermore, in the human adult testes, leptin was found in seminiferous tubules, whereas Ob-R was detected in Leydig cells (Glander et al., 2002; Soyupek et al., 2005; Ishikawa et al., 2007).

In the present investigation, successful Western blot of pig testicular samples evidenced the expected 16 kDa band for leptin and revealed six isoforms for leptin receptor showing molecular weight consistent with the Ob-Rb long form (~120 kDa), with the Ob-Ra short form (~90 kDa), and with shorter isoforms. Therefore, leptin receptor expression could mediate the hormone effects

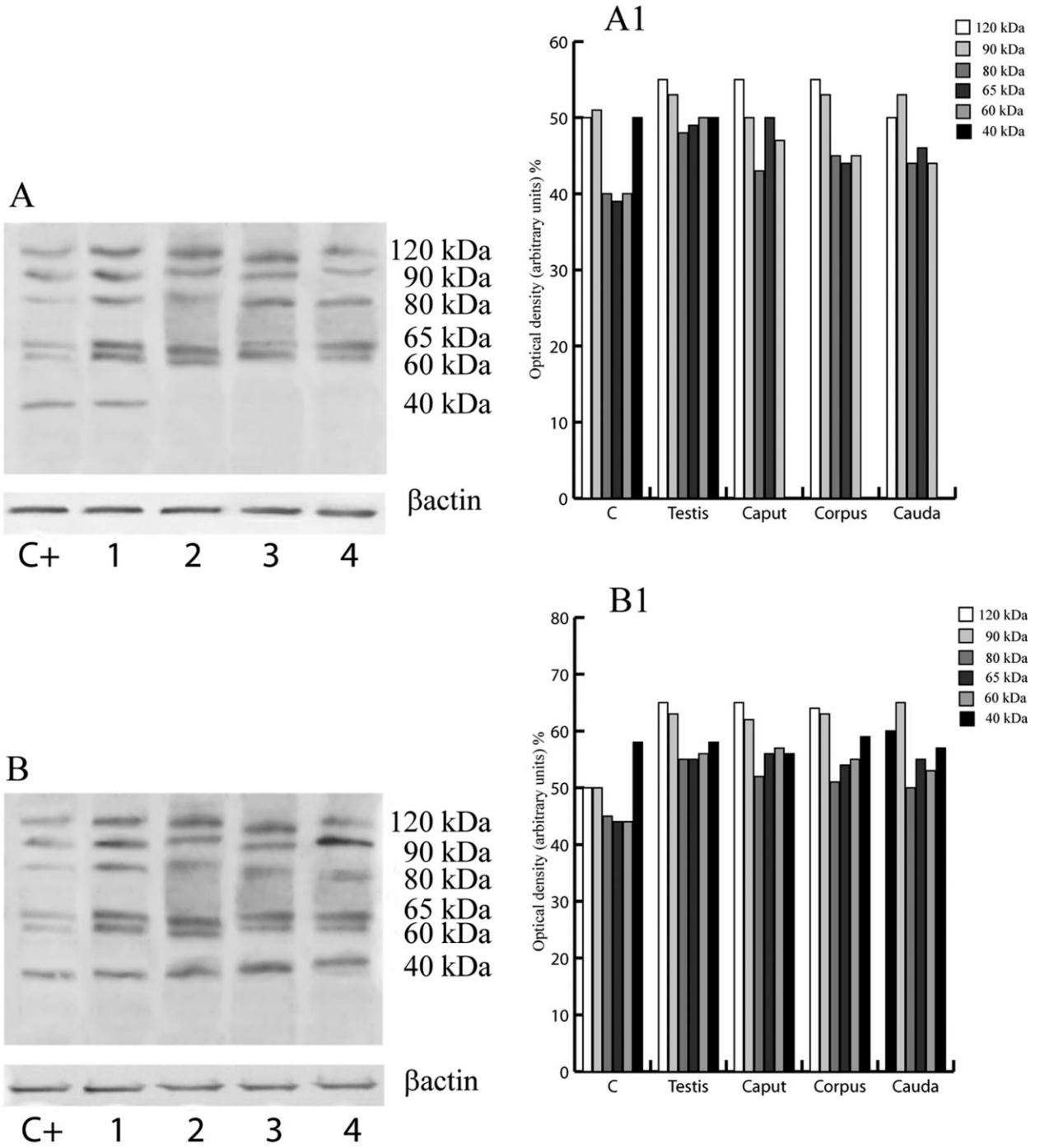

Fig. 5. Western blot results of leptin receptor in testicular and epididymal lysates from representative immature (**A**) and mature (**B**) pigs. lane C+: ejaculated pig sperm used as positive control; lane 1: testis; lane 2: caput; lane 3: corpus; lane 4: cauda. β-actin served as loading control. Numbers on the right-hand side correspond to molecular weights of detected proteins. (**A1**) and (**B1**): band intensities were evaluated in terms of arbitrary densitometric units. Values are as mean ± SEM.

on testes by mediating the direct leptin action, the leptin transport, and in other still not defined ways.

To our knowledge, the presence of leptin and Ob-R in the mammalian epididymis has not been demonstrated, so the present study explored the expression of the two proteins in the porcine genital duct. Firstly, we have identified the hormone and its receptor in pig epididymal tissues with an age-specific localization. In fact, leptin was observed in epithelial cells of the adult epididymis, but not in the young one, leading us to speculate a possible role of the hormone in the functional activity of the genital duct. Epithelial cells of the adult

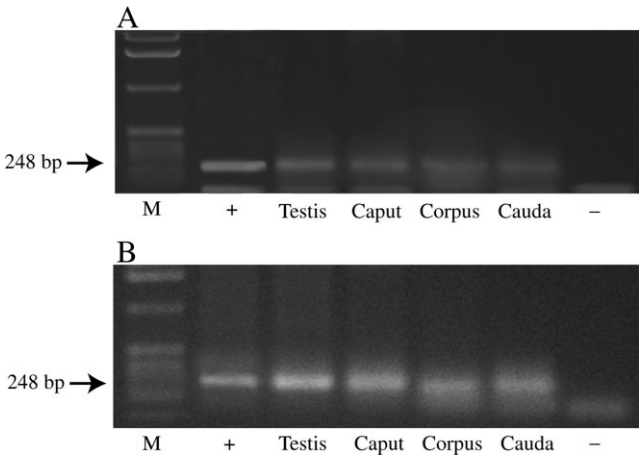

Fig. 6. RT-PCR results of leptin in testis and epididymis from representative immature (**A**) and mature (**B**) pigs. lane M: markers, lane +: positive control (adipocytes), lane –: negative control.

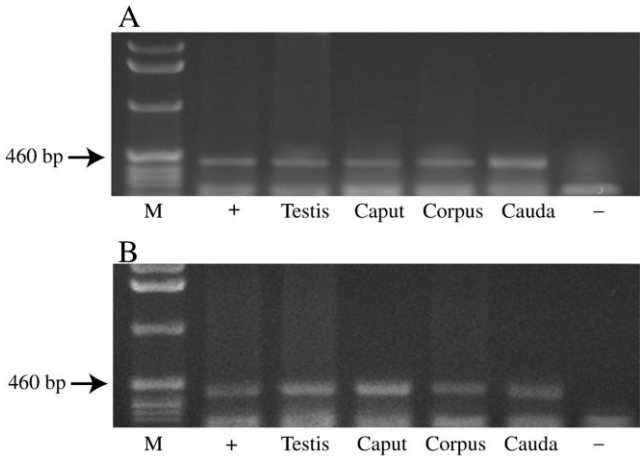

Fig. 7. RT-PCR results of leptin receptor in testis and epididymis from immature (**A**) and mature (**B**) pigs. lane M: markers, lane +: positive control (pig sperm), lane –: negative control.

epididymis are characterized by high secretory activity which is responsible for the post-testicular sperm maturation, due to complexes and sequential interactions between luminal proteins and spermatozoa in transit (Sullivan, 1999; Dacheux et al., 2005). Intriguingly, the secretory activity of the three epididymal regions overlaps the pattern of leptin immunostaining, which was intense in the caput and in the corpus but weak in the cauda.

Conversely, Ob-R isoforms were evidenced in the three epididymal regions of both young and adult animals, suggesting a direct targeting of leptin on the epithelial cells during the growth and the function of the genital duct. However, the identification of a 40 kDa Ob-R isoform exclusively in the mature epididymis is a new finding which remains to be elucidated.

In addition, epididymal connective tissue has showed leptin and Ob-R expression in both immature and mature pigs, so it is reasonable to hypothesize a role of stromal cells in development and physiology of epididymis through leptin signalling.

In conclusion, our results revealed a differential cell-type expression of leptin and of its receptor in testis and epididymis of young and adult pigs, supporting a possible role of the two proteins in the development and functional activity of these genital structures. Therefore, taken together our findings suggest a possible involvement of leptin in the control of porcine male reproduction.

ACKNOWLEDGMENTS

The authors thank Dr. Rocco Panza, chief of the Swine Artificial Insemination Centre, APA, Cosenza (Italy), who provided animals for sample collections.

LITERATURE CITED

Ahima RS, Flier JS. 2000. Leptin. Ann Rev Physiol 62:413–437.

Amstalden M, Garcia MR, Williams SW, Stanko RL, Nizielski SE, Morrison CD, Keisler DH, Williams GL. 2000. Leptin gene expression, circulating leptin, and luteinizing hormone pulsatility are acutely responsive to short-term fasting in prepubertal heifers: relationship to circulating insulin and insulin-like growth factor I. Biol Reprod 63:127–133.

Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V, Andò S. 2005. Leptin secretion by human spermatozoa. J Clin Endocrinol Metab 90:4753–4761.

Aquila S, Rago V, Guido C, Zupo S, Casaburi I, Carpino A. 2008. Leptin and leptin receptor in pig spermatozoa: evidence of their involvement in sperm capacitation and survival. Reproduction 136:23–32.

Barb CR. 1999. The brain-pituitary-adipocyte axis: role of leptin in modulating neuroendocrine function. J Anim Sci 77:1249–1257.

Barb CR, Hausman GJ, Czaja K. 2005. Leptin: a metabolic signal affecting central regulation of reproduction in the pig. Dom Anim Endocrinol 29:186–192.

Bjorbaek C, Elmquist JK, Michl P, Ahima RS, van Bueren A, McCall AL, Flier JS. 1998. Expression of leptin receptor isoforms in rat brain microvessels. Endocrinology 139:3485–3491.

Bouloumie A, Drexler HC, Lafontan M, Busse R. 1998. Leptin, the product of Ob gene, promotes angiogenesis. Circ Res 83:1059–1066.

Breidert M, Miehlke S, Glasow A, Orban Z, Stolte M, Ehninger G, Bayerdörffer E, Nettesheim O, Halm U, Haidan A, Bornstein SR. 1999. Leptin and its receptor in normal human gastric mucosa and in Helicobacter pylori-associated gastritis. Scand J Gastroenterol 34:954–961.

Budak E, Fernández Sánchez M, Bellver J, Cerveró A, Simón C, Pellicer A. 2006. Interactions of the hormones leptin, ghrelin, adiponectin, resistin, and PYY3-36 with the reproductive system. Fertil Steril 85:1563–1581.

Caprio M, Fabbrini E, Ricci G, Basciani S, Gnessi L, Arizzi M, Carta AR, De Martino MU, Isidori AM, Frajese GV, Fabbri A. 2003. Ontogenesis of leptin receptor in rat Leydig cells. Biol Reprod 68:1199–1207.

Caprio M, Isidori AM, Carta AR, Moretti C, Dufau ML, Fabbri A. 1999. Expression of functional leptin receptors in rodent Leydig cells. Endocrinology 140:4939–4947.

Casanueva FF, Dieguez C. 1999. Neuroendocrine regulation andactions of leptin. Front Neuroendocrinol 20:317–363.

Chen B, Guo JH, Lu YN, Ying XL, Hu K, Xiang ZQ, Wang YX, Chen P, Huang YR. 2008. Leptin and varicocele-related spermatogenesis dysfunction: animal experiment and clinical study. Int J Androl, in press.

Cioffi JA, Shafer AW, Zupancic TJ, Smith-Gbur J, Mikhail A, Platika D, Snodgrass HR. 1996. Novel B219/OB receptor isoforms:

possible role of leptin in hematopoiesis and reproduction. Nat Med 2:585–589.
Cunningham MJ, Clifton DK, Steiner RA. 1999. Leptin's Actions on the Reproductive Axis: perspectives and Mechanisms. Biol Reprod 60:216–222.
Dacheux JL, Castella S, Gatti JL, Dacheux F. 2005. Epididymal cell secretory activities and the role of proteins in boar sperm maturation. Theriogenology 63:319–341.
De Ambrogi M, Spinaci M, Galeati G, Tamanini C. 2007. Leptin receptor in boar spermatozoa. Int J Androl 30:458–461.
Dieterich KD, Lehnert H. 1998. Expression of leptin receptor mRNA and the long form splice variant in human anterior pituitary and pituitary adenoma. Exp Clin Endocrinol Diabet 106:522–525.
El-Hefnawy T, Ioffe S, Dym M. 2000. Expression of the leptin receptor during germ cell development in the mouse testis. Endocrinology 141:2624–2630.
Foster DL, Nagatani S. 1999. Physiological perspectives on leptin as a regulator of reproduction: role in timing puberty. Biol Reprod 60:205–215.
Freije WA, Pezzi V, Arici A, Carr BR, Rainey WE. 1997. Expression of 11 beta-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) in the human fetal adrenal. J Soc Gynecol Investig 4:305–309.
Fruhbeck G. 2006. Intracellular signaling pathways activated by leptin. Biochem J 393:7–20.
Glander HJ, Lammert A, Paasch U, Glasow A, Kratzsch J. 2002. Leptin exists in tubuli seminiferi and in seminal plasma. Andrologia 34:227–233.
Glasow A, Haidan A, Hilbers U, Breidert M, Gillespie J, Scherbaum WA, Chrousos GP, Bornstein SR. 1998. Expression of Ob receptor in normal human adrenals: differential regulation of adrenocortical and adrenomedullary function by leptin. J Clin Endocrinol Metab 83:4459–4466.
Hellman B. 1965. Studies in obese-hyperglycemic mice. Ann NY Acad Sci 131:541–558.
Herrid M, O'Shea T, McFarlane JR. 2008. Ontogeny of leptin and its receptor expression in mouse testis during the postnatal period. Mol Reprod Dev 75:874–880.
Ishikawa T, Fujioka H, Ishimura T, Takenaka A, Fujisawa M. 2007. Expression of leptin and leptin receptor in the testis of fertile and infertile patients. Andrologia 39:22–27.
Jones N, Ainsworth-Harrisson G. 1957. Genetically determined obesity and sterility in the mouse. Stud Fertil 9:51–64.
Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ. 1997. Acute stimulation of glucose metabolism in mice by leptin treatment. Nature 389:374–377.
Kastin AJ, Pan W, Maness LM, Koletsky RJ, Ernsberger P. 1999. Decreased transport of leptin across the blood-brain barrier in rats lacking the short form of the leptin receptor. Peptides 20:1449–1453.
Lollmann B, Gruninger S, Stricker-Krongrad A, Chiesi M. 1997. Detection and quantification of the leptin receptor splice variants Ob-Ra, b, and, e in different mouse tissues. Biochem Biophys Res Commun 238:648–652.
Margetic S, Gazzola C, Pegg GG, Hill RA. 2002. Leptin: a review of its peripheral actions and interactions. Int J Obes Relat Metab Disord 26:1407–1433.
Moschos S, Chan JL, Mantzoros CS. 2002. Leptin and reproduction: a review. Fertil Steril 77:433–444.
Mounzih K, Lu R, Chehab FF. 1997. Leptin treatment rescues the sterility of genetically obese ob/ob males. Endocrinology 138:1190–1193.
Murakami T, Yamashita T, Iida M, Kuwajima M, Shima K. 1997. A short form of leptin receptor performs signal transduction. Biochem Biophys Res Commun 231:26–29.
Rossetti L, Massillon D, Barzilai N, Vuguin P, Chen W, Hawkins M, Wu J, Wang J. 1997. Short term effects of leptin on hepatic gluconeogenesis and in vivo insulin action. J Biol Chem 272:27758–27763.
Sirotkin AV, Chrenková M, Nitrayová S, Patras P, Darlak K, Valenzuela F, Pinilla L, Tena-Sempere M. 2008. Effects of chronic food restriction and treatments with leptin or ghrelin on different reproductive parameters of male rats. Peptides 29: 1362–1368.
Smith JT, Waddell BJ. 2002. Leptin receptor expression in the rat placenta: changes in ob-ra, ob-rb, and ob-re with gestational age and suppression by glucocorticoids. Biol Reprod 67:1204–1210.
Soyupek S, Armagan A, Serel TA, Hoscan MB, Perk H, Karaöz E, Candir O. 2005. Leptin expression in the testicular tissue of fertile and infertile men. Arch Androl 51:239–246.
Sullivan R. 1999. Interaction between sperm and epididymal secretory proteins. In: Gagnon C, editor. The male gamete, from basic science to clinical applications. Vienna: Cache River Press. p 93–104.
Takaya K, Ogawa Y, Isse N, Okazaki T, Satoh N, Masuzaki H, Mori K, Tamura N, Hosoda K, Nakao K. 1996. Molecular cloning of rat leptin receptor isoform complementary DNAs–identification of a missense mutation in Zucker fatty (fa/fa) rats. Biochem Biophys Res Commun 225:75–83.
Tartaglia LA. 1997. The leptin receptor. J Biol Chem 272:6093–6096.
Tena-Sempere M, Barreiro ML. 2002. Leptin in male reproduction: the testis paradigm. Mol Cell Endocrinol 188:9–13.
Thomas T. 2004. The complex effects of leptin on bone metabolism through multiple pathways. Curr Opin Pharmacol 4:295–300.
Uotani S, Bjorbaek C, Tornoe J, Flier JS. 1999. Functional properties of leptin receptor isoforms internalization and degradation of leptin and ligand-induced receptor downregulation. Diabetes 48:279–286.
Wauters M, Considine RV, van Gaal LF. 2000. Human leptin: from an adipocyte hormone to an endocrine mediator. Eur J Endocrinol 143:293–311.
Zastrow O, Seidel B, Kiess W, Thiery J, Keller E, Böttner A, Kratzsch J. 2003. The soluble leptin receptor is crucial for leptin action: evidence from clinical and experimental data. Int J Obes Relat Metab Disord 27:1472–1478.

Journal of Anatomy

J. Anat. (2008) **213**, pp555–564 doi: 10.1111/j.1469-7580.2008.00975.x

Human sperm anatomy: ultrastructural localization of 1α,25-dihydroxyvitamin D_3 receptor and its possible role in the human male gamete

Saveria Aquila,[1,2] Carmela Guido,[1,3] Ida Perrotta,[4] Sandro Tripepi,[4] Alfonso Nastro[5] and Sebastiano Andò[2,3,4]

[1]*Department of Pharmaco-Biology,* [2]*Centro Sanitario,* [3]*Faculty of Pharmacy,* [4]*Department of Ecology,* [5]*Department of Territorial Planning, Faculty of Chemical Engineering, University of Calabria, 87036 Arcavacata di Rende, Cosenza, Italy*

Abstract

Previous studies have suggested that 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] has a role in reproductive function. Gonadal insufficiencies were observed as a result of 1,25$(OH)_2D_3$ deficiency and in 1,25$(OH)_2D_3$ receptor (VDR) null mutant mice. To study human sperm anatomy at the molecular level, we first evaluated the ultrastructural localization of VDR by immunogold electron microscopy using a monoclonal antibody against amino acids 344–424 of human VDR, in normozoospermic samples. Intriguingly, VDR was associated predominantly with the cell nucleus. In fact, it is known that VDR is a transcription factor, and that in vitamin-D-depleted animals, VDR is largely localized in the cell nucleus. To assess the significance of VDR in the male gamete, we investigated the role of 1,25$(OH)_2D_3$/VDR in sperm survival and capacitation. Our results revealed that the action of 1,25$(OH)_2D_3$ depended on its concentration because although lower doses induced cholesterol efflux, protein phosphorylation and sperm survival, a higher concentration seemed to be ineffective or did not show an increased effect. These results increase our knowledge of human sperm anatomy at the molecular level and suggest that 1,25$(OH)_2D_3$/VDR may have an important role in sperm survival and the acquisition of fertilizing ability.

Key words 1,25$(OH)_2D_3$ receptor (VDR); 1α,25-dihydroxyvitamin D_3. sperm; male reproduction; nuclear receptors.

Introduction

Vitamin D_3 is synthesized in the epidermis. The hormone is then metabolized in the liver and afterwards in the kidney to give 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), which is the most biologically active metabolite of vitamin D (Kuritzky et al. 2008). The functional activities of 1,25$(OH)_2D_3$ require a high-affinity receptor, the 1,25$(OH)_2D_3$ receptor (VDR), which is a member of the superfamily of nuclear steroid hormone receptors. In addition to its well-known effects on calcium and phosphate homeostasis, 1,25$(OH)_2D_3$, which is also known as calcitriol, acts on a variety of tissues (Johnson & DeLuca, 2001). This may be due to the presence of VDR in more than 30 tissues, which include the brain, pancreas, pituitary, skin, muscle, placenta, immune cells, parathyroid, ovary, prostate, and testis (Habib et al. 1990; Johnson et al. 1996; Chatterjee, 2001).

Correspondence
Sebastiano Andò, Faculty of Pharmacy-University of Calabria, Arcavacata di Rende (Cosenza) 87036, Italy. T: +39 0984 496201
E: sebastiano.ando@unical.it

This work was supported by PRIN – MIUR and Ex 60% -2007.

Accepted for publication *22 July 2008*

In the male genital tract of rodents, VDR has been found in the smooth muscle of the epididymis, spermatogonium and Sertoli cells. The extensive presence of binding sites for 1,25$(OH)_2D_3$ detected in Sertoli cells and the caput epididymis at the time of spermiogenesis suggests that 1,25$(OH)_2D_3$ is involved in spermatogenesis and in sperm maturation in rats (Johnson et al. 1996). VDR null mutant mice demonstrate significant gonadal insufficiency, with decreased sperm count and motility, and histological abnormalities of the testis (Kinuta et al. 2000). The testes of 1,25$(OH)_2D_3$-depleted animals show incomplete spermatogenesis, impaired development, and degenerative changes in the seminiferous tubules. These results suggest that 1,25$(OH)_2D_3$ plays a very important role in the maturation of germ cells. Therefore, it can be concluded that 1,25$(OH)_2D_3$ is involved in male reproduction, presumably at the molecular level.

The VDR mediates 1,25$(OH)_2D_3$-dependent responses through both genomic and non-genomic pathways (Norman et al. 1997, 2004). With regard to the latter, 1,25$(OH)_2D_3$ can rapidly stimulate phosphoinositide metabolism, protein kinase C (PKC), and mitogen-activated protein (MAP) kinases, as well as increase cytosolic calcium and cGMP levels (Barsony & Marx, 1991; Massheimer et al. 1994;

Buitrago et al. 2002; Rebsamen et al. 2002). Several nuclear receptors (El-Hefnawy et al. 2000; Aquila et al. 2004) have been reported to be expressed in ejaculated human spermatozoa, and to regulate cellular processes through non-genomic mechanisms. Recently, it has been shown by Western blotting and immunohistochemistry that human sperm express the VDR (Corbett et al. 2006). However, the significance of $1,25(OH)_2D_3$ and its receptor in male reproduction needs to be investigated further, and the ultrastructural localization of VDR in the male gamete has not yet been reported in the literature.

The subcellular localization of VDR in the sperm nucleus is reported here. We employed immunogold electron microscopy to gain an improved understanding of sperm anatomy at the molecular level. In addition, we studied the possible involvement of $1,25(OH)_2D_3$/VDR in important biological functions of human sperm, such as capacitation and survival.

Materials and methods

Chemicals

Percoll (PVP-coated colloidal silica particles for cell separation), sodium bicarbonate, sodium lactate, sodium pyruvate, dimethyl sulfoxide (DMSO), Earle's Balanced Salt Solution (EBSS), 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and all other chemicals were purchased from Sigma Chemical (Milan, Italy). Acrylamide/bisacrylamide was purchased from Labtek Eurobio (Milan, Italy). Triton X-100 and Eosin Y were purchased from Farmitalia Carlo Erba (Milan, Italy). The ECL Plus Western blotting detection system, Hybond™ ECL™, and HEPES sodium salt were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). The cholesterol-oxidase (CHOD)-peroxidase (POD) enzymatic colorimetric kit was from Inter-Medical (Biogemina Italia Srl, Catania, Italy). The colloidal gold-conjugated goat anti-mouse IgG secondary antibody (Ab) was purchased from Sigma-Aldrich (Milan, Italy). The goat polyclonal actin Ab (1–19), mouse monoclonal anti-human VDR (D-6) Ab, mouse monoclonal anti-phosphotyrosine PY99 Ab, peroxidase-coupled anti-mouse and anti-goat IgG secondary Abs were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal anti-phosphothreonine Ab was purchased from Calbiochem (Nottingham, UK).

Semen samples and spermatozoa preparations

Human semen was collected according to the recommended procedure of the World Health Organization (WHO) by masturbation from healthy volunteer donors of proven fertility, who were undergoing semen analysis in our laboratory. Spermatozoa preparations were made as described previously (Aquila et al. 2005). Briefly, semen samples with normal measurements for volume, sperm count, motility, vitality and morphology, according to the WHO Laboratory Manual (WHO, 1999), were included in this study. For each experiment, three normozoospermic samples were pooled. Pooled sperm were washed, subjected to the indicated treatments, and incubated at 37 °C and 5% CO_2. Prior to centrifugation to collect sperm, several aliquots were used to analyse sperm viability. The study had been approved by the local medical ethics committee and all participants gave their informed consent.

Processing of ejaculated sperm

After liquefaction, normal semen samples were pooled and subjected to centrifugation for 7 min at 800 *g* on a discontinuous Percoll density gradient (80 : 40% v : v) (WHO, 1999). The 80% Percoll fraction was examined using an optical microscope that was equipped with a ×100 oil immersion objective to ensure that a pure sample of sperm was obtained. An independent observer inspected the cells and examined several fields for each slide. Percoll-purified sperm were washed with non-supplemented EBSS (incapacitating medium) and were incubated for 30 min at 37 °C and 5% CO_2, either with no additional treatment (control, NC) or with treatment (experimental). The treatments were as follows: increasing doses of $1,25(OH)_2D_3$ (0.01, 0.1 and 1 nM), or anti-VDR Ab (1 μg mL^{-1}) combined with 0.1 nM $1,25(OH)_2D_3$. When the cells were treated with the anti-VDR Ab, a pretreatment of 15 min was performed.

Immunogold labelling of VDR

Sperm that had been fixed overnight in 4% paraformaldehyde were washed in phosphate-buffered saline (PBS) to remove excess fixative, and dehydrated in a graded series of alcohol. They were then infiltrated with LR white resin, which was polymerized in a vacuum oven at 45 °C for 48 h. Ultrathin 60-nm sections were cut and placed on coated nickel grids for post-embedding immunogold labelling with a mouse anti-human VDR monoclonal Ab VDR (D-6), which was raised against amino acids 344–424 of human VDR. Potential non-specific labelling was blocked by incubating the sections in PBS containing 5% normal goat serum, 5% bovine serum albumin and 0.1% cold water fish gelatine at room temperature for 1 h. Sections were then incubated overnight at 4 °C with the mouse monoclonal anti-VDR primary Ab at a dilution of 1 : 500 in PBS. This was followed by incubation with 10 nm colloidal gold-conjugated goat anti-mouse IgG secondary Ab at a dilution of 1 : 50 for 2 h at room temperature. The sections were subsequently washed in PBS, fixed in glutaraldehyde, counterstained with uranyl acetate and lead acetate, and examined using a Zeiss EM 900 transmission electron microscope. To assess the specificity of the immunolabelling, a negative control was performed in which sperm sections were incubated with the colloidal gold-conjugated secondary Ab but not the primary Ab.

Evaluation of sperm viability

Sperm viability was analysed by red-eosin exclusion, using Eosin Y, to evaluate the potentially toxic effects of the treatments. An aliquot of each sperm sample, which had been incubated in the absence (NC) or presence of increasing concentrations of $1,25(OH)_2D_3$ (0.01–1 nM) or 1 μg mL^{-1} anti-VDR Ab combined with 0.1 nM $1,25(OH)_2D_3$, was examined by optical microscopy after incubation with Eosin Y.

An independent observer scored 200 cells for stain uptake (dead cells) or exclusion (live cells), and sperm viability was expressed as the percentage of total live sperm.

Measurement of cholesterol in sperm culture medium

Cholesterol levels in the medium in which the sperm had been incubated were measured in duplicate by a CHOD-POD enzymatic colorimetric method according to the manufacturer's instructions

(Aquila et al. 2006). Sperm samples that had been washed twice with incapacitating medium were incubated in the same medium with or without the above-described treatments for 30 min at 37 °C and 5% CO_2. At the end of the incubation period, the culture media were recovered by centrifugation and lyophilized. The samples were subsequently dissolved in 1 mL reaction buffer, and incubated for 10 min at room temperature. The cholesterol content was measured by spectrophotometry at 505 nm. A cholesterol standard of 200 mg dL^{-1} was used. The limit of sensitivity for the assay was 0.05 mg dL^{-1}. Inter- and intra-assay variations were 0.04 and 0.03%, respectively. The results were presented as mg cholesterol per 1×10^7 spermatozoa.

Western blot analysis of sperm proteins

Sperm samples that had been washed twice with EBSS were incubated in the absence (NC) or presence of the indicated treatments, and then centrifuged for 5 min at 5000 *g*. The pellets were resuspended in lysis buffer as described previously (Aquila et al. 2002). Equal amounts of sperm protein (80 μg) were boiled for 5 min, separated by electrophoresis on a 10% polyacrylamide gel, transferred to nitrocellulose sheets, and probed with an appropriate dilution of Ab. Binding of the secondary Ab was revealed using the ECL Plus Western blotting detection system (GE Healthcare), according to the manufacturer's instructions. For an internal control, all the membranes were stripped (0.2 M glycine, pH 2.6 for 30 min at room temperature) and re-probed with an anti-β-actin Ab.

Statistical analysis

The immunogold labelling was repeated on at least three independent occasions, whereas Western blot analysis was performed in a further six independent experiments. The data that were obtained from the CHOD-POD enzymatic colorimetric analysis (six replicate experiments using duplicate determinations) and the viability analysis (six replicate experiments using duplicate determinations) were presented as the mean ± SE. The differences in mean values were calculated using analysis of variance (ANOVA) with a significance level of $P \leq 0.05$.

Results

Immunogold localization of VDR in human sperm

The general appearance of the human whole sperm mount revealed good structural preservation (Fig. 1): the cytoskeletal elements, the neck (segmented columns), and the outer dense fibres were all well defined. The majority

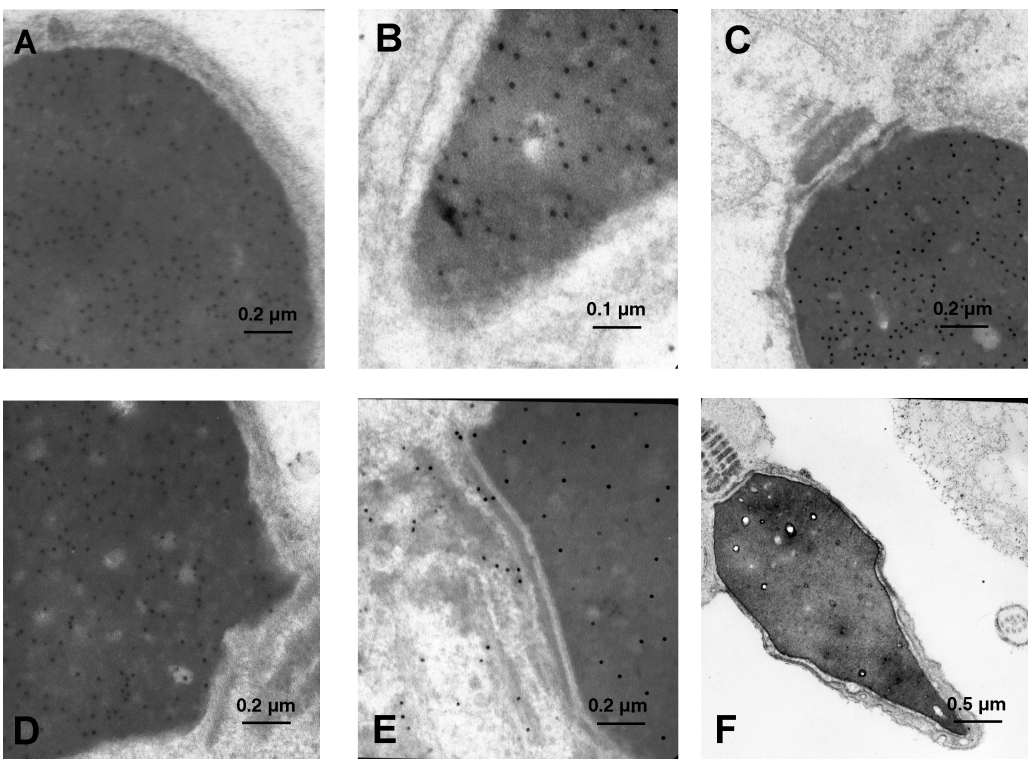

Fig. 1 Ultrastructural localization of VDR in human ejaculated spermatozoa. Subcellular localization of VDR in human ejaculated spermatozoa by immunoelectron microscopy. Electron micrographs of sperm allowed to react with Ab directed against VDR. (A) Region of the head (×50 000, scale bar = 0.2 μm). (B) Region of the apical head (×81 000, scale bar = 0.1 μm). (C and D) Region of the neck (×29 000–50 000, scale bar = 0.2 μm). (E) Region of the neck in which few isolated gold particles are present (×50 000, scale bar = 0.2 μm). (F) Electron micrograph of control section incubated without the primary Ab, in which the sperm are totally without reaction product (×12 000, scale bar = 0.5 μm).

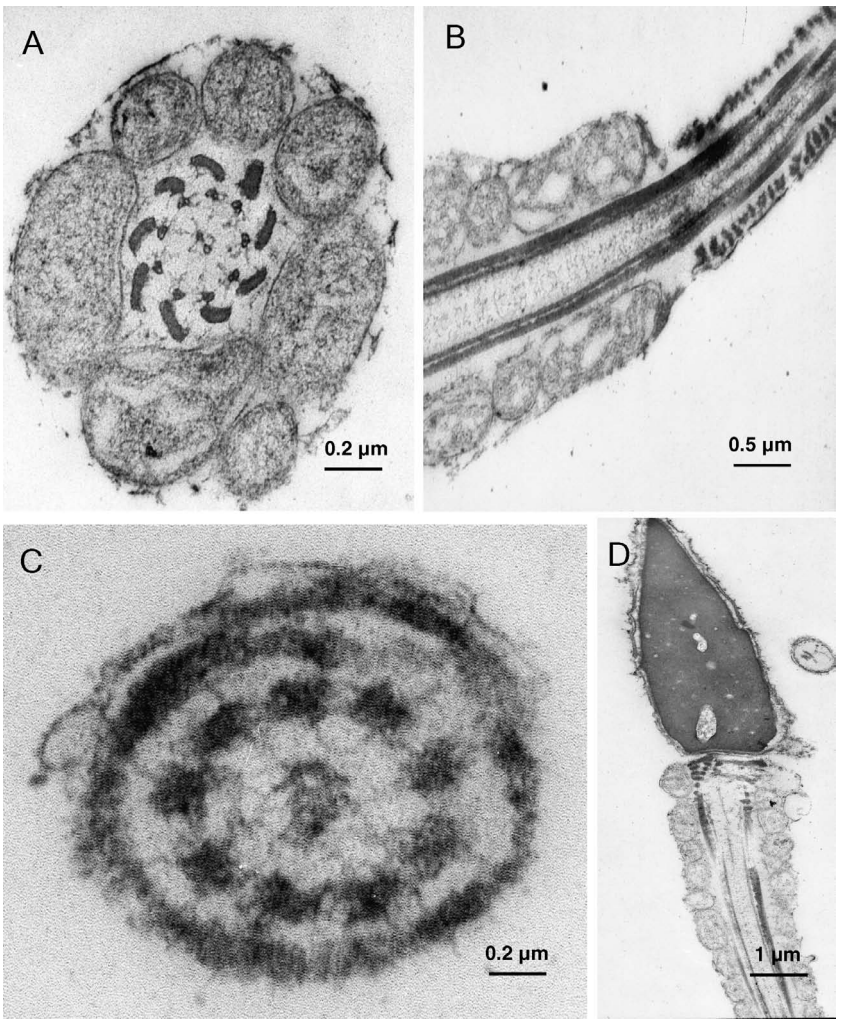

Fig. 2 VDR is absent in the midpiece and along the tail in human spermatozoa. Subcellular localization of VDR in human ejaculated spermatozoa by immunoelectron microscopy. Electron micrographs of sperm allowed to react with Ab directed against VDR. (A) Electron micrograph of a cross-section of the tail probed with anti-VDR Ab, which shows a region of the midpiece (×30 000, scale bar = 0.2 μm). (B) Sagittal section through the tail probed with anti-VDR Ab, which shows the transition between the middle piece with its mitochondria and the principal piece with its fibrous sheath (×18 000, scale bar = 0.5 μm). (C) Electron micrograph of a cross-section of the tail probed with anti-VDR Ab, which shows a region of the end piece (×65 000, scale bar = 0.2 μm). (D) In the negative control section, no immunoreaction was present (×4000, scale bar = 1 μm).

of the sperm showed normal morphological features and the general cell ultrastructure corresponded exactly to that described previously (Friedlaender, 1952). Ultrastructural immunogold analysis of 60-nm ultrathin sections revealed that the VDR was associated particularly with the sperm head. In fact, the gold particles appeared to be clustered in the sperm nucleus (Fig. 1A–D) and were not present in the cytoplasm or the membranes in the head. The chromatin was homogeneous and showed no remarkable changes; there were some clear areas as described previously (Lung, 1972). All the samples showed an identical staining intensity and localization of the immunoreactive components. Some particles also decorated the neck (Fig. 1E). In all the samples, neither the axoneme and outer dense fibres of the mid-piece, which are covered by the mitochondria, nor the tail of the sperm were labelled (Figs 1C and 2A–C). This was observed in transverse and sagittal sections throughout the end-piece. Simultaneous negative control experiments with the secondary Ab alone did not show any labelling (Figs 1F and 2D).

$1,25(OH)_2D_3$ modulates cholesterol efflux in human sperm

Due to the fact that it had never been investigated previously, we evaluated whether $1,25(OH)_2D_3$ could influence extratesticular sperm maturation by examining its effect on capacitation.

Capacitation is a post-ejaculation process that is associated with the acquisition of the ability to fertilize an egg, and encompasses different features. Cholesterol efflux across the sperm membrane contributes to one signalling mechanism that controls sperm capacitation (Aquila et al. 2006 and references therein). To evaluate cholesterol efflux, the sperm were collected by centrifugation after they had been treated as described above. Cholesterol levels in the incubation medium were measured, and the sperm were lysed to evaluate protein phosphorylation. Our results showed a significant increase in cholesterol efflux (Fig. 3) upon treatment with 0.01 and 0.1 nM $1,25(OH)_2D_3$, but 1 nM $1,25(OH)_2D_3$ did not induce a further

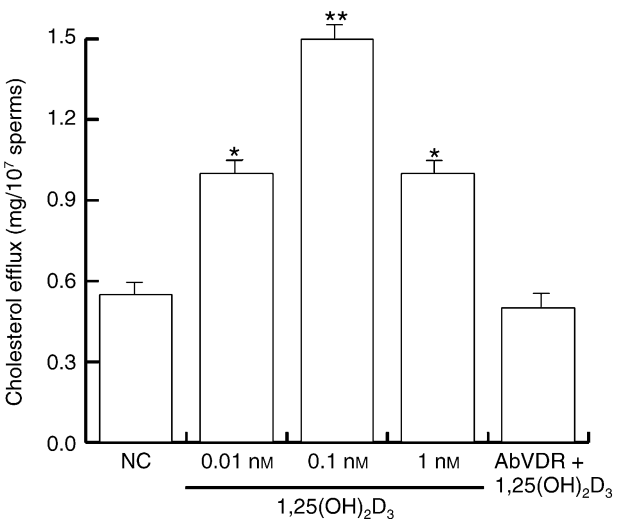

Fig. 3 $1,25(OH)_2D_3$ modulates cholesterol efflux in human sperm. Washed spermatozoa were incubated in non-supplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or presence of increasing $1,25(OH)_2D_3$ concentrations (0.01, 0.1 and 1 nM) or with 0.1 nM $1,25(OH)_2D_3$ combined with anti-VDR Ab. Cholesterol in culture medium from human ejaculated spermatozoa was measured by enzymatic colorimetric assay. Columns are mean ± SE of six independent experiments performed in duplicate. Data are expressed in mg/10^7 sperms. $*P < 0.05$ vs. control, $**P < 0.01$ vs. control.

increase. This effect was reversed by co-treatment with the anti-VDR Ab, which suggested that VDR was involved in the induction of sperm cholesterol efflux.

$1,25(OH)_2D_3$ influences the phosphorylation of proteins on tyrosine and threonine residues in human sperm

Cholesterol efflux across the sperm membrane is a key priming event in the capacitation process because it initiates signalling events that lead to the phosphorylation of sperm proteins (Visconti et al. 1995; Osheroff et al. 1999). Therefore, we investigated whether $1,25(OH)_2D_3$ was also able to influence this important feature of the capacitation process. A significant increase in both tyrosine (Fig. 4A) and threonine phosphorylation (Fig. 4B) was observed with increasing concentrations of $1,25(OH)_2D_3$ (0.01–0.1 nM), whereas 1 nM $1,25(OH)_2D_3$ did not induce a significant increase. In both cases, the anti-VDR Ab abolished the 0.1 nM $1,25(OH)_2D_3$-induced effect. It is important to point out that, under our experimental conditions, the anti-phosphoprotein Abs that were used recognized protein bands that were predominantly within the same size ranges as those that have been documented previously (Naz, 1999). For tyrosine phosphorylation we measured the density of the 95-kDa band; for threonine phosphorylation we used the 110-kDa band.

To investigate whether the protein phosphorylation in sperm that was induced by 0.1 nM $1,25(OH)_2D_3$ represented an early event, we performed a time-course study (0, 5, 10, 30 and 60 min). This experiment revealed that both types of phosphorylation followed a similar pattern: they were observed at 5 min, increased at 10 min and were sustained until 30 min, and then dropped significantly at 1 h (Fig. 5A,B).

Effect of $1,25(OH)_2D_3$ on sperm survival

As shown in Fig. 6, sperm viability was significantly increased in the presence of 0.01 and 0.1 nM $1,25(OH)_2D_3$, whereas 1 nM $1,25(OH)_2D_3$ did not affect sperm viability as compared to the controls. Interestingly, the effect that was induced by 0.1 nM $1,25(OH)_2D_3$ was reversed in the presence of the anti-VDR Ab, which suggested that the effect was mediated by VDR.

Discussion

The archetypal target tissues of $1,25(OH)_2D_3$ include bone, intestine and kidney. Recently, it has become clear that $1,25(OH)_2D_3$ has many additional functions that are not closely linked to its classical role as a skeletal regulator. The importance of this secosteroid in reproduction has emerged from studies that have demonstrated that $1,25(OH)_2D_3$ deficiency results in reduced fertility in rats (Kinuta et al. 2000). However, the function of $1,25(OH)_2D_3$ in the genitourinary organs is unknown, even though this has become a widely researched area. Here, with the aim of studying human sperm anatomy at the molecular level, we investigated for the first time the ultrastructural compartmentalization of VDR in the human male gamete. In addition, we evaluated the molecular mechanisms through which $1,25(OH)_2D_3$ may affect important sperm functions such as the acquisition of fertilizing ability and viability.

The intracellular localization of VDR in somatic cells has been an issue of some controversy because there is not complete agreement about whether VDR is translocated to the cell nucleus in response to $1,25(OH)_2D_3$, or whether it shuttles continuously between the cytosol and nucleus (Shaffer et al. 2005). In addition, it appears that the classic VDR is also associated with the caveolae that are present in the plasma membrane in some cell types, as has been reported for other steroid receptors (Levin, 2002; Huhtakangas et al. 2004). The structure of human spermatozoa has proved rather difficult to elucidate because of the opacity of the head to electron penetration. Therefore, in spite of active interest, our understanding of the ultrastructural organization of ejaculated human sperm is still incomplete, and our knowledge of the molecular anatomy of sperm is very limited.

In our study, immunogold analysis showed that VDR was localized uniformly in the sperm nucleus, although some

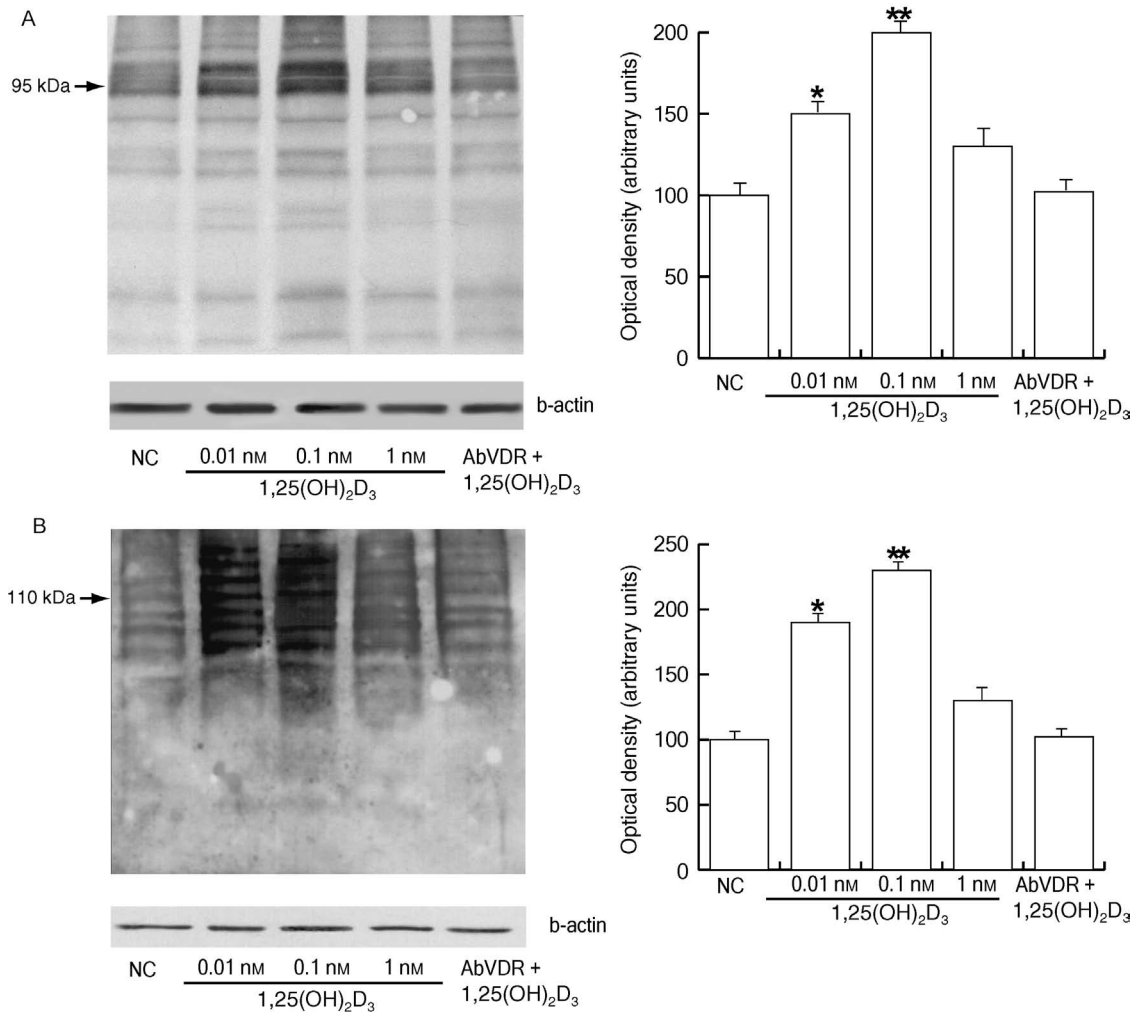

Fig. 4 $1,25(OH)_2D_3$ affects tyrosine and threonine phosphorylation of the human sperm proteins. Washed spermatozoa were incubated in non-supplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or presence of increasing $1,25(OH)_2D_3$ concentrations (0.01, 0.1 and 1 nM) or with 0.1 nM $1,25(OH)_2D_3$ combined with anti-VDR Ab. Sperm lysates of 80 μg were used for Western blot analysis. Actin was used as a loading control. (A) Protein tyrosine phosphorylation. On the right, quantitative representation after densitometric evaluation of the 95-kDa band. (B) Protein threonine phosphorylation. On the right, quantitative representation after densitometric evaluation of the 110-kDa band. Autoradiographs presented are representative examples of experiments that were performed at least six times with repetitive results. $*P < 0.05$ vs. control, $**P < 0.01$ vs. control.

particles also decorated the neck of the sperm. In somatic cells, the receptor–hormone complex becomes localized to the nucleus and then interacts with the $1,25(OH)_2D_3$-responsive element. This binding modifies transcription of the target genes. In addition to their classic genomic action, nuclear receptors regulate cellular processes through a non-genomic mechanism (Losel et al. 2003; Norman et al. 2004). It is generally accepted that the sperm nucleus is transcriptionally inactive due to the highly condensed architecture of its chromatin (Grunewald et al. 2005). In this study, we investigated the rapid effects of the VDR, which we have also observed previously for other nuclear receptors in human sperm (Aquila et al. 2004, 2006, 2007). Indeed, this mode of action seems to be particularly appropriate in the male gamete because sperm functions need to be activated rapidly to accommodate dynamic changes in the surrounding milieu. In addition, sperm have a highly differentiated architecture, and their peculiar anatomy compartmentalizes proteins and other molecules within the areas in which they are needed.

An interesting finding in somatic cells may provide a possible explanation for the significance of the presence of VDR in the sperm nucleus. It has been reported that the VDR is closely related to the nuclear matrix, or part of it, and has a role in ensuring genomic stability (Nangia et al. 1998). DNA is generally considered to be the most critical cellular target for the lethal mutagenic effects of drugs, radiation and environmental chemicals. As Chatterjee has

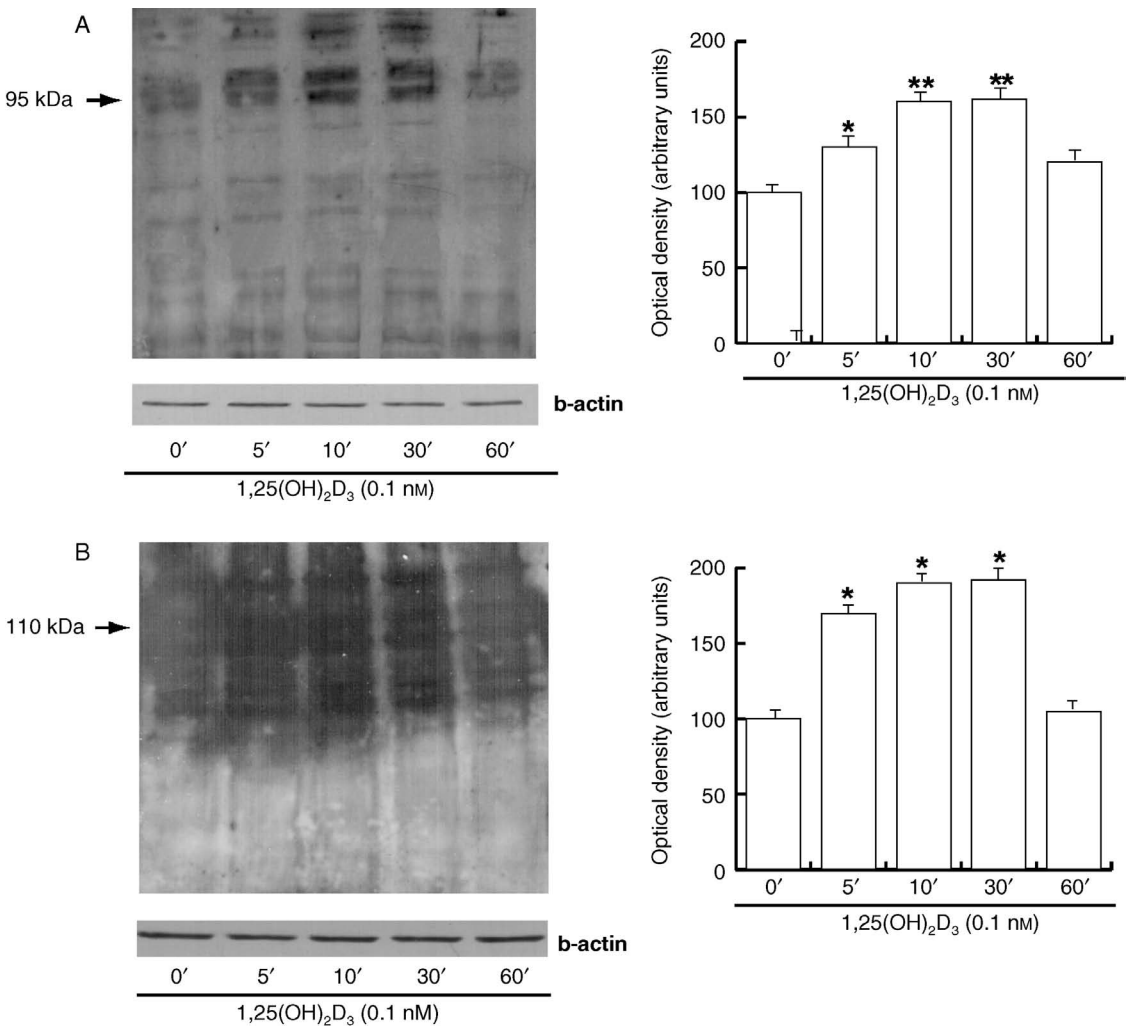

Fig. 5 Time course of the effect of $1,25(OH)_2D_3$ on tyrosine and threonine phosphorylation of sperm proteins. Time-course study (0, 5, 10, 30 and 60 min) in sperm treated with 0.1 nM $1,25(OH)_2D_3$. Actin was used as a loading control. On the right side are reported the quantitative representations after densitometric evaluation as indicated above. Autoradiographs presented are representative examples of experiments that were performed at least six times with repetitive results. $*P < 0.05$ vs. control, $**P < 0.01$ vs. control.

reported, vitamin D stabilizes chromosomal structure and prevents the induction of DNA double-strand breaks by endogenous or exogenous factors (Chatterjee, 2001). Recently, it has been suggested that the sperm nuclear matrix plays a crucial role in the regulation of DNA fragmentation and degradation, both before and after fertilization (Shaman et al. 2007). The accurate transferral of genetic information to progeny is a prerequisite for the conservation and evolution of a species. Therefore, proper control of the integrity of sperm DNA is critical for the maintenance of genome stability, and the speculation that VDR in the sperm nucleus acts as a protective genomic factor is very intriguing.

Successful sperm maturation depends on sequential steps in both the male and female reproductive tracts. After ejaculation, the male gamete must undergo capacitation to be capable of fertilizing an egg; this is a prerequisite for fertilization by mammalian spermatozoa. Capacitation, which *in vivo* occurs within the female reproductive tract, and which can also be accomplished in defined media *in vitro*, confers on the sperm the ability to undergo the acrosome reaction (Yanagimachi, 1994; Visconti et al. 1995). Capacitation has been associated with modifications in cholesterol efflux, plasma membrane fluidity, intracellular ion concentrations, metabolism, and motility, and with the increased phosphorylation of a number of proteins. It has been demonstrated that cholesterol efflux is a priming event that induces changes in the fluidity of the plasma membrane, and sequentially stimulates the activation of sperm adenylyl cyclase and phosphorylation of sperm proteins at tyrosine and threonine residues (Visconti et al. 1995; Visconti & Kopf,

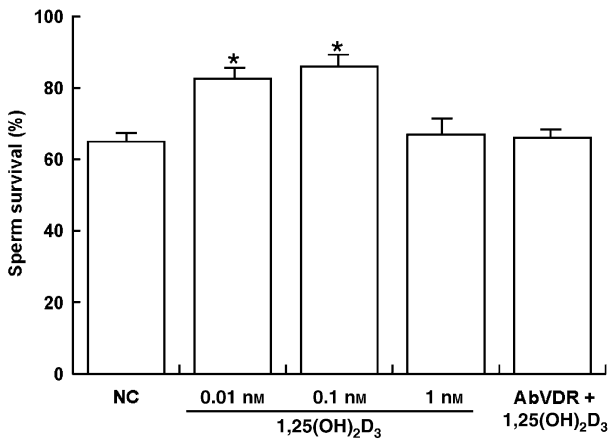

Fig. 6 $1,25(OH)_2D_3$ modulates human sperm survival. Washed spermatozoa were incubated in non-supplemented Earle's medium for 30 min at 37 °C and 5% CO_2, in the absence (NC) or presence of increasing $1,25(OH)_2D_3$ concentrations (0.01, 0.1 and 1 nM) or with 0.1 nM $1,25(OH)_2D_3$ combined with anti-VDR Ab. Columns are mean ± SE of six independent experiments performed in duplicate. $*P < 0.05$ vs. control.

1998; Osheroff et al. 1999; Naz, 1999). Recently, treatment with $1,25(OH)_2D_3$ has been shown to elevate intracellular cAMP levels within 10 min in human syncytiotrophoblast cells (Avila et al. 2007). In sperm, $1,25(OH)_2D_3$ might play a role in capacitation by inducing firstly an increase in cAMP levels and then phosphorylation of certain proteins. It is known that phosphorylation and cholesterol efflux are early phases of the capacitation process; however, the molecular mechanisms and signal transduction pathways that are involved have only been defined partially.

In our study, certain concentrations of $1,25(OH)_2D_3$ were able to modulate both cholesterol efflux and tyrosine and threonine phosphorylation. The effects of $1,25(OH)_2D_3$ appeared to be very dependent on the concentration of the hormone. Intriguingly, the $1,25(OH)_2D_3$-mediated biphasic rapid response that was obtained for cholesterol efflux in our study was reminiscent of the $1,25(OH)_2D_3$-mediated stimulation of the rapid transport of Ca^{2+} (transcaltachia) in the perfused chick intestine, which also follows a biphasic dose–response curve (De Boland & Norman, 1990; Norman, 2006). The outcome of signalling activation can depend on differences in ligand concentration, and this effect has also been recently demonstrated in human sperm (Aquila et al. 2004, 2006, 2007; Andò & Aquila, 2005).

In different mammalian species, sperm may utilize distinct survival strategies, which are probably related to the differing management of energy metabolism. To date, two distinctive phenotypes have been observed, which are characterized by sperm survival under laboratory and *in vivo* conditions. These can be represented by the boar sperm phenotype, which corresponds to a short survival time inside the female vaginal tract (~48 h) (Viring & Eirnasson, 1981), and by the human sperm phenotype, which corresponds to a long survival time inside the female vaginal tract (~ 1 week) (Feldman & Nelson, 1987). Long-term sperm viability in humans may be an important evolutionary adaptation because it is necessary for the sperm to reach the oocyte whilst it occupies the correct position in the fallopian tubes. As the oocyte may not be in this specific location when the sperm reach it, the sperm may have to wait until this event occurs. It emerged from our study that VDR has the ability to modulate sperm survival, which suggests that it could play an important role in this aspect of the physiology of the human male gamete We observed that low doses of $1,25(OH)_2D_3$ stimulated the effect, whereas a higher concentration was ineffective.

Most of the classical physiological actions of $1,25(OH)_2D_3$ have been known since the early part of this century, when dietary vitamin D deficiency was first demonstrated. However, non-classical roles have emerged from studies that have probed the mechanism of $1,25(OH)_2D_3$ action at the molecular level, and from studies of the VDR knockout mouse. Furthermore, the ubiquitous distribution of VDR opens the possibility of unforeseen biological functions of $1,25(OH)_2D_3$. From our results, it emerged that VDR was involved both in the early phases of the functional maturation of ejaculated sperm and in sperm survival. Interestingly, these effects of the hormone were observed at lower doses of $1,25(OH)_2D_3$, whereas a higher concentration was ineffective or did not have a greater effect. It is important to note that human physiological serum levels of $1,25(OH)_2D_3$ are between 37.5 and 150 pM (15–60 pg mL^{-1}) (Masuda & Jones, 2006), which correlates well with our results.

As concerns the rapid effects that we observed, it is possible that sperm might respond to $1,25(OH)_2D_3$ by a VDR-independent mechanism. Schwartz et al. (2002) have observed, in the growth zone of chondrocytes, that $1,25(OH)_2D_3$ activates PKCα by activating phospholipase C-β1 and -β3 through the G-protein Gq, and this activation is not reduced in cells from VDR null mice. A similar mode of action of $1,25(OH)_2D_3$ is also proposed in sperm, on the basis of the predominantly nuclear anatomic location of the VDR.

The discovery that $1,25(OH)_2D_3$ influences sperm function may be useful for the development of novel therapeutic approaches to the treatment of male reproductive disorders. However, the physiological significance and the specific roles of $1,25(OH)_2D_3$/VDR in the human male gamete require further investigation.

Acknowledgements

Our special thanks to Dr Vincenzo Cunsulo (Biogemina Italia Srl, Catania, Italy). We would also like to thank Perrotta Enrico for

excellent technical assistance, and Serena and Maria Clelia Gervasi for language revision.

References

Andò S, Aquila S (2005) Arguments raised by the recent discovery that insulin and leptin are expressed in and secreted by human ejaculated spermatozoa. *Mol Cell Endocrinol* **245**, 1–6.

Aquila S, Sisci D, Gentile M, Middea E, Siciliano L, Andò S (2002) Human ejaculated spermatozoa contain active P450 aromatase. *J Clin Endocrinol Metab* **87**, 3385–3390.

Aquila S, Sisci D, Gentile M, et al. (2004) Estrogen receptor (ER)alpha and ER beta are both expressed in human ejaculated spermatozoa: evidence of their direct interaction with phosphatidylinositol-3-OH kinase/Akt pathway. *J Clin Endocrinol Metab* **89**, 1443–1451.

Aquila S, Gentile M, Middea E, et al. (2005) Leptin secretion by human ejaculated spermatozoa. *J Clin Endocrinol Metab* **90**, 4753–4761.

Aquila S, Bonofiglio D, Gentile M, et al. (2006) Peroxisome proliferator-activated receptor (PPAR)gamma is expressed by human spermatozoa: its potential role on the sperm physiology. *J Cell Physiol* **209**, 977–986.

Aquila S, Middea E, Catalano S, et al. (2007) Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway. *Hum Reprod* **22**, 2594–2605.

Avila E, Díaz L, Barrera D, et al. (2007) Regulation of Vitamin D hydroxylases gene expression by 1,25-dihydroxyvitamin D_3 and cyclic AMP in cultured human syncytiotrophoblasts. *J Steroid Biochem Mol Biol* **103**, 90–96.

Barsony J, Marx SJ (1991) Rapid accumulation of cyclic GMP near activated vitamin D receptors. *Proc Natl Acad Sci U S A* **15**, 1436–1440.

Buitrago C, Gonzalez PV, Russo DB, Boland R (2002). Activation of RAF-1 through RAS and PKC mediates 1,25(OH)2D3 regulation of the map kinase pathway in muscle cells. *J Biol Chem* **278**, 2199–2205.

Chatterjee M (2001) Vitamin D and genomic stability. *Mutat Res* **18**, 69–87.

Corbett ST, Hill O, Nangia AK (2006) Vitamin D receptor found in human sperm. *Urology* **68**, 1345–1349.

De Boland AR, Norman AW (1990) Influx of extracellular calcium mediates 1,25-dihydroxyvitamin D3-dependent transcaltachia (the rapid stimulation of duodenal Ca2+ transport). *Endocrinology* **127**, 2475–2480.

El-Hefnawy T, Manna PR, Luconi M, Baldi E, Slotte PJ, Huhtaniemi (2000) Progesterone action in a murine Leydig tumor cell line (mLTC-1), Possibly through a nonclassical receptor type. *Endocrinology* **141**, 247–255.

Feldman EC, Nelson RW (1987) In *Canine and Feline Endocrinology and Reproduction* (ed. Pedersen E), pp. 420–421, Philadelphia: WB Saunders.

Friedlaender MH (1952) Observations on the structure of human spermatozoa; an electron microscope inquiry. *Proc R Soc Lond B Biol Sci* **27**, 60–69.

Grunewald S, Paasch U, Glander HJ, Anderegg U (2005) Mature human spermatozoa do not transcribe novel RNA. *Andrologia* **37**, 69–71.

Habib FK, Maddy SQ, Gelly KJ (1990) Characterisation of receptors for 1,25-dihydroxyvitamin D_3 in the human testis. *Steroid Biochem* **35**, 195–199.

Huhtakangas JA, Olivera CJ, Bishop JE, Zanello LP, Norman AW (2004) The Vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 alpha,25(OH)2-vitamin D3 *in vivo* and *in vitro*. *Mol Endocrinol* **18**, 2660–2671.

Johnson JA, Grande JP, Roche PC, Kumar R (1996) Immunohistochemical detection and distribution of the 1,25-dihydroxyvitamin D3 receptor in rat reproductive tissues. *Histochem Cell Biol* **105**, 7–15.

Johnson LE, DeLuca HF (2001) Vitamin D receptor null mutant mice fed high levels of calcium are fertile. *J Nutr Jun* **131**, 1787–1791.

Kinuta K, Tanaka H, Moriwake T, Aya K, Kato S, Seino Y (2000) Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads. *Endocrinology* **141**, 1317–1324.

Kuritzky LA, Finlay-Jones JJ, Hart PH (2008) The controversial role of vitamin D in the skin: immunosuppression vs. photoprotection. *Clin Exp Dermatol* **33**, 167–170.

Levin R (2002) Cellular function of plasma membrane estrogen receptor. *Steroids* **67**, 471–475.

Losel RM, Falkeistein E, Feuring M, et al. (2003) Nongenomic steroid action: controversies, questions and answers. *Physiol Rev* **83**, 965–1016.

Lung B (1972) Ultrastructure and chromatin disaggregation of human sperm head with thioglycolate treatment. *Journal of Cell Biology* **52**, 179–186.

Massheimer V, Boland R, De Boland AR (1994) Rapid 1,25$(OH)_2$-vitamin D_3 stimulation of calcium uptake by rat intestinal cells involves a dihydropyridine-sensitive cAMP-dependent pathway. *Cell Signal* **3**, 299–304.

Masuda S, Jones G (2006) Promise of vitamin D analogues in the treatment of hyperproliferative conditions. *Mol Cancer Ther* **5**, 797–808.

Nangia AK, Butcher JL, Konety BR, Vietmeie BN, Getzenberg RH (1998) Association of vitamin D receptors with the nuclear matrix of human and rat genitourinary tissues. *Steroid Biochem* **66**, 241–246.

Naz RK (1999) Involvement of protein serine and threonine phosphorylation in human sperm capacitation. *Biol Reprod* **60**, 1402–1409.

Norman AW (2006) Minireview: Vitamin D receptor: new assignments for an already busy receptor. *Endocrinology* **147**, 5542–5548.

Norman AW, Okamura WH, Hammond MW, et al. (1997) Comparison of 6-*s-cis* and 6-*s-trans* locked analogs of 1,25$(OH)_2$-vitamin D_3 indicates that the 6-*s-cis* conformation is preferred for rapid nongenomic biological responses and that neither 6-s-*cis* nor 6-s-*trans* locked analogs are preferred for genomic biological responses. *Mol Endocrinol* **11**, 1518–1531.

Norman AW, Mizwicki MT, Norman DP (2004). Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. *Nat Rev Drug Discov* **3**, 27–41.

Osheroff JE, Visconti PE, Valenzuela JP, Travis AJ, Gregory JA, Kopf S (1999) Regulation of human sperm capacitation by a cholesterol efflux-stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. *Mol Hum Reprod* **11**, 1017–1026.

Rebsamen MC, Sun J, Norman AW, Liao JK (2002) 1α,25-Dihydroxyvitamin D_3 induces vascular smooth muscle cell migration via activation of phosphatidylinositol 3-kinase. *Circ Res* **91**, 17–24.

Schwartz Z, Sylvia VL, Larsson D, et al. (2002) 1α,25$(OH)_2D_3$ regulates chondrocyte matrix vesicle protein kinase C (PKC) directly via G-protein-dependent mechanisms and indirectly via incorporation of PKC during matrix vesicle biogenesis *J Biol Chem* **277**, 11828–11837.

Shaffer PL, McDonnell DP, Gewirth DT (2005) Characterization of transcriptional activation and DNA-binding functions in the hinge region of the vitamin receptor. *Biochemestry* **44**, 2678–2685.

Shaman JA, Yamauchi Y, Ward WS (2007) Function of the sperm nuclear matrix. *J Reprod Syst* **53**, 135–140.

Viring S, Eirnasson S (1981) Sperm distribution within the genital tract of naturally inseminated gilts. *Nord Vet Med* **33**, 145–149.

Visconti PE, Kopf GS (1998) Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod* **59**, 1–6.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS (1995) Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121**, 1129–1137.

World Health Organization (1999) *Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interactions*, 4th edn. Cambridge, UK: Cambridge University Press.

Yanagimachi R (1994). Mammalian fertilization. In *The Physiology of Reproduction* (eds Knobil E, Neill JD), pp. 189–317. New York: Raven Press.

REPRODUCTION
RESEARCH

Leptin and leptin receptor in pig spermatozoa: evidence of their involvement in sperm capacitation and survival

Saveria Aquila[1,3], Vittoria Rago[2], Carmela Guido[1,3], Ivan Casaburi[1,3], Silvia Zupo[1,3] and Amalia Carpino[2]

[1]*Department of Pharmaco-Biology,* [2]*Department of Cell Biology, Faculty of Pharmacy and* [3]*Centro Sanitario, University of Calabria, 87036 Arcavacata di Rende, Cosenza, Italy*

Correspondence should be addressed to A Carpino at Dipartimento di Biologia Cellulare, Università della Calabria, 87030 Arcavacata di Rende, Cosenza, Italy; Email: am_carpino@yahoo.it

Abstract

Several studies have recently investigated the role of leptin, the adipocyte-secreted hormone, in the growth and reproduction of rodents, humans, and domestic animals. The present study was designed to explore the expression of leptin and its receptor in pig spermatozoa. Successful Western blot evidenced a 16 kDa band for leptin and six isoforms, ranging from 120 to 40 kDa, for the leptin receptor. Both leptin and leptin receptor were interestingly located at sperm acrosomal level, suggesting their involvement in the oocyte fertilization events. In fact, both capacitation indexes and acrosin activity were enhanced by leptin, and these effects were reduced by the anti-leptin receptor antibody. Afterwards, we investigated the main transduction pathways regulated by the hormone. Our results showed that, in pig sperm, leptin can trigger the signal transducer and activator of transcription 3, a classical component of cytokine signal transduction pathways, whose expression has not been previously reported in male gamete; in addition it was found constitutively activated. Besides, leptin was able to induce the activation of phosphatidylinositol phosphate kinase 3 and MAP kinase pathways as well as of BCL2, a known antiapoptotic protein. These data address to a role of leptin and its receptor on pig sperm survival. The presence of leptin and its receptor in pig sperm suggests that they, through an autocrine short loop, may induce signal transduction and molecular changes associated with sperm capacitation and survival.

Reproduction (2008) **136** 23–32

Introduction

Leptin (LEP) is known to be the hormonal link between energy stores and several vital functions, including food intake and energy homeostasis. It is a 16 kDa adipocyte-derived hormone that suppresses food intake, stimulates energy expenditure, increases metabolic rate, and ultimately causes loss of body fat (Kamohara *et al*. 1997, Rossetti *et al*. 1997, Bouloumie *et al*. 1998). The effects of LEP as a central satiety agent have been focused on, and its involvement in the regulation of fatness via feed intake was reported. Although LEP was originally thought to act largely via the central nervous system (CNS), recent studies have demonstrated that LEP exerts a wide repertoire of peripheral effects, through direct actions on target tissues. These effects include the stimulation of fatty acid oxidation in adipocytes (Muller *et al*. 1997, Bouloumie *et al*. 1998, Lord *et al*. 1998, Sierra-Honigmann *et al*. 1998, Zhao *et al*. 1998) as well as the increase of glucose uptake in skeletal muscle and brown adipose tissue (Kamohara *et al*. 1997, Yaspelkis *et al*. 1999).

The pig is emerging rapidly as an important biomedical research model, and whereas genetic influences may impact the degree of similarity between pig and human systems, the regulation of some endocrine and metabolic processes in the pig may be more similar to humans than to rodents (Tsiaoussis *et al*. 2001, Vilei *et al*. 2001). LEP plays a key role in the regulation of energy homeostasis and is also linked to the mechanisms controlling reproductive processes. However, very limited data have reported the involvement of LEP in controlling pig reproductive functions at the level of hypothalamus and pituitary. The relationship between nutrition and reproduction in swine has been extensively reviewed (Quesnel & Prunier 1995, Zurek *et al*. 1995). Nutrition may influence the reproductive performance by a number of mechanisms, including central effects on gonadotropin secretion (Booth *et al*. 1994) and local effects on ovarian function (Cosgrove *et al*. 1992, Booth *et al*. 1996). According to Zak *et al*. (1997), the different feeding regimens caused differential sow body weight changes. In pig, nutritional signals, such as LEP, are detected by the CNS and translated, by the neuroendocrine system, into signals that regulate appetite, gonadotropin-releasing hormone release,

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-07-0304
Online version via www.reproduction-online.org

and subsequent luteinizing hormone (LH) secretion. Changes in body weight or nutritional status are characterized by altered adipocyte function, by a reduction of leptin expression in adipose tissue and a decrease in LH secretion.

In both male and female mice it has been shown that leptin has direct effects on fertility; in fact the hormone is able to reverse the infertility of *ob/ob* mice lacking the leptin gene (Cunningham *et al*. 1999). The biological actions of LEP on body weight homeostasis, neuroendocrine function, and fertility are carried out through interactions with its specific obese receptor (OBR, now known as leptin receptor, LEPR) in target tissues. The pattern of leptin receptor expression in the testis is species specific. There are several isoforms of OBR, which are different mainly in the cytoplasmic domain length (Lee *et al*. 1996, Takaya *et al*. 1996, Lollmann *et al*. 1997, Murakami *et al*. 1997, Bjorbaek *et al*. 1998, Dieterich & Lehnert 1998, Yamashita *et al*. 1998). The OBR, a single membrane-spanning glycoprotein, belongs to the class I cytokine receptor superfamily and shares sequence homologies for interaction with Janus kinase (JAK) as well as STATs (Tartaglia 1997). To date, the human OBR is identified as a full-length OBRb form (Tartaglia *et al*. 1995) and also as several short forms generated by alternative splicing, OBRa, OBRc, OBRd, OBRf, and OBRe (Bennett *et al*. 1996, Cioffi *et al*. 1996, Tartaglia 1997). The hypothalamus is considered the only tissue expressing predominantly the full-length OBR. However, the long form and several short isoforms are expressed in an increasing number of peripheral tissues (Cioffi *et al*. 1996, Tartaglia 1997, Glasow *et al*. 1998, Breidert *et al*. 1999), including liver, heart, kidneys, lungs, small intestine, pituitary cells, testes, ovaries, spleen, pancreas, adrenal gland, and adipose tissue (Margetic *et al*. 2002). Furthermore, studies on different OBR splice variants indicated that many tissues may contain a heterologous mix of OBR subtypes. *OBR* mRNA (long form) has been detected in a variety of porcine tissues, including adipose tissue (Lin *et al*. 2000). Using *in situ* hybridization techniques, other groups have shown that leptin receptor mRNA is expressed in Sertoli cells of adult rats (Tena-Sempere *et al*. 2001).

Rodent testis revealed the OBR expression but with a differential cellular site; in fact, the OBR immunoreactivity was confined in Leydig cells of rat but in germ cells of mouse (El-Hefnawy *et al*. 2000, Caprio *et al*. 2003). This discrepancy may be due to interspecies difference. LEP and OBR were found in human seminiferous tubules (Soyupek *et al*. 2005), human seminal plasma (Jope *et al*. 2003), human sperm (Aquila *et al*. 2005) and, recently, OBR was also identified in boar sperm (De Ambrogi *et al*. 2007). In the present study, we investigated LEP and OBR expression in pig sperm, evaluating their potential role on sperm capacitation and survival. Besides, we investigated the main pathways involved in LEP/OBR signaling in pig sperm.

Results

LEP expression in pig sperm

To determine whether mRNA for LEP is present in pig spermatozoa, RNA isolated from Percoll-purified sperm was subjected to reverse PCR. The primer sequences were based on the pig gene sequence and the RT-PCR amplification revealed the expected PCR product size of 280 bp (Fig. 1A). This product was sequenced and found to be identical to the classical pig gene sequence.

The presence of LEP protein in pig spermatozoa was also investigated by Western blotting (WB) using an antibody raised against the carboxyl terminus of the protein. One immunoreactive band was observed at 16 kDa in the lysates from pig sperm samples at the same mobility of the adipocyte extract used as positive control (Fig. 1B).

OBR expression in pig sperm

To evaluate mRNA for OBR in pig spermatozoa, RNA isolated from Percoll-purified sperm was subjected to reverse PCR. The primer sequences were based on the *OBR* pig gene sequence of the transmembrane region and the RT-PCR amplification revealed the expected PCR product size of 460 bp (Fig. 2A). This product was sequenced and found to be identical to the pig gene sequence considered.

WB of pig sperm extracts showed six different immunoreactive *OBR* isoforms (120, 90, 80, 65, 60, and 40 kDa) (Fig. 2B), such as the WB of pig epididymal tissue used as positive control (Fig. 2B). The bands were not detected by non-immune rabbit serum indicating that these proteins are specific for OBR (Fig. 2B1).

Immunolocalization of LEP and OBR in pig sperm

An intense red fluorescence localized leptin in the acrosome of pig sperm while the other cellular regions were unlabeled (Fig. 3A). In addition, a brilliant green

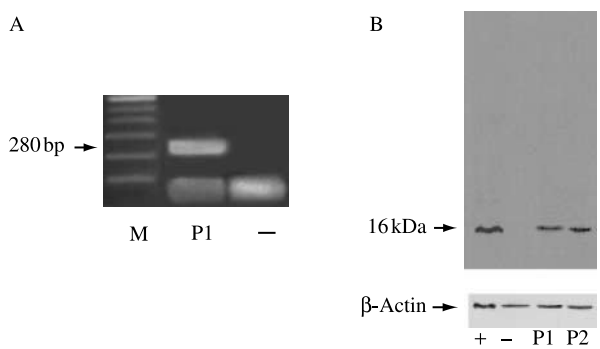

Figure 1 Leptin expression in pig sperm. (A) RT-PCR result in one representative pig sperm sample (lane P1), negative control (lane−), and markers (lane M). (B) Immunoblot of leptin from representative pig sperm samples (lanes P1 and P2), adipocyte cells used as positive (lane+), negative (lane−) controls. β-Actin served as loading control.

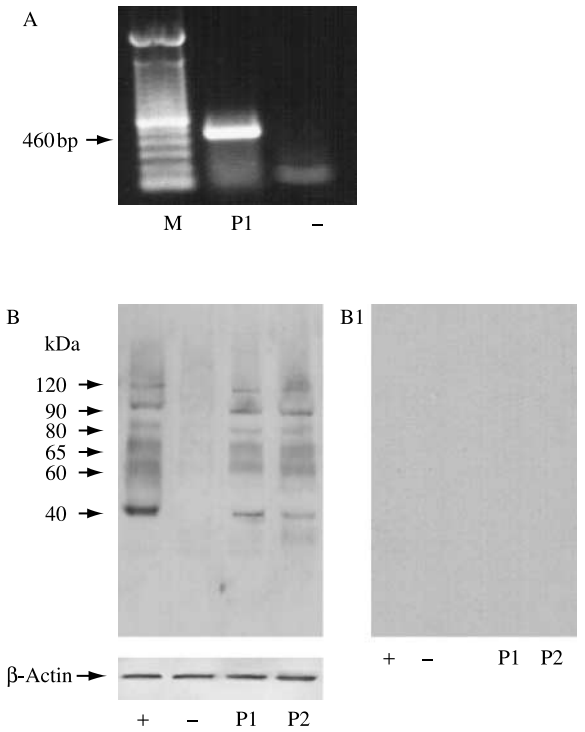

Figure 2 OBR expression in pig sperm. (A) RT-PCR result in one representative pig sperm sample (lane P1), negative control (lane−), and markers (lane M). (B) Immunoblot of OBR from two representative pig sperm samples (lanes−P1, P2), pig epididymis extract (lane+), and negative control (lane−). (B1) Immunoblot of the negative control (membrane incubated with normal rabbit serum). β-Actin served as loading control.

light revealed that OBR was confined in the apical portion of sperm acrosome (Fig. 3B). No fluorescent signal was obtained when primary Abs (anti-LEP Ab or anti-OBR Ab) were omitted (Fig. 3 inserts) thus confirming the specificity of the Ab binding.

LEP influences pig sperm capacitation enhancing both cholesterol efflux and protein tyrosine phosphorylation

One of the early events associated with the capacitation of mammalian spermatozoa is the cholesterol efflux, followed by protein phosphorylation. Our results showed a significant increase in cholesterol efflux upon

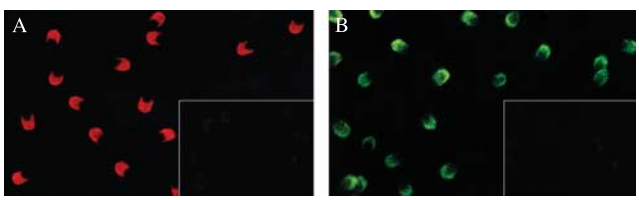

Figure 3 Representative immunofluorescence labeling of leptin and leptin receptor (OBR) in pig spermatozoa. (A) A red intense fluorescence localized leptin in the sperm acrosomal region. (B) A green brilliant light showed OBR in the apical acrosome. Inserts: immunonegative controls. Scale bars: 5 μm.

1 and 10 nM LEP (Fig. 4A). Protein tyrosine phosphorylation pattern of pig sperm was also induced by 1 and 10 nM LEP treatment (Fig. 4B), but 60 nM LEP did not produce any effect. Further, anti-OBR Ab was able to abolish 10 nM LEP effect.

LEP stimulates pig sperm acrosin activity

Acrosin is a sperm representative acrosomal enzyme. Acrosin activity showed that both 1 and 10 nM LEP were able to stimulate the enzymatic activity, while no effect was observed with 60 nM LEP (Fig. 5). Anti-OBR Ab combined with 10 nM LEP was able to abolish LEP effect (Fig. 5).

STAT3 is activated by LEP/OBR in pig sperm

STAT3 is a critical mediator of LEP action. Our results showed the expression of STAT3 (Fig. 6A) in pig sperm

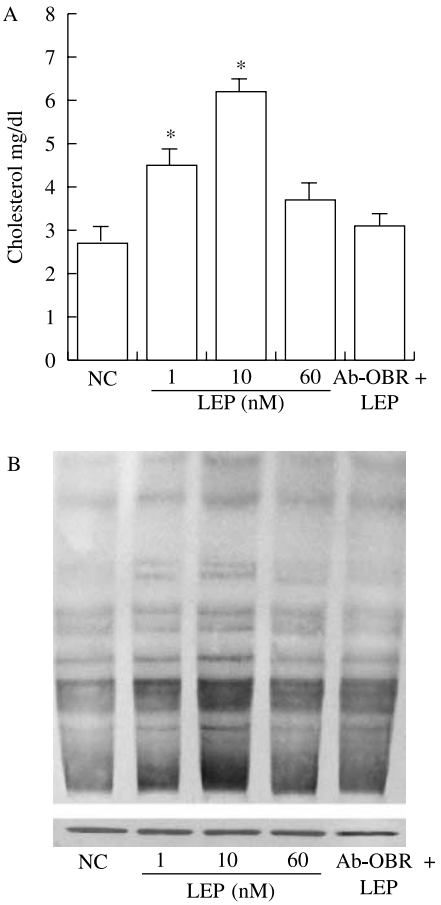

Figure 4 Leptin affects cholesterol efflux and protein tyrosine phosphorylation of pig sperm. Spermatozoa were incubated in the absence (NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin. (A) Cholesterol in culture medium from pig sperm. Results are presented as mean±S.E.M. and are given per 10×10^6 spermatozoa. *$P<0.05$ versus control. (B) Western blot analysis of protein tyrosine phosphorylation from sperm lysates.

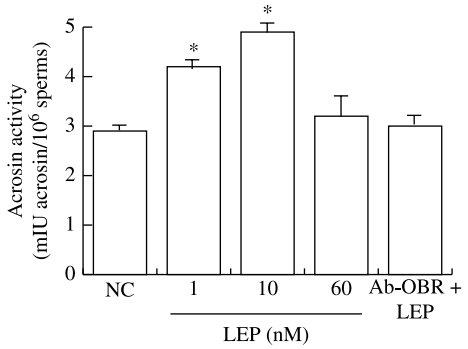

Figure 5 Leptin affects acrosin activity of pig sperm. Spermatozoa were incubated in the absence (NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody + 10 nM leptin. Values are mean ± S.E.M. *$P<0.05$ versus control.

and that it is constitutively activated (phosphorylated) (Fig. 6B). Furthermore, 1 and 10 nM LEP treatments induced a significant increase in STAT3 activation that was reversed by the anti-OBR Ab (Fig. 6B and C).

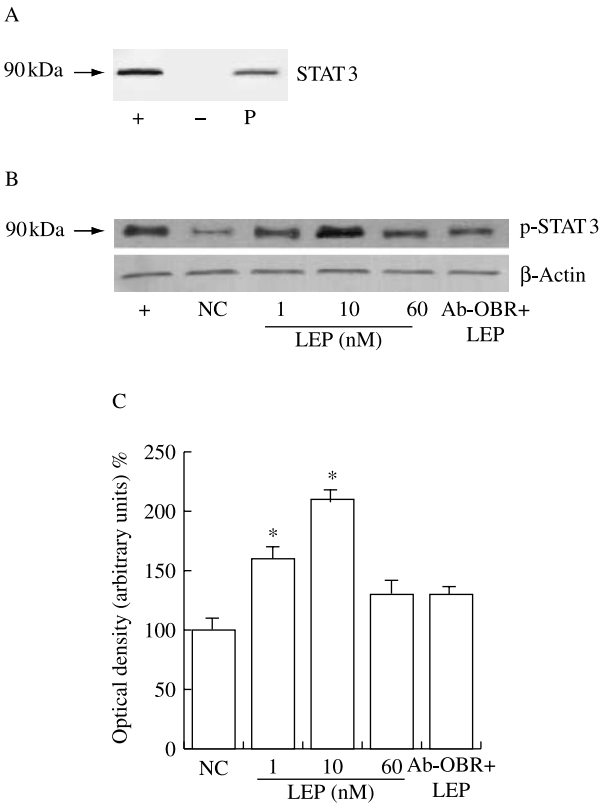

Figure 6 Western blot analysis of STAT3 from one representative pig sperm lysate. (A) Immunoblot of STAT3 in the pig sample (lane P), MCF7 used as positive (lane +) and negative (lane −) controls. (B) p-STAT3 band in sperm incubated in the absence (lane NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin (Ab OBR + LEP). MCF7 cell lines were used as positive controls (lane +). β-Actin served as loading control. (C) Band intensities were evaluated in term of arbitrary densitometric units. Values are as mean ± S.E.M. *$P<0.05$ versus control.

ERK1/2 is activated by LEP/OBR in pig sperm

Components of the ERK family of MAPK are involved in sperm motility and capacitation. Our results showed the expression of ERK1/2 in pig sperm (Fig. 7A) and evidenced that LEP, through its receptor, is able to activate ERK1/2 (Fig. 7B and C). The maximal effect was observed with 1 and 10 nM LEP, while 60 nM LEP did not result in further activation (Fig. 7B and C).

LEP/OBR signaling modulates pig sperm survival

Our data revealed that pig sperm express BCL2, a key protein in sperm survival signaling (Fig. 8A). Furthermore, leptin induced the Serine 70 phosphorylation of BCL2, necessary for its full antiapoptotic function, and this effect was inhibited by the anti-OBR Ab (Fig. 8B and C).

PI3-K plays also an important role in sperm survival, and its main downstream effector is the Akt. Our results showed the expression of Akt (Fig. 8D) and phosphorylated Akt (S473 and T308 phosphorylations) in pig sperm (Fig. 8E). In addition, LEP induced an increase in the kinase phosphorylation, which was reduced by using anti-OBR Ab (Fig. 8E and F). The specific PI3-K inhibitor, 10 μM

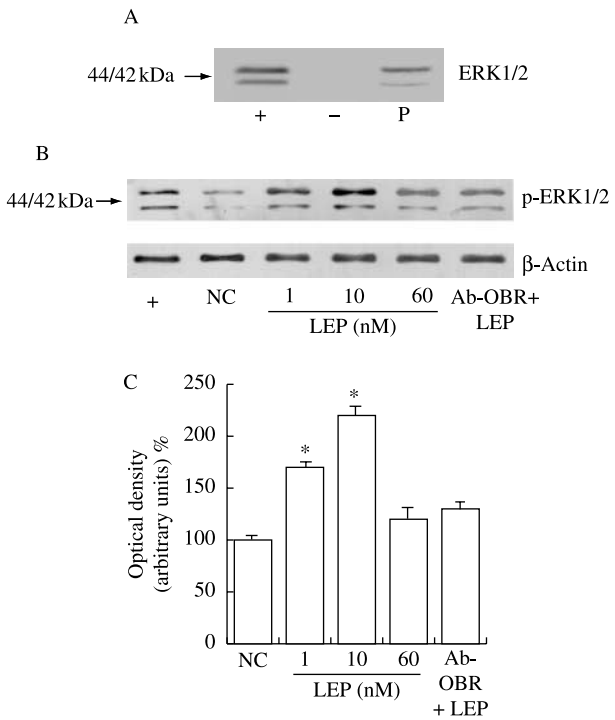

Figure 7 Western blot analysis of ERK1/2 from one representative pig sperm lysate. (A) Immunoblot of ERK1/2 in the pig sample (lane P), MCF7 used as positive (lane +) and negative (lane −) controls. (B) p-ERK1/2 band in spermatozoa incubated in the absence (lane NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin (Ab OBR + LEP). MCF7 cell lines were used as positive controls (lane +). β-Actin served as loading control. (C) Band intensities were evaluated in term of arbitrary densitometric units. Values are as mean ± S.E.M. *$P<0.05$ versus control.

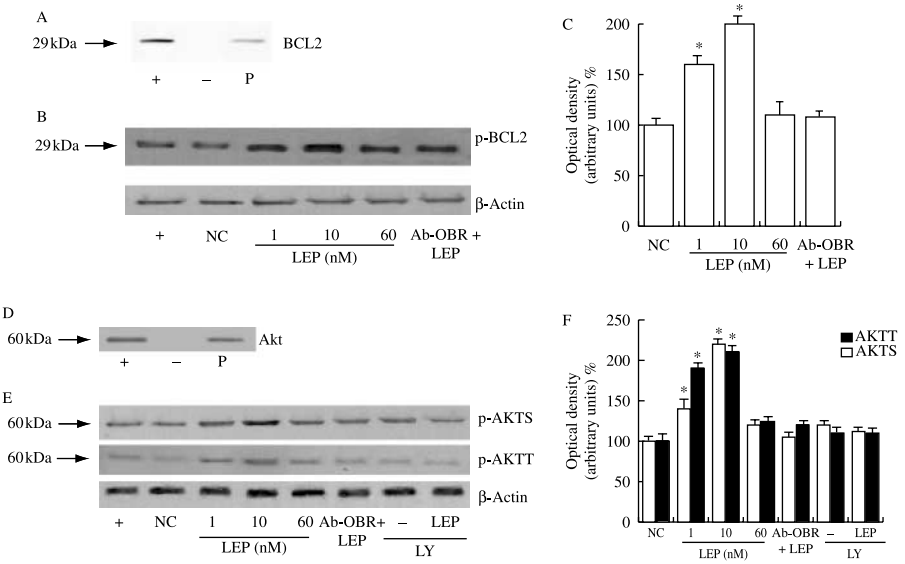

Figure 8 Western blot analysis of BCL2 and Akt from one representative pig sperm lysate. (A and D) Immunoblots of BCL2 and Akt, in the pig sample (lane P), MCF7 used as positive (lane+) and negative (lane−) controls. (B) p-BCL2 band when sperm cells were incubated in the absence (lane NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin (Ab OBR+ LEP). (E) p-AKTS (Akt ser) and p-AKTT (Akt thr) bands in sperm incubated in the absence (lane NC) or presence of leptin (1, 10, and 60 nM) as well as in the presence of anti-OBR antibody +10 nM leptin (Ab OBR+LEP) or LY294002 (LY). (C and F) Band intensities were evaluated in terms of arbitrary densitometric units. Values are as mean±S.E.M. $*P<0.05$ versus control. β-Actin served as loading control.

LY294002, abolished LEP-induced Akt phosphorylations (Fig. 8E and F), confirming the PI3K/Akt activation. Also in the PI3K/Akt pathway, the 60 nM LEP did not induce a significant effect (Fig. 8E and F).

Discussion

In recent years, it has been suggested that LEP is a metabolic signal to the reproductive system (Barash *et al.* 1996). To date, the mechanisms by which LEP regulates reproductive function remain to be determined. LEP and OBR were recently found in human sperm (Jope *et al.* 2003, Aquila *et al.* 2005) and the OBR in boar sperm (De Ambrogi *et al.* 2007). However, LEP and its signaling in the male gamete function are still unknown in non-human species. In the present study, we have investigated the presence of LEP and OBR in pig sperm evaluating, for the first time, their potential action on capacitation, and sperm survival. The main aim of our study was also to identify the potential signaling molecules that are set in motion by LEP in pig sperm.

First, we have demonstrated the presence of LEP and OBR in pig sperm at different levels: mRNA expression, protein expression, and immunolocalization. Pig sperm LEP, as protein, was evidenced at the same size as human sperm leptin (Aquila *et al.* 2005). In addition, we detected the *LEP* and *OBR* mRNAs in pig perm. Other authors reported the presence of different mRNAs in mammalian ejaculated spermatozoa; however, the significance of mRNA in these cells is currently under investigation (Miller 2000, Andò & Aquila 2005). WB analysis revealed six different OBR isoforms, among these the120 kDa band is consistent with the OBRb isoform while the other bands could correspond to short isoforms. A partially similar pattern of OBR has been detected in the adult mouse testis (El-Hefnawy *et al.* 2000), but the pattern of OBR expression is species specific.

Interestingly, immunofluorescence assays localized both LEP and OBR exclusively at acrosomal level of pig sperm. Spermatozoa are highly polarized cells, thus they compartmentalize specific metabolic and signaling pathways where they are necessary. Therefore, a role of LEP/OBR in the events leading to the sperm becoming able to fertilize the oocyte can be hypothesized. Our data on OBR agree with the De Ambrogi *et al.* (2007) findings, showing the receptor prevalently located on the acrosome of boar sperm, whereas in human sperm the receptor was visualized at the tail (Jope *et al.* 2003). These differences may be due to sample management or they could be species specific. Despite the use of the same antibody, the different leptin localization in human and pig sperm is particularly intriguing. In fact, the hormone was specifically compartmentalized in the equatorial segment and in the mid-piece of human sperm (Aquila *et al.* 2005). We can only hypothesize that leptin is differently involved in the regulation of sperm events in the two species.

In order to fertilize oocyte in the female genital tract, the mammalian spermatozoa must undergo the capacitation process that is a prerequisite for the acrosome reaction. Capacitation induces various biochemical and biophysical changes in the sperm plasma membrane, such as the efflux of cholesterol (Visconti *et al.* 1999*a*, 1999*b*, Shadan *et al.* 2004). Cholesterol efflux from spermatozoa destabilizes lipid raft structures in the plasma membrane initiating the protein phosphorylation and the acquisition of a capacitated status. Tyrosine phosphorylation of sperm proteins during capacitation has been reported in mouse, human, bull, hamster (Visconti *et al.* 1995, Leclerc *et al.* 1996, Galantino-Homer *et al.* 1997), and also in pig (Kalab *et al.* 1998,

Flesch *et al*. 1999, Tardif *et al*. 2003). Our data have shown that, in pig sperm, leptin increased cholesterol efflux, protein tyrosine phosphorylation, and the acrosin activity. Therefore, LEP, through its receptor, appears to affect both capacitation and acrosome reaction, suggesting its role in the acquisition of fertilizing ability of pig sperm.

The signaling events, derived from LEP binding to its receptor, have been recently investigated at biochemical and molecular level. The JAK/STAT pathway is one of the main signaling cascades activated by LEP (Thomas 2004). The OBR, which belongs to the class I cytokine receptor superfamily, binds cytoplasmic kinases, mainly JAK2 (Ghilardi & Skoda 1997). Activated JAK2 auto-phosphorylates numerous tyrosine residues and, at the same time, it phosphorylates tyrosine residues on the functional OBR. Then the phosphorylated intracellular domain of the receptor provides a binding site for STAT proteins (particularly STAT3), which are activated and translocated to the nucleus where they stimulate transcription of target genes. In our study, for the first time, we have demonstrated that STAT3 is expressed in the pig sperm, it is constitutively activated, and its phosphorylation increases following LEP binding to its receptor. Therefore, we provide new information to indicate that LEP can stimulate the JAK-STAT pathway in pig sperm.

Since capacitation is a crucial step in the acquisition of sperm fertilizing ability, it is likely controlled by redundant mechanisms, with cross-talks between different pathways (de Lamirande *et al*. 1997, Leclerc *et al*. 1998). The components of the extracellular signal-regulated kinase family of MAPK have been detected in spermatozoa affecting their motility and capacitation (Naz *et al*. 1992, Luconi *et al*. 1998, de Lamirande & Gagnon 2002). Our results have shown the expression of ERK1/2 in pig male gamete and have evidenced that LEP, through its receptor, was able to positively activate ERK1/2, suggesting that MAPK-dependent processes are involved in the hormone action.

Several pathways are also activated by JAKs including PI3K/Akt. PI3K plays an important role in the survival and metabolism of somatic and sperm cells (Fisher *et al*. 1998, Luconi *et al*. 2001, Aquila *et al*. 2004, 2005, Aparicio *et al*. 2006). The PI3K main downstream effector is the Akt, identified as a serine/threonine protein kinase, which was found in human ejaculated spermatozoa (Aquila *et al*. 2004) and in boar spermatozoa (Aparicio *et al*. 2006).

From our results it emerges that PI3K and Akt are implicated in mediating LEP signals in pig sperm, suggesting that this hormone may be involved in sperm survival. Concomitantly, BCL2 (Ito *et al*. 1997), a key protein in survival signaling, is enhanced upon LEP exposure and this effect was reduced by the anti-OBR Ab. Previous works have shown that LEP attenuates apoptosis of different cell types, such as osteoblasts, granulosa cells, and pancreatic islet cells (Almong *et al*. 2001, Okuya *et al*. 2001, Gordeladze *et al*. 2002). Moreover, recently, LEP has been found to inhibit stress-induced apoptosis of T lymphocytes *in vivo* (Fujita *et al*. 2002). Data presented here clearly demonstrate that LEP, by inducing the phosphorylation of classical key survival proteins, such as ERK1/2, PI3K, Akt, and BCL2, aids the pig sperm survival process.

It is important to point out that in all our experiments, we obtained different responses by using low or high LEP doses; in fact 1 nM and 10 nM were stimulatory, whereas the higher LEP concentration seemed to be ineffective (similar to the control). The outcome of signaling activation can depend on differences in ligand concentration as it was demonstrated in human sperm (Aquila *et al*. 2005). Besides, recently it was hypothesized that the net effect of LEP upon male reproductive function may depend on its circulating level (Caprio *et al*. 2001, Tena-Sempere & Barreiro 2002). The effect obtained with the higher LEP dose may be also due to the down-regulation or internalization of the OBR depending on the ligand concentration as previously demonstrated (Uotani *et al*. 1999).

In addition, it has been demonstrated that pig seminal plasma contains a significant amount of LEP, which decreases considerably in the follicular fluid (Lackey *et al*. 2002). As sperm leave seminal plasma during their transit in the female reproductive tract, they are exposed to decreased LEP concentrations. From our results, it may be speculated that the high LEP in seminal plasma may contribute to maintain sperm in a quiescent metabolic condition. Instead, the low LEP concentrations in the pig female reproductive tract (Gregoraszczuk *et al*. 2004) secretions could contribute to sperm activation, by facilitating their capacitation and acquisition of fertilizing ability.

Fatness in pigs has economical importance due to market incentives for the production of lean pork and also because elevated fatness increases feed costs. LEP was identified as a metabolic signal affecting central regulation of reproduction in the pig. Our data have shown that this hormone affects pig sperm acquisition of fertilizing ability. Therefore, further studies, addressed to the knowledge of the balance between local and systemic leptin, will clarify whether the manipulation of LEP concentration, as a strategy to alter body composition, may affect pig reproduction.

Materials and Methods

Chemicals

BSA protein standard, Laemmli sample buffer, pre-stained molecular weight marker, Percoll (colloidal PVP coated silica for cell separation), sodium bicarbonate, dimethyl sulfoxide, Earle's balanced salt solution, triethanolamine buffer, $MgCl_2$, propidium iodide, LY294002 (PI3K inhibitor), and all other chemicals were purchased from Sigma. Recombinant porcine leptin was purchased from Protein Laboratories Rehovot

(Rehovot, Israel) and acrylamide bisacrylamide was from Labtek Eurobio (Milan, Italy). Triton X-100, ECL Plus WB detection system, Hybond ECL were purchased from Amersham Pharmacia Biotech. Antibodies (Abs) polyclonal rabbit anti-leptin (A-20), rabbit anti-OBR (H-300), rabbit anti-p-Akt1/Akt2/Akt3 (Ser473), rabbit anti-p-Akt1/Akt2/Akt3 (Thr308), rabbit anti-phosphotyrosine (PY99), mouse ant-p-signal transducer and activator of transcription-3 p-STAT3 (B-7), peroxidase-coupled anti-rabbit, and FITC/Texas Red conjugated anti-rabbit IgG were from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit anti-p-BCL2 and p-ERK1/2 (p42/44 kDa) Abs were from Cell Signaling (Milan, Italy). Cholesterol-oxidase (CHOD)–peroxidase (POD) enzymatic colorimetric kit was from Inter-Medical (Biogemina Italia Srl, Catania, Italy). Total RNA Isolation System kit, enzymes, buffers, nucleotides 100 bp ladder used for RT-PCR were purchased from Promega. Moloney murine leukemia virus (M-MLV) was from Gibco-Life Technologies Italia. Oligonucleotide primers were made by Invitrogen.

Animals and semen samples

The investigation has been conducted on semen from seven fertile male pigs (*Sus scrofa domestica*, Large White) kept at the 'Swine Artificial Insemination Centre' (Rende, Cosenza, Italy). The animals were 24 to 30-month old and their weights were from 280 to 320 kg. Individual fresh ejaculates were collected by the gloved-hand method and filtered immediately by Universal Semen bags (Minitub, Tiefenbech, Germany). Semen was transported within half an hour to the laboratory, diluted 1:10 with TBS buffer, and centrifuged on a discontinuous Percoll density gradient (72%/90%) to remove bacteria and debris (Kuster *et al.* 2004). Epididymides were obtained from two out of the seven animals after castration at local animal hospitals. All surgical procedures followed approved guidelines for the ethical treatment of animals. Epididymides were brought immediately to the laboratory, then were carefully dissected, freed from sperm, homogenized, and lysed for WB analysis.

Evaluation of sperm viability

Viability of pig sperm was assessed using the DNA-specific fluorochrome PI. Sperm suspension (1×10^6 ml) was exposed to PI (12 μmol/l) for 5 min at room temperature. Then spermatozoa were fixed by adding 1 ml of 12.5% (w/v) paraformaldehyde in 0.5 mol Tris/l (pH 7.4) and the slides were immediately examined under an epifluorescence microscope (Olympus BX41) observing a minimum of 200 spermatozoa×slide (100× objective).

Sample treatments

Percoll-purified sperm were washed with unsupplemented Earle's medium (uncapacitating medium) and were incubated for 30 min at 39 °C and 5% CO_2 without (control) or with increasing concentrations of LEP (1, 10, and 60 nM). These doses were chosen on the basis of physiological concentrations to reproduce the environment of the sperm journey. Some cells were also pretreated (15 min) with the inhibitor, LY294002, or with the anti-OBR Ab (autocrine blockage). No adverse effects among the different treatments have been observed on pig sperm viability.

Then, samples were centrifuged (3000 ***g***×5 min) using the upper phase for further determinations and sperm pellet for WB analysis.

Western blot analysis of sperm proteins

WB analysis was used to identify LEP and OBR in sperm samples obtained from all the seven animals. Sperm samples were washed twice with Earle's balanced salt solution (uncapacitating medium) and then centrifuged for 5 min at 5000 ***g***. The pellet was resuspended in lysis buffer as previously described (Aquila *et al.* 2002). Equal amounts of proteins (80 μg) were boiled for 5 min, separated by 10% PAGE, transferred to nitrocellulose sheets, and probed with an appropriate dilution of the (indicated) specific Ab. The bound of the secondary antibody was revealed with the ECL Plus WB detection system according to the manufacturer's instructions. The negative control was performed using a sperm lysate that was immunodepleted of LEP or OBR (i.e., lysates pre-incubated with anti-leptin Ab or anti-OBR Ab for 1 h at room temperature and immunoprecipitated with Protein A/G-agarose).

To further validate the results for OBR, as negative control, non-immune rabbit serum, instead of the first Ab, was used at the same dilution ratio (1:1000). Epididymal extract was used as pig control tissue.

WB was also performed to identify STAT3, BCL2, ERK1/2, Akt1/Akt2 in pig sperm extracts and evaluate the cell signaling induced by LEP (p-Akt1/Akt2/Akt3, p-STAT3, p-BCL2, and p-ERK1/2). MCF7 cell lines were used as positive controls while negative controls were performed as indicated above. The blots were stripped (glycine 0.2 M (pH 2.6) for 30 min at room temperature) and reprobed with anti-β-actin Ab as loading control.

The experiments were repeated four times for each sample.

RNA isolation, reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from pig spermatozoa purified as previously described (Aquila *et al.* 2002). Before RT-PCR, RNA was incubated with RNase-free DNase (DNase) I in single-strength reaction buffer at 37 °C for 15 min. This was followed by heat inactivation of DNase I at 65 °C for 10 min. Two micrograms of DNase-treated RNA samples were reverse transcribed by 200 IU M-MLV reverse transcriptase in a reaction volume of 20 μl (0.4 μg oligo-dT, 0.5 mM 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 cDNA was performed with 2 U of Taq DNA polymerase, 50 pmol primer pair for both LEP and OBR in 10 mM Tris–Hcl (pH 9.0) containing 0.1% Triton X-100, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.25 mM each dNTP. The applied PCR primers and the expected lengths of the resulting PCR products are the following: 5′ATTCCTGGCTTGGCCC 3′ and 5′ AAGGCAGACTGGTGAGGATCTGTT 3′ for LEP with a product size of 248 bp; 5′ ACTTCCTCTTGCCTGCTGGAATCT 3′ and 5′ GACACAGGCACATGGCATTCACAA 3′ for OBR with a

product size 460 bp. Cycling conditions were: 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min for LEP; 95 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min for OBR. For all PCR primer amplifications, negative RT-PCR was performed without M-MLV reverse transcriptase. The PCR-amplified products were subjected to electrophoresis in 2% agarose gels stained with ethidium bromide and visualized under u.v. transillumination.

Immunofluorescence assay

Following Percoll separation, sperm cells were rinsed three times with 0.5 mM Tris–Hcl buffer (pH 7.5); then 10 µl of concentrated cell suspension was added to 250 µl drop of warm (37 °C) TBS and allowed to settle onto slides in a humid chamber. The overlying solution was carefully pipetted off and replaced by absolute methanol for 7 min at −20 °C. After methanol removal, sperm cells were washed in TBS, containing 0.1% Triton X-100 and were treated for immunofluorescence.

Leptin and OBR stainings were carried out, after blocking with normal goat serum (10%), using anti-leptin (1:100) and anti-OBR (1:100) as primary Abs, followed by anti-rabbit IgG Texas Red-conjugated/FITC-conjugated Abs (1:200) respectively. Sperm cells incubated without the primary Abs were utilized as negative controls. The slides were immediately examined under an epifluorescence microscope (Olympus BX41), observing a minimum of 200 spermatozoa×slide (100×objective).

Measurement of cholesterol in the sperm culture medium

Cholesterol was measured (in duplicate) in the incubation medium from pig spermatozoa obtained from all the seven animals. The CHOD–POD enzymatic colorimetric method was used according to the manufacturer's instructions. Sperm samples, washed twice with uncapacitating medium, were incubated in the same medium (control, NC) for 30 min at 39 °C and 5% CO_2. Other samples were incubated in the presence of 1, 10, and 60 nM LEP concentrations. Some samples were incubated with anti-OBR Ab combined with 10 nM LEP. At the end of the sperm incubation, the culture media were recovered by centrifugation, lyophilized, and subsequently dissolved in 1 ml of the buffer reaction. The samples were incubated for 10 min at room temperature and then the cholesterol content was measured with the spectrophotometer at 505 nm. Cholesterol standard used was 200 mg/dl. The limit of sensitivity for the assay was 0.05 mg/dl. Inter- and intra-assay variations were 0.71% and 0.57% respectively.

The experiments were repeated four times for each sample.

Acrosin activity assay

Acrosin activity was assessed by the method of Glogowski *et al.* (1998). Briefly, sperm samples from all the seven animals were washed in Earle's medium and centrifuged at 800 ***g*** for 20 min, then they were resuspended (final concentration of 100×10^3 sperm/ml) in different tubes containing no treatment (control) or the indicated treatments (experimental). One milliliter of substrate–detergent mixture (23 mmol/l N alpha-benzoyl-DL-arginine p-nitroanilide in DMSO and 0.01% Triton X-100 in 0.055 mol/l NaCl, 0.055 mol/l HEPES (pH 8.0) respectively) was added for 1 h at room temperature. After incubation, 0.5 mol/l benzamidine was added (0.1 ml) to each of the tubes and then they were 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 pro-acrosin activable. The acrosin activity was expressed as mIU/10^6 sperms. The experiments were repeated four times for each sample.

Statistical analysis

Data, presented as mean±S.E.M., were evaluated by the one-way ANOVA. The differences in mean values were calculated at a significance level of $P\leq0.05$.

Acknowledgements

Our special thanks to Dr Vincenzo Cunsulo (Biogemina Italia Srl, Catania, Italy). This work was supported by MURST 2006 (Ex-60%). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Almong B, Gold R, Tajima K, Dantes A, Salim K, Rubistein M, Barkan D, Homburg R, Lessing JB, Nevo N *et al.* 2001 Leptin attenuates follicular apoptosis and accelerates the onset of puberty in immature rats. *Molecular and Cellular Endocrinology* **183** 179–191.

De Ambrogi M, Spinaci M, Galeati G & Tamanini C 2007 Leptin receptor in boar spermatozoa. *International Journal of Andrology* **30** 458–461.

Andò S & Aquila S 2005 Arguments raised by the recent discovery that insulin and leptin are expressed in and secreted by human ejaculated spermatozoa. *Molecular and Cellular Endocrinology* **21** 245–246.

Aparicio IM, Bragado MJ, Gil MC, Garcia-Herreros M, Gonzalez-Fernandez L, Tapia JA & Garcia-Marin LJ 2006 Phosphatidylinositol 3-kinase pathway regulates sperm viability but not capacitation on boar spermatozoa. *Molecular Reproduction and Development* **74** 1035–1042.

Aquila S, Sisci D, Gentile ME, Middea E, Siciliano L & Andò S 2002 Human ejaculated spermatozoa contain active P450 aromatase. *Journal of Clinical Endocrinology and Metabolism* **87** 3385–3390.

Aquila S, Gentile M, Middea E, Catalano S & Andò S 2004 Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* **146** 552–557.

Aquila S, Gentile M, Middea E, Catalano S, Morelli C, Pezzi V & Andò S 2005 Leptin secretion by human spermatozoa. *Journal of Clinical Endocrinology and Metabolism* **90** 4753–4761.

Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, Kuijper JL, Clifton DK & Steiner RA 1996 Leptin is a metabolic signal to the reproductive system. *Endocrinology* **137** 3144–3147.

Bennett BD, Solar GP, Yuan JQ, Mathias J, Thomas GR & Matthews W 1996 A role for leptin and its cognate receptor in hematopoiesis. *Current Biology* **6** 1170–1180.

Bjorbaek C, Elmquist JK, Michl P, Ahima RS, van Bueren A, McCall AL & Flier JS 1998 Expression of leptin receptor isoforms in rat brain microvessels. *Endocrinology* **139** 3485–3491.

Booth PJ, Craigon J & Foxcroft GR 1994 Nutritional manipulation of growth and metabolic and reproductive status in pre-pubertal gilts. *Journal of Animal Science* **72** 2415–2424.

Booth PJ, Cosgrove JR & Foxcroft GR 1996 Endocrine and metabolic responses to re-alimentation in feed-restricted pre-pubertal gilts: associations among gonadotropins, metabolic hormones, glucose, and utero ovarian development. *Journal of Animal Science* **74** 840–848.

Bouloumie A, Drexler HC, Lafontan M & Busse R 1998 Leptin, the product of Ob gene, promotes angiogenesis. *Circulation Research* **83** 1059–1066.

Breidert M, Miehlke S, Glasow A, Orban Z, Stolte M, Ehninger G, Bayerdorffer E, Nettesheim O, Halm U, Haidan A *et al.* 1999 Leptin and its receptor in normal human gastric mucosa and in *Helicobacter pylori*-associated gastritis. *Scandinavian Journal of Gastroenterology* **34** 954–961.

Caprio M, Fabbrini E, Isidori AM, Aversa A & Fabbri A 2001 Leptin in reproduction. *Trends in Endocrinology and Metabolism* **12** 65–72.

Caprio M, Fabbrini E, Ricci G, Basciani S, Gnessi L, Arizzi M, Carta AR, De Martino MU, Isidori AM, Frajese GV *et al.* 2003 Ontogenesis of leptin receptor in rat Leydig cells. *Biology of Reproduction* **68** 1199–1207.

Cioffi JA, Shafer AW, Zupancic TJ, Smith-Gbur J, Mikhail A, Platika D & Snodgrass HR 1996 Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nature Medicine* **2** 585–589.

Cosgrove JR, Tilton JE, Hunter MG & Foxcroft GR 1992 Gonadotropin-independent mechanisms participate in ovarian responses to re-alimentation in feed-restricted prepubertal gilts. *Biology of Reproduction* **47** 736–745.

Cunningham MJ, Clifton DK & Steiner RA 1999 Leptin's actions on the reproductive axis: perspectives and mechanisms. *Biology of Reproduction* **60** 216–222.

Dieterich KD & Lehnert H 1998 Expression of leptin receptor mRNA and the long form splice variant in human anterior pituitary and pituitary adenoma. *Experimental and Clinical Endocrinology and Diabetes* **106** 522–525.

El-Hefnawy T, Ioffe S & Dym M 2000 Expression of the leptin receptor during germ cell development in the mouse testis. *Endocrinology* **141** 2624–2630.

Fisher HM, Brewis IA, Barratt CL, Cooke ID & Moore HD 1998 Phosphoinositide 3-kinase is involved in the induction of the human sperm acrosome reaction downstream of tyrosine phosphorylation. *Molecular Human Reproduction* **4** 849–855.

Flesch FM, Colenbrander B, van Golde LM & Gadella BM 1999 Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. *Biochemical and Biophysical Research Communications* **262** 787–792.

Fujita Y, Murakami M, Ogawa Y, Masuzaki H, Tanaka M, Ozaki S, Nakao K & Mimori T 2002 Leptin inhibits stress-induced apoptosis of T lymphocytes. *Clinical and Experimental Immunology* **128** 21–26.

Galantino-Homer HL, Visconti PE & Kopf GS 1997 Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3959-monophosphate-dependent pathway. *Biology of Reproduction* **56** 707–719.

Ghilardi N & Skoda RC 1997 The leptin receptor activates Janus Kinase 2 and signals for proliferation in a factor-depended cell line. *Molecular Endocrinology* **11** 393–399.

Glasow A, Haidan A, Hilbers U, Breidert M, Gillespie J, Scherbaum WA, Chrousos GP & Bornstein SR 1998 Expression of Ob receptor in normal human adrenals: differential regulation of adrenocortical and adrenomedullary function by leptin. *Journal of Clinical Endocrinology and Metabolism* **83** 4459–4466.

Glogowski J, Demianowicz W, Piros B & Ciereszko A 1998 Determination of acrosin activity of boar spermatozoa by the clinical method: optimization of the assay and changes during short-term storage of semen. *Theriogenology* **50** 861–872.

Gordeladze JO, Drevon CA, Syversen U & Reseland JE 2002 Leptin stimulates human osteoblastic cell proliferation, *de novo* collagen synthesis, and mineralization: impact on differentiation markers, apoptosis, and osteoclastic signalling. *Journal of Cellular Biochemistry* **85** 825–836.

Gregoraszczuk EL, Ptak A, Wojtowicz AK, Gorska T & Nowak KW 2004 Estrus cycle-dependent action of leptin on basal and GH or IGF-I stimulated steroid secretion by whole porcine follicles. *Endocrine Regulations* **38** 15–21.

Ito T, Deng X, Carr BK & May WS 1997 Bcl-2 phosphorylation required for anti-apoptosis function. *Journal of Biological Chemistry* **272** 11671–11673.

Jope T, Lammert A, Kratzsch J, Paasch U & Glander HJ 2003 Leptin and leptin receptor in human seminal plasma and in human spermatozoa. *International Journal of Andrology* **26** 335–341.

Kalab P, Peknicova J, Geussova G & Moos J 1998 Regulation of protein tyrosine phosphorylation in boar sperm through a cAMP-dependent pathway. *Molecular Reproduction and Development* **51** 304–314.

Kamohara S, Burcelin R, Halaas JL, Friedman JM & Charron MJ 1997 Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* **389** 374–377.

Kuster CE, Hess RA & Althouse GC 2004 Immunofluorescence reveals ubiquitination of retained distal cytoplasmic droplet on ejaculated porcine spermatozoa. *Journal of Andrology* **25** 340–347.

Lackey BR, Gray SL & Henricks DM 2002 Measurement of leptin and insulin-like growth factor-I in seminal plasma from different species. *Physiological Research* **51** 309–311.

de Lamirande E & Gagnon C 2002 The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. *Molecular Human Reproduction* **81** 24–35.

De Lamirande E, Leclerc P & Gagnon C 1997 Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Molecular Human Reproduction* **3** 175–194.

Leclerc P, de Lamirande E & Gagnon C 1996 Cyclic adenosine 39,59 monophosphate dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biology of Reproduction* **55** 684–692.

Leclerc P, de Lamirande E & Gagnon C 1998 Interaction between Ca^{2+}, cyclic 3′,5′ adenosine monophosphate, the superoxide anion, and tyrosine phosphorylation pathways in the regulation of human sperm capacitation. *Journal of Andrology* **19** 434–443.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI & Friedman JM 1996 Abnormal splicing of the leptin receptor in diabetic mice. *Nature* **379** 632–635.

Lin J, Barb CR, Matteri RL, Kraeling RR, Chen X, Meinersman RJ & Rampacek GB 2000 Long for leptin receptor mRNA expression in the brain, pituitary and other tissues in the pig. *Domestic Animal Endocrinology* **19** 53–61.

Lollmann B, Gruninger S, Stricker-Krongrad A & Chiesi M 1997 Detection and quantification of the leptin receptor splice variants OBRa, b, and, e in different mouse tissues. *Biochemical and Biophysical Research Communications* **238** 648–652.

Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR & Lechler RI 1998 Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* **394** 897–901.

Luconi M, Barni B, Vannelli GB, Krausz C, Marra F, Benedetti PA, Evangelista V, Francavilla S, Properzi G, Forti G *et al.* 1998 Extracellular signal-regulated kinases modulate capacitation of human spermatozoa. *Biology of Reproduction* **58** 1476–1489.

Luconi M, Marra F, Gandini L, Filimberti E, Lenzi A, Forti G & Baldi E 2001 Phosphatidylinositol 3-kinase inhibition enhances human sperm motility. *Human Reproduction* **16** 1931–1937.

Margetic S, Gazzola C, Pegg GG & Hill RA 2002 Leptin: a review of its peripheral actions and interactions. *International Journal of Obesity and Related Metabolic Disorders* **26** 1407–1433.

Miller D 2000 Analysis and significance of messenger RNA in human ejaculated spermatozoa. *Molecular Reproduction and Development* **56** 259–264.

Muller G, Ertl J, Gerl M & Preibisch G 1997 Leptin impairs metabolic actions of insulin in isolated rat adipocytes. *Journal of Biological Chemistry* **272** 10585–10593.

Murakami T, Yamashita T, Iida M, Kuwajima M & Shima K 1997 A short form of leptin receptor performs signal transduction. *Biochemical and Biophysical Research Communications* **231** 26–29.

Naz RK, Ahmad K & Kaplan P 1992 Expression and function of ras proto-oncogene proteins in human sperm cells. *Journal of Cell Science* **102** 487–494.

Okuya S, Tanabe K, Tanizawa Y & Oka Y 2001 Leptin increases the viability of isolated rat pancreatic islets by suppressing apoptosis. *Endocrinology* **142** 4827–4830.

Quesnel H & Prunier A 1995 Endocrine bases of lactational anoestrus in the sow. *Reproduction, Nutrition, Development* **35** 395–414.

Rossetti L, Massillon D, Barzilai N, Vuguin P, Chen W, Hawkins M, Wu J & Wang J 1997 Short term effects of leptin on hepatic gluconeogenesis and *in vivo* insulin action. *Journal of Biological Chemistry* **272** 27758–27763.

Shadan S, James PS, Howes EA & Jones R 2004 Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. *Biology of Reproduction* **71** 253–265.

Sierra-Honigmann MR, Nath AK, Murakami C, Garcia-Cardena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ *et al.* 1998 Biological action of leptin as an angiogenic factor. *Science* **281** 1683–1686.

Soyupek S, Armagan A, Serel TA, Hoscan MB, Perk H, Karaoz E & Candir O 2005 Leptin expression in the testicular tissue of fertile and infertile men. *Archives of Andrology* **51** 239–246.

Takaya K, Ogawa Y, Isse N, Okazaki T, Satoh N, Masuzaki H, Mori K, Tamura N, Hosoda K & Nakao K 1996 Molecular cloning of rat leptin receptor isoform complementary DNAs-identification of a missense mutation in Zucker fatty (*fa/fa*) rats. *Biochemical and Biophysical Research Communications* **225** 75–83.

Tardif S, Cubè C & Bailey JL 2003 Porcine sperm capacitation and tyrosine kinase activity are dependent on bicarbonate and calcium but protein tyrosine phosphorylation is only associated with calcium. *Biology of Reproduction* **68** 207–213.

Tartaglia LA 1997 The leptin receptor. *Journal of Biological Chemistry* **272** 6093–6096.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J *et al.* 1995 Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83** 1263–1271.

Tena-Sempere M & Barreiro ML 2002 Leptin in male reproduction: the testis paradigm. *Molecular and Cellular Endocrinology* **188** 9–13.

Tena-Sempere M, Manna PR, Zhang FP, Gonzalez LC, Dieguez C, Huhtaniemi I & Aguilar E 2001 Molecular mechanism of leptin action in adult rat testis: potential targets for leptin-induced inhibition of steroidogenesis and pattern of leptin receptor messenger ribonucleic acid expression. *Journal of Endocrinology* **170** 413–423.

Thomas T 2004 The complex effects of leptin on bone metabolism through multiple pathways. *Current Opinion in Pharmacology* **4** 295–300.

Tsiaoussis J, Newsome PN, Nelson LJ, Hayes PC & Plevris JN 2001 Which hepatocyte will it be? Hepatocyte choice for bioartificial liver support systems *Liver Transplantation* **7** 2–10.

Uotani S, Bjorbaek C, Tornoe J & Flier JS 1999 Functional properties of leptin receptor isoforms: internalization and degradation of leptin and ligand-induced receptor downregulation. *Diabetes* **48** 279–286.

Vilei MT, Granato A, Ferraresso C, Neri D, Carraro P, Gerunda G & Muraca M 2001 Comparison of pig, human and rat hepatocytes as a source of liver specific metabolic functions in culture systems implications for use in bioartificial liver devices. *International Journal of Artificial Organs* **24** 392–396.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS 1995 Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121** 1129–1137.

Visconti PE, Galantino-Homer H, Ning X, Moore GD, Valenzuela JP, Jorgez CJ, Alvarez JG & Kopf GS 1999*a* Cholesterol efflux-mediated signal transduction in mammalian sperm. b-cyclodextrins initiate transmembrane signalling leading to an increase in protein tyrosine phosphorylation and capacitation. *Journal of Biological Chemistry* **274** 3235–3242.

Visconti PE, Ning X, Fornes MW, Alvarez JG, Stein P, Connors SA & Kopf GS 1999*b* Colesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Developmental Biology* **214** 429–443.

Yamashita T, Murakami T, Otani S, Kuwajima M & Shima K 1998 Leptin receptor signal transduction: OBRa and OBRb of fa type. *Biochemical and Biophysical Research Communications* **246** 752–759.

Yaspelkis BB III, Ansari L, Ramey EL, Holland GJ & Loy SF 1999 Chronic leptin administration increases insulin-stimulated skeletal muscle glucose uptake and transport. *Metabolism* **48** 671–676.

Zak LJ, Cosgrove JR, Aherne FX & Foxcroft GR 1997 Pattern of feed intake and associated metabolic and endocrine changes differentially affect post weaning fertility in primiparous lactating sows. *Journal of Animal Science* **75** 208–211.

Zhao AZ, Bornfeldt KE & Beavo JA 1998 Leptin inhibits insulin secretion by activation of phosphodiesterase 3B. *Journal of Clinical Investigation* **102** 869–873.

Zurek E, Foxcroft GR & Kennelly JJ 1995 Metabolic status and interval to first ovulation in postpartum dairy cows. *Journal of Dairy Science* **78** 1909–1920.

Received 29 June 2007
First decision 27 July 2007
Revised manuscript received 29 February 2008
Accepted 20 March 2008

Al Magnifico Rettore
Università della Calabria

Presentazione al Collegio dei Docenti della Dott. ssa Carmela Guido per il conseguimento del titolo di " Dottore di Ricerca in Biochimica Cellulare ed attività dei Farmaci in Oncologia" (XXIII ciclo)

Nel corso del dottorato di Ricerca in "Biochimica Cellulare ed attività dei farmaci in Oncologia "(XXIII ciclo) gli interessi scientifici della dott.ssa Guido sono stati focalizzati allo studio della regolazione del gene PTEN da parte degli estrogeni in una linea cellulare umana di tumore testicolare derivata da una lesione primaria di seminoma in un paziente di 35 anni.

In particolare, la Dott.ssa Guido dal momento che le cellule di seminoma TCAM2 non sono state completamente caratterizzate dal punto di vista recettoriale nella prima fase del suo lavoro, ha verificato mediante la tecnica del Western-Blotting, la presenza di entrambe le isoforme del recettore estrogenico ossia il ERα ed il ERβ, i quali prevalentemente mediano l'azione degli estrogeni in quasi tutti i tipi cellulari. I risultati ottenuti mostravano come il nostro tipo cellulare esprime, a parità di contenuto proteico, ERβ mentre nessun segnale era presente a livello dell'isoforma a 67 kDa che è il peso molecolare riportato per il classico ERalpha, chiaramente espresso nelle cellule MCF7 di tumore mammario utilizzate come controllo positivo. Questo dato conferma altri risultati della letteratura che riportano come l'isoforma del ERβ sia quella maggiormente espressa nell'intero tratto genitale maschile. La presenza dell'ERbeta nelle TCAM2 suggerisce una estrogeno-dipendenza (o sensibilità agli estrogeni) dei tumori testicolari e supporta una possibile partecipazione di estrogeni endogeni e/o ambientali nella carcinogenesi testicolare.

Allo scopo di valutare una possibile azione dell'ormone e dei suoi recettori sull'espressione di PTEN sono state testate tre diverse concentrazioni di E2 come pure la combinazione di E2 con ICI, specifico antagonista di entrambi i recettori estrogenici.

Sotto stimolazione estrogenica, studi di Western blotting e di RT-PCR hanno attestato un aumento dell'espressione sia della proteina che dell' mRNA di PTEN, una fosfatasi lipidica, che oltre ad essere un importante fattore implicato nei meccanismi che inducono apoptosi, sembra essere coinvolto nella differenziazione delle cellule germinali testicolari tumorali. Questa azione sembra essere mediata dal recettore estrogenico in quanto l'effetto è reversato da ICI, specifico antagonista

dei recettori estrogenici. L'aumentato trascritto di PTEN indotto dall'E2 mediante i suoi recettori ci ha pertanto fatto ipotizzare una regolazione a livello genico.

Per confermare tale ipotesi nella fase successiva del lavoro, mediante saggi di trasfezione transiente abbiamo studiato la possibile azione estrogenica sul promotore del gene PTEN, utilizzando una serie di plasmidi coniugati con luciferasi esprimenti il promotore di PTEN per 'intero' oppure versioni delete. Attraverso lo studio di questi mutanti, abbiamo identificato la sequenza del gene PTEN responsiva agli estrogeni.

Infatti i nostri risultati hanno indicato che l'attività della luciferasi nel costrutto che conteneva il promotore full-length pGL2768, sembrava avere una minore attività rispetto a quella ottenuta con il plasmide deleto pGL3-612 (-1389/-778), suggerendo la presenza di potenziali regioni regolatorie negative all'interno della sequenza che va dal -777/-160. Mediante l'analisi della sequenza di DNA summenzionata abbiamo rilevato che la regione coinvolta nell'attività trascrizionale conteneva diversi siti per i fattori di trascrizione Sp1. La localizzazione di siti Sp1 nella regione regolatrice di PTEN ci ha fatto dunque presupporre un possibile legame del recettore estrogenico a questa regione promoter, suggerendo che questo svolge un ruolo importante nella regolazione trascrizionale del gene PTEN attraverso E2/ER.

La caratterizzazione delle interazioni molecolari tra i fattori di trascrizione che sono coinvolti nella transattivazione del gene PTEN attraverso l'E_2 è stata realizzata con l'EMSA assay, mentre il ChIp assay ci ha confermato l'interazione. Utilizzando un plasmide codificante una sequenza dominante negativa di ERbeta abbiamo bloccato l'up-regolazione di PTEN, confermando che il ERbeta media l'effetto dell'E2 su PTEN. Il gene PTEN codifica per una fosfatasi lipidica che regola negativamente il pathway della PI3K/AKT, classicamente coinvolto nella sopravvivenza cellulare. Nel presente lavoro si è evidenziato che l'espressione di Akt risultava negativamente regolata dall'azione degli estrogeni e che questo effetto è ER-mediato. Attraverso saggi di trasfezione con il plasmide dominante negativo per l'ERbeta e con il plasmide HeGo overesprimente ERalpha abbiamo confermato che l'effetto dell'E2 è ERbeta-mediato in quanto in assenza di ERbeta l'espressione di Akt non mostra alcuna differenza tra i diversi trattamenti, mentre al contrario la presenza ectopica di ERalpha, rivela un incremento di Akt dose-dipendente sotto trattamento con E2.

Inoltre, la candidata dott.ssa Guido allo scopo di approfondire lo studio sull'eventuale apoptosi indotta dagli estrogeni ha studiato una serie di target a valle di Akt, comprendenti proteine proapoptotiche come BAD e FKHR, le quali presentano un aumento del loro contenuto ssotto trattamento con E2, effetto che invece risulta abrogato in seguito a trattamento combinato con ICI. Questi dati potrebbero indicare che l'E2 induca apoptosi nelle cellule TCAM2, quindi abbiamo

valutato gli effetti dello steroide su due marcatori del processo come la caspasi 9 e PARP1 la cui attivazione non risulta particolarmente chiara ed evidente.

Poiché lo stesso pathway della PI3K/AKT è in comune con i meccanismi molecolari che regolano un altro tipo di morte cellulare, cioè l'autofagia, abbiamo voluto verificare l'influenza degli estrogeni su alcune proteine chiave del processo di autofagia, come la PI3KIII, Beclin-1 AMBRA ed UVRAG evidenziando dunque che sotto E2 vi è un aumento di tutte e tre le proteine coinvolte nel pathway autofagico e che gli estrogeni sono in grado di indurre autofagia nel nostro tipo cellulare.

Inoltre, nell'ultima parte del lavoro il correlato biologico per l'evidenziazione dell'autofagia è stato studiato mediante 2 differenti approcci metodologici: il saggio con la monodansylcadaverina MDC in fluorescenza, e l'osservazione al microscopio elettronico (TEM). Il saggio con la MDC chiaramente dimostra inoltre come in cellule transfettate con DN/ERbeta l'autofagia non si verifica. Interessante, il TEM permette la visualizzazione di un numero elevato di doppie membrane avvolgenti vari organelli cellulari, e che costituiscono autofagosomi ed amfisomi. Concludendo, sulla base di questi dati emerge che la morte cellulare indotta da E2/ERbeta nelle cellule TCAM2 potrebbe avvenire tramite autofagia.

Questi studi sono stati presentati sia a congressi nazionali che internazionali (ISCAA 2010, 2nd International Symposium of Clinical and Applied Anatomy. Praga, July 9th -11th, 2010; 30esimo Congresso Nazionale della Società Italiana di Patologia in collaborazione con l'American Society of Investigative Pathology (ASIP) 2010).

La dott.ssa Guido è inoltre coinvolta nel progetto di ricerca riguardante lo studio dei meccanismi di regolazione di PTEN in cellule di carcinoma mammario umano MCF7, ERalpha positive, sensibili e non all'azione del tamoxifene esaminando il ruolo di PTEN come possibile target fisiologico dell'azione del progesterone nella suddetta linea cellulare.

La produzione scientifica della Dott.ssa Guido ha riguardato anche altri importanti aspetti di fisiopatologia endocrina come quella rivolta alla sfera riproduttiva. I contributi sono stati originali ed hanno raggiunto ottimi livelli in quanto ospitati su riviste internazionali di elevato Impact Factor (Aquila et al. J Cell Physiol. 2009 ; Aquila et al. Br J Pharmacol. 2010; Carpino et al. Int. J. Androl. 2010; De Amicis et al. Int.J. Androl. 2010).

Attualmente, la dr.ssa Guido lavora in qualità di Visiting Scholar presso il Dipartimento "Stem cell biology" University of Thomas Jefferson, Philadelphia-USA (Prof. M.P. Lisanti) ad un progetto di ricerca che è finalizzato allo studio dell'autofagia su cancer-associated fibroblast phenotype.

Durante il triennio di Dottorato la candidata ha mostrato notevole competenza ed entusiasmo lavorativo, interesse nell'apprendimento ed autonoma applicazione di numerose tecniche laboratoristiche, capacità critiche nella scelta di protocolli sperimentali e nell'elaborazione dei risultati ottenuti. Ha partecipato con interesse a tutte le attività seminariali svolte presso la Facoltà di FSNS. La Dott.ssa Guido ha, inoltre, espletato attività di formazione in ambito laboratoristico e scientifico sia degli specializzandi afferenti alla Scuola di Specializzazione in Patologia Clinica della Facoltà di FSNS dell'Università della Calabria, che dei numerosi laureandi durante il periodo di svolgimento delle tesi sperimentali. Si è tra l'altro impegnata in attività didattiche integrative per gli insegnamenti di vari settori disciplinari. Pertanto si esprime parere ampiamente positivo sul lavoro svolto durante l'intero periodo del dottorato di ricerca.

Rende

Il Coordinatore del Dottorando
Prof. Diego Sisci

Il Tutor
Ch.mo Prof. Sebastiano Andò