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ORIGINAL ARTICLE

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Asthenozoospermia and membrane remodeling enzymes: a new role for phospholipase A_2

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SUMMARY

Phosholipase A_2 (PLA_2) activity in the seminal plasma and in sperm heads is closely related to sperm motility and male fertility. Therefore, the purpose of this study was to investigate the possible involvement of different isoforms of phospholipase in asthenozoospermia. To accomplish this, cPLA_2, phospho-cPLA_2, iPLA_2, and sPLA_2 were evaluated by immunofluorescence and immunoblot analyses in spermatozoa obtained from 22 normozoospermic men and 28 asthenozoospermic patients. We found significant differences in cPLA_2 and its phosphorylated/activated form, iPLA_2, and sPLA_2 content and distribution in normal and asthenozoospermic patients. cPLA_2 was localized in heads, midpieces, and tails of all spermatozoa as constitutive enzyme, less expressed in the tail of spermatozoa with low progressive motility. While active phospho-cPLA_2 distribution was homogeneous throughout the cell body of control-donor spermatozoa, lower levels were detected in the tails of asthenozoospermic patients, as opposed to its strong presence in heads. Low immunofluorescence signal for iPLA_2 was found in astenozoospermic patients, whereas sPLA_2 was significantly lower in the heads of asthenozoospermic patients. Spermatozoa with low progressive motility showed differences both in terms of total specific activity and of intracellular distribution. cPLA_2, iPLA_2, and sPLA_2 specific activities correlated positively and in a significantly manner with sperm progressive motility both in normozoospermic men and asthenozoospermic patients. In conclusion, PLA_2s are expressed in different areas of human spermatozoa. Spermatozoa with low motility showed differences in total specific activity and enzyme distributions. We speculated that PLA_2 expression and/or different distribution could be potential biomarkers of asthenozoospermia, one of the major causes of male factor infertility.

INTRODUCTION

One of the major causes of male infertility is asthenozoospermia which, in particularly severe cases, may even influence the pregnancy success rates following assisted reproductive techniques (Tubman *et al.*, 2013). Similar to other cell types, in spermatozoa enzymes are expressed or translocate in different compartments of the cell body, depending on the type of reaction they catalyze or their function. A large amount of data has shown a close correlation between the deficit or absence of sperm motility with presence/absence of specific enzymatic proteins and/or their different distribution within the sperm cell body. For example, motility is a process which requires a substantial amount of ATP, which is hydrolyzed by dynein ATPases, to support coordinated movement (Nakano *et al.*, 2011).

Potential sources of ATP are compartmentalized in distinct regions along the length of the flagellum, while mitochondria are confined to the middle piece of the sperm tail, presenting a challenge to the metabolic machinery to provide sufficient ATP, to support motility along the entire length of the flagellum (Nakano *et al.*, 2011). Moreover, several glycolytic enzymes are localized predominantly in the principal piece, including spermatogenic cell-specific hexokinase 1 variants (HK1S) (Nakamura *et al.*, 2008, 2010), glyceraldehyde-3-phosphate dehydrogenase (Welch *et al.*, 2000; Chaikuad *et al.*, 2011), enolase (Gitlits *et al.*, 2000), and lactate dehydrogenase C (Odet *et al.*, 2011). The functional relationship between capacitation-dependent cell signaling and compartmentalized metabolic pathways has been shown in murine spermatozoa

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(Travis *et al.*, 2001). In sea urchin spermatozoa, several flagellar proteins become phosphorylated by cAMP-dependent protein kinase (PKA) when treated with egg jelly (Su *et al.*, 2005). Flagellar creatine kinase (Sp-CK) uses phosphocreatine for ATP regeneration and could play roles in creatine rephosphorylation and in removing ADP, which is a potent inhibitor of dynein ATPase (Kinukawa *et al.*, 2007). A total of 34 sperm proteins (20.6% of the total) were found to be related to sperm motility (Vicens *et al.*, 2014). Several factors have been reported to regulate sperm motility, such as, for instance, high bicarbonate and calcium (Xie *et al.*, 2013). Regulation of sperm motility has been linked to cAMP signaling pathways in different animal species, including mammals (Lasko *et al.*, 2012) and amphibians (O'Brien *et al.*, 2011), and it has been shown that a decrease in the phosphorylated status of PKC substrates is linked to the regulation of motility. Both PKC substrates and calcineurin are observed in the tail (Krapf *et al.*, 2014).

Flagellar motility is regulated by signaling events which control sperm ion milieu, energy production, and classical second messenger-dependent phosphorylation cascades. In sea urchin spermatozoa, some mitochondrial proteins regulated by PKA or PKC may influence motility (Loza-Huerta *et al.*, 2013). During human spermiogenesis, the enzyme 3-hydroxyisobutyrate dehydrogenase (HIBADH), and its enzymatic ability, is down-regulated in low motility spermatozoa, suggesting that HIBADH plays an important role in regulating sperm function (Tasi *et al.*, 2013). Phospholipases A_2 (PLA_2s) have been implicated in the cascade of signaling events underlying acrosomal exocytosis generating important messengers and metabolites (Roldan, 1998). PLA_2 activity in human seminal plasma is closely related to male fertility and PLA_2 deficiency in the head of spermatozoa may cause male infertility (Wang *et al.*, 2003).

PLA_2 can be broadly classified into four major categories: cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), secretory Ca^{2+}-dependent PLA_2 ($sPLA_2$), Ca^{2+}-independent PLA_2 ($iPLA_2$), and platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein associated (Lp)PLA_2s (Alberghina, 2010). Both arachidonic acid (AA) and lysophospholipids are generated by PLA_2 activity, and they may either act directly or serve as substrates for the generation of other metabolites with important roles in the final stages of membrane fusion (Riffo & Parraga, 1997). Both of them can also stimulate or induce acrosomal exocytosis and it is released from phospholipids by the action of the different isoforms of PLA_2s (Lessig *et al.*, 2006). In mammalians, AA is the precursor molecule of eicosanoids, and converted to prostaglandins or leukotrienes by the action of COXs and 5-lipoxygenase, respectively. $cPLA_2$, iPLA, and $sPLA_2$ differ from each other in terms of substrate specificity, calcium requirement, lipid modification, translocation to cellular membranes, and AA release (Alberghina, 2010). It has been shown that the presence of PAF-acetylhydrolase PLA_2 family (PAFah) in human semen is significantly and negatively correlated with sperm motility, whereas PAF is qualified to be a candidate for sperm capacitation factors (Zhu *et al.*, 2006).

On this basis, this study was undertaken to evaluate the presence of the three isoforms of phospholipase in spermatozoa obtained from normozoospermic donors and from patients with asthenozoospermia.

MATERIALS AND METHODS

Chemical and antibodies

Reagent-grade chemicals were purchased from Sigma (St. Louis, MO, USA) or E. Merck (Darmstadt, Germany). Percoll, protease, and phosphatase inhibitor cocktail and calcium-independent phospholipase A_2 inhibitor bromoenol lactone (BEL) were from Calbiochem (La Jolla, CA, USA). AACOCF3 and rabbit polyclonal against $iPLA_2$ antibody were from Cayman Chemical Co. (Ann Arbor, MI, USA). $cPLA_2$ (*mouse monoclonal*), phospho-$cPLA_2$ (*rabbit polyclonal*), group II $sPLA_2$ (*rabbit polyclonal*), and anti-mouse rhodamine-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). $cPLA_2$, $iPLA_2$, and $sPLA_2$ specific activity assay kits were purchased from Cayman Chemical Co (cat. n. 765021 and n. 765001, respectively).

Semen sample collection

Semen samples were collected from 22 healthy men with normal sperm concentration, total number and total and progressive mobility, and 28 asthenozoospermic patients by masturbation after 3 days of abstinence in the course of infertility work-up. The protocol was approved by the Internal Review Board and an informed written consent was obtained from controls and patients.

After liquefaction, each semen sample was analyzed according to the World Health Organization criteria (WHO, 2010). Samples with abnormal viscosity, increased number of leukocytes or of immature germ cells were not included. Sperm samples were then washed with 0.1 M phosphate-buffered saline (PBS) and centrifuged at 500× ***g*** for 10 min and then the seminal plasma was removed. The pellet was then overlaid on a discontinuous 40% and 80% (v/v; 1 mL each) Percoll gradient and centrifuged at 500× ***g*** for 25 mi, and the 80% fraction was collected (Calogero *et al.*, 1998). 5 μL of this fraction, diluted 1:4 in PBS, were placed on a glass slide and then examined under phase-contrast microscope to ensure that it was free from contaminating cell types. Each fraction was then washed three times with 0.1 M PBS, pH 7.4, and pelleted at 200× ***g*** for 10 min (Calogero *et al.*, 1998).

Confocal microscopy and cell fluorescence analyses

Following Percoll separation, spermatozoa were allowed to settle onto a sterile circular cover glass, fixed with 2% paraformaldehyde-0.5% glutaraldehyde in 0.12 M phosphate buffer (pH 7.2) for 30 min, washed two times (5 min each) with phosphate-buffered saline (PBS), permeabilized by 0.2% Triton X-100 in PBS for 10 min. The non-specific sites were then blocked by incubation in 5% bovine serum albumin (BSA) in PBS at room temperature for 30 min. Subsequently, spermatozoa were incubated with primary antibodies (1:200 dilution for all antibodies, anti-phospho-$cPLA_2$, $sPLA_2$, $iPLA_2$, and $cPLA_2$) overnight at 4 °C in a moist chamber. Following three PBS-washings, anti-rabbit FITC-conjugated secondary antibody and anti-mouse rhodamine-conjugated secondary antibody (both 1:100 dilution) were added for 1 h at room temperature. The specimens were washed three times for 3 min each in PBS and mounted in a 50% glycerol solution. Sperm cells incubated without the primary antibodies served as negative controls.

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Confocal microscopy was performed with an Olympus FV1000 confocal laser scanning microscope (LSM) equipped with UV/visible lasers: 405 nm (50 mW), 20 mW Multiline Argon laser (457 nm, 488 nm, 515 nm, total 30 mW), HeNe (G) laser (543 nm, 1 mW), HeNe (R) laser (633 nm, 1 mW), and spectral filtering system. Acquisition parameters, kept constant for all the samples, were as follows: 488 nm excitation at 20% laser power with emission filter SDM560, emission range 500–530 nm, PMT voltage at 510 V (channel 1, green) and 543 nm excitation line at 31% laser power, emission filter BFA560IF, PMT 435 V (channel 2, red). The detector gain was fixed at a constant value and emitted light was detected in sequential mode (Kalman integration type). Micrographs were acquired with oil immersion objective (60xO PLAPO) and 1× zoom. Images were taken at five random locations throughout the area of the sample. Experiments were performed in triplicate.

Image analysis was carried out using Image J (version 1.46e, public domain, Image Processing and Analysis in Java, National Institutes of Health). For each condition a total number of ~120 cells (~8 cells/field) was analyzed. Specifically, after background subtraction, three regions of interest (ROIs) were selected for each individual cell in the three different cellular regions of the head, the midpiece and the tail. Hence, the measured fluorescence intensity was normalized to the area of the corresponding ROI, the values averaged and the associated standard error were calculated.

Phospholipase A_2 assay for specific activity evaluations

An aliquot of sperm samples, from all single controls and patients, in each of which progressive motility had been evaluated, were lysed as previously described (Harlow & Lane, 1988). For c- and $iPLA_2$ specific activities, equal amounts of lysates were incubated with the substrate arachidonoyl-thio-phosphatidylcholine (ATPC), using the specific assay kit and following the manufacturer's instructions. Arachidonyl trifluoromethyl ketone (AACOCF3, 50 μM) was used as competitive inhibitor of both c- and $iPLA_2$-specific activities. EDTA (1 mM) and bromoenol lactone (BEL, 2.5 μM) allowed to discriminate between c- and $iPLA_2$ activitiy (Anfuso *et al.*, 2014; Lupo *et al.*, 2014). None of these components affected cell viability, as verified by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue) or trypan blue exclusion test (data not shown). The results for c- and $iPLA_2$-specific activities were expressed as pmol of ATPC hydrolyzed per minute and per milligram of protein (pmol/min/mg). To evaluate $sPLA_2$ enzyme activity, a $sPLA_2$ enzyme-linked immunosorbent assay (ELISA) kit was used with the substrate 1,2-dithio analog of diheptanoyl phosphatidylcholine, following the manufacturer's instructions. $sPLA_2$-specific activity was expressed as pmol of diheptanoyl Thio-PC (DTPC) hydrolyzed per minute and per milligram of protein (pmol/min/mg).

Immunoblotting

After Percoll separation, spermatozoa from normozoospermic and asthenozoospermic samples were washed twice in 0.1 M PBS, pH 7.4 and suspended in RIPA (radio immunoprecipitation assay) lysis buffer containing protease and phosphatase inhibitor cocktail and incubated for 1 h at 4 °C followed by sonication (three pulses of 5 sec each) at 20% amplitude (Harlow & Lane, 1988). The protein concentration in lysates was assayed by the BCA (bicinchoninic acid assay) method. Membranes were incubated with primary antibodies against total $cPLA_2$ (1:500), phospho-$cPLA_2$ (1:1000), $iPLA_2$ (1:1000), and $sPLA_2$ (1:500).

Statistical analysis

Data are reported as mean ± SEM throughout the study. Statistical analysis was performed using GraphPad Prism software by one-way analysis of ANOVA, followed by Tukey's post hoc test or Student's t test, as appropriate. Correlation analysis was performed using GraphPad Prism software by Pearson's correlation analysis. A p-value <0.05 was considered statistically significant.

RESULTS

Sperm parameters

The main sperm parameters from both normozoospermic men and asthenozoospermic patients are reported in Table 1. Seminal fluid volume, sperm concentration, progressive motility and normal forms were significantly lower in the latter compared with the former ones.

Immunolocalization of phospholipases A_2

All immunofluorescence experiments were performed analyzing different microscopy fields in all 22 healthy men and 28 asthenozoospermic patients, with similar results within each group (Figs 1 and 2). Figure 1, right panels showed the respective densitometric quantifications in normozoospermic men and in asthenozoospermic patients. Figure 1 (panel A) reports c-PLA_2 immunolocalization in normozoospermic men and asthenozoospermic patients. In the former, the $cPLA_2$ signal was bright red, localized in the head, midpiece, and tail. Differently, the enzyme was less strongly expressed, by 3.3-fold, in the tail of the spermatozoa with low motility.

The distribution of activated phospho-$cPLA_2$ was almost homogeneous throughout the cell body of control spermatozoa, well-detected in the head and in the tail, less in the midpiece (Fig. 1, panel B). Interestingly, whereas significant 1.76-fold higher phospho-$cPLA_2$ green signals were detected in sperm heads of asthenozoospermic samples compared with controls, the presence of activated enzyme was significantly lower in asthenozoospermic midpieces and tails, by 73% and almost by 98%, respectively. Green immunofluorescent signals related to $iPLA_2$ are reported in Fig. 2, panel A. $iPLA_2$ was evident in the heads and in the midpieces of both normozoospermic and asthenozoospermic samples. The images show a significant lower signal extension in the tails of spermatozoa with low

Table 1 Main sperm parameters of the normozoospermic healthy men and in infertile patients with asthenozoospermia included in the study

Parameter	Normozoospermic men (n = 22)	Asthenozoospermic patients (n = 28)
Seminal fluid volume (mL)	2.6 ± 0.13	2.0 ± 0.11*
Sperm concentration (mil/mL)	60.3 ± 7.8	28.7 ± 6.1*
Total motility (%)	56.8 ± 1.8	38.8 ± 2.0*
Progressive motility (%)	37.7 ± 1.3	12.2 ± 1.3*
Normal forms (%)	3.59 ± 0.44	1.93 ± 0.16*
Seminal fluid leukocytes (mil/mL)	0.95 ± 0.17	0.64 ± 0.20

Data are expressed as mean ± SEM. p < 0.005 vs. normozoospermic men (Student's t-test). *p<0.005.

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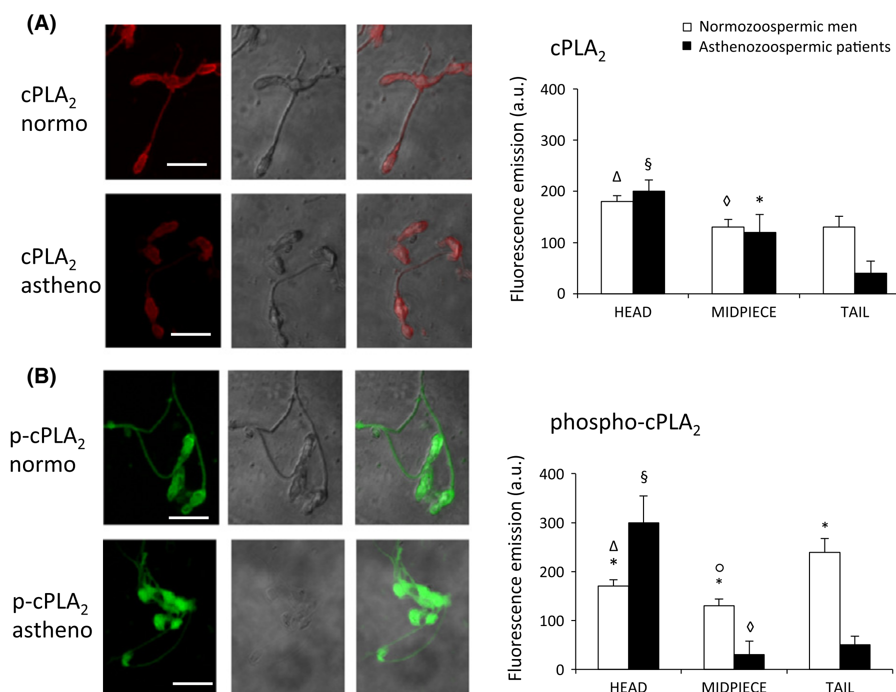
Figure 1 $cPLA_2$ (Panel A) and phospho-$cPLA_2$ (p-$cPLA_2$, Panel B) immunolocalization in spermatozoa isolated from normozoospermic men and asthenozoospermic patients. Percoll-purified spermatozoa were fixed and analyzed by staining with rhodamine- ($cPLA_2$) or FITC (p-$cPLA_2$)-secondary antibodies. The images are also reported in phase contrast (in the middle) and merge (right), to highlight the phenotype and cell architecture. On the right, graphical representations with respective densitometric quantifications (ImageJ program) in normozoospermic men (n = 22) and in astenozoospermic patients (n = 28) are reported. Values are expressed as mean ± SEM; statistically significant differences, by one-way ANOVA and the Tukey post-test ($p < 0.05$) are indicated. Panel A: (*), normozoospermic vs. asthenozoospermic group; (Δ), head vs. midpiece and tail in normozoospermic group; (§), head vs. midpiece and tail in asthenozoospermic group; (◊), midpiece vs. tail in asthenozoospermic group. Panel B: (*), normozoospermic vs. asthenozoospermic group; (Δ), head vs. midpiece in normozoospermic group; (○), midpiece vs. tail in normozoospermic group; (§), head vs. midpiece and tail in asthenozoospermic group; (◊), midpiece vs. tail in asthenozoospermic group.



motility. Quantifications of the immunosignals revealed a significant lower (1.65-fold) $iPLA_2$-related fluorescence in low motile sperm tails, in comparison to healthy donors.

The different expression/localization of $sPLA_2$ is shown in Fig. 2 panel B. The enzyme was strongly present in the head of spermatozoa from normal donors compared with the levels of localization in the intermediate section and in the tail. No significant differences in the signals from the intermediate section and the tail of both groups were evident, whereas the fluorescent signal in the heads of asthenozoospermic samples was significantly fainter (by 37.1%) than the signal found in healthy donors.

PLA_2s enzyme activities

Figure 3, panel A reports PLA_2-specific activity values in normozoospermic and asthenozoospermic samples. EDTA and BEL allowed us to discriminate between the contributions of $cPLA_2$ and $iPLA_2$ in AA release. Without inhibitors, the specific activity of total $cPLA_2$ and $iPLA_2$ was 38% lower in asthenozoospermic samples compared with controls. A proportional decrease in the $cPLA_2$ and $iPLA_2$ occurred in the presence of specific inhibitors. BEL decreased $iPLA_2$ activity by 1.43-fold and 1.21-fold, respectively, in spermatozoa isolated from normozoospermic and asthenozoospermic samples. The sharp decrease in the $cPLA_2$ activity caused by EDTA (65% and almost 68%, respectively) was attributed to the calcium-dependent form of the two enzymatic activities, having a greater role in the release of AA in spermatozoa. AACOCF3, a competitive inhibitor of both enzyme activities, decreased the activity by 90% and almost 92%, respectively. $sPLA_2$ activity (panel A') was 1.88-fold lower in spermatozoa from asthenozoospermic samples compared with normozoospermic ones. Because the changes in PLA_2 activity and the progressive motility parameters varied between samples from normozoospermic and asthenozoospermic donors, the question of whether the variation in the motility parameters in a given sample might be attributable to the observed variation in PLA_2 activity arose.

A multiple linear regression was used to assess whether PLA_2 activity correlated with progressive motility. Strong correlations between progressive motility and specific enzymatic activities were found, both in normozoospermics and asthenozoospermics (Fig. 3, panel B). The correlation coefficients for $cPLA_2$ almost reached the highest values in both groups. A good positive correlation was found for $iPLA_2$ in both normozoospermic men and asthenozoospermic patients. The plots showed significant differences in both slopes and elevations.

Western blotting for PLA_2 protein expression/activation

Western blot analyses of $cPLA_2$ and phospho-$cPLA_2$, $iPLA_2$, $sPLA_2$ in sperm lysates from normozoospermic men and asthenozoospermic patients are reported in Fig. 4. Immunoblot analysis revealed a $cPLA_2$ total protein expression higher by almost 1.7-fold compared with spermatozoa with low motility

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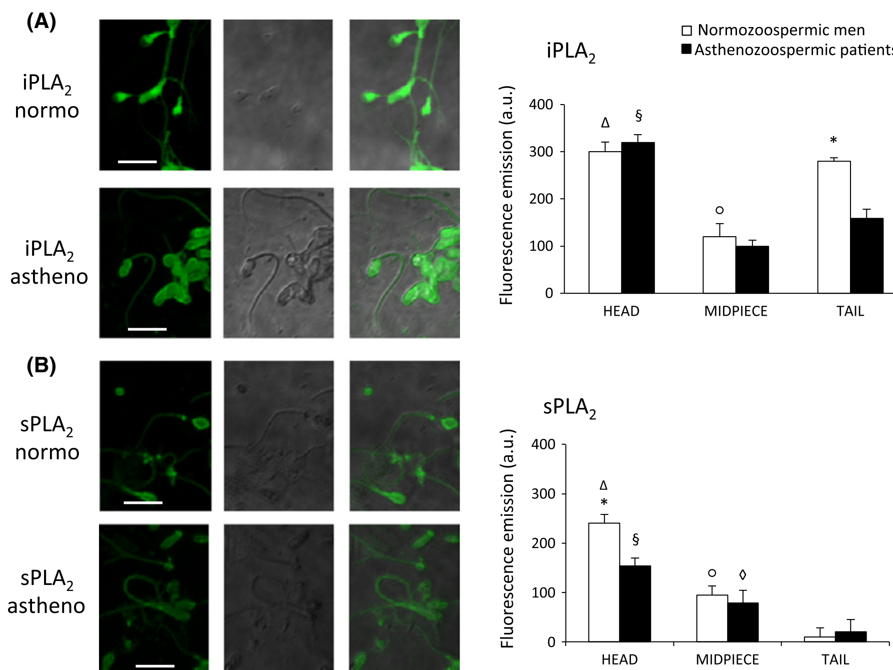
Figure 2 $iPLA_2$ (Panel A) and $sPLA_2$ (Panel B) immunolocalization in spermatozoa isolated from normozoospermic men and asthenozoospermic patients. Percoll-purified spermatozoa were fixed and analyzed by staining with respective FITC-secondary antibodies. The images are also reported in phase contrast (in the middle) and merge (right), to highlight the phenotype and cell architecture. On the right, graphical representations with respective densitometric quantifications (ImageJ program) in normozoospermic men (n = 22) and in astenozoospermic patients (n = 28) are reported. Values are expressed as mean ± SEM; statistically significant differences, by one-way ANOVA and the Tukey post-test ($p < 0.05$) are indicated. Panel A: (*), normozoospermic vs. asthenozoospermic group; (Δ), head vs. midpiece in normozoospermic group; (○), midpiece vs. tail in normozoospermic group; (§), head vs. midpiece and tail in asthenozoospermic group. Panel B: (*), normozoospermic vs. asthenozoospermic group; (Δ), head vs. midpiece and tail in normozoospermic group; (○), midpiece vs. tail in normozoospermic group; (§), head vs. midpiece and tail in asthenozoospermic group; (◊), midpiece vs. tail in asthenozoospermic group.



(panel A). In addition, both total $cPLA_2$ synthesis and phosphorylation were higher suggesting enzyme activation (1.2 p-$cPLA_2$/$cPLA_2$ ratio in normal, 0.7 ratio in asthenozoospermic samples) and an increase in $cPLA_2$ activity. Panels B and C show the immunoblottings relative to $iPLA_2$ and $sPLA_2$ total protein contents, respectively. Once again, both protein expressions were significantly higher in normal samples, suggesting a correlation between lower PLA_2s content/activation and reduced sperm motility.

DISCUSSION

Spermatozoa from mammals are unable to fertilize the oocyte immediately after ejaculation, inasmuch as they undergo a series of biochemical transformations in the female reproductive tract, generally called capacitation. Motility is one of the most important functions of male gametes.

Asthenozoospermia has been reported to account for almost 50% of male infertility (Curi *et al.*, 2003). Up to now, the molecular mechanisms regulating sperm movement remain elusive. Studies of gene knockout mouse models, defects in a single gene, and proteomics of differentially regulated proteins in asthenozoospermia have provided a list of proteins fundamental for sperm motility (Upadhyay *et al.*, 2013). At the cytoskeletal level, sperm flagellar motility is thought to be a result of specific interaction between axonemal microtubular proteins and dynein motors (Bhagwat *et al.*, 2014).

PLA_2s are important enzymes for the metabolism of fatty acids in membrane phospholipids. They cluster in four main categories: secreted-, cytosolic-, calcium-independent PLA_2s, and PAF(Lp)PLA_2s. Each of these types has differently been implicated in lipid metabolism and disease (Burke & Dennis, 2009). Among them, the group IV calcium-dependent cytosolic PLA_2 is widely expressed in nearly all mammalian cells (Van Rossum & Patterson, 2009; Sun *et al.*, 2014).

In this study, we have shown the presence of the three isoforms of PLA_2 in spermatozoa of both normozoospermic men and asthenozoospermic patients by confocal microscopy and specific activity assays. Both total and activated-phosphorylated $cPLA_2$ forms were present, although with significant differences in terms of total content, percentage of phosphorylation/activation and intracellular localization between the two groups of spermatozoa taken into consideration. Total $cPLA_2$ was uniformly localized in heads, midpieces, and tails of control spermatozoa as constitutive enzyme, but it was significantly less expressed in the tail of spermatozoa with low motility. In spermatozoa from normozoospermic men, active phospho-$cPLA_2$ was well detected in the head and in the tail but less in the midpiece; significant lower phospho-$cPLA_2$ levels were detected in the tails of spermatozoa isolated from asthenozoospermic samples, in contrast to its strong presence in the head. At present, we do not know the intrinsic molecular reasons that can give an explanation for the lack of the total $cPLA_2$ and for the different distribution of its phosphorylated form in low motile spermatozoa. It has been reported that $cPLA_2\alpha$ gene disruption impairs female reproductive ability (Uozumi *et al.*, 1997; Achache *et al.*, 2010).

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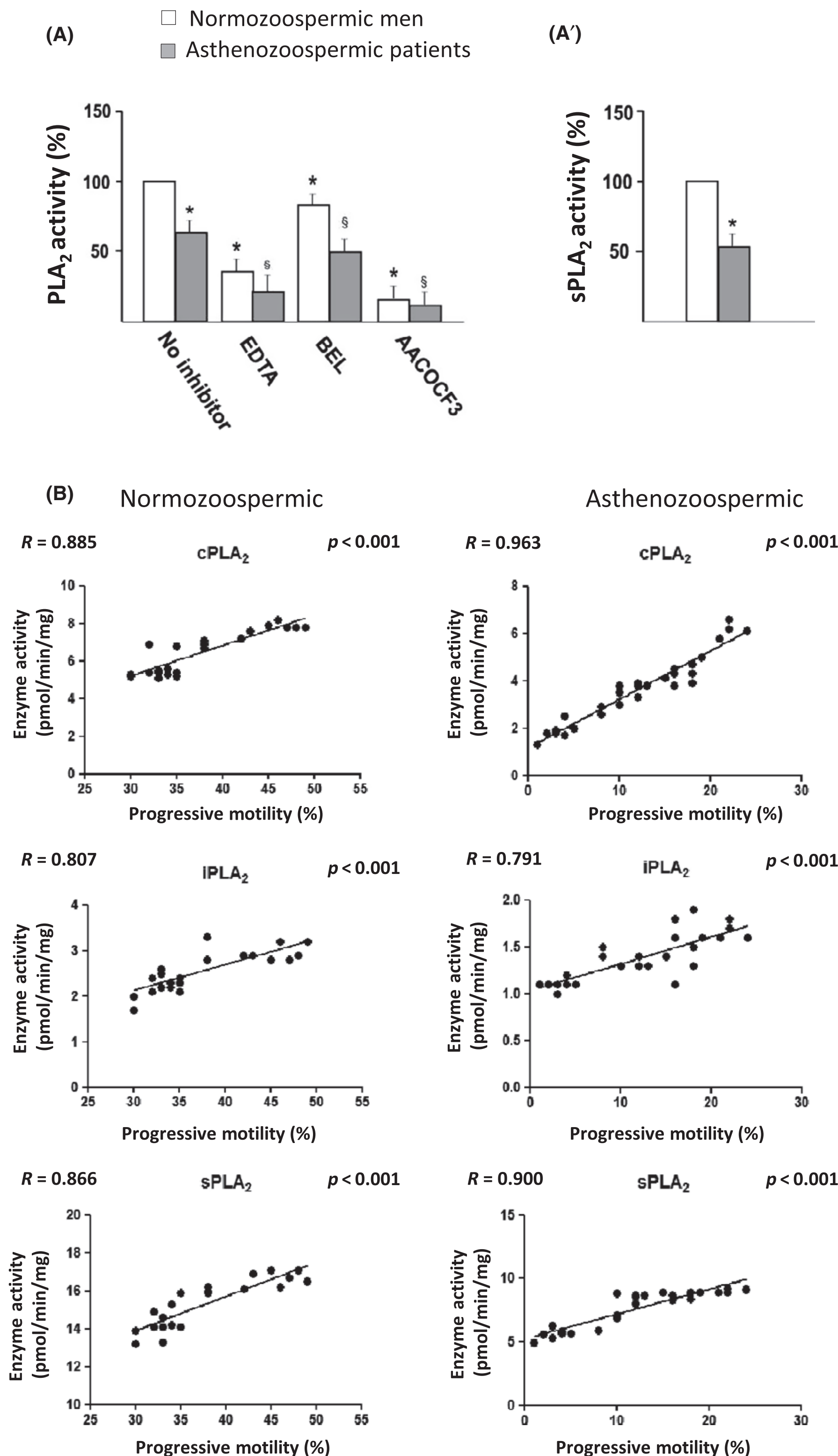

Figure 3 PLA_2s enzyme activities in isolated spermatozoa and their correlation with progressive motility in normozoospermic and asthenozoospermic group. Values are expressed as percentage. Panel A: c- and $iPLA_2$. All incubations were performed with or without 5 mM EDTA or 2.5 μM BEL or 50 μM AACOCF3 (see text). c- and $iPLA_2$ control total specific activity value was 7.4 ± 0.3 pmol/min/mg protein. Panel A′: $sPLA_2$ specific activity; control value was 15.3 ± 1.1 pmol/min/mg protein. Values are expressed as mean ± SEM measured in normozoospermic men ($n = 22$) and in astenozoospermic patients ($n = 28$), performed in triplicate. Statistically significant differences, by one-way ANOVA and the Tukey post-test, are indicated (*, §, $p < 0.05$). (*), vs. total enzyme activity in spermatozoa of normozoospermic men (100% activity); (§),vs. enzyme activity in respective inhibitor-treated spermatozoa of normal men. Panel B: Correlation analysis between sperm progressive motility and $cPLA_2$, $iPLA_2$,and $sPLA_2$-specific activities in normozoospermic healthy men and in infertile patients with asthenozoospermia. Data are expressed as mean ± SEM, $p < 0.001$ vs. progressive motility (Student's *t* test).

As far as phospho-$cPLA_2$, it is phosphorylated on Ser^{505} by the mitogen-activated protein kinases MAPKs, extracellular signal-regulated kinase ERK1/2, and p38 MAPK (Gijon *et al.*, 2000), on Ser^{727} by MAPK-interacting kinase I (Hefner *et al.*, 2000) and on Ser^{515} by CaMK-II (Muthalif *et al.*, 2001). The phosphorylation is essential for the enzyme activation (Alberghina, 2010). Moreover, the intracellular Ca^{2+} concentration is fundamental for $cPLA_2$ translocation on the Golgi, the endoplasmic reticulum, and the perinuclear membranes and activation (Evans *et al.*, 2002). Sperm maturation processes change the response of spermatozoa to calcium ions, and calcium binding substances and calcium transport inhibitors are secreted by male accessory sexual organs and mixed with spermatozoa during ejaculation (Rahman *et al.*,

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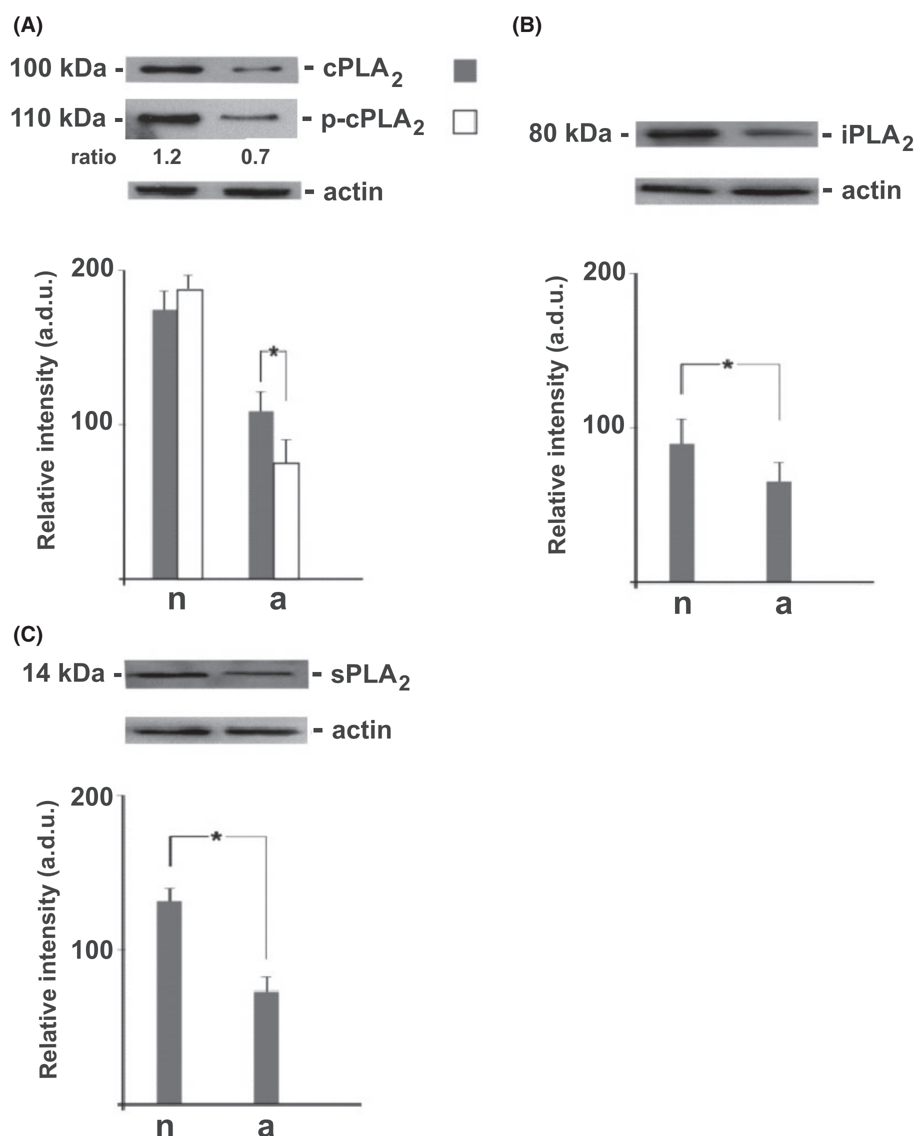

Figure 4 Western blot analyses of $cPLA_2$ and phospho-$cPLA_2$ (panel A), $iPLA_2$ (panel B), $sPLA_2$ (panel C) in sperm lysates from normozoospermic men (*n*) and asthenozoospermic patients (a). Values [mean ± SEM of 22 normozoospermic men and 28 astenozoospermic patients, expressed as arbitrary densitometric units (a.d.u.)], were obtained by ImageJ program. Statistically significant differences, by one-way ANOVA and the Tukey posttest, are indicated (*$p < 0.05$).

2014). It has been proposed that the degree of flagellar pulse asymmetry results from the global Ca^{2+} concentration, and evidence indicated that CaMK-II and CaMK-IV are involved in flagellar bending in mammalian spermatozoa (Ignotz & Suarez, 2005; Marin-Briggiler *et al.*, 2005). It has been reported that CatSper channel (pH-sensitive, voltage-gated calcium channel) is exclusively expressed in the human testis and spermatozoa (Quill *et al.*, 2001) and that its expression and function are associated with progressive motility and may be involved in the pathogenesis of asthenozoospermia (Tamburrino *et al.*, 2014). Therefore, Ca^{2+} and its proper storage being fundamental for the physiological maturation of spermatozoa (consequently, for capacitation and fertilization capability), we do not exclude a correlation between a not precisely correct calcium storage and utilization and the consequent activation of the $cPLA_2$.

One of the functions attributed up to now to $iPLA_2$ has a housekeeping role in control of phospholipid levels (Burke & Dennis, 2009), even if some studies are more consistent with a signaling role (Hooks & Cummings, 2008). It has been shown that the decreased fertilization competence of spermatozoa from male $iPLA_2\beta^{-/-}$ mice is associated to reduced motility, suggesting that $iPLA_2\beta$ is a potential target for developing male contraceptive agents (Bao *et al.*, 2004). In this study, $iPLA_2$ was lower in the midpiece of the cell body, but strongly expressed in sperm heads and tails of spermatozoa isolated from normozoospermic men. On the contrary, low motile spermatozoa showed a significant lower immunofluorescence signal for $iPLA_2$ in the tails than spermatozoa isolated from controls. This data were confirmed by c- and $iPLA_2$-specific activity values in normal and asthenozoospermic men, calculated in samples in which head and tail were not separated (total lysates) and represent the total protein expression values.

Moreover, the use of BEL allowed us to discriminate between the contributions of $cPLA_2$ and $iPLA_2$, and this showed that $iPLA_2$ activity was lower by 1.43-fold in normal, and by 1.21-fold in low motile spermatozoa. The higher $cPLA_2$ activity attributed to this enzymatic form, the greater its role in the release of AA in spermatozoa. Data from multiple linear regression analysis demonstrated the strong correlations between progressive motility and

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all PLA_2-specific enzymatic activities, both in the normozoospermic and asthenozoospermic samples, as shown by the high correlation coefficient values.

Western blot analysis of sperm total lysates confirmed immunofluorescent and enzyme assays data. The different patterns of distribution for both isoforms and, between them, the different distribution of the total and activated form of $cPLA_2$ within the cell body, is very interesting and it needs further study. The deficiencies and/or the different distributions of both PLA_2s are certainly the cause of the different production and accumulation of AA and lysophospholipids. Recently, it has been shown that changes in the lipid composition of murine spermatozoa from the different epididymal regions during maturation are likely to be of physiological relevance and potentially useful as diagnostic markers of sperm maturation and motility acquisition (Pyttel *et al.*, 2014). For this reason, one would expect that an imbalance in phospholipids or their derivatives ratio may cause an abnormal maturation and, therefore, the increase in membrane fluidity and flexibility, required for motility in mature spermatozoa (Salvolini *et al.*, 2013). In addition, AA and docosahexaenoic acid (DHA) levels are significantly lower in spermatozoa of oligoasthenoteratozoospermic patients than in those of normozoospermic men, suggesting that sperm motility and morphology are positively correlated with these fatty acid levels (Khosrowbeygi *et al.*, 2008). Moreover, derivatives of the phospholipid hydrolysis, such as lysophosphatidic acid, play a focal role in motility in various cell types (Liao *et al.*, 2013; Wu *et al.*, 2014). Another aspect to take into consideration is that sperm motility depends upon a certain amount of PGs (Robert, 1976); the inhibition of cyclooxygenase prevents biosynthesis of PGs, causing a rapid fall in sperm motility (Kennedy *et al.*, 2003).

The $sPLA_2$ family contains low molecular weight (typically 14–18 kDa) enzymes with Ca^{2+} dependence and a His-Asp catalytic dyad (Kudo & Murakami, 2002). $sPLA_2$s exhibit distinct localization in spermatozoa, indicating that they could have redundant functions (Masuda *et al.*, 2004). It has been reported that Group III $sPLA_2$ regulates epididymal sperm maturation and fertility in mice (Sato *et al.*, 2010). Escoffier and colleagues have shown that $sPLA_2$ is released during sperm acrosome reaction and controls fertility outcome in mice (Escoffier *et al.*, 2010), while $sPLA_2$-IID is present in mature human spermatozoa, playing a physiological role during acrosome exocitosis and in sperm hyperactivation (Li *et al.*, 2012). Confocal immunofluorescent images of this study showed that, both in normozoospermic and asthenozoospermic groups, $sPLA_2$ is mainly localized in heads (significantly lower in spermatozoa from asthenozoospermic patients), less in midpieces, but no signal was found in tails. Regarding its distribution in spermatozoa, data already published have shown that $sPLA_2$ was present in the acrosomal region and in the midpiece of hamster (Riffo & Parraga, 1997), mouse, and human normal spermatozoa (Masuda *et al.*, 2004).

Similar to the other two PLA_2 forms, the *s*-isoform activity in total lysates of spermatozoa allowed us to detect a significantly lower (1.8-fold) total activity in the asthenozoospermic samples than normal ones, confirmed by immunoblots and related densitometric quantifications. The $sPLA_2$-X gene disruption experiments showed that it regulates the fertility of spermatozoa, acting beyond the step of flagellar motility (Sato *et al.*, 2011).

In conclusion, these findings showed that the three catalytically active $cPLA_2$, $iPLA_2$, and $sPLA_2$ are expressed in different areas within the human spermatozoon cell body. Spermatozoa with a significant low motility showed strong differences both in terms of total specific activity and of different intracellular distribution, compared with normal spermatozoa. These findings identify the PLA_2 expression/distribution as potential biomarker molecules of asthenozoospermia, one of the major causes of male factor infertility.

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AUTHORS' CONTRIBUTION

C.D.A. and G.L. designed the experiments; M.O., C.M., M.Sc., and N.B. performed the experiments; C.D.A., G.L., M.O., M. Sa., S.B., S.L.V., M. Sc., and A.E.C. analyzed the data; C.D.A. and G.L. wrote the manuscript; A.E.C. revised the final draft of the manuscript. All authors discussed the results and commented on the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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