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ORIGINAL RESEARCH

Peroxiredoxin 6 regulates the phosphoinositide 3-kinase/AKT pathway to maintain human sperm viability

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ABSTRACT: Peroxiredoxins (PRDXs) are antioxidant enzymes proven to control the levels of reactive oxygen species (ROS) and to avoid oxidative damage in the spermatozoon. Previously, we have shown that low amounts of PRDXs are associated with male infertility and that PRDX6 is the primary antioxidant defense in human spermatozoa, maintaining survival and DNA integrity (Gong *et al.*, 2012, Fernandez and O'Flaherty, 2018). Oxidative stress can trigger different pathway cascades in the spermatozoa, including truncated apoptosis. It has been reported that the phosphorylation status of phosphoinositide 3-kinase (PI3K) and its target AKT (protein kinase B) prevent the spermatozoon from entering the truncated apoptotic cascade. Here, we aim to study the regulation of the PI3K/AKT pathway by PRDX6 and assess its role in maintaining sperm viability. Human semen samples were obtained over 1 year from 20 healthy non-smoking volunteers aged 22–30 years. Sperm viability, lipid peroxidation and apoptosis-like changes were determined by flow cytometry while phosphorylation of PI3K and AKT substrates were assessed by immunoblotting using anti-phospho-PI3K and anti-phospho-AKT substrates antibodies. We found that the addition of arachidonic acid and lysophosphatidic acid, products of PRDX6 calcium-independent phospholipase A_2 (Ca^{2+}-iPLA$_2$), prevented loss of sperm viability and maintained the phosphorylation of PI3K. Antioxidant compounds such as D-penicillamine partially prevented the oxidative damage on spermatozoa that led to a reduction of their viability. Thus, other pathways can also participate in sperm survival and be regulated by PRDXs. In conclusion, PRDX6 contributes to the regulation of ROS production and the PI3K/AKT pathway for the maintenance of sperm survival.

Key words: peroxiredoxins / oxidative stress / PI3 kinase / AKT-Substrates / arachidonic acid / lysophosphatidic acid / sperm viability / apoptotic-like changes

Introduction

Approximately 15% of couples worldwide are infertile (World Health Organization, 1997; Evers, 2002; Bushnik *et al.*, 2012), and the male factor is the sole contributing cause of infertility in approximately half of these cases (Abid *et al.*, 2008; World Health Organization, 2010). Human spermatozoa are extremely sensitive to oxidative stress, a condition resulting from an imbalance between reactive oxygen species (ROS) production and the antioxidant defense system within the cell (Gagnon *et al.*, 1991; Halliwell and Gutteridge, 2007). Currently, oxidative stress is an important and plausible cause of idiopathic male infertility (Anderson and Williamson, 1988; Agarwal *et al.*, 2008; Agarwal *et al.*, 2014).

High concentrations of ROS can cause a wide range of damage to spermatozoa such as peroxidation of unsaturated fatty acids of the plasma membrane (Storey, 1997), single-stranded or double-stranded DNA breaks (Wang *et al.*, 2003), reduced mitochondrial membrane potential (Gallon *et al.*, 2006; Koppers *et al.*, 2008) as well as low levels of energy production that lead to an impairment in sperm motility (de Lamirande and Gagnon, 1992a, b). In general, high ROS levels are associated with a decrease in semen quality and male infertility (de Lamirande and Gagnon, 1995b; Pasqualotto *et al.*, 2000). On the other hand, low ROS concentrations have a beneficial role in regulating several transmembrane signal transduction pathways in somatic cells (Rhee, 2007) and capacitation, acrosome reaction and motility in spermatozoa (de Lamirande and Gagnon,

1993, 1995b, a; O'Flaherty et al., 2003; de Lamirande and O'Flaherty, 2012).

Human spermatozoa have limited antioxidant protection. There are low concentrations of reduced glutathione (Li, 1975) and small amounts of cytoplasm containing superoxide dismutase 1 (SOD1). Ejaculated human spermatozoa do not have catalase (Zini et al., 1993; O'Flaherty, 2014b), glutathione peroxidase (GPX) 1, GPX2, GPX3 or GPX5 (Williams et al., 1998; Chabory et al., 2009), and mitochondrial mGPX4 is enzymatically inactive in mature spermatozoa (Ursini et al., 1999; Foresta et al., 2002; Conrad et al., 2005). Then, the functional antioxidant system in human spermatozoa is composed of SOD1 and 2, nuclear nGPX4 and all six peroxiredoxins (PRDXs). Spermatozoa are also equipped with enzymes to reduce the cysteine residues in the PRDX active site to make them active after they reacted with ROS (thioredoxin (TRX), TRX reductase and the specific sperm TRX1 (SPTRX1) and 2 (SPRTX2) (O'Flaherty and de Souza, 2011; O'Flaherty, 2014b, a). Therefore, the presence of antioxidant enzymes is important to circumvent oxidative damage in the spermatozoa, and the PRDX family appears to be a major player in the antioxidant defense in spermatozoa (O'Flaherty, 2014b, a). PRDXs are selenium-free peroxidases that are found in high abundance in human spermatozoa (O'Flaherty and de Souza, 2011; O'Flaherty, 2014b). The six members of the family are differentially localized in compartments of the human spermatozoon, with PRDX6 being the most abundant and present in all compartments (O'Flaherty and de Souza, 2011; O'Flaherty, 2014b; Fernandez and O'Flaherty, 2018). PRDX1–5 have two catalytically active cysteines per molecule (2-Cys PRDXs), while PRDX6 has only one. All PRDXs react with hydrogen peroxide, organic peroxides and peroxynitrite (Peshenko and Shichi, 2001; Dubuisson et al., 2004; Trujillo et al., 2007). Interestingly, PRDX6 has GPX, calcium-independent phospholipase A_2 (Ca^{2+}-iPLA$_2$) and lysophospholipid acyltransferase (LPCAT) activities (Fisher et al., 1999; Peshenko and Shichi, 2001). This isoform provides a complete system for the repair of peroxidized cell membranes. PRDX6 can reduce peroxidized cell membrane phospholipids (peroxidase activity) and replace the oxidized Sn-2 fatty acyl group through hydrolysis/reacylation (PLA$_2$ and LPCAT activities) (Fisher, 2017).

We have previously shown that PRDX6 is the primary antioxidant defense in human spermatozoa (Fernandez and O'Flaherty, 2018). Indeed, we recently reported that PRDX6 Ca^{2+}-iPLA$_2$ and peroxidase activities and, to a lesser extent, 2-Cys PRDXs are important to maintain viability and DNA integrity in human spermatozoa (Fernandez and O'Flaherty, 2018). The inhibition of PRDX6 Ca^{2+}-iPLA$_2$ with the transition state analog 1-hexadecyl-3-(trifluoroethyl)-*sn*-glycero-2-phosphomethanol (MJ33) promoted high levels of mitochondrial superoxide anion production and a dose-dependent increase in 4-hydroxynonenal (4-HNE) levels, a product of lipid peroxidation. However, the inhibitor of 2-Cys PRDXs, conoidin A, promoted similar levels of mitochondrial superoxide anion production only at the highest concentration tested (80 μM) without increasing 4-HNE levels. Taken together, these data confirm the primary action of PRDX6 Ca^{2+}-iPLA$_2$ to regulate oxidative stress and prevent lipid peroxidation in human spermatozoa. The inhibition of glutathione-S-transferase Pi, the enzyme required to re-activate PRDX6 peroxidase activity, generated a significant increase in endogenous peroxynitrite ($ONOO^-$) that was not produced when spermatozoa were treated with MJ33 or conoidin A. This finding demonstrates the need for PRDX6 peroxidase activity to regulate the levels of $ONOO^-$ in human spermatozoa (Fernandez and O'Flaherty, 2018).

The role of oxidative stress in triggering truncated apoptosis in spermatozoa has been previously reported (Aitken, 2011). The phosphorylation status of two key defenders of cellular integrity, namely PI3 kinase (PI3K) and its target AKT (protein kinase B), has been identified to prevent the spermatozoa from undergoing the truncated apoptotic pathway (Aitken et al., 2011). However, the molecular mechanism involved in the regulation of these kinases in human spermatozoa is unknown. Moreover, how the spermatozoon fights against the oxidative stress-dependent apoptosis is yet to be unveiled. It is known that PRDX6 activates the PI3K/AKT pathway to promote cancer invasion (Seung et al., 2009) and that overexpression of PRDX6 protects against cisplatin-induced apoptosis of human ovarian cancer cells (Pak et al., 2010). Knowing that PRDX6 is important to maintaining human sperm viability, we wanted to study further the molecular mechanisms involved. Thus, we hypothesize that inhibition of the PRDX6 iPLA activity will prevent phosphorylation of the PI3K/AKT pathway and subsequently will negatively impact on sperm survival. In the present study, we aimed to elucidate the role of PRDX6 in protecting human spermatozoa against oxidative stress by activating the PI3K/AKT pathway to maintain viability.

Materials and Methods

Materials

Probes used for sperm viability (Sytox Blue), lipid peroxidation (BODIPY C11) and apoptosis-like changes (Annexin V–Alexa Fluor 647) were purchased from Life Technologies (Burlington, ON, Canada). Percoll was obtained from GE Healthcare (Baie d'Urfe, QC, Canada). For western blot assays, nitrocellulose membranes (pore size, 0.22 mm) were purchased from Osmonics, Inc. (Westborough, MA, USA) and the ECL Kit Lumi-Light from Roche Molecular Biochemicals (Laval, QC, Canada). Radiographic films obtained from Fuji (Minami-Ashigara, Japan) were used for immunodetection of blotted proteins. The rabbit polyclonal anti-phospho-PI3 Kinase p85 (Tyr 458/p55, Tyr 199) and the anti-phospho-(Ser/Thr) AKT substrate antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The secondary antibodies, peroxidase-conjugated goat anti-mouse or anti-rabbit, were purchased from Jackson ImmunoReseach laboratories, Inc. (West Grove, PA, USA). The mouse monoclonal anti-α-tubulin antibody, lysophosphatidic acid (LPA) and arachidonic acid (AA) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Mouse anti-tubulin antibody, D-penicillamine (PEN), *N*-acetyl cysteine (NAC) and MJ33 were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

Subjects

Semen samples were collected on-site at the Royal Victoria Hospital (Montreal, QC, Canada) from healthy volunteers ($n = 20$) aged 22–30 years by masturbation after an abstinence period of 72 h. Donors were living in the Montreal area and were healthy men and students attending the local universities. After collection, samples were incubated at 37°C for 30 min to induce liquefaction and analyzed by computer-assisted semen analysis system (Sperm vision HR

Software v1.01, Penetrating Innovations, Ingersoll, ON, Canada). Only semen samples that fulfilled the quality according to the parameters set out by the World Health Organization guidelines (World Health Organization, 2010) and with progressive motility greater than 70% were included in this study. This study was approved by Ethics Committee of the McGill University Health Centre, as we previously described (Morielli and O'Flaherty, 2015; Lee *et al.*, 2017) following the suggested guidelines for human semen studies (World Health Organization, 2010; Sanchez-Pozo *et al.*, 2013; Bjorndahl *et al.*, 2016). All donors provided written consent to participate in this study.

Semen sample preparation

After 30 min of semen liquefaction, the samples were centrifuged on a four-layer Percoll gradient (95%–65%–40%–20%) to select the highly motile spermatozoa and separate them from the abnormal sperm cells, seminal plasma and white blood cells, as previously described (O'Flaherty *et al.*, 2004; Lee *et al.*, 2017). Spermatozoa were recovered from the 95% layer, and the 65–95% interface and concentration was determined using a Neubauer chamber. Samples were then diluted to 50×10^6 cell/ml in Biggers, Whitten and Whittingham (BWW, pH 7.8) medium and used for experimentation. Sperm were incubated in the presence or absence of different concentrations of MJ33, a transition state analog that specifically inhibits PRDX6 Ca^{2+}-$iPLA_2$ activity (Fisher *et al.*, 1992; Lee *et al.*, 2017), in the presence or absence of 2 mM PEN (Lee *et al.*, 2017), 4 μM AA or 5 μM LPA for 4 h at 37°C. The safety of MJ33 was demonstrated under *in vitro* or *in vivo* conditions. There was no decrease in viability of mouse perivascular endothelial cells that were treated with 5–25 μM MJ33 during 24 h. A549 human lung epithelial cells were cultured for 7 days and continuously exposed to 10–25 μM MJ33, and no signs of decrease in cell viability was observed during this period (Lee *et al.*, 2013). Mice treated with 12.5–125 μmol MJ33/kg body weight, either by the i.v. or intratracheal route, and observed for 7 weeks did not display any symptoms associated with toxicity such as hyperactivity, aggressive behavior, alopecia, dehydration, salivation, nasal discharge, constipation or diarrhea (Lee *et al.*, 2013).

The concentrations of AA and LPA used here were obtained by preliminary dose-dependent studies (Supplementary Fig. S1). Moreover, the low AA concentration used here does not induce significant increases of ROS and the superoxide anion levels were similar to that of untreated controls (Aitken *et al.*, 2013). PEN, AA or LPA was added 30 min before the addition of MJ33 to assure the incorporation of these compounds into the spermatozoa before adding the inhibitor.

Electrophoresis and immunoblotting

We evaluated the phosphorylation levels of PI3K and AKT substrates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in samples with entire spermatozoa. Briefly, 5× sample buffer containing 0.4 M dithiothreitol were added to the samples and boiled for 5 min before centrifuging them at 15 000*g* for 5 min at room temperature to extract the proteins into the supernatant. Then, 10 μL of supernatant was loaded on a 10% bis-acrylamide gel, electrophoresed and transferred to nitrocellulose membranes. The membranes were incubated for 30 min in 5% skimmed milk and 0.1% Tween 20 in 2 mM Tris buffer saline (TTBS) before the incubation with anti-PI3K (1:2000) or anti-AKT substrate (1:1000) antibodies in 1% skimmed milk TTBS overnight at 4°C. The following day, membranes were washed three times with fresh TTBS and incubated with the appropriate secondary antibody horseradish peroxidase-conjugated (1:2500) at room temperature for 1 h. Positive immunoreactive bands were detected using chemiluminescence (Lumi-light; Roche Molecular Biochemicals). As a loading control, α-tubulin (1:10 000) was detected using a specific antibody on the same membranes and the intensity of protein bands was determined, as we previously described (O'Flaherty *et al.*, 2004; O'Flaherty *et al.*, 2005; Morielli and O'Flaherty, 2015).

Flow cytometry analysis

Sperm viability, lipid peroxidation and apoptosis-like changes were analyzed using a MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Inc., Auburn, CA, USA) where a minimum of 10 000 events were gated for spermatozoa according to the side and forward scatter density plot. Sperm viability was measured using Sytox Blue at a final concentration of 0.2 μM after incubation for 15 min. The percentage of live cells (Sytox Blue-negative) from the total sperm population (Sytox Blue-negative + Sytox Blue-positive (dead sperm)) was recorded. Determination of levels of lipid peroxidation was performed as we previously described using a BODIPY 581/591 C11 probe (Lee *et al.*, 2017). Results were expressed as relative intensity of green fluorescence/red+green fluorescence.

Annexin V externalization

One of the apoptosis-like changes occurring in spermatozoa is the externalization of phosphatidylserine that can be detected by labeling cells with Annexin V conjugated with a fluorochrome. One million spermatozoa were incubated with Annexin V conjugated with Alexa Fluor 647 (1:100 dilution) for 15 min at 37°C in Annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4), as described by the manufacturer. Results were expressed as the percentage of Annexin V-positive cells.

Statistical analysis

All graphical data was plotted as mean ± SEM, and statistical differences between groups were determined using ANOVA and Tukey test, using Sigma Systat 13 (Systat Software Inc., San Jose, CA, USA). Normal distribution of data was confirmed using the Shapiro–Wilk test. Differences with a P value of ≤ 0.05 were considered significant.

Results

The inhibition of PRDX6 Ca^{2+}-$iPLA_2$ promotes the loss of phosphorylated PI3K and AKT substrates and leads to a decrease in sperm viability

Activation of the PI3K/AKT pathway by phosphorylation is associated with sperm survival (Koppers *et al.*, 2011). Here, the inhibition of the PRDX6 Ca^{2+}-$iPLA_2$ activity with MJ33 significantly decreased sperm viability and increased lipid peroxidation in spermatozoa (Fig. 1A and B). MJ33 treatment also significantly decreased the phosphorylation of PI3K and AKT substrates at concentrations of 20 μM or higher (Fig. 2A and B and Supplementary Fig. S2).

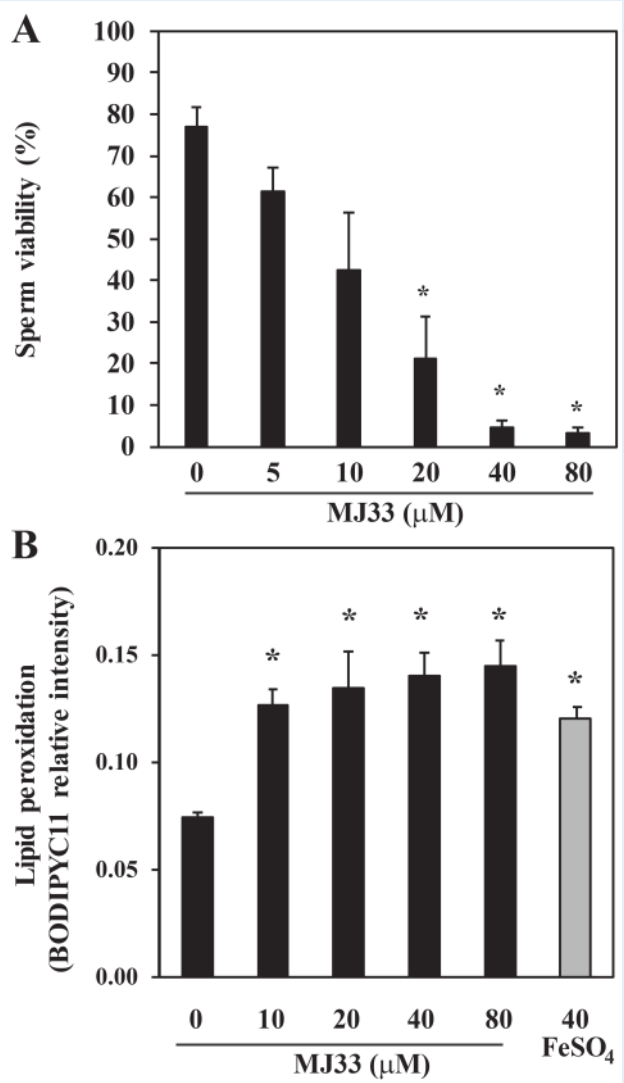

Figure 1 PRDX6 Ca^{2+}-$iPLA_2$ prevents lipid peroxidation and maintains human sperm viability. (**A**) The effect of inhibition of peroxiredoxin 6 calcium-independent phospholipase A_2 (PRDX6 Ca^{2+}-$iPLA_2$) activity by 1-hexadecyl-3-(trifluoroethyl)-*sn*-glycero-2-phosphomethanol (MJ33) was assessed. Human spermatozoa were treated with increasing concentrations of MJ33 for 4 h at 37°C before being stained with Sytox Blue (a marker of cell viability) and analyzed by flow cytometry. (**B**) Spermatozoa were treated with increasing concentrations of MJ33 for 4 h at 37°C and then loaded with the BODIPY C11 probe and analyzed by flow cytometry. Iron(II) sulfate was added to an aliquot as a positive control. *Denotes different from control (0 μM MJ33) (ANOVA and Tukey test, $P < 0.05$; results are presented as mean ± SEM ($n = 8$ different donors).

LPA and AA prevent the damage caused by the inhibition of PRDX6 Ca^{2+}-$iPLA_2$ on sperm viability

PRDX6 Ca^{2+}-$iPLA_2$ can generate both unesterified fatty acid (e.g. AA) and lysophospholipid (e.g. LPA) that have been reported by others to activate different survival pathways in different cell lines (Hernandez

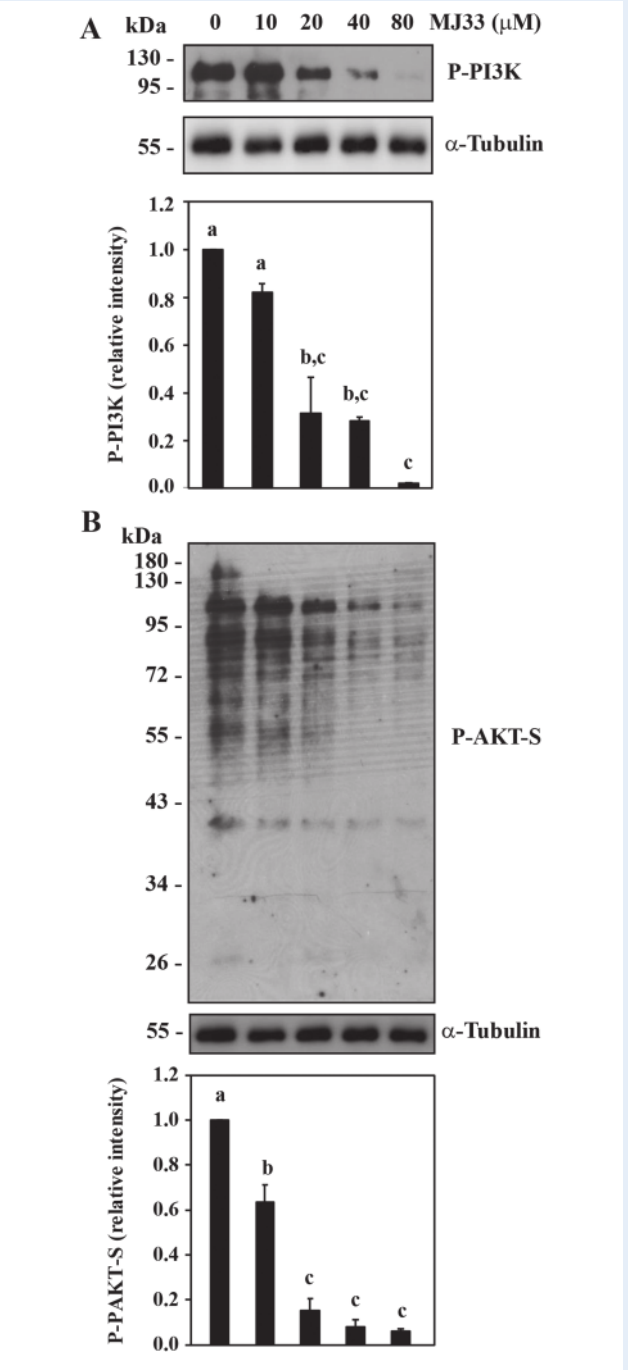

Figure 2 Phosphorylation of PI3K and AKT is compromised by the inhibition of the PRDX6 Ca^{2+}-$iPLA_2$. Spermatozoa were treated with increasing concentrations of MJ33 for 4 h at 37°C, and sperm proteins were electrophoresed and immunoblotted with (**A**) the anti-phospho-phosphoinositide 3-kinase (pPI3K) or (B) anti-phospho-AKT (protein kinase B) antibodies. The membranes were stripped and reblotted with the anti-tubulin antibody to confirm equal loading among lanes. The relative intensity of p-PI3K or p-AKT substrates bands was normalized to that of tubulin band and then normalized again to the control (0 μM). Letters denote significant differences (ANOVA and Tukey test, $P < 0.05$; results are presented as mean ± SEM ($n = 4$ different donors).

et al., 1998; Du et al., 2011; Hii et al., 2001). We explored whether LPA or AA prevented the impairment of sperm viability caused by the inhibition of the PRDX6 Ca^{2+}-$iPLA_2$ activity with MJ33. Both AA and LPA present in the incubation medium prevented the MJ33-dependent impairment of sperm viability (Fig. 3).

To further explore the pathway involved in sperm survival, spermatozoa were incubated with PEN, a drug with antioxidant properties (De Iuliis et al., 2012), with or without MJ33 in the incubation medium. As shown in Fig. 3A, the percentages of viable sperm were similar to control when spermatozoa were incubated with MJ33 in the presence of PEN. AA or LPA prevented the increase of Annexin V-positive cells promoted by MJ33 treatment (Fig. 3B). PEN alone partially decreased Annexin V-positive spermatozoa in MJ33-treated samples compared to spermatozoa treated with MJ33 alone.

The addition of LPA, AA or PEN to the incubation medium prevented the decrease of PI3K phosphorylation in MJ33-treated spermatozoa compared to untreated controls (Fig. 4 and Supplementary Fig. S3).

Discussion

In the present study, we provided evidence for the role of PRDX6 Ca^{2+}-$iPLA_2$ in regulation of the PI3K/AKT pathway to maintain sperm viability. Oxidative stress is known to play a major role in the etiology of male infertility (Tremellen, 2008; Aitken et al. , 2014) and is capable of triggering apoptosis-like changes affecting different survival pathways and sperm health (Ricci et al., 2002; Aitken, 2011; Koppers et al., 2011; Aitken and Baker, 2013). We reported previously that low levels of PRDXs, particularly of PRDX6, are associated with male infertility (Gong et al., 2012). One of the roles of PRDXs in spermatozoa is to maintain low levels of ROS to avoid oxidative damage and assure capacitation in human and mouse spermatozoa (Ozkosem et al., 2016; Lee et al., 2017; Moawad et al., 2017).

Recently, we reported that PRDX6 Ca^{2+}-$iPLA_2$ is the primary antioxidant defense in human spermatozoa since its inhibition leads to lipid peroxidation, mitochondrial dysfunction, DNA oxidation and a decrease in viability (Fernandez and O'Flaherty, 2018). Moreover, the absence of PRDX6 in spermatozoa compromises fertilization *in vivo* and *in vitro* (Ozkosem et al., 2016; Moawad et al., 2017) and increases the levels of lipid peroxidation and post-translational protein modifications (S-glutathionylation and carbonylation) and damages the sperm chromatin, leading to a reduction in the number of pups compared to wild-type controls (Ozkosem et al., 2016). Noteworthy is that sperm quality and fertility worsen as the male ages (Ozkosem et al., 2015).

The increased levels of lipid peroxidation observed in MJ33-treated spermatozoa compared to untreated controls support the essential role of PRDX6 Ca^{2+}-$iPLA_2$ in the protection of human spermatozoa against oxidative stress (Fernandez and O'Flaherty, 2018) (Fig. 1B). The unique lipid composition of the plasma membrane with high levels of polyunsaturated fatty acids makes it particularly susceptible to the damage induced by excessive ROS production (Sanocka and Kurpisz, 2004; Aitken and Baker, 2006; Aitken et al., 2014).

LPA signaling is important for maintaining germ cell viability (Ye et al., 2008). This pathway is driven by G protein-coupled receptors and is involved in vertebrate reproduction (Ye and Chun, 2010). Five receptors (LPA1–5) has been described (Contos et al., 2000). LPA1–4 have been detected in human testes (Noguchi et al., 2003; Choi et al., 2010). LPA1–3 are expressed in mouse testes, and

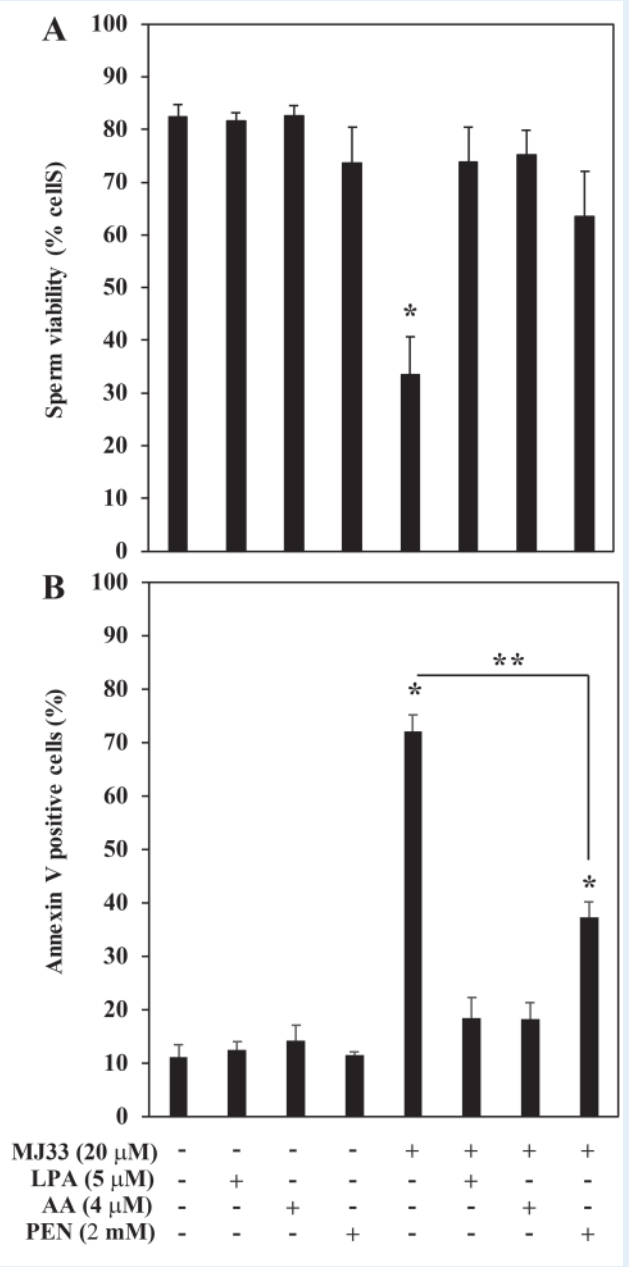

Figure 3 LPA and AA completely, and PEN partially, prevented the loss of human sperm viability when MJ33 is present. Spermatozoa were incubated with 5 μM lysophosphatidic acid (LPA), 4 μM arachidonic acid (AA) or 2 mM D-penicillamine (PEN) for 4 h at 37°C in the presence or absence of 20 μM MJ33 (added 30 min after the initiation of incubation). Spermatozoa were then stained with Sytox Blue (**A**) or Annexin V conjugated with Alexa Fluor 647 to determine sperm viability or externalization of phosphatidylserine, respectively, for flow cytometry analysis. *Means different from control (0 μM). **Denotes significant differences ($P < 0.05$) (ANOVA and Tukey test, $P < 0.05$; results are presented as mean ± SEM ($n = 13$ different donors).

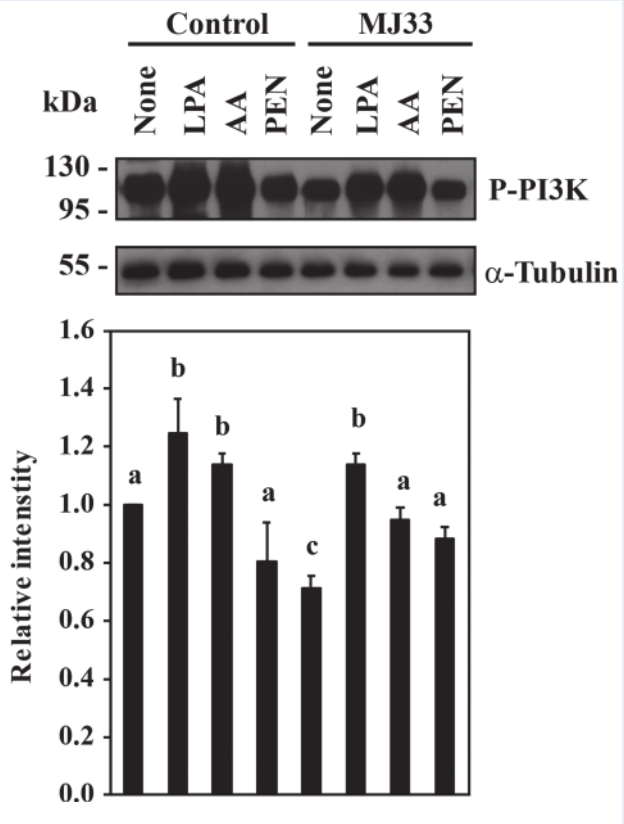

Figure 4 LPA and AA completely, and PEN partially, prevented the loss PI3K phosphorylation due to the inhibition of PRDX6 Ca^{2+}-$iPLA_2$. Spermatozoa were incubated with 5 μM LPA, 4 μM AA or 2 mM PEN for 4 h at 37°C in the presence or absence of 20 μM MJ33 (added 30 min after the initiation of incubation). Sperm proteins were electrophoresed and immunoblotted with the anti-phospho-PI3K (pPI3K) antibody (top panel). The membranes were stripped and reblotted with the anti-tubulin antibody to confirm equal loading among the lanes. The relative intensity of p-PI3K bands (lower panel) was normalized to that of tubulin band and then normalized again to the control (0 μM). Letters denote significant differences (ANOVA and Tukey test, $P < 0.05$, results are presented as mean ± SEM ($n = 4$ different donors).

their absence has been associated with loss of germ cells and, thus, a reduction of sperm production (Ye *et al.*, 2008). Moreover, mice overexpressing lipid phosphate phosphatase 1, an enzyme that degrades LPA to monoacylglycerol, showed impaired spermatogenesis (Yue *et al.*, 2004).

Products of PRDX6 Ca^{2+}-$iPLA_2$, LPA and AA prevented apoptosis in fibroblasts (Fang *et al.*, 2000) and increased the phosphorylation of PI3K leading to an increase in proliferation of lung cancer cells (Ho *et al.*, 2010). Here, the addition of LPA and AA to the incubation medium bypassed the inhibition of PRDX6 Ca^{2+}-$iPLA_2$ caused by the treatment of spermatozoa with MJ33. These lipids maintained high levels of phosphorylated PI3K with a significant decrease in apoptosis-like changes and thus support sperm viability. These results are in concordance with others showing an improvement in boar sperm viability, motility and acrosome reaction when unsaturated fatty acids were present in the incubation medium (Hossain *et al.*, 2007). LPA receptors can activate different pathways, including the PI3K/AKT pathway (Riaz *et al.*, 2016). LPA promotes cell survival in different cell types including macrophages, fibroblasts, cardiac myocytes and mesangial cells (Koh *et al.*, 1998; Fang *et al.*, 2000; Inoue *et al.*, 2001; Karliner *et al.*, 2001). Further studies are required to confirm whether activation of the PI3K/AKT pathway by LPA involves the activation of the LPA receptors.

The semen cryopreservation process generates high levels of ROS (O'Flaherty *et al.*, 1997). The addition of AA to the semen extender (diluent to cryopreserve spermatozoa) during cryopreservation increased the viability, motility and DNA integrity of thawed buffalo spermatozoa compared to untreated controls (Ejaz *et al.*, 2014). AA improved the motility, membrane integrity and viability of boar spermatozoa (Hossain *et al.*, 2007). Oxidative stress is associated with a loss of viability and activation of the apoptosis-like cascade in human spermatozoa (Aitken, 2011). The observation that PI3K phosphorylation was maintained in MJ33-treated spermatozoa incubated in the presence of PEN suggests that oxidative stress promotes the loss of viability by altering the PI3K/AKT pathway in human spermatozoa. The inhibition of PRDX6 Ca^{2+}-$iPLA_2$ generates high levels of ROS due to the dysregulation of mitochondrial activity (Fernandez and O'Flaherty, 2018). The antioxidant and protective effect of PEN depends on the ROS levels present in spermatozoa; whereas in this study 2 mM PEN was sufficient to maintain viability in MJ33-treated spermatozoa, the addition to 2 mM hydrogen peroxide to these spermatozoa promoted a stronger oxidative stress that could not be prevented by the presence of PEN in the incubation medium (Lee *et al.*, 2017). The strong oxidative stress generated promotes the inactivation of peroxidase activity of PRDX6 and possibly of 2-Cys PRDXs. Hydrogen peroxide or organic hydroperoxides reacted with the thiols of the active sites of PRDXs and promoted their thiol oxidation and the subsequent enzymatic inactivation (O'Flaherty and de Souza, 2011; O'Flaherty and Matsushita-Fournier, 2017). Indeed, male rats challenged with tert-butyl hydroperoxide, a compound that generates oxidative stress *in vivo*, had spermatozoa with thiol oxidized and therefore inactive PRDX1 and PRDX6 (Liu and O'Flaherty, 2017). Moreover, we demonstrated that the inhibition of glutathione-S-transferase and depletion of reduced glutathione, both necessary players in the re-activation system of PRDX6 peroxidase activity, promotes a significant increase in mitochondrial superoxide anion production. Noteworthy is the high concentrations of conoidin A, an inhibitor of 2-Cys PRDX peroxidase activity, that was necessary to promote similar levels of superoxide production (Fernandez and O'Flaherty, 2018). The spermatozoon is very sensitive to oxidative stress owing to the limited antioxidant enzymes present in their cytoplasm and the inactivation of PRDXs caused by high levels of ROS (O'Flaherty, 2014b, a, 2018). The inhibition of PI3K with wortmannin promotes increased levels of mitochondrial ROS associated with apoptotic-like changes in human spermatozoa (Koppers *et al.*, 2011), reinforcing the need for an active PI3K/AKT pathway to allow sperm survival, and highlighting the importance of PRDX6 Ca^{2+}-PLA_2 activity to activate the PI3K/AKT pathway to assure sperm survival.

In our proposed model represented in Fig. 5, PRDX6 Ca^{2+}-$iPLA_2$ has an important role in preventing apoptosis-like changes and thus maintaining sperm survival through activation of the PI3K/AKT pathway. PRDX6, through its Ca^{2+}-PLA_2 and peroxidase activities, prevent

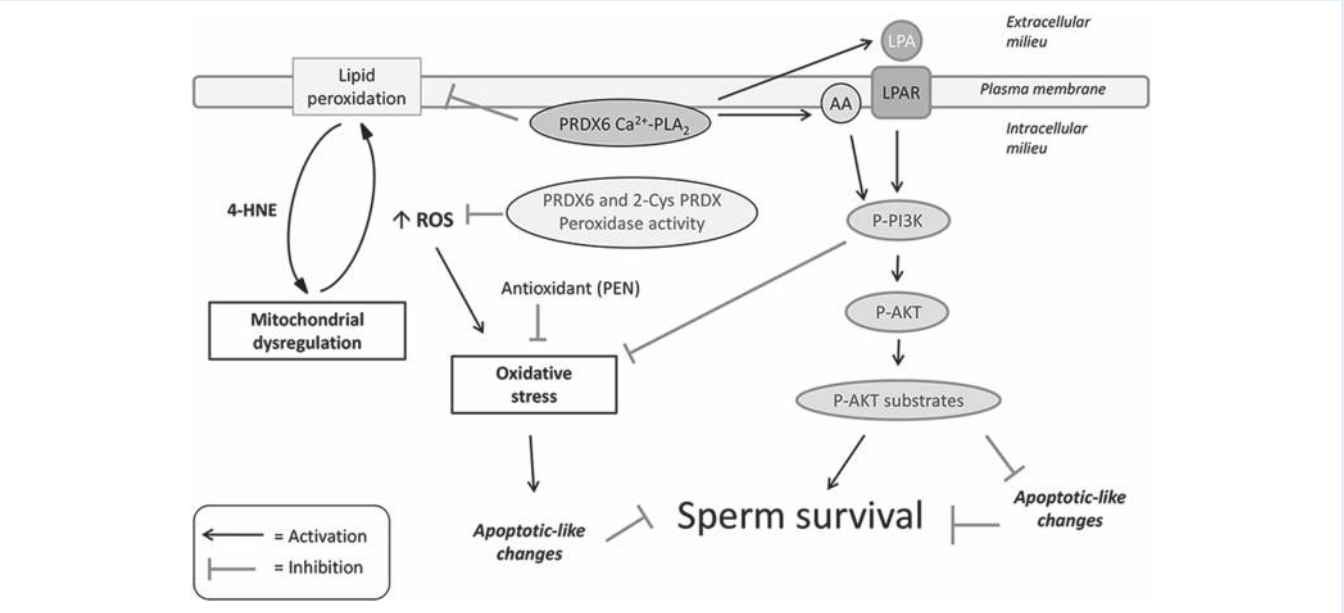

Figure 5 Role of PRDX6 in the maintenance of sperm viability. PRDX6 Ca^{2+}-$iPLA_2$ and peroxidase activities are important to protect the spermatozoon against lipid peroxidation and high levels of reactive oxygen species (ROS), respectively. 2-Cys PRDX peroxidase activity contributes to fighting against oxidative stress. PRDX6 Ca^{2+}-$iPLA_2$ activity produces unesterified fatty acid, such as AA and lysophospholipids, such as LPA. It is known that the positive effect of AA and LPA in activating the PI3K/AKT pathway is necessary to maintaining cell viability. The inhibition of PRDX6 iPLA2 by MJ33 leads to an increase in lipid peroxidation and ROS levels, generating the oxidative stress associated with the apoptotic-like changes observed in dying spermatozoa. Lipid peroxidation products, such as 4-hydroxynonenal, will promote mitochondrial dysfunction (by inactivating enzymes such as succinate dehydrogenase) leading to the production of ROS that will increase the oxidative stress and lipid peroxidation levels. Then, a vicious circle is established where more lipid peroxidation promotes higher levels of mitochondrial ROS. The antioxidant, PEN, partially prevents the effects of oxidative stress. However, due to the inactivation of PRDX6 and 2-Cys PRDX peroxidase activity by ROS (thiol oxidation), the spermatozoon lacks sufficient protection to fight against the oxidative stress established. PI3K is important to fight against oxidative stress, as its inhibition by wortmannin increases the levels of mitochondrial ROS. Thus, inactivation of the PI3K/AKT pathway caused by a lack of AA or LPA to activate the pathway will promote apoptotic-like changes, leading to cell death. LPAR: lysophosphatidic acid receptor.

lipid peroxidation and regulate the intracellular levels of ROS. To a lesser extent, 2-Cys PRDX peroxidase activity acts to prevent oxidative stress (Fernandez and O'Flaherty, 2018). When PRDX6 Ca^{2+}-PLA_2 is inhibited (by MJ33), then it becomes impossible to reduce the levels of lipid peroxidation that will start to build up due to the endogenous production of ROS by the mitochondria. This event creates a vicious circle raising, even more, the lipid peroxidation levels that will generate, among other products, 4-HNE. The levels of 4-HNE increase intracellularly and promote inactivation of mitochondrial enzymes such us the succinate dehydrogenase (SDH) of complex II in the electron transport chain (Aitken *et al.*, 2012). Because of SDH inhibition, the electron transport chain is dysregulated leading to the production of more superoxide anion that will dismutate to hydrogen peroxide. This increase in ROS promotes a stronger oxidative stress that is partially reduced by the antioxidant PEN.

PRDX6 Ca^{2+}-PLA_2 activity is necessary to produce AA and/or LPA, metabolites that will activate the PI3K/AKT pathway and prevent the apoptotic-like changes that lead to spermatozoon death. The inactivation of the PI3K/AKT pathway will lead to an increase in oxidative stress (Koppers *et al.*, 2011) that contributes to the fate of the spermatozoon i.e. to undergo apoptotic-like changes the lead to cell death.

In conclusion, our results indicate that PRDX6 Ca^{2+}-PLA_2 represents the primary antioxidant defense to maintain human sperm survival by generating LPA and/or AA to prevent the apoptosis-like changes, such as ROS production and the externalization of phosphatidylserine, by activating the PI3K-AKT pathway. These results could contribute to the design of new therapeutics that would help to enhance human sperm quality.

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Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Authors' roles

M.C.F., A.Y. and A.R.M. carried out the experiments and analyzed the data. M.C.F. and A.R.M. wrote the draft of the manuscript and

A.Y. revised the manuscript. C.O. conceived and designed the study, analyzed the data and wrote the manuscript.

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Conflict of interest

None declared.

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