Mitochondrial membrane potential disruption pattern in human sperm

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BACKGROUND: Loss of mitochondrial membrane potential (ΔΨm) in spermatozoa is correlated with high levels of reactive oxygen species in semen, abnormal spermogram parameters, and low success rates of IVF. In somatic cells, the loss of ΔΨm is primarily associated with several mechanisms of cell death, mainly the activation of caspases. The impact of mitochondrial dysfunction on sperm function is still not fully elucidated, although disruption of ΔΨm and activation of caspases are processes thoroughly studied in human ejaculates. Disruption of ΔΨm in sperm can be externally triggered by the antineoplastic agent betulinic acid (BA). In this study, we determined whether caspase activation is necessary for the BA-induced disruption of ΔΨm in human sperm.

METHODS: Viable and highly motile sperm cells were selected through a swim-up process and incubated with 90 μg/ml BA. To elucidate the caspase dependency of BA-triggered disruption of ΔΨm, we used the pan-caspase inhibitor zVAD-fmk and the caspase-3/7 inhibitor DEVD-cho.

RESULTS: Exposing highly motile sperm to BA caused a specific disruption of ΔΨm (P < 0.001 versus control) and a corresponding increase in caspase-3/7 activity (P < 0.001 versus control). Pre-incubation of the sperm with zVAD-fmk or DEVD-cho only partially inhibited BA-induced loss of ΔΨm (P < 0.05 versus control).

CONCLUSION: We found that caspases directly participate in the loss of ΔΨm caused by BA in human sperm cells. However, caspase-independent pathways may also be present.

Key words: human spermatozoa / mitochondrial membrane potential / caspases / betulinic acid

Introduction

Studies of mitochondrial membrane potential (ΔΨm) in human spermatozoa have become more common in the last decade, as this measurement can be used as a reliable indicator of sperm quality owing to the correlation of ΔΨm with other sperm functions (Troiano et al., 1998; Donnelly et al., 2000; Marchetti et al., 2002, 2004b; Barroso et al., 2006). Spermatozoa that exhibit high ΔΨm generally have intact acrosome function and high fertilizing capacity as well as normal motility and morphology (Gallion et al., 2006; Grunewald et al., 2008). Likewise, low ΔΨm sperm cells are of low quality, and are associated with reduced IVF rates (Kasai et al., 2002; Marchetti et al., 2004b) and elevated levels of reactive oxygen species (Wang et al., 2003a).

In somatic cells, loss of ΔΨm is preceded by permeability transition pore (PTP) formation. The PTP is a multi-component protein that regulates oxidative phosphorylation, aggregates in mitochondrial membranes and induces cell death when it turns into a non-specific channel (Crompton et al., 1992; Chang and Johnson, 2002; Grimm and Brdiczka, 2007). Another mechanism associated with ΔΨm loss is the coordination between numerous Bcl-2 proteins to induce mitochondrial outer membrane permeabilization (Chipuk et al., 2006); this mechanism is strictly associated with caspase activation (Ricci et al., 2003, 2004).

Caspases significantly contribute to the degradative phase of somatic cell apoptosis by cleaving numerous intracellular substrates (Fischer et al., 2003). Caspases have been implicated in spermatogenesis and in the pathogenesis of several andrological pathologies, including varicocele, immunological infertility and reduced sperm fertilizing potential (reviewed in Said et al., 2004). At the level of ejaculated human sperm, caspase activity has been associated with cell immaturity (Cayli et al., 2004), low cell concentration, reduced motility (Weng et al., 2002; Marchetti et al., 2004a; Taylor et al., 2004), decreased fertilization rates (Grunewald et al., 2008), and plasma membrane deterioration as evidenced by phosphatidylserine externalization (Paasch et al., 2003, 2004).

Experimental approaches have allowed the induction of various signals which are associated with cell death, including those induced
by the herbicide alachlor (Grizard et al., 2007), the cytokine tumour necrosis factor-α (Perdichizzi et al., 2007) and betulinc acid (BA), a triterpene with antineoplastic properties (Paasch et al., 2004). When added to human spermatozoa, BA induces a reduction in $\Delta \Psi_m$ accompanied by an increase in caspase activity (Paasch et al., 2004; Grunewald et al., 2005a; Perticarari et al., 2007). Together, these results suggest the existence of caspase-dependent, apoptotic-like mechanisms associated with mitochondrial functionality in sperm, possibly similar to those found in somatic cells (Boise and Thompson, 1997; Ricci et al., 2003, 2004; Lakhani et al., 2006). In some models of somatic apoptosis, the dissipation of $\Delta \Psi_m$ occurs in the absence of caspase activity (Lesage et al., 1997; Lavoie et al., 1998), and inhibition of the PTP is known to protect against $\Delta \Psi_m$ loss (Crompton et al., 1992; Chang and Johnson, 2002). Although caspase-dependent and -independent mechanisms of $\Delta \Psi_m$ disruption have been thoroughly described in somatic cells, the possible origins of $\Delta \Psi_m$ loss in human sperm cells still remain largely unstudied. Elucidation of the mechanisms responsible for this mitochondrial dysfunction will improve the understanding of a phenomenon strongly associated with male infertility.

In this study, we characterized apoptosis signalling pathways through specific induction and inhibition of key players in the caspase-dependent pathway in functional and morphologically normal human sperm selected from healthy donors.

Materials and Methods

This study was approved by the Institutional Review Board of Medical Faculty at the Universidad de La Frontera. Written consent was obtained from donors for research use of the sperm.

Materials

Human tubal fluid (HTF) medium was prepared as previously described (Quinn et al., 1985). Human albumin was purchased from Behring (Marburg, Germany). BA, JC-1, the pan-caspase inhibitor z-VAD-fmk and the caspase 3/7 inhibitor DEVD-cho were purchased from Biomol Research Lab (Plymouth Meeting, PA, USA). Propidium iodide (PI) was purchased from Nexins Research B.V. (Hoeven, The Netherlands) and DMSO was obtained from Sigma (Saint Louis, MO, USA).

Semen samples and sperm selection by swim-up

Semen samples were obtained from young healthy donors via masturbation into sterile containers (mean age = 24 years), and samples were assessed as normal according to World Health Organization criteria (WHO, 1999). The samples were equilibrated at 37°C for 20 min to allow liquefaction. Sperm were then washed twice by centrifugation at 500 x g for 8 min in HTF medium supplemented with 0.1% human serum albumin (HTF-HSA) and warmed to 37°C. After the second washing, the cell pellet was overlaid on 1 ml of HTF supplemented with 1% HSA in a 15 ml conical tube, and incubated at 37°C for 60 min. The spermatozoa were then collected from the supernatant and maintained at 37°C until use. In each experiment, sperm from two to three donors were pooled in order to achieve the required concentration of sperm cells. The characteristics of the selected sperm used in each experiment were: viability higher than 90%, motility higher than 75%, caspase 3 activity lower than 7% and a $\Delta \Psi_m$ consistently near to $10^4$ in the channel FL-2.

Induction of caspase activity and loss of $\Delta \Psi_m$

BA is an inducer of caspase activity and $\Delta \Psi_m$ disruption in ejaculated human sperm (Paasch et al., 2004; Grunewald et al., 2005b). We first evaluated the effects of different BA concentrations on integrity of the plasma membrane, disruption of $\Delta \Psi_m$, and caspase activity in order to determine the concentration of BA at which these effects could be monitored in swim-up-selected sperm without altering cell viability. For this purpose, 5 x $10^6$ spermatozoa were incubated with 60, 90 or 120 $\mu$g/ml of BA in HTF-HSA. In addition, controls containing only spermatozoa and HTF-HSA (Medium) or spermatozoa with 1.5% v/v of dimethylsulphoxide (DMSO) in HTF-HSA (Vehicle) were run in parallel. All samples were incubated at 37°C for 120 min. In subsequent experiments, 90 $\mu$g/ml BA was used. Before adding BA, the sperm samples (5 x $10^6$ spermatozoa/ml) were incubated with 100 $\mu$M z-VAD-fmk, a pan-caspase inhibitor (Gamen et al., 2000), or 20 $\mu$M DEVD-cho, the specific caspase-3/7 inhibitor (Kuzelova et al., 2007) for 60 min. After adding BA, the cells were incubated at 37°C for 120 min, washed twice and analysed for caspase activity, plasma membrane permeability and $\Delta \Psi_m$. In all the analyses, a concentration of 1.5% v/v DMSO was maintained.

Measurement of plasma membrane integrity

We evaluated plasma membrane integrity using vital staining, a valid criterion for identifying dead cells (Galluzzi et al., 2007). In order to determine cell viability, plasma membrane integrity was evaluated with PI, which is impermeable to the plasma membrane of living cells, at a final concentration of 6 $\mu$g/ml. Cells were incubated with PI for 15 min at 37°C and subsequently analysed using flow cytometry through FL2 channel.

Measurement of $\Delta \Psi_m$

The $\Delta \Psi_m$ was measured using the cationic lipophilic dye JC-1. In recent years, JC-1 has been used to evaluate mitochondrial function of human spermatozoa as an indicator of sperm quality (Troiano et al., 1998; Kasai et al., 2002; Marchetti et al., 2004b). Since there is a direct correlation between fluorescence intensity of the sample and $\Delta \Psi_m$ of the cells, JC-1 accumulation within the mitochondria allows measurement of $\Delta \Psi_m$ (Smiley et al., 1991). For analysis of $\Delta \Psi_m$, we have used the mean fluorescence intensity (MFI) because it allows a better estimation of the progressive changes in the whole cell population instead of the percentage of cells with ‘high’ or ‘low’ $\Delta \Psi_m$, as commonly used in sperm (Troiano et al., 1998; Marchetti et al., 2004b; Gallon et al., 2006). The $\Delta \Psi_m$ was evaluated following the supplier instructions. Briefly, 3 x $10^6$ selected sperm were re-suspended in 250 $\mu$l of HTF containing 1 $\mu$l of the JC-1 solution, incubated at 37°C for 15 min, washed twice, and evaluated by flow cytometry through the FL1 and FL2 channels. The MFI from the FL2 channel was used for statistical analysis.

Evaluation of caspase activity

Caspase-3/7 activity was assessed using the fluorescent substrate fluoroecein isothiocyanate (FITC)-DEVD-fmk, a cell-permeable peptide that acts as an irreversible inhibitor of caspases 3 and 7 (Caspase-3 Detection Kit, Calbiochem, EMD Biosciences Inc., Darmstadt, Germany). The procedure was performed according to the supplier’s instructions. About 1 $\mu$l of the FITC-DEVD-fmk substrate was added to 300 $\mu$l of a sperm suspension at a concentration of 1 x $10^6$/ml in HTF-HSA. After 60 min incubation at 37°C, the cells were washed twice and evaluated using flow cytometry through FL1 channel.
Flow cytometry
Flow cytometry analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 15 mW air-cooled 488 nm argon-ion laser as light source. We performed and analysed 10 000 events using the CellQuestPro software (Becton Dickinson, San Jose, CA, USA).

Statistical analysis
We report results as mean ± SD. The data were analysed by one-way analysis of variance followed by the post hoc Tukey test. P-values below 0.05 were considered significant. The software GraphPad Prism® version 5.0 was used for statistical evaluation (GraphPad Software, San Diego, CA, USA).

Results
Effect of BA on plasma membrane integrity
PI was used to measure cytotoxic effects of BA. Neither the control containing the vehicle nor the addition of BA at 60 or 90 µg/ml altered the integrity of the membrane (Fig. 1A). However, BA at a concentration of 120 µg/ml induced a significant reduction in the proportion of PI negative cells.

Effect of BA on ΔΨm
The addition of vehicle did not cause any alterations of ΔΨm as compared with the control with medium only (Fig. 1B). The MFI for the vehicle was 2729 ± 524 and lower values were considered as reduction or loss of ΔΨm. In this regard, the addition of BA resulted in a reduction of fluorescence intensity in a concentration-dependent manner. At concentrations of 90 and 120 µg/ml, BA induced significant losses of ΔΨm in the cells.

Effect of BA on caspase activity
Caspase-3/7 activity was evaluated using the fluorescent substrate FITC-DEVD-fmk. The control with vehicle showed no significant effect on caspase activity as compared with the control with medium only. In contrast, there was a significant increase in the percentage of positive FITC-DEVD-fmk cells at BA concentrations of 90 and 120 µg/ml (Fig. 1C).

Microscopic analysis demonstrated that the fluorescent substrate was distributed throughout the sperm cell, though most intense in the sperm midpiece (Fig. 2). This pattern, indicating caspase 3/7 activity, was observed in all the cells positive for FITC-DEVD-fmk of both control and treated cells.

Effect of z-VAD-fmk and DEVD-cho on the loss of ΔΨm induced by BA
In order to evaluate the putative contribution of caspase activation to the loss of ΔΨm, swim-up-selected sperm cells were pre-treated with inhibitors of caspase activation and then incubated with 90 µg/ml BA. In this set of experiments, the MFI for the control vehicle was 2468 ± 208 and lower values were considered as loss of ΔΨm. No significant effects on ΔΨm were induced by the addition of vehicle or by separate applications of 100 µM z-VAD-fmk or 20 µM DEVD-cho (Fig. 3A and B). In concordance with the results shown in Fig. 1B, the addition of 90 µg/ml BA induced a significant loss of ΔΨm. This effect was partially inhibited by the addition of either z-VAD-fmk or DEVD-cho (Fig. 3A), as evident in the flow cytometry histogram overlay of a representative experiment (Fig. 3B), where the addition of the inhibitors blocked approximately 15% of the decrease in JC-1 MFI as compared with BA treatment alone.

Discussion
The results obtained in this study show that the loss of ΔΨm induced by BA in sperm may be at least partly mediated by pathways sensitive

Figure 1 Effect of betulinic acid on human spermatozoa.
Evaluation of (A) plasma membrane integrity as the percentage of PI negative cells; (B) integrity of ΔΨm, as mean of fluorescence intensity of JC-1; (C) active caspase-3/7 as a percentage of FITC-DEVD-fmk positive cells. Highly viable sperm cells were incubated with different concentrations of BA at 37°C for 2 h. Control sperm cells were incubated in medium only or with 1.5% v/v dimethylsulphoxide (DMSO, vehicle). Cytometric analysis was performed using the FL2 channel for JC-1 and PI, and the FL1 channel for FITC-DEVD-fmk. Results are expressed as mean ± SD of three experiments each run in duplicate.
to pan-caspase (z-VAD-fmk) and caspase 3/7 (DEVD-cho) inhibitors. This suggests that caspases interfere with $\Delta \Psi_m$ following their activation by pro-apoptotic molecules released from mitochondria, a phenomenon which occurs in the mitochondrial apoptotic pathway. Similar effects on $\Delta \Psi_m$ have been previously described in somatic cells where caspase activity was blocked during apoptosis induction (Ricci et al., 2003, 2004). Additionally, somatic cells from mice lacking caspase-3 and -7 were highly resistant to apoptosis and exhibited stable $\Delta \Psi_m$ (Lakhani et al., 2006). In another study, $\Delta \Psi_m$ was not dissipated by apoptosis inducers in cells lacking caspase 9. When caspase 9 expression was restored, the cells became responsive to apoptotic stimuli that dissipate $\Delta \Psi_m$, thus linking mitochondrial dysfunction with caspase activity (Samraj et al., 2007). On a molecular level, activated caspases contribute to decreased $\Delta \Psi_m$ by interfering with electron transport chain complexes I and II (Ricci et al., 2003), which can be partially explained by the fact that the complex I p75 subunit serves as a substrate for caspase-3 (Ricci et al., 2004). Here, we demonstrate for the first time that in ejaculated sperm, amplification of the feedback mechanism through caspase activation that follows treatment with BA leads to terminal disruption of $\Delta \Psi_m$. As summarized in the flow diagram in Fig. 4, the evidence obtained in somatic cells indicates that BA drives caspase 3 activation, possibly through proteins of the Bcl-2 family, followed by releasing of mitochondrial cytochrome c and the concomitant activation of caspase 9 (Fulda et al., 1997; Fulda and Debatin, 2005). Both caspase 3 and caspase 9 are activated in vitro in sperm by BA (Grunewald et al., 2005a). Once activated, caspase 3 can exercise its catalytic activity inside the mitochondrion of spermatozoa and lead to the disruption of $\Delta \Psi_m$ in a similar way as observed previously in somatic cells (Ricci et al., 2003, 2004).

The general findings of this study are consistent with earlier reports where BA was described as an in vitro apoptosis inducer in somatic cells, via a mitochondrial pathway (Pisha et al., 1995; Fulda et al., 1997). With respect to other apoptosis inducers that we could have used, the inductive effects of tumour necrosis factor-α, a physiological inducer of apoptosis that exists in seminal plasma (Pollitch et al., 2007), may be observed only at high, non-physiological concentrations (Perdichizzi et al., 2007), and we have not found previous studies of this cytokine that identify it as a caspase activator in sperm. The Fas receptor, a molecule capable of inducing apoptosis, is absent in sperm (Perticarari et al., 2008). TRAIL, another apoptosis inducer,
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2006), and can be induced in vitro and the origin of the 2007). However, in the case of human sperm, the function of caspases et al Grizard 2004; Marchetti 2004; Marchetti 2007). Despite the fact that human sperm cells exhibit a number of features similar to those of apoptotic somatic cells (Donnelly et al., 2000; Marchetti et al., 2002; Wang et al., 2003; Marchetti et al., 2004a, b; Taylor et al., 2004; Barroso et al., 2006), previous research has been insufficient to determine whether sperm cells undergo apoptosis in the same manner as somatic cells (Marchetti and Marchetti, 2005). In the present study, we have described a possible apoptotic-like biochemical mechanism in human sperm.

Acknowledgements

This work was supported by Dirección de Investigación, Universidad de La Frontera, D107-1011.

Funding

JAE was granted by Fondo de Desarrollo Institucional, Ministerio de Educación, Chile.

References


Boise LH, Thompson CB. Bcl-x(L) can inhibit apoptosis in cells that have undergone Fas-induced protease activation. Proc Natl Acad Sci USA 1997; 94:3759–3764.


Submitted on September 30, 2008; resubmitted on March 26, 2009; accepted on April 9, 2009.