Phosphoinositide 3-kinase signalling pathway involvement in a truncated apoptotic cascade associated with motility loss and oxidative DNA damage in human spermatozoa

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INTRODUCTION

Defective sperm function is the single most common defined cause of human infertility [1]. Furthermore, the DNA damage commonly associated with defective human spermatozoa is known to have a significant impact on fertility, rates of miscarriage, and the health and wellbeing of the offspring following assisted conception [2,3]. Given this background and the high current rates of recourse to assisted reproductive technologies, insights into the aetiology of DNA damage and defective functionality in the male germ line are urgently needed.

Apoptosis is a major developmental mechanism that is known to play a key role in normal spermatogenesis, the testicular response to toxic injury and the pathogenesis of male infertility [4–7]. On the one hand, the fact that these cells to apoptosis is negatively regulated by PI3K (phosphoinositide 3-kinase)/AKT. If PI3K activity is inhibited, then spermatozoa default to an apoptotic cascade characterized by rapid motility loss, mitochondrial reactive oxygen species generation, caspase activation in the cytosol, annexin V binding to the cell surface, cytoplasmic vacuolization and oxidative DNA damage. However, the specialized physical architecture of spermatozoa subsequently prevents endonucleases activated during this process from penetrating the sperm nucleus and cleaving the DNA. As a result, DNA fragmentation does not occur as a direct result of apoptosis in spermatozoa as it does in somatic cells, even though oxidative DNA adducts can clearly be detected. We propose that this unusual truncated apoptotic cascade prepares spermatozoa for silent phagocytosis within the female tract and prevents DNA-damaged spermatozoa from participating in fertilization.

Key words: apoptosis, DNA damage, mitochondrion, phosphoinositide 3-kinase (PI3K), reactive oxygen species (ROS), spermatozoa.
the embryo [15]. In the present paper we report, for the first time, that these highly specialized cells, lacking both cell-cycle checkpoints and any form of transcriptional activity, can default to a truncated intrinsic apoptotic cascade, characterized by mitoty loss and oxidative DNA damage, following dephosphorylation of PI3K (phosphoinositide 3-kinase) and AKT. Furthermore this process may be clearly distinguished from apoptosis in somatic cells because the physical architecture of these cells prevents the physical translocation of nucleases from the mitochondria and cytoplasm to the sperm nucleus. These results explain why the promotion of PI3K activity is so critical to sperm survival and why DNA damage in spermatozoa is largely oxidative [14,16].

MATERIALS AND METHODS

Chemicals and culture medium

Unless stated otherwise, all chemicals were purchased from Sigma–Aldrich, whereas all fluorescent probes were purchased from Molecular Probes. Spermatozoa were cultured in BWW (Biggers–Whitten–Whittingham) medium supplemented with 0.1% polyvinyl alcohol, 5 units/ml penicillin and 5 mg/ml streptomycin [17]. The antibodies used in the present study were all from Abcam, with the exception of the anti-phospho-AKT (Thr308) antibody, which was purchased from Genesearch, and the anti-PTEN (phosphatase and tensin homologue deleted on chromosome 10) antibodies (anti-PTEN and anti-phospho-PTEN, pSer40, which were purchased from Calbiochem. The final concentrations at which the various antibodies were used were as follows: anti-phospho-PI3K targeting Tyr308 and Tyr34 (1:500), anti-PI3K (1:100), anti-phospho-AKT antibodies targeting pThr308 (1:20), pThr38 (1:20), pSer124 (1:20) and pSer473 (1:20) respectively, anti-pan-AKT1 and anti-pan-AKT2 (1:50), anti-AIFM (apoptosis-inducing factor, mitochondrion-associated) (1:500) anti-cytochrome c (1:50), anti-DIABLO [direct IAP (inhibitor of apoptosis)-binding protein with low pI] homologue (Drosophila) [DIABLO (SMAC (second mitochondrial-derived activator of caspase))] (1:50), anti-ENDO-G (endonuclease G) (1:50), anti-phospho-BAD (Bcl-2-associated death promoter) targeting Ser136 (1:50), anti-PI3K (1:50), monoclonal anti-phospho-PI3K targeting pSer380 (1:50) and anti-CAD (cadmoprotein-activated DNase) (1:50). Although we did not independently validate these commercially available antibodies, transcripts for all of these proteins are known to be present in the male germ cells because the physical architecture of these cells prevents the physical translocation of nucleases from the mitochondria and cytoplasm to the sperm nucleus. These results explain why the promotion of PI3K activity is so critical to sperm survival and why DNA damage in spermatozoa is largely oxidative [14,16].

Preparation of human spermatozoa

Institutional and State Government ethical approval was secured for the use of donated human semen samples for the purposes of this research. Samples from unselected normozoospermic donors of unknown fertility were collected into sterile containers prior to immediate transportation to the laboratory. Following initial inspection for liquefaction, consistency, debris and volume, assessments of cell count and motility were conducted and cell viability was measured using the eosin exclusion test [23]. After allowing at least 30 min for liquefaction to occur, the spermatozoa were fractionated on a discontinuous two-step Percoll gradient (90%/45%), as described previously [24]. The spermatozoa used in these experiments were recovered from the base of the high-density portion of the gradient. These cells were then washed with 5 ml of BWW, centrifuged at 600 g for 15 min and finally resuspended in BWW. Contaminating leucocytes were removed from all samples using magnetic Dynabeads coated with a monoclonal antibody directed against the common leucocyte antigen CD45 (Dynal) and confirmed using a zymosan provocation assay [24].

SDS/PAGE and Western blot analysis

Following treatment, spermatozoa were centrifuged (600 g for 5 min) and protein was extracted by solubilizing the cells in an SDS extraction buffer [2% SDS and 10% sucrose in 0.1875 M Tris/HCl (pH 6.8)] containing a protease inhibitor cocktail (Roche) for 5 min at 100°C. The spermatozoa were then centrifuged at 10 000 g for 15 min in order to remove insoluble components. Quantification of the isolated protein supernatant was achieved using a BCA (bicinchoninic acid) protein assay kit (Pierce) according to the manufacturer’s instructions. Equal amounts of total protein (5 μg, equivalent to approximately 750 000 cells) were boiled in SDS/PAGE sample buffer (SDS extraction buffer as above supplemented with 2% 2-mercaptoethanol and Bromophenol Blue) for 5 min and resolved on 10% polyacrylamide gels. The resolved proteins were then transferred on to nitrocellulose membranes under a constant current of 300 mA for 1 h.

Nitrocellulose membranes were blocked overnight at 4°C with 3% BSA (Research Organics) in TBS (Tris-buffered saline: 100 mM Tris/HCl, pH 7.6, and 150 mM NaCl) (pH 7.4) supplemented with 0.1% Tween 20. Membranes were rinsed in TBS and then probed with the appropriate concentration of primary antibodies in 1% BSA and 0.1% Tween 20 in TBS for 2 h at room temperature (22°C). Following incubation, membranes were washed three times in TBST (TBS containing 0.01% Tween 20) for 10 min. Membranes were then further probed for 1 h with a 1:1000 dilution of HRP (horseradish peroxidase)-conjugated secondary antibody at room temperature. Following a further three washes in TBST, cross-reactive proteins were visualized using an ECL (enhanced chemiluminescence) kit (GE Healthcare) according to the manufacturer’s instructions.

Immunolocalization of proteins on fixed spermatozoa

Following treatment, spermatozoa were fixed in 4% paraformaldehyde, washed three times with PBS, plated on to poly-L-lysine-coated glass slides and placed in a humid chamber at 37°C. The cells were permeabilized with 0.2% Triton X-100 for 15 min, rinsed with PBS and blocked with 10% serum/3% BSA for 1 h. Slides were then washed three times with PBS for 5 min and incubated in a 1:50 dilution of primary antibody overnight at 4°C, before being subjected to three 5 min washes with PBS and incubation in a 1:100 dilution of FITC-conjugated secondary antibody for 2 h at 37°C. Slides were again washed three times for 5 min and mounted in 10% Mowiol 4-88 (Calbiochem) with 30% glycerol in 0.2 M Tris/HCl (pH 8.5) with 2.5% DABCO [1,4-diazobicyclo-(2.2.2)-octane]. Spermatozoa were then imaged on a Zeiss LSM510 confocal microscope (Carl Zeiss) using argon laser excitation (488 nm) with emission collection at 500–530 nm (green).

MitoSOX Red assay

Intracellular generation of mitochondrial superoxide anion was determined using MitoSOX Red (Molecular Probes). For this assay, MitoSOX Red (5 mM in DMSO) and the cell viability stain SYTOX Green (0.125 mM in DMSO) stock solutions were diluted in BWW and added to spermatozoa at 1 × 10^7 cells/ml to give...
final concentrations of 2 mM and 0.05 mM respectively. After an incubation period of 15 min at 37°C, samples were centrifuged for 5 min at 600 g; the pellets were then resuspended in 1 ml of BWW and transferred to a 5 ml FACS tube. The MitoSOX Red (red) and SYTOX Green (green) fluorescence was finally measured on a FACSCalibur flow cytometer (Becton Dickinson). Argon laser excitation at 488 nm was coupled with emission measurements using 530/30 band pass (green) and 585/42 band pass (red) filters. Non-sperm-specific events were gated out and 10 000 cells were examined. The results were expressed as the percentage of cells that were alive and stained positively with the probe. Non-viable cells were excluded from the analysis because commercial preparations of MitoSOX Red contain trace amounts of ethidium bromide that can generate spurious signals if the integrity of the plasma membrane is compromised in any way.

**JC-1 assay**

JC-1 (Molecular Probes) is a fluorescent dye that can report on the state of the mitochondrial membrane potential (ΔΨ). JC-1 stock solution (7.5 mM in DMSO) was diluted in BWW and added to spermatozoa at 1 × 10⁶ cells/ml to give a final concentration of 2 mM. For a positive control, cells were incubated for 15 min with 10 μM CCCP (carbonyl cyanide m-chlorophenylhydrazone) prior to JC-1 staining. After incubation with JC-1 for 15 min, cells were centrifuged (600 g) and the pellet was resuspended in 1 ml of BWW. This suspension was then transferred to a 5 ml FACS tube, where PI (propidium iodide) (10 μg/ml) was added just prior to analysis. The JC-1 and PI fluorescence was then measured on a FACSCalibur flow cytometer (Becton Dickinson). Argon laser excitation at 488 nm was coupled with emission measurements using >670 nm long-pass filter (far red; PI) to exclude dead cells from analysis, followed by 530/30 band pass (green; low ΔΨ) and 585/42 band pass (red; high ΔΨ) filters. Non-sperm-specific events were gated out and 10 000 cells were examined.

**CASA (computer-assisted sperm analysis)**

Sperm motility parameters were analysed by CASA using a Hamilton Thorne Version 12 IVOS (Hamilton Thorne Biosciences). For each sample measurement, a 2.5 μl aliquot of spermatozoa was loaded on to a standard four-chamber slide (Leja). A minimum of 200 spermatozoa were examined for each sample using standard settings (30 frames acquired at a frame rate of 60 Hz and a temperature of 37°C in 20 μm-deep chambers).

**Measurement of activated caspase**

FLICA (fluorescently labelled inhibitor of caspase assay; Immunochemistry Technologies) was used to measure active pan-caspases in whole cells. The FLICA dye was stored as a 150× stock solution in DMSO, according to the manufacturer’s instructions. This stock solution was diluted in BWW and added to spermatozoa to give a final concentration of 1×FLICA dye. Spermatozoa were incubated for 1 h with the dye, and in the last 15 min SYTOX Green was added at a final concentration of 0.05 mM, in order to monitor cell viability. This was followed by centrifugation for 5 min at 600 g and re-suspension in 1 ml of BWW. Flow cytometric analysis was performed as described for the MitoSOX Red Assay.

**Annexin V externalization**

The externalization of PS from the inner-to-outer leaflet of the plasma membrane is a feature of apoptosis. The annexin V–FITC apoptosis detection kit (Sigma–Aldrich) allows the detection of PS on the cell surface through FITC-conjugated annexin V. The addition of a cell-viability indicator, PI, allows the differentiation between apoptotic cells (annexin V positive, PI negative), necrotic cells (annexin V positive, PI positive) and viable cells (annexin V negative, PI negative). For this analysis, spermatozoa were centrifuged (600 g for 5 min), and the pellet was resuspended in buffer containing FITC-conjugated annexin V according to the manufacturer’s instructions. Spermatozoa were incubated with the antibody for 1 h prior to centrifugation at 600 g for 5 min and re-suspension in 1 ml of BWW. Just prior to flow cytometric analysis, the sample was fixed and PI fluorescence was finally measured on a FACSCalibur flow cytometer (Becton Dickinson). Argon laser excitation at 488 nm was coupled with emission measurements using a >670 nm long-pass filter (far red; PI) to exclude dead cells from the analysis, followed by 530/30 band pass (green) filters. Non-sperm-specific events were gated out and 10 000 cells were examined.

**Immunofluorescence assay for 8OHdG (8-hydroxy-2′-deoxyguanosine)**

The formation of 8OHdG was measured using a specific antibody (Biotrin OxyDNA Test Kit, Biotrin International) conjugated to FITC. For the positive control, spermatozoa were incubated for 1 h with H₂O₂ (2 mM) and FeCl₃·3H₂O (1 mM) in a final volume of 200 μl of BWW. The H₂O₂ concentration was determined by measuring the absorbance at 240 nm. The cells were then washed twice in BWW, resuspended in 100 μl of 2 mM DTT (dithiothreitol) in BWW and incubated for 45 min at 37°C to relax the chromatin. After centrifugation at 600 g for 5 min, the cells were fixed by resuspending the pellet in 200 μl of 2% paraformaldehyde and incubating at 4°C for 15 min. The cells were subsequently washed in PBS and stored in 200 μl of 0.1 M glycine at 4°C for a maximum of 1 week. Fixed cells were ultimately washed and resuspended in 100 μl of 0.2% Triton X-100. Cells were then incubated at room temperature for 15 min, centrifuged (600 g for 5 min) and washed in wash solution (from the Biotrin OxyDNA Test Kit); 50 μl of blocking solution (3% BSA) was then added before incubation at 37°C for 1 h. The anti-8OHdG antibody was further purified by adding approximately 1 mg of activated charcoal powder, incubated at room temperature for 1 h and centrifuged twice at 600 g for 5 min. The supernatant containing the purified antibody was then added at a 1:50 dilution to the fixed cells in enough wash solution to achieve a final volume of 100 μl. This was incubated for 1 h and, finally, the cells were washed twice, resuspended in 1 ml of PBS and transferred to 5 ml FACS tubes for flow cytometry analysis. Fluorescence was then measured on a FACSCalibur flow cytometer (Becton Dickinson); argon laser excitation at 488 nm was coupled with emission measurements using 530/30 nm band pass (green). Non-sperm-specific events were gated out and 10 000 cells were examined.

**TUNEL (terminal TdT (deoxyxucleotidyltransferase)-mediated dUTP nick-end labelling) assay**

DNA cleavage may yield double-stranded and single-stranded DNA breaks. Both types of break can be detected by labelling the free 3′-OH terminal with fluorescent nucleotides (fluorescein-labelled dUTPs) in an enzymatic reaction catalysed by TdT in the TUNEL assay. Following treatment, spermatozoa...
were centrifuged (600 g for 5 min) and resuspended in 2 mM DTT in BWW for 45 min to relax the chromatin [25]. After centrifugation at 600 g for 5 min, the cells were fixed by resuspending the pellet in 2 % paraformaldehyde and then incubating at 4°C for 15 min. The cells were subsequently washed in PBS and stored in 200 μl of 0.1 M glycine at 4°C. For analysis, spermatozoa were centrifuged (600 g for 5 min) before resuspending the pellet in 100 μl of permeabilization solution (0.02% Triton X-100 in PBS) and incubating for 2 min at 4°C. The cells were then centrifuged (600 g for 5 min) and the pellets resuspended in PBS. The positive control was treated with 100 μl of DNase I (1 mg/ml) and 10 μl of MgSO4 (10 mM) for 30 min at 37°C. TUNEL labelling was achieved with the In Situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer’s instructions. Cells were then washed twice in PBS, diluted to a final volume of 500 μl in PBS and kept in the dark for analysis using flow cytometry. Flow cytometric analysis was performed as described for the 8OHdG assay.

**Ultrastucture**

Spermatozoa were centrifuged at 1000 g for 10 min, and then resuspended and fixed in 500 μl of 2% formaldehyde/2.5% glutaraldehyde in 0.1 M PBS (pH 7.3) for 2 h at 4°C. To remove the fixative, spermatozoa were washed three times by centrifugation at 1000 g for 5 min and resuspended in 0.1 M PBS. After centrifugation, the spermatozoa were submitted to successive dehydration steps. Cells were immersed progressively in increasingly concentrated ethanol solutions (v/v) at 50%, 75% and 95% for 5 min each, then in three changes of 100%, ethanol for 5 min each, and in two changes of 100% acetone for 5 min. Then, successive Spurr’s resin filtration steps were carried out by immersing the dehydrated samples in progressively more concentrated resin solution in acetone (v/v), beginning with 33% and 50% resin for 1 h each at room temperature, then 66% and 100%, each overnight at room temperature, and finally transferring to fresh 100% resin and polymerizing for 24 h at 60°C in a laboratory oven.

Sections (70 nm) of the embedded tissue were cut on an Ultracut E ultramicrotome (Reichert-Jung), floated out on Milli-Q water and transferred on to 100-mesh copper grids. Grids were then stained with 0.5% uranyl acetate in 30% ethanol for 10 min and rinsed with distilled H2O; further staining was achieved with lead citrate for 10 min. Lead precipitates on grid sections were removed by rinsing in 0.05 M NaOH before further rinsing in distilled H2O. Grids were left at room temperature to dry and the stained sections were examined using a JEOL-1200EX electron microscope operating at 80 kV.

**Statistical analysis**

All experiments were repeated at least three times on independent samples and the results were analysed by ANOVA or a paired Student’s t test using the SuperANOVA and Statview programs respectively (Abacus Concepts); post hoc comparison of group means was by Fisher’s protected least significant difference test. Differences with a P value of <0.05 were regarded as significant.

**RESULTS**

Elements of the PI3K signalling complex are localized to specific domains in human spermatozoa

Western blot analyses using anti-AKT and anti-PI3K (p85 subunit) polyclonal antibodies clearly demonstrated the presence of these key enzymes in human spermatozoa (Figure 1A). Immunocytochemical studies not only confirmed their presence in human spermatozoa, but also revealed highly specific patterns of subcellular localization. Thus an antibody against the p100 kDa catalytic subunit of PI3K revealed this enzyme to be located in the principal piece of the sperm tail, the neck and the acrosome, but to be excluded from the midpiece of the cell where the mitochondria and residual cytoplasm are located (Figure 1B, indicated with arrows). An antibody against the phosphorylated form of the p85 regulatory subunit of PI3K, targeting phospho-Tyr402 and phospho-Tyr406, also revealed the activated form of this component of the heterodimer to be largely located in the principal piece of the sperm tail and absent from the midpiece (Figure 1C).

In most cells the ability of PI3K to generate a powerful signalling molecule, PIP3 [phosphatidylinositol (3,4,5)-trisphosphate], is in dynamic equilibrium with a phosphatase that negatively regulates the bioavailability of PIP3 by catalysing its dephosphorylation to the corresponding bispahosphate, PIP2 [phosphatidylinositol (4,5)-bispahosphate]. This phosphatase is known as PTEN. Immunocytochemical localization of PTEN generated a very weak signal, predominantly in the midpiece of the cell (Figure 1D). By contrast, an antibody against the phosphorylated, stabilized, form of this enzyme (pSer380), indicated that a majority of the PTEN in spermatozoa exists in a phosphorylated state and is localized in the equatorial segment of the sperm head, distant from the PI3K in the principal piece of the tail (Figure 1E). This physical separation of PI3K and phosphorylated PTEN is absolutely unique to spermatozoa and would ensure that the former is free to generate PIP3 without any interference from PTEN phosphatase activity. In keeping with this conclusion we found that addition of the inhibitor bpV (a general vanadate-based kinase inhibitor with demonstrable activity against PTEN) [26] did not promote the pro-survival effects of prolactin or insulin on spermatozoa (results not shown), and actually induced a dose-dependent suppression of motility and progressive motility (Figure 1F), rather than the enhancement of motility that might have been anticipated if PTEN suppression had led to a corresponding increase in PI3K activity.

A major target for PIP3 is the serine/threonine kinase AKT. Two of the three major isoforms of AKT expressed in the testes are AKT1 (RAC-α serine/threonine-protein kinase), a recognized inhibitor of apoptosis and AKT2 (RAC-β serine/threonine-protein kinase), an important constituent of the insulin signalling pathway. Immunocytochemistry revealed that AKT1 was strongly present throughout the sperm tail, including the principal piece and the midpiece where the machinery for apoptosis is located, in the form of mitochondria and the only significant quantity of cytoplasm present in this cell type (Figures 2A and 2B). AKT2 was also strongly present in the midpiece of the cell and, to a lesser extent, the proximal part of the flagellar principal piece; however, in contrast with AKT1, AKT2 also gave a very strong signal in the sperm head, particularly in the vicinity of the acrosome (Figure 2C). The activated phosphorylated forms of this kinase including pThr34-AKT (present in AKT1 but not AKT2) as well as pThr308, pSer373, and pSer124-AKT (represented in both AKT1 and AKT2) were also detectable in human spermatozoa. Significantly, all antibodies exhibited strong staining in the sperm midpiece, in close proximity to the sperm mitochondria (particularly pThr34 and pThr308; Figures 2D and 2E). In addition, pThr308 gave a particularly strong signal in the acrosome (Figure 2E), whereas anti-pThr373 also gave a signal along the principal piece of the tail (results not shown).

In order to determine whether PIP3 generated by PI3K in the principal piece of the sperm tail could be responsible for activating AKT in this and other regions of the cell, the PI3K inhibitor wortmannin was used. Addition of wortmannin led to the highly
Truncated apoptosis in human spermatozoa

Figure 1 Presence of the PI3K, AKT and PTEN in human spermatozoa

(A) Demonstration that both PI3K (regulatory subunit) and AKT are present in human spermatozoa by Western blot analysis. The molecular mass in kDa is indicated on the right-hand side. (B) Immunocytochemical localization of PI3K (p110 catalytic subunit) to the principal piece, the neck and the acrosomal region of human spermatozoa. Note the absence of staining in the midpiece where the mitochondria are located (indicated by an arrow). (C) Immunocytochemical localization of the phosphorylated activated form of PI3K (p85 regulatory subunit) to the principal piece; again note the lack of staining in the midpiece (indicated by an arrow). (D) Weak immunocytochemical signal generated by PTEN in sperm midpiece. (E) A majority of the PTEN is stabilized by phosphorylation and located in the equatorial segment of the sperm head. This physical separation of PI3K and PTEN is unique to spermatozoa and ensures that the former is maximally active. Scale bar in (B)–(E) = 5 μm. (F) The physical separation of PI3K and PTEN also explains why an inhibitor of PTEN, bpV, which should support sperm function by enhancing PI3K activity, is either inactive or, at high concentrations, inhibitory. Results are means ± S.E.M.

effective suppression of phospho-AKT expression (Figure 2A). A major downstream target of AKT kinase activity, BAD, is a well-characterized regulator of apoptosis, so the status of this protein was also examined before and after exposure to wortmannin. Using an antibody that could recognize the phosphorylated form of BAD (pSer99) by immunocytochemistry (but not Western blot analysis), we demonstrated that although phospho-BAD could clearly be detected in the midpiece and tail of control spermatozoa (Figure 2F), treatment with wortmannin led to complete loss of the pSer99 epitope (Figure 2F, bottom panels), indicating a clear change in the phosphorylation status of this apoptosis mediator.

Since such a change in BAD phosphorylation would be expected to cause this protein to adopt a pro-apoptotic role, we next looked for other signs of an intrinsic apoptotic cascade in wortmannin-treated spermatozoa, including changes in sperm motility, vitality, mitochondrial ROS (reactive oxygen species) generation, mitochondrial membrane potential (ΔΨ), PS exposure, caspase activation and DNA damage.

Suppression of PI3K leads to a loss of motility

One of the most obvious and rapid changes to occur following the induction of apoptosis with wortmannin was a highly significant dose- \( (P < 0.001) \) and time- \( (P < 0.001) \) dependent loss of the percentage of sperm motility and progressive motility (Figures 3A–3D). Thus a combination of dose–response analyses, initially covering a broad array of concentrations (Figures 3A and 3C), followed by a more focused and limited range, demonstrated that within 4 h both motility and progressive motility were significantly suppressed by exposure to >5 μM wortmannin (Figures 3B and 3D) and, at doses above 20 μM, motility was effectively lost and cell vitality exhibited a dose-dependent decline \( (P < 0.001; \text{ Figure } 4\text{A}) \). In addition, parallel studies with another PI3K inhibitor, LY294002, revealed the same highly significant dose- and time-dependent inhibition of sperm movement \( (P < 0.001) \) and vitality \( (P < 0.01) \), reinforcing the notion that suppression of this kinase causes spermatozoa to default to a pathway leading to cell death.

Suppression of PI3K leads to mitochondrial ROS generation

In order to determine whether the loss of motility and vitality observed after disruption of the PI3K/AKT/BAD axis with wortmannin involved the activation of apoptosis in mature human spermatozoa, these cells were examined for activities characteristic of cell entry into an intrinsic apoptotic pathway. As

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Figure 2  Impact of wortmannin on AKT and BAD expression

(A) Western blot analysis indicating the presence of phospho-AKT (top panel) in human spermatozoa and demonstrating the dephosphorylation of this kinase in the presence of the PI3K inhibitor wortmannin; the loading control shown in the bottom panel was an anti-pan-AKT antibody. The molecular mass in kDa is indicated on the right-hand side. (B) Immunocytochemical analyses revealed the presence of AKT1 along the entire length of the sperm tail including the neck, midpiece and principal piece, whereas (C) AKT2 was principally located in the sperm acrosome and the midpiece, where the mitochondria are located. (D) The phosphorylated form of AKT1 (pThr34) generated a strong signal in the sperm tail with clear emphasis on the midpiece, whereas (E) pThr308, (present in both AKT1 and AKT2) was found in the midpiece and in the acrosomal region. (F) The anti-apoptotic factor phospho-BAD was predominantly located in the midpiece of human spermatozoa (indicated by an arrow); however, all cross-reactivity was lost in the presence of 20 μM wortmannin, suggesting the dephosphorylation of this apoptosis regulator. Scale bars = 5 μm.

illustrated in Figure 4(B), the addition of wortmannin to human spermatozoa in vitro resulted in significant dose- (P < 0.001) and time- (P < 0.05) dependent increases in mitochondrial ROS generation, as measured by MitoSOX Red. Mitochondrial ROS generation in live cells increased with wortmannin doses up to 20 μM, but collapsed at higher concentrations as the spermatozoa lost their vitality. Since a loss of ΔΨ is also a common signature of apoptotic cells that has been observed to accompany mitochondrial ROS generation [27], we also examined the effect of wortmannin on this aspect of mitochondrial activity. Use of the fluorescent marker JC-1 revealed that, at wortmannin doses where mitochondrial ROS generation was active and cell vitality was not compromised (≤20 μM), wortmannin had no effect on ΔΨ, even after 24 h exposure (Figure 4C). Parallel studies with LY294002 confirmed that the inhibition of PI3K activity resulted in dose- (P < 0.001) and time- (P < 0.001) dependent increases in mitochondrial ROS generation, in the absence of a significant collapse of mitochondrial membrane potential (results not shown). These results suggested that the rapid stimulation of mitochondrial ROS was not due to an uncoupling mechanism, nor was the latter an early component of this apoptotic pathway in human spermatozoa.

Suppression of PI3K activates the intrinsic apoptotic pathway in human spermatozoa

In order to determine whether the suppression of PI3K/AKT resulted in the expression of additional hallmarks of apoptosis other than mitochondrial ROS generation, these cells were also examined for PS externalization and caspase activation. Wortmannin was found to result in highly significant time- (P < 0.001) and dose- (P < 0.001) dependent increases in PS externalization on the surface of viable human spermatozoa (Figure 4D). PS externalization appeared to be a relatively late event in sperm apoptosis because significant changes were only observed after 24 h, at which point the response was found to increase with wortmannin doses up to 20 μM and then declined in concert with the progressive loss of cell viability (Figure 4A). Using FLICA, wortmannin was also shown to induce a highly significant (P < 0.001) dose-dependent increase in caspase activation in human spermatozoa (Figure 5A). In order to determine the temporal relationship between PS externalization and caspase activation, a time-course analysis was conducted following the activation of apoptosis with wortmannin. The results of this study again emphasized that PS externalization...
Results are means significantly suppressed with analysis utilizing a more limited range of concentrations revealed that progressive motility was μ as a percentage of an untreated control sample, such that a dose of 0 D time-dependent inhibition of sperm movement. ()

Figure 3 Inhibition of PI3K leads to the effective disruption of sperm motility

(A) Dose- and time-dependent inhibition of sperm motility with wortmannin over a wide dose range. (B) Using a more refined range of concentrations, significant effects of wortmannin on sperm motility were evident at 4 h with 5 μM of this inhibitor. (C) CASA analysis of progressive motility (average path velocity > 25 μm/s; straightness > 75 %) revealed a similar dose- and time-dependent inhibition of sperm movement. (D) As with overall motility, a dose–response analysis utilizing a more limited range of concentrations revealed that progressive motility was significantly suppressed with > 0.1 μM wortmannin over a 4 h time scale. Results are expressed as a percentage of an untreated control sample, such that a dose of 0 μM equates with the vehicle-only control. Results are means ± S.E.M. (**P < 0.001 and **P < 0.01 compared with the vehicle-only control).

is a relatively late event in the apoptotic cascade as expressed by spermatozoa. Thus, although significant caspase activation was evident 4 h after the initiation of apoptosis (Figure 5B; P < 0.001), PS externalization was not manifest until 8 h (Figure 5B; P < 0.001). The involvement of PI3K in the activation of this apoptotic cascade was also supported by the fact that highly significant (P < 0.001) changes in caspase activation and PS externalization (P < 0.001) were also observed when PI3K activity was suppressed by LY294002 (results not shown).

Interestingly, the addition of other activators of apoptosis in somatic cells, including staurosporine, lipopolysaccharide, Kdo and genistein, did not elicit the activation of caspases (Figure 5C), or induce any significant changes in motility, vitality or 8OHdG (results not shown) in human spermatozoa over the same time period, emphasizing the dramatic differences that exist between the apoptotic pathway(s) expressed in these cells relative to other cell types.

The appearance of DNA strand breaks is another major hallmark of an apoptotic cell. Such apoptosis-associated DNA fragmentation is induced by endonucleases which are either released from the mitochondrial inter-membranous space, (AIFM or ENDOG), or activated in the cytosol (CAD), prior to their translocation to the nucleus. The presence of DNA strand breaks in human spermatozoa has been widely observed in defective sperm populations and is known to be induced by a wide variety of stimuli, including various forms of electromagnetic radiation and xenobiotics [15,28,29]. However, within the time frame of these studies, the addition of wortmannin was unable to induce DNA strand breaks as measured by the TUNEL assay, even 24 h after exposure (Figure 5D).

The reason for this lack of DNA fragmentation became evident following immunocytochemical localization of three major nucleases in human spermatozoa, CAD (Figures 6A and 6B), ENDOG (Figures 6C and 6D) and AIFM (Figures 6E and 6F). This analysis revealed that these effectors of apoptosis were predominantly located in the sperm midpiece, where the mitochondria and a majority of the sperm cytoplasm are located. Stimulation of apoptosis with wortmannin did not induce the translocation of these nucleases from the sperm midpiece to the nucleus because the physical architecture of the cell, featuring mitochondria and residual cytoplasm in one subcellular compartment (midpiece) and nuclear DNA in another (sperm head), does not permit it. Other effectors of the apoptotic cascade, such as cytchrome c (Figures 6G and 6H) or DIABLO(SMAC) (Figures 6I and 6J) were also confined to the midpiece of the cell, before and after activation of the intrinsic apoptotic cascade with wortmannin.

Recent studies have provided evidence highlighting the clinical importance of oxidative stress in the aetiology of DNA damage in human spermatozoa [14]. So, even though the impeded migration of nucleases from the sperm midpiece to the nucleus precluded endonuclease-mediated DNA cleavage during apoptosis, the activation of mitochondrial DNA repair could have induced oxidative DNA damage that might, in time, have generated the expected strand breaks. To examine this possibility the formation of 8OHdG, a marker of oxidative DNA damage, was analysed following wortmannin exposure. In this analysis, wortmannin was shown to induce a highly significant (P < 0.001) dose-dependent increase in the prevalence of oxidative DNA damage in human spermatozoa after 24 h incubation (Figure 6K).

Finally, we examined whether the induction of this apoptotic cascade was associated with ultrastructural changes similar to those seen in somatic cells following the initiation of this process. Ultrastructural analysis of human spermatozoa, under conditions that resulted in both caspase activation and PS externalization, revealed several morphological changes compared with control cells (Figure 7). Specifically, activation of the intrinsic apoptotic cascade with wortmannin resulted in the appearance of large vacuoles and membrane protrusions in the sperm midpiece, where the machinery for expressing this process (mitochondria and a significant cytoplasmic space) is located.

DISCUSSION

Several authors have identified PI3K in the spermatozoa of a variety of mammalian and avian species and assigned to this molecule roles in such cellular processes as the acrosome reaction, capacitation and motility regulation [30–35]. Yet others have identified AKT and PTEN in this cell type [36–38], supporting the notion that biochemical pathways involving PI3K, AKT and PTEN are centrally involved in sperm cell biology. However, the present study is the first to fully investigate the role of this pathway in the modulation of apoptosis in mature human spermatozoa. The results of this analysis revealed that wortmannin could successfully inhibit PI3K phosphorylation and thereby disrupt the ability of this kinase to phosphorylate its downstream target, AKT, on Thr108.
Exposure to wortmannin did not have an effect on sperm vitality until doses in excess of 100 μM were reached (*P < 0.05 and ***P < 0.001 compared with the vehicle-only control). (B) At doses of inhibitor that did not compromise cell vitality (≤ 20 μM), a highly significant impact on mitochondrial ROS generation was observed in live cells (SYTOX green negative) with MitoSOX Red in a dose- and time-dependent manner (*P < 0.05 and **P < 0.01 compared with the vehicle-only control). (C) The induction of mitochondrial ROS production was achieved in the absence of any significant change in mitochondrial membrane potential as measured with JC-1; as a positive control, incubation with the proton ionophore CCCP was shown to completely silence the JC-1 signal. (D) Analysis of PS externalization using annexin V in conjunction with PI demonstrated minimal change within 4 h, but a clear dose-dependent response was observed at 24 h, peaking at 20 μM (*P < 0.05 and ***P < 0.001 compared with the vehicle-only control).

Thus the present study demonstrates, for the first time, that mature functional human spermatozoa can undergo an intrinsic apoptotic cascade. Indeed the latter appears to be a default pathway for these cells. As soon as conditions arise that compromise the phosphorylation status of the PI3K/AKT complex, then these cells enter an intrinsic apoptotic pathway associated with the dephosphorylation of BAD and the induction of mitochondrial permeability. Interestingly, most of the stimuli that induce apoptosis in somatic cells are completely without effect in human spermatozoa, including staurosporine, lipopolysaccharide, Kdo and genistein (Figure 5B). However some features of apoptosis can apparently be induced in these cells by exposure to a variety of non-receptor-mediated stimuli that ultimately create a state of oxidative stress, including exposure to radiofrequency electromagnetic radiation [44], cryostorage [45], heat [44] or the direct addition of H2O2 [46]. As far as we are aware, there are no reports of receptor-activated apoptosis in human spermatozoa. The only possible exception could be the apoptosis-inducing properties of progesterone [46]; however, this steroid probably exerts its pro-apoptotic effect indirectly by mobilizing calcium, which then secondarily induces ROS formation and oxidative stress [47].

Since we can find no evidence to indicate that apoptosis is actively induced via a receptor-mediated mechanism in mature human spermatozoa, we propose that these cells revert to this pathway in response to stress, as a form of programmed senescence. Although we fully acknowledge that the inhibitor we have used extensively in the present study, wortmannin, lacks specificity, it has proven to be a particularly effective chemical inducer of apoptosis in human spermatozoa, and has allowed us to study the biochemical features of this process. Although the range of apoptotic responses that spermatozoa can express is limited by their physical architecture, two features appear to be critical. The first is the expression of annexin V binding as a consequence of the externalization of PS (Figure 5B). The significance of this change may be found following insemination when the female reproductive tract contains millions of moribund and senescent spermatozoa that must be removed by phagocytic leucocytes. Accordingly, there is a massive leucocytic infiltration into the

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Figure 4 Changes in vitality, mitochondrial activity and PS externalization in response to PI3K inhibition

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lower female reproductive tract post coitum [48]. The phagocytic activity exhibited by these cells must be silent; in other words, the spermatozoa must be efficiently phagocytosed and removed, but this activity must not be accompanied by an oxidative burst or the production of pro-inflammatory cytokines. There are many examples of silent phagocytosis in biology, and a common feature of this phenomenon is the expression of apoptotic markers, such as PS, on the surface of the phagocytosed cell. This apoptotic marker is thought to instruct the phagocyte that the target cell should be engulfed in a non-phlogistic manner [49]. We therefore speculate that the activation of this apoptotic cascade in senescent cells is an adaptation that permits the efficient removal of spermatozoa from the female tract by phagocytic leucocytes without provoking a pro-inflammatory response.

In parallel with the surface expression of annexin V-binding sites, apoptotic spermatozoa also exhibit signs of caspase activation (Figure 5A) and a concomitant loss of motility (Figure 3). Whether there is a causal relationship between caspase activation and motility loss has not yet been established; however, these two parameters are highly correlated [50]. Moreover isolation of apoptotic spermatozoa using magnetic beads coated with annexin V revealed a clear association between annexin V binding, caspase activation and impaired motility [51]. The motility loss exhibited as part of this apoptotic cascade in senescent cells is an adaptation that permits the efficient removal of spermatozoa from the female tract by phagocytic leucocytes without provoking a pro-inflammatory response.

Whether there is a causal relationship between caspase activation and motility loss has not yet been established; however, these two parameters are highly correlated [50]. Moreover isolation of apoptotic spermatozoa using magnetic beads coated with annexin V revealed a clear association between annexin V binding, caspase activation and impaired motility [51]. The motility loss exhibited as part of this apoptotic cascade could again be an adaptive response to ensure that moribund, oxidatively stressed spermatozoa with possible DNA damage, cannot participate in the fertilization process. In most cell types the activation of the intrinsic apoptotic cascade culminates in extensive DNA fragmentation and cell death. It is at this point in the apoptotic pathway that the highly specialized anatomy of human spermatozoa starts to play a limiting role. Although in other cell types apoptosis is associated with the movement of endonucleases (AIFM, CAD, ENDOG) into the nucleus, this does not, and cannot, occur in spermatozoa. Following the induction of apoptosis, these proteins [as well as cytochrome c and DIABLO(SMAC)] remain resolutely locked within the midpiece region of the cell (Figure 6). Translocation of nucleases to the sperm nucleus is presumably impeded by the compacted nature of sperm chromatin and the highly compartmentalized architecture of mature spermatozoa, which separates the nucleus in the sperm head from the cytoplasm and mitochondria in the midpiece. The immediate, practical consequence of endonuclease exclusion from the sperm nucleus is that these apoptotic cells do not rapidly exhibit high levels of DNA fragmentation, as measured in the TUNEL assay (Figure 5D). However, the concomitant generation of mitochondrial ROS does result in the induction of oxidative DNA damage, as reflected by 8OHdG formation (Figure 6K). Since the spontaneous formation of 8OhdG in human spermatozoa is highly correlated with DNA damage, as measured with the TUNEL assay [14], it seems probable that these two events are related and sequential. Thus the formation of oxidative base adducts will affect DNA integrity by labilizing the glycosyl bond that attaches the base to the ribose unit, leading the generation of an abasic site. Abasic sites have a strong destabilizing effect on the DNA backbone, which can then result in strand breaks [52]. In light of these considerations we conclude that, ultimately, apoptosis in human spermatozoo does result in DNA fragmentation, but it is a prolonged process driven by oxidative stress rather than endonuclease activity. This notion does not exclude the possibility that extracellular nucleases are released by the reproductive tract that dismantle the DNA of apoptotic spermatzoa post mortem when the integrity of
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Figure 6 Localization of apoptosis mediators and induction of oxidative DNA damage following inhibition of PI3K

Immunocytochemistry revealed that all of the apoptosis mediators examined were confined to the midpiece/principal piece of the spermatozoa prior to the activation of apoptosis (left-hand panels) and remained fixed in this position 24 h after the induction of apoptosis with 20 μM wortmannin (right-hand panels). (A) and (B) represent CAD, (C) and (D) represent ENDOG, (E) and (F) represent AIFM, (G) and (H) represent cytochrome c, and (I) and (J) represent DIABLO(SMAC). (K) Although apoptosis in human spermatozoa was not associated with translocation of nucleases to the sperm nuclei or positive TUNEL signals, the concomitant generation of ROS did result in a significant dose-dependent increase in oxidative damage to the DNA, as measured by 80HdG formation (**P < 0.001 and *P < 0.05 compared with the vehicle-only control). Scale bars = 5 μm.

In summary, the present study provides definitive evidence that mature human spermatozoa can be induced to undergo a limited form of apoptosis characterized by mitochondrial ROS generation, PS externalization, caspase activation, motility loss, cytoplasmic vacuole formation and oxidative DNA damage. The physical architecture of the spermatozoon subsequently prevents endonucleases released from the mitochondria or activated in the cytosol from translocating to the nucleus. As a consequence, DNA fragmentation, one of the hallmarks of apoptosis in somatic cells, cannot occur immediately, but may be precipitated secondarily as a result of oxidative DNA adduct formation. Entry into this apoptotic pathway is actively prevented by the phosphorylation of PI3K and AKT that, in turn, maintain BAD in a phosphorylated anti-apoptotic state. Conversely, dephosphorylation of the PI3K/AKT axis leads to the activation of apoptosis. We propose that this is an adaptive physiological response to oxidative stress, rather than a receptor-mediated event. Furthermore, the purpose of this apoptotic response may be to both facilitate the silent phagocytosis of senescent moribund spermatozoa following insemination, and to prevent oxidatively damaged spermatozoa from participating in the fertilization process. In light of these findings, we would expect that growth factors/cytokines that stimulate PI3K/AKT would have a powerful pro-survival effect on human spermatozoa, as described recently for prolactin [16]. Furthermore, the active selection of

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the plasma membrane has been compromised, as suggested by Dominguez and Ward [53].

Like DNA damage, the changes in mitochondrial activity observed during apoptosis may also be the result of oxidative stress. Thus, when this process is triggered by wortmannin or LY294002, the mitochondria instantly generate ROS, but there is no immediate loss of ΔΨ. Formation of a mitochondrial pore without loss of ΔΨ, although uncommon, has been described previously [54]. In a possible sequence of events, the dephosphorylation of BAD leads to a loss of cytochrome c from the mitochondria, which then activates caspases by participating in apoptosome formation, and alters the activity of the mitochondrial electron transport chain from the normal four-electron reduction of O2 to a one-electron process that produces superoxide anion rather than water [55]. Reverse electron flow to complex I appears to be responsible for the superoxide generation observed under these circumstances [56]. This is significant because we have previously demonstrated that electron leakage from this site is particularly damaging to spermatozoa, promoting extensive lipid peroxidation and progressive motility loss [57]. The fact that the ΔΨ remains unchanged during the induction of mitochondrial ROS generation indicates that depolarization is not a prerequisite for superoxide production. However, as the spermatozoa lose vitality, ΔΨ may be secondarily lost as a result of ROS-induced oxidative damage [58].
non-apoptotic cells should represent an effective means of limiting the presence of senescent DNA-damaged spermatozoa in sperm populations intended for use in assisted conception therapy [10]. Although the present studies were conducted on human spermatozoa, it is likely that the fundamental features of the apoptotic process are similar across all mammalian species. Thus a highly compartmentalized subcellular architecture featuring a nucleus that is physically separated from the mitochondria and most of the cytoplasm, is common to all mammalian spermatozoa, as is the need to undergo regulated senescence within the female reproductive tract post-insemination. Elements of the apoptotic process, such as caspase activation, PS externalization and mitochondrial ROS production, have been observed in the spermatozoa of a wide variety of species, including bull, boar, stallion and mouse, in addition to human spermatozoa [59–61]. However, the truncated nature of the apoptotic process in these cells has not previously been discussed, nor has the concept that apoptosis represents a default pathway for these cells in response to oxidative stress that can only be counterbalanced by pro-survival factors acting through the PI3K/AKT pathway.

**AUTHOR CONTRIBUTION**

Adam Koppers and Lisa Mitchell conducted all of the cell biology studies; Ping Wang and Minjie Lin performed the ultrastructural analysis; and John Aitken designed the study and prepared the manuscript. All authors contributed to the final version of the paper.

**ACKNOWLEDGEMENTS**

We thank Jody Powell for orchestrating our semen donor panel.

**FUNDING**

This work was supported by the ARC Centre of Excellence in Biotechnology and Development (grant number CE00348239) and NSF Department of State and Regional Development.

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Figure 7. Electron microscopy of human spermatozoa treated with wortmannin

(A) Untreated morphologically normal spermatozoa. (B and C) Treatment with wortmannin (20 μM) for 24 h resulted in major morphological changes to these cells, including distension of the cytoplasm-rich midpiece region of the cell and accompanying vacuole formation, as indicated by the arrows. Scale bars = 1 μm.
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Received 18 January 2011/25 March 2011; accepted 7 April 2011
Published as BJ Immediate Publication 7 April 2011, doi:10.1042/BJ20110114
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