SPINK3 modulates mouse sperm physiology through the reduction of nitric oxide level independently of its trypsin inhibitory activity

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Abstract

Serine protease inhibitor Kazal-type (SPINK3)/P12/PSTI-II is a small secretory protein from mouse seminal vesicle which contains a KAZAL domain and shows calcium (Ca2+) transport inhibitory (caltrin) activity. This molecule was obtained as a recombinant protein and its effect on capacitated sperm cells was examined. SPINK3 inhibited trypsin activity in vitro while the fusion protein GST-SPINK3 had no effect on this enzyme activity. The inactive GST-SPINK3 significantly reduced the percentage of spermatozoa positively stained for nitric oxide (NO) with the specific probe DAF-FM DA and NO concentration measured by Griess method in capacitated mouse sperm; the same effect was observed when sperm were capacitated under low Ca2+ concentration, using either intracellular (BAPTA-AM) or extracellular Ca2+ (EDTA) chelators. The percentage of sperm showing spontaneous and progesterone-induced acrosomal reaction was significantly lower in the presence of GST-SPINK3 compared to untreated capacitated spermatozoa. Interestingly, this decrease was overcome by the exogenous addition of the NO donors, sodium nitroprusside (SNP), and S-nitrosoglutathione (GSNO). Phosphorylation of sperm proteins in tyrosine residues was partially affected by GST-SPINK3, however, only GSNO was able to reverse this effect. Sperm progressive motility was not significantly diminished by GST-SPINK3 or BAPTA-AM but enhanced by the addition of SNP. This is the first report that demonstrates that SPINK3 modulates sperm physiology through a downstream reduction of endogenous NO concentration and independently of SPINK3 trypsin inhibitory activity.


Introduction

In mammals, regulation of sperm physiology is mostly limited to posttranslational mechanisms controlled by signal transduction, since the transcription/translation level is restricted (Shima et al. 2004). Upon ejaculation, sperm are first capacitated and then they exhibit hyperactivated motility and undergo a physiological acrosome reaction (AR; Yanagimachi 1994). Capacitation can be described as an event naturally occurring within the female duct, associated with an increase in plasma membrane fluidity due to the efflux of cholesterol, removal of surface attached proteins, and redistribution of membrane phospholipids (Patrat et al. 2000). These changes in its membrane structure induce calcium (Ca2+) influx, increases in intracellular pH (Phillips & Bedford 1988), intracellular cAMP concentration ([cAMP]i), and protein phosphorylation, as well as the production of reactive oxygen species such as nitric oxide (NO; Visconti et al. 2002, Aitken & Baker 2004, Harrison & Gadella 2005, O’Flaherty et al. 2006). The above-mentioned molecules are part of sperm signaling cascades involving numerous interconnected and also some redundant pathways which ensure acquisition of sperm fertilizing ability (Muratori et al. 2011).

Autocrine production of NO by sperm from the beginning of capacitation controls the increase in cAMP/cGMP and most of the known downstream serine, threonine, and tyrosine phosphorylation events, as well as tyrosine nitration of proteins; however high NO concentration blocks all sperm functions (Machado-Oliveira et al. 2008, de Lamirande et al. 2009, Roessner et al. 2010). NO is synthesized in mature sperm by the NO synthase (NOS), which exists as three
isoforms – neural NOS (nNOS), endothelial NOS (eNOS), and inducible NOS – and localize in sperm head and midpiece (Meiser & Schulz 2003). The constitutively expressed nNOS and eNOS isoforms are Ca^{2+}/calmodulin (CaM) dependent (Herrero & Gagnon 2001). Thus, considering the activity of these isoforms, the time-related intracellular Ca^{2+} increase occurring during capacitation should be necessary to induce NO production (de Lamirande et al. 2009, Muratori et al. 2011). The mechanisms involved in Ca^{2+} regulation during capacitation, despite poorly known, are believed to be related to inhibition of Ca^{2+} ATPase, increase in membrane permeability and Ca^{2+} release from intracellular Ca^{2+} stores (Pons-Rejraji et al. 2009). Before capacitation, this Ca^{2+} entry should be impaired.

A well-known Ca^{2+}-transport inhibitor I (caltrin; Chen et al. 1998, Luo et al. 2004), also known as P12 and pancreatic secretory trypsin inhibitor, II, is a secretory serine protease inhibitor Kazal-type (SPINK3, NCBI ID: NP_033284.1) that is constitutively expressed in pancreas and its expression is androgen dependent in seminal vesicle and prostate of mice, rat and human (Mills et al. 1987, Novella et al. 1999). This protein, as other secretory proteins from the accessory glands, attaches to the sperm surface during sperm transit along the male duct and consequently, it is found on the surface of ejaculated sperm (Dematteis et al. 2008). Caltrin-like proteins are known to modulate Ca^{2+} influx (Clark et al. 1993, Coronel et al. 1993). However, the physiological role, the mechanism by which they prevent intracellular Ca^{2+} increase and the signaling pathways that they modulate are still unknown. In addition, although some of them have trypsin inhibitor activity (Lardy 2003), whether their caltrin function is related to its trypsin inhibitory activity remains to be demonstrated. Therefore, our first aim was to study the possible interaction between SPINK3 Ca^{2+} uptake inhibition and the levels of NO in mouse sperm during capacitation. We tested whether trypsin inhibitor activity of SPINK3 was necessary to modulate sperm physiology. Finally, we determined the effect of SPINK3 in the presence or absence of NO donors on sperm AR, tyrosine phosphorylation, and motility.

Results
Recombinant expression of active SPINK3
To explore the role of SPINK3 in sperm physiology mouse recombinant SPINK3 was synthesized in Escherichia coli (E. coli) cells. The coding sequence of mouse mature SPINK3 (57 aminoacids corresponding to the C-terminus of the protein) was synthesized by RT-PCR from total mRNA of seminal vesicle (Fig. 1A) and this cDNA was cloned into the expression vector (pGEX-4T-3) as a fusion protein at the C-terminal region of glutathione S-transferase (GST) downstream a thrombin cleavage site (see Materials and Methods for details). Two protein bands were differentially expressed in E. coli cells harboring pGEX-4T-3-spink3 after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h which were enriched in the soluble fraction. The upper band showed the expected molecular size for GST-SPINK3 fusion protein (34 kDa; Fig. 1, lane 6). After purification (Fig. 1, lane 7), only the 34 kDa polypeptide was recovered which cross-reacted with anti-SPINK1 antibodies raised against the product of the human orthologue gene (Fig. 1, lane 8). This result suggests that the lower band may correspond to a degradation product of the recombinant protein unable to bind to the column. To obtain SPINK3, the GST-SPINK3 fusion protein was digested with thrombin (Fig. 1B, lanes 1 and 2) and purified as indicated in Materials and Methods (Fig. 1B, lane 3). Anti-SPINK1 cross-reacted with both the 34 and 8 kDa protein species obtained after thrombin cleavage (Fig. 1B, lane 2, Western blot) showing that the digestion was not exhaustive and confirming the identity of the recombinant proteins, GST-SPINK3 and SPINK3.

To assess whether the purified SPINK3 and GST-SPINK3 were active proteins, trypsin inhibitory activity was measured using two substrates, CBZ-Gly-Gly-Arg-4-aminomethylcoumarin (CGGR-MEC) and Boc-Val-Pro-Arg-4-aminomethylcoumarin (BVPR-MEC; Fig. 2). SPINK3 inhibited trypsin activity on both substrates (94.80 ± 4.73 and 53.66 ± 1.28% on CGGR and BVPR respectively). The inhibitory activity of SPINK3 was similar to that of the soybean trypsin inhibitor (SBTI) on CGGR-MEC while it was less effective on BVPR-MEC. In contrast, GST-SPINK3 did not display protease inhibitory activity which was similar to that of the GST tag (control).

In order to separate serine protease inhibitor and caltrin SPINK3 activities the work was conducted with GST-SPINK3 because it lacks trypsin inhibitory activity. Caltrin activity of the recombinant fusion protein GST-SPINK3 was confirmed by measuring intracellular Ca^{2+} concentration [Ca^{2+}]i by the fluorometric probe fluo-3 acetoxymethyl (fluo-3 AM) ester. During capacitation, [Ca^{2+}]i was increased by 79% compared to sperm incubated under noncapacitating conditions (0.64 ± 0.1 and 3.15 ± 0.6 arbitrary units for control sperm under noncapacitating and capacitating conditions respectively; P < 0.01). The concentration used for the recombinant protein (2.8 μM) was capable to bind to the sperm surface (Fig. 3A) and promoted a 31% Ca^{2+} uptake inhibition after 45 min of incubation under capacitating conditions (3.15 ± 0.6 and 2.20 ± 0.2 arbitrary units, for control and GST-SPINK3-treated sperm under capacitating conditions respectively; P < 0.05) comparable to that of 50% previously reported by Chen et al. (1998) after 60 min for the purified native molecule. The reduction of Ca^{2+} was mainly observed at the sperm head (Fig. 3B) as recently reported (Ou et al. 2012).
GST-SPINK3 treatment correlates with reduced NO production in capacitated sperm

By different methodologies electron paramagnetic resonance (EPR) spectroscopy, fluorescent probes, and nitrite quantification an increase in NO production was observed by us (Fig. 4C) and other authors in capacitated compared to noncapacitated sperm (Herrero et al. 2000, de Lamirande & Lamothe 2009, Rodriguez et al. 2011).

In epididymal mouse sperm loaded with 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) the distribution of NO changed from the head and midpiece to the midpiece upon capacitation (Fig. 4A, upper panels a and c), consistent with previously reported NOS relocalization (Herrero et al. 1996).

To investigate whether caltrin activity of SPINK3 affects sperm intracellular signaling by reducing NO concentration we examined the effect of the GST-SPINK3 on NO production in capacitated sperm by measuring the percentage of cells showing the signal of the fluorescent probe DAF-FM DA (Fig. 4B) and nitrite production (Fig. 4C) in sperm incubated with GST-SPINK3 during capacitation.

Both the percentage of capacitated sperm with NO signal and NO concentration were significantly diminished by the addition of GST-SPINK3 compared to untreated capacitated sperm or control with the same concentration of GST (Fig. 4B and C, black bars). No significant changes were observed by the effect of GST-SPINK3 on sperm incubated under noncapacitating conditions (Fig. 4B and C, grey bars). Whether SPINK3 caltrin activity was involved in the reduction of the number of cells with NO, a drop in Ca²⁺ concentration should have similar effect as SPINK3. As expected, the treatment of capacitated sperm with a permeable (BAPTA-AM) or impermeable (EDTA) Ca²⁺ chelators reduced the percentage of capacitated sperm with NO signal (Fig. 4B, right panel).

An NO donor reverses SPINK3 effects on AR

Spontaneous and progesterone (P₄)-induced AR (Fig. 5A and B respectively) were assessed on capacitated sperm by evaluating acrosomal loss as evidenced by fading of the blue stained acrosome (Fig. 5, inset). Noncapacitated sperm were assessed as a control. As expected, when sperm were incubated under capacitating conditions, GST-SPINK3 significantly reduced the percentage of spontaneous AR compared to control sperm without

Figure 1 Recombinant expression and purification of SPINK3. (A) The upper panel shows a schematic representation of SPINK3 structure. The positions of the primers used to amplify mature SPINK3 are indicated below the diagram of SPINK3. Note that most of the protein is occupied by the Kazal-type serine protease inhibitors family signature (Prosite entry PDOC00254, Lin et al. 2008) described in the box below. C, conserved cystein residues; the (X) followed by a number or a letter indicates conserved or variable number of aminoacid residues respectively. The lower panel shows the electrophoretic profile by SDS–PAGE of the soluble (S) and insoluble (I) fractions of E. coli cells harboring pGEX-4T-3-spink3 before (lanes 1 and 2) and after 16 h in the absence (lanes 3 and 4) or presence of 0.1 mM IPTG (lanes 5 and 6). GST-SPINK3 was purified by Glutathione Sepharose 4B affinity chromatography, analyzed by electrophoresis stained with CBB R250 (lane 7) and identified by western blotting with anti-SPINK1 antibody (Western blot (WB), lane 8). (B) Electrophoretic pattern of thrombin cleavage product of GST from the fusion protein GST-SPINK3 (CBB, lane 1) and the corresponding western blotting developed with anti-SPINK1 antibody (Western blot (WB), lane 8). Cleaved protein was purified as described in the Materials and Methods section and analyzed by SDS–PAGE (CBB, lane 1). Protein bands were visualized by CBB staining except WB lanes. The position of molecular mass markers is indicated on the left. Arrows indicate the position of the recombinant proteins. A representative gel of three independent experiments is shown.
GST-SPINK3 (Fig. 5A), indicating that the recombinant protein affects AR as described for purified caltrin (Dematteis et al. 2008). P4-induced AR was also significantly affected by GST-SPINK3 compared to untreated capacitated sperm (Fig. 5B).

If endogenous NO was reduced as a consequence of the presence of GST-SPINK3, an NO donor added simultaneously with GST-SPINK3 should prevent the effect. This hypothesis was confirmed using two different NO donors: sodium nitroprusside (SNP) and S-nitroso-glutathione (GSNO). The concentration of SNP and GSNO used was sufficient to increase NO in sperm, independently of the presence of GST-SPINK3 (Fig. 4C). When sperm were incubated under capacitating conditions in the presence of SNP or GSNO plus GST-SPINK3 the percentages of spontaneous and P4-induced AR sperm returned to the values of untreated capacitated sperm (Fig. 5A and B, black bars).

Sperm incubated under noncapacitating conditions had lower percentages of acrosomal loss than capacitated sperm and no effect was observed with any of the treatments (Fig. 5A and B, grey bars), indicating that nonphysiological AR percentages were very low, which is consistent with sperm viability that was invariable (around 82%) for all the treatments.

**Effect of SPINK3 and NO on tyrosine phosphorylation**

When sperm were incubated under capacitating conditions, western blot analysis showed the characteristic increase in the 105 kDa band of hexokinase (Kalab et al. 1994) and the 82 kDa protein compared to noncapacitated sperm (Fig. 6, lanes 1 and 7, arrows). By this method we wanted to know whether GST-SPINK3 affects the signaling pathways activated by incubation of sperm in a capacitating media. It has been reported that rat caltrin I (Dematteis et al. 2008) and recombinant SPINK3 (Ou et al. 2012) had no effect on protein phosphorylation. Contrary to those reports in this work, even when capacitated sperm with GST-SPINK3 did not reestablish the pattern of tyrosine-phosphorylated proteins observed in untreated noncapacitating sperm, GST-SPINK3 reduced the intensity of the 82 and 105 kDa bands (Fig. 6, lane 8). This effect could not be abolished by SNP plus GST-SPINK3 (Fig. 6, lane 10) but was reversed by GSNO (Fig. 6, lane 12). Conversely, none of the treatments produced modifications in the electrophoretic pattern of tyrosine-phosphorylated proteins of noncapacitated sperm (Fig. 6, lanes 1–6), even NO donors.

**Effect of SPINK3 and NO on sperm motility**

The effect of GST-SPINK3 on sperm motility was evaluated by quantifying total motility (TM) and progressive motility (PM) of epididymal sperm incubated for 30 min under noncapacitating or capacitating conditions with different reagents. After this time, sperm incubated under noncapacitating conditions were immotile in all treatments. The addition of GST-SPINK3 on capacitated sperm produced a nonsignificant reduction of sperm PM compared to untreated sperm at 30 min of capacitation, while the simultaneous addition of both SNP (100 μM) and GST-SPINK3 (2.8 μM) significantly enhanced sperm PM (Fig. 7). Surprisingly, GSNO (100 μM) added together with GST-SPINK3 did not produce any effect. As expected, the external Ca2+ chelator, EDTA, produced a drastic effect showing no sperm with PM, while this parameter was unaffected by BAPTA-AM (Fig. 7). At this concentration, EDTA has no cytotoxic effect as denoted by the maintenance of cell viability (~82% for all treatments) and as previously reported (Lee et al. 1996, Okazaki et al. 2011). TM was maintained without variations for all the treatments, except with EDTA (Fig. 7). These results suggest that the enhancement of cell motility associated with the capacitation induced by BSA was not affected by GST-SPINK3.

**Discussion**

SPINK3 is a secretory serine protease inhibitor expressed in the seminal vesicle of various mammals (Mills et al. 1987, Chen et al. 1998). It can be considered as a multifunctional protein since, in addition to its antiproteolytic activity (Luo et al. 2004), inhibits Ca2+ transport in sperm (Coronel et al. 1992, Dematteis et al. 2008). Because of this latter activity it has been named as caltrin protein.
This work demonstrates that incubation of capacitated mouse sperm with recombinant GST-SPINK3 modulates sperm physiology through a downstream reduction of endogenous NO concentration that is independent of SPINK3 antitrypsin activity. To the best of our knowledge, this is the first report that links SPINK3 activity to a signaling pathway involving NO.
Is SPINK3 antitrypsin activity necessary to modulate sperm function?

SPINK3 binds to the spermatozoa surface by means of an unknown receptor. It can inhibit trypsin in vitro (Fig. 2) as shown with purified P12 (Lai et al. 1994, Winnica et al. 2000). It also inhibits acrosin (Winnica et al. 2000), but the target protease in sperm is still unknown. Recently, a membrane-bound serine protease, TESPL, has been proposed to be the SPINK3 anchoring protein because interaction between these proteins has been demonstrated by yeast two-hybrid assay (Ou et al. 2010). However, it is known that the ability of SPINK3 to bind sperm and its trypsin inhibitory activity are not linked. Single-site mutations in the recombinant mouse protein P12 (SPINK3) demonstrated that aminoacid residue R19 was essential for protease inhibition and D22 and/or Y21 were responsible for the binding of P12 to sperm (Luo et al. 2004, Lin et al. 2006). Accordingly, in this work we showed that a modified recombinant SPINK3 protein that lacks trypsin inhibitory activity (GST-SPINK3) can bind sperm, reduce intracellular Ca\(^{2+}\) concentration, and modulate AR and protein phosphorylation, while it has no effect on sperm motility. All these data taken together show that the serine protease inhibitory activity of SPINK3 might not be necessary in sperm signaling which is consistent with the results obtained by Ou et al. (2012) with the recombinant SPINK3 carrying the point mutation R19L.

Remarkably, SPINK3 is not the only serine protease inhibitor from male glands that modulates sperm physiology. In guinea pig, two different caltrin molecules purified from the seminal vesicle secretion are equally effective inhibitors of sperm Ca\(^{2+}\) uptake, although...
caltrin I binds to the head of epididymis sperm and prevents AR during in vitro capacitation (Winnica et al. 2000); while caltrin II binds to the principal portion of the tail and delays sperm hyperactivation (Coronel & Lardy 1992). Caltrin II has no structural homology with caltrin I (Winnica et al. 2000) even though its gene conserves a whey acidic protein (WAP) domain, which is present in serine protease inhibitors (Furutani et al. 2004).

Other serine protease inhibitors, like SERPINE2 (nexin-1) from seminal vesicle and epididymal protease inhibitor (EPPIN), bind to uncapacitated sperm and are believed to inhibit capacitation. SERPINE2 inhibits both plasminogen activator and thrombin, and also inhibits in vitro BSA-induced tyrosine phosphorylation of sperm under capacitating conditions by unknown mechanisms (Lu et al. 2011). EPPIN, characterized by both WAP-type and Kunitz-type consensus sequence (Wang et al. 2005, 2008, O’Rand et al. 2007), acts a decapacitating factor by modulating the activity of PSA in semenogelin, which inhibits human sperm capacitation (de Lamirande et al. 2001). However, it has been found that anti-EPPIN antibodies significantly inhibited the human sperm AR induced by A23187, reduced intracellular Ca\(^{2+}\) concentration, and did not change tyrosine phosphorylation of sperm proteins (Zhang et al. 2010).

Among all these molecules described as decapacitating factors and also serine protease inhibitors, it is clear that EPPIN and SERPINE2 have a role outside the sperm. This might also be the case for SPINK3 antitrypsin activity.

**SPINK3 caltrin activity modulates sperm physiology through a downstream NO reduction**

In fact, SPINK3 expressed as a GST-fusion protein reduced NO production, spontaneous and P4-induced AR in mice epididymal sperm capacitated in vitro but did not completely affect sperm protein phosphorylation in tyrosine residues and progressive motility.

Extracellular Ca\(^{2+}\) is needed for capacitation and NO production (de Lamirande et al. 2009). It has been reported that NO donors cause an elevation of human sperm [Ca\(^{2+}\)]i coming from Ca\(^{2+}\) internal stores by sperm protein S-nitrosylation (Machado-Oliveira et al. 2008). However, SPINK3 cannot cross the sperm plasma membrane, it binds to the sperm surface during ejaculation and comes off during AR (Dematteis et al. 2008), so one could speculate that the reduction of [Ca\(^{2+}\)]i observed in sperm upon treatment with GST-SPINK3, and also reported for caltrin I (San Agustin et al. 1987, Dematteis et al. 2008), should act upstream of NO. This speculation is consistent with the reduction of NO observed by us and other authors in capacitated sperm treated with either GST-SPINK3 (Fig. 4) or Ca\(^{2+}\) chelators (Fig. 4; de Lamirande et al. 2009). GST-SPINK3 caltrin effect might inhibit the Ca\(^{2+}\)/CaM-dependent brain nNOS, as reported for bovine caltrin (Schaad et al. 1996); yet, under capacitating standard conditions, extracellular Ca\(^{2+}\) uptake would activate NOS, increasing NO levels and continuing through a signaling pathway that finally modulates different sperm processes (Fig. 8).

It has been reported that, without external triggers, active NO production by NOS was observed in most mature human spermatozoa (Roessner et al. 2010). This is in agreement with our observation of a strong NO fluorescent signal in all the sperm obtained from cauda epididymis when loaded with DAF-FM DA, where sperm are already mature. Also, the redistribution of NO that we observed upon capacitation from the head and midpiece to the neck is in accordance with the NOS relocalization in mouse and human sperm, observed by
imunofluorescence using antibodies that cannot distinguish among NOS forms (Herrero et al. 1996). However, de Lamirande & Lamothe (2009) observed, using the same probe, an increase of NO signal on the head in human sperm during capacitation. This discrepancy should be attributed to a species-dependent behavior of NO or alternatively, to differences in the methodology, since our experiments did not include fixation of sperm neither we used an antifading agent. Probably, NO production at the sperm head is quenched due to the lack of an antifading agent, so we were able to detect the relocalization of the NOS to the midpiece where NO is being produced, which might occur later after the initiation of capacitation. For this reason we used the Griess reaction to detect total NO.

What is the effect that SPINK3 has on the signaling pathways activated during capacitation? Capacitation is a Ca\(^{2+}\)-dependent process and is known to be prevented by intercellular and extracellular chelators (Leclerc et al. 1998), so one is prompt to hypothesize that SPINK3 should inhibit capacitation. However, previous work with the native rat protein or the recombinant protein showed no effect on protein phosphorylation (Dematteis et al. 2008, Ou et al. 2012). In this work, the change in sperm to capacitating medium triggered the typical increase of tyrosine phosphorylation of sperm proteins of 105 and 81 kDa observed when the assay is performed without the phosphatase inhibitor orthovanadate (Leclerc et al. 1996, Herrero et al. 1999, Kulanand & Shivaji 2001), coincidently with a detectable endogenous NO production (see Figs 4 and 6).

It has been reported that phosphorylation in tyrosine, threonine, and glutamine residues of sperm proteins in human sperm is triggered by NO donors and inhibited by NO scavengers (Herrero et al. 1999, Herrero & Gagnon 2001, Thundathil et al. 2003). Moreover, NO is believed to modulate the increase in cAMP and cGMP that leads to protein phosphorylation (Machado-Oliveira et al. 2008, de Lamirande et al. 2009, Roessner et al. 2010). In particular, Thundathil et al. (2003) demonstrated the increment of the double phosphorylation (P-Thr-Glu-Tyr-P) in p80/105 proteins of human sperm under noncapacitating conditions (containing CaCl\(_2\)) by the addition of spermine NONOate (Thundathil et al. 2003). In contrast, we were able to observe an increase in

![Figure 6](image_url) Effect of GST-SPINK3 and NO on tyrosine phosphorylation of sperm proteins. Cauda epididymis motile mice sperm were incubated under noncapacitating or capacitating conditions in the presence or absence of GST-SPINK3 and with or without the NO donors, SNP, and GSNO. Tyrosine phosphorylation was assessed by western blot analysis of total sperm proteins using a monoclonal anti-phosphotyrosine antibody. Lanes 1, 2, 3, 4, 5 and 6: proteins from noncapacitated sperm; lanes 2 and 8: proteins from sperm treated with GST-SPINK3; lanes 3 and 9: proteins from sperm treated with SNP; lanes 4 and 10: proteins from sperm treated with GST-SPINK3 and SNP; lanes 5 and 11: proteins from sperm treated with GSNO; and lanes 6 and 12: proteins from sperm treated with GST-SPINK3 and GSNO. The positions of molecular mass markers (kDa) are indicated at the left. The immunoreactive bands that change during capacitation are indicated by arrows.

![Figure 7](image_url) Effect of GST-SPINK3 and NO on mice sperm motility. Cauda epididymis motile mice sperm were incubated under noncapacitating (data not shown) or capacitating conditions in the presence or absence of GST-SPINK3 with or without SNP or GSNO, as indicated. Spermatozoa showing any kind of motility (TM) or only PM were quantified after 30 min incubation under brightfield microscope as percentage of total quantified sperm (n=200 per replica). Data are expressed as the mean±S.E.M. of three independent samples. *Value different (P<0.05) from that observed for control spermatozoa under each condition.
tyrosine-phosphorylated proteins caused by either SNP or GSNO merely in sperm incubated with capacitating medium but not changes were provoked by these donors in HM media devoid of bicarbonate, Ca\textsuperscript{2+}, and BSA (Fig. 6).

In addition, under capacitating conditions, SNP did not reverse the effect of GST-SPINK3 on tyrosine phosphorylation, however, the addition of GSNO indeed abolished the effect of GST-SPINK3, which can be explained by the higher levels of NO released by this donor under the assayed conditions (Fig. 4). Whether GSNO effect is through an NO-dependent activation of adenylate or guanylate cyclase or through protein S-nitrosylation cannot be concluded.

In view of these results it can be assumed that NO is not sufficient to mediate protein tyrosine phosphorylation, and maybe it can be directly mediated by Ca\textsuperscript{2+} as also proposed by Muratori et al. (2011). Most of the tyrosine-phosphorylated proteins identified are flagellar proteins, therefore this process might be related to hyperactivation but not with AR.

The effect of SPINK3 on AR might be more clearly related to a reduction in endogenous NO concentration produced by the Ca\textsuperscript{2+} transport inhibiting effect of SPINK3. In agreement with previous results of purified caltrin I from rat (Dematteis et al. 2008) and recombinant SPINK3 (Ou et al. 2012), we observed an inhibition of both spontaneous and P\textsubscript{s}-induced AR mediated by GST-SPINK3, while viability remained invariable. However, the physiological significance of the effect over induced AR is uncertain. It has recently been proposed that SPINK3 does not detach from the sperm surface during capacitation but might be removed by the trypsin-like activity in the uterine fluid of estrous females before the sperm meets the ovum (Ou et al. 2012), still, Ca\textsuperscript{2+} concentrations in this region are low (Abou-haila & Tulsiani 2009).

According to our hypothesis, both NO donors completely reversed the effect of GST-SPINK3, demonstrating that NO was necessary for triggering AR as previously reported (Herrero & Gagnon 2001). The signaling pathway that mediates AR via NO probably involves the activation of a soluble guanylate cyclase since incubation of bovine sperm with SNP caused an increase in cGMP levels during AR, although it was observed in mouse sperm that cGMP levels were not affected by NOS inhibitors (Herrero & Gagnon 2001). The Acrosomal stores of Ca\textsuperscript{2+} have been also proposed to be activated by cGMP (Costello et al. 2009) as part of the pathway. Accordingly, thapsigargin, a molecule that raises cytosolic Ca\textsuperscript{2+} concentration by blocking the ability of the cell to pump Ca\textsuperscript{2+} into the acrosome, was sufficient to prompt AR in the presence of EGTA (Herrick et al. 2005).

Motility was slightly affected by the addition of GST-SPINK3 to capacitating sperm, according to previous

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**Figure 8** Schematic representation of the effect of GST-SPINK3. Cholesterol efflux induced in vitro by BSA or methyl-β-cyclodextrine (Choi & Toyoda 1998) and in vivo by progesterone (Costello et al. 2009) and female tract neighborhood promotes the remodeling of the plasma membrane and triggers signaling pathways leading to different sperm processes that account for sperm fertilizing ability. Extracellular Ca\textsuperscript{2+} influx might induce Ca\textsuperscript{2+}/CaM-dependent NOS activity, increasing NO production. SPINK3 attaches to the sperm surface by unknown receptors (Dematteis et al. 2008) and reduces intracellular Ca\textsuperscript{2+} concentration by acting at the level of the plasma membrane (Breitbart et al. 1990), consequently a reduction of NO is observed (symbolized as ∆). Under capacitating conditions this binding produces the inhibition of the AR that could be overcome by NO donors. SPINK3 also produce a modification in the protein phosphorylation pattern that is also reversed by a strong NO donor, however it was not sufficient to induce protein phosphorylation itself, suggesting a possible control of protein kinases directly by Ca\textsuperscript{2+}.
results with purified rat caltrin I, where no effect was observed (Dematteis et al. 2008). In this sense, it is noteworthy that Ca^{2+} reduction caused by GST-SPINK3 in capacitating sperm was mainly circumscribed to the head region, consistent with other results (Ou et al. 2012), and explaining the low percentage of reduction observed when fluorescence was measured in the sperm suspension loaded with the probe fluo-3 AM (Fig. 3). Besides, a distinct effect was observed in our experiments with both Ca^{2+} chelators: a complete impairment of motility caused by EDTA, as reported (Lee et al. 1996) contrary to a lack of effect of BAPTA-AM (Fig. 7), consistent with the observation that BAPTA-AM did not reduce Ca^{2+}-specific labeling with fluo-3 AM in sperm midpiece (De Blas et al. 2002).

In particular, Ca^{2+} is essential for the development of sperm motility, and its intracellular levels are tightly regulated by several Ca^{2+} channels (Muratori et al. 2011). Signaling pathway that controls flagellar movement is thought to be an NO-dependent mechanism independent of cAMP and cGMP (Machado-Oliveira et al. 2008) and dependent of midpiece stores of Ca^{2+} (Costello et al. 2009). On the contrary, it was reported that NO donors are known to stimulate sperm motility by an increased synthesis of cGMP (Miraglia et al. 2011), however there are some discrepancies regarding the effect of NO donors on motility depending on the doses utilized (Herrero et al. 1999). Surprisingly, in this work SNP plus GST-SPINK3 but not SNP alone or either GSNO enhanced sperm PM, probably due to the differences in NO concentration released under these conditions (Figs 4 and 7).

It was also reported that application of thapsigargin caused elevation of Ca^{2+} in the neck region and hyperactivation in bovine sperm, these effects being independent of Ca^{2+} from the outside (Costello et al. 2009).

Still, several questions remain unanswered and require further investigation: what does relocalization of NO to the midpiece during capacitation means? Is Ca^{2+} compartmentalization the fork in the road that converts a signal to different intracellular pathways? To address these questions real-time imaging should be necessary to understand the timing of these transient signals using intracellular Ca^{2+} chelators and/or ionophores that regulate different Ca^{2+} channels.

**Conclusion**

Recombinant SPINK3 modulates sperm physiology through a downstream reduction of endogenous NO concentration. The effect of SPINK3 can be reversed by the exogenous addition of NO and is independent of SPINK3 antitrypsin activity. However, the effect on sperm protein phosphorylation was not dramatic and almost no effect was observed by SPINK3 on sperm motility.

It can be speculated that SPINK3, a secretory molecule from seminal vesicle, has two different activities: a serine protease inhibitory activity that has not yet been completely related to sperm physiology and caltrin activity that can be considered a checkpoint that turns on or off signaling pathways and thus maintaining sperm in a latent state until it reaches the ovum.

**Materials and Methods**

**Reagents**

Tryptone and yeast extract for growth medium was purchased from OXOID (Basingstoke, Hampshire, UK); Agarose from Genbiotech (Buenos Aires, Argentina); M-MLV Reverse Transcriptase from Promega; Taq polymerase from PB-L (Buenos Aires, Argentina); Quiaguard Gel Extraction and Miniprep kits (Qiagen); random primers DNA labeling system and DNA fluorescent dyes (SYBR Safe and SYBR Gold) from Invitrogen. Restriction enzymes were purchased from Fermentas Inc (Ontario, Canada). DAF-FM DA was purchased from Invitrogen, BAPTA-AM from Calbiochem (Merck, Darmstadt, Germany). Griess colorimetric kit form Promega, SNP from Merck, secondary antibodies, ipegal CA630, and DAPI from Sigma, fluo-3 AM (Molecular Probes F1242; Invitrogen) and 0.05% Silwet L-77 surfactant (Arysta Life Science, Tokyo, Japan). All other chemicals and reagents were of analytical grade and obtained either from Merck or Sigma–Aldrich.

**Animals**

BalbC and CF-1 mice (Mus musculus (M. musculus)) were maintained at 22 °C with a photoperiod of 1200 h light: 1200 h darkness, food, and water ad libitum. Sexually mature (2–3 months old) male mice were killed by cervical dislocation and epididymides and seminal vesicles were immediately removed. All procedures were in agreement with the Local Ethics Committee of the National University of Mar del Plata and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf). Epididymides were processed immediately as described below (Sperm preparation and capacitation) and seminal vesicles were sectioned into 0.5 cm² pieces, immersed in RNA later (Ambion), and stored at −80 °C until used.

**Molecular cloning and heterologous synthesis of recombinant SPINK3 and GST-SPINK3**

Total RNA was extracted from mice seminal vesicles (300 mg) using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Tissue was disrupted by homogenization in an IKA-WERKE T10 basic Ultra-Turrax using an 810N-BG tool for 2 min at 4 °C. First strand cDNA was synthesized by RT with M-MLV Reverse Transcriptase (Promega) according to the manufacturer’s instructions. The reaction mix (25 μl) contained the following: 5 μg RNA, 2 μg/ml oligo (dT) primer, 0.5 mM dNTPs, and 0.2 U enzyme. A DNA fragment corresponding to mature SPINK3 (nucleotides 158–331) that encoded a polypeptide of 34 kDa starting from residue 24 to the stop codon, Fig. 1) was obtained by PCR amplification of single-stranded cDNA using primers designed on the basis of SPINK3 mRNA.
sequence from *M. musculus* (NCBI ID: NM_009258.5). To facilitate cloning of SPINK3 cDNA into the expression vector, the restriction sites BamHI and Xhol were added to the 5’ end nucleotide sequences of the SPINK3-Fw (5’-CGG GGA TCC CTT AAG GTG ACT GGA-3’) and SPINK3-Rev (5’-CGG CTC GAG TCA GCA AGG CCC ACC-3’) primers respectively. The PCR reaction was carried out in a mixture (20 μl) containing 1X buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs mix, 1 μM primers, 3 μl cDNA as template, and 2.5 U Taq DNA polymerase. The PCR amplification protocol was as follows: 35 cycles of denaturation at 94°C for 1 min, primer annealing at 59°C for 1 min, and primer extension at 72°C for 7 min. The PCR product of the expected size (192 bp) was agarose gel-purified according to the MinElute purification kit (Qiagen) and its identity was confirmed by DNA sequence analysis.

The DNA fragment corresponding to SPINK3 was cloned into pGEX-4T-3 expression vector (GE) downstream the GST coding sequence and a thrombin cleavage site to facilitate the purification of the recombinant protein. The cDNA encoding mature SPINK3 and the pGEX-4T-3 expression vector were digested with XhoI and BamHI (Fermentas) following the manufacturer’s instructions and ligated with T4 DNA Ligase (Promega) according to standard protocols. The generated construct, pGEX-4T-3-spink3 which encoded GST-SPINK3, was then amplified in *E. coli* DH5α competent cells and the positive clones were confirmed by DNA sequencing.

For overexpression of GST-SPINK3, *E. coli* Rosetta cells (Novagen, Merck, Darmstadt, Germany) were transformed with the pGEX-4T-3-spink3 recombinant plasmid. *E. coli* cells containing pGEX-4T-3-spink3 were grown overnight at 37°C in Luria Bertani (LB) medium (0.5% yeast extract, 1% tryptone, and 0.5% NaCl) supplemented with 100 μg/ml ampicillin and 20 μg/ml chloramphenicol. This culture was used to inoculate (1/100) fresh LB medium (50 ml) containing ampicillin/chloramphenicol and grown at 37°C under continuous shaking until the optical density at 600 nm (OD₆₀₀) of the culture reached 0.4, then shifted to 18°C and incubated until the OD₆₀₀ was ~0.6. Synthesis of the recombinant protein was induced in the cells harboring pGEX-4T-3-spink3 by the addition of 0.1 mM IPTG for 16 h at 18°C.

Bacteria were harvested by centrifugation (1700 g for 8 min), suspended in PBS (ICN tablet 2810305; 10 mM phosphate buffer (pH 7.4), 137 mM NaCl, and 2.7 mM KCl), containing 1 mg/ml lysozyme, 0.2% (v/v) Triton X-100, and 0.5 mM PMSF and lysed by sonication (6×30 s, 40 W, Cole Parmer 4710 (London, UK)). The soluble (S) and insoluble (I) cell lystate fractions were obtained by centrifugation at 12 000 g for 25 min at 4°C and then analyzed by SDS–PAGE (15% w/v polyacrylamide gel; Laemmli et al. 1970).

Glutathione Sepharose 4B affinity chromatography (G2879; Molecular Probes) was employed for batch purification of GST-SPINK3 from the soluble fraction of induced cells. The column was equilibrated and washed with PBS and eluted with 8 mM reduced glutathione in 50 mM Tris–HCl (pH 8).

Cleavage of GST from the fusion protein was carried out by two different protocols: a) in-slushy digestion: cleavage was performed during purification by incubating the slurry containing GST-SPINK3 bound to the matrix with thrombin (T6634; Sigma; 1 U/50 μg recombinant protein), in buffer T (0.1 M NaCl, 2.5 mM CaCl₂, and 50 mM Tris–HCl (pH 8)) at 18°C for 16 h with gentle agitation. Then, SPINK3 was collected by washing with PBS and released GST was eluted with reduced glutathione. b) After-elution digestion: GST-SPINK3 bound to the matrix was eluted with reduced glutathione and then incubated with thrombin under the conditions previously described.

Protein digests from either protocol (a or b) were fractionated on a 15% polyacrylamide gel, negatively stained with zinc–imidazole and eluted according to Hardy & Castellanos-Serra (2004). Briefly, the gel was soaked for 30 s in double-distilled water, incubated for 15 min in 0.2 M imidazole, 0.1% (v/v) SDS, rinsed with water, and developed for 1 min in 0.2 M zinc sulfate. Proteins, visualized as clear bands on a dark background, were excised from the gel, washed by incubation (2×10 min) in PBS containing 100 mM EDTA (pH 7.4) followed by PBS (2×5 min). Elution was performed by crushing the gel to microparticles suspended in 200 μl PBS, vortexing the suspension for a few seconds and incubating for 20 min to allow passive diffusion of intact biomolecules and proteins were collected by centrifugation.

Efficient digestion of GST-SPINK3 by using protocol (a) was not successful, therefore, as a source of recombinant protein for the following experiments, GST-SPINK3 was purified from the column, SPINK3 without GST was obtained using protocol (b) followed by gel purification, and GST (control) was obtained by protocol (a) followed by gel purification.

These proteins were concentrated by ultrafiltration in YM-3000 membranes (Amicon, Millipore, MA, USA) and analyzed by SDS–PAGE. The identity of SPINK3 was confirmed by western blotting.

**Identification of recombinant proteins by western blotting**

Proteins were fractionated on 15% (v/v) polyacrylamide gels and transferred onto PVDF membranes for 2 h, 75 mA in a TE 70 PWR (GE). Membranes were incubated overnight in blocking solution (3% fish gelatin Sigma G7765 in buffer TBS–T (25 mM Tris, 0.192 mM glycerine, and 0.01% Tween 20)) at 4°C. Incubation with primary antibody was performed for 1 h at 37°C with anti-SPINK1 (1:4500, HPA02749; Sigma) in blocking solution. After washing with TBS–T, membranes were incubated for 40 min with anti-rabbit IgG-biotin conjugated (1:1000, B6648; Sigma–Aldrich) and streptavidin–peroxidase (1:1000, EB386; Sigma–Aldrich) in blocking solution. Development of the membranes was performed by incubation with one volume of solution A (0.1 M Tris (pH 8.5), 2.5 mM luminol, and 0.4 mM coumaric acid) plus one volume of solution B (0.1 M Tris–HCl (pH 8.5), and 0.018% H₂O₂) for 1 min, washed, and analyzed in an LAS-4000 (Fujifilm, Tokyo, Japan).

The expressed SPINK3 sequence has a 67% identity (82% similarity) with the immunogenic sequence of the antibody.

**Determination of trypsin inhibitory activity**

Antitrypsin activity was assessed fluorometrically using two synthetic trypsin-specific substrates: CGGR-MEC and BVPR-MEC. Reaction mixtures containing 0.2 mM substrate,
Immunodetection of GST-SPINK3 in sperm

Washed epididymal sperm were diluted to 5×10^6 cells/ml and incubated with 2.8 μM GST-SPINK3 for 1 h at 37 °C. Then, sperm were washed with PBS by centrifugation (850 g, 15 min), fixed with 4% paraformaldehyde for 30 min. Excess of fixative was removed by washing twice. Immunoreaction was conducted according to reported protocols (Irwin et al. 1983). Briefly, sperm were smeared and dehydrated with absolute ethanol for 20 min, washed with PBS and blocked with 3% BSA for 30 min. Slides were incubated with a 1:50 dilution of anti-SPINK1 (HPA027498; Sigma) for 2 h at 37 °C, washed and incubated with a 1:200 dilution of anti-rabbit IgG (F0382; Sigma) for 2 h at 37 °C. Sperm were washed with PBS by centrifugation (850 g, 5 min), spermatozoa were suspended to a concentration of 20×10^6 cells/ml in HM or HMB medium in the presence or absence of 2.8 μM GST-SPINK3. After a 60-min incubation, an aliquot of each treatment was placed onto a slide and observed under fluorescence microscope (excitation 480 nm; emission 525 nm) at 1000× magnification; spermatozoa showing fluorescence were captured with a Nikon DS-Fi1 camera connected to a computer with analytic software (Nis-Elements F 3.0, Nikon) attached to an inverted microscope Nikon Eclipse E200. Signal intensity was quantified at different sections of single sperm by image analysis using Imagej 1.43 free software (National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/).

Measurement of intracellular Ca^{2+} in sperm

Cauda epididymides were immersed in 1 ml HM medium (without CaCl2). After 10 min, sperm aliquots were transferred to 1.5 ml polypropylene tubes and incubated in the presence of 10 μM fluo-3 AM (Molecular Probes F1242; Invitrogen) and 0.05% Silwet L-77 (Arysta Life Science) as a surfactant in a 1% CO2 at 37 °C for 30 min. After washing by centrifugation (3× 0.7 g, 5 min), spermatozoa were suspended to a concentration of 20×10^6 cells/ml in HM or HMB medium in the presence or absence of 2.8 μM GST-SPINK3. After a 60-min incubation, an aliquot of each treatment was placed onto a slide and observed under fluorescence microscope (excitation 480 nm; emission 525 nm) at 1000× magnification; spermatozoa showing fluorescence were captured with a Nikon DS-Fi1 camera connected to a computer with analytic software (Nis-Elements F 3.0, Nikon) attached to an inverted microscope Nikon Eclipse E200. Signal intensity was quantified at different sections of single sperm by image analysis using Imagej 1.43 free software (National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/). Ca^{2+} fluorescence intensity (excitation 460 nm; emission 525 nm) in the whole sperm suspension was measured immediately using an Ascent Microplate Fluorometer (Thermo Fisher Scientific, USA) at 37 °C for 45 min.

Sperm preparation and capacitation

Cauda epididymides were immersed in 2 ml HM medium (modified Krebs Ringer bicarbonate medium; Visconti et al. 1995; 25 mM Hepes, 109 mM NaCl, 14.77 mM KCl, 1.19 mM MgSO4, 1 mg/ml glucose, 21.18 mM sodium lactate, 1.19 mM KH2PO4, plus 1 mM sodium pyruvate (pH 7.4)) contained by culture dishes on a warm plate at 37 °C. After 10 min tissue debris were removed and aliquots (100 μl) of the sperm suspension were transferred to 1.5 ml polypropylene tubes carefully underlining a layer of 500 μl fresh media.

Samples were incubated in a 5% CO2, 37 °C incubator (precision) for 30 min to perform a swim-up technique for sperm separation (Ren et al. 2004). The supernatants (300 μl) containing the most motile sperm were removed from the top of each tube, collected in a separate tube and concentration was evaluated in a Neubauer chamber. Sperm were pelleted by centrifugation (0.7 g for 10 min) and suspended in HM or HMB (25 mM Hepes, 109 mM NaCl, 14.77 mM KCl, 1.19 mM MgSO4, 1 mg/ml glucose, 21.18 mM sodium lactate, 1.19 mM KH2PO4 (pH 7.4) plus 1 mM sodium pyruvate, 1.7 mM CaCl2, 0.8 mM methyl-β-cyclodextrin, and 25 mM NaHCO3, as indicated in assay conditions. Unless indicated, methyl-β-cyclodextrin was used as a replacement of 3% BSA for capacitation, since the latter may affect the fluorescence in the DAF-FM DA assay. The use of methyl-β-cyclodextrine has been already reported (Choi & Toyoda 1998). Sperm concentration was adjusted to the concentration indicated for each assay.

Sperm incubations were carried out with HM or HMB medium with or without different reagents (Assays Conditions) at 37 °C and 5% CO2 for 1 h.

NO production in sperm

Determination of nitrite concentration by Griess method

After incubation of spermatozoa under noncapacitating or capacitating conditions, aliquots of the sperm suspensions containing 30×10^6 sperm/ml were centrifuged (9700 g for 15 min) and NO production by sperm under different conditions (Assay Conditions) was estimated by assessing the levels of nitrate (NO3^-) and nitrite (NO2^-) in the culture media (supernatant), using a colorimetric kit (G2930; Promega) based on Griess reaction. Nitrite concentration was normalized to 1×10^6 sperm.

NO detected with the fluorescent probe DAF-FM DA

To measure the NO production in sperm cells, the pH insensitive NO indicator, DAF-FM DA cell permeate, was used. Inside the cell, DAF-FM DA is cleaved by esterases to generate intracellular DAF-FM–DA, which is then oxidized by NO to a triazole product accompanied by increased fluorescence. The probe, 10 μM DAF-FM DA, was added either at the beginning, at 30 min, or at 60 min of incubation of sperm in a 96 well microplate under capacitating conditions in HMB medium (100 μl containing 20×10^6 sperm/ml) to evaluate the best time for its addition. NO fluorescence intensity (excitation 480 nm; emission 525 nm) was measured using an Ascent Microplate Fluorometer (Thermo Fisher Scientific) at 37 °C for 230 h. The NO production was the highest during the first
30 min of capacitation (Fig. 4A, lower panel), so the addition of the probe at the beginning of capacitation was the condition adopted for the experiment of the NO production under a fluorescent microscope.

To detect NO production under fluorescence microscope, 10 μM DAF-FM DA were added to motile sperm cells under different conditions (Assays Conditions). Following the incubation period, samples were washed with HM medium, incubated for 5 min and free DAF-FM DA washed by centrifugation (3 x 10 min). After washing, spermatozoa were suspended to a concentration of 5 x 10⁶ sperm/ml in HM medium.

For detection of NO, slides were prepared by placing an aliquot of the sperm suspension on a glass slide and adding a cover slip mounted with glycerol: PBS (9:1). The presence of NO was detected under a fluorescent microscope 490/535 nm (Nikon Eclipse E200) at 1000× magnification by two independent observers. In each treatment, spermatozoa showing fluorescence were quantified as percentage of total sperm evaluated.

**Evaluation of spontaneous and P₄-induced AR**

For this assay CF-1 mice were used due to their higher percentages of AR. Incubation was conducted under non-capacitating or capacitating conditions under different treatments (Assays Conditions) for 1 h at 37 °C in an atmosphere of 5% CO₂. Spontaneous AR was evaluated after this incubation period. For progesterone-induced AR, sperm incubated under each treatment were incubated with 10 μM P₄ for 15 min after capacitation. In both cases, AR was stopped by centrifugation (850 g for 10 min) and pelleted cells were fixed with 4% paraformaldehyde solution in PBS for 2 h. The acrosomal status was evaluated according to the Coomassie Brilliant Blue (CBB) G-250 staining technique (Bendahmane et al. 2002). Briefly, sperm were placed and smeared onto glass slides and stained with a 0.22% CBB G-250 solution prepared in 50% methanol and 10% acetic acid. The slides were washed with distilled water, dried, smeared in glycerol: PBS (9:1), and observed under a light microscope at 1000× magnification by two independent observers. A blue stain over the sperm head dorsal and/or ventral edge was visualized in spermatozoa with intact acrosome, whereas no stain was observed in spermatozoa with reacted acrosome. Spermatozoa with acrosomal loss were quantified as a percentage over 200 sperm cells per replicate.

**Determination of sperm viability**

Sperm viability was evaluated by trypan blue staining technique. After incubation, an aliquot of each sperm strain (see Assays Conditions) was placed onto a glass slide with an equal volume of 0.2% trypan blue and immediately observed under a light microscope at 400× magnification by two independent observers and the percentage of light blue colored spermatozoa (dead) was recorded out of 100 cells.

**Determination of protein tyrosine phosphorylation**

Sperm proteins corresponding to 0.75 x 10⁶ sperm cells incubated under different conditions (Assays Conditions) were disrupted by boiling for 5 min with sample buffer (12 mM Tris–HCl (pH 6.2), 5% glycerol, 0.4% SDS, 0.02% bromophenol blue, and 100 mM diethiothreitol) and separated by electrophoresis on 12% SDS–PAGE. Then, the proteins were electrotransferred onto PVDF membranes in towbin buffer (25 mM Tris, 192 mM glycine, and 20% methanol) using a TE 70 PWRTransfer equipment (Amersham) at 80 mA for 2 h. Nonspecific binding sites on the membrane were blocked with 3% skimmed milk in Tris–buffered-saline (10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 0.1% Igepal CA630–Sigma I3021). Afterward, the membrane was incubated overnight with 1:5000 anti-phosphotyrosine (MP, PY20 691371). The membrane was washed, incubated with 1:5000 anti-mouse IgG labeled with alkaline phosphatase (A3562 Sigma), and developed with 0.33 mg/ml nitro-blue tetrazolium and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphatase buffer (0.1 M Tris (pH 9.5); 0.1 M NaCl, and 5 mM MgCl₂).

**Evaluation of progressive sperm motility**

Cauda epididymis were immersed in 1 ml HM medium contained in culture dishes on a warm plate at 37 °C as described above. After 10 min the sperm suspension was transferred to HMB medium (containing 3 mg/ml BSA instead of methyl-β-cyclodextrine) and distributed for the different treatments (Assays Conditions) at 37 °C and 5% CO₂. Samples presenting ≤35% PM and/or ≤70% TM at time zero were discarded. Motility was evaluated at 0 and 30 min after initiation of capacitation. An aliquot of each treatment was placed onto slides and observed under a light microscope at 400× magnification on a warm surface at 37 °C by two independent observers. Spermatozoa showing PM in different microscopic fields were quantified as percentage of total sperm evaluated (n=200 per replicate).

**Assays conditions**

All the assays (immunocytochemistry, NO detection, Ca²⁺ uptake, tyrosine phosphorylation, and motility) were performed using spermatozoa from BalbC mice, except for AR where CF-1 mice were employed. Viability was evaluated in sperm from both strains under all the treatments.

Motile mice sperm were incubated under noncapacitating (HM) or capacitating (HMB) media with or without either 2.8 μM GST-SPINK3, 2.8 μM GST, 100 μM SNP, 100 μM GSNO, and/or two Ca²⁺ chelators (10 mM EDTA (pH 7.5) and 25 μM BAPTA-AM) as indicated in each assay. Unless specified, the reagents were added at the beginning of capacitation. At the concentrations assayed, no detrimental effect over sperm viability (~82%) was observed with none of the reagents. All treatments were incubated at 37 °C in an atmosphere of 5% CO₂/95% air for the times indicated.

**Statistical analysis**

All data in the text and figures were expressed as means ± S.E.M. Statistical analysis was carried out using the Student’s t-test for paired data (two-tailed), after having normalized the data by
arcsine transformation. Statistical significant differences were determined at P<0.05, unless indicated.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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