Murine binder of sperm protein homolog 1: a new player in HDL-induced capacitation

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Abstract

Binder of sperm (BSP) proteins are ubiquitous among mammals and are exclusively expressed in male genital tract. The main function associated with BSP proteins is their ability to promote sperm capacitation. In mice, two proteins (BSP protein homolog 1 (BSPH1) and BSPH2) have been studied. Using recombinant strategies, BSPH1 was found to bind to epididymal sperm membranes and promote sperm capacitation \textit{in vitro}. The goal of this study was to evaluate the role of native murine BSPH1 protein in sperm capacitation induced by BSA and HDLs. The effect of antibodies, antigen-binding fragments (Fabs), and F(ab\textsubscript{2})\textsubscript{2} specific for murine BSPH1 on BSA- and HDL-induced capacitation was tested. Results indicate that BSPH1 has no direct role in BSA-induced capacitation. However, antibodies, Fabs, and F(ab\textsubscript{2})\textsubscript{2} could block capacitation induced by HDLs and could inhibit the HDL-induced increase in tyrosine phosphorylation, suggesting a specific interaction between HDLs and BSPH1. Results indicate that murine BSPH1 proteins in mice could be a new important piece of the puzzle in sperm capacitation induced by HDLs. As murine BSPH1 is orthologous to human BSPH1, this study could also lead to new insights into the functions and the importance of the human protein in male fertility.

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Introduction

In mammals, for fertilization to occur, sperm have to go through several maturation steps. One of the key maturation steps taking place in the female genital tract is called capacitation. It is a prerequisite for sperm to undergo acrosome reaction (AR), recognize an oocyte, interact with it, and then fertilize it. Although capacitation was first described over 60 years ago, this process is still not fully understood. Capacitation is usually associated with an increase in intracellular pH, an increase in calcium permeability as well as changes in the lipid composition of the sperm plasma membrane, including a decrease in the cholesterol/phospholipid ratio (Go & Wolf 1983, Langlais & Roberts 1985, Suarez 1996, de Lamirande \textit{et al}. 1997, Visconti \& Kopf 1998). It is also accompanied by the time-dependent activation of many different signaling pathways including the protein kinase A (PKA) pathway, the protein kinase C (PKC) pathway, the ERK pathway, and the phosphatidyl-inositol-3-kinase (PI3K)/Akt pathway leading to an increase in the level of protein tyrosine phosphorylation (Breitbart \textit{et al}. 1992, Visconti \textit{et al}. 1995a, de Lamirande \textit{et al}. 1997, Fisher \textit{et al}. 1998, Luconi \textit{et al}. 1998, de Lamirande & Gagnon 2002, Nauc \textit{et al}. 2004).

Capacitation can be induced \textit{in vitro} by incubating sperm in defined media. To induce capacitation, these media require some essential components including calcium, bicarbonate as well as sterol acceptors. In mice, the most commonly used sterol acceptors are BSA and HDLs (Visconti \textit{et al}. 1999a, Xia & Ren 2009).

Some proteins from the male genital tract have been shown to be important for sperm capacitation by preventing a premature capacitation and/or by promoting sperm capacitation. Proteins from the binder of sperm (BSP) superfamily (called bovine seminal plasma proteins prior to the new nomenclature (Manjunath \textit{et al}. 2009)) have been shown to play a role in both prevention and promotion of capacitation. They were first identified in the bovine seminal plasma, while BSP1, BSP3, and BSP5 (previously called PDC-109 or BSP-A1/A2, BSP-A3, and BSP-30K respectively) represent ~60% of the total seminal plasma proteins (Manjunath 1984, Manjunath \textit{et al}. 1987, Seidah \textit{et al}. 1987). These three proteins are secreted by the seminal vesicles and can bind to sperm via an interaction with choline phospholipids (Desnoyers & Manjunath 1992). They have been
identified as important players in the glycosaminoglycan (GAG)- and HDL-induced capacitation in bovine (Thérien et al. 1995, 1997, 2005).

Other members of the BSP superfamily have been identified in species such as boar, ram, goat, stallion, and bison and more recently in human and mice (Calvete et al. 1995, 1997, Menard et al. 2003, Vilemure et al. 2003, Boisvert et al. 2004, Bergeron et al. 2005, Fan et al. 2006). The BSP proteins are all structurally similar as they are composed of a variable N-terminal domain followed by two fibronectin type II (Fn2) domains arranged in tandem (Manjunath et al. 2009). However, the two BSP homologs in mouse (BSP protein homolog 1 (BSPH1) and BSPH2) and the BSP homolog in human (BSPH1) are slightly different from their other counterparts as they are expressed exclusively in the epididymis and represent only a small quantity of the total seminal plasma proteins (Lefebvre et al. 2007). Despite those differences, recent studies using recombinant proteins have demonstrated that all three proteins share many biochemical characteristics with the BSP proteins expressed by the seminal vesicles including binding to gelatin, GAGs, and sperm membrane (Plante et al. 2012, 2014a,b). These studies also demonstrated that incubation of sperm with an excess of murine or human rec-BSPH1 but not rec-BSPH2 was sufficient to induce sperm capacitation (Plante et al. 2012, 2014a,b).

Although our previous studies on murine and human BSP seemed to indicate a possible role of considerable importance in capacitation, studies were performed using an excess of proteins and as such could not reflect entirely in vivo conditions. However, some results demonstrated that incubation of murine sperm with antibodies specific for BSPH1 to block the native protein caused a dose-dependent inhibition of BSA-induced capacitation (Plante et al. 2012). In the current study, we attempted to get further insight into the molecular mechanism involving murine BSPH1 in capacitation induced by BSA and HDLs.

Preparation of fragment antigen-binding (Fab) and F(ab')2

Affinity-purified antibodies raised against (His)6-tagged recombinant BSPH1 (anti-BSPH1) were prepared as described previously (Plante et al. 2012). Following purification by affinity chromatography on a Protein A sepharose column and a rec-BSPH1-Affi-gel 15 column, anti-BSPH1 antibodies were concentrated (~1 mg/ml). Fabs were prepared using these antibodies (Kontou et al. 1996, Lane et al. 1999). Briefly, papain from papaya latex (Sigma–Aldrich) was activated for 15 min at 37 °C in digestion buffer (PBS containing 10 mM EDTA and 2 mM l-cysteine). The enzyme was added to the antibodies in a dilution of 1:15 (papain:antibody). The reaction mixture was incubated at 37 °C for 7 h. To stop the reaction, the digestion mixture was incubated at 4 °C overnight with 25 mM iodoacetamide. The digestion product was then dialyzed against PBS (pH 8.0) and purified on a Protein A sepharose column. Unbound material containing the Fab was washed from the column with ten volumes of PBS and concentrated (~1 mg/ml).

F(ab')2 were prepared using the Pierce F(ab')2 preparation kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. Control Fab (Fab-IgG) and F(ab')2 (F2-IgG) were prepared similarly using IgGs purified from pre-immune normal rabbit serum (IgG).

Preparation of HDLs

HDLs were isolated from human serum by density gradient ultracentrifugation as described previously (Thérien et al. 1997). They were dialyzed against buffer containing 0.9% NaCl, 1 mM EDTA, and 25 mM HEPES, pH 7.4. HDLs were subsequently kept under nitrogen at 4 °C. The purity of the lipoproteins was verified by agarose gel electrophoresis using the Paragon lipoprotein (Lipo) electrophoresis kit (Beckman Instruments, Fullerton, CA, USA) and following the protocol described by the manufacturer. The concentration of lipoproteins was measured according to the modified Lowry procedure (Markwell et al. 1978).

Preparation of sperm

Male mice (10–18 weeks old) were killed by cervical dislocation. Cauda epididymides were removed and placed in warm PBS. Epididymides were then cleaned to remove fat and blood. They were then cut four to six times with scissors and placed in 1 ml of pre-warmed modified Krebs-Ringer medium (Whitten’s HEPES (WH); 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 5.5 mM D-glucose, 1 mM sodium pyruvate, and 4.8 mM L(+)-lactic acid hemicalcium salt in 20 mM HEPES) buffered medium, pH 7.4 (osmolality 315 mOsm/kg), and placed at 37 °C.

Materials and methods

Animals

Pathogen-free CD-1 outbred mice were purchased from Charles River Laboratories (Kingston, NY, USA) and were kept in the animal care facility of the research center. Animals were given food and filtered tap water and were allowed to feed ad libitum, and maintained under a 14 h light:10 h darkness cycle. Studies were approved by the Maisonneuve-Rosemont Hospital Ethics Committee (Protocol #2014-10) and mice were treated according to the guidelines of the Canadian Council of Animal Care.

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for 10 min. Following incubation, epididymal debris was removed and sperm were resuspended by gentle swirling (Moore et al. 1994). For each experiment, sperm from three to four mice were pooled and used for the different assays.

### Inhibition of capacitation assay
For this set of experiments, sperm were collected as described earlier, washed once with 5 ml of WH medium (10 min at 200 g), and resuspended in 500 μl of WH medium. For BSA-induced capacitation studies, 2 × 10⁶ washed sperm were then incubated for 1 h at 37 °C in 1 ml WH medium containing 5 mg/ml of BSA alone or in 1 ml WH medium containing BSA and different concentrations of Fab, F(ab’)₂, or anti-BSPH1. As negative controls, sperm were also incubated under non-capacitating conditions in WH medium depleted of bicarbonate and BSA, with BSA and 5.6 μg/ml of Fab-IgG or with BSA and 5.6 μg/ml of F₂-IgG. For HDL-induced capacitation, different concentrations of HDLs were tested to determine optimal conditions. Then, same conditions as the ones used for BSA-induced capacitation were tested, but instead of BSA, 25 μg HDL were used.

Following the incubation, 200 μl of the sperm suspension were incubated for an additional 30 min at 37 °C with or without 5 μM of calcium ionophore A23187 (Sigma–Aldrich). Sperm were then fixed with 200 μl of 8% paraformaldehyde for 30 min at room temperature, centrifuged for 2 min at 8000 g, and washed two times with 0.1 M ammonium acetate (pH 9.0). They were finally resuspended in a final volume of 100 μl of the same solution and 20 μl of this suspension were finally boiled for 10 min and 1/10th of each sample buffer were added to each fraction. Fractions of 200 μl were collected from the top to the bottom of the gradient. The pellet at the bottom of the tube was resuspended in 200 μl of 10 mM Tris–HCl, pH 7.4, and kept for the analysis. Fifty microliters of 5 × sample buffer were added to each fraction. Fractions were finally boiled for 10 min and 1/10th of each fraction was analyzed on 15% polyacrylamide gels.

### Isolation of light buoyant-density detergent-resistant membrane fractions
Isolation of detergent-resistant membrane (DRM) was performed as described previously (Sleigh et al. 2005). A total of 60 million sperm isolated from cauda epididymides were washed twice with 1 ml of WH media and resuspended in 400 μl of TEN buffer (25 mM Tris–HCl (pH 7.3), 150 mM NaCl, and 5 mM EDTA) containing 0.5% Triton X-100. Complete Mini, EDTA-free protease inhibitor tablet (1 tablet/10 ml; Roche) and 1 mM of phenylmethylsulphonyl fluoride (PMSF). The pellet was Dounce homogenized (30 strokes). It was then sonicated for 1 s at 50% five times and kept on ice for 1 min between each burst. Cell lysate was then rotated at 4 °C for 45 min. To separate the DRM according to their density, sperm lysate was adjusted to 40% sucrose with the addition of 400 μl 80% sucrose in TEN buffer and placed at the bottom of a 2-ml centrifuge tube. This solution was gently overlaid with 800 μl of 30% sucrose in TEN buffer and 400 μl of 5% sucrose in TEN. The sucrose gradient was then centrifuged at 100 000 g for 18 h. Fractions of 200 μl were collected from the top to the bottom of the gradient. The pellet at the bottom of the tube was resuspended in 200 μl of 10 mM Tris–HCl, pH 7.4, and kept for the analysis. Fifty microliters of 5 × sample buffer were added to each fraction. Fractions were finally boiled for 10 min and 1/10th of each fraction was analyzed on 15% polyacrylamide gels.

### Tyrosine phosphorylation
Tyrosine phosphorylation experiments were performed as described previously (Visconti et al. 1995b). 1 × 10⁶ sperm collected as described earlier were incubated for 90 min at 37 °C in 500 μl WH medium containing 12.5 μg/ml of HDL alone or with 12.5 μg/ml of HDL in the presence of different concentrations of Fab or 11.4 μg/ml of Fab-IgG. Following incubation, sperm were pelleted by centrifugation at 16 000 g for 1 min. Sperm pellet was washed with 1 ml of PBS containing 1 mM orthovanadate and centrifuged for another 1 min. Pellet was resuspended in 25 μl of Laemmli sample buffer without mercaptoethanol and boiled for 5 min (Laemmli 1970). After one last centrifugation at 16 000 g for 1 min, the supernatant was transferred to a new tube, boiled in the presence of 5% β-mercaptoethanol for 5 min, and then subjected to SDS–PAGE (10% gels).
Statistical analysis

Data are expressed as the mean ± S.E.M. Differences were analyzed by one-way ANOVA followed by the Bonferroni post hoc test using GraphPad Prism 5 (version 5.03; GraphPad inc., San Diego, CA, USA).

Results

BSA-induced capacitation

Previous experiments using anti-BSPH1 antibodies demonstrated that they could cause dose-dependent inhibition of BSA-induced capacitation (Plante et al. 2012). To investigate this inhibition further, similar experiments were performed using Fab and F(ab′)2. As Fab and F(ab′)2 accounts for approximately two-thirds of an IgG, concentrations used in these experiments were two-thirds of the concentration of anti-BSPH1 used previously (Plante et al. 2012). In general, only capacitated sperm can undergo the AR. Capacitation was therefore assessed by the ability of sperm to undergo AR induced by calcium ionophore A23187. As shown in Fig. 1, under non-capacitating conditions, the basal level of AR in the absence of A23187 was 18 ± 2%. This level was unaffected by the addition of A23187 under non-capacitating conditions and was constant under all conditions tested in the absence of A23187 (gray bars). Following the addition of 5 mg/ml BSA, in the presence of A23187, the level of AR increased to 73 ± 1%. As shown previously (Plante et al. 2012), in the presence of 8.4 μg/ml anti-BSPH1, the percentage of AR sperm was comparable with the basal level. Addition of Fab did not affect the level of AR at any of the concentrations tested (Fig. 1A). However, incubation with F(ab′)2 caused a significant decrease in the level of AR at concentrations as low as 0.3 μg/ml (Fig. 1B). Control using Fab-IgG and F2-IgG produced using IgG from pre-immune normal rabbit serum did not alter the levels of AR.

To verify that the effect observed on capacitation and AR was not due to the death of sperm, spermiogram viability was assessed based on sperm motility. In the beginning of the experiments, 90–95% sperm were motile. Following incubation, before the addition of A23187, motility decreased slightly (5–10%), but this decrease was the same for all conditions tested. This was also observed for HDL-induced capacitation. Results are based on percentages of all cells (motile and immotile).

HDL-induced capacitation

Previously published articles, in which HDLs were used to induce murine sperm capacitation, assessed the capacitated state of sperm by tyrosine phosphorylation and used high concentrations of sperm during incubation (Visconti et al. 1999b). Therefore, before testing the effect of anti-BSPH1 antibodies on HDL-induced capacitation, several concentrations of HDLs were tested to determine the appropriate conditions necessary to induce capacitation in murine sperm using 2 × 10^6 sperm/ml and assess capacitated state by the ability of sperm to undergo AR (Fig. 2). Following incubation with A23187, under non-capacitating conditions, the level of AR was of 25 ± 1%. Addition of 5 μg/ml of HDL did not cause any significant increase in the AR level but the addition of 25 μg/ml of HDL increased it to 54 ± 1%. Similar percentages of AR sperm were obtained with higher concentrations of HDLs. Therefore, 25 μg/ml of HDL were used for the inhibition studies.

In the presence of anti-BSPH1 antibodies, results obtained for HDL-induced capacitation were similar to those obtained for BSA-induced capacitation (Fig. 3A). The addition of 0.5 μg/ml anti-BSPH1 decreased the level of AR significantly and the addition of 8.4 μg/ml anti-BSPH1 dropped the percentage of AR back to the
basal level. However, different results were obtained when sperm were incubated with HDLs in the presence of Fab (Fig. 3B) as the addition of the antibody fragments caused a dose-dependent decrease in the level of AR. Decrease in the percentages of AR sperm was significant following the addition of 1.4 \( \mu g/ml \) of antibody fragments. Identical results were obtained when sperm were incubated with F(ab\(^{0}\))\(_2\) (Fig. 3C). Addition of IgG, Fab-IgG, or F2-IgG had no effect on sperm AR.

**Tyrosine phosphorylation**

HDL-induced capacitation has been shown to increase the phosphorylation of tyrosine residues in signaling proteins. Therefore, the effect of Fab on the phosphorylation induced during incubation of sperm with HDLs was tested. As shown in Fig. 4A, at the beginning of the incubation or 90 min following incubation without HDL, levels of phosphorylation were very low addition of 12.5 \( \mu g/ml \) of HDL increased the phosphorylation of sperm proteins, which was inhibited by the addition of 11.4 \( \mu g/ml \) of Fab. Lower concentrations of antibody fragments had no effect. Furthermore, 11.4 \( \mu g/ml \) of Fab-IgG caused a slight decrease in the phosphorylation, but not as significant as the one observed for specific BSPH1 Fab. A decrease in phosphorylation was also observed when sperm were incubated with 25 \( \mu g/ml \) of HDL, but the effect was not as pronounced as when experiments were performed with 12.5 \( \mu g/ml \) of HDL (not shown). Equal loading of proteins on acrylamide gel was verified by staining the membrane with Amido Black (Fig. 4B). The amount of protein loaded for sperm incubated in the absence of HDLs was lower than that for sperm incubated with HDLs. However, the difference in proteins loaded is not sufficient to explain the absence of tyrosine phosphorylation in those two samples. No difference in loading was observed for sperm incubated with HDLs in the presence of different concentrations of Fab and antibodies.

Figure 2 Effect of HDL on capacitation in murine sperm. Capacitation was assessed by the ability of sperm to undergo the AR induced by ionophore A23187. Epididymal sperm were incubated for 60 min either alone or with different concentrations of HDLs or with 5 mg/ml of BSA as a positive control. They were then incubated with (black bars) or without (gray bars) calcium ionophore A23187 for 30 min. Sperm were smeared on slides and analyzed by Coomassie Blue staining. A minimum of 400 sperm/condition were evaluated. Data are expressed as means ± S.E.M. of four independent experiments. *Significant difference compared with BSA alone (control, \( P<0.001 \)).

Figure 3 Effect of anti-BSPH1, Fab, and F(ab\(^{0}\))\(_2\) on murine sperm capacitation induced by HDLs. Capacitation was assessed by the ability of sperm to undergo the AR induced by ionophore A23187. Epididymal sperm were incubated for 60 min alone or with 25 \( \mu g/ml \) of HDL in the presence of different concentrations of anti-BSPH1, Fab, or F(ab\(^{0}\))\(_2\). As controls, sperm were incubated in the presence of 5.6 \( \mu g/ml \) of Fab-IgG, 5.6 \( \mu g/ml \) of F2-IgG, or 8.4 \( \mu g/ml \) of IgG. Sperm were then incubated for 30 min with (black bars) or without (gray bars) calcium ionophore A23187. Sperm were smeared on slides and analyzed by Coomassie Blue staining. (A) Effect of anti-BSPH1 antibodies. (B) Effect of Fab on sperm capacitation. (C) Effect of F(ab\(^{0}\))\(_2\) on sperm capacitation. A minimum of 400 sperm/conditions were evaluated. Data are expressed as means ± S.E.M. of four independent experiments. *Significant difference compared with HDL alone (control, \( P<0.001 \)).
on sucrose density gradient. Isolation of DRM has not been found in the lipid rafts. It was observed solely in the pellet indicating that it is control, BSPH1 was not found in the light fractions containing cell debris. As opposed to this positive blot, CAV1 was observed in fractions 4–10 and in the fractions of the density gradient. Following western in lipid rafts. As such, CAV1 was found in the light membrane, a fraction of the CAV1 protein is found were analyzed by western blot (Fig. 5). In the sperm plasma membrane contains domains called lipid rafts that are highly enriched in cholesterol and sphingolipids. These domains are known to be insoluble in detergent at 4 °C and possess light buoyant density on sucrose density gradient. Isolation of DRM has often been used to identify raft (Thaler et al. 2006, Miranda et al. 2009). In order to determine whether murine BSPH1 is located in those domains, DRM fractions of epididymal sperm membranes were separated on sucrose density gradient and different fractions were analyzed by western blot (Fig. 5). In the sperm membrane, a fraction of the CAV1 protein is found in lipid rafts. As such, CAV1 was found in the light fractions of the density gradient. Following western blot, CAV1 was observed in fractions 4–10 and in the pellet containing cell debris. As opposed to this positive control, BSPH1 was not found in the light fractions and was observed solely in the pellet indicating that it is not found in the lipid rafts.

**Figure 4** Effect of Fab on tyrosine phosphorylation during HDL-induced capacitation. Epididymal sperm were incubated for 90 min alone or with 12.5 μg/ml of HDL in the presence of different concentrations of Fab or 11.4 μg/ml of Fab-IgG. (A) Proteins were separated by SDS–PAGE on 10% acrylamide gel, transferred onto a PVDF membrane, and probed with an anti-phosphotyrosine antibody. (B) Equal loading of proteins on acrylamide gel was verified by staining the membrane with Amido Black.

**Discussion**

**BSA-induced capacitation**

BSA is often used to induce capacitation in vitro. It acts as a sterol acceptor and removes cholesterol from sperm membranes. This causes a destabilization of the membrane and renders it more fluid, leading to tyrosine phosphorylation and calcium intake (Salicioni et al. 2007). Our previous studies had demonstrated that co-incubation of sperm with BSA and anti-BSPH1 antibodies could inhibit completely the capacitation induced by BSA (Plante et al. 2012). Three molecular explanations were raised to justify this inhibition. The first explanation was that the big size of the antibodies bound to native BSPH1 on sperm membrane blocked access of BSA to the membrane cholesterol (Fig. 6A). The second explanation was that antibodies were preventing a direct interaction between BSA and BSPH1 (Fig. 6B). The last explanation derived from the ability of antibodies to bind to two different antigens. Bovine BSP proteins are known to coat the sperm surface, prevent free movement of the phospholipids, and stabilize sperm membrane (Muller et al. 1998, Manjunath & Thérien 2002). Therefore, in a similar manner, binding of antibodies to the native BSPH1 bound to the sperm surface could have created a stable network between BSPH1 proteins, thereby stabilizing the sperm membrane and preventing BSA-induced capacitation (Fig. 6C). The third explanation at the time was favored, but in the current study, experiments were designed to confirm it.

F(ab′)2 and Fab are digested products of antibodies. F(ab′)2 are less bulky than antibodies as they do not possess the Fc fragment but they can still bind two antigens. Fab on the contrary are only composed of one antigen-binding domain. Repeating the inhibition experiments using these tools had three possible outcomes. The first hypothesis would be confirmed if only anti-BSPH1 antibodies could inhibit BSA-induced capacitation. The second hypothesis would be confirmed if

**Figure 5** Identification of murine BSPH1 in the non-raft fraction of the sperm plasma membrane. Epididymal sperm were lysed in TEN buffer containing 0.5% Triton X-100 and DRM lipid rafts were isolated by sucrose gradient. Following centrifugation, 200 μl fractions were collected from top to bottom and the pellet was resuspended in 200 μl of Tris-HCl. One-tenth of each fraction was separated on 15% acrylamide gel, transferred onto a PVDF membrane, and probed with anti-CAV1 or anti-BSPH1 antibodies. This experiment was repeated four times with similar results.
Fab, F(ab')₂, and antibodies could inhibit capacitation and the third hypothesis would be verified if antibodies and F(ab')₂, but not Fab could cause inhibition. Results obtained indicate that the third hypothesis is the most probable one to explain the inhibition of BSA-induced capacitation by anti-BSPH1 antibodies. They also indicate that BSPH1 is most probably not directly involved in capacitation induced by BSA. Furthermore, these results suggest that BSPH1 could be implicated in the stabilization of the sperm membrane to prevent premature capacitation, as is the case for bovine BSP proteins.

**HDL-induced capacitation**

HDL is a macromolecule composed of phospholipids, triglycerides, cholesterol, and apoproteins. Its major protein is apolipoprotein A-I (apoA-I). It is a major factor implicated in cholesterol transport and, as such, has also been shown to induce capacitation. Similar to BSA, HDLs can induce in vitro capacitation by creating cholesterol and phospholipid efflux from sperm membrane (Thérien et al. 1998). In many species, HDL is found in the follicular and oviductal fluid making it an ideal candidate to promote capacitation in vivo (Travis & Kopf 2002). Previous studies had reported that to induce capacitation-associated tyrosine phosphorylation in 1×10⁷ sperm, a concentration of 300 µg/ml of HDL were necessary (Visconti et al. 1999a). In the current study, under the conditions used (1×10⁶ sperm), a minimum of 12.5 µg/ml of HDL were necessary to induce tyrosine phosphorylation and 25 µg/ml of HDL were necessary to obtain an increase in the level of AR following incubation with A23187.

In bovine, it has been demonstrated that BSP1, BSP3, and BSP5 are all able to bind HDLs and more specifically to apoA-I (Manjunath et al. 1989). HDL also contains choline phospholipids, which could also be responsible for the BSP binding. Through this interaction, bovine BSP proteins can increase the stimulation of capacitation caused by HDLs (Thérien et al. 2001). To determine whether murine BSPH1 could be implicated in a similar mechanism, the effect of anti-BSPH1 on the HDL-induced capacitation in murine sperm was tested. Interestingly, as opposed to what was observed during BSA-induced capacitation, Fab alone was able to decrease capacitation to a level comparable with the inhibition caused by the full antibodies. This result suggested a more direct role of BSPH1 in the HDL-induced capacitation process. As BSP proteins are known to interact directly with HDLs and no direct interaction with other sperm protein has ever been reported for members of the BSP superfamily, results suggest that a direct interaction between HDLs and BSPH1 could be necessary for capacitation.

**Tyrosine phosphorylation**

To confirm the inhibitory effect of anti-BSPH1 on HDL-induced capacitation, the effect of Fab on tyrosine phosphorylation was tested. As was observed before, lower concentrations of sterol acceptors were necessary to detect an increase in tyrosine phosphorylation when compared with concentrations needed to induce AR (Visconti et al. 1995b). The addition of 11.4 µg/ml of Fab decreased the tyrosine phosphorylation back to what was observed without the addition of HDLs. Previous studies in human and bovine demonstrated that BSP proteins do not seem to be directly implicated in the phosphorylation cascade (Lane et al. 1999, 2000).
Plante et al. (2014b). In mice, it is believed that sterol acceptors such as HDL or BSA change the fluidity of the membrane by removing cholesterol and phospholipids. The changes in fluidity lead to an increase in the uptake of calcium and bicarbonate stimulating the production of cAMP. This increase then causes the activation of the PKA pathway and, subsequently, an increase in tyrosine phosphorylation (Visconti et al. 1999b). Based on this theory, it is possible that, as observed in bovine, BSPH1 could help the transfer of cholesterol and phospholipids to the HDLs. However, in the murine model, there is no evidence at the moment showing that BSP proteins would be loaded on HDLs to help remove phospholipids and cholesterol. Experiments performed using recombinant proteins rather suggest that the proteins remain on the surface of the sperm, change location on the surface of the head, and could be implicated in other steps of fertilization such as sperm–egg interaction.

**Lipid rafts**

Sperm membranes contain domains that are enriched in cholesterol, gangliosides, and sphingolipids (Sleight et al. 2005). These domains called lipid rafts also contain many proteins implicated in the regulation of cell signaling, AR, and cumulus- and/or zona pellucida-binding processes (Bou Khalil et al. 2006, Thaler et al. 2006). Many of the proteins found in lipid rafts are acquired during epididymal maturation (Gadella et al. 2008). Furthermore, recent studies have demonstrated that the removal of cholesterol by BSA and apoA-I as well as the removal of phospholipids by apoA-I during sperm capacitation occurs from non-raft fractions (Mendez et al. 2001, Boerke et al. 2013). Most proteins implicated in the cholesterol/phospholipid removal were also located in non-raft fractions. Results obtained in the current study also placed BSPH1 in non-raft fraction, suggesting once again a role for BSPH1 in the cholesterol/phospholipid removal, which occurs during capacitation.

Based on our studies on murine BSPH1, we propose the following mechanism of sperm capacitation (Fig. 7). During epididymal maturation, sperm enter in contact with BSPH1, which binds to the sperm surface via an interaction with choline phospholipids. This interaction stabilizes the membrane and prevents premature capacitation and AR. Following ejaculation, sperm enter the oviduct where they encounter HDLs. BSPH1 transfers phospholipids and cholesterol to HDL particles via a specific interaction, causing a decrease in the cholesterol/phospholipid ratio that destabilizes the membrane. This causes an increase in intracellular pH, calcium, and cAMP, which in turn causes an increase in protein tyrosine phosphorylation and subsequently capacitation.

![Proposed mechanism of sperm capacitation by BSPH1](image)

**Figure 7** Proposed mechanism of sperm capacitation by BSPH1. During epididymal maturation, sperm enter in contact with BSPH1, which binds to the sperm surface via an interaction with choline phospholipids. This interaction stabilizes the membrane and prevents premature capacitation and AR. Following ejaculation, sperm enter the oviduct where they encounter HDLs. BSPH1 transfers phospholipids and cholesterol to HDL particles via a specific interaction, causing a decrease in the cholesterol/phospholipid ratio that destabilizes the membrane. This causes an increase in intracellular pH, calcium, and cAMP, which in turn causes an increase in protein tyrosine phosphorylation and subsequently capacitation.
it is highly possible that this mechanism would also apply to human fertility processes.

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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