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Modifications of the 26S proteasome during boar sperm capacitation

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Abstract

Protein ubiquitination is a stable, reversible posttranslational modification, targeting proteins for degradation/recycling by 26S proteasome in a well characterized enzymatic cascade. Studies revealed the role of UPS in the regulation of fertilization, including sperm-zona pellucida (ZP) interactions and the early event of sperm capacitation. The present study investigates the changes in proteasome compartmentalization, subunit composition and posttranslational modifications during *in vitro* capacitation of fresh boar spermatozoa.

We observed capacitation dependent shedding of both 20S core and 19S regulatory particle from acrosome that was associated with decreased plasma membrane integrity, independent of proteasomal inhibition. Subunits PSMA1–7 of the 20S core did not appear to undergo post-translational modifications during capacitation, based on invariant molecular masses before and after capacitation; however, we observed multiple PSMD4 forms of 19S regulatory particle (50, 53, 70, 115–140, 160, and >176 kDa) sequentially released from spermatozoa. PSMD4 subunit was found to be post-translationally modified during the course of capacitation, resulting in change of apparent molecular mass, some of which were dependent on proteasomal inhibition. These results show that the sperm proteasomes are being modified during sperm capacitation. Additional studies of individual 26S proteasome subunits will be required to elucidate these modifications and to understand how UPS modulates the sperm capacitation.

Keywords

ubiquitin; 26S proteasome; PSMA1–7; PSMD4; sperm capacitation; fertilization; boar

1. Introduction

Ubiquitin-proteasome system (UPS) plays an important role in protein degradation and turnover in all living organisms. Protein ubiquitination is a stable, reversible

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posttranslational modification of target proteins by covalent ligation of the small chaperone protein ubiquitin. Conjugation of ubiquitin to its substrates requires the participation of ubiquitin activating (E1), conjugating (E2), ligating (E3) and deubiquitinating enzymes (DUBs). Linear polyubiquitin chains, resulting from tandem ligation of ubiquitin to its substrates, are recognized by 26S proteasome, a multi-catalytic protease with specific affinity for ubiquitinated proteins, which degrades them to small peptides. Canonical 26S proteasome comprises a hollow 20S core, capped with a 19S regulatory particle on either one or both ends. The 19S particle recognizes, engages and removes the multi-ubiquitin chain ligated to a protein destined for proteasomal degradation. The substrate protein undergoes unfolding and translocation to the 20S core for proteasomal degradation into small peptides that are afterwards released from the 20S core. Polyubiquitin chain is disassembled into single molecules that re-enter the ubiquitination cycle (Glickman and Ciechanover 2002; Sutovsky 2011).

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Studies using human and non-human mammalian spermatozoa (Morales et al. 2007; Sasanami et al. 2012; Sawada et al. 1998; Sutovsky et al. 2004; Yokota and Sawada 2007; Zimmerman et al. 2011) revealed the role of UPS in the regulation of fertilization, including sperm-zona pellucida (ZP) interactions and the preceding event of sperm capacitation, responsible for the remodeling of sperm plasma membrane (PM) and acrosome, necessary for sperm fertilizing ability (Sutovsky 2011; Yi et al. 2007; Zimmerman and Sutovsky 2009). More recent studies provide compelling evidence of UPS being a regulating element of sperm capacitation (Kerns et al. 2016). Capacitation is one of the important steps of sperm maturation, during which the spermatozoon gains the ability to recognize and bind to ZP, and to penetrate it prior to fusing with the oocyte plasma membrane (Austin 1952; Chang 1951). An inseparable part of sperm capacitation is hyperactivation, during which the flagellar beating changes from symmetrical low amplitude bending to vigorous, deep, asymmetric bending (Yanagimachi 1994). While there are several studies regarding possible UPS engagement during capacitation, and thus modulating processes involved in sperm-oocyte interactions (Miles et al. 2013; Sanchez et al. 2011; Yi et al. 2012), and hyperactivation (Mochida et al. 2000), only few studies are available regarding UPS regulation during sperm capacitation, particularly in regard to the possibility of posttranslational modification of proteasomal subunit proteins. It has been reported that during sperm capacitation, the 26S proteasome is being phosphorylated (Diaz et al. 2007; Kong et al. 2009; Morales et al. 2007) by protein kinase A (PKA) which results in decreased chymotrypsin like activity of the 20S core (Kong et al. 2009). Once activated by PKA, the proteasome could directly or indirectly modulate substrate protein phosphorylation at serine and threonine residues through a feedback loop (Kong et al. 2009). Furthermore, it was reported that in ascidian spermatozoa, the PSMA1 subunit is post-translationally modified in its C-terminal domain by proteolytic cleavage of 16 terminal residues (Yokota et al. 2011). To our knowledge, other post-translational modifications to the 26S proteasome subunits in spermatozoa have not been reported.

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Based on previous studies indicating the involvement of UPS in sperm capacitation and regulation of 26S proteasome during capacitation, which is poorly understood, the objective of this study is to elucidate the changes in proteasome compartmentalization, subunit

composition and posttranslational modifications of 26S proteasome during sperm capacitation.

2. Materials and Methods

Semen collection and processing

All studies involving vertebrates were completed under the strict guidance of a protocol approved by the Animal Care and Use Committee (ACUC) of the University of Missouri. Fresh boar spermatozoa were collected weekly from the same, healthy fertile boar also used for routine in vitro fertilization trials with high blastocyst yield, and the sperm-rich fraction was used for the study purposes. Concentration and motility of ejaculates were evaluated by conventional andrological methods with the use of light microscope. Sperm concentration was measured by hemocytometer (Fisher Scientific) and ranged from 250 to 350 million/mL; only ejaculates with >80% motile spermatozoa were used for the study. Ejaculates were free of contaminants other than the expected minimal content of cytoplasmic droplets, thus not necessitating gradient purification. Ejaculates were centrifuged (2,000 RPM ~ 400 g, 10 min; Fisher Scientific) to separate seminal plasma from spermatozoa. Spermatozoa were either diluted tenfold with pre-warmed (within 2°C of boar semen) BTS boar semen extender supplied with gentamicin (IMV Technologies, Maple Grove, MN, USA) and let to cool down to room temperature for further use, or used fresh directly for capacitation studies, described below.

Sperm capacitation studies with proteasomal modulation

Fresh, non-extended spermatozoa separated from seminal plasma were immediately washed three times in warm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered Tyrode lactate medium supplied with polyvinyl alcohol (TL-HEPES-PVA), containing 10 mM Na-lactate, 0.2 mM Na-pyruvate, 2 mM NaHCO₃, 2 mM CaCl₂, 0.5 mM MgCl₂ and 0.01 % (w/v) polyvinyl alcohol (PVA); pH = 7.4, t = 37°C. After final wash, spermatozoa were resuspended in TL-HEPES-PVA medium supplied with 2% (w/v) bovine serum albumin (BSA) to a final concentration not exceeding 15×10⁶ sperm/ml, distributed in 15 ml Falcon tubes; and three treatment groups were initiated: i) without proteasomal inhibition; ii) with proteasomal inhibition, supplied with 100 µM MG132 (ENZO Life Sciences, Farmingdale, NY, USA) in ethanol (EtOH); and iii) 0.2% (v/v) EtOH as a vehicle control. All three treatment groups were left to capacitate for 4 hrs at 37°C, 5% (v/v) CO₂. Sperm samples after capacitation were washed from BSA and used for flow cytometric quantification and protein extraction.

Sample preparation and labeling for flow cytometric analysis

Ejaculated spermatozoa, stored in the BTS medium at room temperature; and fresh, non-extended capacitated spermatozoa with or without proteasomal inhibitors or vehicle solutions, were washed three times with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂HPO₄, pH=7.4), and fixed in 2% formaldehyde for 45 min at room temperature. In preparation for flow cytometry, washed and fixed spermatozoa were sequentially treated in following seven steps: i) Tris buffered saline (TBS; 50 mM TRIS-HCl, pH = 7.4, 137 mM NaCl, as a base buffer for all the following sperm

treatments), ii) 0.75 M KCl, iii) 30 mM n-octyl- β -D-glucopyranoside (OBG), iv) 1% (v/v) Triton X-100 (TrX-100), v) 2 % (v/v) acetic acid (2 % AcOH), vi) sonication (1 min, 30 % amplitude) in modified RIPA buffer (1 % (v/v) TrX-100, 1 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 1 mM DTT, 2 mM EDTA), and vii) SDS-PAGE loading buffer (Laemmli 1970). In general, approximately 100 million spermatozoa were used in a single treatment, which was conducted by adding of 200 μ l of a relevant reagent (i–vii), proteinase and phosphatase inhibitors (Thermo Scientific, Rockford, IL, USA). Sperm pellets were resuspended and incubated on ice for 30 min with periodical agitation. Approximately 10 million spermatozoa were removed from the pool, washed in PBS and used for flow cytometric measurements. The residual spermatozoa were spun (500g, 4°C, 10 min), the supernatant was discarded and the next reagent in the treatment sequence was added. This procedure follows consistently throughout the first five treatment steps up to step vi) where the spermatozoa were sonicated on ice for 1 min, after which approximately 10 million spermatozoa were removed and residual supernatant was discarded; the leftover spermatozoa were boiled in SDS loading buffer in the last, seventh step. Ejaculated and capacitated spermatozoa (both with and without proteasomal inhibition, including vehicle control) after each treatment step were blocked with PBS with 0.1% Tween 20 (PBST) supplemented with 5% normal goat serum (NGS; Sigma, St. Louis, MO) for 30 min at room temperature. Primary antibodies used for 26S proteasome subunits detection were anti-PMSA1–7 subunits of 20S core subunit (1:200 dilution; BML-PW8155, Enzo), and anti-PSMD4 subunit of 19S regulatory particle (1:200 dilution; ab20239, Abcam). Antibodies were diluted with PBST supplemented with 1% NGS. Primary antibodies were added to sperm sample tubes, and incubated overnight at 4°C. For negative controls, non-immune mouse and rabbit sera of comparable globulin concentrations were used instead of primary antibodies, and processed in the same fashion. The following morning, spermatozoa were washed twice with PBST with 1 % NGS, and appropriate species-specific secondary antibodies such as goat anti-rabbit or anti-mouse conjugated to Cyanine5 (Life technologies, Carlsbad, CA) were added at the final 1:150 dilution in PBST with 1 % NGS, and allowed to incubate for 40 min at room temperature. For acrosome integrity assessment, peanut agglutinin lectin conjugated to Alexafluor 488 (PNA-AF488) (1:2,500 dilution; Molecular Probes, Eugene, OR) was used, and 4',6-Diamidino-2-Phenylindole Dilactate (DAPI) DNA stain (1:1500 dilution; Molecular Probes, Eugene, OR) was used as reference and contrast stain; both PNA-AF488 and DAPI were coincubated with secondary antibodies. After incubation with secondary antibodies, spermatozoa were washed twice with PBST with 1 % NGS. Prior to performing flow cytometry, the sample aliquots were checked for fluorescence labeling under a Nikon Eclipse 800 epifluorescence microscope (Nikon Instruments, Melville, NY). After positive labeling was confirmed, the final concentration was adjusted to 5 million spermatozoa per 100 μ l.

Image based flow cytometry

The fluorescently labeled samples were processed on an Amnis FlowSight Imaging Flow Cytometer (EMD Millipore Corp.) fitted with a 20 $\times$ microscope objective (numerical aperture of 0.5) with an imaging rate of up to 2,000 events/sec. The sheath fluid was Dulbecco's PBS (without Ca²⁺ or Mg²⁺). The flow-core size and speed was 10 μ m diameter and 66 mm/sec, respectively. The raw image acquisition was obtained using INSPIRE®

software. The camera was set to 1.0 μm per pixel of the charged-coupled device. The image display dimension for field of view was 60 μm and 8 μm depth of field. Samples were analyzed using four lasers concomitantly: a 405 nm line with intensity set to 50 mW, 488 nm line with intensity set to 50 mW, 642 nm line with intensity set to 20 mW, and a 785 nm line (side scatter) with intensity set to 5 mW. A total of 10,000 events were collected per sample, and the images were compensated for channel crossover by using single-color controls (i.e., DAPI-only; AF488-only; and Cy5-only labeling of spermatozoa) that were merged to generate a multi-color matrix. The compensation matrix file was then applied to an experimental raw-image file, yielding a color-compensated image file. Data analysis of the raw images was accomplished using IDEAS® software (Version 6.2.64.0). Displaying spermatozoa using Gradient RMS for the brightfield channel allowed the gating of focused spermatozoa. A combined Area $\times$ Aspect Ratio scatter plot display allowed us to gate for single-cell events. Single cell population gate was used for histogram display of mean pixel intensities by frequency for following channels: AF488 (channel 2), DAPI (channel 7), and Cy5 (channel 11). Intensity histograms of individual channels were then used to draw regions of sub-populations with varying intensity levels, and for visual confirmation. Intensity of DAPI (channel 7) was used for histogram normalization among different treatment groups. Appropriate masks were applied to all relevant channels to exclude fluorescently positive debris from features' calculation. Feature finder tool was utilized to find the most relevant feature of sub-populations difference, where mean pixel intensities were not sufficiently distinctive. Negative controls of normal mouse and normal rabbit sera are included in suppl. Figs. 1 and 2, respectively.

Sequential protein isolation from the spermatozoa

Ejaculated spermatozoa stored in BTS extender and all three sperm treatment groups after capacitation (proteasomally inhibited, non-inhibited and vehicle control) were washed three times with TBS. Proteins were sequentially isolated from spermatozoa in the order i–vii) as described in the previous section. Approximately 200 million non-fixed spermatozoa were used per single extraction, which was conducted by adding of 100 μl of a relevant reagent (i–vii), proteinase and phosphatase inhibitors (Thermo Scientific, Rockford, IL, USA); sperm pellet was resuspended and incubated on ice for 30 min with periodical agitation. Spermatozoa were spun (500g, 4°C, 10 min), and extracts were stored at -25°C for further analysis. For sequential (stepwise) isolation of proteins, in the first step TBS was added to each pellet, allowed to incubate on ice for 30 min, centrifuged and the extracts saved. In the second step, pellets were reused and 0.75 M KCl was added to each sperm pellet, incubated on ice for 30 min and spun, after which extract was saved, and pellets were washed once with TBS, and reused in the third step for extraction with 30 mM OBG. This procedure was repeated in the fourth step (treatment with 1 % TrX-100), and fifth step (treatment with 2 % AcOH). After the fifth step, modified RIPA buffer was added and spermatozoa were sonicated on ice (60 sec, 30 % amplitude). Lastly, sperm pellets were boiled in SDS-PAGE loading buffer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (WB)

All sperm protein extracts except those obtained with the SDS sample buffer, were mixed with 4×concentrated SDS-PAGE loading buffer in ratio 3:1, boiled for five minutes and briefly spun at 5,000g. Total protein equivalent to 20 million spermatozoa was loaded per single lane. SDS-PAGE was carried out on 8–20% gradient gels (PAGEr Precast gels; Lonza Rockland Inc., Rockland, ME, USA) as previously described (Miles et al. 2013). The molecular masses of the separated proteins were estimated by using prestained Prosieve protein colored markers (Lonza Rockland Inc., Rockland, ME, USA) run in parallel. After SDS-PAGE, proteins were electro-transferred onto a PVDF Immobilon Transfer Membrane (Millipore, Bedford, MA, USA) using an Owl wet transfer system (Fisher Scientific) at a constant 50 V for 4 h for immunodetection (Miles et al. 2013; Zimmerman et al. 2011), according to the method described by (Towbin et al. 1979).

Protein immunodetection

Duplicate polyvinylidene fluoride (PVDF) membranes (Millipore) with the transferred proteins were sequentially blocked with 10 % (w/v) non-fat milk in TBS with 0.05% (v/v) Tween 20 (TBST; Sigma-Aldrich), and incubated in parallel with anti-PSMA1–7 subunits of 20S core subunit (1:4,000 dilution; mouse IgG; BML-PW8155, Enzo) and anti-PSMD4 subunit of 19S regulatory particle (1:2,000 dilution; affinity purified rabbit serum, ab20239, Abcam), overnight. The membranes were incubated for 40 min with an appropriate species-specific secondary antibody such as the horseradish peroxidase (HRP)-conjugated goat-anti-rabbit (GAR IgG-HRP) or mouse (GAM-IgG-HRP) antibodies (1:10,000 dilution). The membranes were reacted with chemiluminescent substrate (Luminata Crescendo Western HRP Substrate; Millipore). Blots were screened with ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, USA) to visualize the protein bands and analyzed by Image Lab Touch Software (Bio-Rad, Hercules, CA, USA). Unless otherwise specified, procedures were carried out at room temperature. Residual gels and membranes after chemiluminescence detection were stained with Coomassie Brilliant Blue (CBB) R-250 (both Thermo Scientific, Rockford, IL, USA) for protein normalization.

Statistical analysis

In order to optimize experimental protocol, three pilot trials were done to establish optimal reagent conditions, and two independent, optimized replicates were conducted afterwards and used for statistical analysis. Each data point is presented as the mean $\pm$ SD. Data were processed using one-way analysis of variance (ANOVA) using the GraphPad Prism 7.03 (GraphPad Software, Inc., La Jolla, CA, USA) in a completely randomized design. Sidak's multiple comparison test was used to compare mean values of individual treatments with 95% confidence interval.

3. Results

Modifications of proteasomal 20S core during sperm capacitation

Formaldehyde fixed ejaculated spermatozoa and those capacitated under proteasome permissive vs. inhibiting conditions were sequentially treated as described above. Spermatozoa were labeled with anti-PSMA1–7 antibody after each step, to monitor changes in compartmentalization, and posttranslational modifications of the 20S core subunit, and with PNA lectin to monitor outer acrosomal membrane (OAM) /acrosome integrity status. Flow cytometric analysis of PSMA1–7 and PNA positive spermatozoa was performed before and after capacitation, with or without proteasomal inhibition (Fig. 1a). With the mild treatment steps (step 1–step 5), the majority of ejaculated spermatozoa were positively labeled for 20S core subunits in acrosomal region, with high levels of OAM/acrosome integrity. Furthermore, in treatment steps 1–5, a portion of 20S core/PSMA1–7 labeling was lost during capacitation ($p < 0.05$, suppl. Fig. 3), with simultaneous decrease in OAM/acrosome integrity ($p < 0.05$, suppl. Fig. 4). The PSMA1–7 signal was retained in the acrosomal region after sperm capacitation. With introduction of more harsh extraction conditions (sonication in RIPA buffer) in step 6, the decrease of the PSMA1–7 positive was observed both in ejaculated (96.5% after step 5 vs. 56.5% after step 6, $p < 0.001$) and capacitated spermatozoa (65.5% after step 5 vs. 23.5% after step 6, $p < 0.001$) in the absence of proteasomal inhibition. A drop from 51.5% after step 5 to 21.8% ($p < 0.001$) was observed after step 6 in proteasomally inhibited spermatozoa. The same trend was observed in OAM/acrosome integrity, with 49.5% PNA positive ejaculated spermatozoa vs. <2% PNA positive capacitated spermatozoa after sixth treatment step, regardless of proteasomal inhibition). After last treatment step, less than 2% of measured spermatozoa remained both PSMA1–7 and PNA positive (Fig. 1a). Both PSMA1–7 and PNA labeling were consistent up to step 5, and proteasomal inhibition did not affect 20S core subunit removal trend after the treatment of capacitated spermatozoa nor affected the decrease in PM integrity (Fig. 1a).

Flow cytometric scatter plot measurements (Fig. 1a') of PSMA1–7 and PNA double labeled spermatozoa across all treatments showed that spermatozoa were either labeled as *double* positive (possessing both the 20S core subunits and intact OAM), *double* negative (with defect or no OAM and lacking detectable 20S core subunits), *20S only* positive (possessing 20S core subunits, but lacking OAM), and *PNA only* positive. However, PNA labeling in *PNA only* subpopulation was localized throughout the whole sperm head region (suppl. Fig. 5) and OAM/acrosome integrity of such spermatozoa was considered as compromised and therefore disregarded. A similar trend can be observed from scatter plots in Fig. 1a', where a consistent pattern can be observed throughout steps 1–5: the majority of ejaculated spermatozoa are *double* positive while capacitated spermatozoa are distributed among *double* positive and *double* negative subpopulations. After treatment step 6, *double* positive labeling shows only a portion of ejaculated spermatozoa, while capacitated spermatozoa are being either *double* negative (majority) or *20S only* positive. After step 7, all spermatozoa were *double* negative.

WB detection of PSMA1–7 was performed on sequentially isolated proteins from ejaculated spermatozoa and spermatozoa capacitated under proteasome permissive vs. inhibiting

conditions (Fig. 2). As described earlier (Miles et al. 2013), PSMA1–7 subunits of 20S core co-migrated at ~25 kDa, with a higher relative abundance in ejaculated vs. capacitated spermatozoa ($p<0.05$, suppl. Fig. 6). No posttranslational modifications during capacitation that would change the molar mass were observed; however, a statistically significant accumulation of PSMA1–7 in capacitated spermatozoa with proteasomal inhibition vs. no inhibition ($p<0.001$) as well as vs. vehicle control ($p<0.001$) was observed in the last extraction step (suppl. Fig. 6). Degradation products of PSMA1–7 subunits were detectable at molar weights of 23 kDa and 15 kDa. The lower band (15 kDa) could be a result of proteolytic cleavage of one of the PSMA subunits, previously reported in ascidian spermatozoa (Yokota et al. 2011).

Modifications of proteasomal 19S regulatory particle during sperm capacitation

Formaldehyde fixed ejaculated spermatozoa and those capacitated under proteasome permissive vs. inhibiting conditions were sequentially treated as described above, and, after each treatment step, spermatozoa were labeled with antibodies against the PSMD4 (aliases RPN10, S5A) subunit of 19S regulatory particle to monitor changes in compartmentalization, and posttranslational modifications of 19S regulatory particle during capacitation. In parallel, PNA lectin was used to monitor OAM/acrosome integrity. The 19S and PNA positive spermatozoa were counted by flow cytometry in all samples before and after capacitation and after each treatment step (Fig. 3a). With the mild protein extraction (step 1–step 5), the majority of ejaculated spermatozoa remained positive for 19S regulatory particle in acrosomal region, with high level of OAM/acrosome integrity. In steps 1–5, a portion of 19S regulatory particles were lost during capacitation and extraction ($p<0.05$, suppl. Fig. 7), with simultaneous decrease in OAM/acrosome integrity ($p<0.05$, suppl. Fig. 4). The 19S subunit localization remained confined to acrosomal region after sperm capacitation. With introduction of more harsh conditions (sonication in RIPA buffer) in step 6, the decrease was observed in the incidence of 19S positive ejaculated spermatozoa (96.8% after step 5 vs. 59.9% after step 6, $p<0.001$) as well as capacitated spermatozoa (45.9% after step 5 vs. 17.9% after step 6, $p<0.001$) in the absence of proteasomal inhibition; and in proteasomally inhibited spermatozoa (46.1% after step 5 vs. 18.6% after step 6, $p<0.001$). Same trend was observed in OAM/acrosome integrity level after sixth treatment step (49.5% ejaculated vs. <2% capacitated spermatozoa had intact PM, both proteasomally inhibited and non-inhibited. After the last treatment step, there were less than 2% spermatozoa positive both for 19S and PNA. Fig. 3a shows that both 19S and PNA labeling was consistent up to step 5, and proteasomal inhibition did not affect 19S core subunit removal trend after the treatment of capacitated spermatozoa, or hinder the decrease in PM integrity (PNA labeling was consistent between Figs. 1a and 3a).

Flow cytometry of 19S and PNA double labeled spermatozoa across treatments (Fig. 3a') showed the presence of *double* positive (possessing 19S regulatory particle labeling and intact OAM/acrosome integrity), *double* negative (with defective or no OAM and lacking 19S regulatory particle), *19S only* positive (possessing 19S regulatory particle, but missing acrosome), and *PNA only* positive. As previously explained, PNA labeling in *PNA only* subpopulation of spermatozoa, localized throughout the whole sperm head region was considered as OAM/acrosome compromised (suppl. Fig. 5) and therefore disregarded. A

similar trend is shown in Fig. 3a', where consistent labeling pattern can be seen throughout steps 1–5, wherein majority of ejaculated spermatozoa is *double* positive while capacitated spermatozoa are distributed among *double* positive and *double* negative subpopulations. After step 6, *double* positive labeling showed only in a portion of ejaculated spermatozoa, while capacitated spermatozoa were either *double* negative (majority) or *19S only* positive. After step 7, all spermatozoa were *double* negative.

Western blotting of PSMD4 subunit, performed on sequentially isolated proteins from ejaculated and capacitated spermatozoa (with and without proteasomal inhibition (Fig. 4), showed a single 50 kDa band as previously reported (Deveraux et al. 1994; Yi et al. 2010b). In the first step of extraction, PSMD4 subunit was detected only in capacitated spermatozoa at approximately 160 kDa, even after signal overexposure (data not shown), and no difference was observed between capacitated spermatozoa with vs. without proteasomal inhibition. After treatment with 0.75 M KCl, the same band of 160 kDa was observed in capacitated spermatozoa, in addition to bands ranging from 115–140 kDa with band patterns altered by proteasomal inhibition. Bands which were detected after longer exposure and were uniquely present in the capacitated spermatozoa in a proteasomal inhibition-dependent manner included a 35 kDa band present only in capacitated spermatozoa without proteasomal inhibition, and bands of 24 kDa and 63 kDa detected only in spermatozoa capacitated with proteasomal inhibition. In all OBG sperm extracts, PSMD4 was detected above 179 kDa and the densitometric analysis revealed that this band was accumulated in capacitated spermatozoa with proteasomal inhibition ($p < 0.05$, suppl. Fig. 8). Moving on to the TrX-100 extracts, PSMD4 band > 179 kDa was detected in spermatozoa capacitated with proteasomal inhibition, suggesting that this isoform of PSMD4 is retained in TrX100 extracts after this treatment. Other PSMD4 isoforms detected in TrX100 extracts were 53 kDa in ejaculated sperm and 70 kDa in both ejaculated and capacitated spermatozoa, with higher abundance in capacitated spermatozoa. A unique band was detected at 24 kDa only in capacitated spermatozoa with proteasomal inhibition. After 2% AcOH extraction, PSMD4 was detected in both ejaculated and capacitated spermatozoa at 50 kDa, with higher abundance in ejaculated spermatozoa, and without any observable differences in capacitated sperm populations. Within the limit of detection, PSMD4 was detected in ejaculated spermatozoa only at 50 kDa after step 6 (sonication in RIPA buffer). In the last extraction step, PSMD4 was detected in both ejaculated spermatozoa (with higher abundance) and capacitated spermatozoa at 50 kDa.

4. Discussion

This study monitored the subunit composition and abundance of 26S proteasomes during boar sperm capacitation, focusing on changes in proteasome compartmentalization, subunit composition and posttranslational modifications. We indeed found such changes when we separately monitored 20S core particle and the 19S regulatory particle in sequentially extracted ejaculated spermatozoa and in spermatozoa capacitated under proteasomally permissive and inhibiting conditions, analyzed by image-based flow cytometry and Western blotting.

We monitored the 20S core using an antibody recognizing all seven PSMA subunits, whereas we used a single subunit-targeting anti-PSMD4 antibody for 19S regulatory particle. We observed that the labeling of both 20S core and 19S regulatory subunits was partially lost during the sperm capacitation. Beyond intact (as in not-extracted) spermatozoa, such a trend was also observed after the sperm proteins were extracted with mild to moderate conditions in the first five steps of sequential extraction. After applying harsher conditions in step 6, we observed the loss of both 20S and 19S subunit proteins; and finally, after treatment with SDS loading buffer, both 20S and 19S signals ceased. Proteasome inhibition does not seem to affect the removal of proteasome subunits during the capacitation. Simultaneously with proteasome subunits screening, we also monitored OAM/acrosome integrity with PNA lectin labeling during capacitation. We observed high OAM/acrosome integrity throughout the first five treatment steps (mild to moderate extraction conditions) in ejaculated spermatozoa and its loss after capacitation. This can be most likely attributed to cholesterol efflux during the capacitation (Yanagimachi 1994), when PM becomes destabilized and is more easily compromised during the processing and fixation of capacitated spermatozoa. Furthermore, the vehicle (0.2% EtOH) seems to further destabilize PM/OAM during capacitation, resulting in additional decrease of PNA labeling in capacitated spermatozoa after treatment with proteasomal inhibition or vehicle. Applying sperm sonication in sixth treatment step (Figs. 1 and 3), we observed dramatic decrease in PNA labeling in ejaculated spermatozoa and no PNA labeling in capacitated spermatozoa; accordingly, sperm sonication is used routinely for acrosomal content isolation. Decrease in PM integrity, therefore, seems to be the prerequisite for 20S and 19S particle shedding during capacitation, as both 20S and 19S labeling decreases along with the decreasing PNA labeling. Similarly, the presence of 20S or 19S single-labeled spermatozoa (no PNA labeling) suggests an intra-acrosomal localization of proteasomes, which is in agreement with previous studies. However, this is not a novel finding as we already reported 26S proteasomes to be localized in sperm acrosomes in our previous studies (Miles et al. 2013). We did not observe changes in sperm compartmentalization of 20S or 19S subunits during the course of sperm capacitation. Interestingly though, the ratio of positively labeled 20S core to 19S regulatory particle was roughly 1:1 in ejaculated spermatozoa and it dropped to 8:10 in capacitated spermatozoa, which suggests that the acrosome contains functional 26S proteasomes.

For monitoring posttranslational modifications of 20S and 19S subunits, we sequentially treated the spermatozoa in an ascending order of extraction strength and used WB to screen for protein modification, which would result in molar weight change. While our flow cytometric studies were performed on formaldehyde fixed spermatozoa, spermatozoa used for WB were not fixed; therefore, these two techniques are complementary. While in flow cytometric studies we assessed whether the extraction treatments would result in decreased 20S/19S signal, as well as the changes in proteasome compartmentalization, we used WB to screen for sequential release of 20S core and 19S regulatory particle from spermatozoa as the strength of isolation conditions was increased with each treatment step. Nevertheless, at least the first step in both flow cytometric study and WB detection can be correlated as both experiments start with fresh spermatozoa. In the first step, 2% formaldehyde fixed spermatozoa were labeled with anti PSMA1–7 antibody, and 96.3% of ejaculated

spermatozoa were positive (Fig. 1a). On the contrary, in the WB detection, some of the 20S core particles were released into the TBS buffer (Fig. 2), indicating that a subpopulation of sperm proteasomes are loosely associated with the sperm surface, as also suggested by previous studies in which cell-impermeable proteasomal substrate peptides and biotinylated proteasomal inhibitors such as ZL3Vs readily bound to sperm acrosomal surface (Yi et al. 2010b; Yi et al. 2009). Analogously, after a portion of 20S core particles were shed during capacitation, 68.9% of capacitated spermatozoa were 20S positive by flow cytometry; reduced 20S subunit band density was also observed by WB in capacitated spermatozoa compared to ejaculated spermatozoa. Interestingly, 20S core seems to be released from non-fixed, ejaculated and capacitated spermatozoa by mere incubation in cold TBS, despite the fact that the first treatment step was rather intended to be a control, and only a negligible amount of 20S core unit was expected to be released. The 19S regulatory particles were released into TBS only from capacitated spermatozoa (Fig. 4), suggesting that PSMD4 release in the first two extraction steps is capacitation dependent. However, it remains unclear which capacitation-induced changes are responsible for this 19S release in the first two extractions steps. From the second treatment step on, a portion of proteins were prone to extraction from non-fixed spermatozoa in every treatment step; while in formaldehyde fixed spermatozoa, treatment conditions did not seem to affect the proportion of 20S:19S positive spermatozoa until the sonication step. However, a consistent pattern was observed in both flow cytometric and WB detection throughout all treatment steps at least for the 20S core subunits, which were more abundant in ejaculated compared to capacitated spermatozoa. Furthermore, PSMA1–7 subunits were found to be continuously released in all extraction steps, and proteasomal inhibition decelerated this release, when in the last extraction step, higher PSMA1–7 signal intensity was observed in capacitated spermatozoa with proteasomal inhibition. We previously co-purified sperm proteasomes with spermadhesins AWN and AQN-1 (Miles et al. 2013; Yi et al. 2010a), and also reported that the shedding of spermadhesin AQN-1 was hindered when acrosomal protein ubiquitination was inhibited by E1 inhibitor PYR-41 (Yi et al. 2012). We can speculate that by helping with shedding of spermadhesins, 20S core subunits are also lost in the process; and proteasomal inhibition can therefore reduce this loss. PSMA1–7 subunits were not found to be post-translationally modified during the capacitation in such manner that would result in change in their molecular masses. However, the antibody against a shared domain of subunits PSMA1–7 detected a protein band of ~ 23 kDa in both ejaculated and capacitated spermatozoa after last extraction step. This might suggest a post-translational modification of one of the PSMA subunits as it was described in ascidian spermatozoa (Yokota et al. 2011) even before commencing capacitation. Further proteomic studies are required to confirm this hypothesis. Considering previous reports that multiple proteasomal subunits are being constitutively phosphorylated (Bose et al. 2004; Feng et al. 2001; Fernandez Murray et al. 2002; Satoh et al. 2001), phosphorylation of sperm proteasomes during sperm capacitation warrants further study. Except for first two extraction steps from ejaculated spermatozoa, PSMD4 subunit was found to be released from spermatozoa in all treatment steps, and besides canonical 50 kDa form (Deveraux et al. 1994), we also detected higher mass bands which seem to emerge in a capacitation dependent manner. Non-immune serum control, done as described previously (Yi et al. 2010b) ruled out non-specific labeling. Furthermore, proteasomal inhibition during capacitation affected PSMD4 modification, as well as its accumulation or

release, which is indicative of proteasome autoregulation during capacitation. It has been previously reported that PSMD4 subunit is mono-, di-ubiquitinated (Isasa et al. 2010), and poly-ubiquitinated (Crosas et al. 2006; Isasa et al. 2010) to regulate PSMD4 ability to recruit substrates to the 26S proteasome in yeast cells. Further, polyubiquitinylation was reported to regulate ubiquitin receptors in third-instar larvae of *D. melanogaster* (Lipinski et al. 2012) and to regulate proteasome activity in human HEK 293T and HeLa cells (Jacobson et al. 2014). Therefore, the observed multi-ubiquitin chain-like laddering of PSMD4 after capacitation comes as no surprise and suggests that 26S proteasome may also be regulated by polyubiquitinylation of PSMD4 subunit also in porcine spermatozoa, while increase of apparent molar weight from 53 kDa to 70 kDa during *in vitro* capacitation in TrX-100 extracts indicates addition of two ubiquitin residues to PSMD4 subunit (molar weight of ubiquitin ~ 8.5 kDa).

The present study investigated the changes in proteasome compartmentalization, subunit composition and posttranslational modifications during *in vitro* capacitation of fresh boar spermatozoa. Partial release of both 20S core and 19S regulatory particle from the acrosome, associated with the remodeling of sperm PM/OAM is obvious from our results, and corroborates previously published work. The functionality of these processes remains to be elucidated in the future, with focus on whether the modifications are functional in sperm capacitation or in later events of fertilization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Austin CR. The capacitation of the mammalian sperm. *Nature*. 1952; 170:326. [PubMed: 12993150]
- Bose S, Stratford FL, Broadfoot KI, Mason GG, Rivett AJ. Phosphorylation of 20S proteasome alpha subunit C8 (alpha7) stabilizes the 26S proteasome and plays a role in the regulation of proteasome complexes by gamma-interferon. *Biochem J*. 2004; 378:177–184. [PubMed: 14583091]
- Crosas B, Hanna J, Kirkpatrick DS, Zhang DP, Tone Y, Hathaway NA, Buecker C, Leggett DS, Schmidt M, King RW, Gygi SP, Finley D. Ubiquitin chains are remodeled at the proteasome by opposing ubiquitin ligase and deubiquitinating activities. *Cell*. 2006; 127:1401–1413. [PubMed: 17190603]
- Deveraux Q, Ustrell V, Pickart C, Rechsteiner M. A 26 S protease subunit that binds ubiquitin conjugates. *J Biol Chem*. 1994; 269:7059–7061. [PubMed: 8125911]
- Diaz ES, Kong M, Morales P. Effect of fibronectin on proteasome activity, acrosome reaction, tyrosine phosphorylation and intracellular calcium concentrations of human sperm. *Hum Reprod*. 2007; 22:1420–1430. [PubMed: 17347169]

- Feng Y, Longo DL, Ferris DK. Polo-like kinase interacts with proteasomes and regulates their activity. *Cell Growth Differ.* 2001; 12:29–37. [PubMed: 11205743]
- Fernandez Murray P, Pardo PS, Zelada AM, Passeron S. In vivo and in vitro phosphorylation of *Candida albicans* 20S proteasome. *Arch Biochem Biophys.* 2002; 404:116–125. [PubMed: 12127076]
- Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev.* 2002; 82:373–428. [PubMed: 11917093]
- Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature.* 1951; 168:697–698.
- Isasa M, Katz EJ, Kim W, Yugo V, Gonzalez S, Kirkpatrick DS, Thomson TM, Finley D, Gygi SP, Crosas B. Monoubiquitination of RPN10 regulates substrate recruitment to the proteasome. *Mol Cell.* 2010; 38:733–745. [PubMed: 20542005]
- Jacobson AD, Macfadden A, Wu Z, Peng J, Liu CW. Autoregulation of the 26S proteasome by in situ ubiquitination. *Mol Biol Cell.* 2014; 25:1824–1835. [PubMed: 24743594]
- Kerns K, Morales P, Sutovsky P. Regulation of Sperm Capacitation by the 26S Proteasome: An Emerging New Paradigm in Spermatology. *Biol Reprod.* 2016; 94:117. [PubMed: 27053366]
- Kong M, Diaz ES, Morales P. Participation of the human sperm proteasome in the capacitation process and its regulation by protein kinase A and tyrosine kinase. *Biol Reprod.* 2009; 80:1026–1035. [PubMed: 19144957]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
- Lipinski Z, Kovacs L, Deak P, Udvardy A. Ubiquitylation of *Drosophila* p54/Rpn10/S5a regulates its interaction with the UBA-UBL polyubiquitin receptors. *Biochemistry.* 2012; 51:2461–2470. [PubMed: 22364263]
- Miles EL, O'gorman C, Zhao J, Samuel M, Walters E, Yi YJ, Sutovsky M, Prather RS, Wells KD, Sutovsky P. Transgenic pig carrying green fluorescent proteasomes. *Proc Natl Acad Sci U S A.* 2013; 110:6334–6339. [PubMed: 23550158]
- Mochida K, Tres LL, Kierszenbaum AL. Structural features of the 26S proteasome complex isolated from rat testis and sperm tail. *Mol Reprod Dev.* 2000; 57:176–184. [PubMed: 10984418]
- Morales P, Diaz ES, Kong M. Proteasome activity and its relationship with protein phosphorylation during capacitation and acrosome reaction in human spermatozoa. *Soc Reprod Fertil Suppl.* 2007; 65:269–273. [PubMed: 17644968]
- Sanchez R, Deppe M, Schulz M, Bravo P, Villegas J, Morales P, Risopatron J. Participation of the sperm proteasome during in vitro fertilisation and the acrosome reaction in cattle. *Andrologia.* 2011; 43:114–120. [PubMed: 21382065]
- Sasanami T, Sugiura K, Tokumoto T, Yoshizaki N, Dohra H, Nishio S, Mizushima S, Hiyama G, Matsuda T. Sperm proteasome degrades egg envelope glycoprotein ZP1 during fertilization of Japanese quail (*Coturnix japonica*). *Reproduction.* 2012; 144:423–431. [PubMed: 22859519]
- Satoh K, Sasajima H, Nyomura KI, Yokosawa H, Sawada H. Assembly of the 26S proteasome is regulated by phosphorylation of the p45/Rpt6 ATPase subunit. *Biochemistry.* 2001; 40:314–319. [PubMed: 11148024]
- Sawada H, Pinto MR, De Santis R. Participation of sperm proteasome in fertilization of the phlebobranch ascidian *Ciona intestinalis*. *Mol Reprod Dev.* 1998; 50:493–498. [PubMed: 9669533]
- Sutovsky P. Sperm proteasome and fertilization. *Reproduction.* 2011; 142:1–14. [PubMed: 21606061]
- Sutovsky P, Manandhar G, Mccauley TC, Caamano JN, Sutovsky M, Thompson WE, Day BN. Proteasomal interference prevents zona pellucida penetration and fertilization in mammals. *Biol Reprod.* 2004; 71:1625–1637. [PubMed: 15253927]
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A.* 1979; 76:4350–4354. [PubMed: 388439]
- Yanagimachi, R. Mammalian fertilization. In: Knobil, E., Neill, JD., editors. *The physiology of reproduction*. Vol. 1. Raven Press; New York: 1994. p. 189–317.

- Yi YJ, Manandhar G, Oko RJ, Breed WG, Sutovsky P. Mechanism of sperm-zona pellucida penetration during mammalian fertilization: 26S proteasome as a candidate egg coat lysin. *Soc Reprod Fertil Suppl.* 2007; 63:385–408. [PubMed: 17566286]
- Yi YJ, Manandhar G, Sutovsky M, Jonakova V, Park CS, Sutovsky P. Inhibition of 19S proteasomal regulatory complex subunit PSMD8 increases polyspermy during porcine fertilization in vitro. *J Reprod Immunol.* 2010a; 84:154–163. [PubMed: 20004025]
- Yi YJ, Manandhar G, Sutovsky M, Zimmerman SW, Jonakova V, Van Leeuwen FW, Oko R, Park CS, Sutovsky P. Interference with the 19S proteasomal regulatory complex subunit PSMD4 on the sperm surface inhibits sperm-zona pellucida penetration during porcine fertilization. *Cell Tissue Res.* 2010b; 341:325–340. [PubMed: 20526895]
- Yi YJ, Park CS, Kim ES, Song ES, Jeong JH, Sutovsky P. Sperm-surface ATP in boar spermatozoa is required for fertilization: relevance to sperm proteasomal function. *Syst Biol Reprod Med.* 2009; 55:85–96. [PubMed: 19462288]
- Yi YJ, Zimmerman SW, Manandhar G, Odhiambo JF, Kennedy C, Jonakova V, Manaskova-Postlerova P, Sutovsky M, Park CS, Sutovsky P. Ubiquitin-activating enzyme (UBA1) is required for sperm capacitation, acrosomal exocytosis and sperm-egg coat penetration during porcine fertilization. *Int J Androl.* 2012; 35:196–210. [PubMed: 21950462]
- Yokota N, Kataoka Y, Hashii N, Kawasaki N, Sawada H. Sperm-specific C-terminal processing of the proteasome PSMA1/alpha6 subunit. *Biochem Biophys Res Commun.* 2011; 410:809–815. [PubMed: 21703233]
- Yokota N, Sawada H. Sperm proteasomes are responsible for the acrosome reaction and sperm penetration of the vitelline envelope during fertilization of the sea urchin *Pseudocentrotus depressus*. *Dev Biol.* 2007; 308:222–231. [PubMed: 17585894]
- Zimmerman S, Sutovsky P. The sperm proteasome during sperm capacitation and fertilization. *J Reprod Immunol.* 2009; 83:19–25. [PubMed: 19853307]
- Zimmerman SW, Manandhar G, Yi YJ, Gupta SK, Sutovsky M, Odhiambo JF, Powell MD, Miller DJ, Sutovsky P. Sperm proteasomes degrade sperm receptor on the egg zona pellucida during mammalian fertilization. *PLoS One.* 2011; 6:e17256. [PubMed: 21383844]

Fig. 1. (a) Flow cytometric measurements of boar spermatozoa, after each treatment step, before and after capacitation under proteasomal activity permissive/inhibiting conditions, including vehicle control. Spermatozoa were labelled with an antibody against a shared domain of 20S proteasomal core subunits PMSA1–7) to screen for proteasome modifications, and lectin PNA for outer acrosomal membrane integrity screening. DNA was stained with DAPI. Representative fluorescence images of spermatozoa with double positive labelling of 20S core and OAM (PNA lectin labelling) are shown in a FlowSight image gallery. (a') Scatted plot representation of 20S and PNA double-labelled spermatozoa, representative of each treatment step, before and after capacitation with/without proteasomal inhibitors, including vehicle control. Each scatter plot represents 10,000 properly focused single cell flow cytometric events. The experiment was replicated twice with comparable results.

Fig. 2.
(a) Western blot of 20S core subunits PMSA1–7 in sequentially isolated proteins from ejaculated and capacitated spermatozoa, with/without proteasomal inhibitors, and vehicle control. (a') PVDF membrane stained with CBB after chemiluminescence detection shows comparable protein loads per lane. (a'') Residual gel after electro-transfer, for protein normalization purposes. Proteins were resolved on 8–20% gradient gel under non-reducing conditions, and protein equivalent of 20 million spermatozoa was loaded per single lane. The experiment was replicated twice with comparable results.

Fig. 3.
(a) Flow cytometric measurements of boar spermatozoa, after each treatment step, before and after capacitation under proteasomal activity permissive/inhibiting conditions, including vehicle control. Spermatozoa were labelled with a polyclonal antibody against 19S regulatory particle subunit PMSD4 to screen for proteasome modifications, and lectin PNA for outer acrosomal membrane integrity assessment during capacitation. Representative epifluorescence image of 19S regulatory particle and PNA labelling is from FlowSight image gallery. (a') Scatter plot representation of PMSD4 and PNA double labelled spermatozoa, representative of each treatment step, before and after capacitation with proteasomal inhibitors, including vehicle control. Every scatter plot represents 10,000 properly focused single cell flow cytometric events. The experiment was replicated twice with comparable results.

Fig. 4.
(a) WB detection of 19S regulatory particle subunit PMSD4 in sequentially isolated proteins extracted from ejaculated and capacitated spermatozoa under proteasome permissive/ inhibiting conditions and vehicle control. Red rectangles highlight protein bands with differential density in capacitated spermatozoa with vs. without proteasomal inhibition; such bands became more evident when the blot was overexposed (inset, representing densitometric measurement of bands detected above 179 kDa in OBG fraction) (a') PVDF membrane stained with CBB after chemiluminescence detection shows comparable protein loads per lane within each treatment, (a'') residual gel after electrotransfer for protein load

normalization purposes. Proteins were resolved on 8–20% gradient gel under non-reducing conditions, and protein equivalent of 20 million spermatozoa was loaded per single lane. The experiment was replicated twice with comparable results.