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Original article

Organization of planar rafts, caveolae and steroid receptors on spermatozoa during development

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ABSTRACT

Sperm membranes undergo progressive structural and functional modifications during epididymal maturation, capacitation and acrosome reaction. Though sperm membranes have raft microdomains, our understanding on the reorganization of membrane rafts during these events is limited. Using caveolin1 (CAV1) and ganglioside GM-1 as markers of membrane rafts, we studied the organization of sperm membrane rafts during epididymal maturation, in vitro capacitation and progesterone-induced acrosome reaction. We also evaluated the effect of raft disruption by methyl beta cyclodextrin on sperm hyperactivation, progesterone induced acrosome reaction and sperm-zona pellucida binding in vitro. CAV1 and ganglioside GM-1 showed transient non-overlapping localization pattern on sperm head during the intermediate phase of epididymal maturation and ended with co-localization on the acrosomal region toward the advanced stages of epididymal maturation and subsequent capacitation. CAV1 was excluded from sperm membrane rafts during capacitation and acrosome reaction. Progesterone receptor (PR) was present in the caveolar rafts of spermatozoa from testis, caput epididymidis and corpus epididymidis, but was not detected in that from cauda epididymidis. Further, capacitation was associated with the appearance of PR in non-caveolar rafts. Estrogen receptors ERα and ERβ, which were located in the caveolar rafts of spermatozoa from testis and epididymides, appeared in the non-caveolar rafts during capacitation and acrosome reaction. It appears that PR, ERα and ERβ were sequestered in the caveolar rafts of spermatozoa during epididymal maturation, and the exit of CAV1 from the membrane rafts overlying the acrosome during capacitation might free them from the sequestration that might trigger PR into action.

1. Introduction

Membrane rafts are ordered sphingolipids and cholesterol enriched microdomains which function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction.[1] Membrane rafts are reported in the sperm membrane[2] and have been proposed to play important roles during sperm capacitation, acrosome reaction and sperm oocyte fusion. It is known that sperm membranes undergo physico-chemical, structural and functional modifications during epididymal maturation and capacitation.[3] The distribution and reorganization of membrane rafts during capacitation have been reported in boar,[4] human,[5] mouse[6] and porcine[7] spermatozoa. Membrane raft proteome underwent changes in composition affecting sperm motility, ability to penetrate the zona pellucida (ZP), ZP responsiveness, and other capacitation-dependent changes.[8] Protein acquisition during epididymal maturation is proposed to be mediated by membrane rafts.[9]

Using two well known membrane raft markers, viz., Caveolin 1 (CAV1) and ganglioside GM-1, we studied the organization and changes in the distribution of sperm membrane rafts during epididymal maturation, in vitro capacitation and progesterone-induced acrosome reaction. As methyl-beta cyclodextrin (MBCD) is known to extract cholesterol from cell membranes[10,11] that disrupts membrane rafts, we also evaluated the effect of MBCD on sperm hyper-activation, acrosome reaction and sperm-oocyte interactions. Since progesterone induces acrosome reaction in capacitated spermatozoa, acting through its membrane receptors, activating a rapid non-genomic signaling cascade, we further examined the association of steroid hormone receptors with sperm membrane rafts during the functional sperm development.

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2. Materials & methods

2.1. Reagents

Estrogen, progesterone, dimethyl sulfoxide (DMSO), trizma hydrochloride, FITC-conjugated cholera toxin B (FITC-CTB) subunit, methyl beta cyclodextrin (MBCD) and hyaluronidase (sheep testes type II) were purchased from Sigma Chemical Company, Milwaukee, WI. Analytical grade sucrose for density gradients was from Hi Media Laboratories, Mumbai, India. Triton X-100 was from Amersham Life Sciences, Cleveland, OH. DC Protein Assay Kit was purchased from Bio-Rad Laboratories, Hercules, CA. Rabbit polyclonal CAV1-1 (N-20), Estrogen receptor alpha (ERα) (MC-20), Estrogen receptor beta (ERβ) (H-150), Mouse monoclonal Progesterone receptor (PR) (C-262), GAPDH (G9), Goat anti Rabbit IgG-HRP, Goat anti Mouse IgG-HRP, Goat anti Rabbit IgG-FITC and Goat anti Mouse IgG-FITC were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. All the other reagents were purchased from local suppliers and were of analytical grade.

2.2. Sperm preparation

Testes from adult goat (*Capra hircus*) were obtained from local slaughterhouses. All procedures involving animals were approved by the Institutional Animal Ethics Committee of Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India. Testis, caput epididymidis, corpus epididymidis and cauda epididymidis were excised and minced in 5 ml Hank's balanced salt solution (HBSS) (KCl – 5.36 mM, KH_2PO_4 – 0.44 mM, NaCl – 136.8 mM, $NaHCO_3$ – 4.1 mM, Na_2HPO_4 – 0.63 mM, Glucose – 5.5 mM, pH 7.2) and the spermatozoa were allowed to disperse for 10 min at 37 °C. The sperm suspension was filtered through a Nitex membrane (80 μM) and sperm were washed twice by pelleting at 500 × *g* for 10 min.

In vitro capacitation was induced by incubating an aliquot of the cauda epididymidis spermatozoa in 2 ml Kreb's ringer bicarbonate (KRB) buffer (sodium lactate 60% w/v −0.55 ml/100 ml, sodium pyruvate – 0.409 mM, NaCl – 94 mM, KCl – 4.6 mM, $CaCl_2$ – 1.27 mM, KH_2PO_4 – 1.19 mM, $MgCl_2{\cdot}7H_2O$ – 1.45 mM, $NaHCO_3$ – 11.5 mM, Glucose – 5.5 mM, BSA – 4 g/L, pH 7.4) for 3 h at 37 °C. At the end of incubation, capacitation was assessed by observing hyperactivated motility.[12] Acrosome reaction was induced by treating the capacitated sperm with 3.14 μM progesterone (final concentration) for 1 h.[12] The progesterone treated sample was assessed for incidence of acrosome reaction by FITC Con-A assay.[13]

2.3. Raft isolation

The spermatozoa were resuspended in 2 ml cold TNE buffer (25 mM TrisHCl, 150 mM NaCl, 5 mM EDTA, pH 7.5) containing 1% Triton X-100, 0.01% PMSF and Complete Mini Protease inhibitor tablet (Roche Diagnostics, Mannheim, Germany). After incubation for 30 min at 4 °C, the sperm suspension was homogenized (5 strokes of 30 s duration, each at 5000 rpm) using a Teflon-glass mechanical homogenizer (Remi Motors, Mumbai, India). The sperm homogenate (3 ml) was mixed with an equal volume of 90% sucrose solution and was overlaid with 3 ml 35% sucrose and 3 ml 5% sucrose in a 12 ml ultracentrifuge tube. The samples were centrifuged at 240,000 × *g* in a Beckman L8-80M ultracentrifuge using SW41 rotor for 15 h at 4 °C. After centrifugation, the content of the tube was fractionated into 1 ml aliquots. The fractions 3 and 4 from the top of the tube which cover the interface between 5% and 35% sucrose layers contained the detergent resistant light membrane (DRM) fraction. This fraction was used for western blot analysis, after normalizing the protein concentration following quantitation using DC protein assay kit (Bio-Rad, Hercules, CA).

2.4. SDS-PAGE and western blotting

Proteins were separated on a 12% polyacrylamide gel and electrophoresis was performed at 100 V according to the standard protocols.[14] Western blotting was performed using a mini trans-blot apparatus (BioRad, Hercules, CA) as described previously.[15] After protein transfer, the membrane was blocked in 5% non-fat skimmed milk for 1 h, washed for 5 min each in three changes of PBST and was incubated with 1:500 dilution of primary antibody in PBST for 2 h. After another series of washing as described earlier, the membrane was incubated with 1:1000 dilution of secondary antibody in PBST for 1 h. The blots were thoroughly washed and were developed by incubation in PBS containing 0.05% diaminobenzidine (DAB), 0.1% H_2O_2 and 0.04% nickel chloride until the desired contrast was obtained. The blots were imaged on a Versadoc MP-2000 gel documentation system (Bio-Rad, Hercules, CA). Intensities of bands of interest were calculated using Advanced Phoretix 1.0 (Nonlinear Dynamics, Tucson, AZ).

2.5. Immunofluorescence assay

Sperm suspension was smeared onto clean poly-L-lysine coated cover-slips, fixed by immersing the coverslips in 3% formalin for 10 min, neutralized with 0.5 M ammonium chloride, and permeabilized in 0.25% TritonX-100 for 10 min. These coverslips were incubated for 2 h in PBS containing 2 mg/ml BSA and 100 mM glycine to block the non-specific binding of cellular proteins to the primary antibodies. The coverslips were washed in 3 changes of PBS and were subsequently incubated with respective primary antibodies at a dilution of 1:200 in PBS for 2 h at room temperature. The cover slips were again washed 3 times in PBS and were incubated with FITC-conjugated secondary antibody for 1 h in dark. The cover slips were washed with PBS and stored in dark till imaging. For imaging, the cover slips were mounted on a clean slide and the immunofluorescence images were taken on a Leica TCS SP-II AOBS system confocal laser scan microscope (Leica TCS SP-II AOBS system, Wetzlar, Germany).

The distribution of ganglioside GM1 on sperm surfaces was visualized using FITC-conjugated Cholera toxin b (CTB-FITC) following published protocols.[2] Briefly, the sperm suspension was incubated with CTB-FITC at 16 °C for 40 min followed by fixation in 3% formalin for 10 min. Thereafter, the spermatozoa were washed by pelleting at 500 × *g*, resuspended in PBS and spread on the Poly-L lysine coated slides. Fluorescence images were acquired on a Leica TCS SP-II AOBS confocal microscope (Leica TCS SP-II AOBS system, Wetzlar, Germany).

2.6. Methyl beta cyclodextrin treatment

1 ml aliquots of 50×10^6 cauda epididymis sperm were treated with 1 mM and 10 mM MBCD dissolved in HBSS for 30 min at 37 °C. An equal number of spermatozoa incubated in HBSS in the absence MBCD (0 mM MBCD) served as control. After incubation, the spermatozoa were pelleted by centrifugation at 800 × *g* for 10 min. The pellet was reconstituted in KRB and was incubated for 3 h at 37 °C in a CO_2 incubator to induce capacitation. The incidence of capacitation was assessed by counting the sperm showing hyperactivated motility. Only those preparations in which the incidence of capacitation was greater than 90% were taken forward for induction of acrosome reaction. Acrosome reaction was induced by treating the capacitated spermatozoa with 3.14 μM of progesterone for 1 h and acrosome reaction was assessed by ConA-FITC binding assay.[13]