Animal Reproduction Science 145 (2014) 29–39

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Thiol-disulfide proteins of stallion epididymal spermatozoa

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ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form
14 December 2013
Accepted 17 December 2013
Available online 31 December 2013

Keywords:
Equus caballus spermatozoa
Sperm maturation
Sulphydrylated proteins
Thiol oxidation

ABSTRACT

Thiol groups of cysteine residues represent redox centers involved in multiple biological functions. It has been postulated that changes in the redox status of mammalian epididymal spermatozoa contribute to the sperm maturation process. The present work shows the thiol-disulfide protein profile of stallion epididymal spermatozoa achieved by two-dimension electrophoresis and MALDI-TOF/TOF mass spectrometry of proteins labeled with a thiol-reactive fluorescent tag, monobromobimane. Our results have shown the formation of disulfide bonds in several sperm protein fractions during the epididymal maturation process. The majority of the oxidized thiol sperm proteins identified correspond to structural molecules of the flagellum (as the outer dense fiber-1 protein – ODF1), followed by glycolytic enzymes (as glyceraldehyde-3-phosphate dehydrogenase spermatogenic), antioxidant protectors (as glutathione S-transferase and phospholipid hydroperoxide glutathione peroxidase – PHGPx). The magnitude of the thiol oxidation differs between proteins, and was more drastic in polypeptides with molecular weights of up to 33 kDa, identified as ODF1 and PHGPx. A kinase anchor protein, a voltage-dependent anion channel protein and a zona pellucida-binding protein were also found in the polypeptide samples that contained oxidized —SH groups. These proteins may be modified or controlled by the mechanisms involved in the cysteine-redox changes, corroborating the belief that a correct degree of protein oxidation is required for the stabilization of sperm structure, protection against oxidative damage, induction of progressive sperm motility and fertilization.

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1. Introduction

The thiol groups of cysteine residues represent redox centers involved in protein structure, enzyme catalysis, signal transduction and regulation of the transcriptional activity of different cell types (Barford, 2004; Fomenko et al., 2008; Jones, 2010). The redox-based regulation of gene expression has emerged as a fundamental regulatory mechanism in cell biology. A variety of proteins, including transcription factors, molecular chaperones, protein tyrosine phosphatases and protein tyrosine kinases, are activated or regulated via redox processes. Conformational changes and/or other post-translational modifications may result from the oxidation of thiols within the protein itself or belonging to interacting proteins, thus mediating diverse responses according to the cell physiological state (Jones, 2010; Monteiro et al., 2008; Sen, 2000).

During the epididymal transit, spermatozoa complete a maturation process that enables these cells to interact with the oocyte at the appropriate time and place. This process is the result of a complex cascade of post-translational changes that involve the rearrangement of the sperm membrane components, the induction of chromatin condensation, the stabilization of the tail components, the processing of different proteins and others modifications

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0378-4320/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.anireprosci.2013.12.007

that lead to the acquisition of motility and the development of the fertilization potential (Cornwall, 2009). It has been postulated that a correct degree of protein —SH oxidation is required not only for these maturation events, but also to prevent oxidative damage that can leads to infertility (Aitken and Vernet, 1998; Dias et al., 2006; Seligman et al., 2005, 2004; Shalgi et al., 1989).

In stallions, we have demonstrated differences in the nuclear and plasma membrane characteristics of spermatozoa obtained from the caput, corpus and cauda regions of the epididymis (Dias et al., 2006; López and De Souza, 1991; López et al., 1987, 2007; Retamal et al., 2000, 2012). The highly packed chromatin of the mature sperm results from changes in the thiol-disulfide status of this structure. The nuclei of immature sperm cells, obtained from the *ductuli efferentes*, contain mostly thiol groups belonging to cysteine residues, while in mature cells, obtained from the cauda epididymides, these groups are oxidized. Concomitantly, a reduction in the Zn^{2+} content which favors the oxidation of the —SH groups was observed. Thus, Zn^{2+} binding sites of sulphydrylated proteins could be related with the redox sensitivity of the protein (Dias et al., 2006). Recently, it has been suggested that zinc can occupy multiple sites in redox sensitivity proteins, increasing the susceptibility of zinc coordinating cysteine residues to oxidation (Heo et al., 2013).

Although there is some evidences of a functional role for the cysteine modifications, the identity, the significance of most protein thiol alterations and the pathway involved in the physiological sperm thiol oxidation in the epididymis remain unknown. The present work describes the thiol-disulfide protein profile of stallion epididymal spermatozoa achieved by two-dimension gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization time of flight/time of flight (MALDI TOF/TOF) mass spectrometry. The redox changes of cysteine residues of sperm proteins occurring during the epididymal transit are highlighted.

2. Materials and methods

2.1. Chemicals

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA) and Merck AG (Darmstadt, Germany). Reagents for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA).

2.2. Sample collection and processing

The epididymides were obtained by surgical castration from six healthy mature stallions, aged between 3 and 8 years old, according procedures described in McKinnon et al. (2011). The connective tissue and superficial blood vessels surrounding the epididymis were removed through careful dissection. The caput, corpus and cauda epididymal regions were separated, gently minced and squeezed in phosphate buffered saline (PBS) pH 7.2. The luminal content released from each epididymal region was centrifuged at 760 × *g* for 30 min (4 °C). The pellets obtained were resuspended with an erythrocyte lysis solution (0.2 M NH_4Cl, 0.1 M $KHCO_3$, 0.1 M EDTA), incubated in an ice bath for 5 min and centrifuged (760 × *g* for 10 min at 4 °C). The supernatants above the sperm pellets were removed and discarded. Afterward, the spermatozoa were gently resuspended in PBS and centrifuged again like described above. This washing procedure was done three times before the subsequent processing.

2.3. Labeling of the thiol groups with mBBr

Epididymal sperm samples were treated with monobromobimane (mBBr) (Calbiochem, San Diego, CA, USA) following a previously described protocol (Dias et al., 2006; Kosower and Kosower, 1987). This procedure allows the determination of the —SH and S—S bonds (after the reduction of S—S by dithiothreitol [DTT]). Briefly, the washed epididymal spermatozoa were incubated in PBS with and without 1 mM DTT for 10 min. Afterward, the sperm cells were incubated with 2 mM of mBBr-solution (prepared from a stock solution of 50 mM mBBr in acetonitrile [ACN]) for 20 min and washed twice in PBS.

2.4. Protein extraction and gel electrophoresis assays

Sperm proteins were obtained by sonication in a 5% sodium dodecyl sulfate (SDS) and 1 mM DTT (1:2) solution for one-dimension polyacrylamide gel electrophoresis (1D-PAGE), and in a 20 mM Tris–HCl, 4% triton X-100, 8 M urea, and 30 mM DTT solution for 2D-PAGE assays. After the addition of protease inhibitors (10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 μM bestatin hydrochloride), the samples were sonicated at 10 W with a Sonic Dismembrator (model F60-Thermo Fisher Scientific, Rockford, IL, USA) for 10 cycles of 30 s in an ice bath, and then centrifuged (15,000 × *g*) for 30 min at 4 °C. The supernatant containing the solubilized sperm proteins was stored at −70 °C for further analysis.

1D-PAGE: Equal amounts of the thiol-labeled sperm proteins from the caput, corpus and cauda epididymal regions were subjected to 12–20% denaturing gradient gel electrophoresis (DGGE) under reducing (10% β-mercaptoethanol) conditions. The electrophoresis was performed in mini-gels using the Mini-Protean III system (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, the gels were thoroughly washed in distilled water and photographed using a UV-light transilluminator (312 nm) coupled to a photographic camera. The gels were stained with Coomassie brilliant blue (CBB) in 40% v/v methanol and 10% v/v acetic acid and then destained in the same solution without CBB. Molecular weight marker kits (Bio-Rad Laboratories, Hercules, CA, USA), ranging from 6.5 to 200 kDa, were used as standards. The relative amount of protein was quantified by densitometry, using a computational program, from a TIFF gray image obtained with a commercial scanner at 400 dpi. The "Gel Perfect" software calculated the relative mobility of each stained band and the area occupied by it, and provided a diagrammatic presentation of the protein bands and their relative concentrations in relation to the total protein content by lane (Retamal et al., 1999).