Serine/threonine phosphorylation associated with hamster sperm hyperactivation

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Background and Aims: Mammalian sperm activation and hyperactivation is regulated by protein phosphorylation. Although tyrosine phosphorylation is considered very important, several studies have investigated whether serine and threonine phosphorylation are also associated with sperm activation and hyperactivation, and that was also the aim of the present study.

Methods: Protein phosphorylation of hamster spermatozoa was detected by Western blotting using antiphospho-amino acid monoclonal antibodies after tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid sequences were analyzed using a peptide sequencer.

Results: Four proteins were phosphorylated at serine residues during hyperactivation via activation and their approximate molecular weights were 90, 38, 32 and 10 kDa, respectively. Five proteins were phosphorylated or dephosphorylated at threonine residues and their approximate molecular weights were 90, 70, 65, 35 and 10 kDa, respectively. The 10-kDa protein corresponded to a previously reported 10-kDa tyrosine phosphoprotein. N-terminal sequences of the 10-kDa protein were similar to carcinustatin, which is a neuropeptide.

Conclusions: During hyperactivation, four serine phosphorylation and five threonine phospho- or dephosphorylations occurred, which suggested that the 10-kDa protein was phosphorylated at tyrosine residues when spermatozoa were activated and then dual-phosphorylated at the serine and threonine residues during hyperactivation. (Reprod Med Biol 2004; 3: 223–230)

Key words: dephosphorylation, hamsters, hyperactivation, phosphorylation, spermatozoa.

INTRODUCTION

Ejaculated mammalian spermatozoa are not able to fertilize eggs1 until they become capacitated, which occurs after activation. Activated spermatozoa can be prepared in vitro by adding activating factors, such as calcium and/or bicarbonate, and are moved progressively with a high-beat frequency.1–3 Capacitated spermatozoa are prepared in vitro by adding calcium, albumin, glucose, etc., and capacitation involves an acrosomal reaction in the sperm head, which is very important for fertilization, and hyperactivation of motility,1 which comprises a large bend amplitude, whiplash and frenzied flagellar movements.4–6

The most recent understanding is that the changes in sperm motility are closely associated with phosphorylation of flagellar proteins,3 particularly serine, threonine and tyrosine; for example, 175-, 93-, 44-, 40-, 38-, 20-kDa proteins have been detected in boar sperm,7 90-, 80-, 62-, 48-kDa proteins in hamsters8,9 and 190-, 110-, 94-, 43–55-, 35-, 18-kDa proteins in humans.10 From these results, it has been assumed that tyrosine phosphorylation plays an especially important role in the hyperactivation of mammalian spermatozoa.1,7,8,11–13

The phosphorylation at the tyrosine residues of a flagellar protein, A-kinase anchor protein (AKAP), has also been reported in detail.14 AKAP binds to the RII subunit of A-kinase15,16 and is a component of the fibrous sheath.17,18 It has been suggested that the phosphorylation of AKAP is regulated through calcium-calmodulin dependent signal transduction,14 and it has been demonstrated that the phosphorylation of AKAP is also regulated by protein phosphatase.8 However, the regulatory mechanism of capacitation is still unclear.

Because we had investigated tyrosine phosphorylation in a previous study,9 in the present experiment we investigated serine and threonine phosphorylation associated with motility activation and hyperactivation in hamster spermatozoa.
MATERIALS AND METHODS

Reagents

Adenosine triphosphate, cyclic AMP, antiphosphoserine monoclonal antibody and antiphosphothreonine monoclonal antibody were purchased from Sigma (St Louis, MO, USA). A Histofine SAB-PO (M) kit was purchased from Nichirei (Tokyo, Japan). Other chemicals of reagent grade were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of spermatozoa

Spermatozoa were obtained from the cauda epididymis of sexually mature (12–16 week old) male golden hamsters (Mesocricetus auratus) as described previously.19 Immotile spermatozoa were prepared according to our previous method.19 In brief, one volume of cauda epididymal spermatozoa was suspended directly in 10 volumes of ice-cold pure water, and then homogenized in 50 volumes of homogenizing buffer (200 mmol/L sucrose, 25 mmol/L glutamic acid, 25 mmol/L KOH and 20 mmol/L Tris-HCl [pH 7.9]) using a Teflon homogenizer. Activated and hyperactivated spermatozoa were prepared according to the methods described previously, using modified Tyrode's albumin lactate pyruvate (m-TALP) medium.20 An aliquot of cauda epididymal spermatozoa was placed in a test tube, several milliliters of m-TALP medium was then carefully added and the mixture was overlayed with mineral oil. The tubes were incubated for 10 min to allow the activated spermatozoa to swim up. The supernatant containing activated spermatozoa was collected and homogenized in 50 volumes of the homogenizing buffer using a Teflon homogenizer. Spermatozoa exhibiting approximately 90% motility were used in the experiment (Table 1).

Table 1 Motility, activation and hyperactivation of hamster spermatozoa

<table>
<thead>
<tr>
<th></th>
<th>Motility (%)</th>
<th>Activation (%)</th>
<th>Hyperactivation (%)</th>
</tr>
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<tbody>
<tr>
<td>Activated</td>
<td>95</td>
<td>93</td>
<td>0</td>
</tr>
<tr>
<td>Hyperactivated</td>
<td>91</td>
<td>12</td>
<td>83</td>
</tr>
</tbody>
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Preparation of demembranated sperm flagella

Demembranated sperm flagella were prepared from immotile, activated and hyperactivated spermatozoa according to the method described previously.19 Each sperm suspension prepared as described above was centrifuged at 5500 g for 5 min at 4°C. The pelleted spermatozoa were suspended in 100-fold of the homogenizing buffer and after centrifugation at 750 g for 5 min at 4°C, the precipitate was resuspended in a 20-fold volume of the homogenizing buffer supplemented with 2 mmol/L phenylmethanesulfonyl fluoride (PMSF) and 20 µg/mL leupeptine. The suspension was homogenized with 100 strokes of a Teflon homogenizer in order to separate flagella from heads. The homogenate was then diluted in a fourfold volume of the homogenizing buffer supplemented with 0.5 mmol/L PMSF and 5 µg/mL leupeptine. After centrifugation at 750 g for 5 min at 4°C, the supernatant was collected and centrifuged at 5500 g for 5 min at 4°C. The precipitate, which contained isolated flagella, was suspended in a 20-fold volume of demembranation buffer containing 200 mmol/L sucrose, 25 mmol/L glutamic acid, 25 mmol/L KOH, 1 mmol/L dithiothreitol, 0.1% (w/v) Triton X-100 and 20 mmol/L Tris-HCl (pH 7.9), and incubated for 30 s at room temperature. The flagellar suspension was centrifuged at 5500 g for 5 min at 4°C. The protein concentration of the pelleted flagella was determined by the method of Bradford and adjusted to a final protein concentration of 1 mg/mL with homogenization buffer.