Molecular Biology Reports **30:** 165–172, 2003.
© 2003 *Kluwer Academic Publishers. Printed in the Netherlands.*

Mouse lipocalin as an enhancer of spermatozoa motility

Ying-Chu Lee[1], Chi-Jr Liao[2], Pei-Tzn Li[3], Woan-Fang Tzeng[2] & Sin-Tak Chu[1,4]
[1]*Institute of Biological Chemistry, Academia Sinica and* [2]*Department of Life Science, Fu-Jen Catholic University,* [3]*Institute of Biological Science, College of Science, National Taiwan University, Taipei, Taiwan;* [4]*Author for correspondence: Institute of Biological Chemistry, Academia Sinica, and P.O. Box 23-106, Taipei, Taiwan (Phone: 886-2-23620261 Ext. 4531; Fax: 886-2-23635038; E-mail: stc316@gate.sinica.edu.tw)*

Accepted 4 February 2003

Key words: acrosome reaction, capacitation, intracellular pH, spermatozoa motility, tyrosine phosphorylation

Abstract

The 24p3 protein is a 25 kDa glycoprotein that is secreted into the uterine fluid during the proestrous phase of mice. We assessed the effects on spermatozoa motility and on the functions of mouse spermatozoa using the computer-assisted sperm analysis method, cytochemical staining and detection of the protein tyrosine phosphorylation pattern. Compared with the control cells, sperm motility was stimulated by the addition of 24p3 protein into the medium. Introducing 24p3 protein enhanced progressive motility but did not promote the appearance of hyperactivated movement. The presence of 24p3 protein in the medium did not allow the cells to undergo the capacitated protein tyrosine phosphorylation pattern and acrosome reaction. The tyrosine phosphorylation pattern shows phosphoproteins in the range of Mr 50000–106000 correlated with the sperm progressive motility after the addition of 24p3 protein into the medium. Using flow cytometry, we assessed the changes in the intracellular pH and measured the intracellular cAMP concentration with an immunodetection kit. The results indicated that the elevation in intracellular pH from 6.67 to 6.89, increase of intracellular cAMP accumulation, and protein tyrosine phosphorylation might be the factors in enhancement of sperm motility as the 24p3 protein bound to the spermatozoa. The 24p3 protein may have a role in regulating flagellar motility.

Abbreviations: BCECF AM – 2,7′-bis-(2-carboxyethyl)-5-(-6)-carboxyfluorescein tetraacetoxymethyl ester; BSA – bovine serum albumin; CASA – computer-assisted sperm assay; DES – diethylstilbestrol dipropionate; ECL – enhanced chemiluminescence; NGAL – neutrophil gelatinase associated lipocalin.

Introduction

24p3 cDNA was originally cloned from cultured mouse kidney cells, infected with a polyoma virus or simian virus-40 [1]. Protein derived from 24p3 cDNA is classified as a member of the lipocalin family [2,3] and has been found in lipopolysaccharide-stimulated mouse PU5.1.8 macrophage cells [4] and bFGF-stimulated 3T3-fibroblasts [5]. As stated by Liu and Nilsen-Hamilton [6], the 24p3 protein is an acute-phase protein detected in the liver. Thus, it has been suggested that the 24p3 protein has important biological significance. The 24p3 protein also shows a high degree of similarity to the human neutrophil gelatinase associated lipocalin (NGAL), which exists on the uterus. The two proteins have 70% similar identities [7,8]. Lipocalins are a large and diverse group of small, extracellular, hydrophobic molecule transporting proteins. Several lipocalins have been implicated in the modulation of cell growth, metabolism and cell regulation [9]. Indeed, many lipocalins need their biological function to be elucidated, and an understanding of the particular specialized function of each individual lipocalin should be rewarding. The results of our previous studies have demonstrated that mouse uterine fluid contains a group of secretory proteins including the 24p3 protein, some of which are produced in the pro-estrus phase of the estrous

cycle [10,11]. Chu et al. [11] found that antiserum against the 24p3 protein was immunoreactive with an estrogen-stimulated glycoprotein found in the mouse uterus and immunoreactive to a caput-initiated protein found in the mouse epididymis [12]. The 24p3 protein is unique to the reproductive organ of the male mouse caput. *In vivo*, the 24p3 protein progressively vanishes from mouse spermatozoa during epididymal transit [12] but is associated with the surface of caudal spermatozoa *in vitro*. The binding of the 24p3 protein to these spermatozoa may be why it is secreted from the uterus during the proestrous phase. Thus, when ejaculated spermatozoa with no 24p3 protein bound enter the genital tract, the 24p3 protein will associate with the sperm surface. Despite the possible physiological significance, a significant biological function for this protein in the reproductive tract has not been reported to date.

Sperm motility is considered one of the major criteria in semen analysis that predicts male fertility [13]. To ensure successful fertilization, capacitation, hyper activation and the acrosome reaction must occur sequentially [14,15]. In order to understand the biological functions of the 24p3 protein on sperm activity, it is necessary to assess sperm motility and physiological changes associated with it. The biochemical events that have been identified during spermatozoa motility initiation include intracellular pH elevation, intracellular cAMP accumulation [16], and protein tyrosine phosphorylation [17]. Knowing this, we conducted *in vitro* tests assessing motility, capacitation and the acrosome reaction by examining the significance of sperm tyrosine phosphorylation, intracellular pH, and cAMP concentration to further define the mechanisms of the actions of the 24p3 protein. In this report, we are the first to identify the significant effects of lipocalin on spermatozoa activities.

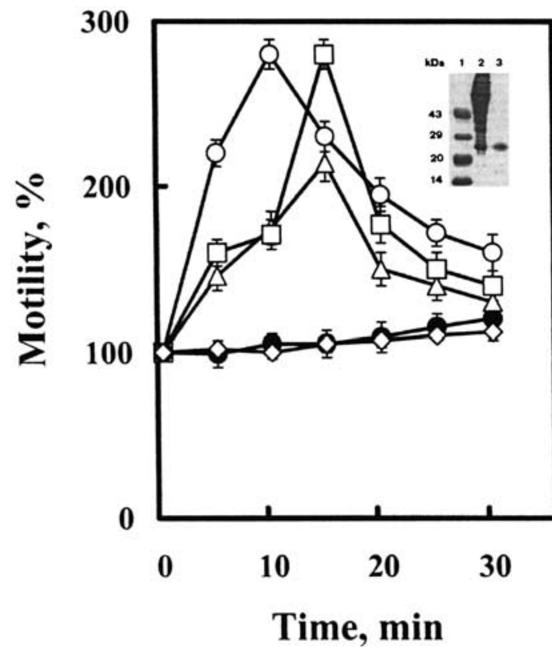

Figure 1. Mouse spermatozoa motility in 24p3 protein medium at various times. Fresh prepared spermatozoa from 10 mice that had been dispersed into the HM medium (10^7 cells/ml) at 37 °C. Spermatozoa motility was observed in the absence or presence of the 24p3 protein or BSA. The cell motility was determined after incubation and was expressed as a percentage of control cell motility measured at zero time incubation. Control, ●; 10 μM 24p3 protein, △; 20 μM 24p3 protein, □; 40 μM 24p3 protein, ○; 0.3% BSA, ◊. Points are means ± SEM for 10 replicate experiments (n=10; $p<0.01$). In right corner of the figure is the image of the purified 24p3 protein. Lane 1, protein markers; lane 2, crude uterine luminal proteins; lane 3, purified 24p3 protein.

Materials and methods

Materials

The bovine serum albumin (BSA, fatty acids free) and diethylstilbestrol dipropionate (DES) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). The antiphosphotyrosine monoclonal antibody (clone 4G10) was acquired from UBI Co. (Lake Placid, NJ, USA). An Enhanced Chemiluminescence plus kit (ECL) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). BCECF AM (2,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein tetraacetoxymethyl ester) were obtained from Molecular Probes (Eugene, Ore). The Biotrak™ cAMP competitive enzyme immunoassay system was bought from Amersham Pharmacia Biotech (Germany). All chemicals were of reagent grade.

Animals

Outbreed ICR mice were purchased from the Charles River Laboratories (Wilmington, Mass. USA). They were bred in the animal center at the College of Medicine (National Taiwan University, Taipei, Taiwan). Animals were treated in accordance with the institutional guidelines for care and use of experimental animals. The test animals were kept under controlled lighting (14 h light/10 h dark) at a constant temperature (23±2 °C) with a supply of water and NIH 31 laboratory mouse chow *ad libitum*. The 24p3 protein

was purified from the uterine fluid of DES-stimulated 3-week-old female mice as previously described [11]. The purified 24p3 protein was shown in figure 1 and the protein purity indicates in single band. Adult male mice (12–16 weeks) were killed by cervical dislocation in order to provide the spermatozoa.

Preparation of spermatozoa

The culture medium for the sperm preparation was a modified HEPES medium (HM), as previously described by Lee and Storey [18]. The modified HM medium consisted of 120 mM NaCl, 2 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 0.36 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, 5.6 mM glucose, 1.1 mM sodium pyruvate, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. The pH of all media were adjusted to 7.2 by aeration with humidified air/CO_2 (19:1) in an incubator at 37 °C for 24 h prior to use. The caudal spermatozoa were removed from this segment of the epididymis because the spermatozoa from this region lack the 24p3 protein [12]. After the spermatozoa were separated from the connective tissue, the spermatozoa were extruded from the distal portion of the tissue at 37 °C for a period of 10 min. The spermatozoa were filtered through nylon gauze and washed with HM medium twice and collected using centrifugation at 60 $\times$ g for 10 min at room temperature. Cell pellets were resuspended in HM medium for further study.

Measurement of sperm motility

When using a mouse model, characteristics of the trajectories of single spermatozoon have been studied and various swimming patterns have been described [19,20]. The motility and movement characteristics of the spermatozoa were assessed following incubation in medium under several different conditions. The sperm concentration was adjusted to approximately 10^7 cells/ml in the medium. A 10 μl sample was placed in a 20 μm deep Dual Sided Sperm Analysis Chamber (2X-CEL) at 37 °C. The analyzer was set as follows: negative phase-contrast optics and recording set to 60 frames/sec, minimum contrast set at 40, minimum cell size was set to four pixels, low-size gate set to 0.2, high-size gate set to 1.5, low intensity gate set to 0.5, high intensity gate set to 1.5, non-motile head size set to 29, non-motile head intensity set to 76, medium average path velocity (VAP) was adjusted to 50 μm/s and the low VAP to 7.0 μm/s, slow motile cells were selected, and threshold STR set to a level greater than 80%. The motile sperm is expressed in percentage (%). Fifteen fields were assessed for each sample. The parameters relating to the spermatozoa motility were analyzed using computer-assisted sperm assay (CASA) with a sperm motility analyzer (IVOS version 10; Hamilton-Throne Research, Beverly, Mass, USA). The sperm movement characteristics measured included curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN; VSL/VCL) and straightness (STR; VSL/VAP).

Cytological observation

For the Coomassie blue staining method, the spermatozoa were air-dried (on a glass slide) and dual washed with PBS (5 mM Na-phosphate buffer containing 150 mM NaCl, pH 7.4) before cytochemical staining. The slides were immersed in 3.5% percholoric acid containing 0.04% Coomassie blue G-250 for 5 min to manifest the intact acrosomes of the spermatozoa, according to previously described methods [21, 22]. The slides were twice moderately washed with PBS and covered with PBS/glycerol (1:1 by volume) before observation. This was carried out by observation under a microscope (Olympus, Tokyo, Japan).

Flow cytometric analysis

The spermatozoa were loaded with intracellular fluorescent probe before intracellular pH measurement. The ester form of BCECF (5 μM BCECF AM) was added to the washed spermatozoa suspension (5×10^8 cells/ml). This was then incubated in the dark at room temperature for 30 min under 5% CO_2 in air and 100% humidity. After the incubation, the suspension was then layered over 0.8 ml of 75% Percoll medium and centrifuged for 30 min at 275 $\times$ g to remove the extracellular BCECF AM. The spermatozoa layer was harvested and washed twice with HM medium. The preliminary experiments showed that the spermatozoa remained stable and unchanged for several hours. Analysis was performed on a COULTER® EPICS® XL™ flow cytometry (Beckman-Coulter company, Fla, USA). The fluorescence was excited at 488 nm and measured via a 525 nm filter. PMT voltages and gains were set to maximize the dynamic range of the signal. The fluorescence intensity of the BCECF and proton complex was quantified for 5000 individual spermatozoa. The intracellular pH was calculated according to the

method of Màriàn et al. [23] and Cross et al. [24] with BCECF loaded spermatozoa.

Cyclic AMP measurement

The intracellular cAMP of the spermatozoa was determined using a cAMP enzyme immunoassay system (Amersham Pharmacia biotech). In the system, a cAMP specific antibody was immobilized on to pre-coated microplates using of a peroxidase-labeled cAMP conjugate as a legend. This assay was based on competition between the unlabeled cAMP and a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites on a cAMP-specific antibody. The spermatozoa ($2x10^7$ cells/ml) were lysed using 0.25% dodecyltrimethyl ammonium bromide in assay buffer at 4 °C for 15 min with mild rotating. Then the cell debris was removed by centrifuging at $14,000 \times g$ for 10 min. After obtaining 100 μl (10^6 cells/well), the extracted cAMP was processed using the enzyme assay system. The blue color that developed was read at 630 nm. The cAMP contents of the samples were extrapolated from a standard curve previously generated by dilution of known cAMP standards. Total cAMP measurements were expressed in fmole/10^6 cells. The assay and method of calculating the results followed the Biotrak cellular cAMP assay protocols.

Detection of protein tyrosine phosphorylation of the spermatozoa

After incubation of the spermatozoa in the HM medium under different conditions, the soluble fraction of the cell lysate was prepared according to the methods described by Visconti et al. [25]. Resolution of the soluble protein components of the cell lysate on SDS-PAGE was performed using 8% polyacrylamide gels (12.0 × 10.0 × 0.075 cm), according to the methods described by Laemmli [26]. The proteins of the cell lysate on the gel were transferred to a nitrocellulose membrane using the electrophoretic method described in Towbin et al. [27] at 35 volts for 6 hours at 4 °C. The protein blots were immunodetected using a monoclonal antibody against phosphotyrosine as the primary antibody, and antimouse IgG conjugated with HRP as the secondary antibody. The enzyme-staining bands were enhanced by chemiluminescence detection, using an ECL-plus kit (RPN2132, Amersham Pharmacia Biotech UK Limited) according to the instructions provided and then exposed with X-ray film.

Results

Effects of the 24p3 protein on the spermatozoa movement characteristics

Comparisons of the increase in motility of the mouse spermatozoa that resulted from incubation for 30 min in a culture medium, reflecting either the absence, or the presence of 24p3 protein are shown in Figure 1 and Table 1. At 5-min intervals, the percentage of the cell motility was determined using the CASA method. The spermatozoa revealed a slight variation in their motility when incubation in HM for 30 min. The 24p3 protein enhanced motility during the initial 5 min of incubation and remained increasingly active over a period of 10 min to 15 min compared with the control cells (Figure 1, ●). The increase in the mean proportion of spermatozoa motility was statistically significant over a dose range from 10–40 μM (the physiological concentration of 24p3 protein is 10–50 μM in uterine fluid of the mature female mice, data not shown) (Figure 1) ($P<0.01$). Although their relative motility reflected a consistent increase during incubation when the spermatozoa were incubated with 24p3 protein, the maximum value was achieved at 10 min (in 40 μM 24p3 protein; Figure 1, ○) or 15 min (in 10 and 20 μM 24p3 protein; Figure 1, △ and □) of incubation and a subsequent decline in motility was detected during the subsequent incubation. The adding of 0.3% BSA to the HM medium, as a negative control test, showed insignificant effects on the spermatozoa motility (Figure 1, ◊). The motility enhancement suggested that there might be a possible modification of the spermatozoa by the 24p3 protein after ejaculation into the uterine lumen.

According to Cancel et al. [28], the expected motility kinematics of hyperactivated sperm is increased amplitude of the ALH and VCL, but a decreased LIN and STR. To examine how the spermatozoa motility was affected, the movement characteristics of the control cells were compared with the spermatozoa treated using 24p3 protein (Table 1). Sperm treated with 0.3% BSA and 1.8 mM $CaCl_2$ in HM medium was positive control. Based on these characteristics, the changes in sperm motion were identified and interpreted. The greater the VCL value, the greater the circular movement of the spermatozoa had increased, and the lower the STR value, the less the forward movement of the spermatozoa had decreased. Hyperactivated (mouse) spermatozoa are defined as those where the tracks exhibited a STR of $<70\%$ and a VCL of $>200\mu$m/sec

Table 1. The effects of different media on caudal sperm motility parameters. The mean CASA values of mouse spermatozoa were present

	Incubation Period (min)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	LIN (%)	STR (%)	BCF (HZ)	ALH (μm)
control								
	0	133 ± 10	110 ± 10	167 ± 10	70 ± 7	87 ± 9	13 ± 5	24 ± 3
	10	125 ± 8	105 ± 5	167 ± 5	68 ± 6	78 ± 10	18 ± 6	19 ± 3
	20	112 ± 3	110 ± 7	170 ± 8	66 ± 5	88 ± 7	15 ± 3	25 ± 5
	30	109 ± 5	103 ± 5	139 ± 10	70 ± 7	76 ± 5	18 ± 2	20 ± 3
40 μM 24p3								
	0	140 ± 10	109 ± 7	170 ± 8	64 ± 6	78 ± 7	19 ± 8	19 ± 5
	10	145 ± 8	125 ± 7	180 ± 9	69 ± 7	85 ± 9	23 ± 7	18 ± 2
	20	148 ± 10	130 ± 7	165 ± 6	78 ± 5	88 ± 8	23 ± 5	17 ± 3
	30	130 ± 9	110 ± 8	158 ± 7	70 ± 5	85 ± 7	21 ± 5	16 ± 5
*0.3% BSA								
1.8 mM $CaCl_2$	0	140 ± 5	115 ± 5	178 ± 8	65 ± 3	80 ± 5	20 ± 8	24 ± 3
	10	140 ± 7	101 ± 6	170 ± 8	59 ± 2	72 ± 2	23 ± 6	25 ± 5
	20	155 ± 8	107 ± 5	210 ± 7	51 ± 6	69 ± 3	25 ± 7	28 ± 5
	30	191 ± 7	119 ± 7	238 ± 9	50 ± 5	62 ± 3	27 ± 5	29 ± 3

Data are expressed as means ± SEM ($n = 10$).
The abbreviations were described as follows.
The significant difference were described as follows:curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN; VSL/VCL) and straightness (STR; VSL/VAP).
*The positive vontrol was present here.

[19,20]. Table 1 shows the average values of VCL and STR for the specified conditions at 30 min. The hyperactivated sperm had the VCL and STR values of 238 ± 9 μm/sec and 62 ± 3%, respectively. The VCL values in the control cells and the 24p3 protein treated cells were verified insignificance (139±10 μm/sec and 158±7 μm/sec) and a higher STR value was retained for the control cells and the 24p3 protein treated cells (76±5% and 85±7%). During the 30 min of culture, the control cells showed no significant changes in the hyperactivation parameters. No statistically significant differences were found for the 24p3 protein incubated cells compared with the control cells for the mean values of the parameters ($p>0.05$). The values of VSL, LIN and STR progressively estimated the motility of the spermatozoa. The hyperactivated sperm have the decreased LIN value for 50±5% compared with the zero time sperm. The control cells and 24p3 protein treated sperm kept the LIN values similar to the zero time sperm (Table 1). Based on the results, the data showed that 24p3 protein enhanced the motility and maintained the progressive motility during 30 min of incubation, however it did not possess the ability to force the spermatozoa to undergo hyperactivation.

The effects of 24p3 protein on spermatozoa intracellular pH (pHi) and cAMP accumulation

Adding 24p3 protein to spermatozoa loaded with BCECF, we further characterized the effects of the protein on intracellular pH variation of the cells. Figure 2 shows that the pHi of the mouse sperm increased from 6.67±0.05 to 6.89±0.06 by adding the 24p3 protein during 10 min of incubation (Figure 2, ▲). Populations incubated in a HM medium, as the control cells, exhibited the pHi variation from 6.67±0.05 to 6.62±0.06 (Figure 2, ◊). Comparing the data with two populations was statistically significance in the pHi variation of the spermatozoa ($p<0.01$). Increases in intracellular pH may initiate the spermatozoa motility.

Since the spermatozoa motility has been shown to be associated with adenylyl cyclase [29], we determined whether the presence of the 24p3 protein in the medium altered intracellular cAMP production. When compared with the control treatment group (Figure 3, ◊, cells treated *in vitro* for 30 min with 40 μM 24p3 protein showed an increase in their cAMP accumulation by more than 2-fold ($p<0.01$) (Figure 3, ■). The data show an association of changes in cAMP accumulation with the elevation of the pHi in the spermatozoa. The results imply that the 24p3 protein elevated the

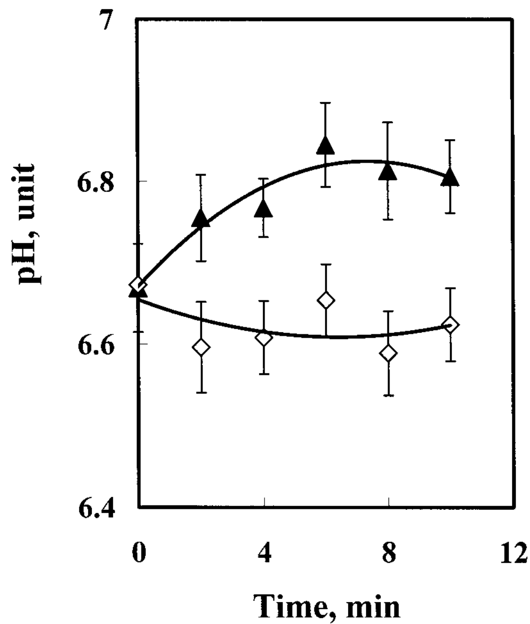

Figure 2. Time course of 24p3 protein-induced change in the intracellular pH. Spermatozoa (5×10^8 cells/ml) were loaded with BCECF prior to measurement using flow cytometry after various treatments. The intracellular proton concentrations were measured using flow cytometry after incubation in HM media or HM medium containing 40 μM 24p3 protein. Spermatozoa were incubated for 10 minutes in a medium that initiated the motility. BCECF-loaded cells were diluted in HM medium (◇) or 24p3 protein solution (▲) (final concentration is 40 μM) at t = 0 time, and the kinetics of pHi change were recorded. Points are mean ± SEM for 10 replicate experiments (n=10; p<0.01).

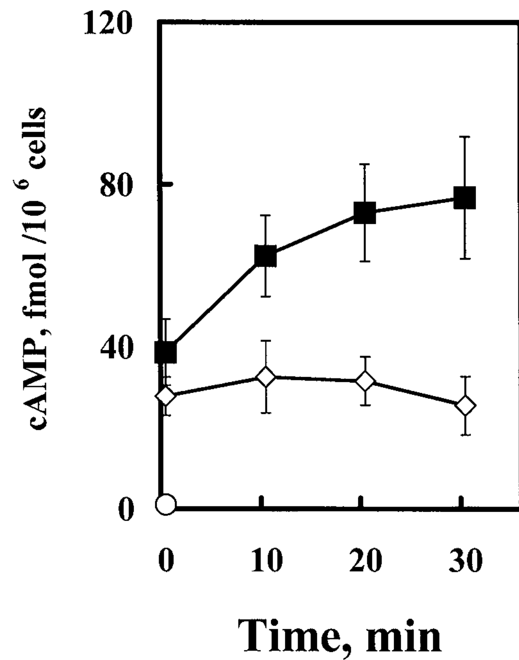

Figure 3. Intracellular cAMP accumulation in the spermatozoa. Spermatozoa (10^6 cells) were incubation in HM or 24p3 protein containing media. The intracellular cAMP content (fmole) of mouse spermatozoa from the caudal epididymis was determined during 30 min of incubation. Control, ◇; 40 μM 24p3 protein, ■. The results are presented as the mean ± SEM. (n=10, p<0.01)

pHi and increased cAMP accumulation then triggered downstream reactions.

Acrosomal reacted state of spermatozoa

The changes in the intracellular pH (pHi) of sea urchin sperm associated with motility initiation and acrosome reaction were investigated [30]. To determine whether the 24p3 protein-enhanced spermatozoa motility could be correlated with acrosome reaction of the spermatozoa, the cells were incubated in the absence or presence of 24p3 protein. The sperm in 0.3% BSA and 1.8 mM $CaCl_2$ containing HM medium as a positive control triggered the acrosome reaction about 50% during 30 min of incubation (Table 2). The acrosomes of the spermatozoa were almost all intact during this incubation period (86±5%) and the acrosome reaction did not occur in the HM medium. Spermatozoa incubated with the 24p3 protein alone resulted in maintaining the intact acrosome (90±3%) during the incubation period (Table 2). No acrosome-reacted cells with the 24p3 protein supplement medium appeared during the incubation period. This evidence suggests that spermatozoa undergo progressive motility after incubation with the 24p3 protein but cannot undergo an acrosome reaction. It might result from the absence of hyperactivation in the 24p3 protein treated cells.

Table 2. The percentage for intact acrosome of spermatozoa in different media

Time (min)	0	15	30	60
Control	96 ± 2	94 ± 3	90 ± 4	86 ± 5
40 μM 24p3 protein	97 ± 2	95 ± 4	92 ± 2	90 ± 3
0.3% BSA/1.8 mM $CaCl_2$	95 ± 3	82 ± 5	72 ± 7	50 ± 6

Freshly prepared caudal spermatozoa from 10 mice (10^7 cells/ml) were incubated in media at 37 °C for various time, Coomassie Blue staining was used to score the acrosomal status. The data were the average of five determinations. Data represent the means of six individual trials counting 200 cells/ treatments pretrial, and error bars represent the SEM.

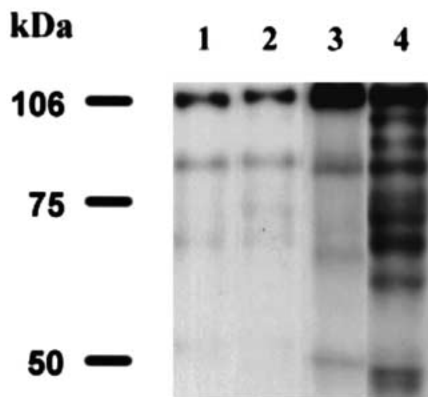

Figure 4. Appearance of protein tyrosine phosphorylation patterns of spermatozoa. The caudal spermatozoa were incubated for 30 minutes conducive to tyrosine phosphorylation in different media. Under those conditions, spermatozoa (5×10^5 cells) were removed, the extracts then resolved using 8% SDS-PAGE. Subsequent to the proteins being transferred to nitrocellulose membrane, any protein phosphotyrosine present on the gel was detected using a monoclonal antibody to phosphotyrosine according to the procedures described in the Materials and Methods section. Lane 1, control cells; lane 2, 0.3% BSA in HM medium; lane 3, 40 μm 24p3 protein in HM medium; lane 4, 0.3% BSA and 1.8 mM $CaCl_2$ in HM medium.

Protein tyrosine phosphorylation in spermatozoa

Tyrosine phosphorylation of specific mouse spermatozoa proteins has been shown to be associated with capacitation and acrosome reaction [25]. Figure 4 displays the protein tyrosine phosphorylation patterns of spermatozoa after incubation with 40 μM 24p3 protein at 37 °C for 30 min. In the control cells and 0.3% BSA-treated cells, phosphorylation was mainly restricted to the protein bands with the molecular mass of approximately 106/95 kDa, which according to Kalab et al. [31] is the 95/106 hexokinase (Figure 4, lane 1 and lane 2). The phosphorylated bands appeared much more significant in the range of Mr 50000–106000 in the 24p3 protein treated spermatozoa (Figure 4, lane 3). Comparing the phosphorylation pattern of the control spermatozoa with the pattern of BSA/Ca-treated spermatozoa, phosphorylation of the proteins in the range of Mr 40000–106000 greatly enhanced [25] a capacitation pattern (Figure 4, lane 4). The data showed that the 24p3 protein induced motility in the spermatozoa while showing a tyrosine phosphorylation pattern, which is different from the capacitated phosphorylation pattern, and neglecting the acrosome reaction. The phosphorylation in these proteins may regulate sperm motility but not sperm capacitation or acrosome reaction.

Discussion

The results from this study demonstrated that functional lipocalin was present in spermatozoa motility initiation, which was followed by maintaining the progressive movement. The elevation of these biochemical events suggested that the 24p3 protein had a role in regulating flagellar motility. The data may give some insights on studying lipocalin within the reproductive system. When capacitated, the spermatozoa of many mammalian species show hyperactivated motility characterized by less linearity, less progressive forward movement and more vigorous movement than that of uncapacitated spermatozoa [32]. Capacitated and hyperactivated sperm will follow with acrosome reactions. Whereas, the results in our study show that the 24p3 protein did not increase the VCL and ALH values or decrease the LIN and STR values [28] but kept the linearity, even with 90 μM of $CaCl_2$ in the medium (unpublished data). This suggests that sperm progressive motility was enhanced by 24p3 protein. The enhancement of sperm progressive motility does not correlate with the hyperactivation. Several modulators of mammalian sperm motility have been identified and studied, except for lipocalin, an increase the internal pH of the sperm motility is one of the events in the stimulating the sperm motility which may trigger the sperm acrosome reaction via changes in endogenous phosphoproteins [30,33]. The protein tyrosine phosphorylation pattern of the capacitated cells showed more phosphorylated protein bands appeared in the 40–106 kDa size range [25]. Cyclic adenosine 3′, 5′-monophosphate (cAMP) and a cAMP-dependent phosphorylation are also though to be involved in the regulation of motility [17] and capacitation [25]. Our findings clearly indicate that the 24p3 protein enhanced spermatozoa motility by elevating intracellular pH and the cAMP accumulation in cytosol. In addition, it showed a phosphotyrosine protein pattern that was distinct from capacitated phosphorylation and inhibited all acrosome reactions. Based on the report by Beltran et al. [34], the 24p3 protein might change the sperm membrane potential and regulated the sperm adenylylcyclase as binding to the sperm. In this experiment, the intracellular cAMP concentration may not have been sufficient to raise the tyrosine phosphorylation which triggers the pathway of capacitation and acrosome reaction, but regulated the motility related phosphorylation. The sperm motility increase via the weak tyrosine phosphorylation implies the additional protein phosphorylation would

occur, such as Thr/Ser phosphorylation to raise the sperm motility [35]. The elevation of intracellular pH may trigger the calcium flux into the cytosol from acrosome [36], and sequentially elevate the intracellular cAMP then increase the sperm motility. However, further studies should be carried out in the future. As for lipocalin [3] being associated with the spermatozoa head [12], the 24p3 protein may influence cell regulation through the system located on the sperm surface, which results in motility enhancement. Further information relating to the molecular events associated with the 24p3 protein in spermatozoa needs to be analyzed in order to gain a greater understanding of the process.

Based on these observations, the enhancement of spermatozoa motility may be one of the factors resulting in functionally effective movement of spermatozoa in the reproductive tract. The 24p3 protein has previously been demonstrated as being secreted into the uterine fluid before ovulation [10,11] and we have illustrated the enhancement of spermatozoa motility, as a result of contact with this protein. Both the reports from the literature and the results from this study imply that the 24p3 protein may promote the ability of spermatozoa to reach the oviduct prior to their losing fertility. More research is required to discover whether the 24p3 protein plays a pivotal role in such events. In summary, all of these observations regarding spermatozoa motility suggest that the 24p3 protein plays an important role both in spermatozoa processing in the female reproductive tract, which warrants further investigation.

Acknowledgment

This work was partially supported by the grant NSC90-2311-B001-131 from the National Sciences Council, Taiwan.

References

1. Hraba-Renevey S, Trler H, Kress M, Saloman C, Weil R (1989). Oncogene. 4: 601–608.
2. Flower DR, North ACT, Attword TK (1991) Biochim. Biophys. Res. Commun. 180: 69–74.
3. Chu ST, Lin HJ, Huang HL, Chen YH (1998) J. Peptide Res. 52: 390–397.
4. Meheus LA, Fransen LM, Raymackers JG, Blockx HA, van Bearrmen JJ, van Ban SM, van de Voorde A (1993) J. Immunol. 151: 1535–1547.
5. Davis TR, Tabatabai L, Bruns K, Hamilto RT, Nilsen-Hamilton M (1991) Biochim. Biophys. Acta. 1095: 145–152
6. Liu Q, Nilsen-Hamilton M (1995) J. Biol. Chem. 270: 22565–22570.
7. Cowland JB, Borregarrd N (1997) Genomics 45: 17–23.
8. Chu ST, Lin HJ, Chen YH (1997) J. Peptide Res. 49: 582–585.
9. Åkerstrom B, Flower DR, Salier J-P (2000) Biochem. Biophy. Acta. 1482: 1–8.
10. Huang HL, Chu ST, Chen YH (1999) J. Endocrinol. 162: 11–19.
11. Chu ST, Huang HL, Chen JM, Chen YH (1996) Biochem. J. 316: 545–550.
12. Chu ST, Lee YC, Nein KM, Chen YH (2000) Mol. Reprod. Dev. 57: 26–36.
13. Pusch HH (1987) Andrologia. 19: 514–527.
14. Fraser LR (1995) Human. Reprod. Suppl. 10: 22–30.
15. Robertson L, Kay VJ (1998) Human. Reprod. Update 4: 776–786.
16. Jaiswal BS, Majumder GG (1998) Reprod. Fertil. Dev. 10: 299–307.
17. Leclerc P, de Lamirande E, Gagnon C (1996) Biol. Reprod. 55: 684–692.
18. Lee MA, Storey BT (1986) Biol. Reprod. 34: 349–356.
19. Neill JM, Olds-Clarke P. (1987) Gamete Res. 18: 121–140.
20. Si Y, Olds-Clark P. (2000) Biol. Reprod. 62: 1231–1239.
21. Aarons D, Battle T, Boettger-Tong H, Holt G, Poirier GR (1991) Mol. Reprod. Dev. 30: 258–264.
22. Moller CC, Bleil JD, Kinloch RA, Wassarman PM (1990) Dev. Biol. 137: 276–286.
23. Màriàn T, Krasznai Z, Balkay L, Emri M, Tròn L (1997) Cytometry 27: 374–382.
24. Cross NL, Razy-Fanlkne RP (1997) Biol. Reprod. 56: 1169–1174.
25. Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS (1995) Development 121: 1129–1137.
26. Laemmli UK (1970) Nature 227: 680–685.
27. Towbin H, Staehelin TH, Gordan J (1979) Proc. Natl. Acad. Sci. USA. 76: 4350–4354.
28. Cancel AM, Lobdell D, Mendola P, Perreault D (2000) Human. Reprod. 15: 1322–1328.
29. Galantino-Homer HL, Visconti PE, Kopf GS (1997) Biol. Reprod. 56: 707–719.
30. Lee HC, Johnson C, Epel D (1983) Dev. Biol. 95: 31–45.
31. Kalab P, Visconti PE, Leclerc P, Kopf GS (1994) J. Biol. Chem. 269: 3810–3817.
32. Fraser LR, McDermott CA (1992) J. Reprod. Fertil. 96: 363–377.
33. Carr DW, Acoh TS (1989) Biol. Reprod. 41: 907–920.
34. Beltran C, Zapata O, Darszon A (1996) Biochemistry. 35: 7591–7598.
35. Tash JS, Bracho GE (1998) Biochem. Biophy. Res. Commun. 251: 557–563.
36. Darszon A, Labarca P, Nishigaki T, Espinosa F (1999) Physiol. Rev. 79: 481–510.