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Effect of platelet activating factor (PAF) on protein tyrosine phosphorylation during acrosome reaction of buffalo spermatozoa

MONIKA GARG[1], SIVARAM VEDANTAM[2], G JAGAN MOHANARAO[3] and S K ATREJA[4]

National Dairy Research Institute, Karnal, Haryana 132 001 India

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ABSTRACT

Ejaculated spermatozoa must undergo 2 important preparatory changes namely capacitation and acrosome reaction to be able to fertilize an egg. Among the biochemical changes that take place during capacitation and acrosome reaction, protein tyrosine phosphorylation has been considered as an important event associated with different sperm functions. PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phophocholine), a potent signaling phospholipid, also regulates different sperm maturational processes. This study was undertaken to determine the effect of different concentrations of PAF as well as its incubation time on protein tyrosine phosphorylation during acrosome reaction of buffalo spermatozoa. Number of protein substrates were tyrosine phosphorylated and their degree of phosphorylation increased with increase in concentrations of PAF. However at higher concentrations of PAF (500 nM) a significant decrease in the extent of tyrosine phosphorylation was observed with respect to 20, 25 and 40 kDa proteins. It was also observed that with the increase in PAF incubation time the degree of protein tyrosine phosphorylation increased during acrosome reaction of buffalo spermatozoa. In conclusion, the effect of PAF on the extent of tyrosine phosphorylation of sperm proteins appeared to be dose related and time dependent and this compound can be utilized for the induction of acrosome reaction in buffalo spermatozoa.

Key words: Acrosome reaction, Buffalo, PAF, Sperm, Tyrosine phosphorylation

The sperm's acrosome reaction is a calcium-dependent process and results from the fusion of sperm outer-acrosomal membrane and plasma membrane with subsequent release of hydrolytic acrosomal enzymes. The hydrolytic action of acrosomal enzymes allows spermatozoa to penetrate zone pellucida and fertilize the egg (Abou-haila and Tulsiani 2009). Both capacitation and acrosome reaction, are regulated by factors such as PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), a potent signaling phospholipid that is involved in reproductive physiology by also influencing ovulation, fertilization, preimplantation embryo development, implantation and parturition (Levine *et al*. 2002). PAF is found in the spermatozoa of many species including the rabbit, mouse, bull and boar (Kumar *et al*. 1988, Parks *et al*. 1990, Mook *et al.* 1998) and is principally of C-16 molecular species. A significant cellular action of PAF or its precursors 1-O-alkylglycerols on spermatozoa is the stimulation of motility, which was demonstrated in boar (Cheminade *et al.* 2002), buffalo (Aravindakshan and Sharma 1996), stallion (Odeh *et al.* 2003) and mouse (Wu *et al.* 2001). PAF acts via a specific G-protein receptor mediated inositol triphosphate-diacylglycerol pathway in spermatozoa ultimately leading to increase in intracellular calcium levels (Purnell *et al*. 2001). PAF receptors are functionally linked to inositol lipid hydrolysis and leading to the mobilization of intracellular free calcium and activation of a tyrosine kinase pathway results into tyrosine phosphorylation of number of protein substrates. The increased calcium also depolymerizes the inter membrane actin network and activates phospholipases, leading to an acrosome reaction. Spermatozoa treatment with PAF antagonists or PAF receptor antibodies, inhibits the motility, acrosome reaction and hamster oocyte penetration, suggesting the presence of receptor for PAF (Sengoku *et al*. 1992). It is suggested that a tyrosine phosphorylated protein in the molecular weight region of 94–97 kDa is probably concerned with the fertilizing ability of mammalian spermatozoa, which is acquired during capacitation in most of the species. Capacitation coupled with an increase in protein tyrosine phosphorylation is considered as a biochemical marker in several mammalian species. Though PAF was found to

Present address: [1]Senior Lecturer, Amity Institute of Biotechnology, Amity University, Noida 201303, Uttar Pradesh, India (monika14aug@gmail.com).[2,3] Ph.D. Animal Biochemistry Division (sivaram3@gmail.com, biochemjagan@gmail.com); [4]Principal Scientist, Animal Biochemistry Division (skatreja@rediffmail.com).

stimulate tyrosine phosphorylation of number protein substrates during capacitation, its effect on acrosome reaction, in particular, has not been studied in large ruminant species like buffalo. In the present study, attempts were made to study the potency of PAF to induce acrosome reaction and to detect the proteins undergoing tyrosine phosphorylation during PAF induced acrosome reaction in buffalo spermatozoa.

MATERIALS AND METHODS

Semen collection and processing: Semen was collected from Murrah buffalo bulls twice a week with an artificial vagina. The bulls were maintained at Artificial Breeding Research Complex, National Dairy Research Institute, Karnal, India, under uniform nutritional conditions. Immediately after collection the volume of the semen was measured and mass motility was assessed by light microscopy at 10× magnification. The semen samples were split into 0.5 mL fractions, and diluted with sp-TALP medium [100 mM NaCl, 10 mM HEPES, 3.1 mM KCl, 0.4 mM EDTA, 0.4 mM $MgCl_2 \cdot 6H_2O$, 0.3 mM $NaH_2PO_4 \cdot 2H_2O$, 21.6 mM Na lactate, 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM Na pyruvate, 25 mM $NaHCO_3$, BSA (1 mg/ml for washing, 6 mg/ml for culturing) with pH 7.4 and osmolarity: 265–270 mOsmol/kg] in 1:6 ratio. The samples were subjected to 3 washings by centrifugation at 275 × g for 5 min to remove seminal plasma. The final sperm pellet was resuspended with 2 mL of sp-TALP and the sperm suspensions of all the tubes were combined. The concentration of sperm was determined by haemocytometer and adjusted to 100×10^6 cells/mL by further dilution with sp-TALP. Final sperm suspensions with >80% forward progressive motility and viability were only used in this study.

Sperm culture and PAF treatment: In vitro capacitation of buffalo spermatozoa was achieved as per Roy and Atreja (2008). The sperm samples after 5 h and 45 min incubation were incubated further for 20 min with PAF in the concentration range of 100 to 500 nM, to induce acrosome reaction. Sperm suspension without PAF treatment served as control. Also to determine the effect of PAF incubation time on protein tyrosine phosphorylation during acrosome reaction, sperm suspensions after 6 h incubation in capacitation media were incubated with 200 nM PAF for different time periods (0, 10, 15, 20 min).

Detection of tyrosine phosphoproteins by immunoblotting: After certain periods of incubation sperm proteins were extracted according to Galantino-Homer *et al.* (1997) and total proteins were resolved by SDS-PAGE on a 10% (w/v) uniform gel at constant voltage of 40 V (Laemmli 1970). After electrophoresis, one gel was subjected to staining with coomassie stain and the other gel was transferred to immobilon-P PVDF membrane. Protein transfer efficiency was assessed by staining the membrane with 0.5% ponceu S dye in 1% glacial acetic acid. The membrane was blocked with 5% (w/v) non-fat dried milk prepared in Tris-buffered saline with Tween-20 and sodium orthovanadate (TBS-TV: 20 mM Tris, 150 mM NaCl, pH 7.6; 0.1% (v/v) Tween-20, 1 mM Na_3VO_4) for overnight at 4 °C. The membrane was incubated with monoclonal antiphosphotyrosine antibody [Clone PT-154, diluted (1:2500) in TBS-TV] for 2 h at room temperature. After washing with TBS-T, membrane was incubated with goat anti-mouse IgG-peroxidase conjugate [A2554, diluted (1:70,000) in TBS-TV] for 1 h and washed again with TBS-T. Subsequently, the bound peroxidase activity was visualized by the enhanced chemiluminescent reagent (western chemiluminescent HRP substrate) according to manufacturer's instructions using X-ray films. After chemiluminescence detection of tyrosine phosphoproteins, the X-ray films were photographed by an image analyser and densitometric analysis was performed with image analysis software. A single band randomly selected from the control lane was assigned a band intensity value of 1.0 arbitrarily. The intensities for the rest of the bands were calculated by the software with respect to the initial band selected.

Statistical analysis: Results were expressed as the mean±SEM. A difference with value $P<0.05$ was considered statistically significant. Data were analyzed by analysis of variance, and statistical differences between the various treatment group means were determined by Duncan's multiple range test (DMRT) using the Statistical Product and Service Solutions, Version 17.0.1 software.

RESULTS AND DISCUSSION

Effect of dose of PAF on protein tyrosine phosphorylation: Our results showed that there is an increase in the extent of specific tyrosine phosphorylated proteins in PAF treated groups as compared to the control. As shown in Fig. 1 and Table 1, PAF at 100 nM concentration itself was able to induce phosphorylation of 20 and 28 kDa proteins, which were absent in the control lane. Proteins with molecular mass 25, 36, 40 were also found to be phosphorylated in the control,

Table 1. Relative band intensities (mean±SEM) of tyrosine phosphorylated proteins in buffalo spermatozoa treated with different concentrations of PAF after 6h of capacitation

Molecular mass	Control	100 nM	200 nM	500 nM
p66	0.13 ± 0.02^{a}	0.431 ± 0.1^{b}	0.74 ± 0.1^{c}	0.70 ± 0.2^{c}
P54.9	-	-	0.4±0.02	0.42±0.06
40	1.00^{a}	1.01 ± 0.5^{a}	1.7 ± 0.4^{ab}	1.8 ± 0.4^{b}
36.3	0.67 ± 0.1^{a}	0.94 ± 0.4^{a}	1.66 ± 0.3^{b}	1.59 ± 0.4^{b}
30	-	-	0.58±0.1	0.50±0.06
28	-	0.5 ± 0.1^{a}	0.8 ± 0.2^{b}	0.82 ± 0.05^{b}
25	0.32 ± 0.1^{a}	0.68 ± 0.2^{b}	1.05 ± 0.02^{c}	0.77 ± 0.08^{b}
20	-	0.16 ± 0.01^{a}	0.71 ± 0.09^{b}	0.68 ± 0.05^{b}

Values are the mean±SEM (n = 3). ^{abc} Within each row means with different letters significantly different ($P < 0.05$).

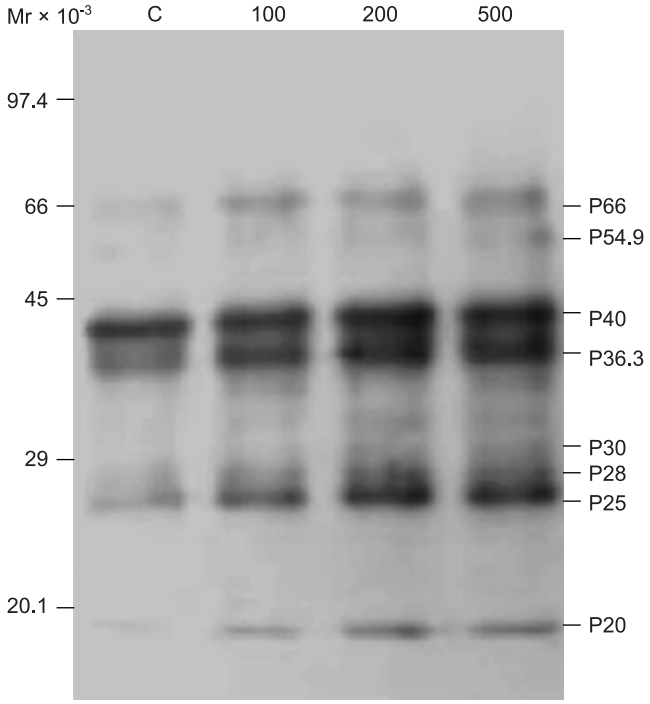

Fig. 1. Effect of different concentrations of PAF (nM) on protein tyrosine phosphorylation during acrosome reaction of buffalo (*Bubalus bubalis*) spermatozoa. Spermatozoa after 6h incubation in capacitation media were further incubated with different concentrations of PAF. Proteins were extracted with Laemmli sample buffer and resolved by 10% SDS-PAGE (10 µg/lane), electrotransferred to a PVDF membrane. Tyrosine phosphorylated proteins were detected using an affinity purified monoclonal antiphosphotyrosine antibody.

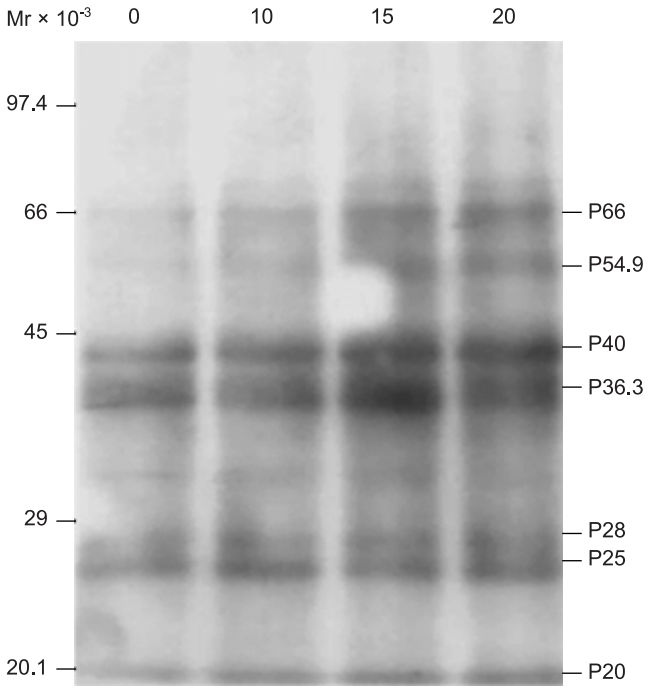

Fig. 2. Effect of time course on protein tyrosine phosphorylation during acrosome reaction of buffalo spermatozoa. Spermatozoa after the 6h incubation in capacitation media were incubated with 200 nM PAF for different time periods (min). Proteins were extracted with Laemmli sample buffer and resolved by 10% SDS-PAGE (10 µg/lane), electrotransferred to a PVDF membrane. Tyrosine phosphorylated proteins were detected using an affinity purified monoclonal antiphosphotyrosine antibody.

but their extent of phosphorylation increased to various levels depending upon the concentration of the PAF. The 20 kDa protein which is absent in the control also increased 4 folds at 200 nM concentration as compared to the 100 nM. In the presence of 200 nM PAF the extent of protein tyrosine phosphorylation was optimal as compared to other concentrations demonstrating the optimal concentration of PAF for tyrosine phosphorylation during buffalo AR. At higher concentrations of PAF (500 nM) a significant decrease in the extent of tyrosine phosphorylation was observed with respect to 20, 25 and 40 kDa proteins.

Table 2. Relative band intensities (mean±SE) of tyrosine phosphorylated proteins in buffalo spermatozoa treated with 200 nM PAF at different time interval of acrosome reaction

Molecular mass	0 min (control)	10 min	15 min	20 min
p66	0.9±0.06	1.3±0.1[a]	1.9±0.5*	2.0±0.6**
p54.9	0.66±0.2	0.98±0.2*	2.3±0.6**	2.26±0.5**
p40	2.1±0.6	2.45±0.8	2.95±0.9*	3.1±0.9*
p36.3	2.0±0.5	1.99±0.5[a]	3.4±1.0**	2.98±0.8*
p30	2.0±0.4	1.1±0.3[a]	1.4±0.3	0.9±0.2
p28	1.5±0.3	1.51±0.4[a]	1.6±0.4	1.56±0.5
p25	1.9±0.4	2.3±0.7*	2.3±0.6*	2.45±0.5*
p20	1.0	1.3±0.4[a]	1.6±0.4*	1.63±0.4*

Values are the mean±SEM (n = 3); *values in a row are significantly higher than the control (P<0.05); **values in a row are significantly higher than the control (P<0.01); [a]values in a row are significantly lower than 15 min PAF treatment (P<0.05).

PAF induced tyrosine phosphorylation of distinct set of proteins that lie in the molecular mass range of 15"110 kDa. The anti-phosphotyrosine antibody used in this study was affinity purified monoclonal antibody and is highly specific for tyrosine phosphoproteins. This was confirmed by skipping primary antibody treatment or pre-incubating it with o-phospho L-tyrosine (blocking agent) before using it for treating the membrane, which resulted in the visualization of no bands, indicating that the proteins identified were indeed phosphorylated at the tyrosine residues. It was found that 200 nM concentration of PAF was optimal in inducing the protein tyrosine phosphorylation in buffalo spermatozoa during AR. We found that 100 nM PAF could induce tyrosine phosphorylation of specific set of proteins as compared to the control. But the extent of tyrosine phosphorylation was optimal with 200 nM PAF. It can be speculated that the

requirement of higher concentration of PAF to induce AR in buffalo sperm might be due to species specific variation and also in part due to the source of PAF used.

Effect of time on PAF induced protein tyrosine phosphorylation: PAF also exerted a time dependent increase in the extent of protein tyrosine phosphorylation during buffalo sperm AR. As shown in Fig. 2 and Table 2, different proteins were phosphorylated to various extents at different time intervals ranging from 0 to 20 min. Though most of the protein bands were present even at 0 min of incubation, the extent of tyrosine phosphorylation increased gradually till 15 min of incubation. Proteins with molecular mass 36, 66 and 54.9 kDa showed 0.75, 1.2 and 2 fold increase, respectively, in their extent of phosphorylation after 15 min of incubation as compared to 0 min. No further rise was observed after 20 min incubation. This clearly showed that 15 min incubation with PAF is sufficient to induce acrosome reaction in buffalo spermatozoa.

PAF has ability to stimulate protein tyrosine phosphorylation in a concentration and time dependent manner (Figs 1, 2). Although the functional characterization of these proteins still remains to be done, quite a few proteins with same molecular weight have already been identified in other species that play a crucial role in sperm functions. In separate studies 2 flagellum associated proteins, namely an isoform of tubulin ß chain (66 kDa protein) and 36 kDa protein were shown to be phosphorylated in hamster spermatozoa and were correlated with the initiation of motility (Fujinoki *et al*. 2004). Two proteins in the molecular weight range of 32 and 30 kDa were reported in bull spermatozoa, which were found associated with calmodulin (CaM). Their association with CaM was inversely correlated with sperm capacitation (Leclerc and Goupil 2000). Nagdas *et al*. (2005) reported that tyrosine phosphorylation of protein less than 24 kDa represents the phosphorylation of an enzyme i.e. phospholipid hydroperoxide glutathione peroxidase ($PHGP_x$). This enzyme is catalytically active in the phosphorylated form and it may interact with other signaling protein(s) that could potentially affect mitochondrial function and/or participate in one of the pathways regulating the hyperactivation of sperm motility. Further a 55kDa protein was found to be tyrosine phosphorylated in bovine spermatozoa, and was linked to sperm motility (Vijayaraghavan *et al*. 1997). In all probability the proteins with same molecular weight identified in our study might be having similar role to play in buffalo spermatozoa also. Further studies are necessary to confirm the identity and their physiological significance during acrosome reaction of buffalo sperm.

In conclusion, PAF was found to be a good inducer of acrosome reaction in buffalo spermatozoa, which was clearly demonstrated with the increase in tyrosine phosphorylation of number of protein substrates. The effect of PAF on the level of phosphorylation of sperm proteins appeared to be dose and time dependent. The concentration of 200 nM PAF with 15 min incubation was optimum for the buffalo acrosome reaction as revealed by tyrosine phosphorylation pattern.

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