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ORIGINAL ARTICLE

Characterization of human sperm *N*-acetylglucosaminidase

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Summary

N-acetylglucosaminidase (NAG) is particularly active in mammalian spermatozoa and appears to be involved in fertilization. Although it is assumed that this enzyme is acrosomal, previous results from our laboratory suggest the presence of NAG at the sperm plasma membrane level. The present study attempted to analyse the subcellular distribution of this enzyme in human spermatozoa. Sperm were incubated under different conditions and NAG activity measured in the soluble extracts and cell pellets using a specific fluorometric substrate. A significant proportion of NAG activity was released when sperm were incubated in culture medium, suggesting a weak association with the plasma membrane. This location was confirmed by western blot analysis of plasma membrane fractions and immunofluorescence on non-permeabilized sperm, which showed a positive signal mainly on the acrosomal domain. The distribution of NAG activity between plasma membrane and acrosome was analysed after cell disruption by freezing and thawing. Triton X-100 stimulated sperm and epididymal NAG activity but not the enzyme obtained from other sources. In addition, biotinylated human recombinant NAG was able to bind to human sperm. Finally, after sperm incubation under capacitating conditions, NAG total activity increased and the sperm enzyme lost its ability to be stimulated by Triton X-100. The possible connection of these results with sperm maturation, capacitation and NAG participation in primary binding to the zona pellucida, was discussed.

Introduction

Carbohydrates are involved in many cellular events, such as differentiation, recognition and adhesion, not only in somatic cells but also in gametes. The enzymes involved in oligosaccharide processing, glycosidases and glycosyltransferases, can be implicated in these events (Rauvala *et al.*, 1981; Shur, 1993). Glycosidases are particularly abundant in the mammalian epididymis as well as in spermatozoa. *β-N*-Acetylglucosaminidase (NAG), also known as Hexosaminidase, is the glycosidase showing the highest activity in both epididymis and sperm (Conchie & Mann, 1957; Chapman & Killian, 1984; Miller *et al.*, 1993). Several reports support the participation of NAG in sperm–egg interaction (Farooqui & Srivastava, 1980; Godknecht & Honegger, 1991; Miller *et al.*, 1993; Perotti *et al.*, 2001). Although sperm NAG has been studied in several species, little is known about the enzyme in human sperm except its presumed acrosomal location (Mack *et al.*, 1983; Tulsiani *et al.*, 1990).

Previous studies in our laboratory on the putative participation of sperm NAG in the induction of acrosome reaction (AR) (Brandelli *et al.*, 1994) lead us to propose that there would be enzyme located at the plasma membrane level. Additional results from our laboratory suggest the participation of NAG in human sperm-zona pellucida (ZP) interaction *in vitro* (Miranda *et al.*, 2000). However, the precise step(s) in which the enzyme is involved (primary binding, secondary binding and/or penetration) remained to be determined. Given that the function exerted by NAG during fertilization is related to its location, we aimed to analyse the subcellular distribution of this enzyme in human spermatozoa.

Materials and methods

Materials

All reagents used were of the highest purity or analytical grade, and purchased from Sigma (St Louis, MO, USA), Fisher (Fairlawn, NJ, USA), Merck (Darmstadt, Germany),

or J.T. Baker (Phillipsburg, NJ, USA). Polyclonal antibody against *N*-acetylglucosaminidase (anti-NAG) was from Nordic Immunological Laboratories (Tilburg, The Netherlands). Anti-SP10 was kindly provided by Dr John Herr from the Department of Cell Biology at the University of Virginia, USA.

Samples

Semen samples from normospermic donors were obtained after 48 h of sexual abstinence. Following liquefaction, 1–2 mL of semen was layered onto a discontinuous gradient of 40/80% Percoll, centrifuged 20 min at 450 ***g*** and washed twice with Biggers-Whitten-Whittingham (BWW; Biggers *et al.*, 1971) medium. A total of four to nine samples from different donors were used for each analysis. Seminal plasma (SP) from normal or vasectomized donors was obtained by centrifugation of semen at 2000 ***g*** for 20 min. For sperm capacitation, cells were incubated in BWW medium supplemented with 35 mg/mL globulin and fatty acid-free BSA at 37 °C under 5% CO_2 for 18 h. Blood plasma was obtained from healthy donors after centrifuging blood in the presence of 0.5% EDTA at 500 ***g*** for 10 min. Human testes and epididymides were obtained from patients undergoing orchydectomy as treatment for prostatic carcinoma (Miranda & Tezon, 1992). Cauda epididymal plasma (CEP) was obtained by perfusion through the vas deferens as previously described (Miranda & Tezon, 1992). Testicular and epididymal cytosols were obtained by tissue homogenization in 50 mM Tris, 0.25 M sucrose, 1.5 mM EDTA, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 0.05% sodium azide, 1 mM mercaptoethanol, pH 7.4, after centrifugation at 105 000 ***g*** for 1 h at 4 °C. All human tissues and fluids were used after informed consent of the patients/donors.

Sperm extracts

Equivalent aliquots of the sperm suspensions were centrifuged and resuspended (2×10^6 sperm/mL) in different isotonic buffers: PBS, BWW medium or 10 mM Pipes, 0.25 M sucrose, pH 7.4. Additionally, two temperatures (4 or 25 °C) and extraction times (30 or 60 min) were tested. Incubation in BWW buffer at room temperature for 30 min was selected as the condition producing the highest cell viability. For the different extractions, sperm were resuspended in BWW alone (basal condition) or supplemented with NaCl (0.5 M, high salt treatment) or 0.1% Triton X-100 (TX100). A cocktail of protease inhibitors (0.8 mg/mL Benzamidine, 5 μg/mL Pepstatin A, 1 μg/mL Leupeptin and 1 μg/mL Bestatin) was added to the different mixtures and sperm suspensions were gently mixed by end over end rotation. After the extraction procedure, suspensions were centrifuged 10 min at 450 ***g***, the supernatant was aspirated and the pellets resuspended in the same buffer. Enzyme activity was measured in both fractions. The procedure described by Pedersen (1972), was used as an alternative approach to obtaining acrosomal extracts. Briefly, sperm were resuspended in medium supplemented with 10% glycerol and frozen by immersing in liquid nitrogen for 5 min. After thawing at room temperature, cell suspensions were centrifuged 15 min at 450 ***g***, the pellet was resuspended in the same medium and NAG activity measured in both fractions. To verify that freezing and thawing were not affecting NAG, a sperm extract was subjected to this procedure and no change in activity was found (data not shown).

Viability and acrosome loss

Sperm viability following the different treatments was determined with eosin. To check for acrosomal status, spermatozoa were fixed 5 min with 2% formaldehyde at 4 °C, immobilized on slides coated with 20 μg/mL polylysine and incubated with fluoresceinated *Pisum sativum* lectin.

Enzyme activity

NAG activity was assayed by measuring the fluorescent signal produced by the cleavage of *N*-acetylglucosamine residues from 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide. Activity was measured by adding 100 μl of 0.25 M sodium citrate buffer pH 4.5 and 100 μl of substrate solution (4 mg/mL) to the enzyme preparation. The reaction mixture was incubated at 37 °C for a convenient period of time (15 min to 5 h depending on the extract). The amount of fluorescent product, methylumbelliferone (MU), was measured in a Hoefer TKO 100 fluorimeter (Hoefer Scientific Instruments, San Francisco, CA, USA) (emission at 380 nm and detection at 460 nm). A calibration curve was plotted using different concentrations of MU. The enzyme activity was determined by interpolation and expressed as nM MU/million sperm/min. To determine whether TX100 was able to stimulate NAG activity, a 0.1% final concentration of this detergent was added to the fluids under study. The results were expressed as Stimulation = 100 × activity with detergent/activity without TX100.

Plasma membrane isolation

Human sperm plasma membranes were isolated following a method previously described (Mack *et al.*, 1986). Briefly, sperm were resuspended in 10 mM Pipes, 0.25 M sucrose, pH 6.8, containing 0.2 mM phenylmethylsolfonyl-

fluoride (PMSF) and subjected to nitrogen cavitation (10 min, 600 psi) in a cell disruption pump (Parr Instruments Company, Moline, IL, USA). After extrusion on EDTA and PMSF (final concentrations 2 mM and 0.2 mM respectively), cell debris was removed by centrifugation at 1000 ***g*** for 10 min. The supernatant was centrifuged 10 min at 6000 ***g***, followed by 60 min at 100 000 ***g***. The crude membrane pellet was then loaded onto a discontinuous gradient of 1, 1.35 and 1.6 M sucrose and centrifuged 90 min at 100 000 ***g***. The band retained above the upper sucrose phase was aspirated and re-centrifuged 30 min at 100 000 ***g***.

Production of recombinant NAG

Human recombinant NAG (hrNAG) was purified from a stably expressing CHO cell line, following a four-step chromatography purification procedure (Miranda *et al.*, 2000). The final hrNAG preparation was analysed by protein staining and western blot with anti-NAG as described below (Fig. 1). Protein concentration was determined using the method described by Bradford.

Electrophoresis and immunoblot

A whole sperm extract was obtained by incubating cells in lysis buffer (50 mM Tris, 1% SDS, 5% β-mercaptoethanol, pH 6.8) at 100 °C for 5 min, followed by centrifuging at 2500 ***g*** for 2 min. Human recombinant NAG was diluted in lysis buffer and boiled for 5 min. The resulting plasma membrane pellet was resuspended in lysis buffer and then boiled for 5 min. Samples and molecular weight markers (BIO-RAD, Hercules, CA, USA) were run in a 10% acrylamide gel. Following electrophoresis, some lanes were separated and fixed for protein staining with Coomasie Blue. The remains were electroblotted to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 20% gelatin, NAG was detected by incubation with a polyclonal antibody developed in rabbits against the enzyme purified from bovine kidney (1 : 1000 in blocking solution). Anti-rabbit-Peroxidase (Sigma) diluted 1 : 10 000 in PBS containing 1 mg/mL BSA was used as secondary antibody. All incubations were carried out at room temperature for 1 h, and all washes were performed with PBS containing 0.1% Tween 20. The ECL detection system (Amersham Pharmacia Biotech Inc., NJ, USA) was used to locate immune complexes.

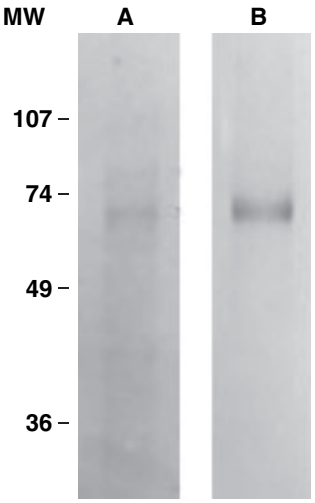

Figure 1 Electrophoretic analysis of human recombinant *N*-acetylglucosaminidase (NAG). The final preparation of human recombinant NAG was subjected to SDS-PAGE and stained with Coomasie Blue (A) or transferred to PVDF and developed with a specific anti-NAG antibody (B). MW: molecular weight standards.

Immunofluorescence

Spermatozoa were fixed 5 min at room temperature using a fresh solution of 0.2% paraformaldehyde in PBS. Following fixing, plasma membrane integrity was verified with eosin and sperm were immobilized on slides. Non-specific binding sites were blocked by 30 min incubation in 30 mg/mL BSA in PBS (blocking solution). Cells were then incubated with the anti-NAG antiserum (1 : 200 in blocking solution) overnight at 4 °C. After three washes with PBS, cells were treated with Alexa555-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) diluted 1 : 2000 in 1 mg/mL BSA in PBS. Following washing and mounting with PBS : glycerol (1 : 9), cells were visualized with a Nikon epifluorescence microscope coupled to an image analyser (IPLab Scientific Imaging Software). As control, anti-NAG was omitted or pre-incubated for 15 min with hrNAG (16 μg/mL) and then added to sperm. Additionally, a monoclonal antibody recognizing an intra-acrosomal antigen, anti-SP 10 (1 : 100), was used to verify that cells were not permeabilized. Anti-SP 10 was detected using FITC-conjugated anti-mouse IgG (Sigma, 1 : 50).

Biotinylation of recombinant NAG

Alpha-amino-terminal residues were biotinylated by treatment with sulfosuccinimidyl-6-(biotinamido)Hexanoate (NHS-LC-Biotin; Pierce, Rockford, IL, USA). Briefly, protein solutions (0.01 mg/mL in 50 mM phosphate buffer pH 6) were supplemented with NHS-LC-Biotin (2.5 μM final concentration) and incubated at 4 °C for 24 h. Biotinylated proteins were isolated by filtration through a membrane with a cut-off value of 10 kDa (Microcon 10; Amicon, Beverly, MA, USA). Medium from CHO cells not expressing human NAG and subjected to an identical procedure, was used as control.

Binding of NAG to spermatozoa

Fresh sperm were incubated with biotinylated-hrNAG (10 000 cells/ng of protein) in 100 μL of BWW containing 1 mg/mL BSA. After 45 min at 37 °C, the suspension was centrifuged 3 min at 600 ***g*** and the sperm pellet washed twice with BWW. These experimental conditions (BWW medium and mild centrifugation) were selected to guarantee cell membrane integrity. The final pellet was resuspended in 100 μL of extravidin-conjugated alkaline phosphatase diluted 1 : 1000 in PBS-0.1% Tween 20. After 45 min incubation at room temperature, cells were washed twice and resuspended with 100 μL of *p*-nitrophenylphosphate (3.8 mM in 10% diethanolamine, 0.5 mM$MgCl_2$, pH 9.8) until colour development. Reaction was stopped with 100 μL of 1 M NaOH and absorbance read at 405 nm. The same procedure was repeated in parallel using biotinylated proteins from control CHO cells. Additional controls omitting different components were also included to verify signal specificity. Results were expressed as the absorbance read at the end of the procedure after subtracting the non-specific binding of biotinylated proteins to sperm-free tubes.

Expression of results and statistical analysis

The use of individual semen samples as experimental units did not allow the measurement of both protein content and enzyme activity. Consequently, the information obtained considering the individual variation was preferred to the expression of results as specific activity. NAG total activity was calculated adding the results obtained for the pellet and supernatant obtained after centrifugation. In the case of capacitated spermatozoa, the capacitation medium was also considered in the calculation of total activity. The enzyme removed by the different treatments (Table 1), was expressed as the percentage of total activity found in the supernatant following extraction. Absolute activity was defined as nM MU/million sperm/min. The fraction of acrosomal NAG was calculated as the difference between the percentage of activity found in the supernatant after freezing and control procedures. Results were expressed as the average value of 4–9 samples ± SEM. Data processing was performed using the GraphPad Prism program (GraphPad Software Graphpad Software Inc., San Diego, CA, USA). Statistical analysis was carried out using a non-parametric Mann–Whitney test for single comparisons, and Dunn's test for multiple comparisons.

Table 1 Extraction Of *N*-acetylglucosaminidase from human spermatozoa

Treatment	Supernatant (%)	Viability	Acrosome loss (%)
Basal	76 ± 4 (62–93)	79 ± 3	26 ± 2
Salt	75 ± 3 (65–87)	37 ± 5	66 ± 4
TX100	89 ± 5 * (63–97)	0	100

Results are expressed as the average percentage of total activity found in the supernatant (SN) after extraction (total activity = SN + pellet). Numbers between brackets represent the range of values obtained for the different samples. Cell Viability and acrosome loss (%) was determined as detailed in materials and methods. *$p < 0.05$ vs. Basal.

Results

In order to analyse the subcellular location of NAG, sperm were incubated in either a high ionic strength (0.5 M NaCl) or detergent (0.1% TX100) solution. A similar tube containing isotonic buffer (medium alone) was run in parallel (basal conditions). Enzyme activity was measured in both the pellet and supernatant obtained after centrifugation and the resulting values were used to calculate total activity. When these procedures were carried out using washed sperm, over 95% of the NAG activity was found in the supernatant. Similar results were obtained with all the conditions tested (data not shown). This result could be due to an inefficient removal of the soluble enzyme, as only a small amount of seminal NAG is associated with sperm. For this reason, Percoll-purified sperm were used throughout the rest of the study. Using this procedure, sperm treatment with TX100 resulted in the highest level of NAG extraction (Table 1). Besides, basal conditions also released a high percentage of NAG total activity. This may be attributed to NAG liberated from disrupted acrosomes. If true, a greater proportion of NAG in the supernatant should accompany the greater acrosome loss produced by sperm treatment with high ionic strength. As the proportion of NAG extracted by basal and high salt buffers were similar (Table 1), the acrosome would not be the main source of the activity found in detergent-free extracts.

An alternative explanation for the presence of a high NAG activity in the basal extract would be the dissociation of an enzyme weakly associated with the plasma membrane. In order to assess this possibility, human sperm plasma membranes were isolated by nitrogen cavitation and ultracentrifugation, and analysed by western blot with an anti-NAG specific antibody. Two bands were recognized by this antibody in total (T) and plasma membrane (M) extracts of human sperm, as well as in the positive control (bovine kidney) (Fig. 2). No reaction was observed when the first antibody was omitted (not shown). The low molecular weight band represents the mature enzyme, and the other polypeptide corresponds to enzyme precursors (usually, the secreted form of NAG also found in the extract containing the recombinant

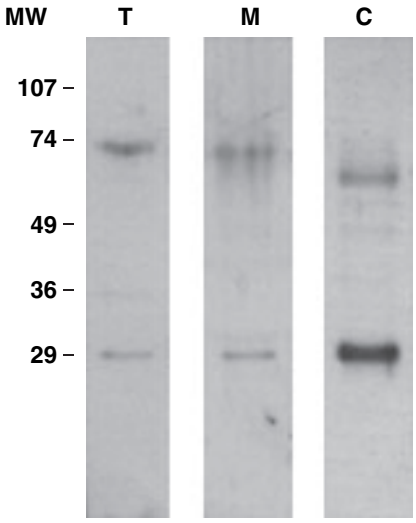

Figure 2 Detection of *N*-acetylglucosaminidase (NAG) in human sperm plasma membrane by western immunoblot. Total (T) and plasma membrane (M) extracts of human sperm were subjected to SDS-PAGE, transferred to PVDF and developed with a specific anti-NAG antibody. The antigen used to raise the antibody (bovine kidney) was run in parallel as control (C). MW: molecular weight standards.

enzyme; see Fig. 1). A similar high molecular weight NAG has been detected in mouse sperm (Miller *et al.*, 1993). The slight difference in the upper band between human sperm and bovine kidney is probably related to a different glycosylation between the two species.

Indirect immunofluorescence assays with anti-NAG resulted in a strong signal in the sperm head (acrosomal and equatorial areas, Fig. 3, Panel a), while anti-SP10 showed no reaction (Panel e). As anti-SP10 recognizes an acrosomal antigen, these results indicate that spermatozoa were not permeabilized, and supported the location of NAG at the plasma membrane. The specificity of the reactions was verified by blocking or omitting anti-NAG (Panel c) and testing anti-SP 10 on permeabilized cells (Panel g).

Subcellular distribution of NAG

As extracts obtained when spermatozoa were disrupted using TX100 contained both the extracellular and the intracellular enzyme, the proportion of acrosomal NAG could be estimated as the difference between the activities released after detergent and isotonic (basal) extraction. Unfortunately, TX100 produced a threefold increase in NAG total activity, evident when results were expressed as absolute activity (Fig. 4). Given TX100 stimulation, the ratio of acrosomal NAG could not be calculated using these results. Accordingly, an alternative procedure to obtain acrosomal extracts was used. After purification and washing, one aliquot of sperm suspension was frozen, while another was kept at room temperature to measure the activity released without acrosomal disruption. Using this procedure total activity was similar in both cases. This result allowed the calculation of acrosomal NAG as the difference between the activities released after both treatments. After processing each individual sample, the resulting average ratio of acrosomal NAG was 38 ± 10% of the total activity.

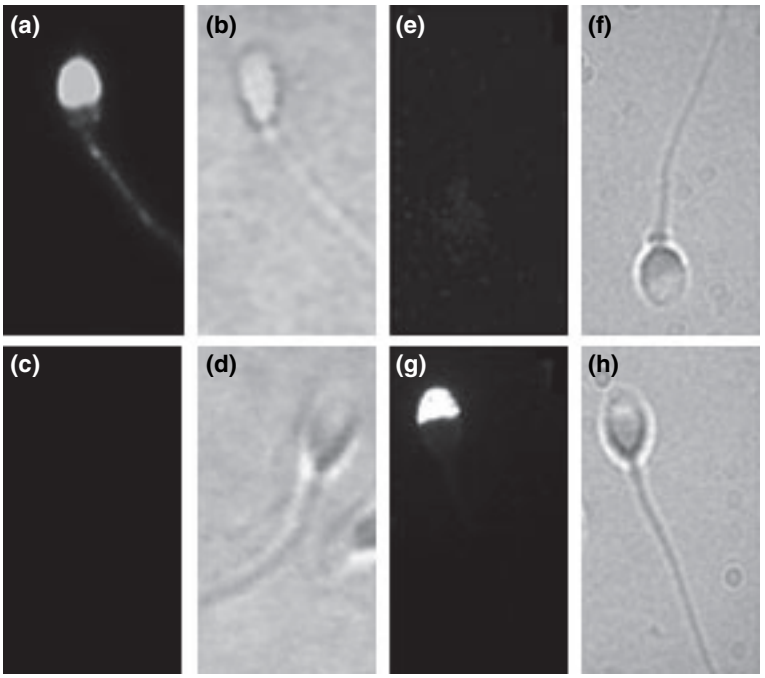

Figure 3 Localization of *N*-acetylglucosaminidase (NAG) in human spermatozoa by immunofluorescence. After mild fixation to avoid permeabilization, human spermatozoa were immobilized on slides and treated with anti-NAG (Panel a) or anti-SP10 (Panel e). Controls included blocked anti-NAG (Panel c) and anti-SP10 on permeabilized cells (g). Pictures in (b), (d), (f) and (h) show the corresponding phase contrast images.

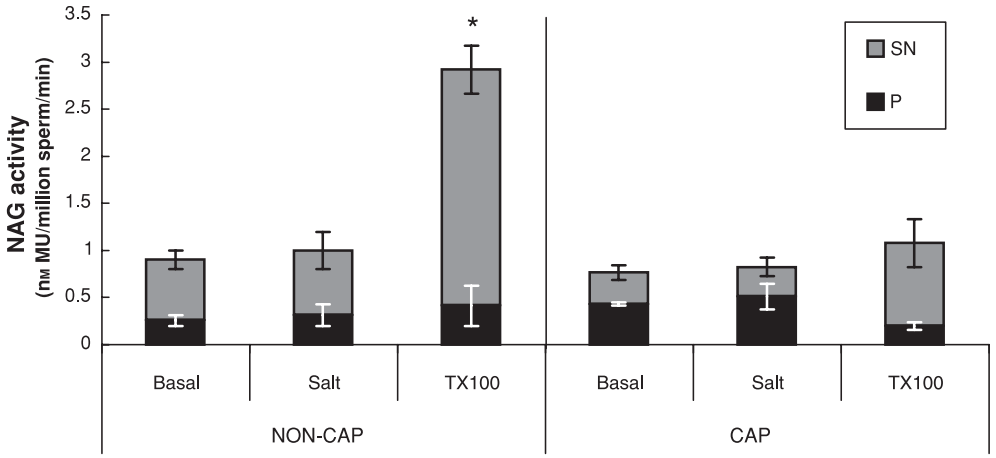

Figure 4 Distribution of *N*-acetylglucosaminidase (NAG) activity between pellet and supernatant after extraction of fresh or capacitated sperm with different buffers. Fresh (NON-CAP) or capacitated (CAP) spermatozoa were incubated in BWW medium alone (Basal) or supplemented with 0.5 M NaCl (Salt) or 0.1% Triton X-100 (TX100). NAG activity was measured in pellet (P) and supernatant (SN) obtained after centrifugation. Activity is expressed as nM MU/million sperm/min. *$p < 0.05$ vs. Basal and Salt.

Sperm incubation under capacitating conditions

Taking into account that a loose association of NAG with the plasma membrane could appear contradictory with its participation in the fertilization process, the effect of sperm capacitation on NAG activity was also determined.

Following sperm incubation under capacitating conditions, the medium was recovered, cell pellet was then subjected to the different extractions described above and enzyme activity was measured in all the fractions obtained. Extraction of capacitated sperm under basal conditions still removed a considerable amount of NAG, although this was lower than the proportion obtained before sperm incubation (50 ± 11% of the whole activity associated with capacitated sperm). Another difference obtained with fresh sperm was that treatment with TX100 did not enhance NAG total activity (Fig. 4).

When extraction of NAG activity under basal conditions was compared in fresh and capacitated cells, sperm-associated activity was found not to decrease as a consequence of incubation (Fig. 5, pellet + supernatant). In addition, the amount of NAG activity found in the pellet (resistant to extraction) was greater than the one observed in fresh sperm. This decrease in the amount of NAG removable by this treatment could be explained by the activity already released into the capacitation medium (Fig. 5). When all fractions (capacitation medium, pellet and supernatant obtained after sperm extraction) were considered, it was evident that NAG total activity increased as a consequence of sperm capacitation. Control experiments indicated that neither BSA nor any other component of the capacitation medium possessed NAG activity or enhanced sperm NAG (data not shown).

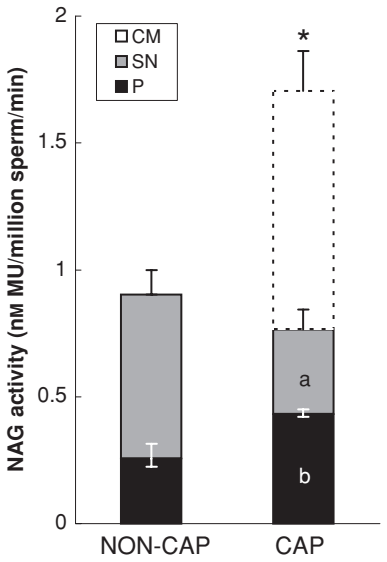

Figure 5 *N*-acetylglucosaminidase total activity before and after sperm capacitation. Spermatozoa were incubated under basal conditions before (NON-CAP) or after (CAP) overnight incubation under capacitating conditions. The activity was measured in the medium removed after sperm incubation (CM), and in pellet (P) and supernatant (SN) obtained following sperm extraction under basal conditions. Results are expressed in nM MU/million sperm/min. *$p < 0.04$ vs. NON-CAP. *(a,b): $p < 0.04$ CAP vs. Non-CAP for SN and P respectively.

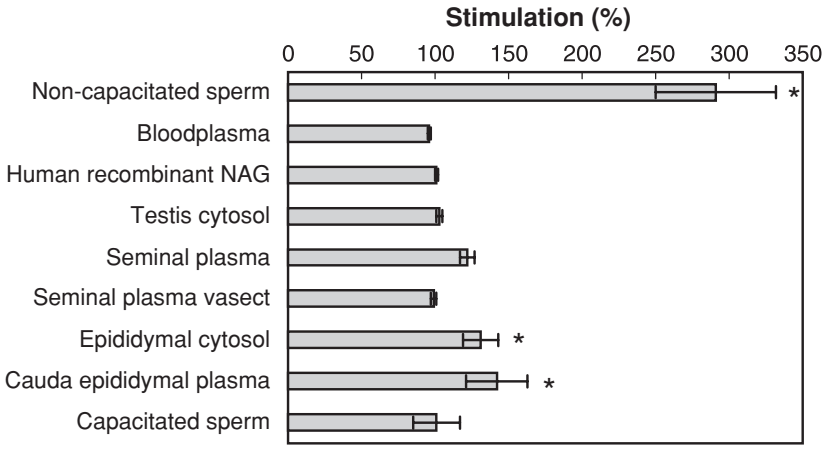

Figure 6 Effect of TX100 on catalytic activity of *N*-acetylglucosaminidase (NAG) obtained from different sources. Protein extracts obtained from different tissues or fluids were supplemented or not with TX100 (0.1% final) and NAG activity measured. Results are expressed as % Stimulation as detailed in materials and methods. $*p < 0.05$ vs. 100%.

Enzyme activation by TX100

The ability of TX100 to increase NAG activity was analysed. For this purpose, TX100 was added to different preparations containing NAG. When the detergent was added to a basal extract of non-capacitated sperm, enzyme activity increased threefold (Fig. 6). However, this characteristic was not detected in NAG coming from any source. The enzyme activity was not enhanced when TX100 was added to human blood plasma or recombinant enzyme expressed by mammalian cells. When NAG from different reproductive sources was analysed, no stimulation by TX100 was found in testicular cytosol or SP from normal or vasectomized donors. On the other hand, enzyme activity was enhanced when TX100 was added to epididymal cytosol and the fluid contained in the lumen of the human epididymis (cauda epididymal). In agreement with the results obtained during the extraction procedures, the addition of TX100 to a basal extract of capacitated sperm did not increase NAG total activity (Fig. 6).

Binding of NAG to human sperm

Binding of NAG to human sperm was carried out to analyse whether the ability of both epididymal and sperm NAG to be stimulated by TX100 could be due to an epididymal enzyme being adsorbed onto sperm during maturation, as reported for the rat (Barbieri *et al.*, 1994). Given the difficulty of obtaining fresh human epididymal sperm and pure epididymal enzyme, this possibility was analysed by carrying out binding assays using ejaculated sperm and recombinant NAG. Human recombinant NAG (hrNAG) produced by CHO cells was biotinylated and incubated with sperm. Proteins from control CHO cells were used in parallel as control. As shown in Fig. 7, sperm incubated with hrNAG produced a significantly greater signal than controls supporting the specific binding of the enzyme to human sperm.

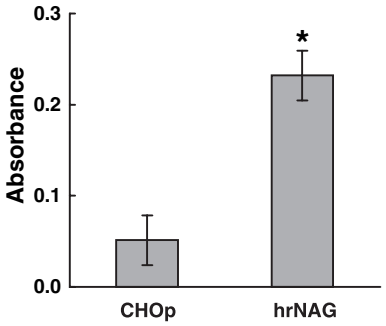

Figure 7 Binding of recombinant *N*-acetylglucosaminidase (NAG) to human sperm. Human recombinant NAG (hrNAG) purified from culture medium of CHO cells with stable expression of the enzyme was biotinylated and incubated with human sperm. Binding of the enzyme to sperm was verified by subsequent incubation with avidin-conjugated alkaline phosphatase and colorimetric development with a specific substrate. Biotinylated proteins secreted by control CHO cells subjected to a similar procedure were used as controls (CHOp). Results are expressed as arbitrary units as detailed in materials and methods. $*p < 0.05$ vs. CHOp.

Discussion

Although many reports support the acrosomal origin of sperm NAG, previous results from our laboratory suggesting its possible role in human sperm maturation (Miranda *et al.*, 1995), induction of acrosome reaction (Brandelli *et al.*, 1994) and binding to the ZP (Miranda *et al.*, 2000), indicated the possibility of a membrane-associated enzyme. As a result, studies were initiated to determine the subcellular location of this enzyme in human spermatozoa.

Results revealed that a high proportion of NAG was released following sperm incubation in isotonic medium. This could be attributed to the liberation of the acrosomal enzyme from disrupted cells and/or to the dissociation of an enzyme weakly associated with the plasma membrane. The results of the western blot and the immunofluorescence on non-permeabilized cells support the presence of NAG in the human sperm plasma membrane.

Other groups also supported our previous suggestion of NAG presence in the plasma membrane of human (Hutchinson *et al.*, 2002) and boar spermatozoa (Flesch *et al.*, 1998). However, NAG was reported to be located at the plasma membrane of somatic cells only recently (Mencarelli *et al.*, 2005).

The extraction of sperm NAG under isotonic (physiological) conditions suggests its low affinity association to sperm plasma membrane. It is unlikely that NAG is bound through its active site, as the presence of its ligand, N-acetyl glucosamine, did not alter extraction efficiency (data not shown).

It is worth noting that TX100 was able to enhance sperm NAG activity. The use of different detergents produced a similar stimulation (data not shown). Interestingly, the activation of a membrane-bound NAG from insect cells by TX100 has been reported (Altmann *et al.*, 1995) and in hamster spermatozoa, a similar effect was also found (unpublished results).

Although the present results appear to disagree with the present view of NAG being primarily an acrosomal enzyme, this contradiction could be only apparent. An enzyme loosely associated with the plasma membrane would be included in the soluble extract obtained after subcellular fractionation and considered cytosolic or acrosomal. In addition, it is usually the extracted rather than the total activity that is measured, or, in some cases, a detergent has been used to assess total activity. If the detergent were to increase total activity, the enzyme liberated under other conditions would be underestimated.

Taking into account that detergents are able to modify conformation and/or environmental conditions and consequently improve enzyme activity, it was initially supposed that stimulation of sperm NAG by TX100 could be a common feature of this enzyme. However, no activation was found when testing blood plasma or human recombinant NAG. To determine whether this attribute was specific to NAG from reproductive organs, the assay was repeated with testicular cytosol and SP, with the same result. Only in those extracts containing epididymal proteins was NAG stimulated by the addition of TX100. The magnitude of activation observed in the epididymal extracts was lower than the one obtained in non-capacitated spermatozoa, possibly resulting from the different nature of both extracts and the wide variability intrinsic in human samples. Taking into account that NAG from the epididymal fluid is contained within SP, the disappearance of the TX100 enhancing effect after ejaculation was surprising. This result could be attributed to the insensitivity of NAG from accessory sex glands to the detergent, as suggested by the results obtained using SP from vasectomized patients. However, epididymal fluid provides a significant proportion of the NAG activity found in human SP (Miranda *et al.*, 1995). The possible existence of a NAG regulatory mechanism in the reproductive tract is currently being determined. Preliminary results from our laboratory suggest that the accessory glands would secrete a component that blocks the effect of the detergent, as the addition of SP from vasectomized donors to cauda epididymal fluid abolishes the TX100 enhancing effect (data not shown).

The weak association of NAG with the sperm plasma membrane would seem more attributable to a decapacitating factor rather than a protein involved in sperm interaction with the ZP. However, although sperm incubation under capacitating conditions released NAG activity to the medium, cell-associated activity did not decrease. Moreover, this activity was more resistant to removal by isotonic medium. These results suggest that NAG is not lost during capacitation, but remains associated with the sperm where it appears to become more tightly bound. This behaviour does not support a role for NAG as a decapacitating factor, but instead suggests its involvement in a later functional event. This possibility is in accordance with a previous report from our laboratory where sperm pre-incubation with NAG did not impair, and in some cases improved, binding to the ZP (Miranda *et al.*, 2000).

Taking into account the enzyme found in the capacitation medium, NAG total activity increased as a consequence of sperm incubation. The requirement of capacitating conditions for this increase was verified by omitting BSA or replacing it with cyclodextrin (data not shown). A similar increase in NAG activity as a consequence of capacitation in dog spermatozoa has also been reported (Kawakami *et al.*, 1999).

Results showing that both TX100 and incubation under capacitating conditions increase sperm NAG total activity, support the possibility that a phenomenon similar to that artificially caused by the detergent would occur in spermatozoa as a consequence of capacitation. This could explain the lack of effect of TX100 on NAG following sperm incubation, as capacitation already increases total enzyme activity (compare the Non-Cap TX100 bar in Fig. 4 with Cap in Fig. 5). This increase in sperm NAG activity could be attributed to an environmental change that improves its catalytic capacity

and/or modifies the interaction with a modulating agent. Alternatively, the changes observed could be produced by improved access to the enzyme. This last possibility would agree with the capacitation-dependent exposure of glycosidades on sperm plasma membrane reported by Tulsiani and co-workers (Abou-Haila & Tulsiani, 2003).

The location of NAG in the sperm plasma membrane and the common detergent sensitivity with the epididymal activity suggested that both enzymes share some particular features. This would agree with previous results from our laboratory that led to the hypothesis of an epididymal NAG being associated with human sperm during maturation (Miranda *et al.*, 1995). Although the pure epididymal enzyme and epididymal sperm were not available for this study, results obtained using ejaculated sperm and recombinant NAG suggest that the enzyme is able to bind to human sperm. This result could be related to the higher ZP-binding ability found in some samples after sperm pre-incubation with hrNAG (Miranda *et al.*, 2000). However, more experiments would be required to support this hypothesis and confirm the participation of NAG in the human sperm epididymal maturation process.

The requirement for NAG during gamete interaction has been questioned following the report that knock out mice were fertile at a young age (Juneja, 2005). However, the functioning of compensatory mechanisms in genetically modified animals should be considered before extrapolating the results to normal individuals, where the disruption of NAG function proved to affect fertilization (Farooqui & Srivastava, 1980; Godknecht & Honegger, 1991, 1995; Miller *et al.*, 1993).

In summary, these results suggest the existence of a population of NAG in the human sperm plasma membrane, as well as enzyme modulation by incubation under capacitating conditions. Both these features would be in accordance with the proposed participation of sperm NAG in human sperm–ZP interaction (Miranda *et al.*, 2000), including primary binding as recently reported for the hamster (Zitta *et al.*, 2006).

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