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Nitric Oxide Regulates Human Sperm Capacitation and Protein-Tyrosine Phosphorylation In Vitro[1]

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ABSTRACT

The aim of the present study was to investigate whether the generation of nitric oxide by human spermatozoa is associated with human sperm capacitation and with the tyrosine phosphorylation of sperm proteins. Human spermatozoa were capacitated in the presence or absence of nitric oxide-releasing compounds or nitric oxide synthase inhibitors, and then the percentage of acrosome loss induced by human follicular fluid or by calcium ionophore was determined. The presence of the nitric oxide-releasing compounds primed spermatozoa to respond earlier to human follicular fluid whereas nitric oxide synthase inhibitors decreased the percentage of acrosome reaction. Moreover, nitric oxide modulated tyrosine phosphorylation of sperm proteins. A tight correlation between capacitation and tyrosine phosphorylation regulated by nitric oxide was observed. Results indicate that nitric oxide is involved in human sperm capacitation and emphasize the importance of oxidoreduction reactions in the fine control of sperm physiology.

INTRODUCTION

Sperm capacitation is a maturational process that occurs in vivo in the female genital tract or can be accomplished in vitro in defined media; the endpoint of this process confers upon the sperm the ability to undergo the acrosome reaction and to fertilize an egg [1]. Many stimuli can induce the acrosome reaction, including the calcium ionophore A23187, follicular fluid, the steroid progesterone, and the zona pellucida [1, 2]. However, discrepancies appear in the literature about the use of some of these inducers. For example, some authors claimed that A23187 is able to bypass capacitation and thus induce the acrosome reaction in noncapacitated sperm [3]. However, in this case, a high concentration of the ionophore (10 μM) was used. Similarly, some studies suggested that spermatozoa are able to respond to human follicular fluid (hFF) after a short period of time (3- to 4-h incubation) [4], while others found that a longer period of time (6 h or more) is necessary for hFF to induce an acrosome loss [5]. These discrepancies could be explained by differences in the chemical composition and protein source in the capacitating medium [6] as well as in the concentrations of the inducers tested.

Capacitation has been shown to be correlated with changes in sperm plasma membrane fluidity, intracellular ion concentration, metabolism, and motility [1], and also with protein tyrosine phosphorylation [7, 8], although some studies suggested that these phosphorylations are not sufficient to achieve the complete process of capacitation [4, 9]. In fact, we believe that protein tyrosine phosphorylation may represent an important pathway that may ultimately regulate events associated with capacitation.

Both capacitation and tyrosine phosphorylation seem to be regulated by oxido-reduction events stimulated by the cellular generation of reactive oxygen species (ROS) [10, 11]. In the last few years, much evidence has emerged to support a role for ROS, mainly superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), on sperm function [12]. Not surprisingly, the free radical, nitric oxide (NO), also appears to be involved in sperm physiology. Synthesis of NO occurs in cells of various types upon arginine oxidation by a specific monooxidase named nitric oxide synthase (NOS), whose activity depends on calmodulin and Ca^{2+} [13]. In spermatozoa, some studies demonstrated that the addition of NO to the capacitating medium affects sperm motility [14–18], hyperactivation [19, 20], and zona pellucida binding [21], while recent reports suggested the presence of NOS in mouse and human spermatozoa [22–24]. However, the role of endogenous NO on human sperm capacitation still remains to be elucidated. Thus, the present study will show the effect of NO on tyrosine phosphorylation of sperm proteins, as well as in the capacitation process, measured by the induction of acrosome loss of two inducers, human follicular fluid (hFF) and the calcium ionophore A23187.

MATERIALS AND METHODS

Chemicals

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): water tissue culture grade, BSA (fraction V), 1,4-diaz-abicyclo[2.2.2.]octane (DABCO), sodium nitroprusside (SNP), calcium ionophore A23187, and *Pisum sativum* agglutinin conjugated to fluorescein isothiocyanate (PSA-FITC). Percoll was obtained from Pharmacia (Dorval, PQ, Canada). Diethylamine-NONOate (ethanamine,1,1-diethyl-2-hydroxy-2-nitrosohydrazine), N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-D-arginine methyl ester (D-NAME), and 7-nitroindazole were bought from Research Biochemicals International (Natick, MA). Monoclonal anti-phosphotyrosine antibody (clone 4G10; Upstate Technology Inc., Lake Placid, NY), nitrocellulose (0.22-μm pore size; Micron Separations Inc., Westboro, MA), goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Kirkegaard and Perry Lab., Gaithersburg, MD), an enhanced chemiluminescence (ECL) kit (Amersham Life Sciences Inc., Oakville, ON, Canada), and x-ray films (Fuji, Minami-Ashigara, Japan) were used for immunodetection of phosphotyrosine-containing proteins. All other chemicals were of reagent grade.

Sperm Preparation

Semen samples were obtained by masturbation from healthy volunteers after 3 days of sexual abstinence. All

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samples had normal semen parameters, according to World Health Organization (WHO) guidelines [25]. The specimens were allowed to liquefy for 30–60 min at room temperature, and then motile spermatozoa were selected by centrifugation through a two-step (40–90%) Percoll gradient as described previously [26]. The pellet was resuspended in modified Tyrode's medium consisting of 117.5 mM NaCl, 0.3 mM NaH_2PO_4, 8.6 mM KCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2 mM glucose, 0.25 mM sodium pyruvate, 19 mM sodium lactate, and 70 μg/ml each of streptomycin and penicillin.

Sperm aliquots (500 μl), at 20×10^6 cells/ml, were incubated in Tyrode's medium plus BSA (Tyrode-BSA) in 1.5-ml micro test tubes for 4 h or 20 h, at 37°C, in 5% CO_2 in air, in the presence or absence of different nitric oxide-releasing compounds (diethylamine-NONOate [da] or SNP) or NOS inhibitors (L-NAME or 7-nitroindazole). Stock solutions of all the components were prepared in Tyrode's medium just before use, except for 7-nitroindazole, which was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 0.1% and did not affect motility or acrosomal integrity. As controls for NO generation from SNP and da, these two compounds were preincubated in Tyrode-BSA for 20 h (to completely exhaust NO formation) and then added to the sperm suspension.

Collection of Human Follicular Fluid

The follicular fluid used in this study was generously provided by the McGill Reproduction Centre at the Royal Victoria Hospital (Montréal, PQ, Canada). The hFF was collected from preovulatory follicles after gonadotropin stimulation, centrifuged for 10 min at 20 000 × *g*, and kept frozen in aliquots at −20°C until use.

Follicular Fluid and Calcium Ionophore-Induced Acrosome Reaction

Spermatozoa were incubated for 4 h or 20 h in the presence or absence of the NO-releasing compounds or NOS inhibitors, washed in Tyrode's medium (500 × *g*, 5 min), and then challenged with 20% hFF (v:v) or 5 μM A23187 for 30 min to induce the acrosome reaction. Sperm were then incubated in 0.2 ml of hyposmotic swelling medium (HOS) for 1 h at 37°C [27] and finally resuspended in 30 μl ice-cold ethanol. The sperm suspension was smeared on a slide, air-dried at room temperature, and stored at −20°C. Sperm acrosome reaction was evaluated by using PSA-FITC as previously described [28] on a total of 100 spermatozoa. Only spermatozoa with curly tails were considered viable and thus scored.

Western Blot Analysis

After the different incubations, spermatozoa were washed twice in Tyrode's medium (500 × *g* × 5 min), and then samples were processed for SDS-PAGE. Briefly, sperm samples containing 20×10^6 cells/ml were resuspended in Laemmli's sample buffer (final concentrations: 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8) and heated at 100°C for 5 min. Proteins were then separated by electrophoresis on 7% SDS-polyacrylamide gels [29] and electrotransferred to nitrocellulose membranes [30]. Nonspecific binding sites on the membrane were blocked with 5% (w:v) skimmed milk in Tris-buffered saline (0.9% NaCl, 20 mM Tris-HCl, pH 7.8) supplemented with Tween 20 (0.1%) (TTBS). The nitrocellulose membrane was incubated for 1 h at room temperature with the anti-phosphotyrosine antibody (1/10 000). The membrane was extensively washed in TTBS, and goat anti-mouse IgG conjugated with horseradish peroxidase (1:5000) was added. After a 45-min incubation period at room temperature, the membrane was extensively washed, and positive immunoreactive bands were detected by chemiluminescence using ECL according to the manufacturer's instructions.

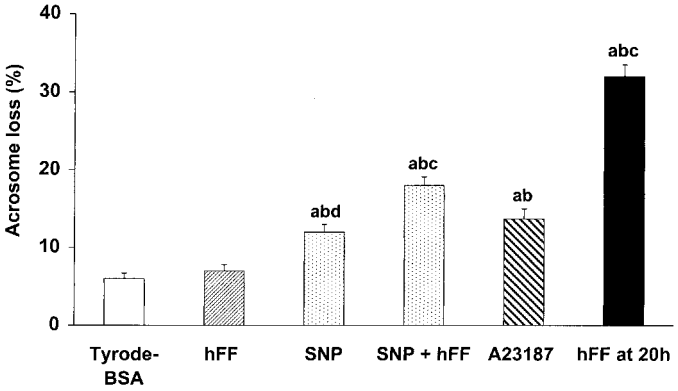

FIG. 1. Effect of SNP on human sperm capacitation. Spermatozoa were incubated in Tyrode-BSA for 4 h in the presence or absence of 0.1 mM SNP. In some groups, 20% hFF was added for another 15 min to induce the acrosome reaction. As positive controls, spermatozoa were incubated for 4 h or 20 h in Tyrode-BSA and then 5 μM calcium ionophore A23187 or 20% hFF was added, respectively, to induce the acrosome loss. The acrosome reaction was then evaluated as described in *Materials and Methods*. Results are mean ± SEM of five different samples. a, Significantly different from Tyrode-BSA ($p < 0.05$); b, significantly different from hFF (4 h; $p < 0.05$); c, significantly different from A23187; d, significantly different from SNP+hFF ($p < 0.05$).

Data Analysis

Statistical differences between treatments were measured by Fisher's protected least-significant difference test after a one-way ANOVA on paired observations.

RESULTS

Nitric Oxide-Releasing Compounds Induced Sperm Capacitation

The potential of NO-releasing compounds to stimulate capacitation was assessed by monitoring the responsiveness of spermatozoa to hFF. After 20 h of incubation, the addition of hFF induced acrosome reaction (positive control), but a 4-h incubation period was inadequate (Fig. 1). However, the presence of low concentrations of NO produced by SNP or da, followed by the addition of hFF at 4 h, caused an increase in acrosome reaction (Figs. 1 and 2). Moreover, after a 4-h incubation, each NO-releasing compound at a concentration of 0.1 mM was able to elicit an acrosome reaction by itself but at a level significantly lower than that induced by treatment with the NO-releasing compound followed by challenge with hFF (Figs. 1 and 2). In all cases, the degradation products of SNP and da (preincubated for 20 h before use) were assayed, and no significant changes were observed compared to the Tyrode-BSA group.

The importance of NO in sperm function was also indicated by the ability of the NO-releasing compounds plus

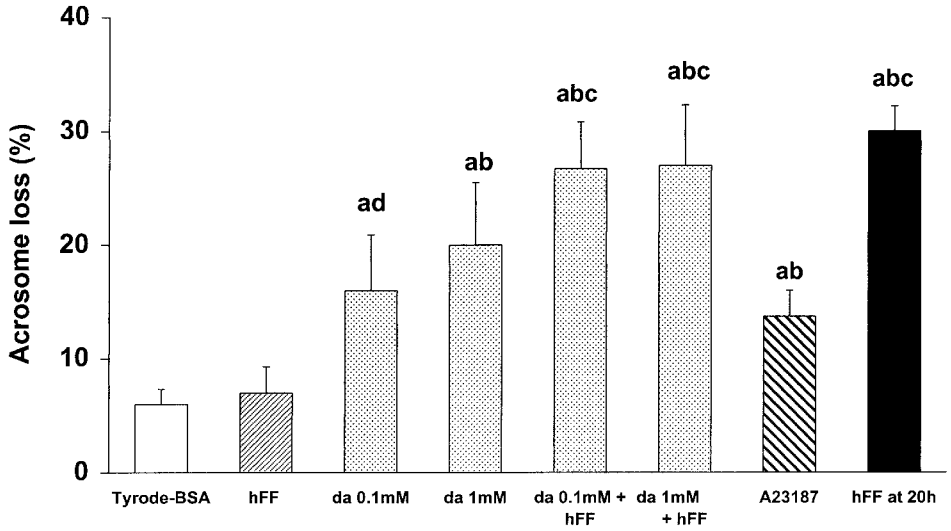

FIG. 2. Effect of da on human sperm capacitation. Spermatozoa were incubated in Tyrode-BSA for 4 h in the presence or absence of 0.1 mM or 1 mM da. In some groups, 20% hFF was added at the end of the incubation for another 15 min. As positive controls, spermatozoa were incubated for 4 h or 20 h in a capacitating medium, and then 5 μM calcium ionophore A23187 or 20% hFF was added, respectively, to induce the acrosome loss. The acrosome reaction was then evaluated as described in *Materials and Methods.* Results are mean ± SEM of five different samples. a, Significantly different from Tyrode-BSA ($p < 0.05$); b, significantly different from hFF (4 h; $p < 0.001$); c, significantly different from A23187 ($p < 0.05$); d, significantly different from da 0.1 mM+hFF ($p < 0.05$).

hFF to induce higher rates of acrosome reaction than that induced by the calcium ionophore A23187 (Figs. 1 and 2).

None of the treatments evaluated had an impact on the percentage of sperm motility, except for 1 mM da, which caused a reduction (25–50%) compared with the control (Tyrode-BSA).

L-NAME and 7-Nitroindazole Inhibited Human Sperm Capacitation

Two different NOS inhibitors (L-NAME and 7-nitroindazole) were used to study the effect of endogenous NO on human sperm capacitation. The presence of 0.1 mM or 1 mM L-NAME did not modify the percentage of acrosome reaction induced by hFF at 4-h of incubation (5 ± 1%). However, the presence of L-NAME, as well as 7-nitroindazole, reduced the percentage of acrosome loss induced by hFF at 20-h of incubation (Figs. 3 and 4, respectively). Moreover, the inhibitory response of L-NAME was dependent on the concentration of this inhibitor, and the inactive enantiomer D-NAME had no inhibitory effect, suggesting once more the specific effect of the NOS inhibitor (Fig. 3).

To determine whether sperm NO synthesis was important early in the capacitation process or whether it is required throughout capacitation, studies were performed in which L-NAME was added at 15, 60, 120, or 240 min after the onset of the incubation. The percentage of acrosome loss induced by hFF at 20 h of incubation decreased in all cases, suggesting that NOS activity was necessary for capacitation, at least during the first 4 h of incubation (Fig. 5, A and B).

L-NAME also inhibited sperm capacitation as measured by the calcium ionophore-induced acrosome reaction. Spermatozoa were incubated in the presence of L-NAME for 4 h, and then 5 μM A23187 was added for another 30 min. Results indicated that the percentage of acrosome loss was significantly reduced compared to that of the A23187 group, suggesting that NOS is involved in capacitation and that it may play its role downstream to the calcium entry (Table 1).

Nitric Oxide Induced Changes in Tyrosine Phosphorylation

To further delineate the involvement of NO on capacitation, the tyrosine phosphorylation of sperm proteins was investigated, since this process appears to be associated with sperm capacitation and regulated by oxidoreduction

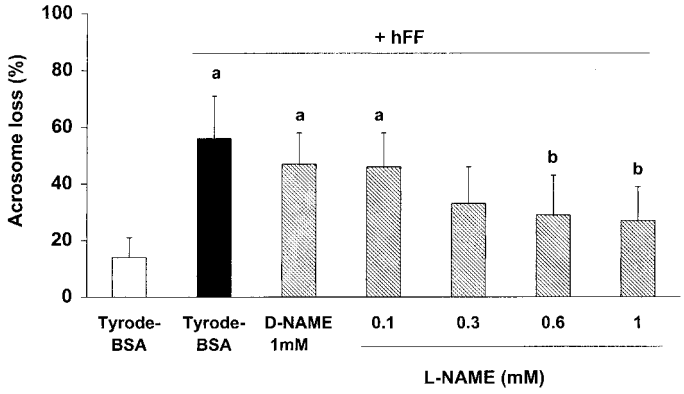

FIG. 3. Effect of different concentrations of L-NAME on human sperm capacitation. Spermatozoa were incubated in Tyrode-BSA for 20 h in the presence or absence of different concentrations of L-NAME or 1 mM D-NAME. At the end of the incubations, 20% hFF was added for another 15 min. The acrosome reaction was then evaluated as described in *Materials and Methods.* Results are mean ± SEM of five different samples. a, Significantly different from Tyrode-BSA ($p < 0.05$); b, significantly different from the Tyrode-BSA + hFF ($p < 0.05$).

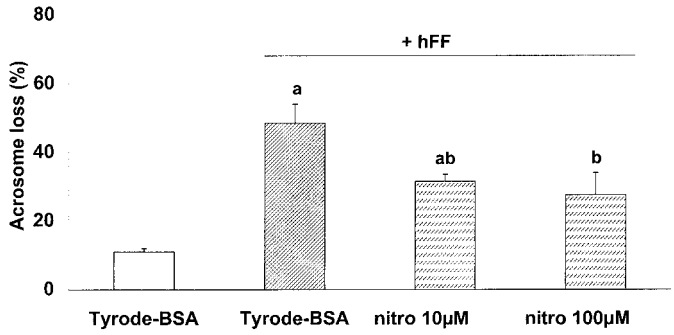

FIG. 4. Effect of 7-nitroindazole (nitro) on human sperm capacitation. Spermatozoa were incubated in Tyrode-BSA for 20 h in the presence or absence of 10 μM or 100 μM of 7-nitroindazole. At the end of the incubations, 20% hFF was added for another 15 min. The acrosome reaction was then evaluated as described in *Materials and Methods.* Results are mean ± SEM of four different samples. a, Significantly different from Tyrode-BSA ($p < 0.01$); b, significantly different from Tyrode-BSA plus hFF ($p < 0.05$).

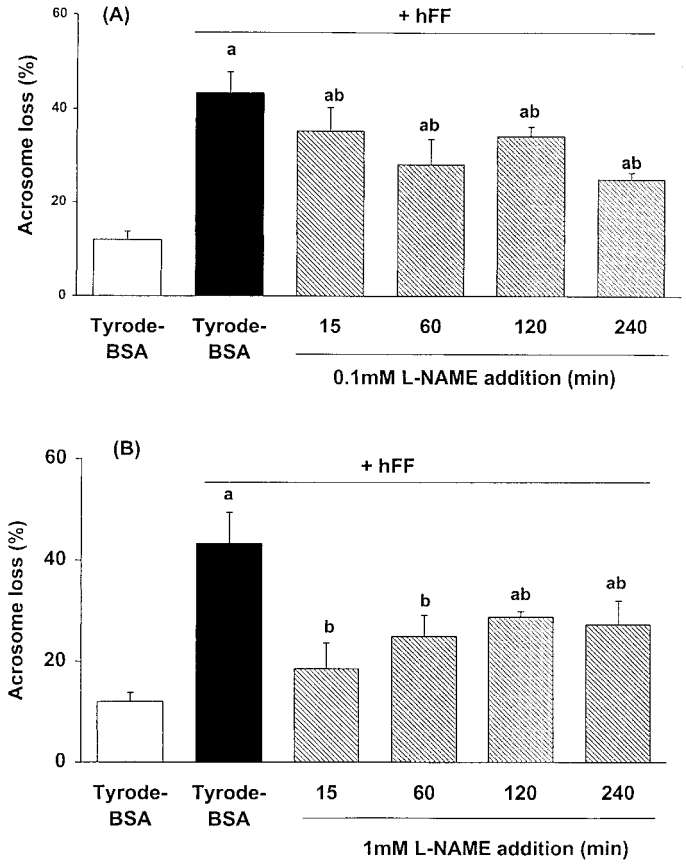

FIG. 5. Effect of L-NAME added at different times during capacitation. Spermatozoa were incubated in Tyrode-BSA for 20 h. At 15, 60, 120, or 240 min during capacitation, **A**) 0.1 mM or **B**) 1 mM L-NAME was added. At the end of the incubations, 20% hFF was added for another 15 min. The acrosome reaction was then evaluated as described in *Materials and Methods.* Results are mean ± SEM of five different samples. a, Significantly different from Tyrode-BSA ($p < 0.01$); b, significantly different from Tyrode-BSA plus hFF ($p < 0.05$).

reactions [9]. After a 4-h incubation, the tyrosine phosphorylation of sperm proteins was enhanced when a high concentration of da was tested in comparison to the control (Tyrode-BSA) and the hFF groups (Fig. 6). Subsequent treatment with da and hFF caused even a greater increase

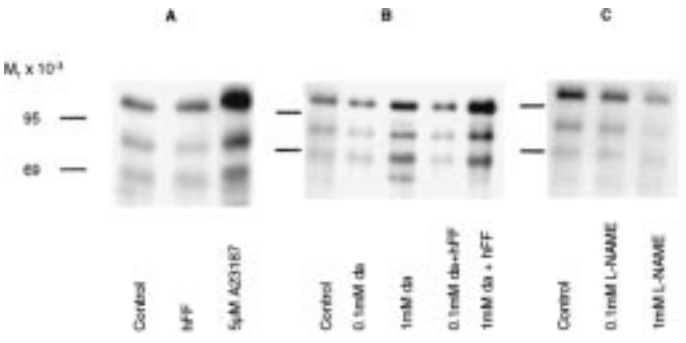

FIG. 6. Tyrosine phosphorylation of proteins in human spermatozoa after 4-h incubation. Effect of L-NAME and da. Spermatozoa were incubated in Tyrode-BSA for 4 h: **A**) medium alone (control), **B**) different concentrations of da, or **C**) L-NAME. In some groups, 20% hFF or 5 μM A23187 was added at the end of the incubation. Sperm proteins were processed as described in *Materials and Methods.* The positions of molecular weight markers are shown on the left. This blot is representative of four assays, and each assay was done with a different sample.

TABLE 1. Effect of L-NAME on human sperm capacitation measured by calcium ionophore A23187-induced acrosome reaction.

Treatment*	Acrosome loss (%)	*P*
Control	6 ± 3	
5 μM A23187	16 ± 4	<0.05[a]
0.1 mM L-NAME + 5 μM A23187	7 ± 3	
1 mM L-NAME + 5 μM A23187	6.2 ± 0.2	

* Spermatozoa were incubated in Tyrode-BSA for 4 h in the presence or absence of 0.1 mM or 1 mM L-NAME. Five micromolar A23187 was then added for another 30 min. The acrosome loss was evaluated as described in *Materials and Methods.* Results are mean ± SEM of three different experiments.

[a] Value significantly different from that obtained in the control ($P < 0.05$).

in protein tyrosine phosphorylation, which was similar to that observed when spermatozoa were challenged with A23187 after a 4-h incubation. The enhancement primarily involved a complex set of protein bands within a molecular weight range of 116–60 × 10^{-3}. In contrast, the presence of different concentrations of L-NAME during the 4-h incubation caused a suppressive effect on the levels of tyrosine phosphorylation in human sperm proteins. It is important to note that the same proteins that were modified with da were also affected by L-NAME (Fig. 6).

Since NO can affect the tyrosine phosphorylation pattern of sperm proteins during capacitation, we then investigated the time at which these modifications occurred. Figure 7 shows that in the control, the level of tyrosine phosphorylation of proteins increased with time. However, treatment of spermatozoa with L-NAME caused a decrease in the level of tyrosine phosphorylation at all time periods tested, and these decreases occurred in a concentration-dependent manner. In contrast, when spermatozoa where incubated in the presence of da, there was an increase in the tyrosine phosphorylation of proteins, which occurred earlier than that of the control (Fig. 7).

DISCUSSION

In the last decade, NO has assumed an important functional role in a variety of physiological systems and different pathways [31]. In spermatozoa, NOS has been localized on the tail and acrosome of human and mouse spermatozoa [23, 24], and it was demonstrated that it plays a role in the mouse fertilization process [22].

In the present paper, we showed through a pharmacological approach that NO is involved in capacitation, and we supported a regulatory role for NO in the tyrosine phosphorylation of sperm proteins.

Similar to the work published by Calvo and coworkers [5], our observations have been that hFF cannot induce the acrosome reaction after a 4-h incubation but only after 6 h or more (our experimental design used 20-h incubation). In addition, our results demonstrate that the presence of NO-releasing compounds in the capacitating medium enables spermatozoa to respond earlier to hFF, that is, after a 4-h incubation. The ability to accelerate the capacitation process varies among NO-releasing compounds. The presence of SNP in the incubation medium accelerates capacitation and thus the ability of sperm to undergo acrosome reaction if stimulated with hFF, although the percentage of acrosome loss is lower than that observed with da. This difference in potency is probably related to the kinetics of NO formed from the NO-releasing compounds, since SNP generates NO instantaneously (3–10 sec) while da releases NO in a

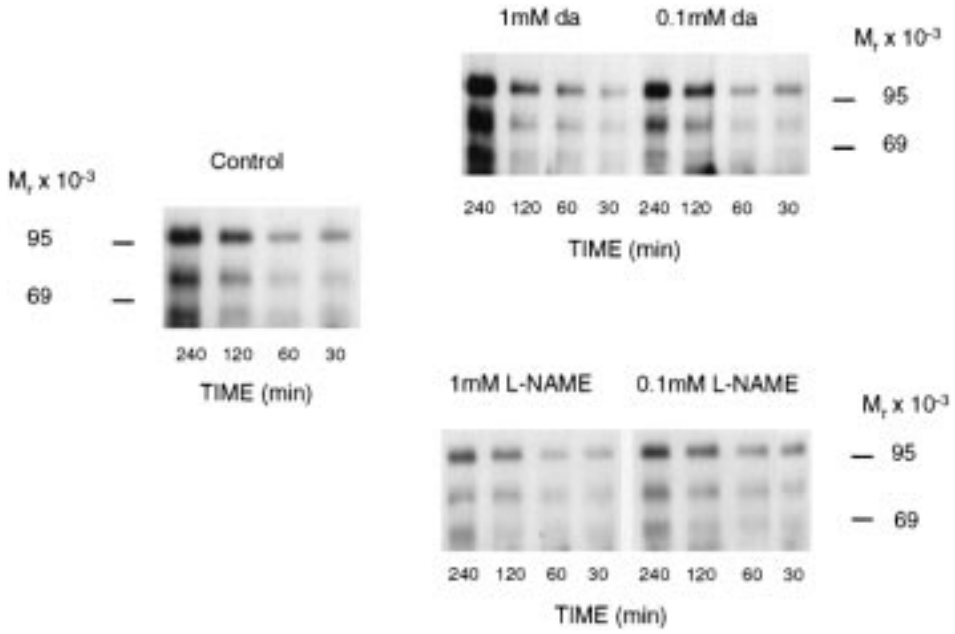

FIG. 7. Time course of protein tyrosine phosphorylation in human spermatozoa. Effect of L-NAME and da. Spermatozoa were incubated in Tyrode-BSA for the times indicated at the bottom of each panel. Left: medium alone; top right: 0.1 mM or 1 mM da; bottom right: 0.1 mM or 1 mM L-NAME. Sperm proteins were processed as described in *Materials and Methods.* This blot is representative of four assays, and each assay was done with a different sample.

longer period of time. Thus, we can postulate that NO requires a short period of time to exert its action on the capacitation process, perhaps initiating a cascade of events that will lead to capacitation in a manner similar to that observed with cAMP in many other systems.

As mentioned in the *Results* section, low concentrations of SNP or da did not affect sperm motility, while sperm motility was reduced 25–50% in the presence of 1 mM da. Several papers have been published on NO and sperm motility, but results are controversial. Our findings are in agreement with those of Weinberg et al. [16], but differ from those of Tomlinson et al.[14], in which even very low concentrations of SNP (10^{-6} M) decreased sperm motility. Differences in sperm preparation methods and in capacitating conditions may have caused these discrepancies.

The addition of the NOS inhibitors L-NAME and 7-nitroindazole at the onset of the incubation period caused an important decrease of the acrosome reaction induced by hFF at 20 h of incubation. Similar results were obtained when the acrosome reaction was induced by A23187 at 4-h incubation. These results indicate that sperm NOS activity and the resulting NO production is necessary for human sperm capacitation and thus to enable spermatozoa to display their full potential to acrosome-react. However, at this point it is not clear how NOS is activated during capacitation. It might be expected that sperm NOS, which seems to be a constitutive isoform [24], is activated by Ca^{2+} during capacitation, although the involvement of Ca^{2+} in initiating and/or regulating capacitation is still uncertain. Nevertheless, the action of Ca^{2+} at the level of effector enzymes involved in sperm signal transduction suggests that this divalent cation is likely to play an important role in capacitation. Clearly, more work is needed to define NOS regulation in spermatozoa.

NO produced by spermatozoa during at least the first 4 h of incubation appeared to be essential for capacitation to proceed. This observation is different from that made with $O_2^{\cdot -}$, which indicated that $O_2^{\cdot -}$ generated by spermatozoa during capacitation was needed only for the first 30 min of incubation [4]. Therefore, NO and $O_2^{\cdot -}$ may have different roles in promoting sperm capacitation. To our knowledge, no evidence has yet been published to support the possibility that NO may modulate $O_2^{\cdot -}$ production. However, it was recently shown that under certain conditions, NOS can synthesize $O_2^{\cdot -}$ [32, 33]. On the other hand, in a recent paper it was demonstrated that $O_2^{\cdot -}$ can potentiate NOS expression in interleukin 1β-stimulated rat mesangial cells [34], and it was also demonstrated that xanthine oxidase can generate NO in the absence of NOS activity [35]. In addition, it is well known that $O_2^{\cdot -}$ and NO can react to form peroxinitrite [31]. Furthermore, it was also demonstrated that H_2O_2 is involved in capacitation, as well as in tyrosine phosphorylation [10, 20]. Thus the question that arises is whether all three ROS share common or different targets during capacitation and whether there is a tight regulation among these three ROS.

The importance of NO on sperm capacitation was also reflected on the levels of tyrosine phosphorylation of sperm proteins. A correlation between sperm capacitation and tyrosine phosphorylation was observed: when capacitation was accelerated by a NO-releasing compound, there was an increase in tyrosine phosphorylation, whereas when sperm capacitation was inhibited by L-NAME, there was an attenuation in the tyrosine phosphorylation of sperm proteins. Therefore, tyrosine phosphorylation of sperm proteins appears to be regulated by NO. However, the stimulation of tyrosine phosphorylation by NO is not specific to sperm cells since it has been demonstrated in the rat cerebral cortex as well [36].

The mechanism by which NO can modulate tyrosine phosphorylation still remains unknown. Perhaps the most important intracellular signaling role for NO is its capacity to activate guanylate cyclase [37]. In other tissues, submicromolar concentrations of NO cause a rapid increase in cGMP levels in target cells, which leads to physiological responses. Many of these responses are mediated by the activation of the protein serine/threonine kinase, the cGMP-dependent protein kinase (PKG) [38]. Whether or not this represents a physiologically important signaling mechanism for NO in spermatozoa is not known at this time.

The signaling pathways by which NO affects cell function are by no means limited to the stimulation of guanylate cyclase. Nitric oxide has been shown to act via the cAMP/protein kinase A (PKA) [39], and since cAMP is the best-

established messenger stimulating the tyrosine phosphorylation event during capacitation [8, 40], it is conceivable that a linkage between NO and the cAMP pathway could account for the stimulatory effect of NO on protein tyrosine phosphorylation. Nitric oxide could cause a stimulation of PKA either through a transactivation of cGMP protein kinase or through a cGMP-induced inhibition of cAMP degradation via cyclic nucleotide phosphodiesterase type 3 as demonstrated in other tissues [41].

Alternatively, the stimulatory effects of NO on tyrosine phosphorylation might involve the tyrosine kinase-phosphatase system. Zini and coworkers [20] suggested that hydrogen peroxide is needed for the action of NO in human sperm capacitation [20]. These data together with the fact that hydrogen peroxide, produced in different tissues, inhibits phosphatase activity and stimulates tyrosine kinase activity may suggest that NO could modulate these enzymes.

In conclusion, our results demonstrate that endogenous NO produced by human spermatozoa is involved in capacitation and the associated tyrosine phosphorylation of proteins. These results emphasize the importance of oxidoreduction reactions in the fine control of sperm physiology. Further investigations are currently in progress to elucidate the possible role of NO as an intracellular messenger during sperm capacitation.

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