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Effects of Nitric Oxide Modulating Drugs on Acrosome Reaction in Mouse Spermatozoa

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Nitric oxide (NO) is a reactive free radical which plays important roles in animal physiology. To investigate involvement of NO in acrosome reaction (AR), effects of drugs which modulate the intracellular NO level were examined in mouse spermatozoa. N (G)-nitro-L-arginine (L-NA), a potent inhibitor of NO synthesis, decreased AR in a reversible manner. On the other hand, sodium nitroprusside (SNP), an NO generating agent, increased spontaneous AR. Preincubation of sperm in the presence of L-NA potentiated AR after sperm transfer into plain- or SNP-media. Methylene blue, a NO scavenging agent, decreased spontaneous AR. Taken together, it is concluded that NO positively controls AR.

In mammalian fertilization, the sperm undergoes physiological changes called "capacitation" in the female genital tracts or in vitro (Austin, 1951; Chang, 1951). Acrosome reaction (AR), a Ca^{2+}-dependent exocytotic process, occurs just before the capacitated sperm penetrates zona pellucidae of the oocyte (Yanagimachi, 1994). Sperm capacitation and AR are regulated by a number of molecules present in the female genital tracts, and extracellular signals generate various intracellular chemical messengers (Ward and Kopf, 1993).

Nitric oxide (NO) is a reactive free radical which plays an important role in animal physiology (Lowenstein and Snyder, 1992). Under physiological conditions NO reacts with sulfhydryl groups to form S-nitrosothiol derivatives, which exhibit different stabilities and spontaneously decompose or react with each other (Ignarro, 1990). On the other hand, NO, by the formation of S-nitrosocysteine or S-nitrosoproteins, exerts a long-lasting and potent effect on cells (Myers et al., 1990). NO is synthesized by two major classes of nitric oxide synthase (NOS) in neurons, macrophages and endothelial cells (Nathan, 1992). Intracellular targets of NO include guanylate cyclase (Arnold et al., 1977) and glyceraldehyde-3-phosphate dehydrogenase on which NO enhances auto-ADP-ribosylation (Dimmeler and Brune, 1992). It was reported that the mouse spermatozoa contain a NOS isoform and have a potential ability to synthesize NO, suggesting a role for endogenous NO in the mammalian sperm functions (Herrero et al., 1997b). The present study aims to verify the regulation of capacitation and AR of mouse sperm atozoa by NO. Effects of N (G)-nitro-L-arginine (L-NA), an inhibitor of NO production, methylene blue (MB), an intracellular NO scavenger, and sodium nitroprusside (SNP), a NO donor (Snyder, 1992; Wolin et al., 1990; Pandol and Schoeffield-Payne, 1990) on AR were examined in mouse epididymal spermatozoa.

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Materials and Methods

Chemicals

SNP and L-NA were obtained from Research Biochemical International (RBI, USA). Other chemicals were of the highest purity available commercially and were purchased from Sigma.

Sperm preparation

Cauda epididymis was removed from a 3 month-old male mouse (ICR strain), placed in modified Tyrode solution (Parrish et al., 1988) and incubated for 10 min with gentle squeezing. Motile spermatozoa were collected and sperm concentration was adjusted to 10^6 sperm/ml with fresh medium. One ml aliquots were placed in 4-well culture dishes and incubated for 120 min under 5% CO_2 in air.

Drug treatment

L-NA stock solution (50 mM) was prepared in a plain medium and added to sperm suspension in the beginning of the incubation in various concentrations (0, 5, and 50 μM). To test whether the effect of L-NA on AR was reversible after incubation for 60 min, an aliquot of the sperm suspension was centrifuged at 1000 g for 10 min. The pellet resuspended in 10 volumes of plain media or media containing SNP and incubated for 60 min.

SNP stock solution (50 mM) was prepared in a plain medium. Working solution was added to the sperm

suspension from the beginning of the incubation or after incubation for 60 min in the presence or absence of L-NA (50 μM).

MB stock solution (10 mM) was prepared in a plain medium. Working solution was added to the sperm suspension and incubated for 180 min.

Evaluation of acrosome reaction

The method of Moller et al. (1990) was partially modified to stain the acrosome. After the incubation, the sperm suspension was fixed with 10 volumes of 5% formaldehyde in phosphate buffered saline (PBS) for 30 min. After centrifugation at 1,000 g for 10 min, the pellet was washed with PBS. A drop of the sperm suspension was smeared on a slide and air-dried. After drying, the sample was stained with protein assay reagent (Bio-rad) for 2 min. After two washes in PBS, the sample was dehydrated in graded ethanol and permanently mounted. The absence of blue stain on the acrosome was regarded as a sign of AR (Fig. 1). The AR rate (acrosome-reacted sperm / total sperm counted) x 100) was obtained by counting more than 200 sperm per slide. Statistical significance was analyzed by ANOVA.

Results

Initially, inhibition of NO on AR was examined. The continuous presence of L-NA caused a decrease in AR in a concentration dependent manner compared to drug-free control. At lower concentration of L-NA (5 μM), an increase in AR during the incubation 60 min was apparent. But no significant difference in the AR rate was observed between the control and the L-NA treated at the end of the incubation at 120 min. At higher concentration (50 μM), an increase in AR was apparent throughout the incubation (Fig. 2).

Secondly, the effect of the NO donor SNP, was examined. SNP present from the beginning of the incubation increased AR compared to the drug-free control during the 120 min. To test effect of SNP on the AR of capacitated spermatozoa, sperm preincubated for 60 min were challenged to SNP. There was a significant increase in AR in the SNP-treated spermatozoa (Fig. 3).

In the next series, effects of L-NA on AR of capacitated spermatozoa was examined. When the sperm suspension was preincubated with L-NA (5 μM) for the first 60 min and transferred to the drug-free medium for the next 60 min, AR was increased relative to the drug-free control (Fig. 4 N-C vs. C).

Interestingly, this treatment regime potentiated AR when compared to that of plain- to plain medium transfer (Fig. 4 N-C vs. C-C). Similarly, when the sperm suspension which had heen preincubated for 60 min with L-NA was further incubated in the SNP medium

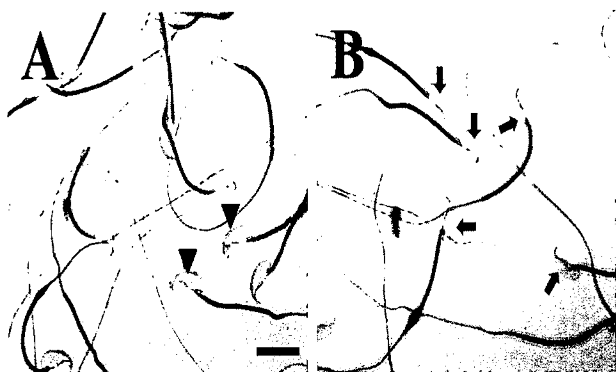

Fig. 1. Microphotographs of mouse spermatozoa stained with Coomassie brilliant blue R250. A, freshly prepared cauda epididymal spermatozoa. Most of spermatozoa have intact acrosomes stained by dye (arrowheads). B, spermatozoa after the Ca^{2+}-ionophore A23187 treatment. Most of spermatozoa lost their acrosomes. Acrosome region of sperm head is free of staining (arrows). Scale Bar=50 μm.

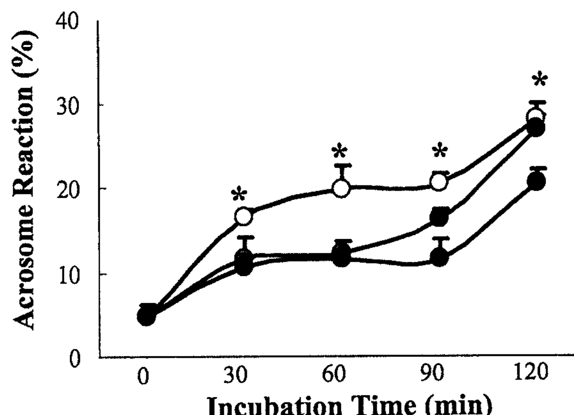

Fig. 2. Effect of L-NA on the acrosome reaction (AR) of mouse epididymal spermatozoa. L-NA was added to sperm suspension at 5 μm ( ) or 50 μm (●) and AR was evaluated at a 30 min interval for 120 min. AR rate was statistically analyzed by one-way ANOVA. Asterisks indicate mean values significantly different from others ($p < 0.05$). Data are mean ± SD (n=4). O; Control.

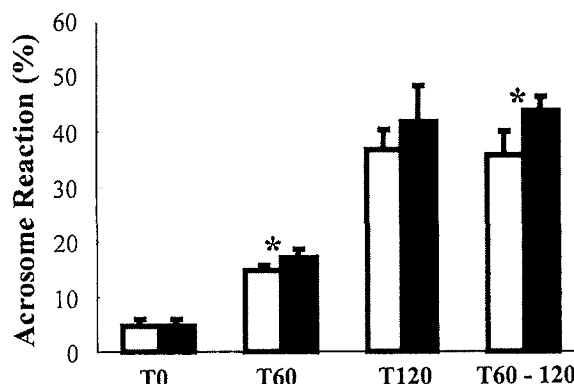

Fig. 3. Effect of sodium nitroprusside (SNP) on the acrosome reaction of mouse epididymal spermatozoa. Mouse epididymal spermatozoa were incubated in the presence (■) or absence (□) of 50 μM SNP for 120 min (T0, T60, and T120). Otherwise, SNP was treated to the sperm suspension preincubated for 60 min in plain medium, and spermatozoa were incubated for 60 min (T60-120). AR was evaluated in 60 min interval for 120 min. AR rate was statistically analyzed by one-way ANOVA. Asterisks indicate mean values significantly different from drug-free control ($p < 0.05$). Data are mean ± SD (n=4).

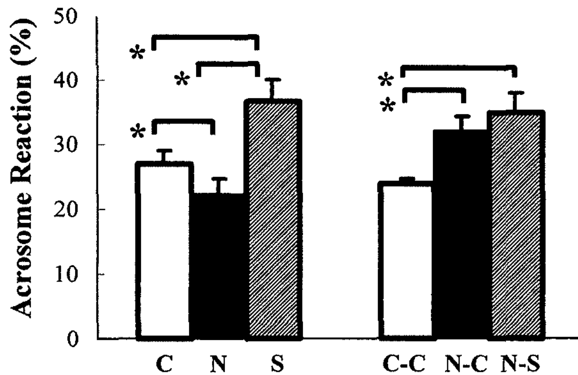

Fig. 4. Effect of L-NA and SNP on the acrosome reaction (AR) of mouse epididymal spermatozoa. Mouse epididymal spermatozoa were incubated in the presence or absence of L-NA or SNP for 120 min. Otherwise, sperm preincubated in the presence or absence of L-NA for 60 min were transferred to plain- or SNP-media and incubated for 60 min. AR was evaluated after incubation for 120 min. The AR rates were statistically analyzed by one-way ANOVA. Asterisks indicate mean values different significantly from each other ($p < 0.05$). Data are mean ±SD (n=4).

for 60 min, AR was increased (Fig. 4 N-S vs. C). Under this condition, however, AR was not significantly increased regardless of the presence or absence of SNP (Fig. 4 N-S vs. N-C). In this series, L-NA significantly inhibited AR (Fig. 4 N vs. C) and SNP increased AR (Fig. 4 S vs. C).

Finally, effect of the NO scavenger MB was examined. There was no obvious difference in AR between control and MB (10 µM)-treated during the 60 min incubation. However, AR was significantly impaired when the incubation was prolonged for 180 min (Fig. 5).

Discussion

Sperm capacitation and AR have been known to be highly dependent on intracellular cyclic nucleotides (Hyne and Garbers, 1979; Santos-Sacchi and Gordon, 1980; Monks and Fraser, 1987; Noland et al., 1988). cAMP and downstream protein kinase system have been known as positive regulators of capacitation and AR (Duncan and Fraser, 1993; Dejong et al., 1991). Increasing bodies of evidence indicate that NO functions as a physiological regulator of AR and that cGMP mediates the NO-induced AR. Recently, a tight correlation of extracellular stimuli such as progesterone (Herrero et al., 1998) and follicular fluid (Anderson et al., 1995; Revelli et al., 1999) with the intracellular NO production in mammalian spermatozoa has been reported. Chatterjee et al. (1996) reported that maximal expression of NOS III in oviduct and uterine during pro-oestrus and oestrus, suggesting that NO was a physiological regulator of sperm function while the sperm resides in the female genital tracts. In our experiments, treatment of L-NA to block production of NO was inhibitory for spontaneous AR in mouse spermatozoa (Fig. 2). This was in agreement with the former study using different NO synthesis inhibitors (Herrero et al., 1998). Conversely, the NO donor SNP, potentiated spontaneous AR during the 60 min incubation of spermatozoa (Fig. 3 T60). Similar results were reported in the human (Sengoku et al., 1998; Joo et al., 1999) and rabbit spermatozoa (Guzman-Grenfell et al., 1999). However, in the prolonged culture the potentiation of AR by SNP was not observed (Fig. 3 T120). When the spermatozoa pretreated with L-NA were transferred to drug-free medium, AR was increased (Fig. 4 N-C), suggesting that the effect of L-NA on AR is reversible and that inhibition of NO production sensitizes the intracellular machinery to NO. Transfer of L-NA-treated sperm to SNP-medium resulted in no significant difference between the drug-free and SNP-medium (Fig. 4 N-C vs. N-S). This suggested that effect the SNP is minute in the prolonged incubation and that particulate GC dominates the soluble GC in spermatozoa. In addition, MB, which scavenges NO and decreases intracellular NO concentration, effectively inhibited the spontaneous AR (Fig. 5). This suggested that blocking or down-regulation of intracellular NO production affect AR. Taken together, it is apparent that NO is a positive regulator of sperm capacitation and AR in mouse spermatozoa.

However, the downstream of NO production leading to AR was unclear in spermatozoa. A tight correlation between tyrosine phosphorylation and NO was reported (Herrero et al., 1999). Progesterone activates NO production and stimulates production of PEG2, an important regulator of AR (Herrero et al., 1995; 1998). An increasing body of evidence indicated the presence of guanylate cyclase (GC) in mammalian spermatozoa and the involvement of NO in regulation of AR by means of regulating the GC activity (Anderson et al., 1995; Zamir et al., 1995). Similarly, the coincident increase in NO and cGMP was reported in Ca^{2+}-dependent membrane fusion (Lee et al., 1994). The similarity in Ca^{2+}-dependency and involvement of cGMP between myoblast fusion and AR led us speculate that

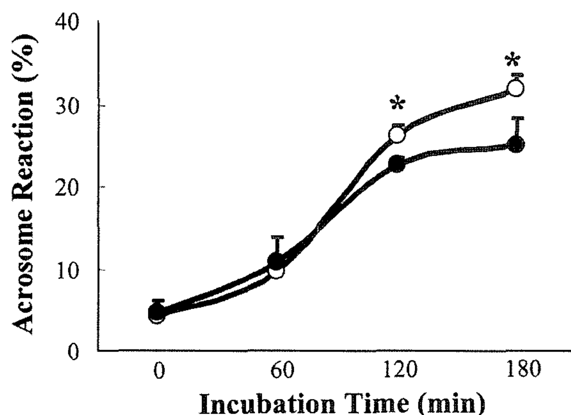

Fig. 5. Effect of methylene blue (MB) on the acrosome reaction (AR) of the mouse epididymal spermatozoa. Methylene blue (10 µM) were added to the sperm suspension (●), and AR was evaluated at a 60 min interval. The AR rates were statistically analyzed by one-way ANOVA. Asterisks indicate mean values different significantly ($p < 0.05$). Data are mean±SD (n=4). O; Control.

biological effect of NO on AR might be mediated by cGMP and Ca^{2+} mobilization. ANP and SNP, which activated particulate GC and soluble GC, respectively, were known to increase intracelullar cGMP (Zamir et al., 1995), suggesting that cGMP may serve as an important second messenger linking the NO production and AR. However, the downstream event of cGMP was not uncovered in spermatozoa. Recently, it was reported that sperm derived from mice lacking cGMP-dependent kinase I (cGKI) can undergo AR and efficiently fertilize eggs (Hedlund et al., 2000). Therefore, it is plausible that at least systems independent of cGMP-GKI system are responsible for AR. In this regard, the cGMP-regulated phosphodiesterase (PDE) system was a possible candidate linking the NO-cGMP-AR. The expression of PDEs including type III (cGMP-inhibited) and type V (cGMP-specific) and their involvement in sperm capacitation were reported in spermatozoa (Richter et al., 1999; Harrison and Miller, 2000) but direct evidence supporting this idea is not available yet. Further works on the NO-induced cGMP production and downstream events linked to AR will be helpful in more comprehensive understanding of regulation of AR by NO.

Acknowledgements

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