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Human seminal plasma inhibits brain nitric oxide synthase activity

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Nitric oxide is a chemical messenger which functions as a neurotransmitter or as a cytotoxic agent. Nitric oxide synthase (NOS) has been isolated from various mammalian reproductive tissues. The presence or absence of NOS in spermatozoa has not yet been reported. We therefore tested human and murine spermatozoa for NOS activity by measuring the conversion of arginine to citrulline. No activity was found either in human or in murine spermatozoa. Human native semen and human seminal plasma exerted an inhibition on brain NOS activity, as assayed on rat brain cytosolic fractions. This inhibitory effect was dependent on the amount of protein present in the human seminal plasma. No inhibitory effect was observed when homogenates of washed spermatozoa were tested. The human seminal plasma did not affect the Michaelis constant (K_m) of NOS for L-arginine (endogenous NOS substrate) whereas the maximal velocity (V_{max}) was reduced, suggesting that it contains a non-competitive inhibitor of brain NOS. This inhibitory component was virtually insensitive to heat; a 10 min treatment to 95°C only slightly reduced its ability to inhibit brain NOS. The physiological relevance of our observations remains to be elucidated. Human seminal plasma may exert an inhibition of nitric oxide synthesis on cells other than spermatozoa or on cells from the male or female genital tract, modulating directly or indirectly (via modulation of reactive oxygen species formation) the functional state of the spermatozoa.

Key words: male infertility/nitric oxide/reactive oxygen species/seminal plasma

Introduction

Defective sperm function has been identified as one of the causes of human infertility. Little is known yet about the biochemical nature of sperm dysfunction. Spermatozoa have to be maintained in a latent state during the periods of epididymal storage and migration to the site of fertilization. Thereafter, spermatozoa undergo a cluster of physiological and biochemical alterations as a consequence of exposure to female genital tract secretions or to an appropriate in-vitro environment. This process is known as capacitation. Capacitation is essential for fertilization and is characterized by hyperactivated motility; it prepares the spermatozoa for the acrosome reaction (Yanagimachi, 1994).

Nitric oxide (NO) was first identified as an endothelium-derived relaxing factor. Its role in cardiovascular regulation, platelet aggregation, and the tumoricidal and bactericidal activities of macrophages is now widely accepted. The autonomous nervous system uses NO as a non-adrenergic, non-cholinergic neurotransmitter in contractile and secretory tissues to decrease muscle tone (for a review, see Schmidt and Walter, 1994).

NO is synthesized by a family of enzymes, the nitric oxide synthases (NOS). Constitutive, Ca^{2+}-dependent enzymes are found in endothelial cells and neurons, and an inducible, Ca^{2+}-independent form is seen in macrophages, neutrophils and also in endothelium (reviewed by Nathan and Xie, 1994).

There is evidence to suggest that NO may be involved in both normal (Hellstrom *et al.*, 1994) and abnormal (Rosselli *et al.*, 1995; Weinberg *et al.*, 1995) sperm functions.

In addition, it is known that NO reacts with the superoxide anion O_2^- and hence can modulate its effects. Superoxide anion or hydrogen peroxide (H_2O_2) can contribute either to peroxidation and toxic effects on spermatozoa (Aitken *et al.*, 1989; Iwasaki and Gagnon; 1992; de Lamirande and Gagnon, 1992) or to their capacitation and hyperactivation (Aitken *et al.*, 1989, 1995; Bize *et al.*, 1991; de Lamirande and Gagnon, 1993, 1995).

The aims of our study were to test human and mouse spermatozoa for the intracellular presence of NOS activity; this study led to the detection of an inhibitory effect of brain NOS in human seminal plasma (HSP). We used rat brain homogenates as a source of high NOS-containing tissue (Salter *et al.*, 1991).

Materials and methods

Mouse spermatozoa

Male BRL mice (4–5 months) were killed by decapitation, and the spermatozoa were collected from two vas deferens in 0.4 ml of a Krebs–Ringer buffer (Sigma K4002) containing NaCl (120 mM), $MgCl_2$ (0.49 mM), KCl (4.56 mM), Na_2HPO_4 (0.7 mM), NaH_2PO_4 (1.5 mM), glucose (10 mM), supplemented with $NaHCO_3$, (25 mM), $CaCl_2$ (1.71 mM), sodium pyruvate (1 mM), sodium lactate (25 mM) and 2% bovine serum albumin (BSA) (buffer A). They were cultured in an atmosphere of 5% CO_2 in air, pH 7.4. Only samples showing >70% motility were used. Spermatozoa were collected by

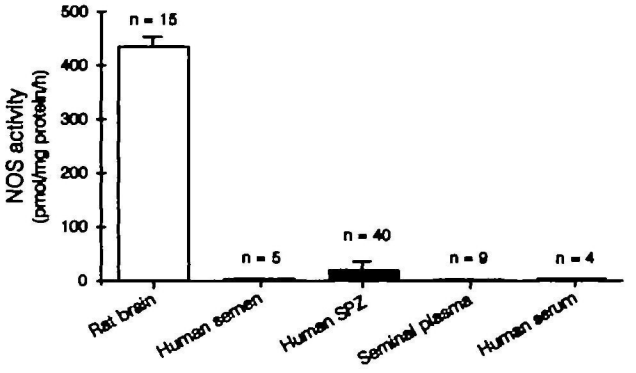

Figure 1. Nitric oxide synthase (NOS) activity in human semen, human seminal plasma and spermatozoa. Samples containing 30 µg of protein were assayed for NOS activity, as described in the text. Data represent the mean ± SEM of the number of determinations indicated in the figure. SPZ = sperm homogenate. Similar negative results were obtained in five samples containing 300 µg of sperm cell protein.

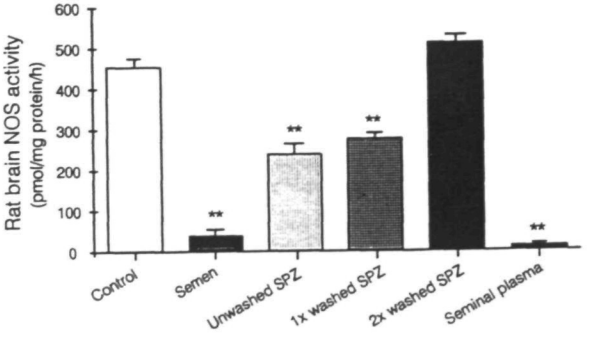

Figure 2. Inhibitory effect of human semen on brain nitric oxide synthase (NOS). Rat brain NOS activity was measured in the presence of 20 µg of human semen, human seminal plasma, and intact or washed sperm homogenate (SPZ), as described in the text. Data represent the mean ± SEM of the number of 9–12 determinations obtained in three or four different experiments. ** = significantly different from control ($P < 0.01$). In a separate set of experiments using six more samples, we separated HSP from sperm cells without freezing and assayed for inhibitory effects: a 95.3 ± 0.8% inhibition was measured.

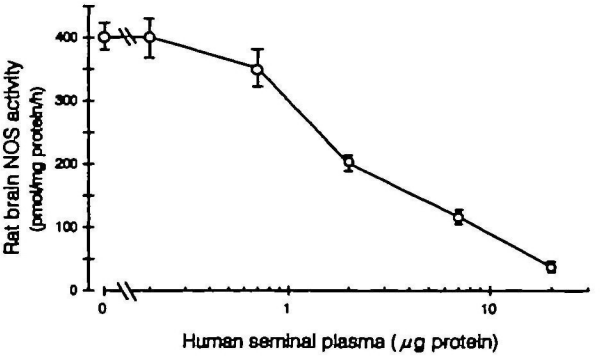

Figure 3. Dose-dependent inhibition of rat brain nitric oxide synthase (NOS) activity. Rat brain NOS (30 µg) was measured in the presence of various amounts of human seminal plasma, as described in the text. Data represent the mean ± SEM of 9–12 determinations obtained in four different experiments. Doses >2 µg inhibited brain NOS activity ($P < 0.01$).

centrifugation (14 000 *g*, 5 min, 4°C) and stored at –80°C until use. The pellet was resuspended in 150 µl of 50 mM HEPES, pH 7.4, containing 1 mM EDTA (buffer B) and homogenized by sonication on ice. Ion-exchange resin (50 mg, AG50WX-8, sodium form) was then added to remove endogenous arginine (Bugnon *et al.*, 1994), and after 5 min, by which time the resin had settled to the bottom of the tube, the supernatant was carefully removed and transferred to another tube. NOS activity was measured in 10 µl samples containing 30 µg of protein (see below).

Human spermatozoa and HSP

Human semen samples were obtained from donors. Ejaculates were collected by masturbation after 4 days of abstinence. Standard routine sperm parameters (volume, pH, sperm concentration, motility, morphology, viability, white blood cells, bacteria) were determined within 2 h of sample collection, and the semen was stored at –80°C. Samples (500–1000 µl) were centrifuged (14 000 *g*, 5 min, 4°C) to separate sperm cells (unwashed spermatozoa) from HSP. Spermatozoa were sometimes resuspended in 0.9% NaCl (1 ml) and centrifuged again before homogenization (washed spermatozoa). The pellet was resuspended in buffer B and homogenized by sonication on ice. Then 50 mg of ion-exchange resin (AG50WX-8, sodium form) was added to remove endogenous arginine. HSP was also treated with resin, as described above. In some experiments that excluded freezing the semen specimens, the spermatozoa were separated from HSP by centrifugation at 300 *g* (10 min) after adding two volumes of standard culture medium (Whittingham's T_6; Quinn *et al.*, 1982). The pellets containing spermatozoa and other cells present in the ejaculate were resuspended in the same medium (300 µl). The motile spermatozoa were obtained by mini-Percoll gradient centrifugation (Ord *et al.*, 1990). They were further washed and centrifuged again, as described above, before biochemical assay. The supernatant containing HSP was recovered for analysis.

Brain homogenate preparation

Animals were decapitated, and their brains were dissected, homogenized at 4°C in buffer B and treated with ion-exchange resin. Cytosolic fractions were prepared by centrifugation (39 000 *g*, 30 min, 4°C). Samples containing 30 µg of protein were routinely used.

NOS assay

NOS activity was assayed in triplicate by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline according to our modification of the Bredt and Snyder (1990) method (Schaad *et al.*, 1994). Briefly, a 10 µl homogenate was added to 15 µl of a reaction mixture containing $CaCl_2$ (final concentration 1.25 mM), EDTA (1 mM), flavin–adenine dinucleotide (FAD; 10 µM), flavin mononucleotide (FMN; 10 µM), 5,6,7,8-tetrahydrobiopterin (THB; 100 µM), calmodulin (10 µg/µl), arginine (25 µM, unless otherwise indicated), dithiothreitol (1 mM) and NADPH (1 mM).

Protein measurement

Protein concentration was determined by a dye-binding method (Bradford, 1976), using BSA as a standard.

Statistics

Data are presented as the mean of the indicated number of determinations. Vertical error bars represent the SEM of the number of determinations, as detailed in the legends. Statistical analysis was performed with unpaired Student's *t*-test or as otherwise indicated, using a graphic software (Fig.P.; Biosoft, Cambridge, UK).

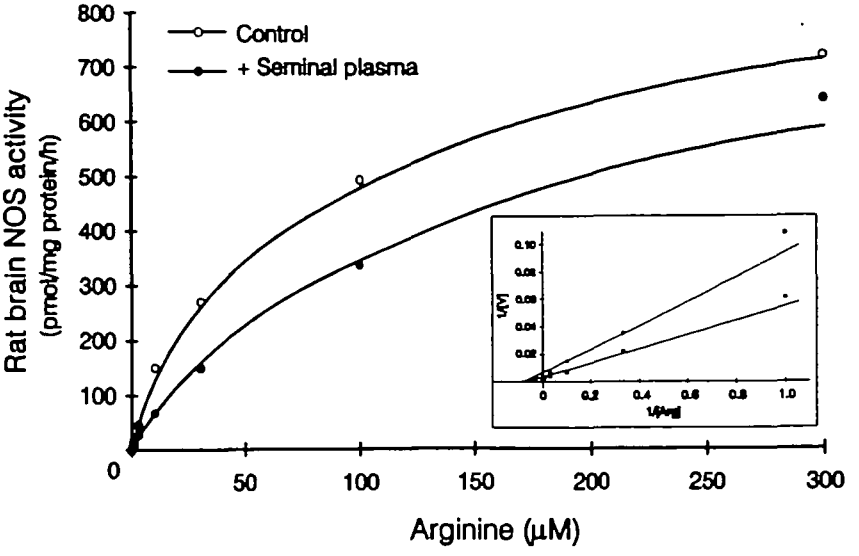

Figure 4. Effect of human seminal plasma (HSP) on rat brain nitric oxide synthase (NOS) K_m and V_{max}. Rat brain NOS activity was measured, as described in the text, in the presence of various concentrations of L-arginine, and in the presence (closed circles) or absence (open circles) of 3.5 μg of HSP protein. Results are representative of three independent experiments.

Material Suppliers

Reagents were purchased from following sources: $NaHCO_3$, sodium pyruvate, sodium lactate, glucose, THB, $CaCl_2$ and HEPES from Fluka (Buchs, Switzerland); Krebs–Ringer buffer, dithiothreitol, arginine, calmodulin, FAD, FMN and EDTA from Sigma (Buchs, Switzerland); AG50WX-8, sodium form resin from BioRad (Glattbrugg, Switzerland); NADPH from Boehringer Mannheim (Rotkreuz, Switzerland); BSA (Fraction V) from Hoechst (Buchs, Switzerland).

Results

When NOS activity was measured in human spermatozoa, no activity was found in 37 samples out of 40. A faint activity was nevertheless observed in three donors whose samples were not associated with any abnormality as estimated by the parameters used in our andrology laboratory (sperm count, motility, morphology, vitality, presence of bacteria or white blood cells). In homogenates of spermatozoa containing a higher (300 μg) protein concentration, NOS activity was also undetectable. HSP or total semen homogenates did not display any NOS activity (Figure 1). Negative results were also obtained in six additional semen specimens that were separated from HSP without any freezing procedure, using a mini-Percoll gradient. Similar negative results were obtained with murine spermatozoa.

We then investigated whether human spermatozoa could inhibit brain NOS activity. We routinely used the cytosolic fraction of rat brain as a NOS-containing tissue to estimate the possible inhibitory activity of various samples. As shown in Figure 2, rat brain NOS was >80% inhibited when 20 μg of proteins contained in whole semen homogenate were added. The origin of the factor responsible for the sperm inhibitory activity was then investigated by separating sperm cells from HSP. HSP provoked a strong inhibition when tested alone. Homogenates prepared from unwashed sperm cells exerted some inhibition on brain NOS activity; however, when they were washed before homogenization, they lost their inhibitory effect. The inhibitory activity of HSP on brain NOS activity was dependent on the concentration of protein, as shown in Figure 3. The half-maximal effect on rat brain NOS (30 μg) was observed using 2 μg of proteins from HSP. Addition of 20 μg of protein from HSP almost completely abolished brain NOS activity. To characterize such an inhibition further, the kinetics of brain NOS were examined using various concentrations of the NOS substrate, L-arginine. In three independent experiments, the addition of HSP (at a submaximal inhibitory concentration) to the reaction mixture did not affect the K_m for brain NOS whereas it reduced the V_{max}, as shown in Figure 4. These results suggest that the component present in HSP is capable of inhibiting brain NOS activity in a non-competitive manner. We then investigated if 20 μg of BSA or 20 μg of proteins from human blood serum could inhibit brain NOS activity, and found that these agents did not share the inhibitory effect of HSP (data not shown).

A brief heat treatment of HSP (10 min boiling) only slightly affects its inhibitory activity, indicating that the active agent is most probably not a protein, or alternatively is a heat-resistant protein or a peptide.

Discussion

Our results show that brain NOS activity (measured by the conversion of arginine to citrulline) is not detectable in human or in murine spermatozoa. These data agree with those recently reported by Zini *et al.* (1995). Interestingly, HSP is shown to contain an endogenous inhibitor of brain NOS. This inhibitor strongly affects brain NOS activity, depending on the concentration of protein present in HSP. The equivalent of 20 μg of protein from HSP inhibits the NOS activity present in 30 μg brain protein.

The physiological relevance of such results is presently unclear, but is certainly related to a reduction in NO synthesis. The importance of NO on sperm function has been poorly investigated. Since spermatozoa do not possess detectable NOS activity, such an inhibitor may only affect NOS from associated cells or leukocytes, preventing their production of NO, or NOS from cells of reproductive tissues (Jovanovic *et al.*, 1994; Telfer *et al.*, 1995). Moreover the main target of NO is known to be the soluble form of guanylate cyclase. However, no convincing evidence for its presence in mammalian sperm cells has been reported as yet. NO elaborated in the female genital tract (Jovanovic *et al.*, 1994; Telfer *et al.*, 1995) *in vivo* has been reported to influence sperm function and fertility adversely (Rosselli *et al.*, 1995; Weinberg *et al.*, 1995) through an inhibition of sperm respiratory function.

In addition, NO can react with reactive oxygen species (ROS) to modulate their functions. The generation of ROS by human spermatozoa has been implicated either in the control of normal sperm function (Aitken *et al.*, 1995) or in the aetiology of male infertility (Jones *et al.*, 1979; Aitken *et al.*, 1989; Alvarez and Storey; 1989; De Lamirande and Gagnon, 1992). The generation of ROS plays a key role in the control of sperm biological responses through the redox regulation of tyrosine phosphorylation (Aitken *et al.*, 1995) that is an essential component of the cascade of biochemical changes that lead to acrosomal exocytosis and fusion with an oocyte. In contrast to their positive effects, the generation of ROS leads to a peroxidation-induced loss of plasma membrane function leading to sperm immobilization (Aitken *et al.*, 1989; de Lamirande and Gagnon, 1992; Iwasaki and Gagnon, 1992). NO and superoxide are not strong oxidants, but they can react together to form peroxynitrite (Koppenol *et al.*, 1992). Peroxynitrite can further release the hydroxyl radical, which is a strong oxidant that can damage cells (Pryor and Squadrito, 1995; Squadrito and Pryor, 1995). By inhibiting NO generation, maybe the amount of hydroxyl radical can be decreased, preventing the subsequent lipid peroxidation involved in cellular damage.

Concerning HSP, it is known that it contains both positive and negative modulators of fertility preventing the development of acrosomal responsiveness or enhancing the acrosome reaction (Cross, 1993). Caltrin (also known as seminal plasmin) and several other seminal proteins have been identified as decapacitating factors (reviewed in Cross, 1993); they include the acrosome-stabilizing factor (Eng and Oliphant, 1978) and spermine (Rubinstein and Breitbart, 1991). An agent inhibiting NO synthesis, such as the one that we report here, could therefore represent an indirect modulator of sperm activation (capacitation and acrosome reaction).

Most probably, the inhibitory component(s) of HSP is not a protein, since pre-heating only weakly attenuates its effect. Several endogenous inhibitors of NOS have been described. These are mainly amino acid derivatives, such as substituted L-arginine: *N*-monomethylarginine, N^G,N^G-dimethylarginine, N^G,N'^G-dimethylarginine or α-guanidinoglutaric acid (Kakimoto and Akazawa, 1970; Yokoi *et al.*, 1994). It has been demonstrated that spermine and spermidine can also reduce brain NOS activity (Hu *et al.*, 1994). We do not think that the inhibitory component present in HSP is either an arginine analogue or spermine, since they appear to be competitive inhibitors, and their inhibition can be overwhelmed by increasing the L-arginine concentration (Hu *et al.*, 1994). Our data show that, even at high arginine concentrations, a clear inhibitory effect is observed, although no change in K_m was observed. This suggests that the inhibitory component probably does not compete with arginine for NO generation. We suppose that the nature of the inhibitory factor present in HSP is a small peptide(s) or any other heat-insensitive substance. Caltrin is an antimicrobial peptide that coats the surface of ejaculated spermatozoa and may promote the acrosome reaction (Clark *et al.*, 1993), an obligatory event in fertilization. It antagonizes selectively and potently the activating effect of calmodulin on target enzymes (Comte *et al.*, 1986). Since brain NOS is a Ca^{2+}/calmodulin-dependent enzyme (reviewed in Nathan and Xie, 1994), caltrin could be responsible for the inhibition of brain NOS.

Since we have described a very potent inhibitory effect of HSP on brain NOS, it is very likely that it plays a physiological role. It could be an important factor contributing to the functional state of spermatozoa, preventing cytotoxicity and infertility associated with microbial infection due to excessive production of NO. Such an hypothesis can be related to the results of Rosselli *et al.* (1995), since these authors found a positive correlation between the seminal plasma concentration of nitrite/nitrate and the percentage of immotile spermatozoa. Moreover, NOS has been shown to be expressed in a variety of cell types in the non-pregnant human uterus (Telfer *et al.*, 1995). A role for the inhibitory effect of HSP inside the uterus may apparently be relevant, since it has already been noted by others that inhibitors of the action of NO might prove useful in the preservation of spermatozoa (Weinberg *et al.*, 1995). Whatever the mechanism of its inhibition of NO formation, HSP could modulate the formation of ROS and thereby modulate either the toxic lipid peroxidation of sperm membranes and/or the sperm cell activation process, preventing its premature capacitation.

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