The nitric oxide synthase of mouse spermatozoa

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Abstract Nitric oxide synthase (NOS) was evidenced in mature mouse spermatozoa by means of biochemical techniques and Western blot. During 120 min of incubation, 10^7 spermatozoa synthesized 7 ± 2 pmol of L-[^{14}C]citrulline. Besides, L-citrulline formation depended on the incubation time and on the concentration of L-arginine present in the incubation medium. Different concentrations of N^G-nitro-L-arginine methyl ester (L-NAME) but not aminoguanidine, inhibited L-[^{14}C]citrulline formation. Western-blot analysis of solubilized sperm proteins revealed a unique band of M_r=140 kDa with the neural, endothelial and inducible NOS antisera tested. These results provide evidence that mature mouse sperm contains a NOS isoform and that spermatozoa have the potential ability to synthesize NO, suggesting a role for endogenous NO on mammalian sperm function.

Key words: Nitric oxide synthase; Western blot; Nitric oxide; Spermatozoa; (Mouse)

1. Introduction

Nitric oxide synthases (NOS) are the enzymes responsible for the conversion of L-arginine to NO and L-citrulline. These enzymes require NADPH and possess binding sites for heme, tetrahydrobiopterin, flavin adenine dinucleotide and flavin adenine mononucleotide. At least three different isoforms of NOS, two constitutives and one inducible, have been described [1]. The isoenzymes isolated initially from the brain (bNOS) and from the endothelium (eNOS) are constitutively expressed, although their activity may be regulated by sex hormones [2]. These isoforms are activated by calcium/calmodulin and are selectively inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (NO_2-Arg) [3]. The inducible isoform (iNOS) was first found in macrophages; it is not normally expressed unless stimulated by cytokines or bacterial products and is selectively inhibited by aminoguanidine [4] and amidines [5].

Nitric oxide is an active free radical and an important physiological intracellular messenger. Several effects of the NO donor sodium nitroprusside on spermatozoa have been previously described. Sodium nitroprusside stimulates hyperactivation and the synthesis of prostaglandins and leukotrienes in mouse sperm [6,7] and increases motility and lipid peroxidation in human spermatozoa [8]. Zamir et al. [9] found that sodium nitroprusside stimulates GMPc formation and acrosome reaction in bovine sperm. Moreover, we have shown that incubation of spermatozoa with L-NAME or NO_2-Arg reduced the outcome of in vitro fertilization in the mouse, suggesting a role for NO in sperm physiology [10]. However, it was not established if the spermatozoon could synthesize NO.

In this study we explore the NOS in mouse spermatozoa; therefore, we applied biochemical and immunochemical methods to evidence the presence of NOS and its activity in the mouse male gamete.

2. Materials and methods

2.1. Culture media and reagents

The standard medium used in this study was Fraser's modification of Whittingham's medium [11], pH 7.4, supplemented with 30 mg/ml bovine serum albumin (BSA) and prepared in culture-grade water with analytical grade reagents. This medium (FM) was equilibrated with 5% CO_2 in air at 37°C.

Both L-NAME and aminoguanidine were obtained from Sigma Chem. Co. (St. Louis, MO). Fresh solutions were prepared daily in fertilization medium.

L-[^{14}C]Arginine monohydrochloride (292 mCi/mmol) was obtained from Amersham Inc. (Arlington Heights, IL) and AG50WX-8 resin was obtained from Bio-Rad.

The Western-blot reagents were obtained from Bio-Rad. Antisera directed to the neural, endothelial or inducible isoforms of NOS were generated in the rabbit against the rat brain NOS [12], endothelial NOS and macrophage NOS, respectively [13]. Enzyme immunoprecipitation assays and cross-reaction of the antisera with extracts of different tissues were previously published [11]. The antisera are encoded NOS-B, NOS-E and NOS-I, respectively, and were obtained from RPMS/Wellcome (Beckenham, Kent, UK).

2.2. Animals and preparation of spermatozoa

Hybrid F1 (C57/B×CBA) sexually mature male mice (aged 3–5 months) were used. Animals were housed at controlled room temperature (22°C) and fed with Purina chow and water ad libitum. Animals were killed by cervical dislocation and the content of each cauda epididymis was immediately removed; the dense sperm mass was allowed to flow out freely. Spermatozoa were incubated into 500 ml drops of FM under paraffin oil at 37°C in 5% CO_2–air atmosphere.

Each drop contained the sperm of a single mouse. Sperm concentration was adjusted to 3–5×10^7 cells/ml.

2.3. Conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline and production of nitric oxide

NOS activity was measured following the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline according to Bredt and Snyder [14]. A time-dependent curve was assayed with the purpose of detecting L-citrulline formation. Spermatozoa were placed into 500 ml drops of fertilization medium as described above. Five drops (one for each time point) were arranged in a Petri dish and a concentration of 5.0 μM L-[^{14}C]arginine was added to each drop at the beginning of the incubation. The reaction was stopped at 0, 20, 60, 120 and 180 min.

Another set of experiments was designed to assess the effect of NOS inhibitors. Different concentrations of L-NAME or aminoguanidine were added to the 500 ml drops 30 min before the addition of 5.0 μM L-[^{14}C]arginine, to allow the inhibitory effect to take place. One drop was used for each experimental condition. Then, the incubation followed for another 120 min.

Finally, a concentration-dependent curve of substrate was assayed:

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sperm drops (one for each experimental condition) were incubated for 120 min in FM at 37°C in a 5% CO_2–air atmosphere. Different concentrations of L-[^{14}C]arginine (5.0–35.0 μM) were added at the beginning of the incubation period.

In all the experiments, the reactions were stopped by three cycles of freeze–thawing and mixtures were centrifuged 10 min at 8000×*g*. Aliquots (200 ml) of the supernatant solutions were mixed with 2 ml of 20 mM HEPES–NaOH buffer, pH 7.4, applied to 2 ml columns of Dowex AG50WX8 resin (Na^+ form) and washed with 2 ml of deionized water.

Percolate plus wash from each column (4 ml) was separated in four vials (1 ml/vial) with 10 ml of liquid scintillation and counted for radioactivity. Measurements are presented as pmol L-[^{14}C]citrulline/10^7 cells. As formation of L-citrulline is stoichiometric with the formation of NO, we could assume that an equal amount of NO was formed [19]. Intra- and interassay variations were less than 10%.

2.4. Western blot

Fresh cauda epididymal spermatozoa were placed in phosphate buffer saline (PBS), pH 7.4, at 37°C and washed 3 times at 700×*g* for 5 min. Spermatozoa (10^8 cells/ml) were resuspended in microcentrifuge tubes and solubilized in 200 ml of 0.0625 M Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 10% (v/v) glycerol and 0.01% bromophenol blue (sample buffer) containing a cocktail of protease inhibitors (10 mg/ml aprotinin, 10 mg/ml leupeptin, 10 mg/ml soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride). The samples were heated in boiled water for 5 min and centrifuged at 12000×*g* for 15 min. Supernatants were then collected and their volumes were recorded. An aliquot was taken and assayed for protein [15] and the remaining supernatant was frozen at −70°C until the time of electrophoresis.

To test antibody recognition of the neural NOS present in rat brain and cerebellum (positive controls) [11], samples of rat brain and cerebellum proteins were extracted by homogenization in 20 mM Tris-buffer, pH 7.4, containing 0.25 M sucrose and a cocktail of inhibitors as previously stated. After centrifugation at 7800×*g* for 10 min, the supernatants were collected and kept at −70°C until used.

SDS–polyacrylamide gel electrophoresis used the discontinuous system of Laemmli [16] with 7.5% polyacrylamide separation gel.

The fractionated proteins (150 mg protein/lane) were electrophoretically transferred to nitrocellulose by the method of Towbin et al. [17]. The nitrocellulose was incubated overnight at 4°C with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, (TBS) with 5% BSA. Once blocked, the nitrocellulose was then incubated at room temperature for 2 h with anti-NOS-B, anti-NOS-E or anti-NOS-I antibodies diluted 1:2000 in TBS/BSA 1%. After washing in TBS +0.05% Tween-20 (TBST) the strips were incubated for 1 h at room temperature with 1:4000 dilution of alkaline phosphatase anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO). Once the strips were thoroughly washed, they were incubated with a mixture of nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt (BCIP) at a 1:1 molar ratio under alkaline conditions for 30 min.

2.5. Statistical analysis

The Instat program (GraphPAD Software Co., San Diego, CA) was used for statistical analysis. ANOVA and Student–Newman–Keuls tests were used for comparisons between groups. Differences were considered significant when $P < 0.05$.

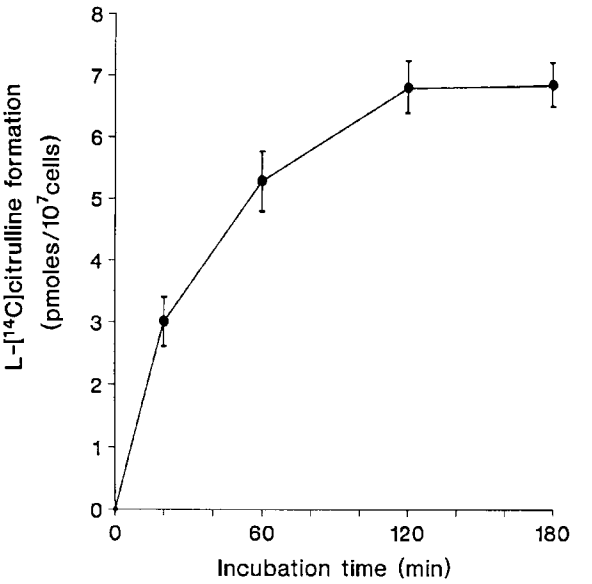

Fig. 1. Measurement of NO formation in spermatozoa at different time intervals. Spermatozoa were incubated in 500 ml of FM with 5.0 μM L-[^{14}C]arginine. At 0, 60, 120 and 180 min of the incubation, L-citrulline formation was determined following the stoichiometric conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline and NO (see Section 2 for details). Data are expressed as pmol L-[^{14}C]citrulline/10^7 cells. Each point represents the mean ± SEM of five assays.

3. Results

3.1. Conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline and production of nitric oxide by spermatozoa

Kinetic assays were done to detect formation of L-[^{14}C]citrulline. L-[^{14}C]arginine was added at the beginning of the incubation period and then L-[^{14}C]citrulline production was measured at different time intervals. Fig. 1 shows that L-[^{14}C]citrulline formation increased during the 120 min of incubation and reached a plateau at 120–180 min. During this period, 10^7 spermatozoa produced 7.0 ± 2 pmol L-[^{14}C]citrulline in the presence of 5.0 μM L-[^{14}C]arginine.

L-NAME, a specific inhibitor of NOS, significantly reduced L-[^{14}C]citrulline production in these cells: 0.6 mM L-NAME (44% of inhibition) and 0.1 mM (22% of inhibition) L-NAME but not with 0.01 mM L-NAME (Table 1). This specific inhibition confirms that the conversion of L-arginine to L-citrulline under our experimental conditions was due to NOS activity. However, when a specific inhibitor for the inducible

Table 1
Effect of NOS inhibitors (N^G-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine) on sperm L-citrulline formation, measured as described in Fig. 1

Treatment	No. of experiments	L-[^{14}C]citrulline formation (pmol/10^7 spermatozoa)	*P*
Control	9	6.8 ± 0.6	
L-NAME 0.01 mM	9	5.8 ± 0.7	n.s.
L-NAME 0.1 mM	9	5.1 ± 0.5*	< 0.05
L-NAME 0.6 mM	9	3.5 ± 0.5*	< 0.01
Aminoguanidine 0.5 mM	9	6.3 ± 1.4	n.s.

Spermatozoa were incubated in 500 ml of FM in the absence or presence of different concentrations of L-NAME or aminoguanidine (see Section 2 for details). After 30 min of incubation, 5.0 μM L-[^{14}C]arginine was added to the sperm suspensions and incubated for another 120 min. Data are expressed as pmol L-[^{14}C]citrulline/10^7 cells produced in 120 min. Each group represents the mean ± SEM of nine assays.
*Significantly different from control. n.s., not significant.

isoform (aminoguanidine) was tested, no inhibition was observed during the incubation period (Table 1).

The specific formation of L-citrulline by sperm was further confirmed when different concentrations of L-arginine were added to the incubation medium. This resulted in a concentration-dependent increase in L-citrulline production (Fig. 2).

3.2. Immunoblotting with NOS antibodies

When protein extracts of epididymal spermatozoa were incubated under denaturing and non reducing conditions and then subjected to immunoblotting assay, a protein fraction of M_r=140 kDa was recognized by the three anti-NOS antisera (neural, endothelial and inducible isoforms) (Fig. 3).

In parallel, rat brain and cerebellum solubilized proteins revealed a main band of 150 kDa and they were used as positive controls.

4. Discussion

Recent data from our laboratory have shown that antibodies raised against the neural isoform of NOS could label the acrosome and tail of murine and human spermatozoa [18]. Therefore, this study provides further evidence that NOS is present in mouse spermatozoon and shows for the first time that NOS activity can be detected in this gamete.

We could detect NOS activity only by incubating intact, living spermatozoa. A similar approach was used by Paveto et al. to detect NOS activity in *Trypanosoma cruzi* [17]. Previously, some authors have shown that NOS activity could not be detected in mouse and human spermatozoa under their in vitro experimental conditions (permeabilized spermatozoa) [20,21]. NOS is a very labile enzyme that requires several cofactors and cell integrity may be a requisite to detect NOS activity.

The maximum level of L-[^{14}C]citrulline production was reached at 120 min. This peak corresponded with the expected time of capacitation in the murine male gamete. Therefore, it is tempting to relate this time course of NOS activity to the capacitation process. Zini et al. showed that low concentrations of a NO donor induced capacitation of human sperm in vitro [20]. Moreover, in recent studies it was shown that NOS inhibition during sperm capacitation impaired fertilization in vitro and spontaneous acrosome reaction in the mouse [10,22].

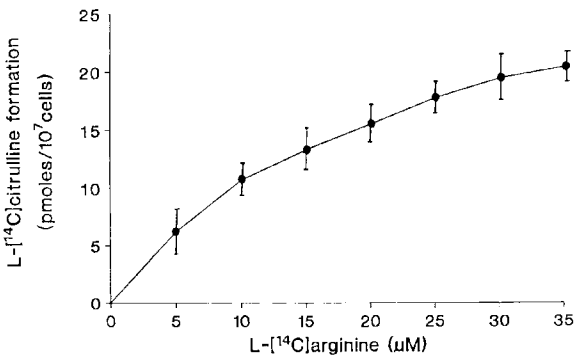

Fig. 2. Concentration-dependent production of L-citrulline in mouse spermatozoa. Spermatozoa were incubated in 500 ml of FM with L-[^{14}C]arginine (5.0–35.0 μM) for 120 min. L-Citrulline formation was determined as described in Fig. 1. Data are expressed as pmol L-[^{14}C]citrulline/10^7 cells produced in 120 min. Each point represents the mean ± SEM of five assays.

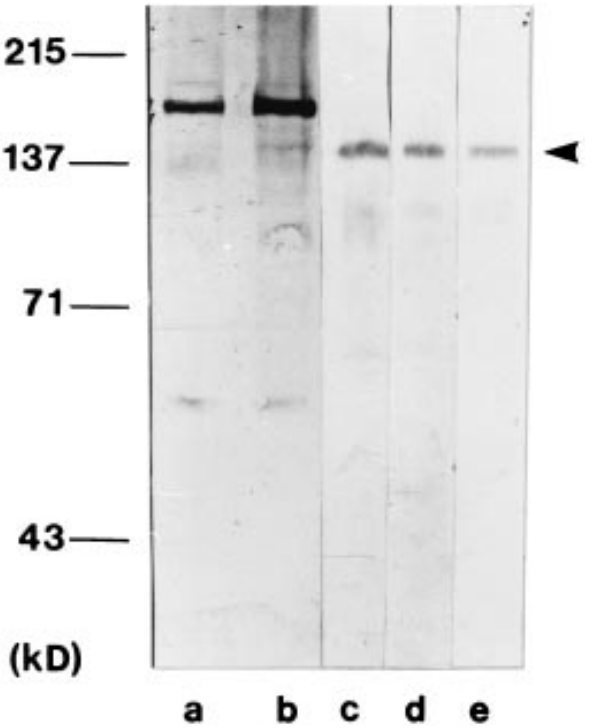

Fig. 3. Immunoblotting of NOS from rat brain, rat cerebellum and murine spermatozoa. Crude extracts from rat brain and cerebellum and solubilized proteins from fresh mature spermatozoa were subjected to electrophoresis on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were then probed with NOS antisera (neural: NOS-B, endothelial: NOS-E and inducible isoforms: NOS-I). Lane a: rat brain; lane b: rat cerebellum; both lanes were revealed with NOS-B and were used as positive controls. Fresh mature mouse spermatozoa were revealed with NOS-B (lane c), NOS-E (lane d) or NOS-I (lane e). Solubilized proteins from lanes a–e were treated under non-reducing conditions. NOS from rat brain and cerebellum homogenates exhibit similar mobility with molecular masses of 150 kDa. The protein bands indicated by an arrowhead at 140 kDa represent NOS from murine spermatozoa revealed with all three antisera. Results are representative of four blots.

However, further investigations are necessary to find out the relationship between sperm NO synthesis and capacitation.

The electrophoretic mobility under non-reducing conditions revealed a unique band corresponding to a sperm protein of 140 kDa after immunoblotting with the neural, endothelial and inducible NOS antisera.

Different isoforms of the enzyme have been described in a variety of cell types [24,25] and tissues such as placenta (125 kDa), spleen (100 kDa), and lung (140 kDa). According to Springall et al., variations in apparent molecular mass may suggest a degree of organ or tissue specificity of the enzyme [12].

Is intriguing that the three NOS antibodies could recognize sperm NOS. It should be remembered that there exists 50% amino acid identity among the three isoforms [23]; therefore, cross-reactions among the antibodies and different isoforms are frequently found. Polyclonal antibodies raised against rat brain constitutive NOS could recognize a similar protein in rat uterus [25] and penis [26]. Springall et al. [12] report that antisera raised against brain NOS cross-reacts with a putative endothelial isoform of human placenta. Recently, Lewis et al. [28] recognized NOS by immunofluorescence in

human sperm using antibodies against NOS-B and NOS-E. The high degree of homology that exists between the neural, endothelial and the inducible isoforms, and the fact that antibodies used in this study were polyclonal may explain why sperm NOS reacted positively with all the three antisera.

Furhter evidences support the notion of sperm NOS being of the constitutive type. First, it is generally accepted that sperm cannot synthesize cytoplasmic proteins de novo [27]; therefore no inducible NOS should be expressed. Besides, the fact that L-NAME but not aminoguanidine inhibited sperm NOS activity also suggests that sperm NOS is presumably of the constitutive type. Furthermore, in a previous paper we showed that L-NAME but not aminoguanidine inhibits in vitro fertilization in the mouse [9]. Finally, in a recent paper Lewis et al. could not find induction of NOS in human sperm [26]. However, only molecular biology techniques will provide more conclusive evidence that spermatozoa have a unique NOS isoform.

In summary, these experiments show new evidence to suggest that mature mouse sperm contain NOS and that spermatozoa have the potential to synthesize NO. These results and recent published data that localized by immunofluorescence sperm NOS [18,22] add further support to the notion that endogenous NO synthesis is related to mammalian sperm function.

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