Characterization of NADPH oxidase 5 in equine testis and spermatozoa

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Abstract

Reactive oxygen species (ROS) play an important role in normal sperm function, and spermatozoa possess specific mechanisms for ROS generation via an NAD(P)H-dependent oxidase. The aim of this study was to identify the presence of an NADPH oxidase 5 (NOX5) in equine testis and spermatozoa. The mRNA of NOX5 was expressed in equine testis as detected by northern blot probed with human NOX5 cDNA and by RT-PCR. Immunoblotting with affinity purified α -NOX5 revealed one major protein in equine testis and other tissues. Immunolocalization of NOX5 showed labeling over the rostral sperm head with some labeling in the equatorial and post-acrosomal regions. In the testis, there was abundant staining in the adluminal region of the seminiferous tubules associated with round and elongating spermatids. The RT-PCR and sequence analysis revealed a high homology with human NOX5. This study demonstrates that NOX5 is present in equine spermatozoa and testes and therefore represents a potential mechanism for ROS generation in equine spermatozoa.

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Introduction

There is substantial evidence that reactive oxygen species (ROS) serve as physiologically relevant signaling molecules that control normal sperm function (Baker & Aitken 2004, Ford 2004). Several laboratories have shown that sperm capacitation, hyperactivation, acrosomal exocytosis, nuclear condensation and mitochondrial stability are redox-regulated events (De Lamirande & Gagnon 1993a, 1993b, Aitken *et al.* 1995, 1998, Baumber *et al.* 2003). Although a number of studies define an important role for ROS generation in the regulation of sperm function, the mechanism(s) responsible for the production of ROS by spermatozoa remains controversial (Baker *et al.* 2003, Ford 2004). ROS can be generated as a consequence of e^- leakage from complex I and III of the mitochondrial e^- transport chain (Turrens & Boveris 1980, Balaban *et al.* 2005), and this source of ROS may be important in oxidative damage to spermatozoa. In addition to mitochondrial sources, an enzymatic system for ROS generation located in the sperm plasma membrane that utilizes the reduced adenine dinucleotides (NAD(P)H) as a substrate via an NAD(P)H-dependent oxidase has been suggested as one mechanism for ROS-mediated signaling in spermatozoa (Aitken *et al.* 1992, 1995, 1997, Ball *et al.* 2001, Armstrong *et al.* 2002, Sabeur & Ball 2006).

In equine spermatozoa, NAD(P)H-dependent generation of ROS can be detected based upon nitroblue tetrazolium (NBT) reduction as well as by cytochrome c reduction by isolated equine sperm membrane preparations (Sabeur & Ball 2006). Cytochemical localization of NAD(P)H-dependent ROS generation by equine spermatozoa demonstrates NBT labeling over both the sperm midpiece and the sperm head, which suggests that superoxide generation may be attributed to two different sources. Labeling over the sperm midpiece may be attributed to the activity of mitochondrial oxidoreductases, whereas labeling over the sperm head may be attributed to a membrane-associated NAD(P)H oxidase (Sabeur & Ball 2006). The generation of superoxide anion in these systems was reduced by the flavoprotein inhibitor, diphenyleneiodonium, suggesting that an NAD(P)H oxidase may be responsible for NAD(P)H-dependent generation of ROS in equine spermatozoa (Sabeur & Ball 2006).

One possible candidate for NAD(P)H-dependent generation of ROS is the family of NADPH oxidases (NOX) that have been characterized for a number of cell and tissue types (Lambeth 2002, Krause 2004, Sabeur *et al.* 2004). The NOX family of NOX consists of seven known members, which are important in a variety of somatic tissues and include NOX 1–5 as well as dual oxidases (DUOX) 1–2 (Lambeth 2002, 2004, Krause

2004). One member of the NOX family, NOX5, is expressed in testis (Banfi *et al.* 2001, Cheng *et al.* 2001) and has been suggested as a possible candidate for low-level ROS generation in spermatozoa (Baker *et al.* 2003). NOX5 is a calcium-activated NOX that has been identified in human testis and lymphocyte-rich areas of spleen and lymph nodes (Banfi *et al.* 2001). NOX5 is expressed in pachytene spermatocytes; however, the function of NOX5 in mature spermatozoa has not been defined. It appears possible that NOX5 acts as a calcium-dependent mechanism for generation of ROS by spermatozoa; however, this remains to be established. Therefore, the objective of the current study was to characterize the presence of NOX5 mRNA and protein in equine testis and spermatozoa.

Materials and Methods

Materials

Fluoresceinated goat anti-rabbit immunoglobulin G (IgG) was purchased from Sigma Chemical. The immobilized glutathione column was purchased from Pierce (Rockford, IL, USA). The electrophoretic markers and the horseradish peroxidase conjugated secondary antibody were purchased from Bio-Rad. The ECL detection kit was from Amersham Pharmacia Biotech. Triazol was from Invitrogen. Animals used in this study were maintained under a protocol approved by the University of California–Davis Institutional Animal Care and Use Committee.

Expression of NOX5 transcript by northern blot analysis of equine testis

The presence of equine NOX5 transcript in equine testis was investigated by Northern blot analysis of polyadenylated (poly(A)) RNA with a labeled human NOX5 cDNA probe. A human NOX5 plasmid DNA insert of 771 nucleotides (Banfi *et al.* 2001) was radiolabeled with ^{32}P -dCTP using a random-primed labeling system (Amersham). Polyadenylated mRNA was extracted from triazol–total RNA using the poly(A) purist kit from Ambion (Austin, TX, USA). RNA samples were denatured and run on a formaldehyde/formamide gel. The resolved RNA was transferred to a nylon membrane (MSI, Westboro, MA, USA) and u.v. cross-linked. After a 2-h prehybridization at 42 °C, the membrane was incubated in a hybridization solution (ExpressHyb; BD Biosciences, Palo Alto, CA, USA) containing ~5 ng of radiolabeled cDNA (1×10^6 c.p.m./ml) at 65 °C overnight. Unbound probe was removed by washing twice with $1 \times \text{SSC}$, 0.1% SDS at 20 °C, then twice with $1 \times \text{SSC}$, 1% SDS at 50 °C. The membranes were exposed for autoradiography at –70 °C for 72 h.

RT-PCR of NOX5 from equine testis

Equine testis poly(A) RNA was isolated from Trizol Reagent as described above. This poly(A) RNA was subsequently used as template for first strand synthesis using oligo-dT primer (0.5 µg/µl, Invitrogen) and AMV reverse transcriptase (Promega). After RT, PCR amplification was conducted with Taq DNA polymerase, and the following primers for the first round PCR, followed by a second round with nested specific human or equine primers at 3' and 5'-end. The equine primers were deduced from the equine genomic sequence for NOX5 β (testis isoform). For the first round of amplification, the primer sequences from 5' to 3' were: NOX700 forward, GACTGCAGCTTCATCGCGGTGCTG and NOX1933; reverse, GTTGGCCAGGAGGT-CAAGGGCCATCTG. For the second round of amplification, primers were NOX700 forward and Nox1806 reverse, CCACTCGAAAGACCGCTGGTCTCT. Sequence was analyzed using the BLAST program (NCBI server at the National Library of Medicine/NIH) and the alignments were performed using multiple sequence alignment (Corpet 1988).

Expression of NOX5 protein by western blot analysis

Tissue extraction

Frozen equine testes and other reproductive and somatic tissues were stored at –70 °C prior to extraction. The tissues (20 g) were cut in small pieces (1 × 1 cm) and homogenized in 50 ml Tris buffer, pH 7.4 using a blender (UltraTurrex 95) at a frequency of 13 500 r.p.m. for 15 s five times. Protease inhibitors were added immediately to the Tris buffer: phenylmethylsulphonyl fluoride (500 µM), pepstatin (1 µM), leupeptin (5 µM) and antipain (25 µM). The homogenized sample was passed through cheesecloth and centrifuged twice at 20 000 *g* for 30 min (Sabeur *et al.* 2001). The resulting pellet was suspended in 6–10 ml Tris buffer with protease inhibitors and triton X-100 was added to a final concentration of 1%. The suspension was mixed overnight (5 °C) and then sonicated using six pulses (15 s each) of a sonic dismembrator 60 (Fisher Scientific, Pittsburgh, PA, USA). The sonicated membrane was centrifuged at 45 000 *g* for 60 min, and the supernatant containing the solubilized protein was stored at –20 °C.

The equine tissues and sperm-detergent extracts were solubilized in SDS and subjected to SDS-PAGE in 12% gels followed by Western blotting. Standard Western blotting procedures were used (Towbin *et al.* 1979). Blots were probed with a α -NOX5 polyclonal antibody (39 µg/ml). Anti-NOX5 was generated in a rabbit against the EF-hand containing region of NOX5, which was generated as a glutathione S-transferase-fusion protein (Banfi *et al.* 2004). The resulting antiserum was purified against immobilized glutathione according to the manufacturer's recommended protocol (Pierce). The

affinity purified antiserum was used for immunoblotting and subsequent immunolabeling experiments in ejaculated spermatozoa. For western blotting, a horseradish peroxidase conjugated secondary antibody (1:5000) was used, and proteins were detected with ECL.

Immunocytochemical localization of NOX5 in equine spermatozoa

Ejaculated equine spermatozoa were diluted in Tyrode's albumin, lactate, pyruvate (72 mM NaCl, 25 mM KCl, 25 mM NaHCO₃, 0.36 mM NaH₂PO₄, 2 mM CaCl₂·2H₂O, 0.4 mM MgCl₂·6H₂O, 25 mM HEPES, 5 mM glucose, 21.6 mM Na lactate and 0.25 mM Na pyruvate) with 1% BSA (w/v), washed by centrifugation (300 *g*, 10 min) and resuspended at 50 million cells/ml in Dulbecco's PBS (DPBS). Washed spermatozoa were permeabilized in 95% methanol for 5 min, centrifuged 8 min at 300 *g*, resuspended in DPBS and centrifuged again for 10 min. The cells were blocked in DPBS-5% BSA for 1 h before incubation with affinity purified α -NOX five antibody (58 μ g/ml in PBS) overnight at 4 °C. Spermatozoa were washed with PBS ($\times 3$, 5 min), and blocked with 5% BSA-PBS ($\times 2$, 5 min, w/v), then incubated with secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG for a 1 h (1:100 in PBS). Samples were then washed twice in PBS and mounted with 1,4-diazabicyclo- (2.2.2) octane, coverslipped, sealed and examined with epifluorescence microscopy. Control experiments were run with secondary antibody only or with replacement of primary antibody with rabbit nonimmune serum.

Immunohistochemical localization of NOX5 in equine testis

The testes of adult stallions were fixed in 4% paraformaldehyde (v/v). After a series of graded ethanol incubations, tissues were embedded in paraffin and sectioned at 5 μ m. The tissue sections were then deparaffinized and hydrated through xylenes and a graded ethanol series. Immunoperoxidase staining was performed with the vectastain ABC-Elite kit (Vector Laboratories, Burlingame, CA, USA). The sections were incubated for 30 min in 0.3% H₂O₂-methanol to quench endogenous peroxidase activity. Antigen retrieval was performed by heating sections for 20 min at 93 °C in unmasking buffer (Vector Laboratories) as previously described (Corbin *et al.* 2003). After a wash in PBS-0.3% TX-100, the sections were incubated for 20 min with diluted blocking serum, washed and incubated with an α -NOX5 antibody (Brar *et al.* 2003; 1:100) overnight, at 4 °C. The sections were then incubated for 30 min with biotinylated horseradish peroxidase linked-secondary antibody (1:200) followed by incubation in ABC reagent and developed with the substrate 3-amino-9-ethylcarbazole according to the manufacturer. The sections were

counterstained with hematoxylin. Normal rabbit serum was used as a negative control.

Results

Northern blot analysis using the radiolabeled human NOX5 cDNA probe detected a single RNA transcript of ~ 2.9 kb in equine and in human testis (Fig. 1). Immunoblotting with α -NOX5 revealed one major protein in equine testis with a molecular mass of ~ 90 kDa. In ejaculated equine sperm, three additional proteins were detected with molecular masses of 70, 35 and 29 kDa (Fig. 2). Immunoblots against other tissues (spleen, kidney, liver, peripheral neutrophils as well as accessory sex glands) recognized a major protein of 90 kDa (Fig. 3).

Immunolocalization of NOX5 in ejaculated equine spermatozoa showed labeling primarily over the rostral sperm head with less consistent labeling noted in equatorial and post-acrosomal regions (Fig. 4). Immunohistochemistry of equine testis with α -NOX5 revealed abundant staining in the adluminal region of the seminiferous tubules associated with the developing acrosomal cap of round spermatids (Fig. 5). Sequence analysis of the RT-PCR product (GenBank Accession # EF152773) from equine testis mRNA demonstrated a high homology with human NOX5 (Fig. 6).

Figure 1 Northern blot analysis of equine testis poly(A) RNA (20 μ g) and human total testis RNA (Ambion, 10 μ g) using a human NOX5 cDNA insert of 771 bp labeled with ³²P. Northern blot analysis using the radiolabeled human NOX5 cDNA probe detected a single RNA transcript of ~ 2.9 kb in equine and in human testis.

Figure 2 Immunoblot of detergent-extracted equine testis and ejaculated spermatozoa using α -NOX5 antibody (39 μ g/ml) and goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP-GAR, 1:5000) followed by detection with ECL. Immunoblots presented are representative of equine testis ($n=3$) and equine sperm ($n=4$) samples probed with α -NOX5 antibody. Western blotting revealed one major protein in equine testis and in spermatozoa with a molecular mass of 90 kDa and three additional proteins were detected with molecular masses of 70, 35 and 29 kDa in equine spermatozoa.

Discussion

The present study provides evidence that NOX5 is present in equine testis and spermatozoa based upon mRNA expression as well as immunolocalization of NOX5. The characterization of NOX5 and its putative role in ROS production are important to understand the relationship between the redox state and the biological function of sperm. A growing body of evidence indicates a role for ROS in a variety of sperm functions, including capacitation (Leclerc *et al.* 1997), tyrosine phosphorylation (Aitken *et al.* 1996, Leclerc *et al.* 1997, Baumber *et al.* 2003, O'Flaherty *et al.* 2006), chromatin condensation (Godeas *et al.* 1997), regulation of intracellular pH (Baker & Aitken 2004) and acrosomal exocytosis (Rivlin *et al.* 2004). Several studies including ours (Aitken & Clarkson 1987, Aitken *et al.* 1995, Tselkas *et al.* 2000, Ball *et al.* 2001, O'Flaherty *et al.* 2003,

Sabeur & Ball 2006) have shown that mammalian spermatozoa are capable of generating ROS but the biochemical mechanisms by which ROS are generated in mammalian spermatozoa are not fully understood. A number of studies demonstrate NAD(P)H-dependent ROS generation in spermatozoa (Aitken *et al.* 1997, Ball *et al.* 2001, Sabeur & Ball 2006), and several investigators have hypothesized that NOX5 is a likely candidate in this role (Banfi *et al.* 2001, Armstrong *et al.* 2002, Baker *et al.* 2003, Ford 2004).

Banfi *et al.* (2001) demonstrated the presence of NOX5 mRNA in human testis with expression detected primarily in pachytene spermatocytes and round spermatids. Ours is the first study to examine expression of NOX5 protein in testis and spermatozoa. NOX5 was localized in round and elongating spermatids in the testis and was localized primarily to the rostral sperm head in ejaculated spermatozoa. The localization of NOX5 protein in equine spermatozoa and spermatids is consistent with expression of mRNA observed by *in situ* hybridization in Banfi's study (Banfi *et al.* 2001). NOX5 mRNA (2.9 kb) was detected by Northern analysis in both human and equine testis and was similar in size to the human testis transcript (Banfi *et al.* 2001). Likewise, the 90 kDa protein detected on immunoblots of equine testis and spermatozoa in the present study compares favorably with the predicted size of human testis isoform of NOX5 (719 amino acids 82 kDa; Banfi *et al.* 2001) as well as an 85 kDa protein detected in human prostatic carcinoma cells (Brar *et al.* 2003). The appearance of lower molecular weight proteins on immunoblots of equine spermatozoa, but not equine testis, probed with α -NOX5 is likely due to proteolytic cleavage.

NOX5 have been recently cloned and identified in a variety of tissues including human testis and lymphocytes as well as in smooth muscle, uterus, ovary, prostate, pancreas and a variety of fetal tissues (Banfi *et al.* 2001, Cheng *et al.* 2001, Brar *et al.* 2003). Two types of NOX5 have been described (Vignais 2002). NOX5-L (long) has calcium-binding EF hands and is present as four different isoforms in testis and spleen (Banfi *et al.* 2001), whereas the short splice variant (NOX5-S) lacks the EF hands and is expressed in fetal kidney and in vascular endothelial cells

Figure 3 Immunoblot of equine testis and somatic tissues using α -NOX5 antibody (39 μ g/ml) and HRP-GAR secondary antibody (1:5000) followed by detection with ECL. Lanes 2 and 10 are testis. Lane 1 is spleen and lanes 3–9 are ampulla of ductus deferens, bulbourethral gland, prostate, seminal vesicle, kidney, liver and peripheral blood neutrophils respectively.

Figure 4 Immunocytochemistry of ejaculated equine spermatozoa labeled with α -NOX5 antibody (58 μ g/ml) and FITC-goat anti-rabbit antibody (1:100). Immunolabeling of equine spermatozoa with α -NOX5 antibody was localized primarily over the sperm head (a and b) with some spermatozoa demonstrating label in the equatorial and post-acrosomal region (c). Control samples in which the primary antibody was omitted did not demonstrate detectable immunofluorescence (d, control phase contrast image, fluorescence image not shown). Bar represents 5 μ m.

(Cheng *et al.* 2001, Bickel *et al.* 2005). Thus, it appears that NOX5 may be important in ROS-mediated cell signaling events in a variety of reproductive and somatic tissues. Consistent with these observations, we detected NOX5 in a variety of reproductive and somatic tissues in the horse.

NOX5 appears unique among the family of NOX in that its activation does not require association with other regulatory subunits (such as p22^{phox}; Geiszt & Leto 2004). NOX5 is activated directly by Ca^{2+} binding to the N-terminal EF hands of the NOX5-L form (Banfi *et al.* 2001, 2004). In mature spermatozoa, NOX5 may act to couple an increase in intracellular Ca^{2+} with other cell-signaling events via an increase in ROS generation. This observation is supported by numerous studies, which have demonstrated that ROS generation by spermatozoa can be stimulated by treatment with calcium ionophore (Aitken *et al.* 1992, Ball *et al.* 2001, Burnaugh *et al.* 2007). Although the ROS generation by NOX5 requires a relatively high $[\text{Ca}^{2+}]$, the concentrations required are at least consistent with those generated during activation of spermatozoa (Banfi *et al.* 2004). Since NOX5 does not require assembly of regulatory subunits (as does NOX2), calcium activation leads to a rapid production of ROS similar to that observed when spermatozoa are treated with calcium ionophore. Phosphorylation of NOX5 appears to further increase the sensitivity of the enzyme to calcium, as evidenced by the increased response to calcium following treatment with phorbol esters (Jagnandan *et al.* 2006). Although studies to date have

Figure 5 Immunohistochemistry of equine testis labeled with α -NOX5 (1:100; a and b) or control (normal rabbit serum; c). Immunolabeling was present primarily over the acrosomal cap of the developing spermatid.

not conclusively demonstrated the role of NOX5 in ROS-mediated signal transduction in spermatozoa, it appears that NOX5 may be a candidate for generation of superoxide anion in equine spermatozoa.

The role of NOX in ROS generation by spermatozoa, however, remains controversial. Several authors suggest that putative ROS generation by spermatozoa in the presence of NAD(P)H is artifactual due to redox cycling of probes such as lucigenin (Ford 2004) and due to the presence of contaminating neutrophils in human semen

Figure 6 Deduced nucleotide sequence (1106 bp) of equine NOX5 (GenBank Accession # EF152773) aligned with the human NOX5 showing high homology (red) with human NOX5. Analysis of the sequence was performed using the BLAST program provided by the NCBI server at the National Library of Medicine/NIH. The alignment was performed using Multalin (<http://prodes.toulouse.inra.fr/multalin/>; Florence Corpet).

or reduction of tetrazolium salts by the activity of P450 reductases in human or rodent spermatozoa (Baker *et al.* 2004). A number of other studies that do not rely on these methodologies, however, demonstrate generation of ROS by spermatozoa in response to NAD(P)H. In equine spermatozoa, NAD(P)H increased H₂O₂ generation as detected by the 'Amplex Red' assay (Ball *et al.* 2001) as well as increased superoxide generation as detected by cytochrome c reduction (Sabeur & Ball 2006). Conclusive resolution of this controversy will likely involve ascribing ROS generation to NOX5 in mature spermatozoa.

In conclusion, this study demonstrates the expression of the NOX, NOX5, in equine testis with immunolocalization of NOX5 protein in round spermatids and in ejaculated equine spermatozoa. NOX5 may play a role in ROS-mediated signaling events in equine spermatozoa although further studies are required to directly establish the specific role of NOX5 in ROS generation by equine spermatozoa.

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