Human spermatozoa possess an IL4I1 l-amino acid oxidase with a potential role in sperm function

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Abstract

Reactive oxygen species (ROS) are known to play an important role in the regulation of human sperm function. In this study, we demonstrate for the first time that human spermatozoa possess interleukin-induced gene 1 (IL4I1), an l-amino acid oxidase (LAAO) which is capable of generating ROS on exposure to aromatic amino acids in the presence of oxygen. The preferred substrates were found to be phenylalanine and tryptophan while the enzyme was located in the acrosomal region and midpiece of these cells. In contrast to equine and bovine spermatozoa, enzyme activity was lost as soon as the spermatozoa became non-viable. On a cell-to-cell basis human spermatozoa were also shown to generate lower levels of hydrogen peroxide than their equine counterparts on exposure to phenylalanine. Stimulation of LAAO activity resulted in the induction of several hallmarks of capacitation including tyrosine phosphorylation of the sperm flagellum and concomitant activation of phospho-SRC expression. In addition, stimulation of LAAO resulted in an increase in the levels of acrosomal exocytosis in both the presence and absence of progesterone stimulation, via mechanisms that could be significantly reversed by the presence of catalase. As is often the case with free radical-mediated phenomena, prolonged exposure of human spermatozoa to phenylalanine resulted in the stimulation of apoptosis as indicated by significant increases in mitochondrial superoxide generation and the activation of intracellular caspases. These results confirm the existence of an LAAO in human spermatozoa with a potential role in driving the redox regulation of sperm capacitation and acrosomal exocytosis.

Introduction

The ability of mammalian spermatozoa to generate reactive oxygen species (ROS) has been appreciated since the pioneering studies of Tosic & Walton (1946) on bull spermatozoa. These authors demonstrated that that the addition of egg yolk-based cryostorage medium to whole bovine semen initially increased oxygen uptake; however, with the passage of time respiration progressively decreased in concert with a decline in sperm motility. Since the inhibitory effect of egg yolk could be reversed by the addition of catalase, it was concluded that the active principle generated on contact with egg yolk was hydrogen peroxide. Tosic & Walton (1950) then went on to demonstrate that under such circumstances the source of ROS involved the oxidative deamination of amino acids by an l-amino acid oxidase (LAAO) in spermatozoa to generate hydrogen peroxide and ammonia. This oxidase was found to have a particular propensity for the deamination of aromatic amino acids, with phenylalanine the preferred substrate (Tosic & Walton 1950). Subsequent studies have confirmed the presence of an LAAO in bull (Shannon & Curson 1972, 1982) and ram (Upreti et al. 1998) spermatozoa with the potential to damage sperm function as a result of oxidative stress. The way in which exposure to hydrogen peroxide leads to a loss of sperm function has also been thoroughly investigated since the pioneering work of Tosic & Walton (1946), and shown to involve a number of pathological processes from the generation of cytotoxic aldehydes as a consequence of lipid peroxidation (Jones et al. 1979, Aitken & Curry 2011, Aitken et al. 2012) to the formation of potentially mutagenic base adducts in sperm DNA (De Iuliis et al. 2009).

Oxidative stress is now known to be a major factor in the etiology of impaired human sperm function responsible for disrupting DNA integrity and the competence of these cells for movement and sperm–oocyte fusion (Aitken & Clarkson 1987, Aitken et al. 1989, 1998a).

Paradoxically, ROS are also known to be important for the physiological promotion of sperm function via the stimulation of tyrosine phosphorylation and the induction of cholesterol oxidation during capacitation (Aitken et al. 1995, Aitken 1997, Leclerc et al. 1997, Lewis & Aitken 2001, O’Flaherty et al. 2006a, b, Awda & Buhr 2010, Brouwers et al. 2011, Donà et al. 2011). The source of reactive oxygen metabolites capable of promoting either the physiological induction, or the pathological...
suppression, of capacitation is currently not understood. The purpose of this study was to determine whether an LAAO might be present in these cells and contributing to the redox balance that defines sperm functionality.

Materials and methods

Chemical reagents

Chemicals and reagents used throughout this study were purchased from Sigma–Aldrich (Sigma Chemical Co.) unless otherwise stated, and were of research grade. The fluorescent probes used were purchased from Invitrogen unless otherwise stated.

Preparation of spermatozoa

Institutional approval for the use of human semen samples in this research project was granted by the University of Newcastle Human Ethics Committee and all donors gave their written informed consent. Samples from unselected donors were collected into sterile containers after at least 48 h abstinence and promptly transferred to the laboratory. Once the semen had liquefied, spermatozoa were isolated by centrifugation on a discontinuous 80/40% Percoll gradient diluted with medium BWW (Biggers et al. 1971) at 500 g for 30 min. High-density spermatozoa were retrieved, washed in 5 ml BWW, and centrifuged at 500 g for 15 min. The resulting pellet was again resuspended in BWW for the assessment of sperm concentration and then diluted to a concentration of 10×10^6/ml. Spermatozoa were assessed in terms of their motility, count, and morphology using guidelines set out by the World Health Organization (2010). Vitality assessments were conducted by the eosin exclusion method (World Health Organization 2010).

Stallion semen was collected from three individual Shetland-cross ponies, aged 6–9 years of proven fertility status, on institutionally approved premises. Semen samples were collected using an artificial vagina lined with a collection bag, diluted in Kenney’s extender 2:1, and transferred to the laboratory for preparation on Percoll gradients, as described above for human spermatozoa. Highly motile equine spermatozoa were recovered from the base of the high-density region of the gradient, washed with medium BWW, and finally resuspended at a concentration of 2×10^7/ml.

Determination ROS production

Generation of hydrogen peroxide by spermatozoa was detected by chemiluminescence using a luminol/peroxidase protocol. Spermatozoa (4×10^6) suspended in BWW with 8 µl of 2 mg/ml HRP (type VI from horseradish, Sigma–Aldrich), 4 µl of 250 µM luminol, and 40 µl of 0–10 mM amino acid treatment in a total volume of 400 µl in BWW. The samples were placed in Rohren tubes (Sarstedt, Nürnberg, Germany) and measured for chemiluminescence over a 2 h time period at 37 °C in a Berthold 9505C luminometer (Berthold, Wildbad, Germany). Control Version 1.04B Software was used.

For certain experiments non-viable spermatozoa were prepared by a single freeze–thaw cycle involving immersion in liquid nitrogen for 10 s followed by rapid thawing at 37 °C. The amino acids were dissolved in BWW, except tyrosine which was dissolved in 1 M ammonium hydroxide and then adjust to pH 7.4 with hydrochloric acid in order to create stocks at a concentration of 100 mM. The experiments to determine the effect of ROS scavengers followed the same outline, with the activation of LAAO activity achieved through the addition of 40 µl of 10 mM phenylalanine to 400 µl reaction volume. Catalase (6000 IU; Sigma–Aldrich) and superoxide dismutase (SOD; 60 IU; Sigma–Aldrich) were added in a volume of 40 µl, immediately before the initiation of the luminometer run or after ~70 min.

Hydrogen peroxide generation was also quantified using the Amplex Red Assay Kit supplied by Invitrogen (catalogue number, A22188). A reaction mixture was made up containing 50 µM Amplex Red reagent and 0.1 U/ml HRP in BWW; 100 µl of this reaction mixture was then added to each well and incubated for 10 min at 37 °C. A suspension of 1×10^6 spermatozoa and the stated concentration of phenylalanine were then added to the reaction wells and the fluorescence emission was monitored using an OPTIMA Fluostar (BMG LABTECH Australia, Mornington, VIC, Australia) spectrophotometer at 4 h and 37 °C.

Determining the localization of LAAO by immunocytochemistry

The samples of 10×10^6 spermatozoa were centrifuged at 500 g for 5 min and fixed in 2% paraformaldehyde for 15 min. The fixed cells were washed in 0.1 M PBS, resuspended in 0.1 M glycine/PBS, and transferred to poly-L-lysine coated coverslips to settle overnight. Triton X-100-PBS (0.2%) was used to permeabilize the spermatozoa for 10 min at room temperature followed by a wash in PBS. Non-specific antibody binding was inhibited with 3% BSA solution (30 mg BSA, 100 µl goat serum, and 900 µl PBS) for 30 min. The coverslips were then rinsed in PBS and treated with 1/25 IL4I antibody (anti-LAAO; Sapphire Bioscience, Waterlooo, NSW, Australia), diluted in PBS overnight at 4 °C in a humidified chamber. They were washed in PBS and treated with AlexaFluor-488 goat anti-rabbit secondary 1/250 in PBS for 1 h at 37 °C and re-washed in PBS, mounted on superfrost slides with mowiol (13% v/v Mowiol 4-88, 33% v/v glycerol, 2.5% w/v – 1,4-diazobicyclo[2,2,2]octane (DABCO), and 66 mM Tris pH 8.5), and sealed with clear nail polish. Fluorescent patterns were observed under a Zeiss fluorescence microscope at 530 nm.

RT-PCR of human LAAO in spermatozoa

Spermatozoa recovered from ejaculates were adjusted to 1×10^7 cells, washed twice in PBS, and pelleted. Total RNA was prepared from the final cell pellets using TRIzol reagent (Life Technologies). RNA was isolated with isopropanol, following exposure to 5 µl of 2 mg/ml glycogen (Life Technologies) to facilitate RNA precipitation.

To determine the presence of human LAAO transcripts in human spermatozoa, 5 µg of total RNA was reverse transcribed with oligo(dT)15 primer (Promega Corporation) and M-MLV Reverse Transcriptase (Promega). RT-PCR for human LAAO was

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then performed to detect the two known isoforms of the LAAO gene. PCR primers were designed to detect isoforms 1 and 2 (GenBank accession numbers: NM_152899 and NM_172374 respectively). For isoform 1 first-round PCR, the forward primer sequence was 5’-CACAAGAGCTGGAGACACC-3’ and the reverse primer sequence was 5’-CCCCGATCCTGTTATCTGCC-3’. These primers were predicted to generate bands of 323 bp. The PCR conditions were as follows: one cycle of 94°C for 5 min; 35 cycles of 95°C for 45 s, 65°C for 45 s, 72°C for 2 min; and one cycle of 72°C for 10 min. For nested PCR, a 1:100 dilution of the first-round product was used as the template and the conditions were the same as above: the forward primer sequence for the nested PCR analysis was 5’-CTGGAGACACCACATCTCCCAC-3’ and the reverse primer sequence was 5’-CAGCGCCAACCACAAATCAC-3’. These primers are predicted to generate bands of 230 bp.

For isoform 2 first-round PCR, the forward primer sequence was 5’-TCCAGTCGAAATGGGCGC-3’ and the reverse primer sequence was 5’-TTTCTGGAAGGGGTCTTGCC-3’. These primers were predicted to generate bands of 372 bp. The PCR conditions were as for isoform 1. For nested PCR, the forward primer sequence was 5’-GGTGAATTTTTGGAAATCAGAGGC-3’ and the reverse primer sequence was 5’-TGAGGATGAGGAGAACGAG-3’. These primers are predicted to generate bands of 252 bp. The PCR conditions were the same as above.

The PCR products were run on a 1.5% agarose gels and the DNA was purified from the gel using the Wizard Gel Clean-Up Kit from Promega. The DNA was sequenced at the Australian Genomic Research Facility (Brisbane, QLD, Australia).

**Determining the effect of phenylalanine on sperm function**

A suspension of 5 × 10⁶ spermatozoa was suspended in 1 ml phenylalanine (0.31–10 mM) or medium BWW as an untreated control. These cells were incubated over 24 h at 37°C and scored for motility and vitality at 0, 2, 4, 6, and 24 h. For experiments on sperm capacitation, 10 × 10⁶ spermatozoa were capacitated by suspending these cells in 1 ml BWW containing 1 mM dibutyryl cAMP (dbcAMP) and 1 mM pentoxifylline (PTX) (Pujianto et al. 2009) or 2.5, 5, and 10 mM phenylalanine in the presence or absence of catalase for 3 h at 37°C. The spermatozoa were then resuspended in 15 μM progesterone (dissolved in DMSO) for 30 min at 37°C to induce the acrosome reaction. The samples for preparing control were suspended in both non-capacitation medium BWW lacking added bicarbonate (Atiken et al. 1998b) and normal unsupplemented BWW. The cells were then resuspended in hypotonic swelling solution for 1 h at 37°C (Atiken et al. 1993). The spermatozoa were then fixed and settled onto slides for cytochemical analysis with a conjugated peanut agglutinin (PNA)–FITC probe (2 μg/ml) targeting the outer acrosomal membrane. The cells were permeabilized with ice-cold methanol for 20 min and then treated with the PNA–FITC conjugate. The percentage of live cells which were acrosome reacted (determined by a loss in acrosomal fluorescence) was recorded by observing under a Zeiss fluorescence microscope at 530 nm.

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**The effect of phenylalanine on tyrosine phosphorylation and phospho-SRC activation in human spermatozoa**

The samples of 10 × 10⁶ spermatozoa were capacitated by suspending them in 1 ml BWW containing 1 mM dbcAMP and 1 mM PTX or 2.5, 5, or 10 mM phenylalanine in the presence or absence of catalase. These cells were then fixed and used for immunocytochemistry to determine phosphorylation rates of tyrosine combined with a PT66 primary antibody (Sigma) diluted 1:2500 in PBS and AlexaFluor-488 goat anti-rabbit secondary 1:250 in PBS. Phospho-SRC activation was determined using a pp60cSRC (z-Tyr418) primary antibody (Calbiochem, San Diego, CA, USA) diluted 1:400 in PBS with an AlexaFluor-488 goat anti rabbit secondary (1:250). The percentage of positive cells was determined by manually counting 100 cells under a fluorescence microscope at a magnification of ×400.

**The effect of phenylalanine on apoptosis**

The spermatozoa were suspended in 0–10 mM phenylalanine at a concentration of 10 × 10⁶ cells/ml for 24 h at 37°C. These cells were then used for cytometry-based determinations of caspase activation (FLICA; ImmunoChemistry Technologies, Bloomington, MN, USA) and ROS generation (MitoSOX Red (MSR)). A FACS-Calibur flow cytometer was used, equipped with a 488 nm argon laser. Cell Quest Software (Becton Dickinson) was used to analyze and acquire the data. Gating was used to prevent incorporation of non-sperm cells, and the evaluations were based on 10,000 gated cells. For MSR assays, cells were centrifuged at 500 g for 5 min and resuspended in a final concentration of 2 μM MSR in addition to a 5 nM SYTOX Green vitality stain. These cells were then incubated for 15 min at 37°C in foil, resuspended in PBS, and transferred to FACS tubes for analysis on the flow cytometer. For the FLICA assay, cells were centrifuged at 500 g for 5 min and resuspended in a final concentration of 5 μM FLICA dye in addition to 2 μM Live/Dead viability stain for 1 h at 37°C in foil. To fix these cells, 2% paraformaldehyde was used. The latter were then transferred to FACS tubes for analysis on the flow cytometer.

**Statistical analyses**

JMP version 10 (SAS, Cary, NC, USA) was used to analyze the data produced from each experiment. Experiments were performed at least three times, using independent donors, unless otherwise stated. Data sets were used for a one-way ANOVA (P<0.05 significance) paired with Fisher’s protected least significant difference (LSD).

**Results**

**LAAO in human spermatozoa**

The expression of the human LAAO, interleukin-induced gene 1 (IL4I1) mRNA, was confirmed in human spermatozoa by nested PCR. Isoform 1 was expressed in spermatozoa from both the low- and high-density Percoll fractions as a single mRNA species of the
expected size (Fig. 1A). Isoform 2 was also expressed in 50 and 100% fractions at the expected amplicon size of 252 bp, but was consistently accompanied by an additional band at 300 bp. On sequencing, this band was found to be a pseudogene made up of a part of IL4I1 isoform 2, a portion of a tRNA gene, and a portion of a noncoding genomic DNA sequence (Fig. 1A). Immunocytochemistry using an antibody against IL4I1 confirmed the presence of LAAO in the acrosomal domain as well as the midpiece of human spermatozoa (Fig. 1B).

**LAAO enzyme activity**

In order to confirm the presence of LAAO enzyme activity in human spermatozoa, luminol–peroxidase mediated chemiluminescence was used to assess the cellular generation of hydrogen peroxide in response to amino acid exposure (Aitken et al. 1992). All naturally occurring L-amino acids were tested at a dose of 1 mM in this analysis, apart from cysteine, which redox-cycled in the presence of this probe to generate a spurious chemiluminescence signal in the complete absence of cells. ANOVA analysis revealed a significant effect ($P<0.001$) associated with the addition of amino acids, while comparison of the group means revealed that the aromatic amino acids phenylalanine and tryptophan generated the highest levels ($P<0.001$) of hydrogen peroxide production (Fig. 2). Interestingly, slight but significant responses ($P<0.05$) were also observed with certain nonaromatic amino acids such as lysine, leucine, isoleucine, and asparagine. However, the L-aromatic amino acids were clearly the preferred substrate.

Studies were subsequently conducted using phenylalanine as a substrate to demonstrate that the LAAO-mediated production of ROS was both time- (Fig. 3A) and dose- (Fig. 3A and B) dependent ($P<0.001$). In addition, the response to phenylalanine was shown to be highly dependent on cell viability (Fig. 3B). Thus, the destruction of cell viability with a single freeze–thaw cycle led to a complete loss of LAAO activity in human spermatozoa (Fig. 3B), in complete contrast to bull and ram spermatozoa, where the redox response to exogenous phenylalanine has been shown to increase following
a loss of plasma membrane integrity (Shannon & Curson 1982). This observation was confirmed with equine spermatozoa (Fig. 3C) and points to a fundamental difference in the stability of LAAO in human spermatozoa compared with other species. In this context, western blot analysis demonstrated a loss of signal when human spermatozoa were frozen—thawed, suggesting a physical loss of the enzyme rather than a simple loss of enzyme activity (Fig. 3B, insert).

In order to confirm that the chemiluminescent signals generated in the presence of phenylalanine were ROS related, the inhibitory impact of SOD and catalase on this activity was examined as depicted in Fig. 4. These data indicated that the chemiluminescent signal is suppressed below control levels if these enzymes are added before or after the induction of chemiluminescent activity with phenylalanine (Fig. 4). The chemiluminescence data presented in Fig. 3 also indicated that the ROS response generated by stallion spermatozoa to phenylalanine was significantly greater than that observed with human spermatozoa on a cell-to-cell basis. In order to confirm this observation, hydrogen peroxide generation by intact human and equine spermatozoa was quantified and compared across a range of phenylalanine doses. This analysis revealed that while neither species exhibited significant responses in the D-phenylalanine control incubations, exposure to L-phenylalanine for 2 h resulted in a dose-dependent generation of hydrogen peroxide that was approximately twice as high as in the stallion spermatozoa compared with their human counterparts (P<0.001; Fig. 5).

**Functional consequences of LAAO activity**

In order to determine how the induction of LAAO activity in human spermatozoa impacted upon the functional attributes of these cells, time- and dose-dependent studies were conducted using phenylalanine as the substrate for this enzyme. Despite the evident

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**Figure 3** Effect of phenylalanine on l-amino acid oxidase activity. (A) Addition of phenylalanine to human spermatozoa results in a dose- and time-dependent increase in oxidase activity as measured luminol–peroxidase chemiluminescence. (B) Dose-dependent increase in ROS generation presented as counts integrated over 2 h and demonstrating the devastating impact of lost viability on the response. Insert depicts a western blot analysis revealing how a loss of cell viability is associated with an apparent reduction in the LAAO (IL4l1) content of the spermatozoa relative to the tubulin loading control (α-T). (C) In contrast, the responsiveness of equine spermatozoa to phenylalanine is significantly enhanced by a loss of cell viability. ***P<0.001. All experiments run on three independent samples.

**Figure 4** Effect of ROS scavenging enzymes on human LAAO activity. Addition of superoxide dismutase and catalase (Phe+SOD/CAT) scavenged the ROS invoked by phenylalanine (Phe) treatment (1 mM). This was observed if the scavengers were applied before the chemiluminescence run or after 70 min. All experiments run on three independent samples, representative example shown.
induction of ROS generation, human spermatozoa remained fully viable and motile for at least 6 h in the presence of up to 10 mM phenylalanine (Fig. 6). However after 24-h exposure to this amino acid, the oxidative stress generated by LAAO resulted in a highly significant dose-dependent loss of both motility (P < 0.001) and vitality (P < 0.001) (Fig. 6). This loss of functionality after prolonged exposure to high doses (10 mM) of phenylalanine was associated with the induction of sperm apoptosis as evidenced by statistically significant increases in two hallmarks of this process, mitochondrial ROS generation (Koppers et al. 2011), and caspase activation (Fig. 7).

In order to determine whether the redox activity is associated with more moderate exposures to phenylalanine-influenced sperm capacitation, two biochemical features of this process were examined, phosphorysine expression and the presence of activated SRC, a protein kinase (Baker et al. 2006). Analysis of activated SRC expression demonstrated a highly significant (P < 0.001) activation of this kinase in the principle piece following exposure to phenylalanine (Fig. 8). A global increase in tyrosine phosphorylation was also noted following exposure to phenylalanine that could be reduced to control levels by the concomitant presence of catalase (Fig. 9A). As the LAAO was present in the acrosomal domain of human spermatozoa, we also examined the impact of phenylalanine on the ability of these cells to undergo acrosomal exocytosis in both presence and absence of progesterone. This analysis demonstrated that the stimulation of hydrogen peroxide generation with phenylalanine significantly increased acrosome reaction rates via mechanisms that could be completely reversed by the simultaneous presence of catalase (Fig. 9B). Such stimulation of acrosome reaction rates was observed at the higher doses of phenylalanine in the absence of progesterone, but with all doses of this amino acid when progesterone was present (Fig. 9B).

Discussion

This study has demonstrated for the first time that human spermatozoa possess an LAAO capable of generating hydrogen peroxide. The existence of this enzyme was initially inferred from the use of a nested PCR strategy to demonstrate the presence of both known isoforms of the mRNA for IL4I1. The existence of the translated gene product was then confirmed by results of immunocytochemistry, which demonstrated the presence of this enzyme in both the acrosomal domain and the midpiece of human spermatozoa. In addition, the biochemical signature of LAAO activity, notably the generation of hydrogen peroxide in the presence of L-amino acids, was confirmed using both luminol–peroxidase-mediated chemiluminescence (Figs 2 and 3) and the Amplex Red Assay (Fig. 5). A survey of potential substrates for this enzyme demonstrated that it was optimally active following exposure to the aromatic amino acids phenylalanine and tryptophan (Fig. 2) in keeping with the known activity of this enzyme in the immune system (Boulland et al. 2007). The localization of this enzyme in the sperm acrosome is also consistent with the observation that this enzyme is maximally active at acidic pH and is preferentially targeted to lysosomes (Mason et al. 2004); the acrosomal vesicle is known to exhibit a low intraacrosomal pH and shares many

![Figure 5](image5.png) Quantification of hydrogen peroxide production by human and equine spermatozoa as a consequence of LAAO activity. The Amplex Red Assay was used to measure hydrogen peroxide production over a 2-h time course in the presence of either (A) L-isomers of phenylalanine or, as controls and (B) the inactive D-isomers. ***P < 0.001. All experiments run on three independent samples.

![Figure 6](image6.png) Time- and dose-dependent analysis of the impact of phenylalanine on the motility and vitality of human spermatozoa. After 24 h, phenylalanine induced a dose-dependent inhibitory effect on (A) sperm motility and (B) vitality. *P < 0.05; **P < 0.01; and ***P < 0.001. All experiments run on three independent samples.
features with cellular lysosomes (Working & Meizel 1983, Moreno & Alvarado 2006). Importantly, the anti-IL4I1 antibody used in this study could not discriminate between the two isoforms of this molecule, because the translated protein generated by the two genes represented in Fig. 1 is thought to be identical after cleavage of the predicted signal peptides (Wiemann et al. 2005); it is just the promoter regions of these genes that differ, allowing differential regulation of their expression. It should be noted that this is the first report of both IL4I1 variants appearing in a single cell type (IL4I1 was previously thought to exclusively expressed in B lymphoblasts), raising the intriguing possibility that these variants are differentially expressed and translated during spermatogenesis possibly contributing to the subcellular localization of the protein in the sperm acrosome and midpiece respectively.

LAAO activity has also been observed in bull, ram, and stallion spermatozoa; however, the enzyme in human spermatozoa exhibits some notable differences with these species. In all other species analyzed, the enzyme has been found to exhibit an exclusive preference for aromatic amino acids (phenylalanine, tryptophan, and tyrosine; Tosic & Walton 1950, Shannon & Curson 1972, 1982, Upreti et al. 1998). In human spermatozoa, tyrosine was not active as a substrate, but other nonaromatic L-amino acids (lysine, isoleucine, asparagine, and leucine) did exhibit a low level of activity (Fig. 2). Another interesting difference is that in all other species that have been examined to date, the enzyme is optimally active in nonviable cells, presumably because under these circumstances the amino acid substrates can gain unimpeded access to the enzyme. However, nonviable human spermatozoa are completely different from all other species examined in that they exhibit no LAAO activity. A possible reason for this loss of activity in non-viable human spermatozoa is shown in Fig. 3, where a single round of freeze–thawing was found to result in a major loss of LAAO protein according to a western blot analysis. Thus, the LAAO in human spermatozoa is not bound to the cell as tightly as in other species. This difference is dramatically illustrated in Fig. 3, where the induction of cell death was associated with a dramatic increase in the LAAO activity detected in equine spermatozoa but with a complete loss of activity in human cells.

This difference in enzyme behavior may have functional consequences particularly in the area of sperm cryostorage. Egg yolk-based cryodiluents have been shown to possess sufficient quantities of phenylalanine to stimulate LAAO activity and the generation of ROS by dead cells in the sperm suspension. The oxidative stress generated in this way has then been shown to compromise the functionality of live spermatozoa in the immediate vicinity (Shannon & Curson 1982). While activation of ROS generation by phenylalanine does ultimately compromise both the motility and the vitality of human spermatozoa (Fig. 6), such stress is evidently not accentuated by nonviable cells present in the same sperm suspension. Rather, the increased ROS production associated with activation of LAAO activity in human spermatozoa eventually overwhelms the cells’ own antioxidant defense capacity and ultimately precipitates a state of oxidative stress that induces apoptosis after 24 h, as reflected in the activation of caspase activity and the promotion of oxidative stress and apoptosis in human spermatozoa. After 24 h exposure to 10 mM phenylalanine, human spermatozoa experience a significant increase in (A) mitochondrial ROS generation and (B) caspase activation. *P<0.01 and ***P<0.001. MitroSOX Red experiment run on three independent samples, FLICA analysis run on three independent samples.
Brouwers et al. 2011). In keeping with these observations, exposure of human spermatozoa to phenylalanine was found to significantly stimulate the ability of these cells to both capacitate and undergo acrosomal exocytosis via mechanisms that could be reversed by the addition of catalase to the medium (Fig. 9). As the latter specifically scavenges hydrogen peroxide, these data support the notion that this oxidant is a physiological mediator of sperm function, driving both the tyrosine phosphorylation events associated with sperm capacitation (Bize et al. 1991, Griveau et al. 1994, Rivlin et al. 2004, O’Flaherty et al. 2006a,b) and facilitating the acrosome reaction (Hsu et al. 1999, O’Flaherty et al. 2005). It is interesting to note that overall, phenylalanine is a much more powerful stimulus for the acrosome reaction than progesterone. However, at the 2.5 mM phenylalanine dose, there may be some synergy between this amino acid and progesterone (Fig. 9).

Interestingly, while LAAO is demonstrably more active in equine than in human spermatozoa, we have not been able to find any significant impact of phenylalanine on capacitation and acrosomal exocytosis in the former (RJ Aitken, unpublished observations). A major difference between human and equine spermatozoa is that the latter are highly dependent on oxidative phosphorylation and have developed a high level of tolerance to oxidative stress (Gibb et al. 2014). Under these circumstances, redox regulation of sperm capacitation and acrosomal exocytosis are less evident and the primary purpose of the LAAO may be to simply deaminate amino acids according to the fundamental equation, \( \text{L-amine acid} + \text{H}_2\text{O} + \text{O}_2 = \text{NH}_3 + \text{H}_2\text{O}_2 + 2\text{-oxo acid} \), before metabolism of their carbon skeletons by the sperm mitochondria. Alternatively, an ancient role for this oxidase may be in the control of local bacterial infections (Puiffe et al. 2013).

In human spermatozoa, it may be significant that metabolism is heavily dependent on glycolysis (Hereng et al. 2011) and, as a result, these cells are exquisitely sensitive to changes in their redox status. Under these circumstances, it is possible that this enzyme has, in human spermatozoa, acquired a new function as a regulator of sperm capacitation and the acrosome reaction. The diminished activity of this enzyme in human spermatozoa compared with their equine counterparts would be in keeping with such a physiological role.

If phenylalanine is physiologically important as a regulator of sperm function, then adequate concentrations of this amino acid would have to be present in the female reproductive tract fluids to support such a role. Mouse oviductal fluid at estrus has been found to contain phenylalanine (202 \( \mu \)M) and tryptophan (50 \( \mu \)M) and facilitating the metabolism of their carbon skeletons by the sperm mitochondria. Alternatively, an ancient role for this oxidase may be in the control of local bacterial infections (Puiffe et al. 2013).

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If phenylalanine is physiologically important as a regulator of sperm function, then adequate concentrations of this amino acid would have to be present in the female reproductive tract fluids to support such a role. Mouse oviductal fluid at estrus has been found to contain phenylalanine (202 \( \mu \)M) and tryptophan (50 \( \mu \)M) and facilitating the metabolism of their carbon skeletons by the sperm mitochondria. Alternatively, an ancient role for this oxidase may be in the control of local bacterial infections (Puiffe et al. 2013).
sufficient redox activity to enhance capacitation and acrosomal exocytosis in spermatozoa approaching the oocyte (Zhang et al. 2014). Furthermore, the concentration of phenylalanine in follicular fluid is known to be significantly greater than that in blood plasma close to the time of ovulation (Jimena et al. 1993). In vivo, the detrimental consequences of prolonged exposure to phenylalanine may not be as significant as we have observed in vitro, because reproductive tract fluids are endowed with a complex array of antioxidant factors that control the levels of oxidative stress experienced by the spermatozoa, but are missing from conventional IVF culture media (Töörä et al. 2003, Huang et al. 2014).

In conclusion, this study has reported for the first time the presence of an LAAO, IL411, in human spermatozoa. The enzyme is maximally active with the aromatic amino acids, tryptophan, and phenylalanine, as substrates, is located in the sperm acrosome and midpiece, and is capable of generating hydrogen peroxide in a dose-dependent manner. The induction of LAAO activity results in the stimulation of several components of capacitation, including phospho-SRC expression and an increase in tyrosine phosphorylation, as well as enhanced rates of acrosomal exocytosis. These findings suggest a possible pathway by which the redox status of human spermatozoa might be physiologically regulated and may find application in the assisted conception industry in the design of optimized media for the production of capacitated spermatozoa.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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