Journal of Cell Science 108, 2017-2025 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function

R. J. Aitken[1,*], M. Paterson[1], H. Fisher[1], D. W. Buckingham[1] and M. van Duin[2]

[1]MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh EH3 9EW, Scotland
[2]Organon International B. V., Scientific Development Group, Department of Immunology, PO Box 20, 5340 BH Oss, The Netherlands

*Author for correspondence

SUMMARY

The redox status of human spermatozoa was found to have a profound influence on the fertilizing potential of these cells in association with qualitative and quantitative changes in the patterns of tyrosine phosphorylation. In general, oxidizing conditions enhanced tyrosine phosphorylation and stimulated sperm function, whereas reducing conditions had the opposite effect. Unstimulated human spermatozoa exhibited low levels of spontaneous acrosomal exocytosis and sperm-oocyte fusion and minimal reactive oxygen species generation, while phosphotyrosine expression was largely confined to a single protein of 116 kDa. However, if the spermatozoa were exposed to oxidizing conditions through the addition of exogenous H_2O_2, or the stimulation of endogenous NADPH-dependent reactive oxygen species generation, then a dramatic increase in tyrosine phosphorylation was observed (major phosphotyrosyl bands at 222 kDa, 200 kDa, 159 kDa, 133 kDa, 116 kDa and 82 kDa) in concert with the functional activation of the spermatozoa. A causal association between reactive oxygen species generation, tyrosine phosphorylation and sperm function was indicated by studies with the ionophore, A23187, which induced high rates of sperm-oocyte fusion together with enhanced rates of reactive oxygen species production and the increased expression of phosphotyrosyl proteins. This functional response to A23187 could be abrogated, without any concomitant change in sperm motility or viability, by using membrane permeant thiols or catalase to suppress the reactive oxygen species-induced increase in phosphotyrosine expression. The fact that the biological responses of human spermatozoa to biological agonists (recombinant human ZP3 and progesterone) could also be inhibited by catalase indicated the general relevance of these findings. These results suggest that the generation of reactive oxygen species by human spermatozoa plays a key role in the control of sperm function through the redox regulation of tyrosine phosphorylation.

Key words: spermatozoon, tyrosine phosphorylation, reactive oxygen species, NADPH oxidase

INTRODUCTION

Fertilization is initiated by a cell specific recognition event involving the sperm plasma membrane and a glycoprotein constituent of the zona pellucida, ZP3 (Wassarman, 1990). Studies employing the mouse as an animal model have indicated that a key mediator of gamete recognition is a 95 kDa tyrosine kinase that is thought to autophosphorylate on being cross-linked by the ZP3 ligand (Leyton and Saling, 1989a,b; Leyton et al., 1992; Bunch et al., 1992) and may exhibit a hexokinase like structure (Kalab et al., 1994). This change is then thought to initiate a cascade of cellular events, including a sudden influx of extracellular calcium and an efflux of protons, that ultimately results in the induction of acrosomal exocytosis and the generation of a fusogenic plasma membrane capable of recognizing, and fusing with, the vitelline membrane of the oocyte. Some evidence has also been obtained for the autophosphorylation of a similar 94 kDa protein in extracts of human spermatozoa incubated with either porcine ZP3 or unfractionated, solubilized, human zona pellucida glycoproteins (Naz et al., 1991). Moreover, the steroid hormone, progesterone, has also been shown to activate human spermatozoa via a non-genomic mechanism that involves the enhanced phosphorylation of a 94 kDa molecule on tyrosine (Tesarik et al., 1993). The fact that the suppression of tyrosine phosphorylation in murine and human spermatozoa by genistein is associated with the inhibition of the acrosome reaction emphasizes the causal nature of the relationship between tyrosine kinase activity and the biological activation of these cells (Tesarik et al., 1993; Bunch et al., 1992).

Recent studies in a variety of cell types have demonstrated the importance of cellular redox status in regulating the levels of tyrosine phosphorylation. For example, it has been appreciated for some time that the autophosphorylation of the β-subunit of the insulin receptor can be induced by exposure to H_2O_2 (Mukerjee et al., 1978; Koshio et al., 1988). There have

also been reports of H_2O_2 acting in synergy with vanadate to induce tyrosine phosphorylation in peripheral blood human T cells (O'Shea et al., 1992). Similarly, the exposure of Ramos cells to H_2O_2 induces the selective phosphorylation of the $p72^{syk}$ (Schieven et al. 1993) while exposure to exogenous oxidizing agents, such as iodoacetamide and diamide, has been shown to influence the activity the tyrosine kinase, Ltk, in lymphocytes (Bauskin et al., 1991). The reactive oxygen species (ROS) generated by the NADPH oxidase system in neutrophils has also been shown to have a profound effect on the levels of tyrosine phosphorylation observed in these cells (Fialkow et al., 1993). Since human spermatozoa may also possess an NADPH oxidase system for the generation of ROS (Aitken and Clarkson, 1987a,b), it is possible that the tyrosine phosphorylation that appears to play such an important role in regulating sperm function is also under redox control. In this report we have examined this hypothesis and produced evidence for the induction of tyrosine phosphorylation in response to the activation of the NADPH oxidase system in human spermatozoa. Such redox regulation is shown to be an essential component of the mechanisms by which human spermatozoa exhibit a biological response to the calcium and pH signals that activate these cells.

MATERIALS AND METHODS

Reagents

All reagents were obtained from Sigma Chemical Co. (St Louis, MO) with the exception of Percoll® (Pharmacia, Uppsala, Sweden), Dulbecco's phosphate buffered saline and medium 199 (Flow Laboratories, Irvine, Scotland), the anti-phosphotyrosine monoclonal antibody (UBI, TCS Biologicals, Buckingham, UK), anti-quenching agent (Citifluor, London, UK), nitrocellulose (Hybond C Super, Amersham, UK) and human serum albumin (Armour Pharmaceutical Co., Eastbourne, UK). Recombinant human ZP3 was produced in CHO cells and affinity purified as described in detail by van Duin et al. (1994).

Sperm preparation

The study population comprised 24 unselected, normospermic donors (World Health Organization, 1992), who had been clinically examined and shown to be free of any detectable pathology, including hepatitis and sexually transmitted diseases such as HIV, which might have influenced the quality or cellular composition of their semen. The semen samples were produced by masturbation and collected into sterile containers for immediate transportation to the laboratory. After allowing at least 30 minutes for liquefaction to occur, the spermatozoa were fractionated on a discontinous 2-step Percoll gradient comprising a 3 ml volume of isotonic Percoll (100 %) overlaid with a further 3 ml volume of 50% Percoll in a 15 ml conical-based sterile centrifuge tube. Isotonic Percoll was created by supplementing 10 ml of 10× concentrated medium 199 with 300 mg human serum albumin, 3 mg sodium pyruvate and 0.37 ml of a sodium lactate syrup followed by the addition of 90 ml of Percoll. This preparation was designated 100% Percoll (Lessley and Garner, 1983) and was subsequently diluted with HEPES-buffered medium BWW (Biggers et al., 1971).

Samples of 1 to 3 ml of semen were layered on the top of each gradient and centrifuged at 500 ***g*** for 20 minutes. Thereafter, the seminal plasma was discarded and the cells collected from the base of the 100% Percoll fraction. The spermatozoa were then resuspended in 7 ml of BWW, centrifuged at 500 ***g*** for 5 minutes and finally resuspended in this medium at a sperm concentration of 20×10^6 cells/ml.

Sperm-oocyte fusion

The ability of human spermatozoa to acrosome react and fuse with the vitelline membrane of the oocyte was examined using the zona-free hamster oocyte penetration assay (Yanagimachi et al., 1976; Aitken et al., 1993a). For certain experiments the spermatozoa were stimulated to acrosome react with A23187-free acid (Aitken et al., 1993a,b). A23187 was prepared as a 100 mM stock solution in dimethylsulphoxide and diluted to 1 mM with BWW prior to storage at 4°C. The ionophore was subsequently diluted to the final working concentration (2.5 μM) immediately before the initiation of each experiment. The spermatozoa were incubated with the ionophore, either alone (positive control) or in association with a variety of reagents designed to modify the redox status of the cells including superoxide dismutase (SOD; 87.5 U/ml), catalase (50 kU/ml), dithiothreitol (0.1 and 1 mM), 2-mercaptoethanol (0.1 and 0.05%) and reduced glutathione (1 mM), for 3 hours at 37°C in an atmosphere of 5% CO_2 in air. Similar experiments were conducted in which the ability of catalase to inhibit the biological responses given by human spermatozoa following a 3 hour incubation with recombinant human ZP3 (50 ng/ml) or progesterone (5 μM) was investigated. In additional studies, A23187 was omitted and the spermatozoa were incubated for 3 hours under oxidizing conditions created by the addition of H_2O_2 (50 and 100 μM), glucose oxidase (12.5 and 25 ng) or NADPH (1.25, 2.5, 5.0 and 10 mM).

At the end of the incubation period, the spermatozoa were pelleted by centrifugation at 500 ***g,*** resuspended in the same volume of fresh medium BWW and distributed as 50 μl droplets under liquid paraffin. At this point, the motility and viability (Jeyendran et al., 1984) of the spermatozoa were assessed in order to exclude the possibility that the treatments employed had had a detrimental effect on the survival of these cells.

Zona-free hamster oocytes were prepared as described in the original publication of Yanagimachi et al. (1976) and dispensed into the droplets at 5 oocytes/drop and 15-20 oocytes /sample. After a further 3 hours, the oocytes are recovered from the droplets, washed free of loosely adherent spermatozoa, compressed to a depth of about 30 μm under a 22 mm × 22 mm coverslip on a glass slide and assessed for the presence of decondensing sperm heads with an attached or closely associated tail, by phase contrast microscopy. The number of spermatozoa penetrating each egg was recorded and the results expressed as the percentage of oocytes penetrated at a motile sperm concentration of 5×10^6/ml.

Acrosome reaction

For certain of the above experiments a sample (200 μl) of the sperm suspension was removed at the end of the 3 hour incubation period in order to assess the acrosome reaction. For this procedure the spermatozoa were pelleted by centrifugation at 500 ***g*** for 5 minutes and resuspended in 200 μl of fresh BWW, prior to the assessment of sperm motility and the acrosome reaction. The protocol developed for assessing the acrosome reaction involved the use of a detection reagent targeting the outer acrosomal membrane (*Arachis hypogaea* lectin), in conjunction with the hypoosmotic swelling test (Jeyendran et al., 1984; Aitken et al., 1993a) to monitor sperm viability. For this procedure, 50 μl of this sperm suspension was added to 500 μl of hypoosmotic swelling medium, comprising 7.35 g sodium citrate and 13.51 g fructose in 1 l of distilled water (Jeyendran et al., 1984), and incubated for 1 hour at 37°C. At the end of this period the spermatozoa were pelleted by centrifugation at 500 ***g*** for 5 minutes and resuspended in 50 μl of ice cold methanol. A 10 μl sample of the fixed cells were subsequently pipetted onto the well of a 4-spot Henley slide (C. A. Henley Ltd, Loughton, England) and allowed to dry. The wells were then overlaid with fluorescein-conjugated peanut lectin (*Arachis hypogaea*) at a concentration of 2 mg/ml and incubated for 15 minutes in the dark (Mortimer et al., 1987). The excess lectin was then gently removed by washing with Dulbecco's phosphate buffered saline and

the slides visualized under a fluorescence microscope in the presence of an anti-quenching agent. As a consequence of exposing the spermatozoa to the hypoosmotic swelling medium, viable cells developed a characteristic coiling of the flagellum. In order to exclude moribund or dead spermatozoa (which may have lost their acrosomes for pathological reasons) from the analysis, only viable cells with coiled tails were scored for acrosomal status (Aitken et al., 1993a). The spermatozoa were classified as non-acrosome-reacted, if the acrosomal region of the sperm head exhibited a uniform, bright fluorescence and the sperm tails adopted the coiled configuration typical of viable cells. However, if the acrosomal region of such viable cells exhibited a punctate labelling pattern or restriction of the fluorescence to the equatorial segment of the sperm head, then they were classified as undergoing the acrosome reaction.

Solubilization of sperm

The spermatozoa were incubated in BWW medium containing the compound(s) under investigation in a total volume of 200 μl. After a 3 hour incubation at 37°C in a 5% CO_2, 95% air atmosphere, the spermatozoa were centrifuged (500 ***g***, 5 minutes) and washed with 3× 1 ml 0.0625 M Tris-HCl, pH 6.8. The spermatozoa were then solubilized in 14 μl SDS-solubilization buffer (0.187 M Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 1 mM vanadate, 1 mM PMSF, 0.02 i.u. aprotinin) for 45 minutes at 25°C with occasional vortexing. The sperm extract was then centrifuged (7,500 ***g***, 20 minutes) and the supernatent added to 14 μl SDS-PAGE buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% 2-mercaptoethanol) and heated to 100°C for 5 minutes. The samples were then loaded onto 5% polyacrylamide gels and electrophoresed. For each lane of the electrophoresis gel, the equivalent of 1.5×10^6 spermatozoa was applied.

Western blotting

After SDS-PAGE the proteins were transferred onto nitrocellulose and then blocked for 1 hour at 25°C with 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.6 (TBS), containing 5% milk protein, to prevent any non-specific binding. The nitrocellulose was then incubated overnight at 4°C in a 1/1,000 dilution of a monoclonal anti-phosphotyrosine antibody in TBS containing 5% milk protein and 0.05% Tween-20. After washing briefly with water, the nitrocellulose was washed 4× with TBS containing 0.05% Tween-20 before incubating in a 1/6,000 dilution of anti-mouse horseradish peroxidase (Amersham, UK) in TBS containing 5% milk protein, 0.05% Tween-20 for 1 hour at 25°C. The nitrocellulose was washed again as described above, then developed using an enhanced chemiluminesence technique (Amersham, UK).

Reactive oxygen species

Superoxide ($O_2^{-}\cdot$) generation in response to NADPH administration was detected by lucigenin-dependent chemiluminescence as described by Aitken et al. (1992) using a Berthold LB 9505 luminometer (Wildbad, Germany). This analysis was conducted on samples that had been purified on Percoll gradients and subjected to an FMLP (formylmethionyl-leucyl-phenylalanine) provocation test in order to ensure that they were free of leucocyte contamination (Krausz et al., 1992). H_2O_2 production in response to A23187 treatment was monitored by chemiluminescence using luminol (25 μM) and horseradish peroxidase (12.4 U) as the probe (Aitken et al., 1992) in leucocyte-free samples that did not respond to FMLP stimulation (Krausz et al., 1992).

Statistics

All experiments were replicated at least 3 times and the statistical significance of any differences observed determined by analysis of variance using the Statview 2 programme (Abacus Concepts Inc., Berkeley, CA). Post hoc testing of differences between group means was accomplished using Fisher's Protected Least Significant Difference (PLSD) with the significance level set at $P<0.05$.

RESULTS

Redox regulation of sperm function

Exposure of human spermatozoa to the divalent cation ionophore, A23187, induced these cells to undergo an exocytotic event, the acrosome reaction, and concomitantly acquire the capacity to fuse with the vitelline membrane of the oocyte (Fig. 1). At the same time, A23187 stimulated human spermatozoa to exhibit a burst of H_2O_2 generation (Aitken and Clarkson, 1987a,b), which appeared to play a key role in mediating the influence of the ionophore on sperm function. This conclusion was based on the fact that treatment of human

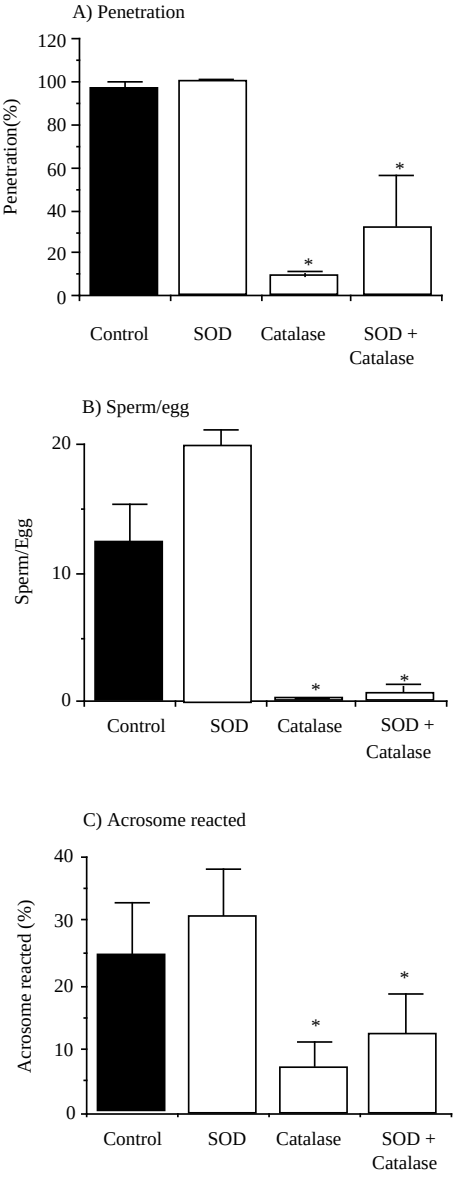

Fig. 1. Influence of superoxide dismutase (SOD) and catalase on the biological responses of human spermatozoa to stimulation with A23187. Data presented for (A) percentage of oocytes penetrated, $n=3$; (B) mean number of spermatozoa fusing with each oocyte, $n=3$; and (C) percentage of viable spermatozoa that had undergone the acrosome reaction, $n=8$. Filled bars, control incubations with A23187 alone; open bars, treatment. *$P<0.05$. Error bars, s.e.m.

Table 1. Influence of membrane permeant and impermeant thiols on the capacity of human spermatozoa for oocyte fusion following activation with A23187

Reagent	Sperm-oocyte fusion (%)	Motility (%)
Control	62.66±25.07	75.0±7.8
DTT (1 mM)	0.0±0.0*	42.3±6.7*
DTT (0.1 mM)	16.3±8.2*	69.3±5.2
2ME (0.1%)	23.0±5.0*	65.7±14.3
2ME (0.05%)	23.0±3.0*	74.7±9.2
GSH (1 mM)	51.3±18.5	68.7±10.1

2ME, 2-mercaptoethanol; GSH, reduced glutathione.
*P<0.05.

spermatozoa with catalase, alone or in the presence of SOD, led to a dramatic and statistically significant decline in their functional response to ionophore (Fig. 1), in terms of both the acrosome reaction and the level of sperm-oocyte fusion (P<0.001 for the overall treatment effect). This inhibition of sperm function was achieved without any detectable decline in the motility or viability of the spermatozoa: indeed, in the presence of catalase alone, the viability of the cells was even significantly improved (P<0.05). In contrast, the addition of exogenous SOD, which converts $O_2^{-\cdot}$ to H_2O_2, induced a slight increase in the capacity of human spermatozoa to undergo the acrosome reaction and fuse with the oocyte (Fig. 1B,C) while the viability and motility of these cells again remained unchanged.

The suppressive effects of catalase on the response of human spermatozoa to A23187 suggest that H_2O_2 is involved in the cascade of biochemical changes that transform the calcium and pH signals generated by the ionophore into a biological response. In order to explore further the influence of redox conditions on the ability of human spermatozoa to respond to ionophore stimulation, the ability of membrane permeant (dithiothreitol, 2-mercaptoethanol) and membrane impermeant (reduced glutathione) reducing agents to modulate the biological responses to A23187 was investigated. Treatment with dithiothreitol and 2-mercaptoethanol resulted in a dramatic decline in the capacity of human spermatozoa to acrosome react and fuse with the vitelline membrane of the oocyte (Table 1) following ionophore challenge. The site(s) of action of these thiol compounds was presumably intracellular, since high concentrations of a membrane impermeant compound such as glutathione (1 mM) had no significant effect on the response to

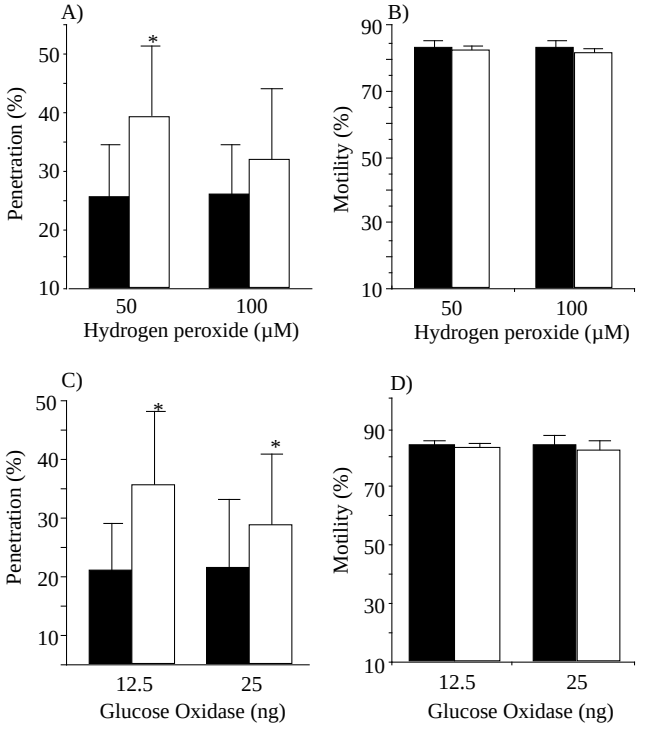

Fig. 2. Influence of oxidizing conditions on the fertilizing potential of human spermatozoa. (A) H_2O_2 significantly enhances the capacity of human spermatozoa for sperm-oocyte fusion without influencing motility (B), n=6. (C) Generation of H_2O_2 exogenously with glucose oxidase is also associated with a significant increase in the rates of sperm-oocyte fusion without influencing motility (D), n=8. Filled bars, control incubations; open bars, treatment. *P<0.05. Error bars, s.e.m.

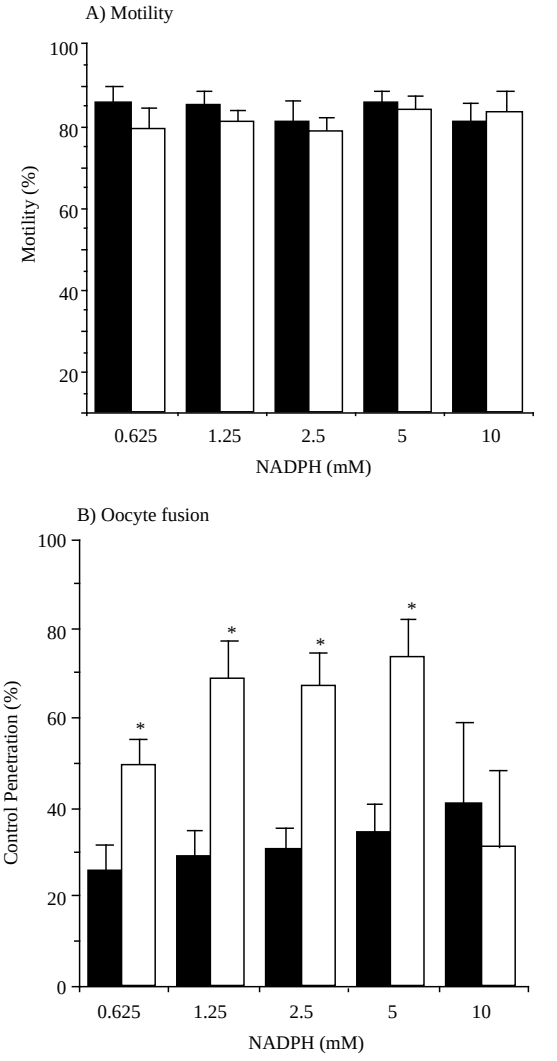

Fig. 3. (A) Over a 3 hour time course, the stimulation of $O_2^{-\cdot}$ generation with NADPH had no effect on sperm motility but (B) significantly enhanced sperm-oocyte fusion, n=7-10. Filled bars, control incubations, open bars, treatment. *P<0.01. Error bars, s.e.m.

A23187 (Table 1). The inhibitory action of the membrane permeant reducing agents was not secondary to a change in the viability or motility of the spermatozoa. Simultaneous assessments of sperm motility only revealed a slight inhibitory effect for the highest concentration of dithiothreitol employed (Table 1), while measurements of sperm viability failed to reveal any detectable changes in this parameter (data not shown). Moreover, it should be emphasized that the sperm-oocyte fusion results presented in Table 1 are expressed for a constant concentration of motile spermatozoa (5×10^6/ml).

In order to determine whether a change in the redox status of human spermatozoa could, of itself, lead to cellular activation, a variety of different strategies were employed to generate oxidizing conditions for these cells. Direct exposure of human spermatozoa to 50 μM H_2O_2 induced a significant rise ($P<0.05$) in the capacity of human spermatozoa to exhibit sperm-oocyte fusion (Fig. 2A) without significantly inhibiting sperm movement. (Fig. 2B) or viability (data not shown). As an alternative strategy for creating an oxidizing environment, the glucose oxidase system was used to generate H_2O_2 enzymatically. Addition of 12.5 or 25.0 ng/ml glucose oxidase again significantly ($P<0.05$) stimulated sperm-oocyte fusion (Fig. 2C) without any change in the motility (Fig. 2D) or the viability of these cells (data not shown).

Stimulation of superoxide generation with NADPH

The above experiments demonstrated that exposure of human spermatozoa to exogenous H_2O_2 stimulated these cells to acrosome react and fuse with the vitelline membrane of the oocyte. In order to determine whether the induction of *endogenous* ROS production could also enhance human sperm function, experiments were conducted involving the addition of NADPH to the culture medium. This cofactor is the substrate for ROS generation by human spermatozoa (Aitken and Clarkson, 1987a,b) and we have recently found that $O_2^{-\cdot}$ generation by these cells can be readily stimulated by the addition of exogenous NADPH to the culture medium (data not shown). In response to the presence of NADPH, a dose-dependent generation of $O_2^{-\cdot}$ was induced which could be detected by lucigenin-dependent chemiluminescence and readily scavenged by the addition of the $O_2^{-\cdot}$ scavenger, SOD. The stimulation of $O_2^{-\cdot}$ generation by such means had no effect on sperm movement (Fig. 3A) but significantly elevated the capacity of the spermatozoa for sperm-oocyte fusion over the dose range 0.625-5 mM ($P<0.01$; Fig. 3B).

Tyrosine phosphorylation

In view of the importance of tyrosine kinase activity in the control of sperm function (Leyton and Saling, 1989a,b; Leyton et al., 1992) and the importance of redox mechanisms in regulating the phosphorylation of tyrosine kinases (Koshio et al., 1988; Bauskin et al., 1991), it was of interest to determine whether the functional changes induced by altering the redox status of human spermatozoa were associated with changes in the pattern of tyrosine phosphorylation.

Western blot analyses with a monoclonal anti-phosphotyrosine antibody revealed that in human spermatozoa cultured for 3 hours in a simple defined culture medium, a single, major phosphotyrosine species could be observed with a molecular mass of 116 kDa. A diffuse band of immunoreactive material could frequently be observed migrating in advance of this protein with a molecular mass of 107 kDa, as well as a weak band at 82 kDa (Fig. 4). The stimulation of sperm function observed when human spermatozoa were exposed to oxidizing conditions, was associated with a dramatic enhancement in the number and intensity of phosphotyrosyl proteins. Regardless of whether the changed redox status of the cells was induced by exogenous oxidants (H_2O_2, glucose oxidase) or endogenous ROS generation (NADPH), a similar pattern of enhanced tyrosine phosphorylation was observed which, in addition to the major band of 116 kDa, involved the expression of a large number of additional phophotyrosyl proteins, the major species exhibiting molecular masses of 222 kDa, 200 kDa, 159 kDa, 133 kDa and 82 kDa, while minor bands were present at 172 kDa, 144 kDa, 125 kDa and 107 kDa (Fig. 4).

The increase in tyrosine phophorylation induced by NADPH was observed over a range of concentrations (1.25-5.0 mM) that had been found to increase the capacity of human spermatozoa for sperm-oocyte fusion. Higher doses of NADPH (10 mM) markedly enhanced the pattern of tyrosine phophorylation (Fig. 4) but did not enhance fertilization rates (Fig. 3). The reason for this discrepancy is that the very high rates of ROS

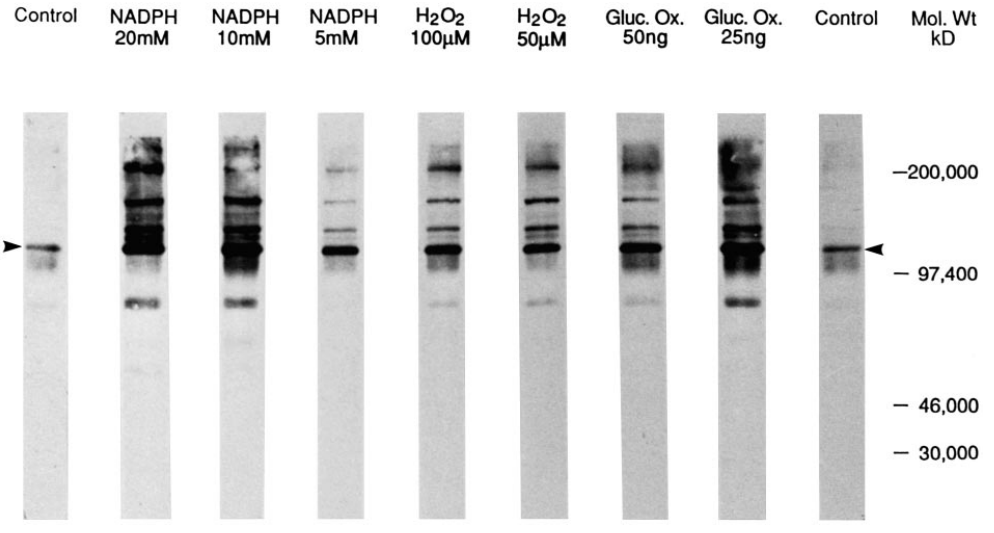

Fig. 4. Phosphotyrosine expression by human spermatozoa according to western blot analysis using a monoclonal antibody. A single major species of phosphotyrosyl protein was observed in unstimulated, control cells of 116 kDa (arrowheads). Following the creation of oxidizing conditions through the stimulation of endogenous reactive oxygen species generation by NADPH, the addition of exogenous H_2O_2 or the synthesis of exogenous H_2O_2 with glucose oxidase (Gluc. Ox.), a marked increase in tyrosine phosphorylation is observed involving major protein species of 222 kDa, 200 kDa, 159 kDa, 133 kDa and 82 kDa.

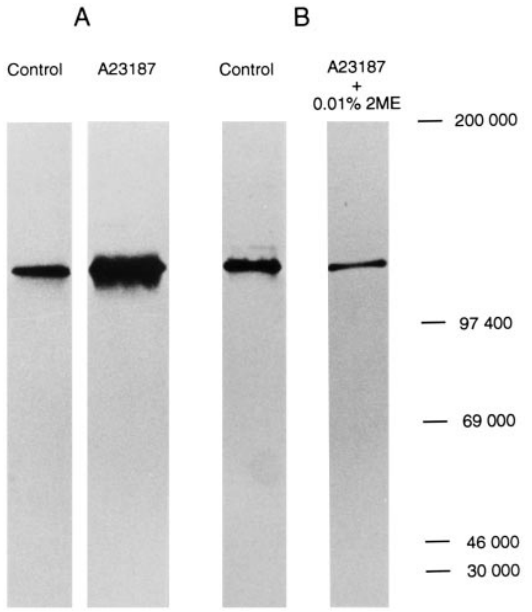

Fig. 5. Influence of membrane-permeant thiols on the pattern of tyrosine phosphorylation observed in response to A23187. (A) The stimulation of sperm function with A23187 was associated with a marked enhancement in tyrosine phosphorylation, particularly with respect to the 116 kDa band. (B) Inhibition of the functional response to A23187 with 2-mercaptoethanol (2ME) or dithiothreitol (not shown) was associated with a corresponding decline in the level of tyrosine phosphorylation.

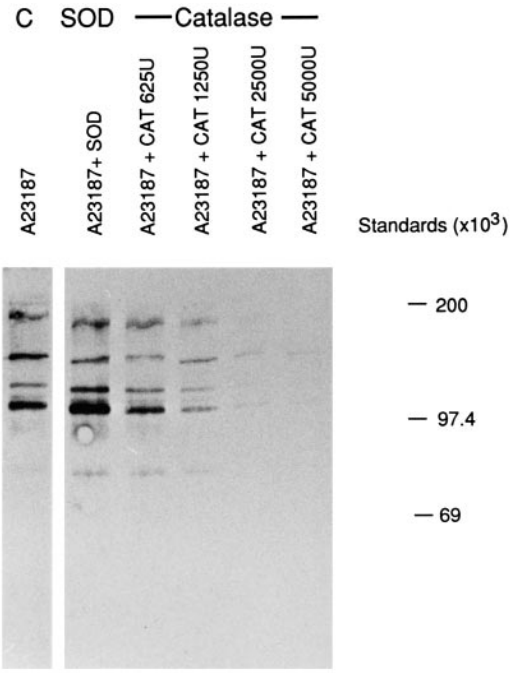

Fig. 6. Influence of catalase (CAT) and superoxide dismutase (SOD) on the patterns of tyrosine phosphorylation observed in response to A23187. Addition of SOD enhanced the expression of phosphotyrosine residues while catalase exhibited a dose-dependent capacity to inhibit this activity.

generation observed with 10 mM or 20 mM NADPH induced peroxidative damage in the sperm plasma membrane, as measured in a malondialdehyde assay (data not shown). As a consequence of this damage the capacity of the spermatozoa for oocyte fusion was reduced (Aitken et al., 1993b).

In contrast to the enhancing effect of oxidizing conditions on tyrosine phsphorylation and sperm function, reducing conditions had the opposite effect. Thus, the stimulatory effect of A23187 on tyrosine phophorylation could be impaired by the concomitant presence of a reducing agent such as 2-mercaptoethanol (Fig. 5) just as the biological responses of human spermatozoa to A23187 had been impaired by this reagent (Table 1). Even more dramatically, the suppressive effects of catalase on the acrosome reaction and sperm-oocyte fusion was associated with a marked dose-dependent decline in the level of phosphotyrosine expression induced by ionophore treatment (Fig. 6). Conversely, the slight enhancement of sperm function observed following the exposure of human spermatozoa to SOD in presence of A23187 (Fig. 1) was associated with a corresponding enhancement in the intensity of tyrosine phosphorylation within these cells (Fig. 6). That such redox mechanisms are involved in the stimulation of human spermatozoa by more biologically relevant agonists was indicated by the ability of catalase to impair the functional responses of human spermatozoa to both progesterone and recombinant ZP3, in the absence of any concomitant change in motility (Fig. 7).

One of the mechanisms by which H_2O_2 generation might enhance tyrosine phosphorylation in human spermatozoa is through the inhibition of tyrosine phosphatase activity (Hecht and Zick, 1992). It was therefore of interest to compare the patterns of phosphotyrosine proteins expressed following exposure to H_2O_2 with the pattern observed after treatment of human spermatozoa with sodium orthovanadate, a known inhibitor of protein tyrosine phosphatases (Grinstein et al., 1990). This experiment revealed a dramatic enhancement in the overall level of tyrosine phosphorylation observed with both vanadate and H_2O_2, with no detectable difference in the spectrum of proteins phosphorylated under these conditions (Fig. 8).

DISCUSSION

The human spermatozoon is characterized by a capacity to generate O_2^- and H_2O_2 using NADPH as the electron donor (Aitken and Clarkson, 1987a,b). Such NADPH oxidase activity is normally associated with phagocytic cells such as neutrophils and macrophages, although there is now a growing list of additional cell types in which this property has been detected (for review see Cross and Jones, 1991). In addition to spermatozoa, ROS generation has been recorded for Epstein-Barr transformed human B lymphocytes (Volkman et al., 1984), fibroblasts (Meier et al., 1989), endothelial cells (Ooi et al., 1983), mesangial cells (Shah and Naum Bedigian, 1981; Basci and Shah, 1987), Leydig cells (Sandhu et al., 1989), thyroid cells (Taurog, 1970; Deme et al., 1985; Dupuy et al., 1986), platelets (Finazzi-Agro et al., 1982), the carotid body (Acker et al., 1989), tumour cells (Borst, 1962) and adipocytes (Mukherjee et al., 1978). In several of these cell types, NA(D)PH oxidase-like activity has been implicated, including

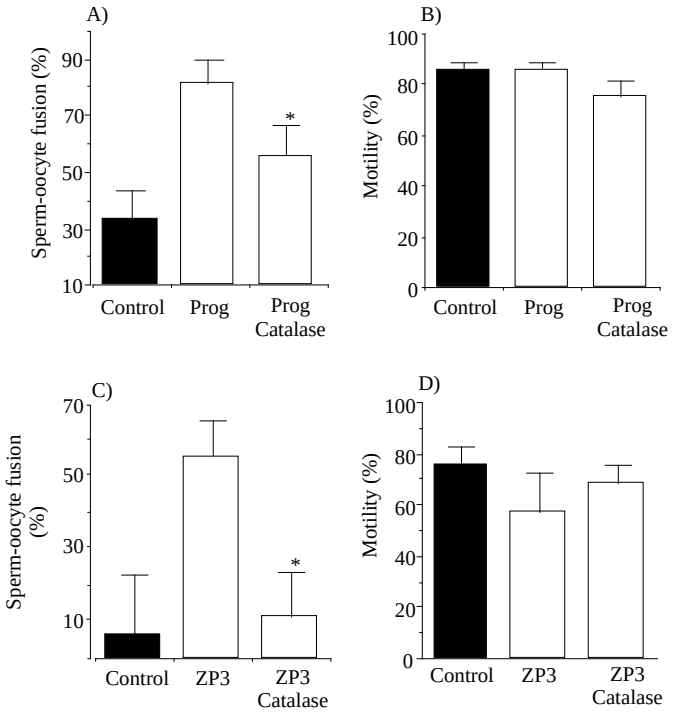

Fig. 7. Influence of catalase on the motility of human spermatozoa and their capacity for sperm-oocyte fusion in response to progesterone (5 μM) and recombinant ZP3 (50 ng/ml). (A) Sperm-oocyte fusion; and (B) motility following exposure to progesterone ± catalase for 3 hours (n=7). (C) Sperm-oocyte fusion; and (D) motility following exposure to ZP3 ± catalase for 3 hours (n=3). Filled bars, control incubations; open bars, treatment. *P<0.05. Error bars, s.e.m.

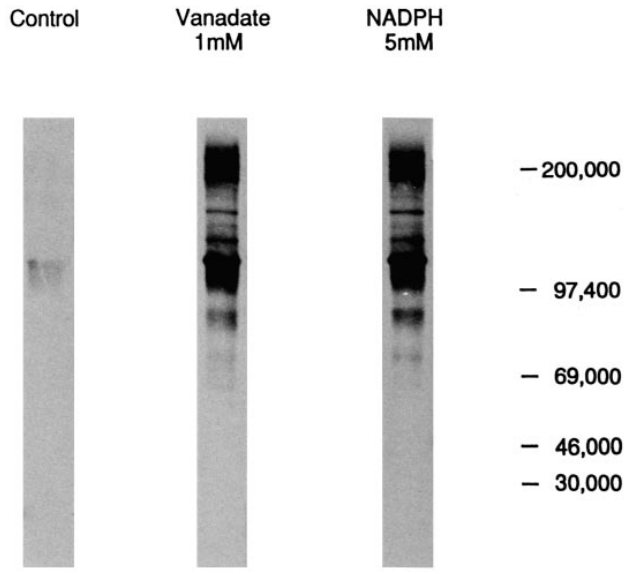

Fig. 8. Comparison of the patterns of tyrosine phosphorylation observed with vanadate and NADPH. The suppression of tyrosine phosphatase activity with vanadate induced an enhancement of tyrosine phosphorylation that was quantitatively and qualitatively identical to that observed following the stimulation of endogenous ROS generation with NADPH.

adipocytes, tumour cells, lymphocytes, thyroid cells and spermatozoa (Cross and Jones, 1991). Despite the widespread nature of such specialized oxidase systems, there is currently no consensus as to their biological purpose. A general role in cellular signalling has been proposed and would be in keeping with the small size, ubiquitous distribution and short half-life of these molecules. Roles in the induction of cell proliferation, the regulation of intracellular pH and the antagonism of nitric oxide have been suggested by various authors (Cross and Jones, 1991). The results obtained in this study clearly suggest that, in the human spermatozoon, the stimulation of ROS generation is associated with the enhancement of tyrosine phosphorylation and that this activity is an essential component of the cascade of biochemical changes that lead to acrosomal exocytosis and fusion with the oocyte.

In the resting spermatozoon only one major species of phosphotyrosyl protein was detected with a molecular mass of 116 kDa. Recent data (Kalab et al., 1994) have been published indicating that the major phophotyrosine protein in murine spermatozoa is a hexokinase-like molecule that exhibits apparent molecular masses of 116 kDa and 95 kDa under reducing conditions and non reducing conditions, respectively. The 116 kDa phosphotyrosine protein in human spermatozoa does not change its electrophoretic mobility under non reducing conditions (data not shown), suggesting a difference from the hexokinase molecule, as discussed by Kalab et al. (1994), although western blot studies will be needed to confirm this point. The presence and molecular mass of the 116 kDa phosphoprotein species was confirmed on many independent samples and contrasts with the report by Naz et al. (1991) suggesting that human spermatozoa possess two major phosphotyrosyl proteins of 94 kDa and 46 kDa. The reason for this discrepancy is unclear but it may reflect differences in extraction conditions, proteolytic cleavage, the physiological status of the sperm suspensions or the resolving power of the electrophoretic analysis. The 5% gels used in this study were clearly able to resolve a number of high molecular mass components (100-200 kDa) that would have been difficult to distinguish on the 12.5% gels employed by Naz et al. (1991).

The pattern and intensity of tyrosine phosphorylation could be dramatically altered by exposing the spermatozoa to oxidizing conditions. These conditions could be created by the addition or generation of exogenous H_2O_2 or by the stimulation of endogenous ROS production by NADPH or A23187. The addition of exogenous NADPH was particularly effective in stimulating high levels of tyrosine phosphorylation in multiple high molecular mass species (Fig. 4). Qualitatively similar patterns of tyrosine phosphorylation could be induced by exogenous H_2O_2, suggesting that this particular oxidant was responsible for the stimulatory effect. This conclusion was reinforced by the finding that the treatment of A23187-activated human spermatozoa with catalase could remove all detectable signs of tyrosine phosphorylation from these cells after 3 hours, while exposure to SOD produced the opposite effect (Fig. 6). A role for H_2O_2 in the control of tyrosine phosphorylation is also supported by recent results obtained with human neutrophils, indicating that tyrosine phosphorylation in these cells could be enhanced by exogenous H_2O_2, providing 3-aminotriazole was present to suppress catalase activity (Fialkow et al., 1993). The paucity of catalase in human spermatozoa (Jeulin et al., 1989) explains the lack of a need for aminotriazole in the experiments reported here.

The mechanism by which H_2O_2 leads to phosphotyrosine accumulation could conceivably involve the stimulation of tyrosine kinase activity, the inhibition of tyrosine phosphatase activity or a combination of both of these effects. The similarity in the tyrosine phosphorylation pattern observed under oxidizing conditions with that generated in the presence of a known tyrosine phosphatase inhibitor, vanadate, suggests that the inhibition of tyrosine phosphatase activity may be an important component of the mechanism of action. This concept is also supported by previous reports indicating that H_2O_2 is an extremely effective inhibitor of tyrosine phosphatases (Hecht and Zick, 1992). This effect is due to the fact that this class of enzyme is critically dependent upon the reduced status of the thiol group on a highly conserved cysteine residue (Cys215 in PTP 1B), which can be directly oxidized by H_2O_2, leading to a loss of phosphatase activity. The reduction in tyrosine phosphorylation observed with intact spermatozoa following exposure to membrane permeant thiols, or the scavenging of H_2O_2 with catalase, is also consistent with the proposal that reducing conditions favour the activity of protein tyrosine phosphatases in these cells.

In addition to the suppression of phosphatase activity, evidence has also been obtained in lymphocytes for the oxidant-mediated activation of tyrosine kinases such as $p72^{syk}$ and Ltk (Bauskin et al., 1991; Schieven et al., 1993). It is well recognized that the activity of receptor tyrosine kinases can be stimulated by an oligomerization process involving the cross linking activity of cellular or proteinaceous ligands. It is also possible that receptor oligomerization could be induced chemically, by the creation of disulphide bridges across the cysteine-rich extracellular domains of adjacent receptors, and that such cross linking activity is induced, or facilitated by, the generation of H_2O_2. Such a mechanism has been proposed for the activation of Ltk (Bauskin et al., 1991) and may apply in the case of human spermatozoa. Evidence has already been obtained indicating that mammalian spermatozoa can be activated by the cross linking of a receptor tyrosine kinase (p95) on the sperm surface, by the zona pellucida glycoprotein, ZP3 (Leyton and Saling, 1989b). The concomitant generation of H_2O_2 by the sperm NADPH oxidase may facilitate this oligomerization process, or represent an alternative route by which the cross linking of receptor tyrosine kinases on the sperm surface can be achieved, in order to activate the spermatozoa.

Regardless of the cellular mechanisms involved, the ROS-induced enhancement of phosphotyrosine expression appears to play a key role in regulating the biological responses of human spermatozoa. Thus, the enhanced expression of phosphotyrosyl proteins observed following the creation of oxidizing conditions with NADPH or H_2O_2 was invariably associated with increased rates of sperm-oocyte fusion without any change in sperm motility or viability. A causative role for H_2O_2 was suggested by the inhibitory effect of catalase on the high levels of phosphotyrosine expression and sperm-oocyte fusion, induced by A23187. The general importance of this peroxide-mediated mechanism for the activation of human spermatozoa was indicated by the suppressive effect of catalase on the responses of human spermatozoa to biological stimulants such as progesterone or recombinant ZP3. Similarly, hamster spermatozoa have been shown to activate in response to H_2O_2, while both spontaneous and lysophosphatidyl choline-induced acrosome reactions can be inhibited by catalase in this species (Bize et al., 1991). Although catalase is membrane impermeant its effectiveness in independent studies suggests that either H_2O_2 must be generated on the outer surface of the sperm plasma membrane or, more likely, the constant scavenging of H_2O_2 from the ambient medium facilitates its outward diffusion from the intracellular space and thence a lowering of its intracellular concentration.

In conclusion, the results obtained in this study indicate that the stimulation of ROS generation by human spermatozoa is associated with the enhanced expression of phophotyrosyl proteins and that this activity appears to be an essential component in the cascade of intracellular changes that lead to the activation of the human spermatozoon. A similar second messenger role has been suggested for the NADPH oxidase of neutrophils (Fialkow et al., 1993) and may also apply to the wide range of nonphagocytic cell types expressing a capacity for ROS generation.

REFERENCES

Acker, H., Dufau, E. Huber, J. and Sylvester, D. (1989). Indications of an NADPH oxidase as a possible pO_2 sensor in the rat carotid body. *FEBS. Lett.* **256**, 75-78.

Aitken, R. J. and Clarkson, J. S. (1987a). Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fertil.* **81**, 459-469.

Aitken, R. J. and Clarkson, J. S. (1987b). Generation of reactive oxygen species by human spermatozoa. In *Free Radicals: Recent Developments in Lipid Chemistry, Experimental Pathology and Medicine* (ed. T. Dormandy and C. Rice-Evans), pp. 333-335. Richelieu Press, London.

Aitken, R, J., Buckingham D. W. and West K. M. (1992). Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. *J. Cell. Physiol.* **151**, 466-477.

Aitken, R. J., Buckingham, D. W. and Huang, G.-F. (1993a). Analysis of the responses of human spermatozoa to A23187 employing a novel technique to monitor the acrosome reaction. *J. Androl.* **14**, 132-141.

Aitken, R. J., Harkiss, D. and Buckingham, D. (1993b). Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *J. Reprod. Fertil.* **31**, 531-537.

Basci, A. and Shah, S. V. (1987). Trypsin and chymotrypsin induced chemiluminescence by isolated rat glomeruli. *Am. J. Physiol.* **252**, C611-C617.

Bauskin, A. R., Alkalay, I. and Ben-Neriah, Y. (1991). Redox regulation of a tyrosine kinase in the endoplasmic reticulum. *Cell* **56**, 685-696.

Biggers, J. D., Whitten, W. K., Whittingham, D. G. (1971). The culture of mouse embryos in vitro. In *Methods in Mammalian Embryology* (ed. J. C. Daniel, Jr), pp. 86-116. Freeman, San Francisco, CA.

Bize, I., Santander, G., Cabello, P., Driscoll, D. and Sharpe, C. (1991). Hydrogen peroxide is involved in hamster sperm capacitation in vitro. *Biol. Reprod.* **44,** 398-403.

Borst, P. (1962). The aerobic oxidation of the reduced diphosphopyridine nucleotide formed by glycolysis in Ehrlich ascites tumour cells. *Biochim. Biophys. Acta* **57**, 270-282.

Bunch, D., Leyton, L. and Saling, P. (1992). Sperm interaction with the zona pellucida: role of a tyrosine kinase receptor for ZP3 in fertilization. In *Spermatogenesis, Fertilization and Contraception,* Schering Foundation Workshop 4 (ed. E. Nieschlag and U. -F. Habenicht), pp. 367-381. Springer-Verlag, Berlin.

Cross, A. R. and Jones, O. T. G. (1991). Enzymic mechanisms of superoxide production. *Biochim. Biophys. Acta* **1057,** 281-298.

Deme, C., Virion, A., Ait Hammou, N. A. and Pommier, J. (1985). NADPH-dependent generation of H_2O_2 in a thyroid particulate fraction requires Ca^{2+}. *FEBS Lett.* **186,** 107-110.

Dupuy, C., Virion, A., Ait Hammou, N. A., Kaniewski, J., Deme, D. and Pommier, J. (1986). Solubilization and characteristics of the thyroid

NADPH-dependent H_2O_2 generating system. *Biochem. Biophys. Res. Commun.* **141**, 839-846.

Fialkow, L., Chan, C. K., Grinstein, S. and Downey, G. P. (1993). Regulation of tyrosine phosphorylation in neutrophils by the NADPH oxidase. *J. Biol. Chem.* **268**, 17131-17137.

Finazzi-Agro, A., Menichelli, A., Persiani, M., Biancini, G. and Del Principe, D. (1982). Hydrogen peroxide release from human platelets. *Biochim. Biophys. Acta* **718**, 21-25.

Grinstein, S., Furuya, W., Lu, D. J. and Mills, G. B. (1990). Vanadate stimulates oxygen consumption and tyrosine phosphorylation in electropermeabilized human neutrophils. *J. Biol. Chem.* **265**, 318-327.

Hecht, D. and Zick, Y. (1992). Selective inhibition of protein tyrosine phosphatase activities by H_2O_2 and vanadate in vitro. *Biochem. Biophys. Res. Commun.* **188**, 773-779.

Jeulin, C., Soufir, J. C., Weber, P., Laval-Martin, D. and Calvayrac, R. (1989). Catalase activity in human spermatozoa and seminal plasma. *Gamete Res*. **24**, 185-96.

Jeyendran, R. S., Van der Ven, H. H., Perez-Palaez, M., Crabo, B. G. and Zaneveld, L. J. D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics, *J. Reprod. Fertil.* **70**, 219-228.

Kalab, P., Visconti, P., Leclerc, P. and Kopf, G. S. (1994). p95, the major phophotyrosine-containing protein inmouse spermatozoa, ia a hexakinase with unique properties. *J. Biol. Chem.* **269**, 3810-3817.

Koshio, O., Akanuma, Y. and Kasuga, M. (1988). Hydrogen peroxide stimulates tyrosine phosphorylation of the insulin receptor and its tyrosine kinase activity in intact cells. *Biochem. J.* **250**, 95-101.

Krausz, C., West, K., Buckingham, D. and Aitken, R. J. (1992). Analysis of the interaction between N-formylmethionyl-leucyl-phenylalanine and human sperm suspensions: development of a technique for monitoring the contamination of human semen samples with leucocytes. *Fertil. Steril.* **57**, 1317-1325.

Lessley, B. A. and Garner, D. L. (1983). Isolation of motile spermatozoa by density gradient centrifugation in Percoll. *Gamete Res*. **7**, 49-61.

Leyton, L. and Saling, P. M. (1989a). Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. *J. Cell Biol*. **108**, 2163-2168.

Leyton, L. and Saling, P. M. (1989b). 95kD sperm proteins bind ZP3 and serve as tyrosine kinase substrates in response to zona binding. *Cell* **57**, 1123-1130.

Leyton, L., LeGuen, P., Bunch, D. and Saling, P. M. (1992). Regulation of mouse gamete interaction by a sperm tyrosine kinase. *Proc. Nat. Acad. Sci. USA* **89,** 11692-11695.

Meier, B., Radecke, H. H., Selle, S., Younes, M., Sies, H., Resch, K. and Habermehl, G. G. (1989). Human fibroblasts release reactive oxygen species in response to interleukin 1 or tumour necrosis factor-α. *Biochem. J.* **263**, 539-545.

Mortimer, D., Curtis, E. F. and Miller, R. G. (1987). Specific labelling by sperm agglutinin of the outer acrosomal membrane of the human spermatozoon *J. Reprod. Fertil*. **81**, 127-135.

Mukherjee, S. P., Lane, R. H. and Lynn, W. S. (1978). Endogenous hydrogen peroxide and peroxidative metabolism in adipocytes in response to insulin and sulfhydryl reagents. *Biochem. Pharmacol.* **27**, 2589-2594.

Naz, R. K., Ahmed, K. and Kumar, R. (1991). Role of membrane tyrosine proteins in human spermatozoal function. *J. Cell Sci*. **99**, 157-165.

Ooi, B. S., MacCarthy, F. P., Hsu, A. and Ooi, Y. M. (1983). Human mononuclear cell modulation of endothelial cell proliferation. *J. Lab. Clin. Med.* **102**, 428-433.

O'Shea, J. J., McVicar, D. W., Bailey, T. L., Burns, C. and Smyth, M. J. (1992). Activation of human peripheral blood T lymphocytes by pharmacological induction of protein-tyrosine phosphorylation. *Proc. Nat. Acad. Sci. USA* **89**, 10306-10310.

Sandhu, S. S., Band, A., Abavasekera, D. R. E., Rice-Evans, C. F. and Cooke, B. A. (1989). A novel superoxide generating system. *Biochem. Soc. Trans.* **17**, 710-711.

Schieven, G. L., Kirihara, J. M., Burg, D. L., Geahlen, R. L. and Ledbetter, J. A. (1993). $p72^{syk}$ tyrosine kinase is activated by oxidizing conditions that induce lymphocyte tyrosine phophorylation and Ca^{2+} signals. *J. Biol. Chem.* **268**, 16688-16692.

Shah, S. V. and Naum Bedigian, S (1981). Light emission by isolated rat glomeruli in response to phorbol myristate acetate. *J. Lab. Clin. Med.* **98**, 46-57.

Taurog, A. (1970). Thyroid peroxidase and thyroxine biosynthesis. *Recent Prog. Horm. Res.* **26**, 189-247.

Tesarik, J., Moos, J. and Mendoza, C. (1993). Stimulation of protein tyrosine phophorylation by a progesterone receptor on the surface of human sperm. *Endocrinology* **133**, 328-335.

van Duin, M., Polman, J. E. M., de Breet, I. T. M., van Ginneken, K., Bunschoten, H., Grootenhuis, A., Brindle, J. and Aitken, R. J. (1994). Recombinant human zona pellucida protein ZP3 produced in Chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion. *Biol. Reprod.* **51**, 607-617.

Volkman, D. J., Buescher, E. S., Gallin, J. I. and Fauci, A. S. (1984). B cell lines as models for inherited phagocytic diseases: abnormal superoxide generation in chronic granulomatous disease and giant granules in Chadiak-Higashi syndrome. *J. Immunol.* **133**, 3006-3009.

Wassarman, P. M. (1990). Profile of a mammalian sperm receptor. *Development* **108**, 1-17.

World Health Organization (1992). *WHO Laboratory Manual For The Examination of Human Semen and Semen-Cervical Mucus Interaction.* Cambridge University Press, Cambridge.

Yanagimachi, R., Yanagimachi, H. and Rogers, B. J. (1976). The use of zona free animal ova as a test system for the assessment of the fertilizing capacity of human spermatozoa. *Biol. Reprod.* **15,** 471-476.

(Received 5 October 1994 - Accepted 14 February 1995)