Evidence for the involvement of PECAM-1 in a receptor mediated signal-transduction pathway regulating capacitation-associated tyrosine phosphorylation in human spermatozoa

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Summary
Mammalian spermatozoa must become ‘capacitated’ in the female reproductive tract before they gain the ability to fertilize the oocyte. The attainment of a capacitated state has been correlated with a number of biochemical changes, the most notable of which is a dramatic increase in the tyrosine phosphorylation status of these cells. Despite its biological importance, the mechanisms responsible for initiating this tyrosine phosphorylation cascade in vivo are unknown. Here, we report that this signalling pathway can be elicited in a rapid, dose-dependent and lectin-specific manner by wheat germ agglutinin (WGA), but none of 18 other lectins assessed. This response was abrogated by prior enzymatic cleavage of either sialic acid or GlcNAc residues from the sperm surface and by treatment with a range of pharmacological inhibitors directed against protein kinase A, protein tyrosine kinases and intermediates including Src. Proteomic analysis of the WGA-binding sites on the sperm surface identified the putative cognate receptor as platelet cell adhesion molecule 1 (PECAM-1/CD31). This conclusion was supported by the following evidence: (i) anti-PECAM-1 antibodies identified a molecule of the correct molecular mass in human spermatozoa, (ii) PECAM-1 could be isolated from a pool of sperm surface proteins using WGA immobilized on a solid phase support, (iii) PECAM-1 and WGA co-localized to the sperm surface and (iv) anti-PECAM-1 antibodies could completely block the ability of WGA to stimulate tyrosine phosphorylation in these cells. Collectively, these data provide the first evidence that a receptor-mediated signal transduction pathway triggers human sperm capacitation and identifies PECAM-1 as the probable initiator of this second messenger cascade.

Key words: Tyrosine phosphorylation, Spermatozoa, Capacitation, Wheat germ agglutinin, PECAM-1

Introduction

The capacity for fertilization is conferred on mammalian spermatozoa during their transit through the female reproductive tract. This process, termed capacitation, represents the culmination of a series of complex physiological and biochemical changes that result in remodelling of the sperm surface and induction of a state of hyperactivated movement. Among the correlates of capacitation, the phosphorylation of tyrosine residues on multiple sperm proteins has recently emerged as a critical factor in the regulation of sperm-egg interaction (Asquith et al., 2004; Urner et al., 2001; Urner and Sakkas, 2003; Visconti et al., 1995a; Visconti et al., 1995b). However, despite the importance of this tyrosine phosphorylation event, the biochemical signals responsible for initiating this process in vivo have yet to be fully elucidated.

Interestingly, the activating role of tyrosine phosphorylation during sperm capacitation, draws a number of close parallels with the activation of other cell types, including human platelets and vascular endothelial cells. In such cells, insights into the molecular mechanisms responsible for the induction of tyrosine phosphorylation have been gained through the judicious use of specific lectins (Ganguly et al., 1985; Higashihara et al., 1985; Hwang and Wang, 1991; Inazu et al., 1991; Palmetshofer et al., 1998; Rendu and Lebret, 1984; Smirnova et al., 1998; Yatomi et al., 1995). Given the complexity of the sperm glycocalyx, this cell type has proved particularly amenable to studies involving lectins. As such, there is an extensive literature documenting the use of these probes to study changes in sperm surface architecture that accompany key biological events such as epididymal maturation and the capacitation of these cells within the female reproductive tract (Bearer and Friend, 1990; Gall et al., 1975; Koehler, 1978; Koehler, 1981; Lee and Damjanov, 1985; Mahmoud and Parrish, 1996; Navaneetham et al., 1996; Nicolson et al., 1972; Nicolson et al., 1977; Nicolson and Yanagimachi, 1972; Nicolson and Yanagimachi, 1974; Olson and Danzo, 1981). More recently, it has also been demonstrated...
that the relative number and distribution of specific lectin binding sites on the sperm surface represents an important prognostic indicator of male infertility (Fei and Hao, 1990; Zhixing and Yifei, 1990). In the current study we have investigated the physiological consequences of sperm-lectin interaction in human spermatozoa and in doing so, obtained the first evidence that a receptor-mediated signal transduction pathway may be involved in the capacitation of these cells. Moreover, our data point to the importance of PECAM-1 in activating the tyrosine phosphorylation cascade that characterizes the capacitation process.

Materials and Methods

Reagents

Chemical reagents were obtained from Sigma (St Louis, MO, USA), Research Organics (Cleveland, OH, USA) or Vector Laboratories (Burlingame, CA, USA) and were of research grade.

Human sperm purification

All experiments described in this study were conducted with human semen obtained from a panel of healthy normozoospermic donors, in accordance with the Institutes' Human Ethics Committee guidelines. Samples were collected after a period of 48 hours abstinence into sterile specimen containers and left for a period of at least 30 minutes to liquefy before processing. Spermatozoa were isolated by discontinuous Percoll gradient centrifugation using a simple 2-step design incorporating 44% and 88% Percoll as described previously (Aitken et al., 1998). Purified spermatozoa were recovered and washed with Hapes-buffered Biggers, Whitten and Whittingham media (BWW) supplemented with 1 mg/ml polyvinyl alcohol (PVA) (Biggers et al., 1971), centrifuged at 500 g for 5 minutes, and resuspended at a concentration of 2×10^6 cells/ml.

To eliminate the confounding effects of contaminating leukocytes within our experiments, comparative studies were performed on subpopulations of fractionated sperm isolated by a swim up procedure. For this purpose, spermatozoa recovered from the pellet of the 88% Percoll fraction were re-centrifuged at 300 g for 5 minutes, over-layered with medium and incubated for 1 hour in an inclined tube. The highly motile, leukocyte free, sperm population was subsequently aspirated and the pellet discarded.

Histochemical labelling of live human spermatozoa

Prior to studying the biochemical consequences of lectin interaction, a panel of 19 lectins were examined for their ability to bind human spermatozoa. For this purpose, spermatozoa were diluted to 5×10^6 cells/ml in BWW and incubated with the fluorescein isothiocyanate (FITC)-conjugated lectins (0.5 μg/ml) listed in Table 1. Labelling was conducted in a dark, humidified chamber at 37°C in an atmosphere of 5% CO₂ in air for 30 minutes. Following incubation, spermatozoa were washed with Hepes-buffered Biggers, Whitten and Whittingham media (BWW) supplemented with 1 mg/ml polyvinyl alcohol (PVA) (Biggers et al., 1971), centrifuged at 500 g for 5 minutes, and resuspended at a concentration of 2×10^6 cells/ml.

To confirm the specificity of the WGA response, spermatozoa (5×10^6 cells/ml) were treated with either neuraminidase and/or N-acetylglucosaminidase (SJA; a lectin that does not interact with the sperm surface) and/or GlcNAc residues, respectively. Following treatment, a portion of the sperm sample was incubated with FITC-conjugated SJA and/or sWGA for 1 hour at room temperature and viewed under a fluorescence microscope to examine the efficacy of sugar removal. The remaining sample was subjected to WGA challenge and prepared for immunoblotting with anti-phosphotyrosine antibodies as indicated above. Finally, as an additional means of investigating the physiological significance of the WGA response, spermatozoa were examined for their ability to undergo the acrosome reaction after lectin challenge using the protocols described by Aitken et al. (Aitken et al., 1993). Briefly, 5×10^6 cells/ml were incubated with 0.5 μg/ml WGA for 30 minutes, then 200 μl aliquots were added to either 200 μl of 5 μM A23187 or the DMSO vehicle control (diluted 1:5000) and
incubated for a further 30 minutes. Spermatozoa were then washed by centrifugation (450 \text{ g} for 2 minutes), resuspended in HOS medium and incubated for 1 hour. Following incubation, spermatozoa were again washed and then plated onto poly-L-lysine-coated slides, air-dried and permeabilized by immersion in methanol for 10 minutes. The slides were finally stained with FITC-labelled \textit{Arachis hypogaea} agglutinin for 30 minutes at 4°C, mounted with 5 \mu\text{M} of anti-fade reagent, and the acrosomal status of viable cells was examined under a fluorescence microscope.

Characterization of the WGA stimulated signal transduction pathway

In order to elucidate the molecular events underlying WGA stimulation of sperm tyrosine phosphorylation, studies were performed to examine the relative contribution of a number of key intracellular signalling pathways that are known to operate in human spermatozoa.

Involvement of reactive oxygen species

In light of previous studies that have documented the generation of reactive oxygen species (ROS) in other cell types following WGA treatment (Kesari et al., 1983; Okamura et al., 1987) and the fact that ROS have previously been reported to play a fundamental role in the regulation of sperm function, including tyrosine phosphorylation (reviewed by Baker and Aitken, 2004), we first sought to investigate whether WGA binding stimulates the production of ROS in spermatozoa. For these studies, we used a combined approach incorporating: (i) lucigenin and luminol-peroxidase-mediated chemiluminescence assays to examine the production of ROS following WGA challenge and (ii) the ROS scavenging agents, superoxide dismutase (SOD) and catalase, to determine if such reagents were capable of abrogating the WGA-induced response.

For the former studies, 400 \mu\text{M} of sperm suspension (5\times10^6 cells/ml), fractionated by either Percoll gradient centrifugation or the swim-up procedure, were added to 5 ml luminometer tubes (Sarstedt, Ingle Farm, SA, Australia). Lucigenin or luminol-peroxidase was added to the appropriate tubes and baseline chemiluminescence measurements were recorded for 20 minutes using an AutoLumat luminometer (Berthold, Bundoora, Vic, Australia) as previously described (Aitken et al., 2003). WGA (0.5 \mu\text{g}/ml) was then added and chemiluminescence emission was recorded for an additional 30 minutes. Each tube was then tested for its ROS-generating potential by the addition of phorbol 12-myristate, 13-acetate (PMA) at a final concentration of 100 nM (Aitken et al., 1996; Krausz et al., 1992).

To examine the effect of SOD and catalase, spermatozoa (5\times10^6 cells/ml) were pre-incubated in these reagents (300 and 3000 U, respectively) for 1 hour prior to the addition of WGA (0.5 \mu\text{g}/ml). Incubations were continued for an additional 30 minutes prior to processing of the sperm cells for phosphotyrosine expression by western blot analysis, using the procedures outlined above.

Involvement of a cyclic-AMP/PKA pathway

We next sought to investigate whether the interaction of WGA with sperm surface proteoglycans was capable of modulating the intracellular cAMP concentration ([cAMP]), and hence promoting an alternative tyrosine phosphorylation cascade involving the activation of protein kinase A (PKA) and downstream protein tyrosine kinase(s) (PTK) (Visconti et al., 1995b).

For this purpose, spermatozoa (10\times10^6 cells) were suspended in 2 ml BWW supplemented with WGA as outlined above. Following incubation, the sperm sample was split and one half was prepared for the measurement of tyrosine phosphorylation levels as previously outlined. The remaining spermatozoa were pelleted (500 g, 3 minutes) and resuspended in 200 \mu\text{L} 0.1 M \text{HCl}. These cells were mixed by vortexing and incubated at room temperature for 20 minutes to effect extraction of intracellular cAMP. Quantitative determination of [cAMP], was performed using the BIOMOL® Format A Cyclic AMP “PLUS” Enzyme Immunoassay Kit (BIOMOL Research Laboratories Inc. Plymouth Meeting, PA, USA) according to the manufacturer’s protocols.

To examine the involvement of signalling molecules within this proposed pathway that act downstream of cAMP, spermatozoa (5\times10^6 cells/ml) were preincubated for 1 hour in BWW supplemented with inhibitors of either PKA (H89; 10 \mu\text{M}) or protein tyrosine kinase [genistein (200 \mu\text{M}) and herbamycin (150 \mu\text{M})], or the vehicle control

\begin{table}
\begin{center}
\begin{tabular}{|l|l|l|l|}
\hline
Lectin & Name & Organism of origin & Affinity* \\
\hline
Head labeling & & & \\
AIL & Jacalin & Artocarpus integrifolia & galactosyl (β-1,3) N-acetylgalactosamine \\
Con A & Concanavalin A & Canavalia ensiformis & α-linked mannose \\
DBA & Dolichos biflorus agglutinin & Dolichos biflorus & α-linked N-acetylgalactosamine \\
\hline
Entire surface labeling & & & \\
LCA & Lens culinaris agglutinin & Lens culinaris & α-linked mannose \\
LEL & Tomato lectin & Lycopersicon esculentum & N-acetylgalactosamine \\
MAA & Maackia amurensis lectin & Maackia amurensis & sialic acid (α-2,3 linkage) \\
PHA-E & Phaseolus vulgaris erythroagglutinin & Phaseolus vulgaris E & Complex oligosaccharides \\
PHA-L & Phaseolus vulgaris leucoagglutinin & Phaseolus vulgaris L & N-acetylgalactosamine (β-1,2) mannose \\
SNA & Elderberry lectin & Sambucus nigra & sialic acid (α-2,6 linkage) \\
sWGA & Succinylated wheat germ agglutinin & Triticum vulgaris & N-acetylgalactosamine \\
WGA & Wheat germ agglutinin & Triticum vulgaris & N-acetylgalactosamine and sialic acid \\
\hline
Weak or no labeling & & & \\
DSL & Datura stramonium lectin & Datura stramonium & (β-1,4) linked N-acetylgalactosamine \\
ECL & Erythrina cristagalli lectin & Erythrina cristagalli & galactosyl (β-1,4) N-acetylgalactosamine \\
GSL II & Griffonia simplicifolia lectin II & Griffonia simplicifolia & α or β-linked N-acetylgalactosamine \\
PHA-E & Peanut agglutinin & Arachis hypogaea & galactosyl (β-1,3) N-acetylgalactosamine \\
SIA & Sophora japonica agglutinin & Sophora japonica & N-acetylgalactosamine and galactose residues \\
STL & Solanum tuberosum lectin & Solanum tuberosum & N-acetylgalactosamine \\
UEA-1 & Ulex europaeus agglutinin-1 & Ulex europaeus & α-linked fucose \\
VVA & Hairy vetch agglutinin & Vicia villosa & α or β-linked N-acetylgalactosamine \\
\hline
\end{tabular}
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\footnote{This table shows the primary sugar specificity of each lectin used in the present study. It should be noted that most lectins have additional structural requirements for binding.}
\end{table}
to the final concentration indicated. Following this initial incubation, WGA (0.5 μg/ml) was added and the incubations were continued for an additional 30 minutes. The sperm sample was then prepared as outlined above for analysis of phosphotyrosine levels.

Involvement of Src family kinases
Using lectin activation of human platelets as a model, we also sought to examine the involvement of the Src protein tyrosine kinase family in WGA-mediated tyrosine phosphorylation in human spermatozoa. These studies were conducted by pre-incubating sperm in the broad spectrum Src family kinase inhibitors, pyrazolopyrimidine-1 and -2 (PP1 and PP2; 10 μM) in addition to SU6656 and subsequently assessing the cells for their ability to undergo tyrosine phosphorylation in response to WGA challenge.

Isolation and sequencing of sperm surface WGA binding proteins
To isolate sperm surface proteins, the outer leaflet of the membrane was vectorially labelled with sulfo-NHS-SS-Biotin (Pierce), a thiol cleavable, membrane-impermeable derivative of biotin. Briefly, cells were vectorially labelled with sulfo-NHS-SS-Biotin (Pierce), a thiol cleavable, membrane-impermeable derivative of biotin. Cells were then recovered by centrifugation at 500 g for 3 minutes and washed twice with BWW. Sperm proteins were extracted with 10 mM CHAPS as outlined above, and biotinylated surface proteins purified using streptavidin-conjugated magnetic beads (Dynal, Oslo, Norway) in accordance with the manufacturer’s protocols. Bound proteins were eluted from the beads by reduction of the disulphide linker arm with 50 mM dithiothreitol in PBS and resolved on duplicate two-dimensional (2D) SDS-PAGE gels as previously described (Asquith et al., 2004). One gel was subsequently stained with Coomassie Brilliant Blue (G-250) whilst the other was transferred to a nitrocellulose membrane as outlined above. The membrane was probed with HRP-conjugated streptavidin (to assess the efficacy of removal of the biotin tag), before being stripped and reprobed with biotinylated WGA and HRP-conjugated streptavidin.

Proteins that cross-reacted with WGA were excised from the Coomassie-stained gel, subjected to an in gel tryptic digest and the resulting peptides were analysed by matrix assisted laser desorption/ionisation–time of flight (MALDI-ToF) mass spectrometry using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences). Briefly, 1 pL fractions of the tryptic peptides were mixed with an equal volume of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% TFA:acetonitrile, 50:50) before being applied to the sample slide. The mass spectra were acquired in the reflectron mode and internal mass calibration was performed with two trypsin auto-digestion fragments (842.5 and 2211.1 Da). Measured monoisotopic masses of tryptic masses of peptide were used as inputs to search the NCBI non redundant database using the Mascot search engine (http://www.matrixscience.com). Up to one missed tryptic cleavage was considered in most cases. A mass accuracy of 50 ppm or lower was used for each search. A minimum of three matching peptides were used as the basis for unambiguous identification of target proteins.

Affinity precipitation
The WGA cross-reactivity of proteins of interest was confirmed by affinity precipitation. Briefly, purified sperm surface proteins (500 μg) were incubated for 30 minutes with biotinylated WGA (0.5 μg) under constant rotation. Following incubation, streptavidin-conjugated magnetic beads (800 μL, Dynal) were added and the suspension was returned to constant rotation for a further 1 hour. The beads, bound to WGA binding proteins, were then isolated by magnetic separation and washed five times in PBS. They were then suspended in SDS-PAGE loading buffer and eluted proteins resolved on SDS-PAGE gels. The proteins were then transferred to membranes and immunoblotted with the appropriate primary antibody and HRP-conjugated secondary antibodies.

Statistics
All experiments were replicated a minimum of three times with pooled semen samples obtained from at least three different donors. Graphical data are presented as mean values ± s.e.m., the standard errors being calculated from the variance between samples. Statistical significance was determined using an analysis of variance (ANOVA). The differences between group means were assessed using Fisher’s protected least significant difference (PLSD) test.

Results
Lectin labelling of live spermatozoa
Prior to studying the biochemical implications of lectin interaction with sperm surface glycoconjugates, we first identified a panel of 11 lectins that were capable of binding to the surface of live human spermatozoa. Histochemical analysis revealed two distinctive patterns of lectin localisation: those that bound uniformly to the entire surface of the cell, and those that bound only to the sperm head (Table 1). In each case, more than 90% of viable, motile cells were labelled. The diversity of the sugar specificities displayed by these lectins (Table 1) demonstrate the complexity of the human sperm surface glycoalyx.

WGA binding elicits an increase in sperm tyrosine phosphorylation
To explore the physiological significance of lectin binding, we examined the tyrosine phosphorylation status of cells following lectin challenge, using α-tubulin expression as an internal loading control. As demonstrated in Fig. 1, only the binding of WGA (GlcNAc and sialic acid) induced an upregulation of tyrosine phosphorylation. Interestingly, the interaction of other lectins that recognise either sialic acid (SNA and MAA) or GlcNAc (sWGA) alone and that displayed a similar pattern of sperm localisation to that of WGA, were incapable of eliciting a similar physiological response (Fig. 1A). Furthermore, incubation of sperm with a combination of such lectins (sWGA + MAA + SNA) also failed to stimulate tyrosine phosphorylation. We inferred from these observations that the activation of tyrosine phosphorylation involved the cross-linking of receptors with terminal sialic acid and GlcNAc residues. In keeping with this concept, the enzymatic cleavage of either sialic acid or GlcNAc residues from the sperm surface prior to WGA challenge abolished the subsequent induction of tyrosine phosphorylation (Fig. 1B).

In terms of the nature of the response to WGA challenge, we noted a global increase in the tyrosine phosphorylation of proteins ranging in molecular mass from approximately 20-210 kDa. This pattern of phosphotyrosine expression appeared to be consistent across individual samples and identical to that induced by the pharmacological agents, dbcAMP and pentoxifylline, in the positive controls (Fig. 2). However, considerable differences in the protein profile were noted...
depending upon the method of cell solubilization employed (Fig. 2). For instance, initial studies conducted with the mild zwitterionic detergent, CHAPS, revealed a complex pattern of phosphotyrosine proteins whereas those performed with a more stringent SDS-based solubilization, were biased by the presence of two predominant proteins of approximately 85 and 105 kDa, as previously reported (Emiliozzi and Fenichel, 1997). Overexposure of the latter blots revealed a more complex pattern of tyrosine phosphorylation, approximating that seen in the CHAPS extract (Fig. 2). The absence of a suitable surface marker to ensure equivalent protein loading between each of our CHAPS extracts dictated the use of SDS solubilization protocols since such samples contain β-tubulin.

Characterization of WGA-induced sperm tyrosine phosphorylation

As anticipated, WGA induction of sperm tyrosine phosphorylation was able to be modulated by both the concentration of the lectin and the period of incubation (Fig. 3). Interestingly, the response appeared to be biphasic, such that increases were noted up to a maximum level at 0.5 μg/ml WGA and thereafter, higher concentrations of WGA induced a sub-maximal tyrosine phosphorylation response (Fig. 3A). Consistent with these data, the level of cellular agglutination increased and consequently the motility of treated samples decreased in the presence of WGA at or above 5 μg/ml (Fig. 3B). Similarly, the viability of sperm, as determined with the HOS assay, was significantly reduced by elevated concentrations of WGA (Fig. 3B). In this context, it was noted that greater than 90% of cells were agglutinated and only 20% of these cells remained viable following treatment with 5 μg/ml WGA. In terms of the kinetics of the response, a biphasic pattern of stimulation was again observed. WGA was shown to elicit a rapid increase in the levels of tyrosine phosphorylation within 5 minutes of treatment. Tyrosine phosphorylation continued to rise to a maximum at 30 minutes,
and thereafter declined. Based on these results, incubation of sperm in 0.5 \(\mu\)g/ml WGA for a period of 30 minutes was established as the optimal conditions for the induction of tyrosine phosphorylation and the avoidance of detrimental effects upon sperm viability. As such, these conditions were employed for the remainder of the studies.

The notion that the WGA induction of tyrosine phosphorylation represents a physiological response to lectin interaction with normal viable spermatozoa was supported by experiments involving morphologically abnormal cells recovered from low-density Percoll fractions. This latter population consistently failed to respond to stimulation by WGA. Furthermore, the possible contribution of contaminating leukocytes to this response was eliminated on the basis that we saw no discernible quantitative or qualitative differences in WGA-induced tyrosine phosphorylation in cells isolated by Percoll fractionation compared to those collected from a swim up procedure. The biological significance of this response was further highlighted by the fact that WGA treatment led to a significant increase in the number of spermatozoa that responded to stimulation by WGA.

**Fig. 3.** Characterisation of the dose and time dependency of WGA-stimulated sperm tyrosine phosphorylation. Purified human spermatozoa were incubated in varying concentrations of WGA (0.005-5 \(\mu\)g/ml) or in positive (+ve) and negative (–ve) control media for 30 minutes. Alternatively, spermatozoa were incubated in 0.5 \(\mu\)g/ml WGA for varying times (5-90 minutes). Following incubation, spermatozoa were either (A) solubilized in SDS extraction buffer and prepared for immunoblotting with anti-phosphotyrosine or (B) examined to determine the status of their motility and viability. This experiment was replicated three times with pooled semen samples obtained from at least three different donors and representative blots are depicted. Graphical data is given as the means ± s.e.m. from the three replicate experiments. *\(P<0.05\), **\(P<0.01\).

**Fig. 4.** Comparison of the WGA-induced tyrosine phosphorylation in normal and defective spermatozoa. (A) Human spermatozoa recovered in the low density (defective) and high density (normal) fractions after Percoll density gradient centrifugation were incubated with either WGA (0.5 \(\mu\)g/ml) or in positive (+ve) and negative (–ve) control media for 30 minutes. An additional control, consisting of sperm incubated in complete BWW supplemented with SJA (a lectin that does not interact with the sperm surface) was also included. Following incubation, spermatozoa were solubilized in SDS extraction buffer and prepared for immunoblotting with anti-phosphotyrosine. This comparison was replicated three times with pooled semen samples obtained from at least three different donors and representative blots are depicted.
of viable cells competent to undergo an induced acrosome reaction in response to A23187. In the absence of WGA, 52.0±2.8% of spermatozoa underwent the acrosome reaction following ionophore exposure compared with 20.0±1.3% in the DMSO controls. However, when capacitation was promoted by a 30 minute exposure to 0.5 g/ml WGA, the percentage of cells undergoing acrosomal exocytosis increased significantly to 62.0±3.4% (n=6; P<0.05).

Characterization of the WGA-stimulated signal transduction pathway

ROS generation

In light of previous studies that have documented the generation of reactive metabolites in other cell types following WGA treatment (Keisari et al., 1983; Okamura et al., 1987) and recent data suggesting that the tyrosine phosphorylation events associated with capacitation are redox regulated (reviewed by Baker and Aitken, 2004), we examined the ability of WGA to trigger an oxidative burst in spermatozoa using luminal peroxidase-dependent chemiluminescence. As shown in Fig. 5A, the addition of WGA to cells isolated from the 88% Percoll fraction elicited a rapid increase in H2O2 generation to levels significantly greater than those observed in untreated cells. By contrast, spermatozoa selected by the swim-up procedure, despite displaying higher motility and viability than sperm obtained from 88% Percoll fraction, did not respond in a similar fashion upon WGA addition. In fact, no increase in H2O2 generation above basal levels was noted in this latter population (Fig. 5A). Taken together, these findings suggest that the observed H2O2 generation was attributable to leukocyte contamination of the former cell population. This interpretation is consistent with the fact that the subsequent addition of PMA elicited an immediate increase in H2O2 generation in cells from the 88% Percoll fraction but not within the swim-up population (Fig. 5A). Consistent with the absence of H2O2 production by spermatozoa, WGA treatment also failed to elicit redox activity in these cells in the presence of lucigenin (Fig. 5B).

The ability of WGA to elicit a ROS-mediated signal transduction pathway was further discounted on the basis that pre-incubation of spermatozoa with either SOD, which catalyses the dismutation of an O2– radical to H2O2 and O2, or catalase, which catalyses the conversion of H2O2 to H2O and O2, failed to suppress the induction of tyrosine phosphorylation following WGA challenge. Similarly, pre-treatment of spermatozoa with a complement of both ROS inhibitors, also failed to suppress WGA-induced tyrosine phosphorylation (Fig. 5C).

Cyclic-AMP/PKA pathway

We next sought to investigate whether WGA enhanced tyrosine phosphorylation by stimulating an increase in intracellular cAMP via mechanisms that were independent of ROS generation. In order to do this, we first examined the [cAMP]i, stimulated in response to WGA binding. As illustrated in Fig. 6, despite the induction of both a time- and dose-dependent increase in tyrosine phosphorylation as previously reported, WGA failed to elicit an increase in [cAMP]i, above that of untreated controls, in identical cell populations. By contrast,
sperm incubated in the presence of dbcAMP and ptx displayed significantly elevated [cAMP]i. Although such results argue against WGA stimulation of [cAMP]i, it is possible that an increase in the synthesis of this metabolite could be masked by its rapid degradation. Therefore, the potential role of the cAMP-stimulated pathway in the WGA response was further investigated using several types of inhibitors.

Interestingly, it was demonstrated that pre-incubation of sperm with the PKA inhibitor, H89 suppressed the subsequent induction of tyrosine phosphorylation in response to WGA challenge. As illustrated in Fig. 7, the tyrosine phosphorylation recorded following this treatment was reduced to levels similar to those observed in vehicle-only control samples. Similarly, genistein and herbamycin, both potent inhibitors of protein tyrosine kinases, were effective in modulating the WGA-induced response (Fig. 7).

Src family kinase pathway
Based on studies of lectin activation in human platelets (Ohmori et al., 2001), we finally sought to examine the involvement of the Src protein tyrosine kinase family by pre-incubating human spermatozoa in the presence of broad spectrum Src family kinase inhibitors, PP1 and PP2, in addition to the more specific inhibitor SU6656. As shown in Fig. 7, PP1, PP2 and SU6656 all potently inhibited the WGA-mediated increase in sperm tyrosine phosphorylation.

Identification of the human sperm WGA receptor(s)
In order to identify the sperm WGA receptor(s) responsible for initiation of the signalling pathway characterised above, the human sperm surface proteome was isolated and resolved using duplicate 2D SDS-PAGE (Fig. 8A-C). Those proteins possessing affinity for WGA were determined by immunoblotting and the corresponding spots excised from the Coomassie-stained gel and identified by MALDI-ToF mass spectroscopy. This approach revealed a complex profile of purified sperm surface proteins ranging in molecular mass from approximately 10 kDa to 125 kDa, of which four displayed significant affinity for WGA (with masses of, 28 kDa, 32 kDa, 52 kDa and 125 kDa). Of these proteins, reliable peptide mass fingerprint data was generated for three and these were each identified as previously characterised proteins (Fig. 8D).

Among these proteins, platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31) was considered to have properties most compatible with those expected of a WGA sperm receptor. However, given the novelty of this finding, further studies were undertaken to confirm the presence of this molecule in human spermatozoa and its cross-reactivity with WGA. For this purpose, polyclonal anti-PECAM-1 antibodies (anti-CD31; R & D Systems, Minneapolis, MN, USA) were used to probe solubilized sperm extracts and to localize the

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**Fig. 6.** Evaluation of cAMP levels and tyrosine phosphorylation status following WGA treatment. To investigate the production of intracellular cAMP following WGA challenge, human spermatozoa were incubated with either WGA (0.5 μg/ml) or SJA (0.5 μg/ml), or in positive (+ve) or negative (–ve) control media for 30 minutes. Following incubation, the sperm sample was split and one half was prepared for (A) the measurement of [cAMP], and the remaining cells were prepared for (B) immunodetection of phosphotyrosine residues (anti-py). This experiment was replicated three times with pooled semen samples obtained from at least three different donors and representative blots are presented. Graphical data represent the means ± s.e.m. from the three replicate experiments, **P<0.01.

**Fig. 7.** Impact of inhibitors on WGA-induced tyrosine phosphorylation. Spermatozoa were pre-incubated for 1 hour in genistein, herbamycin, H-89, PP1, PP2, SU6656 or the vehicle control (DMSO). WGA (0.5 μg/ml) was then added and the incubations continued for an additional 30 minutes. The sperm sample was then prepared for analysis of phosphotyrosine levels by immunoblotting. This experiment was replicated three times with pooled semen samples obtained from at least three different donors and representative blots are depicted.
Human sperm capacitation protein on live human spermatozoa. As shown in Fig. 9A, anti-PECAM-1 displayed cross-reactivity with a single sperm protein of approximately 125 kDa. This protein was of a similar size to that recognised as PECAM-1 in the platelet control. In addition, a protein band of identical molecular mass was also detected using a panel of three additional anti-PECAM-1 monoclonal antibodies (5A2, B2B1, 2BD4) targeting different facets of this molecule, prepared as previously described (Ashman et al., 1991) (Fig. 9B). These results were confirmed by affinity precipitation with WGA from aqueous sperm extracts, which clearly revealed a band of 125 kDa that cross-reacted with the polyclonal anti-PECAM-1 antibody (Fig. 9C). In terms of its pattern of expression on live human spermatozoa, PECAM-1, like WGA, appeared to localise to the majority of the surface of greater than 90% of the cell population (Fig. 9D).

Investigation of the role of PECAM-1 in mediating the WGA response
In order to explore whether PECAM-1 is a functional receptor for WGA in human spermatozoa we examined whether the addition of anti-PECAM-1 antibodies were capable of competitively inhibiting WGA binding and hence compromising its effect. Following treatment, cells were examined for their response by western blot analysis with monoclonal anti-phosphotyrosine antibodies as shown in Fig. 10. As illustrated, treatment with anti-PECAM-1 antibodies prior to WGA challenge completely suppressed the up-regulation of tyrosine phosphorylation normally exhibited following this treatment, supporting a role for this receptor in activating the tyrosine phosphorylation cascade associated with human sperm capacitation.

Discussion
A sudden increase in phosphotyrosine expression is an essential part of the biochemical remodelling of mammalian spermatozoa as they ascend the female reproductive tract and prepare for fertilization. Despite its biological importance, little is known about the physiological mechanisms that regulate this signal transduction pathway in vivo. In order to gain insights into this process we have screened a large panel of lectins to determine whether the targeting of specific sperm surface glycoconjugates could activate the tyrosine phosphorylation cascades associated with capacitation, in much the same way as lectins have been found to induce phosphotyrosine expression in platelets and vascular endothelial cells (Ohmori et al., 2001; Palmetshofer et al., 1998).

Consistent with previous findings (Bains et al., 1992; Lee and Damjanov, 1985; Singer et al., 1985), we identified a large
number of lectins, with distinct sugar specificities, that bound to the surface of human spermatozoa. Although a subpopulation of these lectins was found to display a restricted pattern of localization, the majority bound to the entire sperm surface. Of all the sperm-binding lectins assessed, only the plant lectin WGA, which possesses dual binding specificity for terminal N-acetylglucosamine (GlcNAc) and sialic acid residues, was found to enhance tyrosine phosphorylation, generating patterns of phosphotyrosine expression reminiscent of that observed during cAMP-induced capacitation. Neither the succinylated form of WGA (sWGA), which lacks the sialic acid-binding activity of the native lectin, nor SNA and MAA, which bind to $\alpha$-2,3 and $\alpha$-2,6-linked sialic acid residues, respectively, were able to stimulate a similar response. Interestingly, sperm also proved insensitive to challenge with various combinations of these lectins either in their native form or when presented as cross-linked oligomers. Collectively, these results indicate that the conformation of the WGA molecule is critical for promoting the aggregation of the corresponding sperm receptor(s) that are responsible for potentiating the downstream signalling cascade. Such conclusions are consistent with the fact that this response can be abolished by the removal of either sialic acid or GlcNAc residues from the sperm surface (Fig. 1B).

The presence of WGA receptors on the surface of male germ cells at various stages of differentiation and maturation has been previously reported (Gabriel and Franken, 1997; Gabriel et al., 1994a; Gabriel et al., 1994b; Kallajoki et al., 1985; Lee and Damjanov, 1985). Furthermore, it has also been suggested that the relative number and distribution of WGA receptors may represent an important prognostic indicator of human male infertility (Fei and Hao, 1990; Gabriel and Franken, 1997; Gabriel et al., 1995; Gabriel et al., 1994b; Zhixing and Yifei, 1990); the ability of sperm to bind to WGA-coated microbeads reflecting the quality of sperm morphology and accurately predicting the success of IVF treatment (Gabriel and Franken, 1997). Although these results implicate receptors containing

![Image](https://example.com/image.png)
terminal GlcNAc and/or sialic acid (specific saccharides for WGA) in sperm-egg interaction, these earlier studies failed to identify the precise role of such receptors. To our knowledge, the present study is the first to establish a specific role for WGA-binding sites in the signal transduction events leading to sperm capacitation. Furthermore, our finding that WGA stimulates global protein tyrosine phosphorylation, which is itself a prerequisite for sperm-zona binding (Asquith et al., 2004), explains why cells exhibiting reduced expression of WGA receptors are associated with lowered fertility.

The ability of WGA to promote tyrosine phosphorylation in human spermatozoa has led us to hypothesise that this agonist activates the receptor-mediated signal transduction cascade associated with sperm capacitation. WGA was found to elicit a rapid dose-dependent tyrosine phosphorylation response under conditions where the spermatozoa were still viable, motile and free of significant agglutination. Overstimulation of the spermatozoa with WGA resulted in a clear cytotoxic response similar to that observed in a wide a variety of other cell types exposed to this lectin, including lymphocytes, leukemic cell lines, haemopoietic stem cells, intestinal mucosa and olfactory epithelium (Lorenz-Meyer et al., 1985; Lustig and Pluznik, 1976; Moon and Baker, 2002; Ohba and Bakalova, 2003; Ohba et al., 2003). This cytotoxic effect is reportedly augmented by the generation of ROS following WGA interaction (Keisari et al., 1983; Okamura et al., 1987).

ROS have been shown to produce a biphasic response in human spermatozoa, enhancing tyrosine phosphorylation when produced in low, physiological levels and compromising sperm motility and vitality when exposure levels are high (Aitken and Fisher, 1994; Aitken and Clarkson, 1987; Aitken et al., 1998; Baker and Aitken, 2004). Since WGA also stimulated tyrosine phosphorylation at low levels of exposure and loss of motility and viability when exposure levels were high, we hypothesized that the biological activity of this lectin depended on the stimulation of ROS generation. However, the data gathered in this study did not support this hypothesis, with WGA failing to stimulate any significant changes in redox activity. Furthermore the primary mechanism by which ROS enhances tyrosine phosphorylation in human spermatozoa, through the elevation of intracellular cAMP (Aitken et al., 1998; Aitken et al., 1995), did not account for the stimulatory action of WGA on these cells. However, preincubation of spermatozoa with the specific PKA inhibitor, H89, essentially eliminated the ability of WGA to induce tyrosine phosphorylation. Such results suggest that while cAMP/PKA is an essential component of this unique signal transduction pathway, WGA is modulating tyrosine phosphorylation via an alternative route. For example, the ability of the Src kinase inhibitor SU6656 to suppress WGA-induced tyrosine phosphorylation (Fig. 7) raises the possibility that this promiscuous tyrosine kinase is heavily involved in the tyrosine phosphorylation response associated with sperm capacitation. While Src might normally be activated by PKA (Obara et al., 2004) it can also be activated directly by receptor tyrosine kinases such as ErbB2/HER2 (Roskoski, 2005) as well as immunoglobulin superfamily members such as PECAM-1 (Ohmori et al., 2001). Activated Src could then signal to downstream effectors including members of the extracellular signal-regulated protein kinase (ERK) pathway; Shc, Grb2, Ras(p21), Raf and ERK1 and 2, all of which have been identified in human spermatozoa (de Lamirande and Gagnon, 2002). These signal transduction molecules would then be responsible for phosphorylating key targets, such as AKAPs, on the sperm tail. In support of this concept, inhibitors of Grb2, Ras(p21), Raf and MEK prevent the wave of protein tyrosine phosphorylation associated with the induction of sperm capacitation by foetal cord serum ultrafiltrate (de Lamirande and Gagnon, 2002). These data lend support to our overall hypothesis that WGA is mimicking the actions of an extracellular factor, which originates in the female reproductive tract and is responsible for activating the tyrosine phosphorylation cascade that regulates sperm capacitation in vivo.

The results obtained in the study suggest that this WGA-binding receptor is PECAM-1. Firstly, MALDI-TOF analysis of the WGA-binding proteins in human spermatozoa identified three peptides in a high molecular mass protein that exactly matched the PECAM-1 sequence and exhibited the same molecular mass as this molecule. Moreover a molecule with the same mass was found to cross-react with an anti-PECAM antibody on western blot analysis. In addition, this molecule cross-reacted with a panel of monoclonals directed against different regions of the PECAM-1 molecule (Ashman et al., 1991) (Fig. 9B). Cytological studies also demonstrated that anti-PECAM antibodies co-localized with WGA on the surface of human spermatozoa (Fig. 9D). Furthermore, WGA precipitated a molecule with the same molecular mass as PECAM-1 from human sperm extracts that cross-reacted with the anti-PECAM antibody (Fig. 9C). Finally, this anti-PECAM antibody completely blocked the ability of WGA to induce a tyrosine phosphorylation response in human spermatozoa. Identification of PECAM-1 as the sperm WGA receptor is also supported by studies on human platelets, in which the WGA-induced activation was found to be mediated by PECAM-1 (CD31) (Ohmori et al., 2001). In platelets, WGA engagement of PECAM-1 induces rapid oligomerization and phosphorylation of the receptor. This, in turn, stimulates the recruitment and activation of various downstream signalling molecules including Src family kinases (Ohmori et al., 2001) in keeping with the suggested role of Src in mediating the WGA-activated, SU6656-inhibited increase in tyrosine phosphorylation observed in human spermatozoa. The fact that PECAM-1 can be activated by hydrogen peroxide is also perfectly in keeping with the ability of this oxidant to activate tyrosine phosphorylation in capacitating human spermatozoa (Aitken et al., 1998; Maas et al., 2003).

The significance of this work lies in the fact that PECAM-1 is the first activatable receptor to have been identified on the surface of human spermatozoa. These results will be instrumental in stimulating new research into the factors released by the female reproductive tract that might activate sperm PECAM-1 in vivo. In this context it is of considerable interest that a sialic acid-binding protein (SABP) with lectin-like properties is known to be synthesized in the human endometrium and secreted into uterine fluid (Banerjee and Chowdhury, 1994; Banerjee and Chowdhury, 1995). Intriguingly, absence of SABP from endometrial homogenates has been correlated with cases of unexplained primary infertility (Banerjee and Chowdhury, 1994). In light of such data, SABP warrants further investigation as a possible candidate for mediating the PECAM-1 responses we have observed in the present study. Future research will also address
the pathways through which PECAM-1 signals to the spermatozoon’s tyrosine phosphorylation machinery during capacitation and the extent to which similar pathways are employed by other mammalian species. Ultimately, such studies should make a significant contribution to our understanding of the molecular mechanisms that drive the capacitation process, facilitating the development of novel strategies for both the induction and treatment of male infertility.

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