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Induction of Acrosomal Exocytosis in Chicken Spermatozoa by Inner Perivitelline-Derived N-linked Glycans

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In birds, the ovum is surrounded by a glycoprotein coat known as the inner perivitelline layer (IPVL), which is analogous to the mammalian zona pellucida and, as such, is the site of initial sperm binding and induction of acrosomal exocytosis (the acrosome reaction). In this study, we demonstrate that oligosaccharides isolated from chicken-IPVL glycoproteins are capable of inducing the acrosome reaction in chicken spermatozoa. Preparations containing only O-linked glycans were unable to induce the acrosome reaction whereas N-linked oligosaccharides released from the IPVL by PNGaseF treatment could induce the acrosome reaction. Addition of galactose to terminal *N*-acetylglucosamine residues suppressed the acrosome reaction-inducing capacity of the oligosaccharide preparation; however, this capacity could be restored by co-incubation with β-galactosidase. This evidence suggests that the acrosome reaction-inducing factor is probably an N-linked oligosaccharide with terminal *N*-acetyl-glucosamine residues.

Key Words: **acrosome reaction; chicken; spermatozoa; inner perivitelline layer; oligosaccharide; fertilization; N-linked glycan; lectin.**

Fertilization involves binding and fusion of gametes. In birds, fusion of the sperm plasma membrane to the oolemma is blocked by a glycoprotein layer, the inner perivitelline layer (IPVL), which surrounds the plasma membrane at ovulation (1, 3). This layer is analogous to the mammalian zona pellucida (ZP). Indeed, one of the IPVL glycoproteins has 41% amino acid sequence identity with mouse ZPC (4, 5), a member of the ZPC family of proteins. In both mammals and birds, spermatozoa interact with these glycoprotein layers and undergo an exocytotic event known as the acrosome reaction (AR) (6, 7), resulting in the release of acrosomal contents which facilitate sperm penetration of the egg investments.

In mammals, the ZP family of glycoproteins has been identified as playing a key role in sperm binding and the induction of acrosomal exocytosis (for reviews see 8–10). In the mouse, these activities are associated with oligosaccharides O-linked to the C-terminal portion of ZPC (11). In pigs, oligosaccharides attached to zona pellucida proteins also mediate these activities (12), although there is some debate as to the nature of the oligosaccharides possessing sperm binding activity: conflicting evidence supports both O-linked (13) and N-linked (14) oligosaccharides in this role. In pigs, the nature of the protein anchor is also uncertain—with recent evidence that a ZPB-ZPC complex rather than ZPC alone is responsible for sperm binding (15, 16). In both the mouse and pig, the secretagoguic activities of glycans are dependent on the attachment of glycans to zona proteins. Isolated glycans do not induce the AR in capacitated mouse sperm (17), so that induction of the AR is thought to be dependent upon aggregation of receptors (18) on the surface of capacitated spermatozoa. The complex nature of this interaction has made it difficult to define the nature and structure of glycans responsible for induction of the AR. To date, although several possible candidate oligosaccharides with a role in sperm:egg binding have been suggested: galactose (19, 20); mannose (21, 22); and terminal *N*-acetylglucosamine (23–25); there has been little progress in defining precise structures of oligosaccharides able to induce the AR.

There is considerable evidence that in birds, as in mammals, carbohydrates play a crucial role in sperm: egg interactions (26, 27). The evidence so far presented involves assay of the overall interaction of sperm and IPVL *in vitro*, in which a successful sperm:egg interaction event is manifest as a small hole in the IPVL. However, this interaction involves both binding of spermatozoa to isolated IPVL, induction of the AR fol-

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lowed by hydrolysis of the IPVL by spermatozoa. Thus, using this assay it is not possible to determine whether manipulation of sperm:egg interaction affects one or many of these stages. In this paper, we present evidence that IPVL carbohydrates play a crucial role in induction of the acrosome reaction. To determine the acrosomal status of spematozoa we exploited the differential binding of FITC labelled PNA to acrosome-intact but not acrosome-reacted spermatozoa (28). Using this approach we found that acrosomal excocytosis could be instigated by an isolated fraction of IPVL-derived carbohydrates. These carbohydrates did not need to be attached to protein, were N-linked to the IPVL and required a terminal *N*-acetyl-D-glucosamine residue (GlcNAc) for biological activity.

MATERIALS AND METHODS

Materials. All chemicals were obtained from the Sigma Chemical Company Ltd. (Poole, Dorset), except PNGase F (EC 3.5.1.52), endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97), fucosidase (EC 3.2.1.111), and sialidase (EC 3.2.1.18), Glycoclean R cartridges, UDP β-D-galactose, galactosyltransferase (EC 2.4.1.22), β-galactosidase (EC 3.2.1.23), and an O-GlcNAc detection kit, which were obtained from Oxford Glycosystems (Abingdon, UK).

Birds and bird maintenance. Male chickens, *Gallus domesticus*, were ISA "Grandparent" cockerels from ISA Poultry Services, Peterborough; chickens hens were ISA Brown commercial layers. All birds were caged individually, given a photoperiod of 14 h light:10 h dark and fed a commercial breeder's ration *ad libitum*.

Semen collection and preparation. Semen was collected from chickens by abdominal massage (29). For most experiments, pooled ejaculates from 3–4 males were used to provide each sample. The sample was diluted 1:4 in 0.15 M NaCl with 20 mM TES (*N*-Tris-[hydroxymethyl]-methyl-2-aminoethansulfonic acid) at pH 7.4 (NaCl-TES) and stored aerobically in a shaking water bath at 30°C for up to 1 h before use.

Preparation of perivitelline layers from laid eggs. Inner and outer perivitelline layers were separated using acid treatment (3). IPVLs were prepared for use in the acrosome reaction assay either by dispersion (by homogenising a 0.5 cm $\times$ 0.5 cm square of IPVL in 1 ml NaCl TES) or, proteolysis (2 IPVL ml^{-1} homogenised in dilute HCl at pH 2.0 were incubated overnight with 25 $\mu g\ ml^{-1}$ pepsin followed by gel filtration using a G-25 Sephadex column equilibrated in NaCl-TES).

FITC-lectin staining of acrosome reacted spermatozoa. Samples containing 1.25×10^7 spermatozoa ml^{-1} were incubated with an IPVL preparation (either dispersed or solubilised), the test sample or, in the case of controls, without IPVL, in a final volume of 1 ml in NaCl-TES with 5 mM $CaCl_2$ for 5 min at 40°. Samples were then micro-centrifuged at 1889g for 40 s and the pellet was resuspended in 50 μl of NaCl-TES, 100 $\mu g\ ml^{-1}$ FITC-conjugated *Arachis hypogea* lectin (FITC-PNA). This was then incubated at room temperature for 10 min in light shielded containers, washed with 950 μl NaCl-TES, micro-centrifuged at 1889*g* for 40 s and the pellet resuspended in 50 μl of NaCl-TES. A 10 μl aliquot was removed and examined by phase contrast and epifluorescence microscopy at $\times$1000 and acrosome-reacted spermatozoa were identified by the presence of fluorescent tips (28). At least 100 spermatozoa were counted from each sample. Positive controls, treated with IPVL (either homogenised or solubilised by proteolysis), typically contained between 50 and 70% acrosome-reacted sperm, whereas untreated, negative controls usually contained 1–10% acrosome reacted sperm. Sets of samples with negative controls showing 30% or greater spontaneous AR were discarded. The number of acrosome-reacted sperm in the test samples was expressed as a percentage of acrosome reactable sperm and all assays were carried out in duplicate on each individual sample.

Preparation of glycans from IPVL. Release of mixed (N-linked and O-linked) glycans or O-linked glycans from isolated IPVL was achieved by using reductive β-elimination in mild conditions (O-linked glycans) or mild followed by harsh conditions (for a mixture of N- and O-linked glycans) (30). Typically, 10 IPVL (approximately 0.9 mg of protein) were incubated for 16 h at 45°C in 0.05 M NaOH containing 1 M $NaBH_4$ followed by incubation for 6 h at 100°C in 1.025 M NaOH containing 1 M $NaBH_4$. The solution was cooled on ice and neutralised by the addition of 50% v/v acetic acid, centrifuged at 3000*g* for 10 min and the supernatant retained. This was desalted using a G-25 Sephadex column (40 $\times$ 2.5 cm) equilibrated in distilled water and 5 ml fractions were collected and tested for sugar using the orcinol-sulfuric acid assay (31) and protein using the bicinchoninic acid protein assay (32). Glycan-containing fractions were pooled, lyophilised then re-N-acetylated by dissolving in saturated Na_2CO_3, neutralised with 10 μl aliquots of acetic anhydride, then desalted on an H^+ Amberlite column. Fractions collected from the Amberlite column were lyophilised and stored at -20°C until required.

Release of N-linked glycans was achieved by pre-incubating pepsin-solubilised IPVL (0.511 mg ml^{-1}) in 250 μl of sodium phosphate buffer (20 mM at pH 7.5) containing 0.5% SDS and 50 mM EDTA l^{-1} at 100°C for 2 min. After addition of: Nonidet (1% v/v); 1 mM phenylmethylsulfonyl fluoride; 1 mM leupeptin; 1 mM *o*-phenanthroline; and 16 U ml^{-1} peptide-*N*-glycosidase F (PNGase F); the incubation was continued for a further 18 h at 37°C. Released glycans were separated from IPVL protein with Glycoclean R cartridges according to the manufactures instructions. Glycans were lyophilised and stored at -20°C until required.

Lectin affinity chromatography of mixed glycan samples. Mixed glycans (0.128 mg), prepared by reductive alkolysis, were dissolved in either 200 μl of NaCl-TES (for *Triticum vulgaris* lectin-agarose-(WGA-agarose) affinity chromatography) or 200 μl of 5 mM sodium acetate containing 0.1 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ (for to *Canavalia ensiformis* lectin-agarose (Con-A-agarose) affinity chromatography). They were then mixed with 800 μl of either WGA-agarose suspended in NaCl-TES or 800 μ Con A-agarose suspended in 5 mM sodium acetate containing 0.1 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$ for 18 h at 25°C. These samples were then poured into a 1 ml column and washed with 15 ml NaCl-TES (WGA-agarose) or 15 ml 5 mM sodium acetate containing 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ (Con A-agarose). Fractions were assayed for sugar using the orcinol-sulfuric acid assay (31) and any sugar containing fractions retained. The WGA-agarose column was washed with 10 ml of 0.1 M GlcNAc and fractions collected. Any monosaccharides were removed by passing fractions through individual G-25 Sephadex columns equilibrated in NaCl-TES.

IPVL-glycoprotein modification. IPVL, 29 μl of 0.221 mg ml^{-1} (solubilised by homogenising IPVL in 1 ml of 6 M guanidinium HCl (GuHCl), followed by heating for 1 min at 100°C and then exhaustive dialysis to remove GuHCl), was labelled with UDP-D-[^{3}H]-galactose using an Oxford Glycosystems O-GlcNAc detection kit, according to the manufacturer's instructions, using albumin as a positive control.

Mixed glycan modification. Mixed glycans (0.236 mg ml^{-1}) were incubated in with 5 mg ml^{-1} UDP galactose and 0.33 units ml^{-1} galactosyl transferase in 20 mM HEPES, 0.15 M NaCl, 0.2% w/v sodium azide, pH 7.4, for 3 h at 37°C. Buffer components and enzymes were removed using Glycoclean R cartridges. Samples were lyophilised and tested for their ability to induce the AR (compared to controls incubated with no galactosyl transferase). Modified glycans were then incubated with 1 unit ml^{-1} β-galactosidase from bovine testes in 100 mM sodium citrate/phosphate for 18 h at 37°C. Buffer

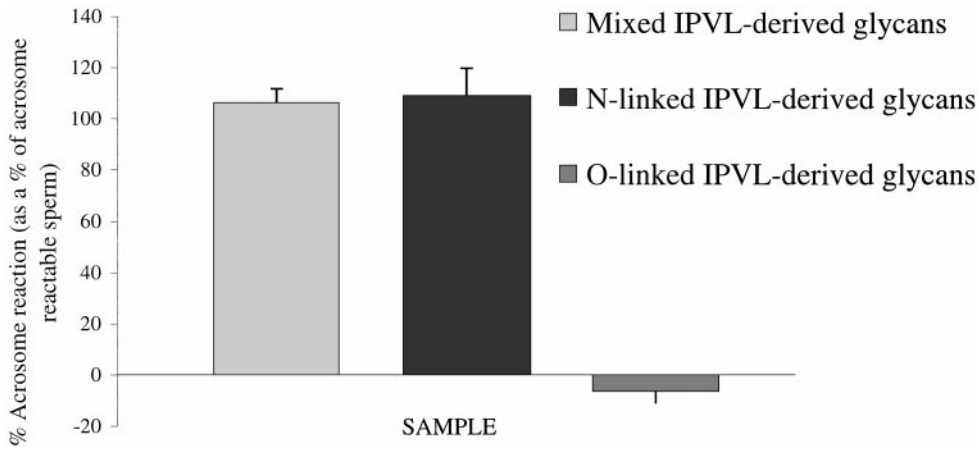

FIG. 1. The acrosome reaction inducing capacity of IPVL-derived mixed glycans, IPVL derived O-linked glycans, and IPVL derived N-linked glycans. Each type of IPVL-derived glycan was tested for the ability to induce the acrosome reaction as detailed under Materials and Methods. The results shown represent the % of acrosome reacted sperm (as a % of acrosome reactable sperm) for IPVL-derived mixed glycans (mean ± SE; $n = 15$), IPVL derived N-linked glycans (mean ± SE; $n = 4$) and IPVL derived O-linked glycans (mean ± SE; $n = 8$).

components and enzymes were removed using Glycoclean R cartridges according to the manufactures instructions and samples lyophilised and tested for their ability to induce the acrosome reaction (compared to controls incubated with no galactosidase).

RESULTS

Preparation of IPVL Derived Glycans

Typically, 0.3 mg of mixed glycans were obtained from 10 IPVL by β-elimination in mild followed by harsh conditions. When only mild conditions were used typically 0.16 mg of O-linked glycans were released from 10 IPVL.

Induction of the Acrosome Reaction by IPVL-Derived Oligosaccharides

No significant difference was seen between the ability of IPVL-derived mixed glycans and homogenised IPVL in their ability to induce the AR in spermatozoa (2-sample unpaired t test $P > 0.5$, n = 15; Fig. 1). No significant difference was observed between the proportion of acrosome-reacted spermatozoa in samples treated with O-linked glycans and negative controls (2 sample unpaired t test $P > 0.05$, $n = 8$). N-linked IPVL derived glycans were able to induce the AR and there was no significant difference between the AR inducing ability of N-linked glycans and homogenised IPVL (2-sample unpaired t test $P > 0.05$, $n = 4$). Sugar concentrations in the incubation mixtures ranged from 0.003–0.0122 mg ml $^{-1}$ (mixed glycans). There was no relationship between glycan concentration and level of AR within this range (concentrations of both N-linked and O-linked glycans were within this range). No induction of the AR (as a % of acrosome reactable sperm) was observed when spermatozoa were incubated with amyloglucosidase (6%; $n = 2$); amyloglucosidase-derived mixed glycans (0.6%; $n = 3$); ovalbumin (0.5%; $n = 2$) and ovalbumin-derived mixed glycans (15%; $n = 2$). Similarly, *N*,*N*-diacetylchitobiose (−7%; $n = 4$) and *N*-acetyl glucosamine (−9.5%; $n = 2$) were also incapable of inducing an acrosome reaction *in vitro.*

Lectin Affinity Chromatography of IPVL-Derived Mixed Glycans

Mixed glycans unable to bind WGA-agarose were found to have no significant ability to induce the AR when compared to positive controls (2 sample t test: $P < 0.0001$; $n = 4$; Table 1), whereas glycans eluted with D-GlcNAc had significant ability to induce the AR when compared to negative controls (2 sample t test: $P > 0.005$; $n = 4$). Mixed glycans with AR-inducing activity were unable to bind Con A-agarose.

Modification of IPVL Glycans

Incubation of solubilised IPVL with H^3GlcNAc led to a significant incorporation of label compared to con-

TABLE 1

Binding of the Acrosome Reaction-Inducing Factor to WGA-Agarose

Sample	% Sperm undergoing the acrosome reaction (as a % of acrosome reactable sperm) ± SE
Fractions eluting with NaCl-TES[a]	−12.7 ± 9.4
Material eluting with 0.1 M GlcNAc[b]	116.0 ± 13.3

[a] Sugar concentration of 0.0028 mg ml^{-1}.
[b] Sugar concentration of 0.0015 mg ml^{-1}.

TABLE 2

The Effect of Glycan Modification on the Ability of IPVL-Derived Glycans to Induce the Acrosome Reaction

Treatment	% Acrosome reaction (as a % of acrosome reactable sperm) ± SE, (n = 3)
Incubation with galactosyl transferase and UDP galactose	4.0 (±4.0)
Control (no galactosyl transferase)	119.1 (±21)
Incubation of galactosylated glycans with β-galactosidase	90.7 (±10)
Control (no β-galactosidase)	3.3 (±3.2)

trols, typically 56536 DMP incorporated into 6.4 μg solubilised IPVL.

Galactosyltransferase-treated mixed glycans lost the capacity to induce the AR (Table 2) compared to controls incubated in the absence of enzyme (2 sample t test $P < 0.05$, $n = 3$), however, after incubation with β-1-4 galactosidase, the ability to induce the AR was restored (Table 2) to a level comparable to untreated samples (2 sample t test $P > 0.05$; $n = 3$).

DISCUSSION

These results show that, in chickens, the AR is induced by N-linked glycans with terminal GlcNAc. Our evidence is that the chicken sperm AR can be induced by oligosaccharides separated from the glycoproteins of the IPVL by chemical or enzymtic hydrolysis and, more specifically, by N-linked, but not O-linked glycans. Glycans derived from ovalbumin and amyloglucosidase were unable to induce the AR. Furthermore, masking of GlcNAc moieties with galactose could be shown to inhibit the AR-inducing properties of IPVL derived glycans and this inhibition could be reversed by subsequent treatment with β-galactosidase.

Induction of the AR by N-linked glycans in solution has not been found in other vertebrates studied. In the mouse, AR-induction requires O-linked glycans attached to the C-terminus of mZP3, a member of the ZPC gene family (11, 33–35), or neoglycoproteins, such as mannose-BSA, *N*-acetylglucosamine-BSA or *N*-acetylgalactosamine-BSA (36). In boar, induction of the AR is also a property of protein-anchored glycans although there is evidence that both N-linked (14) and O-linked (13) oligosaccharides are involved. Thus, even although chicken IPVL contains a protein that has significant homology with mammalian ZPC (4, 5) the glycan array required to induce the acrosome reaction is clearly different.

Previous studies of chicken sperm:egg interactions have indicated that GlcNAc residues on IPVL proteins *in situ* are required for the hydrolysis of the IPVL by chicken spermatozoa *in vitro* (27). However such studies did not differentiate between factors modulating sperm binding, induction of the AR or proteolysis of the IPVL, or whether or not GlcNAc was terminal or within the glycan structure. In this work, we show, by masking and subsequent unmasking of saccharides with galactose, that a terminal GlcNAc is indeed present on, and is essential for the AR-inducing activity of, the isolated glycans. In mice, solubilised ZPC competitively inhibits sperm:egg interaction and preincubation with galactosyl transferase from bovine milk (25) removes this inhibition, indicating that GlcNAc is required for sperm binding. Indeed, a sperm-specific galactosyl transferase has been identified (37) that specifically galactosylates ZP3 (25) and, on aggregation will induce the acrosome reaction (38). An avian equivalent of mouse-sperm galactosyl transferase may thus act as a sperm IPVL-binding protein. However, considering the ability of unanchored glycans to induce the AR in birds, it appears that receptor aggregation is not necessary for induction of the AR.

Binding of the AR-inducing factor to WGA-agarose indicates that the AR- inducing oligosaccharide contains either an N-linked oligosaccharide with core GlcNAc or, as we confirm from the galactose addition experiments, terminal GlcNAc residues (39). This corroborates evidence from both the selective release of AR-inducing activity by PNGase F treatment and the inhibition of sperm:egg interaction by *N*,*N*-diacetylchitobiose and GlcNAc (27). The inability of ConA agarose to bind AR-inducing activity indicates that the active oligosaccharides did not contain terminal mannose, to which Con A has a high affinity, or a terminal α-linked glucose, to which ConA has a lower affinity (39). In mice (and rats) mannose has been proposed to act as a potential zona pellucida-sperm ligand with sperm surface mannosidase acting as a receptor (21, 40, 41). However, our data plus the findings of Robertson *et al.* (27), that D-mannose and mannose binding lectins (ConA and PNA) were shown not to interfere with chicken sperm-egg interaction, would suggest that mannose-containing oligosaccharides do not play a significant role in the induction of an avian AR.

In summary, the avian AR can be induced by oligosaccharides N-linked to the IPVL. The oligosaccharides need to have terminal GlcNAc groups and are capable of inducing the acrosome reaction when detached from their protein anchor, suggesting that induction of the excoytotic process does not involve a receptor aggregation event of the type hypothesized in mammalian spermatozoa.

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