Changes in the properties and composition of zona pellucida of pigs during fertilization *in vitro*

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Summary. Several hundred fertilized pig eggs were prepared by an *in vitro* fertilization (IVF) technique in which follicular phase ovarian eggs were matured *in vitro* to meta-phase II before incubation with capacitated epididymal spermatozoa for 12 h at 39°C. Parthenogenetic eggs were also prepared by stimulation of the mature eggs with an electric pulse. The zonae were solubilized with 0.2% pronase/phosphate-buffered saline (PBS) or lactic acid/PBS. The time taken for solubilization was 30–40% shorter than for unfertilized eggs, indicating that zona hardening was induced during fertilization. At the same time, the sperm receptor activity of the zona was reduced. Electrophoretic analyses of zona glycoproteins from the ovarian, mature and fertilized eggs revealed that the amount of 90 kDa proteins decreased substantially during fertilization. This fraction could barely be detected in the zonae from parthenogenetic eggs. However, modification with a fluorescent probe showed that the general architecture of the zona remained unchanged during fertilization. These results suggest that the minor 90 kDa proteins are specifically degraded by the protease(s) released from the oocyte at fertilization, thereby leading to the block to polyspermy.

Keywords: zona pellucida; fertilization; pig; *in vitro* fertilization

Introduction

The zona pellucida has several roles in fertilization (Nakano, 1989). One function of the zona is to block polyspermy, but the molecular mechanism has still to be clarified. In invertebrate species, a trypsin-like protease may be released from the cortical granules during their breakdown at fertilization, inducing the hardening of the zona (Wolf, 1982). However, only indirect and controversial evidence has been offered in support of participation of cortical granule enzymes in mammalian fertilization (Schmell & Gulyas, 1980; Wolf, 1982). To understand the zona reaction involved in the block to polyspermy, it is necessary to investigate the properties of the zona of the fertilized egg.

Changes in zona proteins of pigs during fertilization *in vivo* have been reported by Brown & Cheng (1986) and Hedrick *et al.* (1987) who analysed [¹²⁵I]-labelled zona proteins by polyacrylamide gel electrophoresis (PAGE) and found additional oviductal glycoproteins binding firmly to the zona derived from the fertilized eggs. However, the limited quantity of zona material and the presence of oviductal proteins preclude accurate analysis of the change in the zona itself. Furthermore, [¹²⁵I]-labelling causes some degradation of zona proteins in the autoradiograms, although this method is effective for analysis of the zona proteins isolated from a single egg (Hedrick *et al.*,

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1987). These experiments, investigated changes in the properties of the pig zona and of zona glycoproteins during *in vitro* fertilization (IVF) i.e. in a situation in which oviductal factors are absent. To exclude contamination by sperm proteins, we also analysed the zonae from parthenogenetic eggs obtained by artificial activation with an electric pulse. Since a large number of fertilized eggs can be obtained by IVF, silver staining of the zona proteins in the acrylamide gels was possible.

Materials and Methods

Preparation of fertilized eggs

Fertilized eggs were prepared by *in vitro* fertilization as reported previously (Nagai *et al.*, 1988). Cumulus-egg complexes isolated from the ovaries of slaughtered gilts were incubated in TCM 199A at 39°C for 48 h under 5% CO₂ in air (Nagai & Moor, 1990) and the eggs that matured to metaphase II were transferred to fertilization medium (Brackett & Oliphant, 1975). An epididymal sperm suspension, preincubated in TCM 199B (2×10^8 spermatozoa ml⁻¹) at 37°C for 70 min under 5% CO₂ in air, was then added to the eggs at a final concentration of 10^6 spermatozoa ml⁻¹. After incubation at 39°C for 12 h, fertilization was assessed by the presence of enlarged sperm heads, male pronucleus(ei) with corresponding sperm tail(s) or both in the oocytes after staining with aceto-orcein (Nagai *et al.*, 1988). The fertilization rate by this method was at least 80%.

Preparation of parthenogenetic eggs

Parthenogenesis of the pig eggs was induced by an electric pulse (Maruyama *et al.*, 1991). The eggs that matured to metaphase II were treated with 0.1% hyaluronidase in TCM 199 to disperse the cumulus cells and transferred to 0.3 mol sucrose l⁻¹ containing 0.05 mmol CaCl₂ l⁻¹ and 0.1 mmol MgSO₄ l⁻¹. Current pulses for stimulation were provided by the use of a cell fusion apparatus (Shimadzu SSH-1). The eggs were stimulated twice by an electric pulse at 25–100 V mm⁻¹ for 25 µs and incubated in Brackett & Oliphant (BO) solution (Brackett & Oliphant, 1975) at 39°C for 6 h under 5% CO₂ in air. Parthenogenesis was established in 80–100% of the eggs.

Spermatozoa-egg binding assay

Boar cauda epididymal fluid containing spermatozoa was kept at 16°C for 16 h. After washing twice with mKRB (Toyoda & Chang, 1974), the spermatozoa were suspended in mKRB (2×10^8 spermatozoa ml⁻¹) and capacitated by incubation at 37°C for 2 h under 5% CO₂ in air. Capacitated sperm suspension, 10 µl, was added to ten eggs in mKRB (200 µl) containing 2 mmol caffeine l⁻¹ under paraffin oil and the mixture incubated at 37°C for 2.5 h. The eggs were washed twice by transfer through fresh mKRB with a pipette (bore size 200 µm) and fixed with glutaraldehyde. The heads of spermatozoa binding to the eggs were stained with 4',6-diamidino-2-phenyl-indole (DAPI) (Russell *et al.*, 1975) and their number was counted under a fluorescence microscope.

Zona solubilization assay

Ten eggs were placed in 0.2% pronase-PBS (pH 7.4) or lactic acid-PBS (pH 3.6) at room temperature and the time required for complete lysis of the zonae was recorded.

Isolation of zonae

The metaphase II eggs, fertilized eggs and parthenogenetic eggs that had been kept frozen at -80°C were thawed in fresh PBS. The eggs were mechanically ruptured by being sucked into a fine glass pipette (bore size less than the diameter of the eggs). The yolk bodies were removed by transfer of the zonae through three changes of PBS.

The zonae from ovarian eggs were isolated as described previously (Nakano *et al.*, 1989).

Heat solubilization of the zonae

The zonae in PBS were transferred to distilled water and solubilized by heating at 70°C for 10 min. The solution was then filtered through a 0.45 µm filter membrane to remove spermatozoa that had adhered to the zonae after fertilization. The filtrates containing the solubilized zona proteins were analysed.

Two-dimensional IEF/SDS polyacrylamide gel electrophoresis

For isoelectric focusing (IEF), the zona proteins from 160–200 zonae were dissolved in 20 µl of an aqueous solution containing 2% ampholine (pH 3.5–10), 5% β-mercaptoethanol and 8.5 mol urea l⁻¹. Gels (2.0 × 70 mm)

were prepared using a solution containing 4% acrylamide, 0.2% *N,N'*-methylenebisacrylamide, 8.5 mol urea l^{-1} , and 2% ampholine (pH 3–10). IEF was performed at 200 V for 15 h. The gel was then soaked in 62.5 mmol Tris-HCl l^{-1} buffer pH 6.8, containing 2% SDS and 5% β -mercaptoethanol for 30 min, before it was placed on the 10% acrylamide slab gel and fixed by agarose gel. Second-dimension SDS electrophoresis was carried out at a constant current of 15 mA for 1 h 45 min. After pretreatment with 10% TCA, the gel was stained using a silver-stain kit (Kanto Kagaku Co.).

Endo- β -galactosidase digestion of zona proteins

The heat-solubilized zona proteins from 70–100 unfertilized and fertilized eggs were digested with *Escherichia freundii* endo- β -galactosidase (0.17 mU) in 20 μ l 0.1 mol ammonium acetate l^{-1} buffer, pH 5.6, at 37°C for 40 h. SDS-PAGE of the digests was performed at 15 mA for 2 h under reducing conditions in 12.5% acrylamide gel. The gel was stained with the silver-based stain, the density of bands monitored by densitometric scanning using a dual-wavelength TLC scanner (Shimadzu CS-910) and the amounts of proteins estimated from peak areas.

Two-dimensional diagonal electrophoresis

Endo- β -galactosidase digests of the zona glycoproteins from 135 mature eggs and 142 fertilized eggs were subjected to SDS-PAGE (Laemmli, 1970) in 10% gel in the glass column (2 $\times$ 70 mm) under nonreducing conditions. After electrophoresis at 1 mA per column for 2 h, the gels were soaked for 30 min in the reducing sample buffer containing 5% mercaptoethanol. The gels were then placed on the second 12.5% gel and fixed with 1% agarose. The second electrophoresis was performed at 15 mA for 1.5 h and the gel stained with the silver-based stain.

Modification of the zonae with fluorescein isothiocyanate

Sixty to 80 unfertilized and fertilized eggs were modified with 50 μ mol fluorescein isothiocyanate (FITC) l^{-1} in PBS at 25°C for 4 h (Hatanaka *et al.*, 1988). After transfer through three changes of fresh PBS, the zonae were isolated and solubilized as described above.

Gel filtration HPLC of zona proteins

The zona proteins were fractionated into three families under nonreducing conditions by gel filtration high performance liquid chromatography (HPLC) on a column of TSK gel G3000SW as described previously (Nakano *et al.*, 1987b).

Results

Changes in properties of zona during fertilization

The sperm-binding capacity of the zona and the time for solubilization by pronase and dilute acid were changed during fertilization (Table 1). The activity of the sperm receptor of the mature egg was significantly reduced after fertilization. The times required for complete lysis of the zonae of fertilized eggs with 0.2% pronase/PBS (pH 7.4) and lactic acid/PBS (pH 3.6) were 40% and 33% slower, respectively, than those for the unfertilized mature egg.

Electrophoretic characterization of the zona glycoproteins from unfertilized and fertilized eggs

Two-dimensional PAGE revealed that amounts of 90 kDa proteins were markedly reduced during fertilization, whereas changes in other protein families were hardly observed (Fig. 1). Figure 1 also shows the actin (42 kDa) is present only in the zonae of the ovarian eggs.

When the sugar chains of zona proteins were partly removed by digestion with endo- β -galactosidase, a better resolution of the protein skeletons of each family was obtained in one-dimensional PAGE than with undigested specimens (Fig. 2). This digestion resulted in a change in the molecular weights of the zona proteins from 90, 65, 55 and 25 kDa to 78, 63, 45 and 14 kDa, respectively (Table 2). Bands representing minor 24 and 18 kDa components and enzyme (38 kDa) were also detected. A linear relationship was observed between the density of silver-stained

Table 1. Changes in the properties of pig zonae pellucidae during fertilization *in vitro*

	Activity of sperm receptor ^a (number of spermatozoa binding per egg)	
Mature eggs	208.7 ± 17.5	
Fertilized eggs	92.7 ± 18.2	
	Solubilization time (s) of the zonae ^b	
Medium	0.2% pronase/PBS (pH 7.4)	Lactic acid/PBS (pH 3.6)
Ovarian eggs		18 ± 3
Mature eggs	133 ± 24	21 ± 5
Fertilized eggs	185 ± 31	28 ± 5

^aTen eggs were incubated with capacitated spermatozoa at 37°C for 2.5 h in mKRB.

^bTen eggs were placed in the medium and the time required for complete lysis of the zonae was recorded.

bands of zona proteins from the ovarian eggs and protein concentration in the range from 0 to 4.0 µg (data not shown). The molar ratio of the endo-β-galactosidase digests (78 kDa:63 kDa:14 kDa) of the components in PZP2 from the mature eggs was estimated to be 2:5:4 from the peak areas of the bands and their apparent molecular weights. Densitometric scanning of the electrograms (Fig. 2) revealed a prominent reduction in the 78 kDa (originally 90 kDa) proteins (Table 2). A decrease in the amount of 63 kDa (originally 65 kDa) proteins and slight increase in the amounts of 45 kDa (originally 55 kDa) proteins and 14 kDa (originally 25 kDa) proteins were also observed together with a prominent reduction in the amount of 90 kDa component in the parthenogenetic eggs (Fig. 2 and Table 2), possibly caused by a protease(s) from the oocyte.

A decrease in the amount of 78 kDa proteins was also observed but no additional spot was detected in the low molecular weight region in the gel of the zonae from fertilized eggs (Fig. 3).

HPLC analysis of zona glycoproteins from unfertilized and fertilized eggs

The zona proteins from unfertilized and fertilized eggs were fractionated into three families by gel filtration HPLC (Fig. 4a, b). The amount of fraction PZP1 was reduced during fertilization, whereas relative amounts of PZP2 and 3 remained unchanged.

The PZP2 family may be located in the outer region of the zona structural unit (Hatanaka *et al.*, 1988). That is, FITC, a fluorescent probe, reacted with PZP2 situated in the ovarian egg and in the isolated zona, but hardly reacted with the major family, PZP3. This property was retained during maturation and fertilization (Fig. 4c, d, e), suggesting that no prominent change in the architecture of the structural unit is caused by the degradation of the 90 kDa component.

Discussion

Modification of sperm heads with DAPI facilitated the determination of the number of spermatozoa binding to the egg. The activity of the sperm receptor retained by the zona of mature eggs was reduced after fertilization (Table 1). Comparison of the time for solubilization between the zonae isolated from mature and fertilized eggs showed that hardening of the pig zonae occurs during fertilization as in the mouse (Inoue & Wolf, 1975). The reduction in activity of the sperm receptor and induction of hardening indicate some structural change in the zona.

Fig. 1. Two-dimensional polyacrylamide gel electrophoresis (PAGE) of unfertilized and fertilized pig zona glycoproteins under reducing conditions. The first dimension (left to right) is isoelectric focusing (IEF) (pH 4–7), and the second dimension (top to bottom) is sodium dodecylsulfate (SDS–PAGE) in 10% acrylamide gels. Heat solubilized zona protein from 200 ovarian eggs (a), from 180 mature eggs (b), from 160 fertilized eggs (c) were applied, and the gels stained with a silver-based stain. a: actin.

Zona glycoproteins from the frozen pig ovary have been fractionated into three families (PZP1–3) by gel filtration HPLC (Nakano *et al.*, 1987b). The pig zona proteins, which were prepared without freezing the eggs or in the presence of an inhibitor of trypsin-like enzyme (phenylmethanesulfonyl fluoride: PMSF), gave similar profiles on HPLC and SDS–PAGE (not shown). However, the possibility that cytoplasmic contents modify the zona components during freezing and thawing cannot be ruled out. The properties of zonae from unfertilized and fertilized eggs kept frozen under the same conditions were therefore investigated.

The highest molecular weight fraction, PZP1, contains quite heterogeneous proteins. They are detected as faint dispersed bands in SDS–PAGE, but emerge as a single sharp peak in HPLC

Fig. 2. SDS-PAGE of the endo- β -galactosidase digests of zona glycoproteins from unfertilized and fertilized eggs. The digests of heat-solubilized ZP from 104 ovarian eggs (lane 1), from 80 mature eggs (lane 2), from 80 fertilized eggs (lane 3), and 68 parthenogenetic eggs (lane 4) were electrophoresed at 15 mA for 2 h in 12.5% acrylamide gel. The gel was stained with a silver-based stain.

Table 2. Changes in the amounts of the zona protein components of pig eggs during fertilization

Protein component (reduced state)	90 kDa	65 kDa	55 kDa	25 kDa
(reduced state after endo- β -galactosidase digestion)	78 kDa	63 kDa	45 kDa	14 kDa
Relative amount (weight %) in the mature eggs ^a	7	16	74	3
Changes in the amount at fertilization ^b				
Mature egg	100	100	100	100
Fertilized egg	27	69	113	120
Parthenogenetic egg	27	91	108	135

^aRelative amounts were calculated by densitometric analysis of silver stained gels (Fig. 2).

^bThe peak area of each component of the mature egg is taken to be 100.

(Fig. 4). PZP2 consists of 90 kDa proteins and the cross-linked 65 kDa and 25 kDa proteins (Hedrick & Wardrip, 1987), whereas PZP3, the major family, consists of two kinds of protein: α - and β -glycoproteins (Yurewicz *et al.*, 1987).

Each family contains heterogeneous charge isomers (Fig. 1) differing mainly in the sulfate content of the sugar moiety (Nakano *et al.*, 1990). Two-dimensional (IEF-SDS) PAGE of these

Fig. 3. Two-dimensional diagonal electrophoresis of the endo- β -galactosidase digests of the zona glycoproteins. The digests of heat-solubilized zonae from (a) 135 mature eggs and (b) 142 fertilized eggs were applied. The gels were stained with a silver-based stain.

original zona proteins and one-dimensional SDS-PAGE of their digests with endo- β -galactosidase revealed that 90 kDa proteins of the PZP2 family are not seen after IVF (Figs 1 and 2). A similar result was obtained in the parthenogenetic eggs prepared by artificial activation, indicating that the degradation is caused by a protease(s) from oocytes. From the fertilization and activation rates, the residual 90 kDa proteins (about 27%) in the zonae from fertilized and parthenogenetic eggs (Table 2) may be from eggs that were not fertilized or activated.

The junctional complex penetrating the zona connects the oocyte with the cumulus cells in developing mammalian ovarian follicles (Anderson & Albertini, 1976). Actin molecules attaching to the junctional complexes would therefore have been introduced during the mechanical preparation of the zonae (Fig. 1a). Since no actin molecules were found in the zona proteins from the mature eggs as well as from the fertilized eggs (Figs 1b, c), disintegration of the junctional complex

Fig. 4. Fractionation of pig zona glycoproteins by HPLC on a column of TSK gel G3000SW. (a) Heat-solubilized zonae from 80 ovarian eggs, (b) from 80 fertilized eggs, (c) from 77 FITC-labelled ovarian eggs, (d) from 62 FITC-labelled mature eggs, and (e) 61 FITC-labelled fertilized eggs were chromatographed as described previously (Nakano *et al.*, 1987b). The absorbance of the effluent at 210 nm (a) and (b), and the fluorescence intensity with excitation at 480 nm and emission at 515 nm for (c)–(e) were monitored. Peak 1: PZP1; 2: PZP2; 3: PZP3; 4: SDS; i: FTC–PZP1; ii: FTC–PZP2; and iii: FTC–PZP3.

must have occurred during egg maturation (Thibault *et al.*, 1987) and the amounts of junctional materials reduced as egg maturation proceeded (Larsen *et al.*, 1986).

PZP1 and PZP2 in the zona structure are shown to be susceptible to proteases added *in vitro*, whereas PZP3 is very resistant (Nakano *et al.*, 1987a, 1989). These results, and the reactivity with FITC, indicate that PZP1 and PZP2 are present in the outer labile region of the zona structural unit, whereas PZP3 is in the inner rigid region (Hatanaka *et al.*, 1988). This general architecture was shown to be retained after fertilization (Fig. 4). Hedrick & Wardrip (1987) reported that the 65 kDa and 25 kDa proteins are derived from a part of the 90 kDa proteins under reducing conditions. Of these three components in the ZP2 family, only the 90 kDa component was missing in the fertilized egg, whereas the 65 kDa component slightly decreased and the 25 kDa component slightly increased (Table 2). The amount of the 45 kDa band from the 55 kDa proteins (PZP3) also slightly increased. No additional spot in the low molecular weight region of the two-dimensional diagonal electrophoretograms was found after fertilization (Fig. 3), indicating that the proteolytic cleavage of the 90 kDa component is quite limited. The changes in the amount of each component (Table 2) suggest that the degradation products of the 90 kDa component are not contained in the band of the 65 kDa component but in those of the 55 kDa or 25 kDa components either alone or in combination.

Changes in the composition of the zona during fertilization have been reported in mice (Bleil *et al.*, 1981), humans (Shabanowitz & O'Rand, 1988) and hamsters (Brown *et al.*, 1990). It has been suggested that the conversion of a major mouse zona protein, ZP2, to ZP2_f by limited proteolysis,

correlates with the hardening of the zona in fertilization (Moller & Wassarman, 1989). In fertilization in pigs, the minor 90 kDa proteins in PZP2 probably play an important role in the block to polyspermy. A specific and limited cleavage of the 90 kDa proteins probably induces a subtle structural change of the zona, leading to hardening. Consequently, the sperm receptor, which is retained by the sugar chain moiety of PZP3 (Sacco *et al.*, 1989) may be buried inside the rigid structure of the zona. Further detailed analysis of the structural changes of the zona during fertilization are needed to elucidate the molecular mechanisms of the block to polyspermy and of sperm-egg recognition. The fertilized pig egg, which can be obtained by IVF in comparatively large amounts, is well suited for this purpose.

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