The distribution and requirements of microtubules and microfilaments during fertilization and parthenogenesis in pig oocytes

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Microtubules and microfilaments are major cytoskeletal elements in mammalian ova and are important modulators of many fertilization and post-fertilization events. In this study, the integrated distribution of microtubules and microfilaments in pig oocytes were examined under a laser scanning confocal microscope, and the requirements of their assembly during *in vitro* fertilization and parthenogenesis in *in vitro* matured pig oocytes were determined. After sperm penetration, an aster of microtubules was produced in the spermatozoon, and this microtubule aster filled the whole cytoplasm during pronuclear movement. During pronuclear formation after activation by insemination, microfilaments became concentrated at the male and female pronuclei and, after electrical stimulation, at the female pronucleus. At metaphase of cleavage, microtubules were detected in the spindle and microfilaments were found mainly in the cortex. At anaphase, microtubule asters assembled at each spindle pole. During cleavage, large asters filled each daughter blastomere and a microfilament-rich cleavage furrow was observed. Cytochalasin B, a microfilament inhibitor, inhibited microfilament polymerization but affected neither pronuclear formation nor movement. However, syngamy and cell division were inhibited in eggs treated with cytochalasin B. Treatment with nocodazole after sperm penetration inhibited microtubule assembly and prevented migration leading to pronuclear union and cell division. These results indicate that microtubule and microfilament assembly in pig oocytes are integrated during fertilization and are required for the union of sperm and egg nuclei and for subsequent cell division.

Introduction

At fertilization, male and female nuclei undergo extensive rearrangements which are necessary for the union of the paternal and maternal genomes. Changes in nuclear structure include male and female pronuclear formation, pronuclear movements, intermixing of paternal and maternal genomes and mitotic processes for the completion of the fertilization processes. In a previous study that used the microtubule inhibitors, colcemid and nocodazole (Schatten *et al.*, 1989), it was shown that microtubule assembly is required for the formation of male and female pronuclei as well as for pronuclear migration in mice. However, microtubules may not be the only cytoskeletal element for pronuclear migration. In experiments using the microfilament inhibitor latrunculin (Schatten *et al.*, 1986), it has been shown that microfilaments also play a role in pronuclear movement. In the mouse oocyte, microfilaments became concentrated around both pronuclei after fertilization (Maro *et al.*, 1984).

Although the changes of chromatin are related to microtubule and microfilament dynamics during fertilization (Maro *et al.*, 1985; Van Blerkom and Bell, 1986; Webb *et al.*, 1986; Kim

et al., 1996a, b), the exact mechanism whereby chromosomes (either male or female) organize cytoskeletons is poorly understood. Furthermore, except in the mouse oocyte, little information is available on the role of microtubules and microfilaments and their interaction during fertilization and parthenogenesis in mammalian ova. Recently, we have reported microtubule assembly during pig fertilization and parthenogenesis, and demonstrated that a functional centrosome is the result of blending of paternal and maternal centrosomal components, and that microtubules are involved in pronuclear migration (Kim *et al.*, 1996b). In the present study, the integrated distribution and role of microtubules and microfilaments in pronuclear migration and two-cell division during fertilization and parthenogenesis in pigs are investigated.

Materials and Methods

In vitro maturation

Pig oocyte–cumulus complexes with uniform ooplasm and a compact cumulus cell mass were prepared in Hepes-buffered TALP medium containing 0.1% polyvinylalcohol (Hepes-TL-PVA) as described by Funahashi *et al.* (1994). Culture medium

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for *in vitro* maturation was BSA-free NCSU23 medium (NCSU23, Petters and Wells, 1993) supplemented with 10% (v/v) pig follicular fluid (pFF), 10 i.u. eCG ml⁻¹ (Intervet America Inc., Millsboro, DE) and 10 i.u. hCG ml⁻¹ (Lympo Med Inc., Rosemont, IL). Fifty oocyte–cumulus complexes were transferred to 500 µl of NCSU23, covered with paraffin oil (light mineral oil, number 0121-4; Fisher Scientific, Fair Lawn, NJ) in a four-well culture plate and then cultured for 22 h at 39°C in an atmosphere of 5% CO₂ in air. The oocyte–cumulus complexes were then transferred to 500 µl of NCSU23 without hormonal supplements and cultured for an additional 22 h at 39°C in 5% CO₂ in air (Funahashi and Day, 1993).

In vitro fertilization

Sperm-rich fractions (15 ml) were collected from boars by the gloved hand method and, after adding antibiotic–antimycotic solution (GIBCO BRL, Life Technology Inc., Grand Island, NY), the semen sample was kept at 20°C for 16 h. The semen was washed three times with 0.9% (w/v) NaCl supplemented with 1 mg BSA ml⁻¹ (Fraction V, Sigma Chemical Co., St Louis, MO) by centrifugation at 600 g for 3 min. At the end of washing, the pellets containing spermatozoa were resuspended at 2×10^8 cells in modified TCM-199 ml⁻¹ (Funahashi *et al.*, 1994; Sigma) at pH 7.8 supplemented with 1% pFF. The sperm suspension was incubated for 90 min at 39°C in an atmosphere of 5% CO₂ in air. Ten oocytes were washed three times with modified TCM-199 supplemented with 10 mmol caffeine sodium benzoate l⁻¹ and 4 mg BSA ml⁻¹ at pH 7.4 and placed into a 50 µl droplet of the same medium under paraffin oil. Fifty microlitres of diluted preincubated spermatozoa was added to 50 µl of the medium containing oocytes so that a final sperm concentration of 1×10^6 cells ml⁻¹ was obtained. Oocytes were co-cultured with spermatozoa for 6 h at 39°C in an atmosphere of 5% CO₂ in air. The oocytes were then transferred to 500 µl of fresh NCSU23 and cultured at 39°C in an atmosphere of 5% CO₂ in air. The oocytes were fixed at 6, 9, 12, 24 and 36 h after insemination.

Oocyte activation

Before electrical stimulation, oocytes matured *in vitro* were denuded of cumulus cells, washed and preincubated for 5 min in electroporation medium (0.25 mol mannitol l⁻¹ supplemented with 0.01% polyvinyl alcohol, 0.5 mmol Hepes l⁻¹, and 100 mmol CaCl₂ · 2H₂O l⁻¹ and 25 mmol MgCl₂ · 6H₂O l⁻¹; pH 7.2). Electrical stimulation to induce activation was delivered via a BTX electrocell manipulator (Biotechnologies

and Experimental Research, Inc., San Diego, CA) to a chamber with two parallel platinum wire electrodes (200 mm outer diameter) spaced 1 mm apart and overlaid with electroporation medium as described above. A single DC pulse of 1.2 kV cm⁻¹ for 30 µs was used for electrical stimulation. After a 2 min recovery, the oocytes were transferred to 500 µl of Whitten's medium and cultured at 39°C in an atmosphere of 5% CO₂ in air. The oocytes were fixed at 1.5, 3, 6, 12, 18 and 24 h after electrical stimulation for immunocytochemistry.

Immunofluorescence microscopy

At specific time points, the oocytes were permeabilized in a modified Buffer M (Simerly and Schatten, 1993; 25% glycerol, 50 mmol KCl l⁻¹, 0.5 mmol MgCl₂ l⁻¹, 0.1 mmol ethylenediaminetetraacetic acid l⁻¹, 1 mol β-mercaptoethanol l⁻¹, 50 mmol imidazole l⁻¹, pH 6.7, 3% (v/v) Triton X-100, and 25 µmol phenylmethylsulfonyl fluoride l⁻¹) for 20 min, fixed in methanol at 20°C for 10 min and stored in PBS containing 0.02% (w/v) sodium azide and 0.1% (w/v) BSA for 2–7 days at 4°C.

Microtubule localization was performed using α-tubulin monoclonal antibody (Sigma). Fixed oocytes were incubated for 90 min at 39°C with antibody diluted 1:300 in PBS. After several washes with PBS containing 0.5% Triton X-100 and 0.5% BSA, oocytes were incubated in a block solution (0.1 mol glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered milk, 0.5% BSA and 0.02% sodium azide l⁻¹) at 39°C for 1 h. The blocking was followed by incubation in Rhodamine-labelled goat anti-mouse antibody (Sigma). DNA was observed by exposure to 5 µg TOTO-3 ml⁻¹ (Molecular probe, Eugene, OR). Stained oocytes were then mounted under a coverslip with antifade mounting medium (Vectashild, Vector Lab. Burlingame, CA) to retard photobleaching.

Slides were examined by a laser-scanning confocal microscope using a BIO-RAD MRC 600 equipped with a Krypton-argon ion laser. Images were recorded digitally and archived on an erasable magnetic optical diskette.

Drug treatments

The effects of nocodazole (a microtubule inhibitor), cytochalasin B (a microfilament inhibitor), and taxol (a microtubule stabilizer on pronuclear formation and movement) were examined during *in vitro* fertilization. Stock solutions of 1 mmol nocodazole l⁻¹ (Sigma), 5 mmol of cytochalasin B l⁻¹ (Sigma) and 1 mmol taxol l⁻¹ (Sigma) in dimethyl sulfoxide were used. The stock solution was stored at -20°C and diluted to 10 µmol nocodazole l⁻¹ and 5 µmol cytochalasin B l⁻¹ in

Fig. 1. Laser scanning confocal microscopic images of microtubules, microfilaments and chromatin in the pig oocyte after fertilization. Green, microtubules; blue, microfilaments; red, chromatin. (a) Microtubules were observed in the meiotic spindle in the oocyte at metaphase II. Two microfilament domains: a microfilament-rich domain and a microfilament-thin domain, were observed. (b) After sperm penetration, microtubules were found in association with the incorporated sperm head. (c, d) The same oocyte observed at different focal points. (c) When focused on the female nucleus (about 50 nm from the surface), microfilaments were concentrated to the female structure. (d) Microfilaments were also observed within the male chromatin (d). (e) At the time of pronuclear centralization, microtubules filled the cytoplasm. (f) Microfilaments were concentrated in both the male and female nuclear structure during pronuclear movements. (g) An astral bipolar spindle was observed at metaphase and microfilaments were also observed in the cortex. (h) At the time of division to the two-cell stage, thick microfilament furrows were observed between blastomeres. Scale bar represents 25 nm.

NCSU23 before treatment of oocytes. Oocytes were treated with drugs between 6 h and 12 h, and between 12 and 24 h after insemination. At least three replicates were conducted for each experiment.

Statistical analysis

Statistical differences were determined by Fisher's protected least significant difference test.

Results

Microtubule and microfilament dynamics during fertilization and parthenogenesis

In oocytes at the metaphase II stage, microtubules were detected in the second meiotic spindle, and two microfilament domains (a microfilament-rich and a microfilament-thin domain) were present in the egg (Fig. 1a). In almost all of the unfertilized mature oocytes (76 of 89, 85%), the metaphase chromatin with spindle was located in the thick microfilament domain (Fig. 1a). Male pronuclear formation was observed between 9 h (24 of 107, 22%) and 12 h (69 of 104, 66%) after insemination. At 9 h after sperm insemination, the sperm asters (55 of 97, 57%) were observed adjacent to the incorporated sperm head (Fig. 1b). Polyspermic penetration was found in 46 (47%) zygotes at 9 h. As observed by Kim *et al.*, (1996b) in polyspermic zygotes, multiple sperm asters were found in association with each penetrated spermatozoon. During pronuclear formation, microfilaments became concentrated to both the male and female chromatin (Fig. 1c, d). At pronuclear apposition (21 of 69, 30% at 24 h after insemination), microtubules organized by sperm components filled the cytoplasm and microfilaments became concentrated to both the male and female pronuclei (Fig. 1e, f). At mitotic metaphase (3 of 69, 4% at 24 h after insemination), microtubules were detected in the spindle and microfilaments were found mainly in the cortex (Fig. 1g). During cleavage (4 of 69, 6%), large asters filled each daughter blastomere (data not shown) and a microfilament-rich cleavage furrow was observed (Fig. 1h). After electrical stimulation, a pronucleus was observed between 3 h (15 of 46, 33%) and 6 h (29 of 43, 67%). During pronuclear formation after electrical stimulation, microfilaments concentrated to the female pronuclear structure and a dense network of microtubules was found throughout the cytoplasm (Fig. 2a, b). Under the same culture conditions, the pig oocytes were cultured to establish

Table 1. Development *in vitro* of pig oocytes after insemination and electrical stimulation

Treatment	Number of oocytes examined	Number of oocytes developed (%)	
		Two-cell cleavage	Morulae/blastocysts
Insemination	130	79 (61)	38 (29)
Electrical stimulation	129	71 (55)	31 (24)

cleavage and developmental ability. The rates of normal cleavage and the developmental ability of pig oocytes to the morula and blastocyst stages after *in vitro* fertilization or electrical stimulation are summarized (Table 1).

Effects of cytoskeletal inhibitors during pronuclear formation and apposition

Treatment with cytochalasin B after electrical stimulation or insemination inhibited microfilament polymerization, but did not affect pronuclear formation or movement (Fig. 2c). However, gamete union and cell division were not observed in the cytochalasin B treated eggs (Table 2). Treatment with nocodazole after sperm penetration inhibited microtubule assembly and prevented migration leading to centralized pronuclei and cell division (Fig. 2d, Table 2). Neither the microtubule nor the microfilament inhibitor inhibited pronuclear formation (Fig. 2e). Taxol, which stabilizes microtubules (Maro *et al.*, 1986; Schatten *et al.*, 1989), did not prevent pronuclear formation but did prevent pronuclear movement. After nocodazole treatment, taxol induced microtubules in the cytoplasm (Fig. 2f). After 3 h recovery from nocodazole, treatment with taxol induced formation of microtubules in the cytoplasm (Fig. 2g). Treatment with cytochalasin B induced multiple pronuclei in the cytoplasm, and a 3 h recovery period allowed the formation of microfilaments in the cytoplasm (Fig. 2h).

Discussion

This study examined the integral distribution of microtubules and microfilaments and demonstrated a need for their assembly for pronuclear formation and migration, as well as cleavage after fertilization and parthenogenesis, in the pig oocyte. The

Fig. 2. Laser scanning confocal microscopic images of microtubules, microfilaments and chromatin in pig oocytes after parthenogenetic activation and fertilization. Green, microtubules; blue, microfilaments; red, chromatin. M, male chromatin; F, female chromatin (a) During pronuclear formation after parthenogenetic activation, microfilaments were concentrated in the pronucleus. (b) A dense network of microtubules was observed in the parthenote during pronuclear movements. (c) Treatment with cytochalasin B after parthenogenetic activation inhibited microfilament polymerization, but did not inhibit female pronuclear formation. (d) During fertilization, nocodazole did not inhibit male and female pronuclear formation, but did prevent centralization of pronuclei during fertilization. (e) During fertilization, treatment with either nocodazole or cytochalasin B did not inhibit pronuclear formation, but did inhibit syngamy. (f) Treatment with taxol induced microtubule foci in the cytoplasm during pronuclear formation after parthenogenetic activation. (g) After recovery from the microtubule inhibition, taxol induced microtubules were seen in the cytoplasm. After parthenogenetic activation, two pronuclei were observed in the cytoplasm (arrows). (h) Recovery from treatment with cytochalasin B allowed microfilament formation in the cytoplasm. Three pronuclei were observed in the cytoplasm during parthenogenesis (arrows). Scale bar represents 25 μ m.

Table 2. Treatment *in vitro* of pig oocytes with nocodazole and cytochalasin B between 12 h and 24 h after insemination

Treatment	Number of oocytes examined	Number of oocytes (%) with			
		Female pronucleus migration	Male pronucleus migration	Syngamy	Mitosis (cell division)
Control	56	42 (75)	32 (57)	16 (29)	4 (7)
Nocodazole	48	15 (31)*	2 (4)	—	—
Cytochalasin B	61	47 (77)	27 (44)	4 (7)	—

* $P < 0.05$ compared with controls.

sperm aster enlarged during sperm decondensation and extended throughout the cytoplasm at the time of pronuclear apposition. Treatment with nocodazole inhibited pronuclear migration, suggesting a role for microtubules during pronuclear apposition in the pig. In most animals, the microtubules of the sperm aster are responsible for moving the male and female pronuclei from the inner face of the oocyte cortex in the egg cytoplasm (Albertson, 1984; Schatten *et al.*, 1986; Le Guen and Crozet, 1989; Yllera-Fernandez *et al.*, 1992; Long *et al.*, 1993; Breed *et al.*, 1994). However, in mice, numerous cytoplasmic microtubule foci have been observed in the cytoplasm (Maro *et al.*, 1985; Schatten *et al.*, 1985). During pronuclear development, the asters containing microtubules increased in size as the cytoplasm became filled with a microtubule matrix. Although the pattern of microtubule configuration during mouse fertilization is atypical, microtubule activity is required to achieve pronuclear union (Schatten *et al.*, 1986). Pronuclei are embedded within the microtubule matrix and, by a process involving both assembly and disassembly, the male and female pronuclei are moved into apposition at the centre of the cell. Rieder and Salmon (1994) proposed that the female nucleus is covered with dynein-like, minus-end directed motors that translocate the female pronucleus from the periphery of the sperm aster towards its centre. While the sperm aster enlarges, it is likely that it moves the decondensing male pronucleus to the centre of the oocyte. The force responsible for male pronuclear movement may be the aster ejection force, which consists of kinesin-like, plus-end directed motors (Schatten, 1994).

The distribution of microfilaments has been studied in mammalian ova. In mature mouse (Maro *et al.*, 1984) and rat (Zernicka-Goetz *et al.*, 1993) oocytes, microfilaments are located mainly in the cell cortex overlying the meiotic spindle. This domain, rich in microfilaments, appears to be responsible for maintenance of the meiotic spindle and chromosomes in a peripheral position (Webb *et al.*, 1986). Maro *et al.* (1984) found that after fertilization of mouse oocytes, this domain disappeared and microfilaments were concentrated around pronuclei. Kim *et al.* (1996c) demonstrated that, in mature pig oocytes, two domains (a thick and a thin microfilament domain) are found in the oocyte cortex. Chromosomes are located in the thick microfilament domain of the cortex, which may be important for polar body extrusion and normal development during fertilization. In the present study, microfilaments in the pig oocyte concentrated in both the male and female chromatin after sperm penetration. Although the mechanism controlling

microfilament polymerization during fertilization is elusive at present, microfilaments above both nuclear structures may arise from a polymerization of actin recruited from the egg cytoplasm. Van Blerkom and Bell (1986) have shown that, in mice, chromosomes gain the capacity to modify the organization of microfilaments in their vicinity during oocyte maturation. After sperm penetration, a rapid series of increases of intracellular calcium ions has been observed in many species (Mivazaki *et al.*, 1993; Machaty *et al.*, 1997). Battaglia and Gaddum-Rosse (1987) showed that an increase in calcium ions in the rat egg cytosol was directly or indirectly associated with changes in the actin cytoskeleton. Therefore, after sperm penetration or parthenogenetic activation an increase in calcium ions may affect cortical microfilament assembly. In this study, treatment with cytochalasin B did not completely inhibit movement of both pronuclei after pronuclear formation, but did prevent union of the nuclei at the oocyte centre. Webb *et al.* (1986) demonstrated that a microfilament-rich domain initially overlying the meiotic spindle disappeared during ageing of the mouse oocyte. In such conditions, the spindle with chromatin migrated toward the centre of the egg. In aged pig oocytes, the metaphase chromatin is also frequently located outside of the microfilament rich domain (Kim *et al.*, 1996a). Migration of chromatin from the dense microfilament domain may be the major cause of formation of two female pronuclei without polar body extrusion in aged eggs since polar body extrusion is dependent upon the organization of the microfilaments (Maro *et al.*, 1986). In sheep oocytes, after extended exposure to cytochalasin D, the metaphase spindle of the second meiotic division migrated toward a subcortical region and became tangentially, instead of radially, oriented (Le Guen *et al.*, 1989). Therefore, previous results and the present study suggest that microfilaments are involved in maintaining the spindle in a cortical position. Disruption of microfilament assembly may result in the release of chromatin from the cortex of the oocyte.

Advances in *in vitro* maturation and *in vitro* fertilization have increased the availability of pig embryos for the study of early zygotic development. However, abnormal spindle formation, asynchronous pronuclear formation and an abnormally high incidence of polyspermy have repeatedly been observed in pig oocytes after *in vitro* maturation and *in vitro* fertilization (Funahashi *et al.*, 1994; 1996; Kim *et al.*, 1996a,b). Normal and abnormal patterns of nuclear structures and cytoskeletons during maturation, *in vitro* fertilization and parthenogenetic activation have been observed in pigs (Kim *et al.*, 1996 a,b,c; Funahashi *et al.*, 1996) suggesting that inadequate culture

conditions during *in vitro* maturation and *in vitro* fertilization impair the function of cytoplasmic organelles, including microtubule and microfilament assembly, and results in abnormal pronuclear formation after fertilization and a lower incidence of early embryonic development. In the present study, the roles of microtubules and microfilaments and their interactions during fertilization and parthenogenesis in pig oocytes were examined. The results help to provide insight into strategies for improving *in vitro* maturation and *in vitro* fertilization conditions in pigs.

In conclusion, the results of the present study suggest that both microtubules and microfilaments are integrated during fertilization and may not be required for pronuclear formation, but are required for the union of sperm and egg nuclei leading to subsequent cell division.

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