MOLECULAR REPRODUCTION AND DEVELOPMENT 71:256–261 (2005)

Small GTPase RhoA Is Required for Ooplasmic Segregation and Spindle Rotation, but not for Spindle Organization and Chromosome Separation During Mouse Oocyte Maturation, Fertilization, and Early Cleavage

ZHI-SHENG ZHONG, LI-JUN HUO, CHENG-GUANG LIANG, DA-YUAN CHEN, AND QING-YUAN SUN*
State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

ABSTRACT RhoA, a small GTPase, plays versatile roles in many aspects of cell function such as stress fiber formation, cytokinesis, and cell polarization. In this study, we investigated the subcellular localization of RhoA and its possible roles during oocyte maturation and fertilization. RhoA was localized in the cytoplasm of eggs from the germinal vesicle (GV) stage to 2-cell stage, especially concentrating in the midbody of telophase spindle when oocyte extruded PB1 and PB2. The RhoA kinases (ROCKs) specific inhibitor Y-27632 blocked GV breakdown (GVBD) and first polar body extrusion, but did not affect apparatus formation and anaphase/telophase I entry. Anti-RhoA antibody microinjection into the oocytes showed similar results. RhoA inhibitor caused abnormal organization of microfilaments, failure of spindle rotation, PB2 extrusion as well as cleavage furrow formation, while sister chromatid separation was not affected. Microinjection of RhoA antibody also blocked PB2 emission. Our findings indicate that RhoA, by regulating microfilament organization, regulates several important events including GVBD, polar body emission, spindle rotation, and cleavage. *Mol. Reprod. Dev. 71: 256–261, 2005.* © 2005 Wiley-Liss, Inc.

Key Words: RhoA; meiosis; oocyte; fertilization; mouse; microfilament

INTRODUCTION

Mammalian follicles grow continuously, but oocytes remain at germinal vesicle (GV) stage until sexual maturation. After sexual maturation, these primary oocytes gain the potential to resume meiosis under the stimulations of hormones, cytokines, and other factors. Extracellular stimulations, such as hormones and cytokines, induce the change of protein kinase/phosphatase activities, and thus trigger oocyte maturation. Following completion of meiosis I, the oocyte will undergo asymmetric cytokinesis, yielding a secondary oocyte and emitting the first polar body (PB1). Oocytes will again arrest at metaphase of meiosis II until the sperm-induced resumption of meiosis occurs. In mouse, fertilization caused 90° spindle rotation and segregation of sister chromatids to opposing poles of the meiotic spindle, followed by subsequent restoration of a diploid genome by syngamy and emission of the second polar body (PB2). Zygote enters the first mitosis after DNA duplication.

The small GTPases RhoA, together with Rac and Cdc42, belongs to the Rho family. RhoA activation leads to the assembly of contractile actin-myosin filaments (stress fibers) and formation of focal adhesion complexes to regulate diverse cellular processes including cell morphology and motility, tumor metastasis, smooth muscle cell contraction, morphogenesis, etc. (reviewed by Hall, 1998; Etienne-Manneville and Hall, 2002).

Recent studies have suggested that the RhoA signaling participates in oocyte maturation, cortical granule exocytosis, protein synthesis control, and cleavage in sea urchin eggs (Mabuchi et al., 1993; Nishimura et al., 1998; Cuellar-Mata et al., 2000; Manzo et al., 2003; Covian-Nares et al., 2004). RhoA is also reported to regulate ooplasmic segregation and cytokinesis at the first cleavage of ascidian eggs after fertilization (Yoshida et al., 2003). RhoA is possibly related to the polarization of early mouse blastomeres (Clayton et al., 1999).

In this study, by using immunestaining, specific inhibitor and antibody microinjection, we investigated the distribution and function of RhoA during mouse oocyte maturation, fertilization, and early embryo cleavage.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 30225010, 30371037; Grant sponsor: Special Funds for Major State Basic Research (973) Project (G1999055902).

*Correspondence to: Qing-Yuan Sun, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China.
E-mail: sunqy1@yahoo.com; sunqy@ioz.ac.cn

Received 10 September 2004; Accepted 12 November 2004
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20253

MATERIALS AND METHODS

Collection of Oocytes

GV oocytes were isolated from 8-week-old female Kunming mice 45–48 hr after injection with pregnant mares' serum gonadotropin (PMSG) and cultured in M2 culture medium supplemented with 150 mM 3-isobutyl-1-methylxanthine (IBMX). The oocytes were washed three times in M2 containing 150 mM IBMX and cultured in the same medium to maintain meiotic arrest. Meiotic maturation was initiated by rinsing the oocytes three times in CZB culture medium without IBMX. After maturing in vitro for 3 hr, GV breakdown (GVBD) stage oocytes were collected. After maturing in vitro for 8 hr, MI stage oocytes were collected. After maturing in vitro for approximately 12 hr, PB1 emission oocytes were collected. MII oocytes were obtained from oviducts 15 hr after human chorionic gonadotropin (Hcg) injection, and then digested by 300 IU/ml hyaluronidase to remove the cumulus cells (Gao et al., 2002). All chemicals from Sigma, St. Louis, MO, unless otherwise specified.

Fertilization In Vitro

Mouse sperm were obtained from the cauda epididymis of 10-week-old Kunming males and collected into CZB medium supplemented with 20 mg/ml BSA and 20 mM HEPES. Cauda epididymal sperm were capacitated in CZB medium supplemented with 20 mg/ml BSA and caffein for 1 hr. Fertilization in vitro was performed at a concentration of $2–3 \times 10^6$ motile sperm/ml (Fraser and Drury, 1975). Eggs and zygotes were cultured in 50-μl drops of CZB medium under paraffin oil at 37°C in a humidified atmosphere containing 5% CO_2 (Chatot et al., 1989).

Antibody Microinjection

Except for GV stage oocyte microinjection, which was performed in M2 medium supplemented with 150 mM IBMX, 10% FBS, microinjection of other stage oocytes was performed in M2 medium supplemented with 10% FBS. RhoA monoclonal antibody (SC-418) was purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA). About 10 pl of solution of anti-RhoA IgG (200 μg/ml) was injected into the cytoplasm. Injected or uninjected oocytes were rinsed three times in M2 medium (Yao et al., 2004). Oocytes microinjected with the same volume of rabbit IgG (200 μg/ml) were used as sham control.

Laser Confocal Microscopy

Zona pellucida of eggs from GV stage to 2-cell stage was removed with acidified M2 (pH 2.5). Oocytes or embryos were fixed in 4% paraformaldehyde solution for 30 min at room temperature (RT). Specimens were permeabilized in permeabilization solution (0.5% Triton X-100, 20 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose, 0.02% NaN_3 in PBS) for 45 min at RT. Oocytes or embryos were incubated at 37°C for 1 hr or 4°C overnight with anti-RhoA antibody diluted 1:100. FITC-conjugated anti-mouse antibody (1:100) was used as secondary antibody (Zhongshan Biological Technology Co., Ltd, Beijing, China). Oocytes or embryos were stained with 10 μg/ml propidium iodide to visualize DNA. Finally, all specimens were mounted on glass slides with DABCO and scanned using a TCS-4D laser confocal microscope (Leica Microsystems, Bensheim, Germany).

Oocytes or embryos were incubated with FITC-conjugated anti-α-tubulin antibody (1:50; Sigma) to tag microtubules. Oocytes or embryos were incubated with FITC-Phalloidin (1:50, Sigma) to tag microfilaments.

Experimental Design

Experiment 1: The distribution of RhoA during mouse oocyte maturation, fertilization, and early embryo cleavage. Ooctyes at GV, GVBD, MI, anaphase I, telophase I, and MII stages, and eggs at 1, 2, 6, 20–24 hr of insemination, as well as 2-cell embryos, were collected for confocal microscopy.

Experiment 2: The effect of Y-27632, a RhoA kinase (ROCK) specific inhibitor, on spontaneous in vitro maturation and fertilization of mouse oocytes. The effect on GVBD, polar body emission, and cleavage were observed especially. Three groups, high concentration group 100 μM, low concentration group 10 μM, and control group were tested according to previous report (Tahara et al., 2002). A total of about 30–50 oocytes were used per group pre experiment. Each experiment was repeated three times. After assessment of GVBD, polar body emission and cleavage, ooctyes, or embryos were fixed for confocal microscopy observation of the change in microfilaments/microtubules or MII spindle rotation.

Experiment 3: The effect of RhoA antibody microinjection on oocyte maturation and fertilization. Each experiment was repeated three times.

Statistical Analysis

Data were analyzed by Chi-square test. If $P < 0.05$, difference was considered as statistically significant.

RESULTS

Distribution of RhoA During Mouse Oocyte Maturation, Fertilization, and Early Cleavage

RhoA was located in the ooplasm of the oocytes from GV stage to 2-cell stage, especially concentrated in the midbody of telophase I and II spindles, and the middle zone of two separated groups of chromosomes during the first cleavage after fertilization (Fig. 1), while RhoA showed no special concentration in the ooplasm when stained with secondary antibody alone (data no shown).

Effect of RhoA Inhibition on Oocyte Maturation

When GV oocytes were treated by Y-27632 for 3 hr, GVBD was inhibited in a dose-dependent manner (Table 1).

GVBD oocytes were collected after maturation in vitro for 3 hr, and randomly assigned into three groups

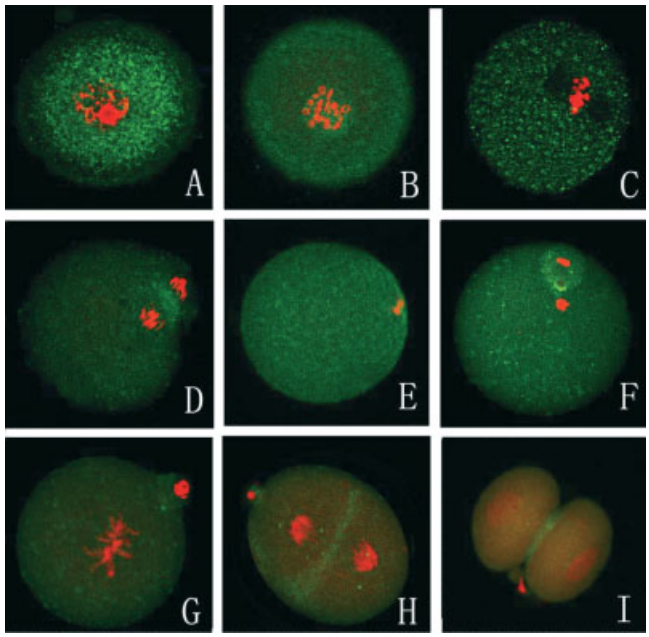

Fig. 1. Localization of RhoA (green) during mouse oocyte maturation fertilization and early cleavage. **A**: RhoA was located in the ooplasm of GV stage oocytes, but less intensive staining in the GV was observed. **B**: After GVBD RhoA concentrated in the region surrounding chromosomes. **C**: RhoA uniformly distributed in the ooplasm except for spindle region in MI stage oocyte. **D**: RhoA concentrated in the telophase I spindle midbody. **E**: RhoA uniformly distributed in the ooplasm of MII oocytes. **F**: Two hours after fertilization, RhoA concentrated at the midbody between eggs and PB2. **G**: RhoA showed no obvious concentration at the first mitotic metaphase. **H**: RhoA concentrated at the middle region between two separated nuclei during the first cytokinesis. **I**: RhoA concentrated in the cell junction of 2-cell stage embryo.

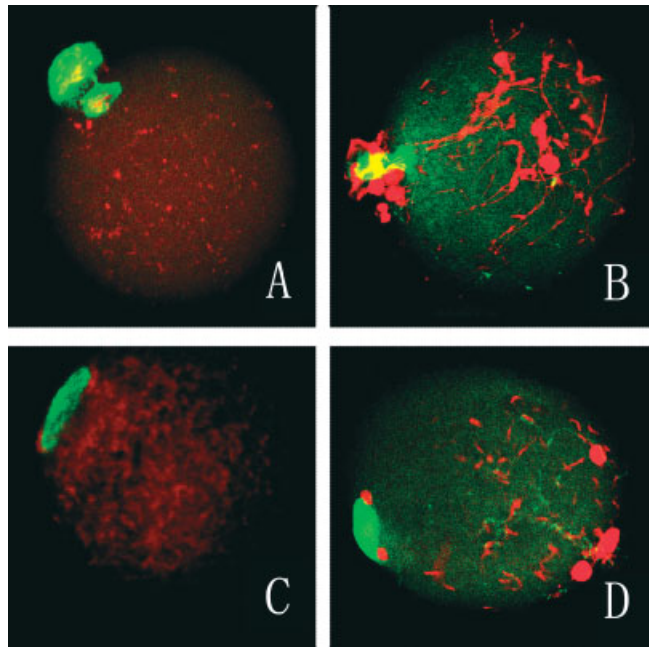

Fig. 3. The influence of Y-27632 treatment on mouse eggs microtubule (green) configuration and spindle rotation. Microtubules configuration of egg extruding PB1 or PB2 were showed in (**A**) and (**B**), respectively. Those spindles were perpendicular to egg membrane (A, B). Oocyte/eggs treated with Y-27632 could not extrude PB1/PB2, but they could assemble spindle, whose microtubule configuration appeared not obviously impaired (**C**, **D**). These telophase spindles were parallel to oocyte/egg membrane (C, D).

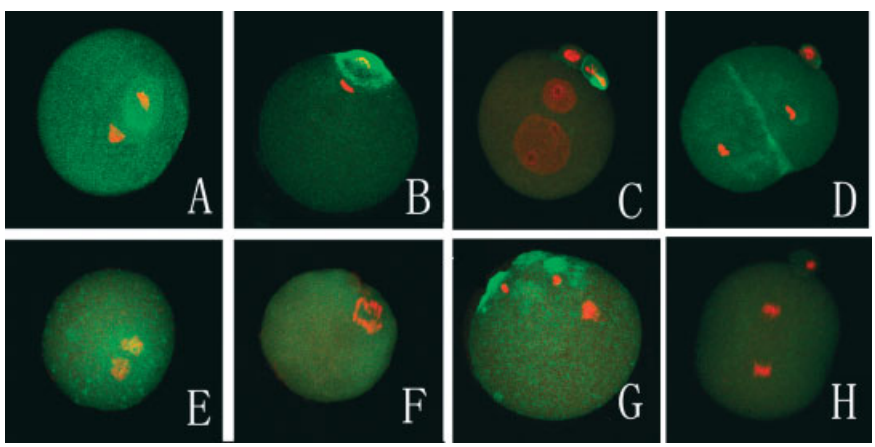

Fig. 2. The influence of Y-27632 treatment on ooytes/zygotes microfilament (green) configuration. **Panels A–D** depicted untreated oocytes. A: Microfilaments were polymerized and formed a capsule, which wrapped one of two separated groups of chromosomes during the first meiotic anaphase. B: Microfilaments polymerization was obviously observed beneath the membrane, where PB1 extrusion would take place. C: Six hours after IVF, when pronuclei had formed, microfilaments showed no obvious concentration in ooplasm except for the membrane region of polar bodies. D: Microfilaments were polymerized and cleavage furrow formed during cytokinesis. **E**: Part of GVBD oocytes treated with Y-27632 could enter the anaphase of meiosis I, but these oocytes showed no obvious concentration of microfilaments in ooplasm. **F**: When treated withY-27632, oocytes showed no obvious microfilament concentration in the membrane region where PB1 would be extruded. **G**: Six hours after fertilization in vitro, microfilaments in some Y-27632 treated zygotes showed microfilament polymerization beneath the membrane near female pronucleus, but PB2 extrusion was not observed in those zygotes. **H**: Y-27632 treated zygotes could completed nuclear division, but could not proceed to go cytokinesis. Those zygotes showed neither obvious microfilament concentration nor cleavage furrow formation.

TABLE 1. Effect of Y-27632 on GVBD During Mouse Oocytes Maturation

Groups	No. of oocytes assessed	GVBD oocytes (%)
Control group	91	73 (80.2%)[a]
10 μM group	89	34 (38.2%)[b]
100 μM group	83	11 (13.3%)[c]

Different superscripts mean statistical difference, $P < 0.05$.

TABLE 3. Effect of Y-27632 on Emission of PB2 After Insemination

Groups	No. of oocytes examined	No. of oocytes with extruded PB2 (%)
Control group	52	32 (61.5%)[a]
10 μM group	41	16 (39%)[b]
100 μM group	46	0 (0%)[c]

Different superscripts mean statistical difference, $P < 0.05$.

according to experimental design for 9 hr further culture. Emission of PB1 was assessed. Results showed that Y-27632 inhibited emission of PB1 in a dose-dependent manner (Table 2).

Effect of RhoA Inhibition on PB2 Emission After Sperm Penetration

In order to exclude the effect of Y-27632 on sperm binding to oocytes, Y-27632 was added into fertilization medium 30 min after insemination. After Y-27632 treatment for 1.5 hr, the emission of PB2 was assessed. Results showed that Y-27632 inhibited emission of PB2 in a dose-dependent manner (Table 3).

Effect of RhoA Inhibition on Cleavage

Y-27632 was added into medium 30 min after fertilization in vitro. After fertilization in vitro for 22 hr, cleavage was assessed. Results showed that Y-27632 inhibited cleavage of embryos (Table 4).

Effect of RhoA Inhibition on Polymerization of Microfilaments

Y-27632 has remarkable effects on the configuration of microfilaments in mouse oocytes/zygotes. After treated with Y-27632, part of GVBD oocytes (2/30) entered anaphase of meiosis I, but no concentration of microfilaments in the middle of two separated groups of chromosomes was observed, while a capsule of microfilaments was observed in the middle of two separated groups of chromosomes in the control oocytes. In Y-27632 treated zygotes, microfilaments were polymerized under the membrane near the female pronuclei, but PB2 was not extruded. Y-27632 also inhibited polymerization of microfilaments and formation of cleavage furrow, although it did not affect separation of chromosomes (Fig. 2).

Effect of RhoA Inhibition on Spindle Function and Rotation

After treated with Y-27632, spindles of meiosis I and II could form, and meiosis could proceed to telophase, but telophase spindles were parallel to oocyte membrane accompanying with failure of the PB1 and PB2 extrusion in all examined oocytes (n = 17, 22) (Fig. 3).

Effect of RhoA Antibody Microinjection on Polar Body Emission

Oocytes completed GVBD were collected at 3 hr and cultured for an additional 6 hr, and then microinjected with RhoA antibody or rabbit IgG. The antibody injected and sham injected oocytes were scored for the emission of PB1.The rate of PB1 emission was decreased from 50% (22/44) in the control group to 28.9% (13/45) in the microinjected group ($P < 0.05$).

MII oocytes were microinjected with RhoA antibody or rabbit IgG, and then cultured for 30 min in CZB. In vitro fertilization was performed by conventional procedure described above. The antibody injected and sham injected oocytes were scored for the PB2 emission. The rate of PB2 emission was decreased from 65.1% (28/43) in the control group to 45.6% (20/47) in the antibody microinjected group ($P < 0.05$).

DISCUSSION

RhoA, a member of small GTPase family, regulates the organization of the actin cytoskeleton (Massoumi et al., 2002; Maddox and Burridge, 2003), and it has an essential role in the accumulation of cortical actin and contractility of cleavage furrow (reviewed by Maddox and Oegema, 2003). It has been reported that RhoA participates in the sea urchin oocyte maturation, cortical granule exocytosis, and cleavage. RhoA was also found to play a role in the GVBD of mouse oocytes

TABLE 2. Effect of Y-27632 on Emission of PBl During Mouse Oocyte Maturation

Groups	No. of oocytes assessed	No. of oocytes with extruded PB1 (%)
Control group	83	63 (75.9%)[a]
10 μM group	77	23 (29.9%)[b]
100 μM group	82	4 (4.9%)[c]

Different superscripts mean statistical difference, $P < 0.05$.

TABLE 4. Effect of Y-27632 on Cleavage After Fertilization In Vitro

Groups	No. of eggs examined	No. of eggs cleaved (%)
Control group	34	28 (82.4%)[a]
10 μM group	28	19 (67.9%)[a]
100 μM group	29	11 (37.9%)[b]

Different superscripts mean statistical difference, $P < 0.05$.

(Cheon et al., 2000). We found that RhoA was expressed in the oocytes from the GV stage to MII stage, and inhibition of RhoA activity blocked GVBD as previously described. The mechanism of RhoA signaling participating in GVBD is not clear. Reactive oxygen species (ROS) may be one of candidate mediators in the RhoA-mediated GVBD. In mitotic cells, Rho is important for ROS production (Adachi et al., 2001). The generation of ROS induces oocyte maturation, whereas antioxidants block the resumption of meiosis (Takami, 2000). Microinjection of C3 transferase, or incubation with ROS scavengers, inhibited the production of ROS and GVBD (Cheon et al., 2000). Mitogen-activated protein kinase (MAPK), also called extracellular regulating kinase (ERK), is an important regulator of oocyte maturation (reviewed by Fan and Sun, 2004). RhoA signaling can activate ERK (Frost et al., 1996; Laboureau et al., 2004). But whether ERK pathway participates in regulation cytoskeleton formation mediated by RhoA is unclear.

Translocation of RhoA from the cytosol to the membrane fraction was considered as the activation of RhoA (Takaishi et al., 1995; Kusama et al., 2001). We observed that RhoA concentrated in the midbody of telophase spindle during meiosis I. Previous researches found that RhoA was located at the cleavage furrow of somatic cells and participated in cytokinsis (Lee et al., 2004; Yonemura et al., 2004). RhoA may be activated and shift to the midbody of telophase spindle and participate in the asymmetric division-emission of the polar body, since we found that the ROCK specific inhibitor, Y-27632, repressed mouse oocyte GVBD and the emission of PB1. Microinjection of RhoA antibody also inhibited emission of PB1.

In this study, we investigated the distribution and function of RhoA in fertilization. Our results showed that RhoA was mainly concentrated in the midbody of telophase II spindles. The inhibition of RhoA by Y-27632 blocked the emission of PB2 and cleavage after fertilization. Microinjection of RhoA antibody showed similar results. Two hours after fertilization, the polymerization of microfilaments was observed beneath the membrane near chromosomes in fertilized eggs treated with Y-27632, but the configuration of microfilaments was different to that of control. The formation of cleavage furrow was also inhibited after treatment with Y-27632. These results implied that RhoA is activated and participates in the extrusion of PB2 and cleavage after fertilization by regulation of microfilaments.

We also showed that the formation of meiosis I and II spindles and the separation of homologous chromosomes/sister chromatids were not affected by treatment with Y-27632, but telophase spindle of meiosis II was kept parallel to ooplasm membrane, with the failure of PB2 emission. In the mouse, the meiosis II spindle rotates 90° and becomes perpendicular to ooplasm membrane after sperm penetration (Maro et al., 1984). Our previous study showed that microfilaments participated in the rotation of spindle and the extrusion of PB (Zhu et al., 2003). Thus, RhoA activity is not required for spindle assembly and chromosomes/chromatids separation, but controls meiosis spindle rotation by regulation of microfilament polymerization.

CONCLUSIONS

RhoA, via action on microfilaments, regulates GVBD, meiosis spindle rotation, polar body emission, and cleavage, but not spindle assembly and chromosome separation during mouse oocyte maturation, fertilization, and early cleavage.

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