Temporary Inhibition of Germinal Vesicle Breakdown by Rho Kinase Inhibitor Y-27632 is Detrimental to Oocyte Maturation

Hiroyuki Suzuki, Kei-ichi Koyama, Katsuya Kabashima, Jiachen Fang and Masatoshi Matsuzaki*

Faculty of Agriculture and Life Science, Hirosaki University, Aomori 036-8561, Japan

Abstract: *In the present study, we investigated the effects of a specific Rho kinase (ROCK) inhibitor, Y-27632, on germinal vesicle breakdown (GVBD), changes of cytoskeletal organization, and the resumption of porcine oocyte maturation after its removal. In Experiment 1, cumulus-oocyte complexes (COCs) cultured in medium containing Y-27632 for 24 h showed GVBD of 98, 88, 84, and 4% (P<0.01) of the oocytes treated with 0, 1, 10, and 100 μM of the drug, respectively. In addition, Y-27632 treatment from 24 to 44 h of incubation significantly inhibited maturation of the oocytes. The metaphase II (MII) rates were 84, 78, 65, and 10% (P<0.01) for the corresponding groups, and 0, 2, 8, and 45% of the oocytes failed to show emission of the first polar body (PB1). In Experiment 2, resumption of meiotic maturation was examined after removal of Y-27632. COCs incubated with Y-27632 for 18 h and then cultured in Y-27632-free medium for an additional 44 h showed a significantly lower MII rate (86 vs. 33%, P<0.01). After the addition of Y-27632, cortical microfilaments of the oocytes decreased in density to approximately 40% of that of the controls. The present results suggest that Y-27632 inhibits GVBD and emission of PB1 in porcine oocytes and that ROCK inhibition for 18 h after the start of maturation incubation may be detrimental to and exert an irreversible effect on the progression of meiosis.*

Key words: *Pig Oocyte, Cytoskeleton, Maturation, Rho kinase inhibitor, GVBD*

Received: April 15, 2011
Accepted: June 6, 2011
*To whom correspondence should be addressed.
e-mail: suzuki@cc.hirosaki-u.ac.jp

Introduction

Follicular oocytes are able to complete nuclear maturation spontaneously *in vitro*, when liberated from their follicular environment and incubated in suitable culture media. Spontaneous oocyte maturation is characterized by germinal vesicle breakdown (GVBD) and chromosome condensation. After completion of the first meiosis, the oocytes reach the second metaphase (MII), which is characterized by emission of the first polar body (PB1) [1–5]. However, detailed mechanisms of meiotic maturation remain unclear.

Recent studies have suggested that RhoA, a member of small GTPase Rho family, may play a role in mouse oocyte maturation including GVBD, polar body extrusion, spindle rotation and cleavage [6, 7]. It has also been reported that RhoA regulates the organization of the microfilaments [8, 9], and it has an essential role in the accumulation of cortical actin and contractility of the cleavage furrow [10]. However, a role of RhoA/ROCK in the meiotic maturation of porcine oocytes has not been reported.

In the present study, we investigated the effects of a specific Rho kinase (ROCK) inhibitor, Y-27632, on GVBD, changes of cytoskeletal structures, and the resumption of porcine oocyte maturation after its removal.

Materials and Methods

Collection of Oocytes

Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) of ovaries collected from slaughtered prepubertal gilts. Only oocytes enclosed by compact cumulus cells with more than three layers were selected for the experiments. A

total of 599 COCs were used in this study. After being washed with Dulbecco's phosphate-buffered saline (DPBS) containing 0.1% polyvinyl alcohol (Sigma Chemical Co., MO, USA), groups of 10–15 COCs were transferred to 100-μl microdroplets of NCSU-23 medium [11] supplemented with 10% (v/v) porcine follicular fluid, 10 i.u./ml eCG (ASKA Pharmaceutical Co. Ltd., Tokyo, Japan) and 10 i.u./ml hCG (ASKA Pharmaceutical). COCs were cultured for the times described below in an atmosphere of 5% CO_2 at 39°C, as reported previously [12].

ROCK inhibition by Y-27632

Porcine oocytes are in the GV stage from 17.6 to 23.4 h of maturation incubation, and GVBD occurs between 17.6–26.4 h [13,14]. In Experiment 1, COCs were cultured in NCSU-23 medium containing 0, 1, 10 or 100 μM of Y-27632 (Sigma, Y0503) either for 0–24 h or for 24–44 h to assess the effects of the inhibitor on the progression of meiosis. In Experiment 2, COCs were treated with or without 100 μM of Y-27632 for 18 h, and then carefully washed to remove the inhibitor and cultured in inhibitor-free medium for an additional 10 h or 44 h to assess the resumption of meiosis and cytoskeletal organization in the oocytes after the Y-27632 treatment.

Fluorescence observations

After incubation, cumulus cells were removed from mature oocytes by vigorously vortexing in a 1.5-ml microtube for 1–2 min in a solution of 0.1% hyaluronidase (Sigma) in calcium-free DPBS. After removing the cumulus cells, the oocytes were processed for fluorescence observation as follows. The nuclear configuration and the distribution of microtubules and microfilaments were observed as reported previously [12]. Briefly, denuded oocytes were fixed in a microtubule stabilization fixative at 37°C for 1 h, washed extensively and blocked overnight at 5°C in the washing medium (calcium-free DPBS containing 2% BSA, 2% goat serum, 0.2% milk powder, 0.2% sodium azide and 0.1% Triton-X). The fixed samples were then exposed overnight to anti-β tubulin primary antibodies (1:200; Sigma) at 5°C, and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200; Sigma) at 37°C for 2 h. After rinsing, the oocytes were stained with rhodamine-phalloidin (1:1000; Molecular Probes, Eugene, OR, USA) to visualize microfilaments for 1 h, and then stained for DNA with Hoechst 33342 (10 μg/ml, Sigma) in mounting medium composed of DPBS and glycerol (1:1).

The samples were viewed on a laser scanning confocal microscope (FLUOVIEW FV1000D, Olympus, Tokyo, Japan) or a conventional fluorescence microscope (BX-FLA, Olympus) [12]. Image analysis was performed using ImageJ 1.45 (Wayne Rasband, NIH, USA) to calculate the average pixel intensities of the cortical microfilaments.

Statistical analysis

Data were subjected to one-way analysis of variance, after arc-sin transformation if needed, and differences between the means were determined using Tukey's multiple range test. Fluorescence intensity differences between the control and Y-27632 groups were analyzed with Student's t-test. The data are presented as the mean ± SEM.

Table 1. Effect of Y-27632 on GVBD in porcine oocytes during 24 h of maturation

Groups	No. of oocytes examined	% of GVBD oocytes
0 μM	40	98 ± 2^{A}
1 μM	41	88 ± 6^{A}
10 μM	42	84 ± 9^{A}
100 μM	44	4 ± 8^{B}

$^{A, B}$Values with different superscripts in the same column differ significantly ($P<0.01$).

Results

Effect of ROCK inhibition on oocyte maturation

The percentage of oocytes treated with Y-27632 for 24 h exhibiting GVBD are summarized in Table 1. Almost all (98%) of GV-stage oocytes without the inhibitor showed spontaneous maturation manifested by GVBD. The oocytes exposed to the lower concentrations of Y-27632 for 24 h after initiation of culture showed similar GVBD rates, 88 and 84% for the 1 and 10 μM groups, respectively, whereas the highest concentration of Y-27632 (100 μM) significantly inhibited GVBD to 4% of the oocytes. When GVBD oocytes were treated with Y-27632 from 24 to 44 h of maturation incubation, emission of PB1 was inhibited in a dose-dependent manner (Table 2). The nuclear configuration of the oocytes possessing no PB1 was sustained at the telophase.

Table 2. Effect of Y-27632 on first polar body (PB1) extrusion during porcine oocyte maturation*

Groups	No. of oocytes examined	% of MII oocytes with PB1	% of MII oocytes failing to emit PB1
0 μM	48	84 ± 5^{A}	0
1 μM	46	78 ± 3^{A}	2 ± 2^{B}
10 μM	49	65 ± 7^{A}	8 ± 4^{B}
100 μM	52	10 ± 1^{B}	45 ± 10^{A}

*Oocytes were cultured in NCSU-23 medium for 24 h and then incubated with different concentrations of the inhibitor for 20 h. [A, B]Values with different superscripts in the same column differ significantly (P<0.01).

Table 3. Effect of 18 h of incubation with Y-27632 (100 μM) on porcine oocyte maturation

Duration of incubation		No. of oocytes examined	% of GVBD oocytes	% of MII oocytes
With Y-27632	Without Y-27632			
–	18 h	34	32 ± 6^{B}	–
18 h	–	39	3 ± 3^{C}	–
–	28 h	37	100	33 ± 3^{B}
18 h	10 h	37	38 ± 2^{B}	0
–	44 h	48	88 ± 4^{A}	86 ± 3^{A}
18 h	44 h	42	89 ± 6^{A}	33 ± 2^{B}

[A, B, C]Values with different superscripts in the same column differ significantly (P<0.01).

Effect of time of ROCK inhibition on GVBD and oocyte maturation

When GV-stage oocytes were incubated in control culture medium for 18 h and 28 h, 32 and 100% of them, respectively, showed GVBD (Table 3). However, treatment with 100 μM of Y-27632 for 18 h inhibited GVBD by 97%. When GVBD-inhibited oocytes treated with Y-27632 for 18 h after initiation of culture were incubated in NCSU-23 medium without Y-27632 for an additional 10 and 44 h, 38 and 89% of them, respectively, showed GVBD. However, only 33% of the oocytes (P<0.01), which resumed meiotic maturation, reached the MII stage in the group incubated for an additional 44 h. Cortical microfilaments of the oocytes treated with Y-27632 decreased in density to approximately 40% of that of the control MII oocytes (19 ± 2 vs. 47 ± 5, P<0.01), even at 44 h after removal of the inhibitor.

Effect of ROCK inhibition on cytoskeletal organization

Porcine spindle orientation was usually perpendicular to the ooplasm membrane in the control MII oocytes (Fig. 1a). However, 47% of the Y-27632-treated oocytes, of which spindle orientation was determinable with certainty (n=17), showed an altered direction of the metaphase plate (Fig. 1b). These observations suggest that microfilament architecture may be affected by the ROCK inhibitor. However, there seemed to be no effect of Y-27632 on the microtubule dynamics, because the chromosomes were evenly arranged at the equatorial plane and the meiotic spindle was normally formed (Fig. 1).

The layer of cumulus cells expanded during maturation incubation, due to elongation of the cytoplasmic projections between the cumulus cells, which consisted of microtubules and microfilaments as reported previously [12]. However, cumulus cells remained compact in the Y-27632-treated COCs irrespective of the duration of maturation incubation.

Discussion

The present study revealed that ROCK is involved in GVBD and the emission of PB1 in porcine oocytes, as in mouse oocytes. It has been shown that RhoA participates in GVBD, polar body emission, spindle rotation, and cleavage of mouse oocytes [6, 7]. The mouse MII spindle rotates 90° and becomes perpendicular to the ooplasm membrane after sperm penetration [1]; microfilaments function as well in the extrusion of the polar body [2–5]. Therefore, RhoA/ROCK may play a role, via organization of

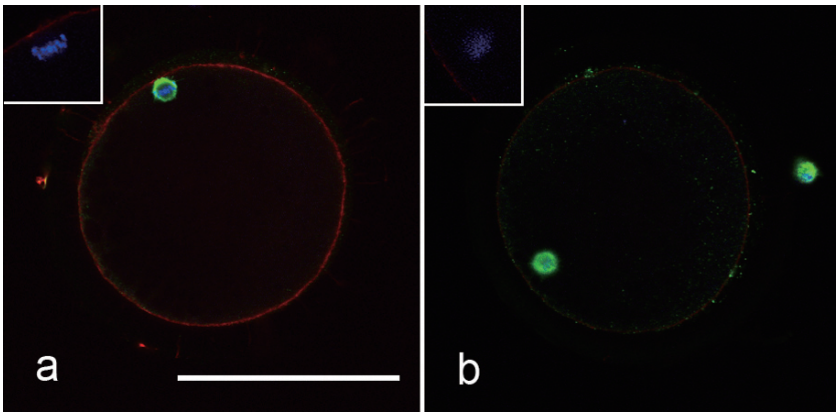

Fig. 1. Confocal images of a control MII oocyte (a) and a Y-27632-treated oocyte (b), which was incubated with 100 μM of Y-27632 for 18 h and then cultured in inhibitor-free medium for 44 h. PB1 is out of focus in each image. Microtubules are green, microfilaments are red, and chromatin is blue. The bar in (a) represents 100 μm. A close-up insertion shows signals for chromatin (blue) and microfilaments (red) only in each oocyte. The control MII oocyte (a) is characterized by a ring of submembranous microfilaments and a perpendicular spindle (at the eleven o'clock position). Note the close-up insertion of (a) indicating a normal arrangement of MII chromosomes. A Y-27632-treated oocyte (b) shows decreased density of the cortical microfilaments and a spindle oriented parallel to the apical surface (at the eight o'clock position). A close-up insertion of (b) demonstrates the altered direction of the metaphase plate (rotated approximately 90° compared to (a)). A cumulus cell is seen on the extreme right of (b). Means of fluorescence intensities of cortical microfilaments are 81 and 21 in (a) and (b), respectively.

microfilaments, in polar body emission, spindle rotation, and cleavage, because RhoA regulates the organization of the microfilaments [8, 9].

In contrast, ROCK inhibition suppressed GVBD in porcine oocytes, probably not via organization of microfilaments. Cheon *et al*. [6] have suggested that RhoA participates in GVBD by producing reactive oxygen species (ROS) in mouse oocytes. Further study of this aspect may be warranted. Cumulus expansion was blocked by the addition of Y-27632 to the incubation medium in this study. Recent research suggests that RhoA/ROCK signaling in cumulus cells may mediate extracellular matrix assembly in mice [15] in addition to reorganizing the cytoskeleton.

The present results demonstrate that the inhibitory effect of Y-27632 on GVBD was reversible in porcine oocytes, because Y-27632-treated oocytes were able to resume meiosis after its removal. Addition of Y-27632 18 h or later from the start of incubation failed to block GVBD. This would be explained by the observation that porcine oocytes are in the GV stage from 17.6 to 23.4 h of maturation incubation and GVBD occurs between 17.6–26.4 h [13, 14]. It is noteworthy that ROCK inhibition during the first 18 h of incubation may exert a detrimental effect on the subsequent progression of meiosis.

Cortical microfilaments of the oocytes treated with Y-27632 decreased in density to approximately 40% (P<0.01) of that of the control MII oocytes, even at 44 h after removal of the inhibitor. Porcine spindle orientation is normally perpendicular to ooplasm membrane [12, 16–18], but 47% of the MII oocytes (n=17) showed abnormal spindle orientation in the Y-27632-treated oocytes. These observations suggest that microfilament architecture may be irreversibly affected by Y-27632. However, there seemed to be no effect of Y-27632 on the microtubule dynamics, because of the even arrangement of chromosomes and normal spindle formation. Our previous reports showed that developmental competence of the oocyte is closely associated with cytoskeletal reorganization during oocyte maturation, which is affected by oocyte diameter [16], cumulus condition [17], and temperature fluctuation [18]. Defective organization of the cytoskeleton in the oocyte may decrease its viability, due to abnormal transport and distribution of the

organelles, such as mitochondria [17].

In conclusion, Y-27632 inhibits GVBD and emission of PB1 in porcine oocytes and ROCK inhibition in the 18 h from the start of maturation incubation may be detrimental and exert an irreversible effect on the progression of meiosis. Moreover, GVBD may be involved in direct signaling through the Rho-ROCK pathway, which may be different from the pathway including organization of microfilaments in polar body emission.

Acknowledgements

The authors thank the staff of the Gene Research Center at Hirosaki University for use of the image analyzing system and the staff of the Inakadate Meat Inspection Office (Aomori, Japan) for supplying pig ovaries. The present work was supported by a Grant-in-Aid for Scientific Research (C) (No. 21580340) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1) Maro, B., Johnson, M.H., Pickering, S.J. and Flach, G. (1984): Changes in actin distribution during fertilization of the mouse egg. J. Embryol. Exp. Morphol., 81, 211–237.
2) Verlhac, M.H., Kubiak, J.Z., Clarke, H.J. and Maro, B. (1994): Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. Development, 120, 1017–1025.
3) Zhu, Z.Y., Chen, D.Y., Li, J.S., Lian, L., Lei, L., Han, Z.M. and Sun, Q.Y. (2003): Rotation of meiotic spindle is controlled by microfilaments in mouse oocytes. Biol. Reprod., 68, 943–946.
4) Kim, N.H., Funahashi, H., Prather, R.S., Schatten, G. and Day, B.N. (1996): Microtubule and microfilament dynamics in porcine oocytes during meiotic maturation. Mol. Reprod. Dev., 43, 248–255.
5) Sun, Q.Y., Lai, L., Park, K.W., Kühholzer, B., Prather, R.S. and Schatten, H. (2001): Dynamic events are differently mediated by microfilaments, microtubules, and mitogen-activated protein kinase during porcine oocyte maturation and fertilization in vitro. Biol. Reprod., 64, 879–889.
6) Cheon, Y.P., Kim, S.W., Kim, S-J., Yeom, Y-I., Cheon, C. and Ha, K-S. (2000): The role of RhoA in the germinal vesicle breakdown of mouse oocytes. Biochem. Biophys. Res. Commun., 273, 997–1002.
7) Zhong, Z-S., Huo, L-J., Liang, C-G., Chen, D-Y. and Sun, Q-Y. (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. Mol. Reprod. Dev., 71, 256–261.
8) Massoumi, R., Larsson, C. and Sjolander, A. (2002): Leukotriene D(4) induces stress-fibre formation in intestinal epithelial cells via activation of RhoA and PKCdelta. J. Cell Sci., 115, 3509–3515.
9) Maddox, A.S. and Burridge, K. (2003): RhoA is required for cortical retraction and rigidity during mitotic cell rounding. J. Cell Biol., 160, 255–265.
10) Maddox, A.S. and Oegema, K. (2003): Closing the GAP: A role for a RhoA GAP in cytokinesis. Mol. Cell, 11, 846–848.
11) Petters, R. M. and Wells. K.D. (1993): Culture of pig embryos. J. Reprod. Fertil. (Suppl), 48, 61–73.
12) Suzuki, H., Jeong, B-S. and Yang, X. (2000): Dynamic changes of cumulus-oocyte cell communication during in vitro maturation of the porcine oocytes. Biol. Reprod., 63, 723–729.
13) Ocampo, M.B., Ocampo, L.T. and Kanagawa, H. (1990): Timing of sequential changes in chromosome configurations during the 1st meiotic division of pig oocytes cultured in vitro. Jpn. J. Vet. Res., 38, 127–137.
14) Wehrend, A. and Meinecke, B. (2001): Kinetics of meiotic progression, M-phase promoting factor (MPF) and mitogen-activated protein kinase (MAP kinase) activities during in vitro maturation of porcine and bovine oocytes: species specific differences in the length of the meiotic stages. Anim. Reprod. Sci., 66, 175–184.
15) Yodoi, R., Tamba, S., Morimoto, K., Segi-Nishida, E., Nishihara, M., Ichikawa, A., Narumiya, S. and Sugimoto, Y. (2009): RhoA/Rho kinase signaling in the cumulus mediates extracellular matrix assembly Endocrinology, 150, 3345–3352.
16) Suzuki, H., Fukudome, H., Takami, A. and Matsuzaki, M. (2008): Cytoskeletal and mitochondrial distributions in porcine oocytes at different germinal vesicle stages. J. Mamm. Ova Res., 25, 177–183.
17) Suzuki, H. and Saito, Y. (2006): Cumulus cells affect distribution and function of the cytoskeleton and organelles in the porcine oocytes. Reprod. Med. Biol., 5, 183–194.
18) Suzuki, H., Kumai, T. and Matsuzaki, M. (2007): Effect of temperature decline on the cytoskeletal organization of the porcine oocyte. J. Mamm. Ova Res., 24, 107–113.