Analysis of the Germinal Vesicle Requirement for the Activation of MPF in Maturation of Porcine Oocytes

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Abstract: *The necessity of the germinal vesicle (GV) for the activation of MPF during porcine oocyte maturation was investigated. Porcine follicular oocytes were enucleated without damaging the oolemma, and the enucleated oocytes without culture or cultured for 30 h were examined for their protein synthesis and MPF activities. The protein synthesis pattern in enucleated oocytes changed between before and after 30 h of culture, and this change was exactly the same as that of intact oocytes between before and after GV breakdown. The MPF activity increased about 7 and 5 times after 30 h of culture in cumulus enclosed and denuded oocytes, respectively. The activation of MPF was also observed in the enucleated oocytes after 30 h of culture and the MPF activity was the same level as that of denuded oocytes. These results indicate that the presence of GV is not required for normal protein synthesis during porcine oocyte maturation and that the generally suggested nucleus-cytoplasmic interactions, cyclin B movement into GV and the mixing of karyoplasm with cytoplasm, are not required for the MPF activation.*

Key words: *MPF, GVBD, Protein synthesis, Porcine oocytes, Enucleation.*

Maturation promoting factor (MPF) was first discovered as an activity that induces maturation in immature *Xenopus* oocytes [1]. Subsequently, this activity was identified in the oocytes of many species including mammals, and in many eukaryotic cells [2–4] during M-phase, and was renamed as M-phase promoting factor. Purified MPF from *Xenopus* oocytes has been shown to be composed of two major proteins [5], catalytic subunit cdc2 and regulatory subunit cyclin B. MPF activity elevates at the beginning of oocyte maturation, when germinal vesicle (GV) of an immature oocyte is broken down and the karyoplasm is mixed with the cytoplasm.

There is evidence which indicates interactions between the nucleus and cytoplasm at GV breakdown (GVBD). For example, cyclin B moves into the nucleus just before M-phase in starfish oocytes [6], mammalian somatic cells [7] and pig oocytes [8], and mixing of the karyoplasm with the cytoplasm is required for sufficient activation of MPF especially in starfish oocytes [9], and *Xenopus* oocytes [10]. In spite of its importance, it has never been investigated in mammalian oocytes, whether cyclin B movement into the nucleus is a prerequisite for the start of MPF activation, and whether the mixing of the karyoplasm with the cytoplasm is required for sufficient activation of MPF.

Here we studied the necessity of GV for MPF activation by removing it from the porcine immature oocyte. Generally, protein synthesis patterns change between before and after GVBD in intact oocytes [11–13] and in enucleated oocytes [14, 15]. We also examined the protein synthesis of enucleated porcine oocytes and discussed the relationship between MPF activation and protein synthesis.

Materials and Methods

Collection and culture of porcine oocytes

Ovaries of prepubertal gilts were collected at a local abattoir and transported to the laboratory at 37–39°C in saline. Cumulus-oocyte complexes (COCs) were aspirated with an 18 gauge needle and a syringe from follicles ranging in size from 2 to 5 mm. COCs with intact unexpanded cumulus cells were isolated from cellular debris and rinsed seven times in a culture medium consisting of modified Krebs-Ringer bicarbonate solu-

Received: July 12, 1999
Accepted: September 1, 1999
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tion (TYH, [16]) containing 20% porcine follicular fluid (pFF), 1.0 IU/ml pregnant mare's serum gonadotropin (Pramex; Sankyo, Tokyo, Japan) and 3.2 mg/ml BSA. Porcine FF was collected as described previously [17]. Some of the oocytes were denuded from the surrounding cumulus cells by pipetting gently with a fine-bore pipette, and some of the denuded oocytes were enucleated as described below. Groups of 20–25 COCs, denuded oocytes and enucleated oocytes were cultured in drops of 0.1 ml culture medium for 30 h at 37°C in an incubator with 100% humidity and 5% CO_2 in air. After culturing, COCs were treated with 150 IU/ml hyaluronidase (type IV-S, Sigma, St Louis, MO) in culture medium for a few minutes at room temperature, and the surrounding cumulus cells were removed as described above. The oocytes before and after culture were used for [^{35}S]-methionine labeling and MPF activity assay.

Enucleation

Denuded non-cultured oocytes were centrifuged at 15000 rpm for 6 min, so that GV became visible. Then the oocytes were incubated in culture medium supplemented with 5 μg/ml cytochalasin B for 15 min. Each oocyte was held with a holding pipette (outer diameter of 150 μm and inner diameter of 75 μm) and its zona immediately above the GV was cut with an injection pipette without damaging the oolemma. The zona cut oocyte was aspirated by the holding pipette, so that GV with small amount of cytoplasm was pushed out of the zona through the slit (Fig. 1). Karyoplast was separated from the enucleated oocytes by pipetting. Oocyte enucleation was performed within 3 h and the time at the end of manipulation was defined as 0 h of culture.

[^{35}S]-Methionine labeling

Intact and enucleated oocytes were labeled in a CO_2 incubator for 3 h (0–3 h or 30–33 h of culture) in culture medium containing [^{35}S]-methionine (1000 Ci/mmol, Amersham) at a radioactive concentration of 500 μCi/ml. Intact oocytes were denuded by pipetting as described above before labeling. Ten labeled oocytes were put in 8 μl of saline supplemented with 0.1% polyvinylpyrrolidone (PVP, Av. Mol. Wt. 10000; Sigma), added to 2 μl of 5 × Laemmli buffer [18], and denatured at 100°C for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, [18]) was performed using 10% polyacrylamide gels. After SDS-PAGE, the gel was dried on a 3MM filter paper (Whatman) and radio labeled bands were detected by the BASS system (FLA-3000; FUJIFILM).

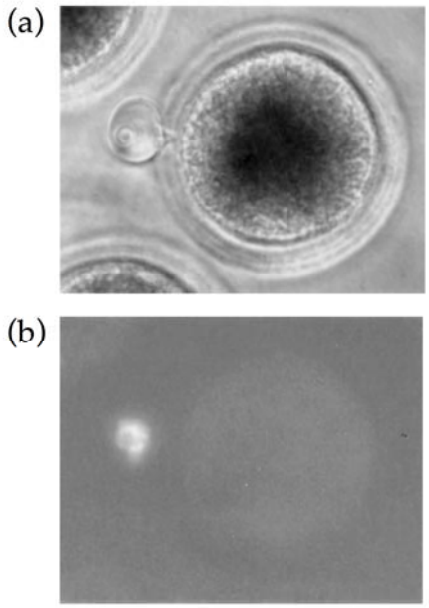

Fig. 1. (a) An enucleated oocyte with a karyoplast. (b) The same oocyte was stained in culture medium supplemented with 10 μg/ml Hoechst 33342 (Sigma) for 10 min, and examined by fluorescent microscope.

Histone H1 Kinase assay

Ten oocytes were lysed in 2.5 μl assay buffer (pH 7.2) composed of 15 mM EGTA, 1% Nonidet p-40, 60 mM sodium β-glycerophosphate, 30 mM *p*-nitrophenylphosphate, 25 mM Mops, 15 mM $MgCl_2$, 0.2 mM Na_3VO_4, 1 mM dithiothreitol (DDT), 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM phenylmethylsulphonylfluoride (PMSF) and 50 μM *p*-aminobenzoic acid (PABA), and frozen at –70°C until used. For assaying the histone H1 kinase activity, the lysate (2.5 μl) was added to 2.5 μl of 2.5 μM cAMP-dependent protein kinase inhibitor (Sigma), 5 μl of 2 mg/ml histone H1 (Sigma) and 5 μl of 0.1 mM [γ-^{32}P]ATP (0.4 mCi/ml), and the reaction was performed at 37°C for 1 h. Then the lysate was added to 5 μl of Laemmli buffer and denatured at 100°C for 5 min. SDS-PAGE was performed as described above. After SDS-PAGE, the gel was dried on a 3MM filter paper (Whatman) and exposed to X-ray film (FUJI MEDICAL) for 12 h. The activity of histone H1 kinase was measured as the density of the histone H1 bands evaluated by NIH image. The experiments were repeated at least 3 times.

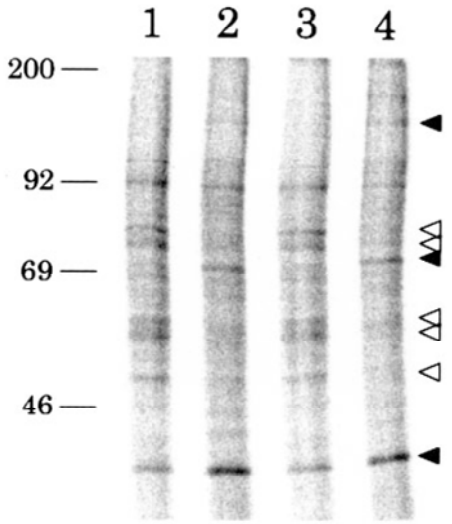

Fig. 2. Protein synthesis during porcine oocyte maturation. Intact (lanes 1, 2) and enucleated (lanes 3, 4) oocytes before culture (lanes 1, 3) and after 30 h of culture (lanes 2, 4) were radiolabeled with [^{35}S]-methionine for 3 h and the synthesized proteins were detected by the BASS system. The arrowheads indicate the major proteins increased (black) and decreased (white) after 30 h of culture, respectively.

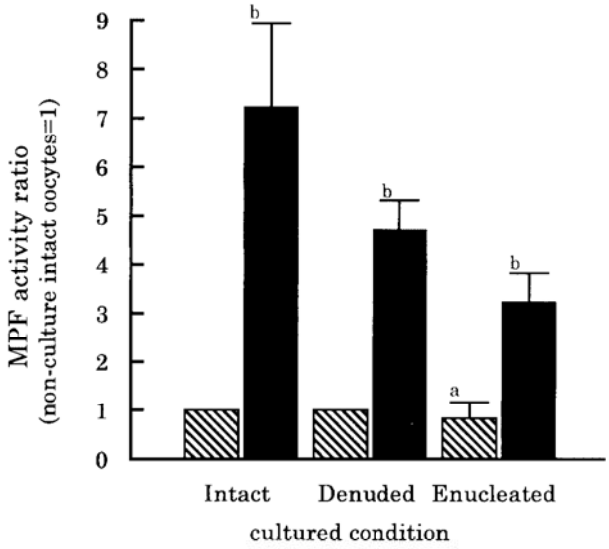

Fig. 3. MPF activity in oocytes cultured with (intact) or without (denuded) cumulus cells and in enucleated oocytes (enucleated). Data are shown as relative activity (mean ± SEM) of that in the non-cultured intact oocytes. The hatched bars indicate MPF activity of non-cultured oocytes, and black bars indicate that of oocytes after 30 h of culture. Bars with different superscripts are significantly different ($P<0.05$). Experiments were repeated at least 3 times.

Statistical analysis

Student's *t* test was used for evaluation of the results. A probability of $P<0.05$ was considered to be statistically significant.

Results

Protein synthesis during maturation

In our *in vitro* maturation system of porcine oocytes [17], GVBD took place mainly after 24 h of culture and most of the oocytes with or without cumulus cells were at the first meiotic metaphase at 30 h of culture (data not shown). As shown in Fig. 2, the protein synthesis pattern in intact oocytes changes dramatically between before (0–3 h: lane 1) and after (30–33 h: lane 2) GVBD. In the enucleated oocytes, the protein synthesis pattern also changes between 0–3 h and 30–33 h of culture and was exactly the same as those of control oocytes.

Histone H1 Kinase activity during enucleated porcine oocyte maturation

In oocytes cultured with and without cumulus cells, histone H1 kinase activity at 30 h of culture was increased about 7 and 5 times compared with the values at 0 h of culture, respectively (Fig. 3). The histone H1 kinase activity just after enucleation was the same as that of non-cultured intact oocytes. The activity was also elevated significantly about 4 times after 30 h of culture and was not statistically different from those of oocytes cultured for 30 h without enucleation ($P>0.05$).

Discussion

The present results showed that MPF activity after 30 h of culture elevates in oocytes cultured with or without cumulus cells as reported previously [19, 20]. Interestingly, in enucleated oocytes, activation of MPF was also observed after 30 h of culture, and the level of activation of MPF in enucleated oocytes was comparable with that of oocytes cultured without cumulus cells. This result indicates that cyclin B transfer into GV and the mixing of karyoplasm with cytoplasm are not required for the activation of MPF during porcine oocyte maturation. In starfish oocytes, it has been suggested that factors inhibiting activation of MPF and other factors inhibiting MPF activation inhibiting factors were present in cytoplasm and

karyoplasm, respectively. Therefore, the mixing of karyoplasm with cytoplasm is believed to be required for MPF activation in this species [9]. However, the present results indicate that porcine oocytes have no such factors as are supposed in starfish oocytes.

The MPF activity at 30 h of culture, and the level of enucleated oocytes was comparable with that of denuded oocytes, but tended to be lower than that of oocytes cultured with cumulus cells. We have previously shown that the MPF activity during porcine oocyte maturation is affected by the composition of maturation medium [21]. A lower activity was observed when oocytes were cultured in the medium from which cumulus cells were removed during maturation culture, indicating the importance of cumulus cells in maintaining sufficient activation of MPF in porcine oocytes. Therefore, the relatively low activity of MPF in 30 h-cultured enucleated oocytes might be due to the removal of cumulus cells rather than the absence of GV.

The present study showed that the protein synthesis pattern changed between before and after GVBD in intact porcine oocytes. Although each protein was not able to be identified in the present study, the patterns agreed well with previous reports [22]. The pattern of protein synthesis in the enucleated oocytes also changed between before and after culture, and the patterns were exactly the same as those of intact oocytes. In *Rana pipiens*, sheep and cattle [14, 15, 23], it has been reported that oocyte enucleation has no effect on the protein synthesis pattern both before and after maturation culture, and our results indicate that this is the case also in porcine oocytes.

It has been reported that a low amount of pre-MPF, the hyperphosphorylated non-active form of MPF, is present in immature porcine oocytes [24], and the low amount of pre-MPF is activated by dephosphorylation at the time of GVBD [25]. Cyclin B synthesis is indicated to start after GVBD, and this cyclin B synthesis increases the MPF activity further until it reaches a level sufficient to maintain the first meiotic metaphase. The present results have shown that proteins were synthesized normally in enucleated porcine oocytes, and therefore it is supposed that cyclin B was also synthesized normally, although the 62 kDa cyclin B band was not visible in the present study as described previously [24].

In summary, the present study shows that GV was not required for the normal protein synthesis during porcine oocyte maturation either before or after GVBD and that cyclin B movement into GV and the mixing of karyoplasm with cytoplasm were not required for the MPF activation.

Acknowledgements

This work was supported by Grand-in-Aid for Scientific Research (no. 10660267 and no. 11556051 to KN) from the Ministry of Education, Science, Sports and Culture of Japan.

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