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Okadaic acid-sensitive phosphatase is related to MII/G1 transition in mouse oocytes

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Summary

It is reported that okadaic acid (OA)-sensitive phosphatase is related to mitogen-activated protein kinase (MAPK)/p90rsk activation in mammalian oocytes. OA is also involved in the positive feedback loop between M phase-promoting factor (MPF) and cdc25c in *Xenopus* oocytes during meiotic maturation. However, the effect of phosphatase inhibition by OA on MPF and MAPK activities at the MII/G1 in oocytes remains unknown. The aim of this study is to clarify the relationship between OA-sensitive phosphatase and mitosis MII/G1 transition in mouse oocytes. MII-arrested oocytes were, isolated from mice, inseminated and cultured in TYH medium (control group) or TYH medium supplemented with 2.5 μM of OA (OA group). Histone H1 kinase and myelin basic protein (MBP) kinase activities were measured as indicators of MPF and p42 MAPK activities after insemination. Phosphorylation of cdc25c after insemination was analized in OA and control group by western blotting. Seven hours after insemination a pronucleus (PN) was formed in 84.1% (69/85) of oocytes in the control group. However, no PN was formed in oocytes of the OA group ($p < 0.001$). Although MPF and MAPK activities in the control group significantly decreased at 3, 4, 5, and 7 h after insemination, these decreases were significantly inhibited by OA addition ($p < 0.05$). Furthermore, OA addition prevented cdc25c dephosphorylation 7 h after insemination. In conclusion, OA-sensitive phosphatase correlates with inactivation of MPF and MAPK, and with the dephosphorylation of cdc25c at the MII/G1 transition in mouse oocytes.

Keywords: Cdc25c, MAPK, Mouse oocyte, MPF, Okadaic acid

Introduction

M phase-promoting factor (MPF) plays a crucial role in GII/prophase transition of *Xenopus* oocytes (Kobayashi *et al.*, 1991; Doree & Hunt, 2002), and it's activity is regulated by dephosphorylation of the cdc2 subunit at Thr14 and Tyr15, two negative regulatory sites (Cyert & Kirschner, 1988; Gautier & Maller, 1991; Kobayashi *et al.*, 1991). Myt1, membrane-associated Wee1 homolog, phosphorylates the cdc2 subunit on both Thr14 and Tyr15, and is suppressed by progesterone treatment through mitogen-activated protein kinase (MAPK)-induced activation of kinase p90rsk (Palmer *et al.*, 1998). In *Xenopus*, mice and pigs, MII-arrested oocytes show higher MPF and p42 MAPK activities than those oocytes not in GII/prophase transition (Kubiak *et al.*1992; Naito & Toyoda, 1992). Furthermore, studies in mice show that fertilization dramatically decreases MPF and p42 MAPK activities, resulting in a second polar body (PB) extrusion and the formation of a pronucleus (PN) (Moos *et al.*, 1995).

Okadaic acid (OA), a specific inhibitor of protein phosphatase (PP)1 and 2A, has been shown to induce an electrophoretic shift in the p42 MAPK band to its slow-migrating form and to increase p42 MAPK activity, resulting in germinal vesicle breakdown in mice (Gavin *et al.*, 1994; Chesnel &

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Eppig, 1995). Fan *et al.* (2003) reported that OA induced the prompt phosphorylation of MAPK and p90rsk in mouse, rat, and pig oocytes. Furthermore, the authors suggested that an OA-sensitive phosphatase-dependent inhibitory mechanism existed and that it prevented MAPK/p90rsk activation. They also observed that p90rsk dephosphorylation occurred following MAPK inactivation after parthenogenetic activation of rat oocytes (Tan *et al.*, 2001). On the other hand, dephosphorylation of Thr14 and Tyr15 on the cdc2 subunit is catalysed by the dual specificity phosphatase, cdc25c, the activity of which is inhibited by PP2A (Dunphy & Kumagai, 1991; Gautier & Maller, 1991; Millar & Russell, 1992). Some investigators have suggested that MPF can phosphorylate cdc25c in reverse to form a positive feedback loop, and amplify MPF activity during GII/prophase transition in *Xenopus* oocytes (Kumagai & Dunphy, 1992; Hoffmann *et al.*, 1993; Izumi & Maller, 1993). Furthermore, PP2A inhibition and Plx1 kinase activity are required for the initiation of this positive feedback loop in *Xenopus* oocytes. However, the effect of OA-induced PP2A inhibition in oocytes during MII/G1 transition and on MPF and MAPK activities remains unknown.

The aim of this study was to evaluate whether OA, a specific inhibitor of PP2A, is related to the MII/G1 transition in mouse oocytes during fertilization.

Materials and methods

Oocyte collection

B6C3F1 hybrid female mice (6–10-week-old) were superovulated by intraperitoneal injections of 10 IU pregnant mare serum gonadotropin (Nakarai Teque, Inc.) and human chorionic gonadotropin (hCG, Mochida Pharmaceutical Co.) given 48 h apart. Mice were anesthetized and sacrificed 14–16 h after hCG injection, and excised oviducts were placed in modified human tubal fluid (mHTF) solution at 37°C. Cumulus–oocyte complexes (COCs) were collected from oviducts and transferred to mHTF. Surrounding cumulus cells were removed by pipetting COCs through a fine-bore pipette in a solution supplemented with 1 mg/ml hyaluronidase (Sigma Chemical Co.). After rinsing denuded oocytes with fresh mHTF solution, oocytes with an extruded PB and without a germinal vesicle were selected for the following experiments.

In vitro fertilization

Epididymal spermatozoa were obtained from the cauda epididymis of 9–10-week-old B6C3F1 hybrid male mice and capacitated in 200 μl of insemination medium (TYH: NaCl 119.37 mM, KCl 4.78 mM, $CaCl_2{\cdot}2H_2O$ 1.71 mM, KH_2PO4 1.19 mM, $MgSO_4{\cdot}7H_2O$ 1.19 mM, $NaHCO_3$ 25.07 mM, sodium pyruvate 1 mM, glucose 5.56 mM, bovine serum albumin (BSA) 4 g/l, penicillin 100 IU/l, streptomycin 50 μg/ml) in 5% CO_2 and humidified air at 37°C for 2 h. Capacitated spermatozoa were transferred to 200 μl of TYH medium, supplemented with 0.4% BSA, containing 40–60 oocytes to make a final sperm concentration of 5×10^5 cells/ml. The inseminated oocytes were cultured in 200 μl of TYH medium supplemented with or without 2.5 μM OA (Chain *et al.*, 1999), and PP inhibitor, which indirectly promotes cdc25c, in 5% CO_2 and humidified air at 37°C for 1, 3, 5, and 7 h. After culture, oocytes were observed at ×400 magnifications under an inverted phase contrast microscope (IMT2-31, Olympus Diaphot). Oocytes with two pronuclei and two polar bodies were referred to as fertilized.

Histone H1 kinase and MBP kinase assay

Ten oocytes after culture were washed three times in assay buffer, which consisted of 60 mM β-glycerol phosphate, 30 mM *p*-nitrophenyl phosphate, 25 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS, pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 0.2 mM sodium vanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 50 μM *p*-aminobenzamidine, and stored at −80°C until use. Before the histone H1 kinase assay, oocyte suspensions were thawed at room temperature and centrifuged (15,000 *g*, 5 min, 4°C). The supernatant was used as the oocyte extract.

MPF and p42 MAPK activities were evaluated in terms of histone H1 kinase and myelin basic protein (MBP) kinase activities. Oocyte extracts (2.5 μl) were mixed with 2.5 μl of 2.5 μM cAMP-dependent protein kinase inhibitor (Sigma), 5 μl of 2 mg/ml of histone H1 solution (Sigma), 2.5 μl of 10 mg/ml MBP solution (Sigma), and 5 μl of 0.1 mM [γ-32P]ATP (0.4 mCi/ml, Amersham Pharmacia Biotech), and the kinase reaction was initiated by the addition of [γ-32P]ATP and performed at 37°C for 60 min. The kinase reaction was stopped by the addition of 5 μl of 5× Laemmli buffer and the mixture was heated at 100°C for 5 min. The mixture was subjected to SDS-PAGE, and the MPF and MAPK band activities were detected by autoradiography and quantified by measuring the band density with NIH Image software. The relative activities of MPF and MAPK were normalized to that of an MII oocyte, arbitrarily defined as 100%.

Western blotting

Phosphorylation of cdc25c was analysed by western blotting. Oocytes (n = 100) were transferred to a

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Table 1 Effects of okadaic acid on second polar body extrusion and pronuclear formation.

	Oocytes (*n*)	Oocytes with the second polar body *n* (%)	Oocytes with pronuclear formation *n* (%)
Control group	85	80 (94.1)	69 (84.1)
Okadaic acid group	53	5 (9.4)[a]	0 (0)

[a] $p < 0.05$ compared with control.

microtube with 2 μl of saline containing 0.1% PVP. A total of 0.5 μl of 5× Laemmli buffer was added to the microtube and proteins were denatured at 100°C for 5 min. Proteins were separated on a modified 10% polyacrylamide gel containing 0.1% SDS and electrically transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences UK). After blocking the membrane with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) at room temperature, the membrane was incubated at 4°C with an anti-cdc25c antibody (Santa Cruz Biotechnology Inc.), diluted 1:200 in TBS-T. After rinsing three times with fresh TBS-T, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (ECL Kit, Amersham Biosciences UK). Signals were visualized using ECL detection reagents (ECL Kit, Amersham Biosciences UK).

Statistical analyses

Data were analysed using Student's *t*-test and the chi-squared test. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of OA on *in vitro* fertilization

Extrusion of the second PB in our *in vitro* fertilization experiments was performed approximately 3 h after insemination. After 3 h, the level of oocytes with the second PB was significantly higher in the control group than in the OA group [91.4% (80/85) vs. 9.4% (5/53), respectively; $p < 0.05$]. Pronuclear formation was observed 7 h after insemination, and 84.1% (69/85) of oocytes in the control group showed pronuclear formation. However, no PN was found in the oocytes of the OA group (Table 1).

Effects of OA on histone H1 kinase and MBP kinase activities during fertilization

Fertilization significantly decreased histone H1 kinase and MBP kinase activities at 3, 4, 5, and 7 h after insemination compared with that of the MII oocyte (100%) (histone H1 kinase activity: 32.6, 26.5, 27.5, and 15%, respectively; MBP kinase activity: 71.0%, 35.0, 35.3, and 22.5%, respectively; $p < 0.05$), and the decrease in histone H1 kinase activity preceded the decrease in MBP kinase activity (Fig. 1). However, OA addition significantly suppressed the decreases in histone H1 kinase and MBP kinase activities during fertilization (histone H1 kinase activity in the control and OA groups: 3 h: 32.6% vs. 102%, 5 h: 27.5% vs. 63.5%, 7 h: 15% vs. 69.5%, respectively: $p < 0.05$; MBP kinase activity in the control and OA groups: 7 h: 22.5% vs. 59.8%, respectively; $p < 0.05$; Fig. 2).

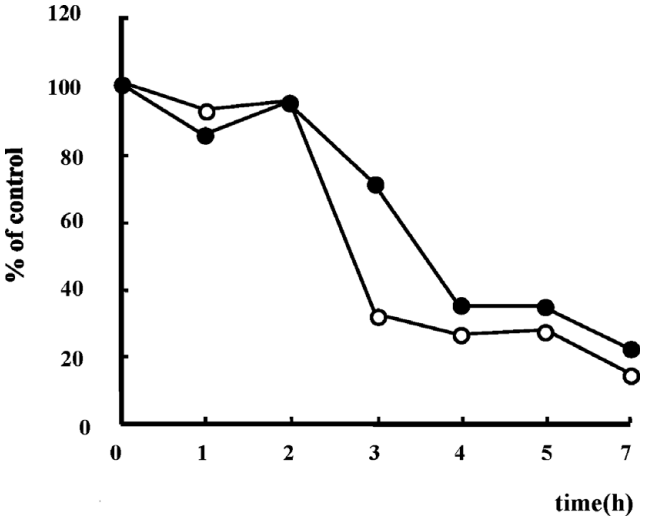

Figure 1 Histone H1 and myelin basic protein kinase activities after insemination. Inseminated oocytes were cultured in TYH medium in 5% CO_2 and humidified air at 37°C for 1, 3, 5, and 7 h. After culture, M phase-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities were evaluated in terms of histone H1 kinase (solid circles) and myelin basic protein kinase (open circles) activity. The relative activities of MPF and MAPK were normalized to those in an MII oocyte, arbitrarily defined as 100%. Data are expressed as the mean ± SD.

Effects of OA on cdc25c

The dephosphorylated form of cdc25c was observed 7 h after insemination in the control group. However, OA addition eliminated its dephosphorylated appearance (Fig. 3).

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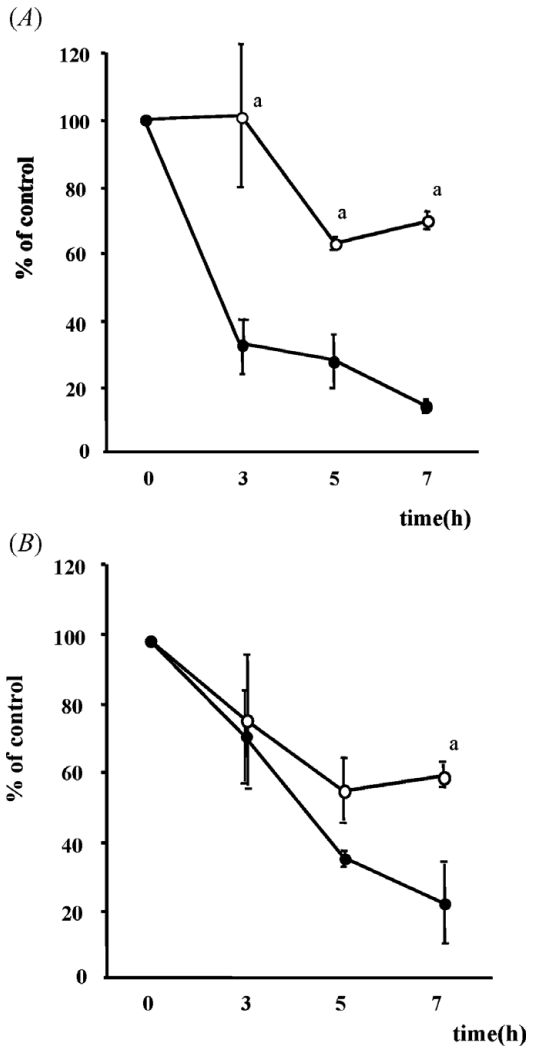

Figure 2 Effects of okadaic acid on histone H1 and myelin basic protein kinase activities after insemination. Inseminated oocytes were cultured in TYH medium with okadaic acid (OA group; open circles) or without okadaic acid (control group; solid circles) in 5% CO_2 and humidified air at 37°C for 1, 3, 5, and 7 h. After culture, M phase-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activities were evaluated in terms of histone H1 kinase and myelin basic protein (MBP) kinase activities. The relative activities of MPF and MAPK were normalized to the MII oocyte, arbitrarily defined as 100%. Data are expressed as the mean ± SD. (*A*) Histone H1 kinase activity. (*B*) MBP kinase activity (a: $p < 0.05$).

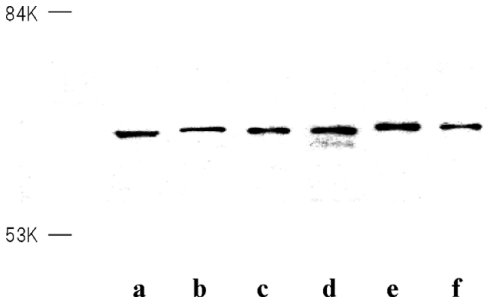

Figure 3 Effect of okadaic acid on cdc25c phosphorylation. Inseminated oocytes were cultured in human tubal fluid medium with or without okadaic acid (OA and control groups) in 5% CO_2 and humidified air at 37°C for 3, 5, and 7 h. The phosphorylation status of cdc25c was analysed by western blotting. Lanes: a, MII oocyte; b–d, 3, 5, and 7 h after insemination in the control group, respectively; e,f, 5 and 7 h after insemination in the OA group, respectively.

Discussion

In this study, OA inhibited second PB extrusion and PN formation is MII-arrested oocytes, and suppressed the decreases in both histone H1 kinase and MBP kinase activities induced during fertilization. By maintaining histone H1 kinase and MBP kinase activity levels during fertilization, the inhibition of both second PB extrusion and PN formation occurred, which suggested that these events were related.

Studies in knockout mice suggest that the role of MAPK appears to be the reactivation of MPF between MI and MII and the maintenance of arrest at MII (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994). It is well established that at the MI/MII transition MAPK activity is maintained at a higher level than in GII-arrested oocytes, despite a decrease in MPF activity, and the nuclear envelope cannot be formed during the same period. Furthermore, Moos *et al.* reported that elevating MAPK activity subsequent to PN formation resulted in precocious pronuclear envelope breakdown prior to entry into M phase (Moos *et al.*, 1995).

In the present study, PN formation was not observed under conditions of low histone H1 kinase activity and high MBP kinase activity 3 h after insemination. Furthermore, PN formation was only observed after histone H1 kinase and MBP kinase activities had been maintained at one-third the level of that in metaphase II oocytes for 7 h. These data indicate that MAPK inactivation after MPF inactivation is essential for pronuclear envelope formation.

The mechanism by which OA suppresses the decreases in histonH1 kinase and MBP kinase activities that are induced by fertilization has not been established. In this study, OA immediately suppressed the decrease in histone H1 kinase activity. Furthermore, suppression of the decrease in MBP kinase activity by OA appeared later than it did for histone H1 kinase. We found that the addition of OA eliminated the appearance of dephosphorylated cdc25c in our study. Dephosphorylation of Thr14 and Tyr15 on the cdc2 subunit is catalysed by the dual specificity phosphatase, cdc25c, the activity of which is prevented

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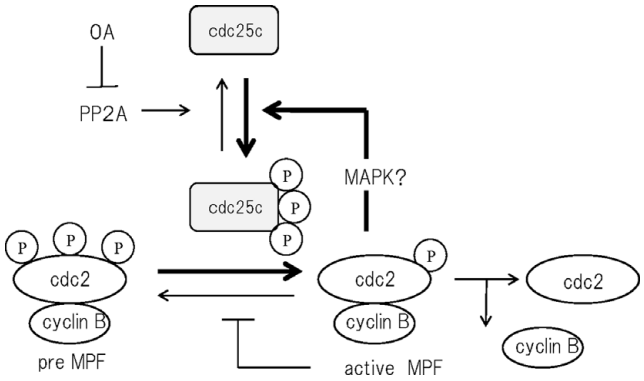

Figure 4 Proposed regulatory pathway for MPF, cdc25c, MAPK, and PP2A and the effect of OA on this pathway. OA is a specific inhibitor of PP2A. PP2A antagonizes cdc25c activation. Pre-MPF requires dephosphorylation of cdc2 on Tyr15 and Thr14 to become active MPF. Active MPF phosphorylates and activates cdc25c.

by PP2A (Dunphy & Kumagai, 1991; Gautier & Maller, 1991; Millar & Russell, 1992). Some investigators have suggested that MPF can phosphorylate cdc25c in reverse, form a positive feedback loop, and amplify MPF activity during GII/prophase transition in *Xenopus* oocytes (Hoffmann *et al.*, 1993; Izumi & Maller, 1993; Kumagai & Dunphy, 1992). At present, there is no reported evidence for the existence of a positive feedback loop between cdc25c and MPF in mouse oocytes. Our findings suggest that MPF, MAPK, cdc25c, and PP2A participate in a series of phosphorylation/dephosphorylation events to form a signaling pathway that regulates the GII/prophase transition in mouse oocytes. We believe that OA can mediate a direct effect on this pathway, as illustrated in Fig. 4.

Considering the early suppression of the decrease in histone H1 kinase activity by OA, the production of active MPF may be enhanced by it controlling cdc25c, despite destruction of cyclin B during fertilization. OA-sensitive phosphatase antagonizes cdc25c activation, and we think that this is an important point in this study.

OA is also believed to prevent PP, which inhibits MAPK/p90rsk activation during meiotic maturation. Our findings show that this is not the case, because suppression of the decrease in MBP kinase activity by OA did not precede that of histone H1 kinase.

It is clear that the inhibition of OA-sensitive phosphatase prevent second PB extrusion and PN formation through the suppression of the decreases in MPF and MAPK activities. The supression of OA-sensitive phosphatase during fertilization seems to inhibit the dephosphorylation of cdc25c, and thereby delay MII/G1 transition. Therefore, we can conclude OA-sensitive phosphatase is directly related to MII/G transition in mouse oocytes.

The mechanism of how a fall in MAPK activity influences MPF levels is currently unknown and so further research is needed to clarify this MAPK/MPF relationship and how they influence cdc25c, during fertilization.

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