

Effects of MEK inhibitor U0126 on meiotic progression in mouse oocytes: microtubule organization, asymmetric division and metaphase II arrest

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ABSTRACT

In this study we used U0126, a potent and specific inhibitor of MEK, to study the roles of MEK/ERK/p90^{rsk} signaling pathway in the meiotic cell cycle of mouse oocytes. The phosphorylation of MAP kinase and p90^{rsk} in the oocytes treated with 1.5 μ M U0126 was the same as that in oocytes cultured in drug-free medium. With 1.5 μ M U0126 treatment, the spindles appeared normal as they formed in oocytes, but failed to maintain its structure. Instead, the spindle lost one pole or elongated extraordinarily. After further culture, some oocytes extruded gigantic polar bodies (>30 μ m) that later divided into two small ones. Some oocytes underwent symmetric division and produced two equal-size daughter cells in which normal spindles formed. In oocytes with different division patterns, MAP kinase was normally phosphorylated. When the concentration of U0126 was increased to 15 μ M, the phosphorylation of both MAPK and p90^{rsk} were inhibited, while symmetric division was decreased. When incubating in medium containing 15 μ M U0126 for 14 h, oocytes were activated, but part of them failed to emit polar bodies. MII oocytes were also activated by 15 μ M U0126, at the same time the dephosphorylation of MAP kinase and p90^{rsk} was observed. Our results indicate that 1) MEK plays important but not indispensable roles in microtubule organization; 2) MEK keeps normal meiotic spindle morphology, targets peripheral spindle positioning and regulates asymmetric division by activating some unknown substrates other than MAP kinase /p90^{rsk}; and 3) activation of MEK/ERK/p90^{rsk} cascade maintains MII arrest in mouse oocytes.

Keywords: *kinases, signal transduction, oocyte development, fertilization, meiosis.*

INTRODUCTION

The meiotic cell cycle of oocytes is regulated by a cascade of protein phosphorylation/ dephosphorylation events. Two isoforms of MAP kinases, ERK1 (p44) and ERK2 (p42), appear to play a pivotal role in this orchestration[1,2]. MAP kinases are a family of Ser/Thr protein kinases that are activated by MEK (MAP kinase kinase) phosphorylation of both tyrosine and threonine residues. The activation of MEK is regulated by MOS, a germ cell-specific Ser/Thr protein kinase. Among many substrates for MAP kinases, p90^{rsk} was the first to be discovered[3]. In *Xenopus* oocytes, the main effects of MAP kinase, including S phase suppression, MII arrest

maintenance, and spindle formation regulation, are mediated by p90^{rsk}[4,5].

There is an evidence suggesting that MAPK pathway is involved in the regulation of microtubule organization during the meiosis of oocytes. Mouse oocytes injected with antibody against MOS failed to assemble a meiotic spindle[6]. In maturing *mos*^{-/-} oocyte MAP kinase could not be activated throughout meiosis, which resulted in the shape alteration of spindle[7,8]. In mouse oocytes, MAP kinase is specifically associated with the microtubule-organizing centers (MTOCs) present at the spindle poles and in the cytoplasm[9], which suggests that MAP kinase might involve in controlling spindle assembly and microtubular configurations. Reports in other species also suggested the important roles of MAP kinase in microtubule organization[10]. Our previous study [11] showed that MAP kinase might be involved in microtubule nucleation by γ -tubulin in pig oocytes. However,

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it was reported that mouse oocytes deficient in MAP kinase activity could still divide and release polar bodies [6,12-14]. Until now, the targets of MAP kinase in the meiotic spindle remain unclear. Therefore, the roles of MAP kinase in spindle organization is still a question to be elucidated.

Oocyte meiosis is characterized by asymmetric division. But the first meiotic division is symmetric in some *mos*^{-/-} oocytes [8]. In wild-type oocytes the spindle formed in the center of oocytes and migrated to the cortex before meiotic division. But in *mos*^{-/-} oocytes that underwent asymmetric division, the centrally formed spindle failed to migrate, instead, it elongated in place [14]. Although many reports show that MOS participates in oocyte asymmetric division, the downstream molecule that mediates this event is still unclear.

The metaphase II arrest is another characteristic of meiosis in almost all mammalian oocytes. It is well established that the MII arrest is maintained by cytostatic factor (CSF) activity, with MOS/MEK/ERK cascade being its essential components [4,10,12,13,15]. Although existing evidence has suggested that MAP kinase is important for metaphase arrest, almost all data come from *mos*^{-/-} oocytes or other models indicating MAP kinase is not activated during the entire maturation process. It is still unknown whether the MII oocytes with activated MAP kinase could be released from the arrest when MEK is specifically inhibited.

U0126, a potent and specific inhibitor of MEK1 and MEK2, has little effect on other kinases such as Cdk2, ERK, JNK, MEKK and Raf [16]. In porcine oocytes, the inhibition of ERK activity with U0126 during the meiosis I/meiosis II transition suppressed chromosome separation, first polar body emission and formation of the metaphase II spindle [17]. When incubated in a medium with U0126, part of porcine oocytes completed the second meiotic division and entered into interphase [18]. In cumulus-enclosed mouse oocytes, U0126 produced a dose-dependent inhibitory effect on FSH-induced meiotic resumption [19].

This report shows the novel effects of U0126 on meiotic cell cycle progression in mouse oocytes, including ERK and p90^{ras} activation, disturbance of microtubule organization, PB extrusion abnormality, and MII arrest. The findings provide further understanding of the roles of MAP kinase signaling pathway in the regulation of meiotic cell cycle progression in mammalian oocytes.

MATERIALS AND METHODS

Oocyte collection and culture

Fully grown germinal vesicle-intact oocytes were collected from ovaries of 4-6 week-old Kunming mice 48 h after the females were injected with 10 IU pregnant mare's serum gonadotropin (PMSG). Cumulus-free and GV-intact oocytes were released from antral follicles by puncturing the follicles with a needle in M2 medium (Sigma) supplemented with 60 mg/ml penicillin and 50 µg/ml streptomycin. All cultures were carried out in M2 medium at 37 °C in a humidified atmosphere of 5% CO₂.

Metaphase II-arrested eggs were obtained from mice of the same strain. Females were superovulated by intraperitoneal injection of 10 IU of PMSG, and 48 h later, they were injected with 10 IU of human chorionic gonadotrophin (hCG). Mice were sacrificed and oviducts were removed at 15 h post-hCG injection. Using a pair of fine forceps to tear the oviducts, cumulus masses were collected in M2 medium. The cumulus cells surrounding the eggs were removed by a brief exposure to 300 IU/ml hyaluronidase and repeated pipetting, followed by three washes in M2 medium.

Parthenogenetic activation

Parthenogenetic activation of mouse oocytes was performed as we previously reported [20]. The denuded eggs were treated with 5 µM A23187 for 5 min, and then incubated in M2 medium containing 10 µg/ml CHX. After 2, 4 and 8 h of culture, treated eggs were examined with a phase contrast microscope. The emission of the second polar body and the formation of pronucleus were examined.

Confocal microscopy

After removal of zona pellucida (ZP) in acidified Tyrode's solution (pH 2.5), eggs were fixed in 3% formaldehyde, 2% sucrose in PBS for 30 min and then incubated in incubation buffer (0.5% Triton X-100 in 20 µM Hepes, pH 7.4, 3 µM MgCl₂, 50 µM NaCl, 300 µM sucrose, 0.02% NaN₃) for 30 min. After being treated in methanol for 5 min at -20 °C, the eggs were washed in washing buffer (PBS with 0.1% Tween 20 and 0.01% Triton X-100) for three times and then incubated with 1:50 diluted FITC-conjugated mouse anti-α tubulin antibody (Sigma) for 1 h. The eggs were rinsed three times and incubated with 10 mg/ml propidium iodide. Finally, the eggs were mounted on glass slides and examined using a TCS-4D laser scanning confocal microscope (Leica Microsystems).

Western blot analysis

Eggs were collected in SDS sample buffer after treatment with different stimuli at different times, heated to 100 °C for 4.5 min, and frozen at -20 °C until use. Each sample contained 30 eggs. The total proteins were separated by SDS-PAGE with a 4% stacking gel and 10% separating gel at 90 V, 0.5 h and 120 V, 2 h respectively and electrophoretically transferred to nitrocellulose membrane (Gelmen, poresize 0.45 µm) for 2 h, 200 mA, at 4 °C. Following transfer, washing in TBS (10 µM Tris, 150 µM NaCl, pH 7.4) twice and blocking in 5% skimmed milk in TBST (TBS containing 0.1% Tween-20) at 4 °C overnight, the membrane was incubated in TBST containing 1:1000 polyclonal anti-phospho-ERK antibody (Santa Cruz

Biotechnology, Santa Cruz, CA) at 37 °C for 2 h. After washing three times in TBST, 10 min each, the membrane then was incubated with HRP-conjugated goat-anti-mouse IgG (Jackson ImmunoResearch Laboratories, INC, PA) diluted 1:1000 in TBST at 37°C for 1 h. The membrane was washed three times in TBST, 10 min each, and was processed by using the ECL detection system.

For reprobing, the membrane was washed in stripping buffer (100 μ M b-mercaptoethanol, 20% SDS, 62.5 μ M Tris pH6.7) at 50°C for 30 min to strip off bound antibody after ECL detection. The membrane was reprobed with polyclonal rabbit anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:300 and polyclonal rabbit anti-p90rsk antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500, using the same procedure as described above. All experiments were repeated at least three times.

Statistical analysis

All percentages from 2 or 3 repeated experiments were expressed as mean \pm SEM and the number of oocytes observed was labeled in brackets as (n=). The rates of PB1 emission and symmetric division were subjected to arcsin transformation. The transformed data were analyzed by ANOVA followed by Student-Newman-Keuls test. Differences at $p < 0.05$ were considered to be statistically significant.

RESULTS

Division patterns and activity of MAP kinase and p90^{rsk} in oocytes treated with U0126

In order to study the effects of MEK/ERK/p90^{rsk} cascade inhibition on meiotic maturation of mouse oocytes, cumulus-free and GV intact oocytes were incubated in M2 medium containing 1.5 μ M U0126[19]. The culture medium containing U0126 was renewed every 12 h to prevent the possible degradation of U0126. Some oocytes were cultured in drug-free medium as control. As shown in Fig 1A, there was no significant difference in PB1 emission rate between oocytes cultured with or without U0126 14 h after maturation culture ($74.9 \pm 2.4\%$ (n=307) in U0126 treated groups, and $75.7 \pm 0.44\%$ (n=162) in drug-free groups. Although part of treated oocytes underwent asymmetric division as in control oocytes, $8.4 \pm 0.32\%$ oocytes (n=307) underwent symmetric division, resulting in the formation of 2-cell-like oocytes, and $36.2 \pm 1.4\%$ (n=307) extruded extraordinarily big polar bodies ($>30 \mu$ m) that did not undergo degeneration but divided in the following 10 h. To detect the changes of the activity of ERK and p90^{rsk}, the oocytes at different developmental stages were collected for Western blot analysis. ERK activity and the full phosphorylation of p90^{rsk} could be detected in oocytes after 4 h incubation with U0126 (Fig 2), the same as in oocytes

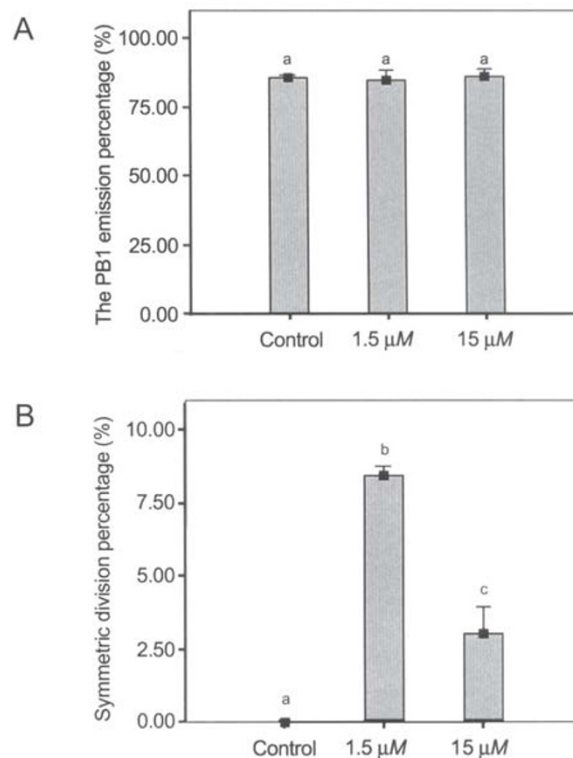


Fig 1. Effect of U0126 on polar body emission and symmetric division of mouse oocytes. Cumulus-free GV oocytes were treated with different concentrations of U0126 (1.5 and 15 μ M) for 14 h. As control, oocytes at GV stage were cultured in a drug-free medium for 14 h. (A) The PB1 emission rates in mouse oocytes treated with U0126. (B) The symmetric division rates in mouse oocytes treated with U0126. The rate was shown as percentage (mean \pm SEM). Different superscripts (a, b, and c) denote statistical difference at a $P < 0.05$ level of significance.

cultured in a drug-free medium (Fig 3). After 8 h incubation, ERK of the oocytes was completely activated and its activation maintained until 24 h after incubation in both 1.5 μ M U0126 treated and drug-free medium. To detect whether the oocytes with unusual division patterns were more sensitive to U0126, the oocytes with different division patterns (2-cell like, 2-PB like, normal-MI like, no-PB) were collected at 15 h incubation with 1.5 μ M U0126 and the ERK activity was analyzed. Just as in the control, high ERK phosphorylation was detected in each sample (Fig 4).

Since 1.5 μ M U0126 does not inhibit the MEK/ERK/p90rsk cascade, higher concentrations of U0126 were used and PB1 emission was observed. We found that there was no significant difference in PB1 emission rates between the oocytes treated with 1.5 and 15 μ M U0126 [$74.9 \pm 2.4\%$ (n=307) and $76.0 \pm 1.8\%$ (n=200), respectively](Fig 1A). However, the rate of symmetric division in oocytes treated with 15 μ M U0126 ($3.02 \pm$

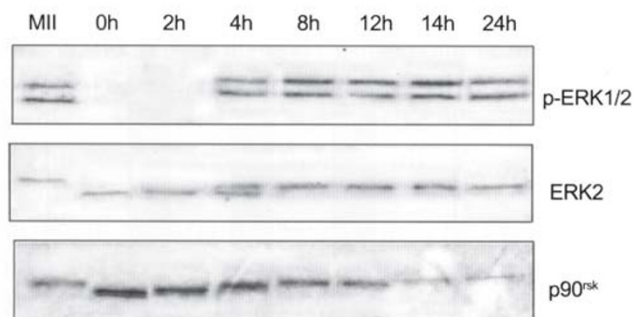


Fig 2. Effect of 1.5 μ M U0126 on MAP kinase and p90rsk phosphorylation in mouse oocytes. Samples for immunoblotting were collected after the GV-intact oocytes were treated with 1.5 μ M U0126 for 0, 2, 4, 8, 12, 14, and 24 h. MII oocytes were also collected as a positive control. MAP kinase was activated 4 h after maturation. A basic phosphorylation of p90^{rsk} was observed in GV oocytes and the full phosphorylation was detected 4 h after culture. Each lane represent the protein extracted from 30 cells.

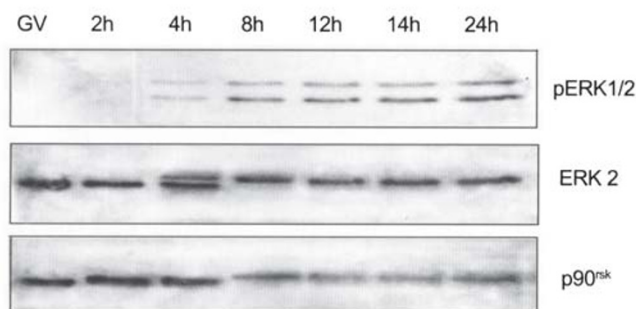


Fig 3. MAP kinase and p90^{rsk} phosphorylation during mouse oocytes maturation. Samples for immunoblotting were collected after the GV-intact oocytes were cultured for 0, 2, 4, 8, 12, 14, and 24 h. MAP kinase was activated 4 h after maturation. A basic phosphorylation of p90^{rsk} was observed in GV oocytes and the full phosphorylation was detected 8 h after culture.

90% (n=200)) was significantly lower than that in those oocytes treated with 1.5 μ M U0126 (Fig 1B). Western blot analysis showed that the activity of MAP kinase and migration of p90^{rsk} could not be detected within 24 h after incubation with 15 μ M U0126 (Fig 5). Therefore, 15 μ M is the effective concentration for U0126 to inhibit MAP kinase and p90^{rsk} activation.

To examine whether both daughter cells of the 2-cell-like oocytes were able to undergo the second meiotic division, the oocytes were treated with A23187+CHX. Both cells divided 2 h after activation and no obvious asymmetric division was observed. (Fig 6)

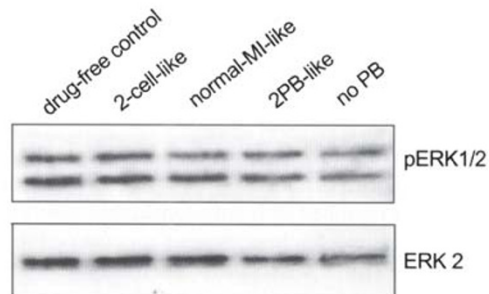


Fig 4. MAP kinase activity in oocytes with different division patterns. Cumulus-free GV oocytes were cultured in medium containing 1.5 μ M U0126 for 15 h. The oocytes with different division patterns (2-cell like, 2-PB like, normal-MI like, no-PB) were collected respectively. GV oocytes cultured in a drug-free medium for 14 h and then collected as control group. MAP kinase phosphorylation in the oocytes with different division patterns. MAP kinase was fully phosphorylated in the oocytes with different division patterns and drug-free control.

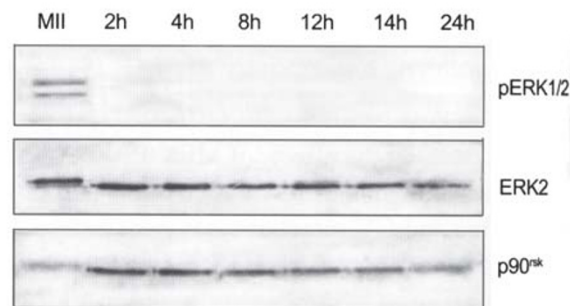
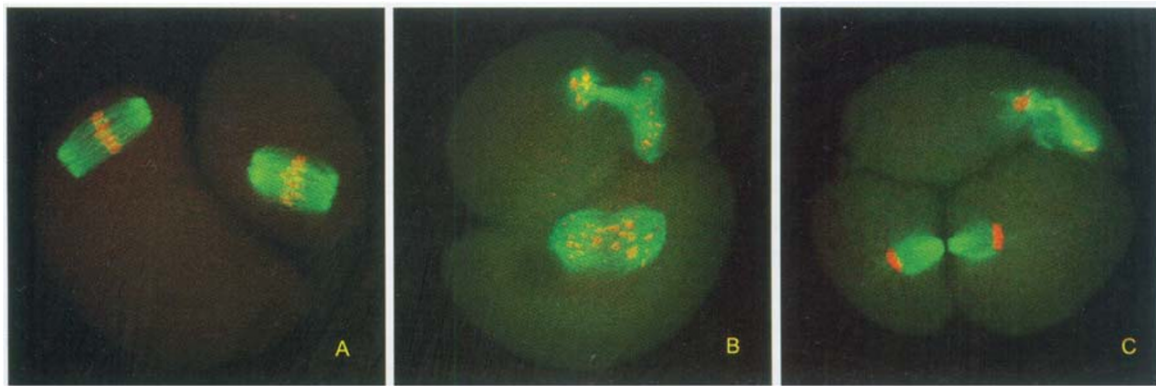


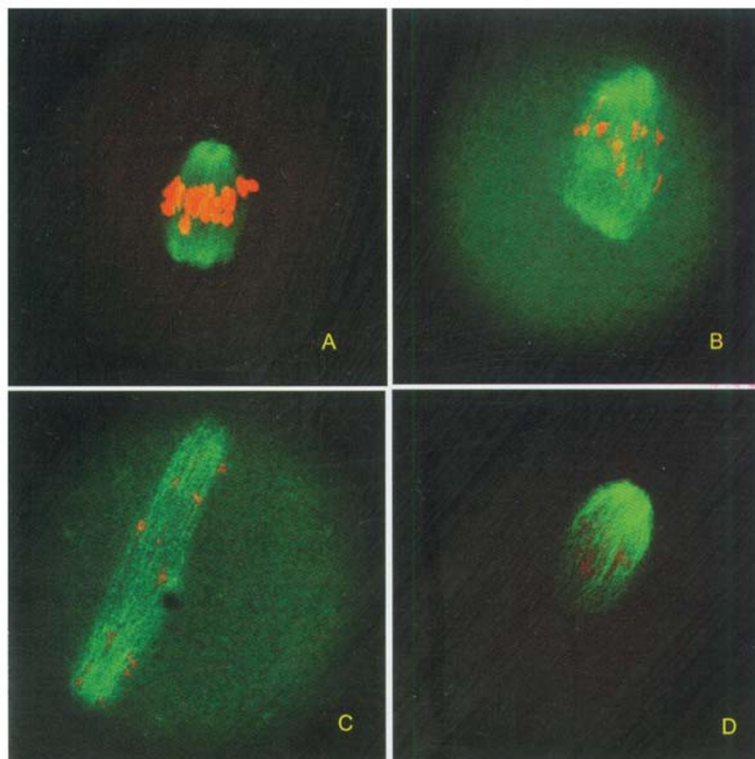
Fig 5. Effect of 15 μ M U0126 on MAP kinase and p90^{rsk} phosphorylation in mouse oocytes. Samples for immunoblotting were collected after the GV intact oocytes were treated with 15 μ M U0126 for 0, 2, 4, 8, 12, 14, and 24 h. MII oocytes were also collected as a positive control. 15 μ M U0126 inhibited MAP kinase activation and full phosphorylation of p90^{rsk}.

Microtubule Dynamics in Oocytes Treated with U0126

Since MEK/ERK/p90^{rsk} cascade affects the division pattern of the oocytes, microtubule organization was examined in oocytes treated with 1.5 or 15 μ M U0126. In oocytes treated with 1.5 μ M U0126, spindles formed normally as in drug-free control 8 h after incubation (Fig 7A). However, when the incubation time was prolonged, the intracellular position or morphology of the spindle changed. Three phenomena existed: 1) the spindle elongated and one pole closest to the cortex moved while 2-



△ **Fig 6.** The second meiotic division of the 2-cell-like oocytes activated by A23187+CHX. Green, α -tubulin; red, chromatin; yellow, overlapping of green and red. (A): A 2-cell like oocyte; (B and C): Both daughter cells were dividing.



◁ **Fig 7.** Microtubule dynamics in the oocytes treated with 1.5 μ M U0126. (A) a spindle in the oocyte treated with 1.5 μ M U0126 for 8 h; (B) an elongated spindle and one pole closest to the cortex while the other pole remained in place; (C) an elongated spindle with each pole moved to its proximal cortex, resulted in a gigantic spindle running through the entire oocyte; and (D) a deformed spindle with only one pole near the cortex.

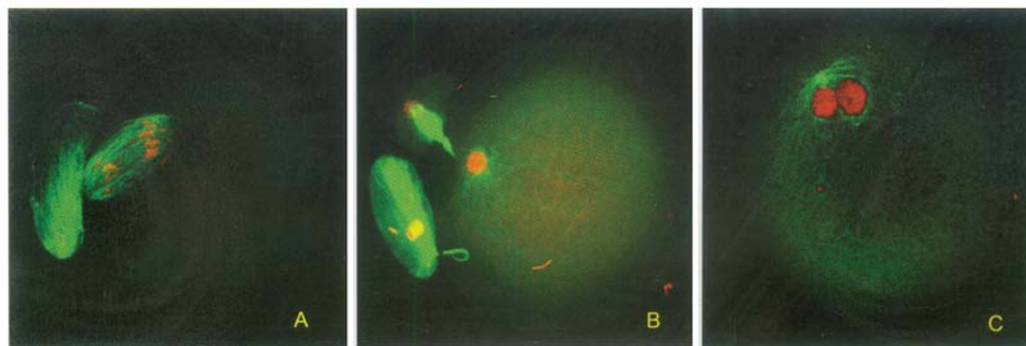


Fig 8. The GV oocytes incubated in a medium containing 15 μ M U0126. (A and B) the oocytes were activated after 14 h incubation; and (C) an oocyte with two pronuclei.

cell-like oocytes or oocytes with abnormally big polar bodies were formed. The oocytes arrested at MII stage, with normal the another remained in place (Fig 7B); 2) the spindle elongated but each pole moved to its proximal cortex, resulting in a gigantic spindle running through the oocyte (Fig 7C); and 3) the spindle was deformed with only one pole near the cortex (Fig 7D). Microtubule dynamics were the same in oocytes treated with 1.5 or

15 μ M U0126 before 12 h incubation (data not shown). However, a large proportion of the oocytes [$68.9 \pm 1.45\%$ ($n=153$)] incubated with 15 μ M U0126 did not arrest at MII stage, but entered interphase with formation of pronuclei and diffusion of microtubules (Fig 8A-B). The PB2 emission was blocked in some activated oocytes [$11.0 \pm 0.15\%$ ($n=91$)]. As a result, two pronuclei were observed in these oocytes (Fig 8C).

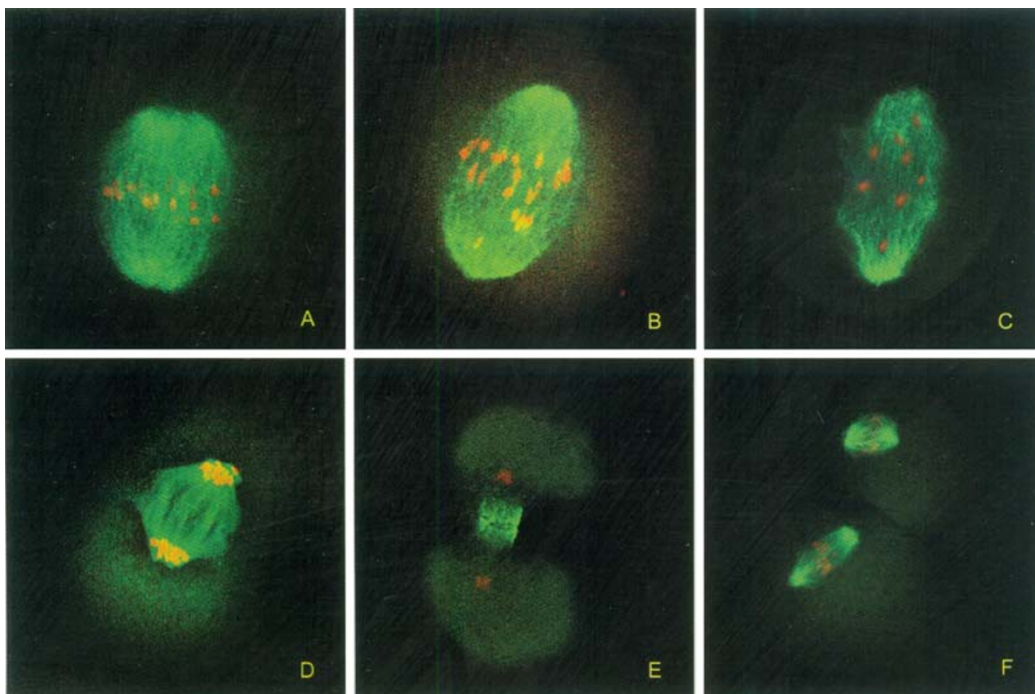


Fig 9. Microtubule dynamics in the oocytes with symmetric division. (A, B and C) spindles elongated extraordinarily; (D and E) oocytes with symmetric division; and (F) each daughter cell formed a normal spindle.

Microtubule dynamics were also traced in the oocytes with equal division. After 8 h incubation in 1.5 μ M U0126, the spindle formed displaying normal morphology (Fig 7A). It elongated during metaphase I/anaphase I transition rather than migrated to the cortex as observed in normal oocytes (Fig 9A-C). As a result, spindles in both daughter cells (Fig 9D-F).

MAP kinase and p90^{rsk} activity in MII eggs treated with U0126

To examine the effect of MEK inhibition on MII arrest and MAP kinase/p90^{rsk} phosphorylation, MII eggs

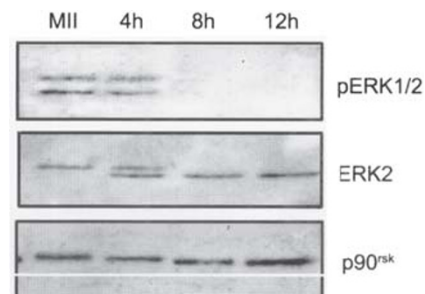


Fig 10. Effect of 15 μ M U0126 on MAP kinase and p90^{rsk} phosphorylation in mouse oocytes at MII stage. Samples for immunoblotting were collected after the MII oocytes were treated with 15 μ M U0126 for 0, 4, 8, and 12 h, respectively. MAP kinase and p90^{rsk} were dephosphorylated after 4 h treatment.

were treated with 15 μ M U0126 and collected every 4 h for nuclear status examination and for Western blot analysis. After 12 h of incubation, pronuclei were formed in $38.8 \pm 7.8\%$ ($n=131$) of the treated eggs. The results of Western blot showed that MAP kinase activity was decreased and the p90^{rsk} was gradually dephosphorylated (Fig 10).

DISCUSSION

In both vertebrates and invertebrates, meiotic divisions of oocytes are typically asymmetric. The size difference between two daughter cells is typically a consequence of asymmetric positioning of the spindle before cytokinesis. In mouse oocytes, the meiotic spindle forms in the center of the cell, which does not elongate during anaphase but migrates to the cortex just before polar body extrusion[21]. In *mos*^{-/-} mouse oocytes, the spindle shape is altered and the spindle fails to translocate to the cortex. As a result, in these oocytes the first meiotic division resembles mitotic cleavage and produces two cells of the same size[22]. Inhibition of protein synthesis frequently results in a similar phenotype[23] in starfish oocytes. In the present study, when the oocytes were treated with 1.5 μ M U0126, abnormal division was similar to that observed in *mos*^{-/-} oocytes. However, in oocytes treated with 1.5 μ M U0126, even in those with typical abnormal division patterns, MAP kinase was still activated as in control oocytes. Furthermore, although MAP kinase could be inhibited by a high concentration of U0126, the rate of symmetric division did not increase. It is apparent that MAP kinase activity does not insure the asymmetric division in oocytes. Interestingly, the *mos*^{-/-} oocytes have the same phenotype as oocytes treated with U0126 shown here. Since MEK is a downstream molecule of MOS and it is the only target of U0126, it is clear that the depletion of MEK activity, rather than MAP kinase activity, is responsible for the abnormality in meiotic division. It is possible that MEK is involved in the asymmetric cytokinesis of oocytes through substrate of unknown nature and MEK could activate it by the domains different from those responsible for MAP kinase activation. Different concentrations of U0126 may exert diverse effects on MEK configuration and make MEK activate one of its substrates, but not the others. This may be the possible reason of why the incidence of symmetrical division was decreased after the treatment of U0126 with high concentration.

In animal cells, the position of spindle determines the

plane of cleavage[24,25]. In our experiments, some spindles in U0126-treated oocytes elongated extraordinarily at metaphase I/ anaphase I transition rather than translocating to the cortex as seen in normal oocytes. As a result, symmetric division took place. During mitosis, spindle positioning involves a cortical-microtubule capture mechanism and several proteins are involved in the interaction between microtubules and the cortex[26,27]. In *Saccharomyces cerevisiae*, microtubule cortical attachment includes mediation by actin, Kar9p, Bud6p and Bni1p[28,29]. Verlhac et al[14] found that actin distribution was disturbed in *mos*^{-/-} oocytes, which lends strong support to our data. The presence of an actin-rich plaque covering the meiotic spindle appears to be a common feature of animal cortices[30-32]. In wild-type oocytes, the actin-rich cortical domain appears progressively, concomitant with spindle migration[33,34]. In *mos*^{-/-} oocytes [14], signs of cortical polarization are present only at anaphase and no obvious actin-rich region is found. MEK and its downstream molecules may act on these proteins and regulate the spindle positioning. Further work is necessary to elucidate these interesting results.

We have shown that the MII eggs were released from MII arrest and MAP kinase and p90rsk were dephosphorylated after MEK had been inhibited, suggesting that MEK activity is necessary for maintaining the activity of MAP kinase and p90rsk as well MII arrest. Since MAP kinase dephosphorylation is protein phosphatase dependent, it is possible that there is an equilibrium between MEK and MAP kinase-specific protein phosphatase in the meiotic cell cycle: if MEK activity overcomes protein phosphatase activity, MAP kinase is phosphorylated; alternatively, MAP kinase is dephosphorylated. Verlhac et al[35] showed that MOS activated MAP kinase through two opposite pathways: activation of MEK and inhibition of protein phosphatase. We and others found that inhibition of protein phosphatase by okadaic acid (OA) induced precocious MAP kinase activation in mouse and pig oocytes [36,37]. OA treatment of fertilized mouse or pig eggs resulted in prompt phosphorylation of MAP kinase, disassembly of microtubules around the pronuclear area, chromatin condensation, and nuclear membrane breakdown[38,39]. Although very little is known about the physiological MAP kinase-specific protein phosphatase, the work in *Xenopus* oocytes shows that a tyrosine phosphatase and a PP2A-like threonine phosphatase mediate MAP kinase inactivation[40]. At MII arrest, both MEK activity and

the MAP kinase-specific protein phosphatase activity may exist, but the MEK activity is in dominance. When treated with U0126, MEK activity was inhibited and thus MAP kinase was dephosphorylated, causing release of MII arrest.

The data in *Xenopus* oocytes suggested that the activity of MAP kinase is essential for maintaining the activity of p90^{rsk} [41,42]. Previous work by us and others also showed that in mouse and rat oocytes, the change of MAP kinase activity is always followed by the change of p90^{rsk} activity during meiotic maturation, fertilization, and parthenogenetic activation [43-45]. However, there is no direct evidence that the inhibition of MAP kinase activity at MII arrest leads to the dephosphorylation of p90^{rsk}. In our experiments, when MAP kinase activity was inhibited by U0126, during the interphase transition of oocytes, dephosphorylation of p90^{rsk} was detected by Western blotting, which supports the previous suggestion that p90^{rsk} is one of the direct targets of MAP kinase and a key mediator of CSF in oocytes.

In conclusion, the meiotic abnormalities caused by U0126, a specific inhibitor of MEK, imply important regulatory roles of MEK in asymmetric division and the release of the MII arrest induced by U0126 implies an important role of the MEK/ERK/p90^{rsk} cascade in MII arrest of mouse oocytes.

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Intermittent hypoxia attenuates ischemia/reperfusion induced apoptosis in cardiac myocytes via regulating Bcl-2/Bax expression

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ABSTRACT

Intermittent hypoxia has been shown to provide myocardial protection against ischemia/reperfusion-induced injury. Cardiac myocyte loss through apoptosis has been reported in ischemia/reperfusion injury. Our aim was to investigate whether intermittent hypoxia could attenuate ischemia/reperfusion-induced apoptosis in cardiac myocytes and its potential mechanisms. Adult male Sprague-Dawley rats were exposed to hypoxia simulated 5000 m in a hypobaric chamber for 6 h/day, lasting 42 days. Normoxia group rats were kept under normoxic conditions. Isolated perfused hearts from both groups were subjected to 30 min of global ischemia followed by 60 min reperfusion. Incidence of apoptosis in cardiac myocytes was determined by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) and DNA agarose gel electrophoresis. Expressions of apoptosis related proteins, Bax and Bcl-2, in cytosolic and membrane fraction were detected by Western Blotting. After ischemia/reperfusion, enhanced recovery of cardiac function was observed in intermittent hypoxia hearts compared with normoxia group. Ischemia/reperfusion-induced apoptosis, as evidenced by TUNEL-positive nuclei and DNA fragmentation, was significantly reduced in intermittent hypoxia group compared with normoxia group. After ischemia/reperfusion, expression of Bax in both cytosolic and membrane fractions was decreased in intermittent hypoxia hearts compared with normoxia group. Although ischemia/reperfusion did not induce changes in the level of Bcl-2 expression in cytosolic fraction between intermittent hypoxia and normoxia groups, the expression of Bcl-2 in membrane fraction was upregulated in intermittent hypoxia group compared with normoxia group. These results indicated that the cardioprotection of intermittent hypoxia against ischemia/reperfusion injury appears to be in part due to reduce myocardial apoptosis. Intermittent hypoxia attenuated ischemia/reperfusion-induced apoptosis via increasing the ratio of Bcl-2/Bax, especially in membrane fraction.

Keywords: *intermittent hypoxia, apoptosis, cardiac myocytes, Bax, Bcl-2.*

INTRODUCTION

Intermittent hypoxia, or periodic exposure to hypoxia interrupted by return to normoxia or less hypoxic conditions, occurs in many circumstances[1]. Many studies showed that intermittent hypoxia might have the cardioprotective effects similar to those observed in ischemic preconditioning[2-8]. A number of studies have

attempted to define the mechanisms of this phenomenon and several potential factors have been proposed to be involved in the protective mechanism afforded by intermittent hypoxia[3,5-7,9-11], however, the precise mechanisms underlying the protective effects of intermittent hypoxia on ischemic hearts are far from clear.

Apoptosis, a form of death characterized by cell shrinkage, plasma membrane blebbing, chromatin condensation and genomic DNA fragmentation, is essential for development and maintenance of tissue homeostasis. On the other hand, apoptosis has been implicated in many diseases such as congestive heart failure and ischemic injury. Although a large portion of the cell loss during cardiac ischemia and reperfusion occurs through

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