

# Mitogen-Activated Protein Kinase and Cell Cycle Progression During Mouse Egg Activation Induced by Various Stimuli

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A very sensitive method was established for detecting the activity of mitogen-activated protein (MAP) kinase in mouse eggs, and used to follow temporal changes of this kinase during fertilization and spontaneous or chemically-induced parthenogenic activation. MAP kinase activity increased between 1 and 2.5 h post-insemination, at which time the second polar body was emitted and sperm chromatin was dispersed; its activity decreased sharply at 8 h, when pronuclei were formed. Both calcium ionophore A23187 and ethanol simultaneously induced pronuclear formation and MAP kinase inactivation in aged eggs 8 h after incubation but less effectively in fresh eggs. The protein kinase inhibitor staurosporine induced pronuclear formation and MAP kinase inactivation more quickly than other treatments, with MAP kinase inactivation occurring slightly preceding pronuclear formation. Okadaic acid, a specific inhibitor of protein phosphatase 1 and 2A, induced increase in MAP kinase activity, and overcame pronuclear formation induced by various stimuli. MAP kinase inactivation preceded pronuclear formation in eggs spontaneously activated by aging *in vitro*, perhaps due to cytoplasmic degeneration and thus delayed response of nuclear envelope precursors to MAP kinase inactivation. These data suggest that MAP kinase is a key protein kinase regulating the events of mouse egg activation. Increased MAP kinase activity is temporally correlated with the second polar body emission and sperm chromatin decondensation. Although different stimuli (including sperm) may initially act through different mechanisms, they finally inactivate MAP kinase, probably by allowing the action of protein phosphatase, and thus induces the transition to interphase.

## Introduction

Mammalian eggs are arrested at meiotic metaphase II (MII) before fertilization or activation by cytostatic factor (CSF) (Masui, 1991). It is believed that CSF promotes meiotic arrest directly or indirectly by stabilizing the activity of maturation promoting factor (MPF), a heterodimeric complex of cyclin B and P34<sup>cdc2</sup> kinase (Murray *et al.*, 1989; Sagata, *et al.*, 1989; Masui, 1991). Recently, mitogen-activated protein (MAP) kinase, also named extracellular regulated kinase (ERK), has also been proved to play an important role in controlling meiotic cell cycle during maturation of mouse, rat, pig, goat and bovine oocytes (Sobajima *et al.*, 1993; Verlhac *et al.*, 1993; Inoue *et al.*, 1995; Dedieu *et al.*, 1996; Fissore *et al.*, 1996; Zernicka-Goetz *et al.*, 1997). More recent research revealed

that decrease in MAP kinase activity has a possible role in pronuclear envelope assembly in mouse eggs following fertilization (Moos *et al.*, 1995, 1996a). However, the function of MAP kinase, a microtubule-associated protein kinase (Verlhac *et al.*, 1993), in the early events of fertilization has not been addressed.

In addition to physiological activation by sperm, mouse eggs can be activated *in vitro* by various physical and chemical stimuli. Ethanol and the Ca<sup>2+</sup> ionophore A23187 can activate mouse eggs by inducing intracellular Ca<sup>2+</sup> rise, although neither stimuli induce Ca<sup>2+</sup> oscillations as a sperm does (Lorca *et al.*, 1993; Yanagimachi, 1994; Kline, 1996). However, cycloheximide and protein kinase inhibitor staurosporine have been proved by us and others to activate eggs with no accompanying Ca<sup>2+</sup> rise (Moses and Kline, 1995; Sun *et al.*, 1997a; Wang *et al.*, 1997a). Mouse eggs also undergo spontaneous parthenogenic activation during aging, but whether this too involves an initial Ca<sup>2+</sup> rise has not been well determined (Yanagimachi,

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1994). It is necessary to clarify further whether different stimuli induce cell cycle progression by acting on MAP kinase, a convergence point for many signaling pathways, as a sperm does.

By using an antibody specific for active MAP kinase, we have established a very sensitive method for detecting MAP kinase activity in mouse eggs, and used to follow the temporal changes of MAP kinase activity in mouse eggs that became activated spontaneously or artificially, as well as in eggs activated by sperm.

## Materials and Methods

### *Preparation of chemical reagents*

Stock solutions of A23187 (free acid, Calbiochem, La Jolla, CA), staurosporine, okadaic acid (OA) were prepared with dimethyl sulfoxide (DMSO) at 20 mM, 2 mM and 0.2 mM, respectively, and stored frozen at  $-20^{\circ}\text{C}$ . Stock solution of cycloheximide in 0.9% saline was stored at  $-20^{\circ}\text{C}$ . Prior to use, the stock solutions were diluted with modified Tyrode's solution (T6) (Wood *et al.*, 1987). An 8% solution of ethanol (Mercaz, Shatner, Jerusalem, Israel) was freshly prepared in T6 medium for every experiment. Hyaluronidase was dissolved in T6 medium at 300  $\mu\text{g}/\text{ml}$  and 0.5 ml aliquots stored at  $-20^{\circ}\text{C}$ .

All the reagents, unless otherwise specified, were from Sigma Chemical Co. (St. Louis, MO).

### *Collection of eggs*

Cumulus cell-enclosed metaphase II-arrested eggs were obtained from 6–10 week-old BALB/c mice. Females were superovulated by injecting 10 iu of pregnant mare serum gonadotrophin (PMSG), and 48 h later they were injected with 10 iu of human chorionic gonadotrophin (hCG). Mice were killed by cervical dislocation at 15 or 19 h post-hCG, and the cumulus cell masses were released from the oviducts. The cumulus cells surrounding the eggs were removed by brief exposure to 300 iu/ml hyaluronidase in T6 medium. After a series of washes, the eggs were cultured as follows.

### *Experimental treatments*

*In vitro* fertilization (IVF): Spermatozoa collected from the cauda epididymis and ductus deferens of 2 adult male mice were mixed and capaci-

tated for 1.5 h in T6 medium containing 20 mg/ml BSA. Cumulus-free eggs collected at 15 h post-hCG injection were inseminated in the same medium containing approximately  $1 \times 10^6$  sperm cells/ml.

*Artificial parthenogenic activation:* Cumulus-free eggs collected at 15 or 19 h post-hCG injection were exposed to 8% ethanol for 7 min or 5  $\mu\text{M}$  A23187 for 5 min, respectively. Treatment was carried out at room temperature in air. After washing 3 time, 5 min each, the treated eggs were transferred to a droplet (200  $\mu\text{l}$ ) of T6 medium containing 20 mg/ml BSA under paraffin oil for further incubation at  $36.5^{\circ}\text{C}$ , 5%  $\text{CO}_2$  and 100% humidity until 8 h. Incubation treatment of eggs collected at 15 h post-hCG injection in 2  $\mu\text{M}$  staurosporine or 500  $\mu\text{M}$  cycloheximide was for the entire period under the same conditions. Eggs cultured in drug-free medium were adopted as controls.

*Spontaneous parthenogenic activation:* Eggs collected at 15h and 19 h after hCG injection were aged *in vitro* in T6 medium containing 20 mg/ml BSA till 72 h or 96 h.

*Effect of okadaic acid:* After the eggs were inseminated for 2 h or treated with 5  $\mu\text{M}$  A23187 for 5 min or 8% ethanol for 7 min, they were cultured further in T6 medium containing 2  $\mu\text{M}$  okadaic acid till 8 h. In other treatment groups, eggs were cultured in the medium containing both 2  $\mu\text{M}$  staurosporine or 500  $\mu\text{M}$  cycloheximide and 2  $\mu\text{M}$  okadaic acid.

All experimental groups in this study were conducted a minimum of 3 times.

### *Confirmation of egg activation*

At 1, 2.5, 4 and 8 h after insemination or treatment with different reagents, or at different times after aging *in vitro*, eggs were observed under an inverted phase-contrast microscope (Leitz, Germany). Extrusion of the second polar body (PB2) and pronucleus (PN) formation were recorded. The criterion for egg activation was pronuclear formation or in some cases the formation of interphase nuclei in both blastomeres.

### *Electrophoresis and immunoblotting*

For all treatments, samples prepared from 20 eggs were loaded per lane. Morphologically normal eggs were randomly collected in sodium

dodecyl sulfate (SDS) sample buffer at 1, 2.5, 4 and 8 h after insemination or treatment with different stimuli or at 28, 48, 72 and 96 h aging after hCG injection, and heated to 100 °C for 4 min. After cooling on ice for 4 min and centrifuging at  $16,000\times g$  for 5 min, samples were frozen at -70 °C until use. The proteins were separated by SDS-PAGE with a 4% stacking gel and a 10% separating gel for 50 min at 188 volts, electrophoretically transferred onto PROTRAN nitrocellulose membrane (Schleicher & Schuell, Germany) for 1 h, 200 mA, at 4 °C. After blocking for 30 min in TBS buffer (20 mM Tris, 137 mM NaCl, pH 7.6) containing 5% BSA and 0.1% Tween-20, the membrane was incubated overnight at 4 °C in TBS buffer containing 1:5000 polyclonal anti-Active<sup>TM</sup> MAP kinase antibody that selectively recognizes the dually phosphorylated form of P42<sup>ERK2</sup> and P44<sup>ERK1</sup> (Promega, Madison, WI USA, Lot No. 64840) (Schaefer and Moravec, 1996; White *et al.*, 1996; Racke *et al.*, 1997). After 2 washes of 15 min each in TBS containing 0.1% Tween-20 (TBS-T), the membrane was incubated for 1 h at room temperature with donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc) diluted 1:6000 in TBS-T. The membrane was washed twice in TBS-T and then processed using the enhanced chemiluminescence (ECL) detection system. The intensity of the bands representing ERK2 and ERK1 in each lane was quantified by densitometry using TINA 2.0 software.

For reprobing, the blots were stripped of bound antibodies by washing in stripping buffer (100 mM  $\beta$ -mercaptoethanol, 20% SDS, 62.5 mM Tris, pH 6.7) at 50 °C for 30 min. The membrane was reprobed with polyclonal anti-ERK2 (Santa Cruz Biotechnology, Inc, Santa Cruz, Lot No. 1276) diluted 1:3000 using the same procedure as described above. All experiments were repeated at least twice.

#### Data analysis

All data on parthenogenic egg activation were evaluated by  $\chi^2$  analysis. Eggs showing signs of degeneration were not included. Student's *t* test was used to compare the mean OD value between different treatment time point and the control. Differences at  $P < 0.05$  were considered significant.

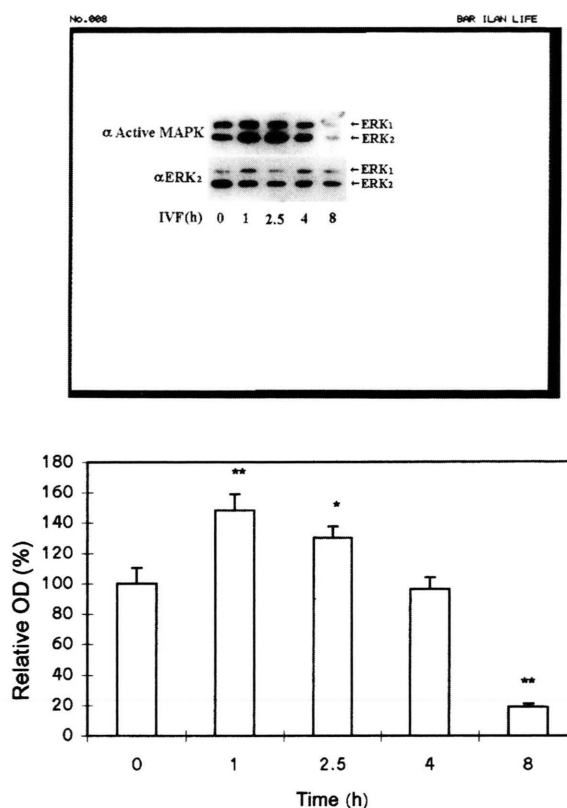


Fig. 1. Changes of MAP kinase after mouse egg fertilization. **A:** Immunoblotting of MAP kinase at different times after insemination. Metaphase II-arrested eggs were inseminated *in vitro*, eggs were collected at different times after insemination, and extracts were separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane. A total of 20 eggs were loaded per lane. Blots (upper) were incubated with a polyclonal anti-Active<sup>TM</sup> MAP kinase antibody and developed as described in Materials and Methods to show MAP kinase activity. The same blots were subsequently stripped and reprobed with polyclonal anti-ERK2 antibody which recognizes both active and inactive forms of MAP kinase to show the existence of the kinase (lower). The positions of ERK1 and ERK2 are indicated on the right. The same treatments were applied in all subsequent figures. Note, there was no mobility and quantity change after MAP kinase was inactivated at 8 h (the same as follows). The immunoblot shown is representative of two similar experiments. **B:** The relative MAP kinase activity at different times after fertilization, shown by relative optical density (OD), was determined by densitometry analysis of the anti-Active<sup>TM</sup> bands in the blot (the same as follows). The OD value at each time point was normalized against the control group. The value expressed by each bar represented the mean  $\pm$  standard deviation (SD) ( $n = 3$ ). \*  $P < 0.05$ , \*\*  $P < 0.01$ , compared with control.

Table I. Mouse egg activation induced by sperm and various chemical stimuli.

Treatments	1 h		2.5 h		4 h		8 h	
	PB2 [%]	PN [%]	PB2 [%]	PN [%]	PB2 [%]	PN [%]	PB2 [%]	PN [%]
Ctrl, 15-h egg	–	–	–	–	–	–	–	8/150 (5.3) <sup>a</sup>
Ctrl, 19-h egg	0/106 (0)	0/106 (0)	0/106 (0)	0/106 (0) <sup>a,d</sup>	–	2/106 (1.9) <sup>a,f,h,i</sup>	–	6/106 (5.7) <sup>a</sup>
IVF, 15-h egg	0/136 (0)	0/136 (0)	50/84 (59.5)	0/84 (0) <sup>a</sup>	79/84 (94)	13/164 (7.9) <sup>a,f,j</sup>	73/80 (91.3)	247/330 (74.8)
IA23, 15-h egg	1/75 (1.3)	0/75 (0)	1/75 (1.3)	0/75 (0) <sup>a</sup>	5/75 (6.7)	2/75 (2.7) <sup>a,l,h</sup>	5/75 (6.7)	6/75 (8.0) <sup>a</sup>
IA23, 19-h egg	0/150 (0)	0/150 (0)	11/150 (7.3)	6/150 (4.0) <sup>a</sup>	–	14/100 (14) <sup>a,g</sup>	–	45/70 (64.3) <sup>b</sup>
EtOH, 15-h egg	0/50 (0)	0/50 (0)	2/50 (4.0)	0/50 (0) <sup>a</sup>	–	–	15/30 (50)	16/50 (32.0) <sup>b,c</sup>
EtOH, 19-h egg	–	–	21/185 (11.4)	6/185 (3.2) <sup>a</sup>	27/75 (36)	31/180 (17.2) <sup>a,c</sup>	35/50 (70)	141/178 (79.2) <sup>b</sup>
CHX, 15-h egg	0/368 (0)	1/368 (0.3)	35/396 (8.8)	17/396 (4.3) <sup>a,e</sup>	193/452 (42.7)	198/572 (34.6) <sup>a,c</sup>	153/260 (58.8)	273/381 (71.7) <sup>b</sup>
STP, 15-h egg	0/175 (0)	3/175 (1.7)	0/118 (0)	35/118 (29.7) <sup>b</sup>	0/298 (0)	231/298 (77.5) <sup>b</sup>	2/171 (1.2)	132/173 (76.3) <sup>b</sup>

The PB2 emission and PN formation were evaluated at different times after each treatment. The denominators represent the numbers of eggs without degenerating signs and the numerators represent the cumulative totals of eggs with PB2 or PNs.

Ctrl, control; IVF, *in vitro* fertilization; IA23, A23187; EtOH, Ethanol; CHX, cycloheximide; STP, staurosporine; PB2, second polar body; PN, pronucleus.

Each experiment was repeated at least 3 times.

a Vs b, b Vs c, a Vs c, b Vs d, e Vs f, g Vs h, P < 0.01, d Vs e, i Vs j, P < 0.05.

## Results

### *Kinetics of mouse egg activation and MAP kinase activity during fertilization in vitro*

MAP kinase activity increased between 1 and 2.5 h after insemination (Fig. 1), just preceding or coinciding with PB2 emission (Table I). Activity remained comparable to levels in control eggs till 4 h post-insemination when PNs were not formed in most of the inseminated eggs (Table I and Fig. 1). At 8 h post-insemination, by which time pronuclei were visible in most of the eggs (Table I), MAP kinase activity sharply decreased (Fig. 1). When the same membrane was stripped, re-blocked and reprobed with anti-ERK2, the band shift of both ERK1 and ERK2 was not evident even 8 h post-insemination when MAP kinase had already been inactivated (Fig. 1A).

### *Egg activation and MAP kinase activity change induced by chemical stimuli*

In our experiment, eggs collected at 15 h or 19 h after hCG injection were rarely activated when they were cultured *in vitro* for 8 h (Table I). In contrast, obvious response was observed when the eggs were treated with ethanol, A23187, cycloheximide and staurosporine. However, these four stimuli induced cell cycle progression with different efficiencies, staurosporine being the strongest stimulus, followed by cycloheximide, ethanol

and A23187. As shown in Table I, when mouse eggs collected at 15 h after hCG injection were treated with 5  $\mu$ M A23187 for 5 min, PN formation was no different from control groups (P > 0.05). However, when aged eggs collected 19 h after hCG injection were treated by the same procedure, 64.3% were activated. Similarly, ethanol treatment induced activation of only 32% of the 15-h eggs, but 79.2% of the 19-h eggs (Table I). Persistent treatment with 500  $\mu$ M cycloheximide was a more effective method for activating 15-h mouse eggs, giving PN formation in 34.6% after 4 h incubation and 71.7% after 8 h. With staurosporine treatment, PN formation began at 1 h and was completed after 4 h. In contrast to the other treatments, activation of eggs with 2  $\mu$ M staurosporine was so fast that nearly all the eggs failed to extrude the PB2. Furthermore, there were usually more (4–6) visible pronucleoli in staurosporine-treated eggs than in other groups.

Eggs collected at different times after each treatment were used to follow the temporal changes of MAP kinase. In each case, PN formation was correlated well with the dephosphorylation (inactivation) of MAP kinase. Treatment of 15-h eggs with 5  $\mu$ M A23187 for 5 min did not influence MAP kinase during incubations up to 8 h (Fig. 2). With ethanol, MAP kinase activity decrease was found after 8 h (Fig. 2). When 19-h eggs were treated by these 2 reagents, MAP kinase



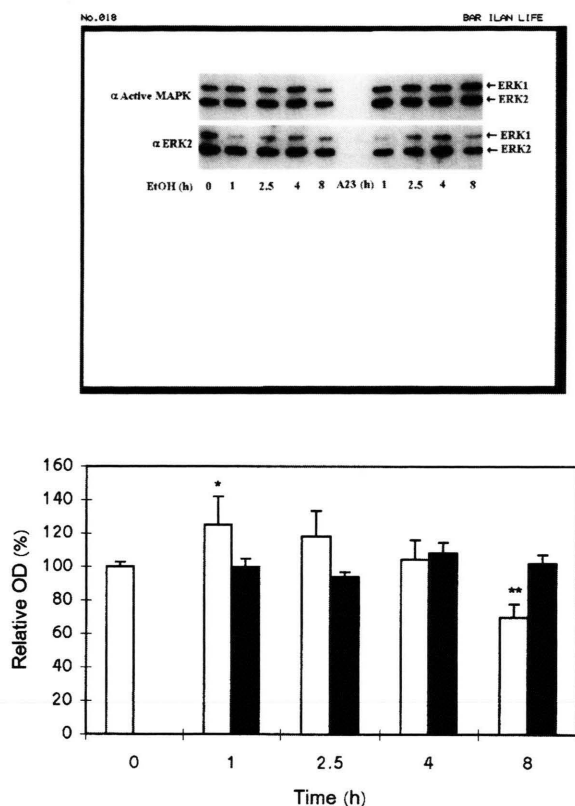


Fig. 2. Changes in MAP kinase in eggs collected 15 h after hCG injection after treatment with ethanol (EtOH) and A23197 (A23). **A**: Immunoblotting of MAP kinase at different times after treatment with EtOH and A23187. Experiments were conducted 3 times, with similar results. **B**: The relative MAP kinase activity shown by relative OD value of the bands at different times after treatment with EtOH (open bars) and A23187 (filled bars). The OD value at each time point was normalized against the first group in each treatment. The value expressed in each bar represented mean  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$ , \*\*  $P < 0.01$ , compared with control.

was completely inactivated after 8 h incubation when the pronuclei were formed in most of the eggs (Table I and Fig. 3). In cycloheximide-treated eggs, MAP kinase activity was significantly decreased at 4 h and was nearly fully inactivated by 8 h; MAP kinase inactivation and PN formation were well correlated in extent and timing (Table I and Fig. 4). Staurosporine induced MAP kinase inactivation concomitantly or slightly preceding PN formation (Table I and Fig. 4). Reprobing the membrane with polyclonal anti-ERK2 revealed that none of the treatments caused evident catabolism of MAP kinase (Figs 3, 4). Fur-

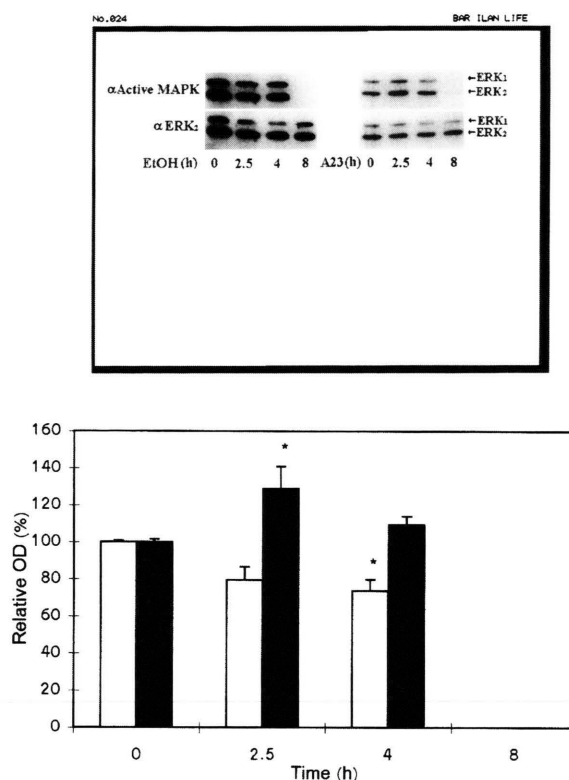


Fig. 3. Changes in MAP kinase in the eggs collected 19 h after hCG injection after treatment with ethanol (EtOH) and A23197 (A23). **A**: Immunoblotting of MAP kinase at different times after treatment with EtOH and A23187. Experiments were conducted 3 times, with similar results. **B**: The relative MAP kinase activity shown by relative OD value of the bands at different times after treatment with EtOH (open bars) and A23187 (filled bars). The OD value in each time point was normalized against the 0 time point in each group. The bar value represents mean  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$ , compared with control.

thermore, no mobility shift of ERK1 and ERK2 was observed under our experimental conditions (Figs 3, 4).

#### *Kinetics of egg activation and MAP kinase activity during spontaneous parthenogenic activation*

When the 15-h eggs were cultured *in vitro*, 7.4% (12 out of 162), 28.9% (33 out of 114) and 90% (45 out of 50) were activated at 28, 48 and 72 h after hCG treatment, respectively. By 96 h, 17.1% (7 out of 41) degenerated and 90.2% (37 out of 41) cleaved and arrested at the 2-cell stage. In con-

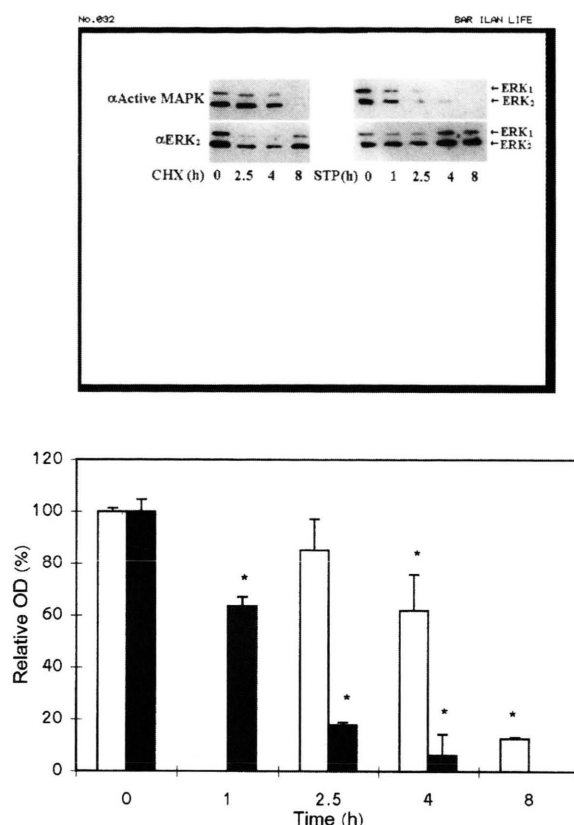


Fig. 4. Changes of MAP kinase in eggs collected 15 h after hCG injection after treatment with cycloheximide (CHX) and staurosporine (STP). **A:** Immunoblotting of MAP kinase at different times after treatment with CHX and STP. This immunoblot is a representative of 3 similar experiments. **B:** The relative MAP kinase activity shown by relative OD value of the bands at different times after treatment of eggs with CHX (open bars) and STP (filled bars). The OD value in each time point was normalized against the 0 time point in each group. The bar value represents mean  $\pm$  SD ( $n = 3$ ). \*  $P < 0.01$ , compared with control.

trast to IVF and artificial parthenogenic activation, MAP kinase inactivation preceded PN formation. At 48-h when PN formation was found only in 28.9% eggs, MAP kinase was already inactivated (Fig. 5). The inactivation of MAP kinase during aging was not due to the degradation, since even at 72 h the amount of ERK1 and ERK2 was not different from that of control group at 18 h (Fig. 5). At 96 h, the amount of ERK1 and ERK2 significantly decreased (data not shown).

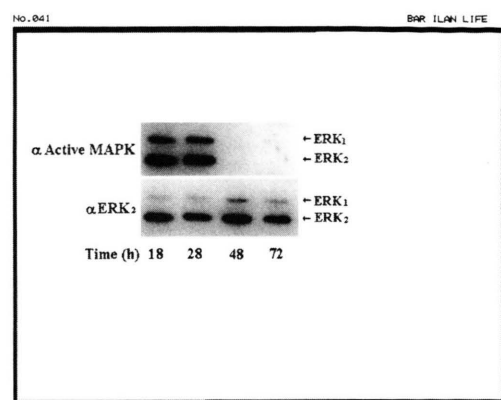


Fig. 5. Immunoblotting of MAP kinase at different times after the eggs were aged *in vitro*. MAP kinase was inactivated by 48 h (upper blots) but the protein still existed after 72 h (lower blots). This immunoblot is a representative of 3 similar experiments.

#### *Effect of okadaic acid on egg activation and MAP kinase activity induced by sperm and various stimuli*

We have previously demonstrated that okadaic acid, an inhibitor of protein phosphatases 1 and 2A, overcame the effect of staurosporine on KunminWhite mouse (Chinese local breed) and por-

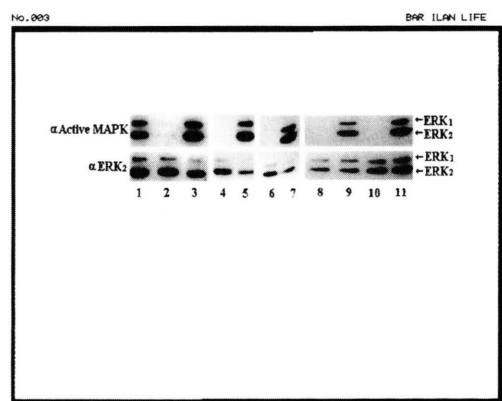


Fig. 6. Immunoblotting of MAP kinase in the eggs at 8 h after different treatments: effect of okadaic acid. Lane 1, control; lane 2, fertilized; lane 3, fertilized + okadaic acid; lane 4, alcohol-treated; lane 5, alcohol-treated + okadaic acid; lane 6, A23187-treated; lane 7, A23187-treated + okadaic acid; lane 8, staurosporine-treated; lane 9, staurosporine-treated + okadaic acid; lane 10, cycloheximide-treated; lane 11, cycloheximide-treated + okadaic acid. Pronuclear formation was inhibited when MAP kinase was superactivated by okadaic acid in different groups. This immunoblot is a representative of 2 similar experiments.

cine egg activation (Sun *et al.*, 1997a; Wang *et al.*, 1997a). OA also induces the precocious breakdown of pronuclear envelopes and inhibits PN formation induced by cycloheximide in CF1 mouse eggs (Moos *et al.*, 1996b). In this experiment, we found that neither sperm nor various stimuli could induce the formation of pronuclei when eggs were cultured in the medium containing 2  $\mu$ M OA. With all treatments (sperm+OA, staurosporine+OA, cycloheximide+OA, A23187+OA and ethanol+OA), MAP kinase remained phosphorylated, and MAP kinase activity was higher than in control eggs. The hyperactivation of MAP kinase by OA was not due to synthesis but to the phosphorylation of ERK1 and ERK2 (Fig. 6).

## Discussion

In the present study, both polyclonal anti-Active<sup>TM</sup> MAP kinase antibody and polyclonal anti-ERK2 antibody were used for immunoblotting. Anti-Active<sup>TM</sup> MAP kinase is an affinity purified antibody made against a dually phosphorylated protein that corresponds to the active form of MAP kinase enzymes, and it specifically recognizes this (active) form of ERK1 and ERK2 (Schaefer and Moravec, 1996; White *et al.*, 1996; Racke *et al.*, 1997; Wang *et al.*, 1997b). Anti-ERK2 is reactive with p42<sup>ERK2</sup> and to a lesser degree with P44<sup>ERK1</sup>. Therefore, it is reasonable that we observed 2 bands corresponding to ERK1 and ERK2 when either antibody was used.

Previously, MAP kinase phosphorylation was assessed indirectly by its mobility shift on SDS-PAGE. In this study, we found no such mobility shift of ERK1 or ERK2 when MAP kinase was inactivated. Other authors also found that the mobility-shift method has lower fidelity (Posda and Cooper, 1992; Verlhac *et al.*, 1994; Scrimgeour *et al.*, 1997). In contrast, the method we established using the phosphorylation-state sensitive anti-Active<sup>TM</sup> MAP kinase antibody is a sensitive and reliable method for measuring mouse-egg MAP kinase activation state. The use of anti-Active<sup>TM</sup> MAP kinase antibody in Western blots, followed by reprobing of the stripped membrane with anti-ERK2 as conducted in this study is an ideal method for detecting MAP kinase changes.

During fertilization, we observed an increase of MAP kinase activity between 1 and 2.5 h after in-

semination when the PB2 is emitted, followed by a sharp decrease at 8 h after insemination by which time pronuclei were formed in most eggs. Since most eggs were not penetrated until 30 min post-insemination and decondensing sperm head were first found at 1h post-insemination in our system (Sun *et al.*, 1997b), it is possible that the increases in MAP kinase activity is associated with sperm chromatin dispersion after penetration. Verlhac *et al.* (1994) also suggested that MAP kinase activity may be required for the remodeling of the sperm nucleus after fertilization. During meiotic maturation of mouse oocytes, MAP kinase is found to be related with the first meiotic spindle and microtubule behavior correlates with MAP kinase activity but not MPF activity. High MAP kinase activity persists when the first polar body (PB1) is emitted (Verlhac *et al.*, 1993). Furthermore, in immature mouse oocytes subjected to the antibody against Mos, the molecule upstream of MAP kinase in the cascade, formation of PB1 was blocked (Zhao *et al.*, 1991). Thus, another possibility is that the increase of MAP kinase activity after fertilization that occurred just around the time of the PB2 emission is related to this event. It is suggested that high MAP kinase activity keeps mammalian eggs arrested in MII stage (Verlhac *et al.*, 1994; Dedieu *et al.*, 1996). We dissent from this view, since our experiments showed that the resumption of meiosis II after fertilization or activation occurred when the MAP kinase activity is still high (4 h after insemination when PB2 were emitted in 94% of the eggs). Furthermore, during oocyte maturation, MAP kinase activity reaches peak level when the oocyte proceeds to the MI stage, but it continues maturation until the MII stage. We propose that high MAP kinase activity is necessary to prevent oocyte microtubules from organizing into the interphase state. Once MAP kinase activity is decreased, microtubule reorganization can proceed.

Consistent with results obtained by Moos *et al.* (1995, 1996a, 1996b), we found that inactivation of MAP kinase temporally correlated with the formation of the pronuclear envelopes induced by sperm and cycloheximide, and elevation of MAP kinase activity by OA treatment inhibited pronuclear formation. Previous experiment also showed that addition of purified MAP kinase to cell-free preparations of frog eggs at interphase induced the

interphase to metaphase transition (Gotoh *et al.*, 1991). OA, a specific inhibitor of PP1 and PP2A, caused retention of phosphorylation on a 70 kD protein which likely represents nuclear lamin in mouse eggs. OA-sensitive protein phosphatases exist in mouse oocytes and 1-cell embryos (Schwartz and Schultz, 1991). It has also been proved that protein phosphatase plays an attenuating role in regulation of MAP kinase activity in many cell types such as ventricular cardiomyocyte (Braconi *et al.*, 1996). All the above evidence suggests that PN formation is probably caused by inactivation of MAP kinase via the activation of OA-sensitive protein phosphatase.

In mouse eggs, staurosporine, shown in this study to inactivate MAP kinase, induced not only the formation of many pronucleoli, but also activation of the egg genome in the absence of fertilization (Sun *et al.*, 1997a). As observed in both *in vitro* fertilization (IVF) and parthenogenic activation, OA-dependent elevation of MAP kinase activity induced the disappearance of pronucleoli. Thus, entry into S phase is attenuated by MAP kinase in mouse eggs. In starfish eggs, it is also suggested that down-regulation of MAP kinase is necessary and sufficient for triggering the G/S phase transition (Tachibana *et al.*, 1997). Thus, we suggest that MAP kinase regulates not only pronuclear assembly/disassembly, but also chromatin behavior after egg activation. The present results also supported the suggestion that the different events after fertilization, for example, the PB2 emission and PN formation, may be uncoupled and controlled by different mechanisms (Moos *et al.*, 1995; Sun *et al.*, 1997c).

Our previous experiment suggested that inhibition of a staurosporine-sensitive protein kinase, possibly MAP kinase is responsible for the transition from metaphase to interphase (Sun *et al.*, 1997a; Wang *et al.*, 1997a). The present study indicated that MAP kinase activity decreased in a time-dependent manner in the eggs that were treated with 2  $\mu$ M staurosporine, beginning 1 h after treatment. There is a good temporal correlation between PN formation and MAP kinase inactivation, with MAP kinase inactivation slightly preceding PN formation. Thereafter, MAP kinase activity decreased so fast that the metaphase spindle was disrupted before the emission of PB2 (Sun *et al.*, 1997a). Protein kinase inhibitors 1-(5-isoqui-

nolinesulfonyl)-2-methylpiperazine (H7) and 6-DMAP were also reported to activate eggs (Mayes *et al.*, 1995; Sun *et al.*, 1995). It is possible that protein kinase inhibitor activates eggs by inactivating kinases, such as MAP kinase, via 6-DMAP sensitive mechanism (Moses *et al.*, 1995), which is required for PN formation.

When freshly ovulated eggs were treated with A23187, few were activated and MAP kinase activity did not decrease. When the freshly ovulated eggs were treated with ethanol, MAP kinase activity slightly decreased at 8 h, which was consistent with the extent of PN formation. Both ethanol and A23187 induced PN formation and MAP kinase inactivation with similar time course to fertilization, and the inactivation of MAP kinase correlated strongly with the extent of PN formation in 19-h eggs. In bovine eggs, ethanol activation causes the disappearance of p39<sup>Mos</sup>, a molecule which acts upstream of MAP kinase (Wu *et al.*, 1997), and Mos disruption was proved to induce parthenogenic development in unfertilized mos-knocked-out (Mos<sup>-/-</sup>) mouse eggs (Colledge *et al.*, 1994; Hirao and Eppig, 1997). We suggested that both A23187 and ethanol bypass the maintenance of MII arrest by targeting a calcium-sensitive mechanism and later induce PN formation by inactivating MAP kinase.

When the eggs were incubated *in vitro*, most of them formed pronuclei and cleaved till 72h and 96 h after hCG injection, respectively, but they did not develop further. Kikuchi *et al.* (1995) reported that histone H1 kinase activity in pig eggs decreased gradually with aging. It was also reported that bovine oocytes aged beyond the optimal time (44–48 h after *in vitro* maturation) for fertilization seemed to lose the capacity for Mos protein synthesis (Wu *et al.*, 1997). We found that MAP kinase activity had significantly decreased 48 h after hCG injection when only part of the eggs formed pronuclei, but most of the eggs formed pronuclei between 48 and 72 h. Although there is no direct comparison among MPF, Mos and MAP kinase changes during egg aging, it seems that MAP kinase is inactivated later than MPF and Mos. It is likely that the inactivation of upstream molecules finally leads to inactivation of MAP kinase. The delay in PN formation compared to MAP kinase inactivation is perhaps due to the delayed response of nuclear envelope precursors to MAP



kinase inactivation, since degenerative changes in various organelles are evident in aged eggs (Yanagimachi, 1994).

Taken together, the data suggest that MAP kinase is a key protein kinase in regulation of mouse egg activation. Increased MAP kinase activity is temporally correlated with the PB2 emission and sperm head decondensation. Although different stimuli (including sperm) may act through different mechanisms at early steps of egg activation, they finally inactivate MAP kinase, probably *via*

the action of protein phosphatase, and thus induces the transition to interphase.

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- Braconi Quintaji S. B., Church D. J., Rebsamen M., Vallotton M. B., Hemmings B. A. and Lang U. (1996), Role of protein phosphatase 2A in the regulation of mitogen-activated protein kinase activity in ventricular cardiomyocytes. *Biochem. Biophys. Res. Commun.* **221**, 539–547.
- Colledge W. H., Carlton M. B., Udy G. B. and Evans M. J. (1994), Disruption of c-mos causes parthenogenetic development of unfertilized mouse eggs. *Nature* **370**, 65–68.
- Dedieu T., Gall L., Crozet N., Sevellec C. and Ruffini S. (1996), Mitogen-activated protein kinase activity during goat oocyte maturation and acquisition of meiotic competence. *Mol. Reprod. Dev.* **45**, 351–358.
- Fissore R. A., He C. H. and Vande Woude G. F. (1996), Potential role of mitogen-activated protein kinase during meiosis resumption in bovine oocytes. *Biol. Reprod.* **55**, 1261–1270.
- Gotoh Y., Nishida E., Matsuda S., Shiina N., Kosako H., Shiokawa K., Akiyama T., Ohta K. and Sakai H. (1991), *In vitro* effect on microtubule dynamics of purified *Xenopus* M phase-activated MAP kinase. *Nature* **349**, 251–254.
- Hirao Y. and Eppig J. (1997), Parthenogenetic development of Mos-deficient mouse oocytes. *Mol. Reprod. Dev.* **48**, 391–396.
- Inoue M., Naito K., Aoki F., Toyoda Y. and Sato E. (1995), Activation of mitogen-activated protein kinase during meiotic maturation in porcine eggs. *Zygote* **3**, 265–271.
- Kikuchi K., Izaike Y., Noguchi J., Furukawa T., Daen F. P., Naito K. and Toyoda Y. (1995), Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged *in vitro*. *J. Reprod. Fertil.* **105**, 325–330.
- Kline D. (1996), Activation of mouse eggs. *Theriogenology* **45**, 358–361.
- Lorca T., Cruzalegui F. H., Fesquet D., Cavadore J. C., Mery J., Means A. and Doree M. (1993), Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* **366**, 270–273.
- Masui Y. (1991), The role of cytotstatic factor (CSF) in the control of oocyte cell cycle: A summary of 20 years of study. *Dev. Growth Differ.* **33**, 543–551.
- Mayes M. A., Stogsdill P. L. and Prather R. S. (1995), Parthenogenic activation of pig oocytes by protein kinase inhibition. *Biol. Reprod.* **53**, 270–275.
- Moos J., Visconti P. E., Moore G. D., Schultz R. M. and Kopf G. S. (1995), Potential role of mitogen-activated protein kinase in pronuclear envelope assembly and disassembly following fertilization of mouse eggs. *Biol. Reprod.* **53**, 692–699.
- Moos J., Xu Z., Schultz R. M. and Kopf G. S. (1996a), Regulation of nuclear envelope assembly/disassembly by MAP kinase. *Dev. Biol.* **175**, 358–361.
- Moos J., Kopf G. S. and Schultz R. M. (1996b), Cycloheximide-induced activation of mouse eggs: effects on cdc2/cyclin B and MAP kinase activities. *J. Cell Sci.* **109**, 739–748.
- Moses R. M., Kline D. and Masui Y. (1995), Maintenance of metaphase in colcemid-treated mouse eggs by distinct calcium- and 6-DMAP-dimethylaminopurine (6-DMAP)-sensitive mechanisms. *Dev. Biol.* **167**, 329–337.
- Moses R. M. and Kline D. (1995), Release of mouse eggs from metaphase arrest by protein synthesis inhibition in the absence of a calcium signal or microtubule assembly. *Mol. Reprod. Dev.* **41**, 264–273.
- Murray A. W., Solomon M. J. and Kirschner M. W. (1989), The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature* **339**, 280–286.
- Posda J. and Cooper J. A. (1992), Requirements for phosphorylation of MAP kinase during meiosis in *Xenopus* oocytes. *Science* **255**, 212–215.
- Racke F. K., Lewandowska K., Goueli S. and Goldfarb A. N. (1997), Sustained activation of extracellular signal-regulated kinase/mitogen-activated protein kinase pathway is required for megakaryocytic differentiation of K562 cells. *J. Biol. Chem.* **271**, 16439–16442.
- Sagata N., Watanabe N., Vande Woude G. F. and Ikawa Y. (1989), The product of the mos proto-oncogene is a cytotstatic factor responsible for meiosis arrest in vertebrate. *Nature* **342**, 512–518.
- Schaefer E. M. and Moravec R. (1996), Specific detection of biologically active MAP kinase using Anti-Active™ MAPK pAb. *Promega Notes* **58**, 34–35.
- Schwartz D. A. and Schultz R. M. (1991), Stimulatory effect of okadaic acid, an inhibitor of protein phos-

- phatases on nuclear envelope breakdown and protein phosphorylation in mouse oocytes and one-cell embryos. *Dev. Biol.* **145**, 119–127.
- Scrimgeour A. G., Blakesley V. A., Stannard B. S. and Leroith D. (1997), Mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathway are not sufficient for insulin-like growth factor I-induced mitogenesis and tumorigenesis. *Endocrinology* **138**, 2552–2558.
- Sobajima T., Aoki F. and Kohmoto K. (1993), Activation of mitogen-activated protein kinase during meiotic maturation in mouse oocytes. *J. Reprod. Fertil.* **97**, 389–394.
- Sun Q. Y., Gao S. R. and Chen D. Y. (1995), 6-Dimethylaminopurine (6-DMAP) spontaneously induces the transition to interphase in metaphase II mouse oocytes. *Reprod. Dev. Biol.* **4**, 14–20.
- Sun Q. Y., Liu H. and Chen D. Y. (1997a), Calcium-independent, egg-age-dependent parthenogenic activation of mouse eggs by staurosporine. *J. Reprod. Dev.* **43**, 189–197.
- Sun Q. Y., Gao S. R. and Chen D. Y. (1997b), Mechanistic studies of sperm head transformation during mouse egg fertilization. *Acta Zool. Sin.* **43**, 382–389.
- Sun Q. Y., Wang W. H., Hosoe M., Taniguchi T., Chen D. Y. and Shioya Y. (1997c), Activation of protein kinase C induces cortical granule exocytosis in a  $\text{Ca}^{2+}$ -independent manner, but not the resumption of cell cycle in porcine eggs. *Dev. Growth Differ.* **43**, 523–529.
- Tachibana K., Machida T., Momura Y. and Kishimoto T. (1997), MAP kinase links the fertilization signal transduction pathway to the G1/S-phase transition in starfish eggs. *EMBO J.* **16**, 4333–4339.
- Verlhac M. H., de Pennart H., Maro B., Cobb M. H. and Clarke H. J. (1993), MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. *Dev. Biol.* **158**, 330–340.
- 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.
- Wang W. H., Sun Q. Y., Hosoe M. and Shioya Y. (1997a), Calcium- and meiotic spindle-independent activation of porcine eggs by the inhibition of staurosporine-sensitive protein kinases. *Zygote* **5**, 75–82.
- Wang X. M., Zhai Y. and Ferrel J. (1997b), A role for mitogen-activated protein kinase in the spindle assembly checkpoint in XTC cells. *J. Cell Biol.* **137**, 1478–1483.
- White M. A., Vale T., Camonis J. H., Schaefer E. M. and Wigler M. H. (1996), A role for the Ral guanine nucleotide dissociation stimulator in mediating Ras-induced transformation. *J. Biol. Chem.* **271**, 16439–16442.
- Wood M. J., Whittingham D. G. and Rall W. F. (1987), The low temperature preservation of mouse oocytes and embryos. In: *Mammalian Development: A Practical Approach* (M. Monk, eds.). IRL Press, Oxford, pp. 255–280.
- Wu B., Ignatz G., Currie W. B. and Yang X. Z. (1997), Expression of Mos proto-oncogene in bovine oocytes during maturation *in vitro*. *Biol. Reprod.* **56**, 260–265.
- Yanagimachi R. (1994), Mammalian fertilization. In: *The Physiology of Reproduction* (E. Knobil and J. D. Neill, eds.). Raven Press, New York, pp. 189–318.
- Zernicka-Goetz M., Verlhac M. H., Geraud G. and Kubiak J. Z. (1997), Protein phosphatases control MAP kinase activation and microtubule organization during rat oocyte maturation. *Eur. J. Cell Biol.* **72**, 30–38.
- Zhao X., Singh B. and Batten B. E. (1991), The role of c-mos proto-oncogene in mammalian meiotic maturation. *Oncogene* **6**, 43–49.