

MAPK Activity in Porcine Oocytes Maturing InVitro

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유사분열 활성화 단백질 효소가 돼지난자의 체외성숙에 미치는 영향

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Abstract In this study, we determined effects of the mitogen-activated protein kinase (MAPK) inhibitor, U0126 on meiotic maturation, microtubule organization and actin filament assembly in the porcine oocyte. The phosphorylated MAPK was first detected at 12 h after the initiation of maturation cultures, fully activated at 24h, and remained until metaphase II. Treatment of germinal vesicle (GV) stage oocytes with 20 μ M U0126 completely blocked MAPK phosphorylation, but germinal vesicle breakdown (GVBD) was normally proceeded. However, the oocytes didn't progress to the metaphase I. The inhibition of MAPK resulted in abnormal spindles. In oocytes treated with U0126 after GVBD, polar body extrusion was normal, but the organization of the metaphase plate and chromosome segregation were abnormal. In conclusion, MAPK activity plays an important regulatory role in GV chromatin configuration and meiotic progress in porcine oocyte maturation.

요 약 본 연구에서는 MAPK 저해제인 U0126이 난자성숙과정에서 특히 감수분열, 미세소관 형성 그리고 액틴 필라멘트 형성에 미치는 영향을 조사하였다. 그 결과 MAPK 단백질은 12시간째에 인산화되기 시작하여, 24시간째에 대부분 인산화 되었고 metaphase II에 이르기 까지 유지되었다. 배포단계(GV)에 있는 난자를 U0126의 20 μ M 농도로 처리하였을 때 MAPK의 인산화가 완전히 억제되었으나 배포의 파열 단계(GVBD)로의 성숙에는 진행하였으나, metaphase I까지는 발달하지 못하였다. 또한 MAPK 저해제로 인해 비정상적인 방추사의 형성을 초래하였다. 난자를 배포의 파열단계(GVBD) 이후에 U0126을 처리하였을 때 극체의 방출은 정상 이었으나 증기 판의 배열과 염색체의 분열은 비정상적 이었다. 결론적으로, 유사분열 활성화 효소단백질인 MAPK의 활성화는 돼지 난자의 체외성숙과정에서 배포단계(GV)의 염색체의 배열과 감수분열의 완성에 중요한 조절 인자임을 이번 연구를 통해 알 수 있었다.

Key Words : oocyte maturation, MAPK, microtubules, actin filaments

1. INTRODUCTION

In most mammals, fully grown follicular GV oocytes are arrested at G2 phase, the so-called germinal vesicle (GV) stage of development. The oocytes resume meiosis in response to specific signals, often hormone, or after being liberated from their follicular environment. Following germinal vesicle breakdown (GVBD), chromatin is condensed, the metaphase I (MI) spindle is

organized [1]. The oocytes develop to metaphase of second meiosis (MII), where it extrudes first polar body and maintains a second meiotic arrest until fertilization. The family of MAPK (p42 and p44), also known as extracellular signal-regular kinase (ERK1 and ERK2), is serine/ threonine kinase that requires threonine and tyrosine phosphorylation to become fully active. Both ERK1 and ERK2 are phosphorylated and activated by MAPK kinase (MEK). The MAPK is activated during the

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whole oocyte maturation process and reaches maximal level at MII. It is proposed that MAPK plays a pivotal role in meiosis. MAPK activity is associated with the microtubule organization and cell cycle progression during pig oocyte [2]. Uring is a potent and specific inhibitor of MEK1 and MEK2, the kinases that phosphorylate and, thus, activates ERK type MAPKs [3]. In porcine oocytes, inhibition of ERK activity using Uring during the meiosis I /meiosis II transition suppressed chromosome separating Urinfirst polar body emission and formation of the MII spindle [3,4].

In contrast, a significant fraction of porcine oocytes treated with U0126 at MII completed the second meiotic division and entered interphase [5]. Although MAPK activity is implicated in controlling meiotic spindle formation, little information is available on the effect of MAPK on cytoskeletal components in mammalian oocytes maturing in vitro. Therefore, the present study examined the effect of the MAPK pathway inhibitor U0126 on microtubule organization, and microfilaments assembly in pig oocytes maturing in vitro.

2. MATERIALS AND METHODS

2.1 Porcine oocyte collection, In vitro maturation, and U0126 treatments

Porcine ovaries were obtained from gilts at commercial slaughterhouse and transported to the laboratory at 25°C in saline. For the collection of porcine cumulus-oocyte complex (COCs) were aspirated from follicles (2-5mm in diameter) with an 18-gauge needle and a syringe. COCs with intact unexpanded cumulus cells were isolated from cellular debris and rinsed three times in HEPES. These experiments use a basic culture medium consisting of TCM199 medium supplemented with 0.4% (w/v) BSA, 10 IU EGF, 10 IU hCG, and 10 IU PMSG. The effects of U0126 (Cell Signaling Technology, Beverly, MA) on IVM of pig oocytes were examined as follows. U0126 was added to the culture medium at concentrations of 10-30µM since these concentrations exerted a significant stimulatory effect on the maturation of pig oocytes in our previous study. COCs were cultured in a 500 µl droplet of TCM 199 in the presence or absence of 20µM U0126

under mineral oil at 39°C in a humidified atmosphere containing 5% CO₂ for 44h.

2.2 Western blot analysis

Oocytes (15 oocytes per sample) were solubilized in 20 µl 1X SDS Sample Buffer (62.5 mM Tris-HCl), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red) and heated for 5min at 95°C. For Western analysis, proteins were resolved on a 5-12% Tris-SDS page gel for 1h 30min at 80-100V. Samples were transferred to a nitrocellulose membrane (Amersham, Hybond-ECL) at 300mA for 2h using transfer buffer. After blocking with 5% skim milk in PBS for 1h, the membrane was incubated for at least 2h with a phospho p44/42MAPK antibody (Cell Signaling Technology, Beverly, MA) diluted 1:300 in blocking solution (1XTBS, 0.1% Tween-20, 5% w/v nonfat dry milk), washed three times in TBST (20mM Tris.HCl (pH7.5), 250mM NaCl, 0.1% Tween 20), and incubated for 1 h with an HRP-linked anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) diluted 1:2000 in blocking solution. After three washes with TBST, antibody binding was visualized using a Chemiluminescence Luminol Reagent (Invitrogen).

2.3 Immunofluorescence and confocal microscopy

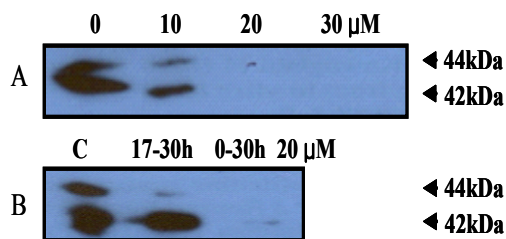
Approximately 30 oocytes in three replicates were collected at various time points during in vitro maturation. The oocytes were fixed with 4% paraformaldehyde, and stained for microtubules, γ -tubulin, actin, and NuMA. The following anti-bodies were used: monoclonal anti- α -tubulin clone B-512 (Sigma, St. Louis, MO), phalloidin-FITC (Sigma), anti-NuMA (BD Biosciences, NJ, USA). The samples were incubated with the secondary antibody, an anti-mouse IgG-FITC. The nuclear status of the oocytes was evaluated by staining with propidium iodide. The phase-contrast images of individual samples were digitized using laser scanning confocal microscopy (Leica Laser Technik GmbH) and MetaMorph software.

3. RESULT

3.1 Effect of U0126 on MAPK phosphorylation of porcine oocyte maturation

In order to determine the concentration of U0126 which would effectively inhibit activation of MAPK, oocytes were cultured for 24 h in medium containing 0, 10 or 20, 30 μ M U0126. The amount of activated MAPK was significantly reduced in a dose dependent manner [Fig 1A]. MAPK activity was completely absent when oocytes were cultured in the presence of 20 μ M U0126 for 14h (Fig 1B). However, when U0126 was added to cultures at 4 h after initiation of in vitro oocyte maturation, the MAPK activity was not completely, but significantly inhibited after 14 h in the maturation culture.

When GV oocytes were cultured in medium containing 20 μ M U0126 for 9h, the oocytes underwent GVBD, but were significantly inhibited in progression to the MI stage as compared to controls (Control; 76.7 \pm 5.66%, 10 μ M U0126; 29.0 \pm 7.35%, 20 μ M U0126; 26.4 \pm 5.74%). Polar body extrusion was observed in most oocytes when cultured in the presence of 20 μ M U0126 at 4h after initiation of in vitro oocyte maturation to 14h into the in vitro maturation incubation (Control ;76.6 \pm 4.21%, 20 μ M U0126; 70.7 \pm 4.61%). However, there was an decrease in normal MII stage oocytes when cultured for 4 to 14h (Control; 87.7 \pm 5.12%, 20 μ M U0126; 32.8 \pm 3.79%), for example of abnormality, polar body lacking a nucleus, as well as abnormal spindle or metaphase plate.



[Fig. 1] Western blot analysis of MAPK in the porcine oocytes during oocyte maturation. A, porcine oocytes were cultured in medium for 44h in the presence of 0, 10, 20, or 30 μ M U0126. B, porcine oocytes were cultured in the presence of 20 μ M U0126 for 0, 17-30, or 0-30h of the in vitro maturation.

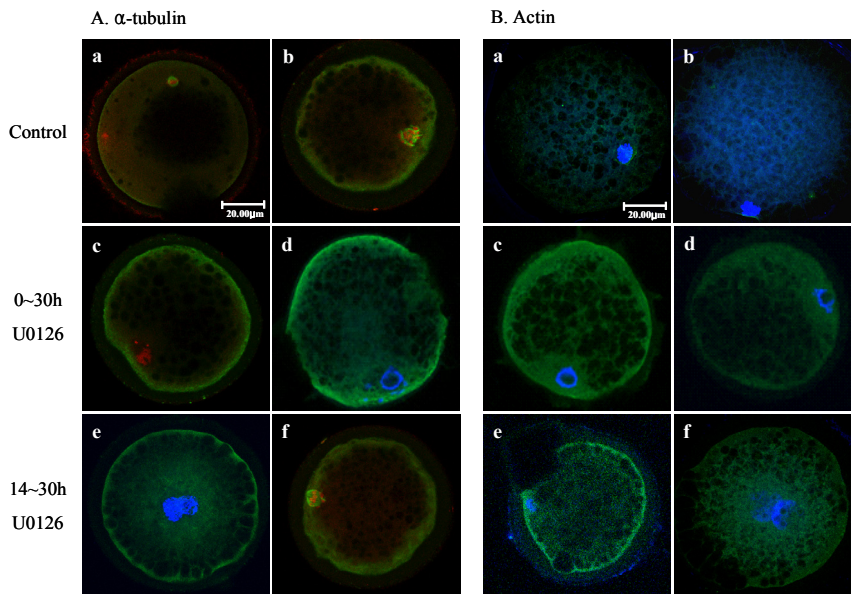
3.2 Effect of U0126 on microtubule and actin filaments

At GV stage oocytes, the microtubules were found in the cortex and some in near the vesicle [Fig. 2Aa]. Following GVBD, numerous small microtubule asters were produced near the chromatin [Fig. 2Ab] and formed a spindle at MI stage oocytes. The oocytes in which GVBD was arrested by U0126 treatment had several types of abnormal microtubule assembly [Fig. 2Ac]. In some cases, a disorganized array of microtubules formed around the chromatin [Fig. 2Ad], some oocytes had microtubules in a side near the chromatin [Fig. 2Ae], and others had incomplete spindles [Fig. 2Af]. Actin filaments were observed in the cytoplasm cortex at the GV stage oocytes [Fig. 2Ba]. Following GVBD numerous actin filaments emanate from the plasma membrane and form a cortical actin-rich layer following GVBD [Fig. 2Bb]. The actin filament network was not altered in U0126 treated oocytes arrested at GVBD [Fig. 2Bc&d]. Furthermore, normal actin filament assembly was observed during polar body emission in oocytes treated with U0126 from 4- to 14h after initiation of in vitro maturation[Fig. 2Be & f].

4. DISCUSSION

In mammalian cells, MAPK is activated by MEK phosphorylation upon stimulation of the cells by various growth factors [6,7]. Activated MAPK that remains in the cytoplasm can regulate mitotic microtubule dynamics by influencing the phosphorylation state of microtubule associated proteins [8]. Our results, which are consistent with previous observations [9], showed that MAPK activation occurred after GVBD, indicating that MAPK is not required for GVBD but is involved in regulating post-GVBD events .

Inhibition of MAPK activity by treatment with U0126 and arrest at the GVBD stage supports the hypothesis that MAPK activity is involved in formation of the metaphase plate. Previous studies have shown that MAPK activity is responsible for the control of microtubule and chromatin organization in mouse and porcine oocytes [4,10]. An upstream activator of MAPK, MOS, has also been shown to control microtubule and chromatin organization.



[Fig. 2] Immunofluorescent localization of microtubules and actin filament in porcine oocytes during in vitro maturation in the presence or absence of 20 μ M U0126. A, microtubule ;B, actinfilament. Green, microtubules (A) or actin filament (B); red or blue, chromatin.

MAPK was not activated in c-mos knockout mouse oocytes and abnormal spindles, loosely condensed chromosomes, and large polar bodies were observed [11,12]. Similarly, in the present study, abnormal assembly of microtubules was detected in U0126 treated, GVBD arrested oocytes.

Actin filaments that are associated with the plasma membrane are important for generating cell-surface specialization areas and also provide the driving force for cell surface remodeling. The mouse oocyte cortex contains numerous actin filaments that emanate from the plasma membrane, forming a uniform layer of F-actin. This layer of actin filaments appears to anchor the spindle and force polar body extrusion. In the present study, we did not detect any disassembly of actin filaments in U0126 treated, GVBD arrested oocytes. Furthermore, polar body extrusion rates were reduced allowing U0126 treatment. This suggests that MAPK is not involved in actin filament assembly or its function in polar body extrusion.

In conclusion, the meiotic abnormalities caused by U0126, a specific inhibitor of MEK, indicate that the MEK/MAPK pathway is an important regulator of microtubule and centrosome assembly, but not actin filament assembly.

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<Research Interests>

Animal Reproductive Physiology