MAP Kinase is Activated dring the Maturation of Porcine Oocytes*

Ki-Hwa Chung and Chul-Wook Kim**
Department of Animal Resources Technology, Jinju National University, Gyeongnam 660-758, Korea

ABSTRACT : In an attempt to evaluate the function of MAP kinase in porcine oocytes and to develop a method of the assessment of its activity, myelin basic protein (MBP) was used as a substrate to detect the MAP kinase activity of porcine oocytes which had undergone maturation *in vitro*. The existence of MAP kinase and MAP kinase kinase (MAPKK) was verified in immature porcine germinal vesicle (GV) oocytes at 0 h culture via Western blotting. Porcine oocytes exhibited a low level of MAP kinase activity during the first 20 h of culture, which increased at 25 h, during which time a breakdown in the nuclear membrane occurred. Significantly higher increases ($p<0.05$) of MAP kinase activity were detected at 30 h of culture. Using the gel phosphorylation method, MBP was phosphorylated at two positions corresponding to mammalian MAP kinase-extracellular signal-regulated kinase (ERK 1) (44 kDa) and ERK 2 (42 kDa). The absolute levels of those proteins did not increase during 40 h of culture, suggesting that the detected increase in MAP kinase activity was the result of phosphorylation rather than changes in the total amount of protein. MAPKK and MAP kinase were dephosphorylated in first-stage (MI) meiotic oocytes by the addition of cycloheximide, a protein synthesis inhibitor. These results of this study indicate that the MAP kinase cascade does exists in porcine oocytes and that its activation leads to oocyte maturation.
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Key Words : Porcine Oocytes, Maturation, Western Blotting, MAP Kinase, Phosphorylation

INTRODUCTION

Mitoge-activated protein (MAP) kinase, also known as mammalian extracellular signal-regulated kinase (ERK) in mammals, has been reported to be produced by a variety of organisms, ranging from yeasts to mammals. MAP is one of a number of mitogen-activated protein kinases. The function has been well reported, as signal transmitters in the cell cycle, the differentiation and proliferation of cells, and the synthesis of cellular proteins and membrane-bound transcription factors such as Elk, p90 ribosome S6 kinase (p90RSK) (Sturgill et al., 1988), MAP 2 (Hoshi et al., 1988; Ray and Sturgill, 1988), and MAP 4 (Hoshi et al., 1992). In PC12 cells, MAP kinase is activated by nerve growth factor (NGF) (Gotoh et al., 1990), and in hippocampal neurons, it is activated by a receptor for asparaginic acid (Bading and Greenberg, 1991).

MAP kinases are activated via the phosphorylation of serine and theronine hydroxyl groups by MAPKK (MAP kinase kinase) (Kosako et al., 1992; Matsuda et al., 1993). MAPKK is the only kinase that phosphorylates MAP kinase; MAPKK may also be auto-activated via the phosphorylation of one of its serine residues by MAPKK-kinase, which located in the upper region of MAPKK (Ahn et al., 1993; Matsuda et al., 1993).

The direct target of MAP kinase within the cell has not yet been definitively established; but, *in vitro* studies have shown that it catalyzes the phosphorylation of the protein, lamin (Peter et al., 1992). Myelin basic protein (MBP) has been used as a substrate for *in vitro* MAP kinase assays (Ohashi et al., 2003).

MAP kinase is activated during the differentiation processes of reproductive cells. It has been shown that during oocyte maturation in *Xenopus*, the activation of maturation promoting factor (MPF) is dependent on the activation of MAP kinase (Gotoh et al., 1991b; Posada and Cooper, 1992), as well as in mouse and porcine (Verlhac et al., 1996; Goto et al., 2002). MAP kinase activation has been observed in maturing mouse oocytes after the breakdown of the germinal vesicle (GVBD) stage (Verlhac et al., 1996). However, relatively little is known whether MAP kinase phosphorylates maturation promoting factor (MPF) in porcine (Ohashi et al., 2003), and bovine (Liu and Yang, 1999; Alberio et al., 2000) oocytes.

The objectives of the present study are therefore, 1) to determine whether the MAP kinase cascade is associated with the termination of GVBD in maturing porcine oocytes via the activation of MPF, 2) to examine the issue of the whether inactivation of MAP kinase by protein synthesis blockers has deleterious effects on the maturation of porcine oocytes, and 3) to establish whether MAP kinase itself is affected when protein synthesis is inhibited in maturing porcine oocytes.

MATERIALS AND METHODS

Media

Chemicals and media were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise

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** Corresponding Author: Chul-Wook Kim. Tel: +82-055-751-3281, Fax: +82-55-759-1893, E-mail: cwkim@jinju.ac.kr
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specified. The medium IVM used for oocyte maturation was supplemented with 1 IU/ml of PMSG (Peamex, Sankyo, Japan), 0.50 mM Na-pyruvate, 1.0% penicillin-streptomycin (10,000 IU; GIBCO, USA), and 10% porcine follicular fluid (pFF). The medium used for oocyte handling was mKRB (modified Krebs'-Ringer Bicarbonate) solution with 4 mg/ml bovine serum albumin (BSA). Both the maturation and handling media were adjusted to pH 7.4 and 280 osmolality.

Oocyte collection and *in vitro* maturation

Ovaries were collected from a local slaughterhouse and transported to the laboratory within two hours of collection at 37-39°C in 0.9% NaCl containing 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulfate. Cumulus-oocyte complexes (COCs) were aspirated from 2-5 mm diameter follicles with a 20 gauge needle attached to a 10 ml disposable syringe and washed three times in the handling medium. After selection of COCs by assessment of their diameter ranging with 120 um, COCs were cultured in IVM medium under mineral oil (Sigma, Co., USA) at 37°C in a humidified atmosphere of 5% CO_2 in air (Song et al., 2002) for 0, 10, 20, 25, 30, 40, 45 and 50 h, respectively.

After *in vitro* maturation, the cumulus cells were removed from COC's by re-pipetting with a narrow glass diameter pipette in handling medium containing 0.1% hyaluronidase. The remaining culture was used for the determination of MAP kinase activation, Western blotting and phosphorylation in gel. A portion of the culture was also used for preparation of whole mount samples for examining the oocyte maturation process *in vitro*. Prepared samples were segregated into the following groups: germinal vesicle (GV), pre metaphase I (PMI), metaphase I (M I), anaphase-telophase I (ATI) and metaphase II (MII) stage. The nuclear status of all samples was observed at all stages (data not shown).

Oocyte preparation for determining activation of MAP kinase

Oocytes at different stages were collected and washed twice in handling medium (mKRB). Groups of 50 oocytes at each stage were allocated and kept at -70°C. Oocytes were examined for maturation following the removal of cumulus cells, transferred to mKRB solution with Hoecst-33342 and then incubation for 15-60 minutes at 37°C. Oocytes were stained for examination of nuclear membrane and nuclear shape under a fluorescent microscope after centrifugation for 5 minutes at 15,000 rpm in order to make them visible (Beckman J2-21M/E, USA). Oocytes at GV, PMI and MI stages were cultured for 28-32 h, and sample AT and MII was incubated for 40-45 h, respectively, for the microscopic observation of oocyte maturation.

Determining MAP kinase activation

MBP (Sigma, Co., USA) was used as a substrate for the activation of MAP kinase in a system for the analysis of H1 kinase activation. About 20 oocytes were added to a vial containing 21.8 μM MBP. The vial was incubated at 30°C and samples were withdrawn at 15 min intervals for quantification of MAP kinase activation by the method employed by Natio and Toyoda (1991).

Analysis of MAP kinase by Western blotting

Twenty-five μl of sample buffer (65 mM Tris-HCl, 25% glycerol, 1 mM DTT, 1% SDS, 0.2 mM EDTA, pH 7.0) was added to 5 μl of a stock solution of oocytes in the handling buffer to a final volume of 30 μl and the resulting solution was denatured by heating at 100°C for 3 minutes. This sample was subjected to electrophoresis in 10% polyacrylamide gel (375 mM Tris-HCl, 0.1% TEMED, 10% acrylamide, 0.13% *bis*-acrylamide, 0.025% ammonium sulfate, 0.1% SDS). The stocking gel for loading the sample was 3% polyacrylamide gel (125 mM Tris-HCl, 0.2% TEMED, 3% acrylamide, 0.13% *bis*-acrylamide, 0.025% ammonium sulfate, 0.1% SDS). The electrophoresed sample was transferred to a nitrocellulose membrane and incubated for 12 h at 4°C in TBS buffer with 5% skim milk to block any non-specific binding. Mouse monoclonal anti-MAP kinase [(ERK 1 and 2), Zymed Laboratory] was diluted 1:2,000 with TBS buffer, and the membrane was incubated for 12 to 15 h at 4°C in the sample buffer for specific binding. For the second binding, the mixture of biotin-conjugated anti-mouse IgG and streptokinase-alkali phosphatase was used, and BCIP/NBT (Biotin, USA) was added as a substrate for the indicator. The commercial kit was used for the ensure experiment.

Analysis of MAP kinase activation by phosphorylation in gel

Putative protein kinase A (PKA) inhibitor was added to 5 μl of a stock solution of oocytes in handling buffer for final concentration of 0.5 μM, and 20 μl sample buffer (65 mM Tris-HCl, 25% glycerol, 1 mM DTT, 1% SDS, 0.2 mM EDTA, pH 7.0) was added to final volume 30 μl. This sample was denatured at 37°C for 3 min and was then subjected to electrophoresis in a polyacrylamide gel containing MBP 2.5 mg/ml. After electrophoresis, this gel was washed 4 times at 4°C for 4 h in 100 ml of solution (20% isopropanol, 50 mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol), and then the denatured MAP kinase in gel was then restored. This gel was submerged in phosphorylating solution (40 mM HEPES, pH 8.0, 2 mM DTT, 0.1 mM EGTA, 5 mM $MgCl_2$, 5 μM [γ-^{32}P]dATP (10 mCi/ml) at 28°C for one hour at for reaction. To remove the free [γ-^{32}P]dATP, it was washed 5 times for 2 h with 30

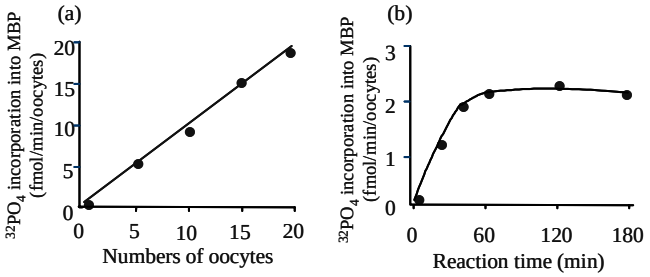

Figure 1. Incorporation of MBP by MAP kinase in porcine oocytes. The cytosol preparations employed were obtained from porcine follicular oocytes cultured for 45 h in pFF-PMSG. (a), Relationship between the numbers of oocytes per one assay vial with kinase activity. (b), time course of MBP phosphorylation at 30°C.

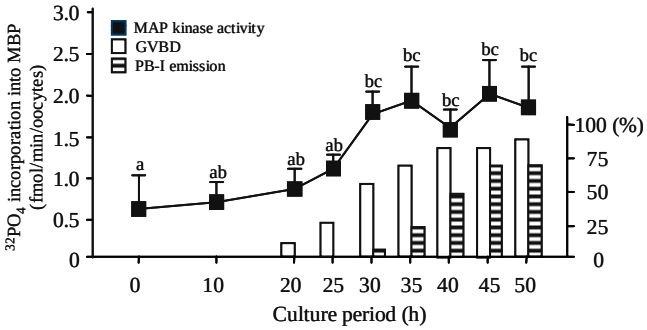

Figure 2. Changes in MAP kinase activity during meiotic maturation in porcine oocytes cultured in pFF-PMSG. After each culture period, the oocytes were collected and MAP kinase activity was assayed. Data are shown as mean±SEM of four experiments. Values with the same letters are significantly different ($p<0.05$) as evaluated by the student *t-test*. The percentages of oocytes which had lost their germinal vesicle (striped columns) and which had extruded their first polar bodies (closed columns) were determined. GVBD: germinal vesicle breakdown, PB-I: first polar body.

minutes interval in 300 ml solution (5% TCA, 1% Na-Pyrophosphate), until all radioactivity in the washed solution had disappeared (Kameshita and Fujisawa, 1989). This washed gel was dried on 3 M filter paper, and developed with X-ray film (Kodak Rochester., USA).

Analysis of MAP kinase and MAPKK activation in oocytes treated with inhibitor of protein synthesis by Western blotting

At 30-40 h after *in vitro* maturation, the COCs were further incubated in IVM medium for 30 to 40 h, supplemented with 10 μg/ml to CHX (cycloheximide) for additional 10 to 20 h. The oocytes were washed in handling buffer, and determined activation of MAP kinase and MAPKK by Western blotting described by Kameshita and Fujisawa (1989). Briefly, for the analysis of MAP kinase activation, rabbit anti-MAP kinase antibody (polyclonal K-23 Santa Cruz., USA) was used at a dilution of 1:500. For the second reaction, biotin-conjugated anti-rabbit IgG (Cappel ICN Pharm. Inc., USA) was used. In the case of the analysis of MAPKK activation, monoclonal anti-MAPKK (clone 4A5, MBL) was used at a dilution of 1:500, and biotin-conjugated-anti mouse IgG (Cappel, ICN Pharm. Inc., USA) was used as a second antibody for binding.

Statistics

All the data were analyzed ANOVA followed by Student's *t-test*. The differences were considered significant at $p<0.05$.

RESULTS

Assay system for MAP kinase activation in *in vitro* oocyte maturation

The incorporation of $^{32}PO_4$ into MBP as an indicator of kinase activation increased in proportion to the absolute number of oocytes in the incubation mixture (Figure 1a). The change of $^{32}PO_4$ incorporation into MBP is shown in Fig. 1b as a function of reaction time, 0, 15, 30, 50, 100 and 150 minutes for mixtures containing 10 oocytes and [γ-^{32}P]dATP at 30°C. Incorporation rate changes were proportional to reaction time for periods up to 50 minutes, but decreased for longer periods of time. The incorporation value of $^{32}PO_4$ at 40 minutes reaction were maximal in this study at 40 minutes.

Changes in MAP kinase activation during oocyte maturation

The overall change of MAP kinase activation is shown as an average of four experimental values in Figure 2. The bar column exhibits the number of oocytes undergoing GVBD, while the striped column represents oocytes that have extruded the first polar antibody. The value of MAP kinase activation of oocytes in the GVBD state or having a polar body was 0%. The value of MAP kinase activation was the lowest for immature oocytes immediately after collection (0 h in culture), and did not increase until after 25 h of culture. A rapid increase in MAP kinase activation was found for oocytes in transition from GVBD to the MI stage; a level of high activation of this enzyme was maintained up to 50 h of culture, with an insignificant decrease ($p<0.05$) at 40 h, when the oocyte extrudes the first polar body. Our experimental results indicated that the activation of MAP kinase is low in oocytes at GV stage and then begins to increase at GBVD stage, reaching high sustained levels during the maturation stage from MI to MII.

Determination of MAP kinase activation by phosphorylation in gel

Myelin basic protein (MBP) has been used as a substrate for the assay of cdc2 and other enzymes in addition to MAP kinase (Pearson and Kemp, 1991).

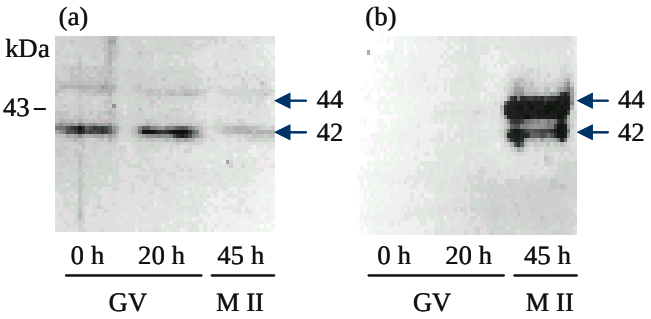

Figure 3. Detection of MAP kinase during metaphase by in-gel assay and Western blotting. (a) Fifty oocytes collected at 0, 20 and 45 h were subjected to SDS-PAGE with MBP-containing gel. The enzymes were renatured, and kinase reaction was performed in the gel with [γ-^{32}P] dATP. The gel was washed, dried and auto-radiographed. (b) Detection of MAP kinase by immunoblotting. The oocytes, collected at 0, 20 and 45 h of culture, were subjected to immunoblotting with anti-MAP kinase monoclonal antibody. GV: germinal vesicle, M II: second metaphase.

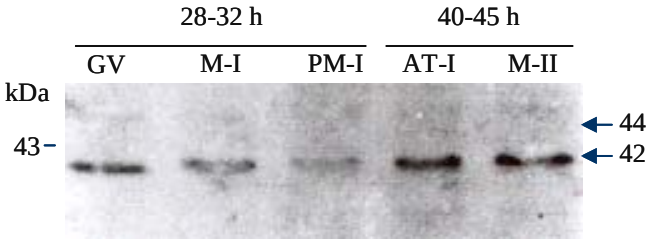

Figure 4. Detection of MAP kinase by immunoblotting. The oocytes collected at 32 h and 45 h of culture were classified according to meiotic maturation stage and subjected to immunoblotting with anti-MAP kinase monoclonal antibody. GV: germinal vesicle, M-I: first metaphase, AT-I: fist anaphase-telophase, M-II: second metaphase.

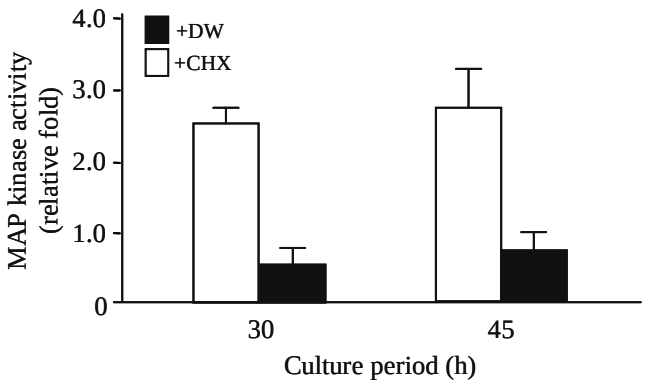

Figure 5. Changes in MAP kinase activity during porcine meiotic maturation. The oocytes were cultured in pFF-PMSG with distilled water (+DW) or with cycloheximide (+CHX, 10 μg/ml). Data are expressed as the fold strength of the control (GV oocytes cultured for 0 h). Data are shown as the mean±SEM from three different replicated experiments.

According to the results of our assay, the activation pattern of MAP kinase is nearly identical to published reports on histone H1 kinase when MBP is used as a substrate. In this assay for the activation of MAP kinase, the MBP reaction mixture was separated by SDS-PAGE and the altered protein was restored and phosphorylated. In this fashion, it was possible to analyze the relationship between molecular weight and the activation of MAP kinase.

Following oocyte collection, three samples were subject to electrophoresis: 50 immature oocytes (0 h culture), 50 oocytes at 20 h culture (just before GVBD) and finally, 50 oocytes at 40 h culture (oocytes at approximately 70% of maturity)(data not shown). In the gel, the band position(s) of phosphorylated MBP were identical to the molecular weight, (44 and 42 kDa), of MAP kinase that was present at MII stage in 45 h culture. The phosphorylated MBP band of MAP kinase in oocytes at GV stage in 0 h culture, as well as that of MAP kinase in oocytes following 20 h of culture was not present in the gel. This result was identical to those obtained for other assay systems for MAP kinase activation. The activation of MAP kinase is detection in the maturation process of porcine oocytes in this experiment only when MBP is used as a substrate for this enzyme.

Analysis of MAP kinase by western blotting

Two protein bands corresponding to 42 and 44 kDa were detected in oocytes cultures at 0 h, 20 h and 45 h (similar to those used for phosphorylation experiment in gel), when they were analyzed by Western blotting with anti-MAP kinase antibody (Figure 3a). The same bands were also observed in oocytes at the GV stage in 0 and 20 h culture and one at the M 2 stage in a 45 h culture. The intensity of the signal band for the MAP kinase in the gel was the same in all lanes except for one in the 45 h culture, in which the band intensity had diminished (Figure 3b). These results indicate that MAP kinase is, in fact activated by phosphorylation. The signal for MAP kinase activation in gel was decreased in all oocytes except those at the GV stage (Figure 4). Since the protein content of MAP kinase in porcine oocytes after the GVBD stage remains constant throughout the remainder of the maturation process, this suggests that the activation of MAP kinase in dependent on the its phosphorylation, rather than an increase in protein content in MAP kinase.

Inactivation of MAP kinase and MAPPK by protein synthesis inhibition

The activation of MAP kinase was not observed in cultured oocytes which has been treated with CHX at 30 h and even 45 h (Figure 5). The phosphorylated form of two MAP kinases, ERK 1 (44 kDa), ERK 2 (42 kDa), were detected in cultured oocytes at 0 hours (lane 1) at the GV stage by Western blotting. The levels of activated MAP kinases were reduced in the 30 h culture (with over 90% of the oocytes at the MI stage - lane 2) and in the 50 h culture

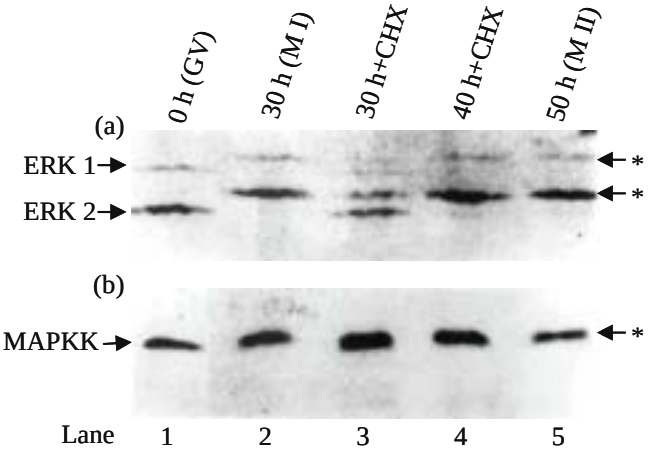

Figure 6. Detection of ERK 1, ERK 2, and MAPKK by immunoblotting. Oocytes were collected at each culture period, and subjected to immunoblotting with anti-MAP kinase polyclonal antibody (a) or anti-MAPKK antibody (b). Lane 1: GV-stage oocytes cultured for 0 h, lane 2: M I oocytes cultured for 30 h, lane 3: oocytes cultured for 30 h and then treated with CHX for 20 h, lane 4: oocytes cultured for 40 h and then treated with CHX for 10 h and lane 5: M II oocytes cultured for 50 h. The electrophoretically retarded bands represent the phosphorylated (activated) forms of MAP kinase and MAPKK (arrow with an asterisk).

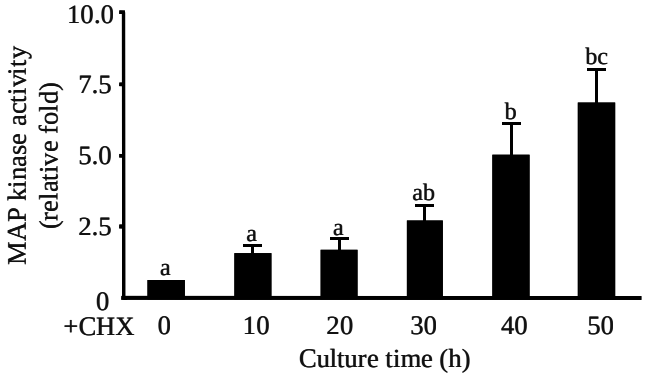

Figure 7. Changes in MAP kinase activity in porcine oocytes, cultured with CHX. Data are expressed as fold strength of control (GV oocytes cultured for 0 h) and as ANOVA results with different letters indicating a statistical difference ($p<0.05$) between treatments. Data are shown as the mean±SEM from three experiments.

(with over 93% oocytes at the MII stage-lane 5). Both active and inactive bands corresponding to MAP kinase were detected in the 30 h culture (MI stage-lane 3); however, the increase in activation of MAP kinase was not considered significant when it was compared to the value for the 0 h culture (GV stage) (Figure 7, column 30+20). In 40 h CHX-treated cultures (oocytes in the MII stage), only the activated form of MAP kinase was observed (Figure 6a, lane 4), and the activation of MAP kinase was increased significantly (Figure 7, column 40+10). The band intensity of activated MAPKK appeared to be decreased in the 30 and 50 h cultures by Western blotting assay with anti-MAPKK antibody (Figrue 6b). The band corresponding to inactivated MAPKK was also detected in 30 h culture of oocytes (MI stage) and activated forms of MAPKK were observed in CHX treated oocytes at the MII stage (40 h culture).

DISCUSSION

MAP kinase activation changes, which occur throughout oocyte maturation processes have been reported in *Xenopus* (Gotoh et al., 1991a; Nebreda and Hunt, 1993), and in mice by Verlhac et al. (1996). This kinase is activated immediately prior to germinal vesicle breakdown (GVBD) in *Xenopus* and after GVBD in mouse. High levels of this enzyme are maintained during meiosis. The results in this study, specifically the activation pattern of MAP kinase, are consist to those reported previously by other investigators (Goto et al., 2002), and high levels MAP kinase activation have been maintained from the MI to MII phase in the maturation process. There are two isoforms of MAP kinase in mammals, the molecular weights of which are 44 kDa (ERK 1) and 42 kDa (ERK 2) (Verlhac et al., 1993, 1996). The results herein serve to verify the presence of isoforms of MAP kinase in porcine oocytes which are similar to those found in mouse oocytes, via phosphorylation of the enzyme in the gel and Western blotting. In addition, the time of activation of this enzyme system and its effects appear to be identical for porcine and mouse oocytes. Evidence of strong MAP kinase activation was detected by the phosphorylation assay in the gel in porcine oocytes following 45 h of culture, but not at 0 or 20 h of culture. These results are in agreement with previous studies which conclude that MAP kinase activation in porcine oocytes is the result of phosphorylation enzyme rather than an increased level of kinase itself, i.e. protein content (Shibuya et al., 1992; Shibuya and Ruderman, 1993).

MAP kinase activation to appears prevalent, likely that it is important in oocyte maturation in all species; however its true function remains unknown. Verlhac et al. (1993) has reported that MAP kinase was present at microtubule-organizing centers (MTOC) or the spindle pole in mouse oocytes, and its activation is involved in changes of chromosomal and that microtubule status. In addition, the same investigator suggests that activation of MAP kinase is responsible for the formation of spindle during the MII stage in mouse oocyte maturation. In *Xenopus* oocytes, MAP kinase activation functions as a cytostatic factor (CSF), which stops the maturation of oocytes at the MII stage (Haccard et al., 1993; Kosako et al., 1994). In c-mos knockout mice, where MAP kinase is not activated, treatment of oocytes with activated enzyme permits the

continuation of continues the maturation beyond the MII stage (Colledge et al., 1994; Hashimoto et al., 1994). The second polar body, released following fertilization of mouse and porcine oocytes, was consistently released at the MII stage, resulting in the arrest of maturation arrest at the MII stage (Verlhac et al., 1996; Goto et al., 2002).

In our study, high levels of MAP kinase activation at the MI stage I were maintained into the MII stage, suggesting that the activation of this enzyme plays a role in CSF (colony stimulating factor). The level of MAP kinase activation is generally high for normal oocytes at the first stage of meiosis, but as shown in this study, it is decreased in CHX-treated maturation-arrested oocytes at the MI stage. The low level of enzyme activation appears to be due to its dephosphorylation by Western blotting rather than absolute decreases in the enzyme itself. A similar explanation is likely for the low level of MAPKK activation in CHX-treated oocytes as well.

In this study, oocyte maturation is suppressed by the dephosphorylation of kinase in CHX-treated oocytes, with a resultant maturation and abnormal spindle formation in the MI stage at 30 h culture. A likely explanation for this phenomenon is the involvement of MAP kinase in microtubule formation at the MI stage, which when inactivated by CHX, an inhibitor of protein synthesis, results in the formation of an abnormal spindle pole and blockage of meiosis.

The results of this study, while they do not elucidate the exact function of MAP kinase in porcine oocytes, strongly suggest an important role for the MAP kinase cascade in porcine oocyte. This study, demonstrates the following in porcine oocyte maturation system *in vivo*: 1) the existence of MAPKK and two types of MAP kinase, ERK 2 and ERK 1 (44 kDa and 42 KDa), in the immature oocyte, 2) MAP kinase activation in meiosis, with the maintenance of activity into the final stages of oocyte maturation, 3) an elevation in kinase activation as a result of phosphorylation, rather than absolute increases in the levels of enzyme itself, and 4) suppression of the MAP kinase cascade activation at the MI stage by inhibitors of protein synthesis.

We conclude that this study definitively proves the existence of the MAP kinase cascade in porcine oocytes and its primary role in oocyte maturation. In addition, the activation patterns of MAP kinase in porcine oocytes appear similar to those reported in other animal species. Further investigations in the role of MAP kinase activation in the suppression of porcine oocyte maturation and induction of GVBD, as has been reported in *Xenopus* is warranted.

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