Activation of mitogen-activated protein kinase during meiotic maturation in porcine oocytes

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Summary

To investigate the involvement of mitogen-activated protein kinase (MAP kinase) in meiotic maturation of porcine oocytes, we assayed MAP kinase activity using myelin basic protein (MBP) as a substrate. MAP kinase activity was low during the germinal vesicle stage, 0—20 h of culture. An abrupt increase was observed at metaphase I (30 h of culture), and activity remained significantly higher than that at 0 h until 50 h of culture, with a transient slight decrease at the time of first polar body extrusion (40 h). Detection of the kinase activity by an in-gel phosphorylation assay confirmed that the 42 and 44 kDa MAP kinases were significantly activated in 45 h cultured oocytes but not in 0 h oocytes, and just slightly in 20 h oocytes. In immunoblotting, however, the 42 and 44 kDa bands were detected in 0, 20 and 45 h cultured oocytes. Furthermore, the signal strength of the two bands did not change during the period of culture, but shifted up to 45 h, indicating that the activation of MAP kinase depended not on the synthesis but on the phosphorylation of this enzyme. These results suggest that the activation of MAP kinase is involved in the regulation of meiotic maturation of porcine oocytes, and especially in the regulation after germinal vesicle breakdown.

Key words: In vitro maturation, MAP kinase, Meiosis, Oocyte, Pig

Introduction

During meiotic maturation, protein phosphorylation and dephosphorylation play key roles in a series of events such as nuclear envelope breakdown, chromosome condensation and cytoskeletal change (Albertini, 1992). It is therefore important to investigate the regulation of protein kinase activation in elucidating the regulatory mechanisms for the induction of M-phase events.

Mitogen-activated protein kinases (MAPKs), also termed extracellular signal-regulated kinases (ERKs), are intermediates in signal transduction pathways that are initiated by a variety of mitogens such as growth factors, tumour promoters and proto-oncogene products in mammalian cultured somatic cells (reviewed in Davis, 1993). MAPKs/ERKs are activated by phosphorylation on both tyrosine and serine/threonine residues by an upstream kinase identified as a dual specific MAP kinase or MAPK/ERK kinase (MEK) (Matsuda et al., 1992, 1993; Kosako et al., 1992). Previous reports suggested that microtubule-associated protein 2 and bovine myelin basic protein (MBP) were phosphorylated by activated MAP kinase in vitro (Ray & Sturgill, 1988; Erickson et al., 1990). The MAP kinase cascade appears to be conserved from yeast to vertebrates and thus might play a critical role in diverse intracellular signalling processes by which external signals trigger the G0-G1 transition of the cell cycle (Nishida & Gotoh, 1993).

MAP kinase is also stimulated during meiotic maturation in sea star (Sanghera et al., 1991), clam (Shibuya et al., 1992a), Xenopus (Gotoh et al., 1991a; Posada et al., 1991) and mouse oocytes (Sobajima et al., 1993; Verlhac et al., 1993, 1994). The mechanisms of MEK–MAP
kinase activation have been well studied, especially in Xenopus, and it is indicated that the activation of this kinase cascade occurs downstream of maturation promoting factor (MPF) (Gotoh et al., 1991a, b), the proto-oncogene product Ras (Shibuya et al., 1992; Hattori et al., 1992) or Mos (Nebreda & Hunt, 1993; Posada et al., 1993; Shibuya & Ruderman, 1993). Moreover, the microinjection study of anti-MEK antibody into Xenopus oocytes showed that no oocytes underwent germinal vesicle breakdown (GVBD) without activation of MAP kinase (Kosako et al., 1994a). While these results showed that MAP kinase activation is indispensable to the resumption of meiosis in Xenopus, the pattern of the activation and function of this kinase in mammalian oocytes is unclear. Previous reports on mouse oocytes showed that MAP kinase activation was observed after GVBD, but the manner in which this occurred was different in each report (Sobajima et al., 1993; Verlhac et al., 1993, 1994). Cultured in vitro, mouse oocytes resume their maturation within 1 h. This quick transition of the cell cycle might cause difficulty in detecting the changes in kinase activation, and result in such a discrepancy. In pig, on the other hand, meiotic resumption occurs after about 24 h of culture (Naito et al., 1988; Naito & Toyoda, 1991; Jung et al., 1993; and in this report as follows). This long duration of the germinal vesicle (GV) stage makes it easy to analyse the meiotic events occurring in the nuclei.

The present study, therefore, was designed to define the pattern of MAP kinase activation during meiotic maturation of porcine oocytes cultured in vitro, in order to study the relationship between the MEK–MAP kinase cascade and mammalian oocyte maturation. The results revealed that MAP kinase activity was low in GV-stage oocytes but significantly increased in mature oocytes, which demonstrates that MAP kinase activation can function as a key step in meiotic events in the pig as well as in Xenopus.

Materials and methods

Preparation of oocytes

Porcine ovaries were obtained from a slaughterhouse and transported to the laboratory in saline at 37–39°C. Follicular oocytes were collected by puncturing medium-sized follicles (2–5 mm in diameter). Each of 10–15 cumulus–oocyte complexes was cultured in 0.1 ml porcine follicular fluid (pFF) collected as described previously (Naito et al., 1988) with 1.0 IU pregnant mares’ serum gonadotrophin per millilitre (Peamex, Sankyo Co., Japan) for various periods up to 50 h at 37°C in 5% CO₂ in air. After culturing, the oocytes were treated with 150 U hyaluronidase (Type IV-S, Sigma, St Louis, MO) in a modified Krebs–Ringer bicarbonate solution (TYH; Toyoda et al., 1971) for a few minutes at room temperature. Thereafter, the surrounding cumulus cells were removed by pipetting gently with a fine-bore pipette. The denuded oocytes were used for MAP kinase assay and immunoblotting, and some of them were mounted on glass slides, fixed in ethanol–acetic acid fixative and stained with 0.75% acetic orcein solution. The stained oocytes were examined under a phase-contrast microscope (Nikon) and classified according to meiotic maturation stages.

Kinase assays

Ten oocytes were lysed in 10 µl extraction buffer (pH 7.2) composed of 15 mM EGTA, 1% Nonidet p-40, 60 mM sodium β-glycerophosphate, 30 mM p-nitrophenylphosphate, 25 mM Mops, 15 mM MgCl₂, 0.2 mM Na₃VO₄, 1 mM dithiothreitol (DTT), 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM phenylmethylsulphonylfluoride (PMSF) and 50 µM p-aminobenzoic acid (PABA), and frozen at −70°C until used. The lysate (10 µl) was added to 5 µl of 2.5 µM cAMP-dependent protein kinase inhibitor (Sigma), 5 µl of 2 mg/ml myelin basic protein (MBP; Sigma) and 5 µl of 0.1 mM [γ-32P]ATP (0.4 mCi/ml). Reactions were performed at 30°C for 40 min and stopped by 0.4 ml of 20% trichloroacetic acid solution (TCA) and 0.1 ml of 1% bovine serum albumin (BSA-FRV; Wako Pure Chemical Ind., Japan) as a carrier protein for precipitation. The assay suspensions were centrifuged at 15,000 rpm for 5 min and supernatants were discarded. The precipitates were washed three times with 0.4 ml of 20% TCA and dissolved in 0.2 ml of 1 M NaOH. To the solution was added 1 ml of scintillation fluid (ACS II; Amersham International, Amersham, UK) and radioactivity measured using a liquid scintillation counter (Aloka, LSC-1000). The value for blank tubes that contained all materials except lysed oocytes was subtracted from each experimental value to obtain the kinase activity, which was represented as 10⁻¹⁶ mol phosphate incorporated per minute per oocyte.

In-gel phosphorylation assay

MAP kinase activity was assayed in polyacrylamide gel containing MBP (2.5 mg/ml) (Gotoh et al., 1990). The cell lysates, prepared as described above, were denatured at 37°C for 3 min in 65 mM Tris-HCl (pH 7.0) containing 25% glycerol, 1 mM DTT, 1% SDS and 0.2 mM EDTA. After electrophoresis at 50 mA for 3 h, SDS was removed by washing the gel at room temperature with two changes of 100 ml each of 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) for 1 h, with 250 ml of 50 mM Tris-HCl (pH 8.0) containing...
5 mM 2-mercaptoethanol for 1 h, and with two changes of 100 ml of 6 M guanidine HCl for 1 h. The enzyme was renatured with five changes of 250 ml each of 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol and 0.04% Tween 40 at 4°C for 16 h. Kinase reaction was performed in the gel by incubating at 28°C for 1 h with 15 ml of 40 mM Hepes (pH 8.0) containing 2 mM DTT, 0,1 mM EGTA, 5 mM MgCl₂ and 5 μM [γ-32P]ATP (10 μCi/ml). The reaction was terminated by washing with 5% (w/v) TCA containing 1% sodium pyrophosphate until the radioactivity of the solution became negligible. The washed gel was dried on filter paper and exposed to Kodak X-ray film at -70°C.

Immunoblotting

Fifty oocytes were lysed in 15 μl extraction buffer, and thereafter immediately 15 μl of sample buffer composed of 65 mM Tris-HCl, 25% glycerol, 1 mM DTT, 1% SDS and 0.2 mM EDTA were added. Prior to electrophoresis, the samples were denatured at 100°C for 3 min. SDS-PAGE was performed according to Laemmli (1970), using modified polyacrylamide gels composed of 375 mM Tris-HCl, 0.1% TEMED, 10% acrylamide, 0.13% Bis, 0.025% ammonium persulphate and 0.1% SDS (running gel); and 125 mM Tris-HCl, 0.2% TEMED, 3% acrylamide, 0.04% Bis, 0.025% ammonium persulphate and 0.1% SDS (spacer gel). After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and detected with the anti-MAP kinase monoclonal antibody (Zymed Laboratory, South San Francisco, CA) using a blotting detection kit in which the streptavidin–alkaline phosphatase conjugate was used as the signal-generating system (Amersham International).

Results

In experiments using 45 h cultured porcine oocytes, the phosphorylation rate of MBP was proportional to the oocyte number up to 20 (Fig. 1a) and almost constant for the first 50 min and then decreased (Fig. 1b). Therefore, 10 oocytes per assay tube and an assay period of 40 min were chosen.

Up to 25 h of culture, MAP kinase activity was low (Fig. 2). In our study, all oocytes had GV until 10 h, 9.8% of them resumed their meiosis at 20 h, and 30% at 25 h (Table 1). These results show that MAP kinase was inactive in GV-stage oocytes, even just before their meiotic resumption. The kinase activity abruptly increased significantly at 30 h (p < 0.05), when more than 60% of oocytes lost their GV and entered first metaphase. Then the high activity was maintained until 50 h, when most oocytes reached second metaphase. MAP kinase activity fell transiently at the time of first polar body extrusion (40 h of culture), though the reduction was not significant.

Since MBP can be the substrate of kinases other than MAP kinase (Peason & Kemp, 1991), kinase activity was examined in the gel containing MBP after separation of oocyte proteins by electrophoresis. Two bands with molecular masses of 42 and 44 kDa were the main bands detected (Fig. 3, left-hand panel). These two bands changed in parallel; both showed a significant increase at 45 h of culture, while they could not be detected at 0 h and just slightly at 20 h. The 42 and 44 kDa bands were also detected in immunoblotting with anti-MAP kinase antibody (Fig. 3, right-hand panel). These two bands were detected in the
Figure 2  Changes in MAP kinase activity during meiotic maturation in porcine oocytes cultured in porcine follicular fluid. After each culture period, the oocytes were collected and activity of MAP kinase was assayed. Data are shown as means ± SEM of four experiments. Values with different letters are significantly different (p < 0.05) as evaluated by Student's t-test. The percentages of oocytes that had lost their germinal vesicles (dotted bars) and that had extruded their first polar bodies (filled bars) were shown. GVBD, germinal vesicle breakdown; PB-1, first polar body.

Table 1  Maturation stages of procine oocytes cultured in vitro

<table>
<thead>
<tr>
<th>Culture period (h)</th>
<th>Total no. of ova examined</th>
<th>No. of experiments repeated</th>
<th>No. (%) of oocytes at the stage of:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Germinal vesicle</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>4</td>
<td>50 (100)</td>
</tr>
<tr>
<td>10</td>
<td>58</td>
<td>3</td>
<td>58 (100)</td>
</tr>
<tr>
<td>20</td>
<td>87</td>
<td>6</td>
<td>79 (90.8)</td>
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<tr>
<td>25</td>
<td>69</td>
<td>4</td>
<td>48 (69.6)</td>
</tr>
<tr>
<td>30</td>
<td>159</td>
<td>6</td>
<td>59 (37.1)</td>
</tr>
<tr>
<td>35</td>
<td>85</td>
<td>4</td>
<td>22 (25.9)</td>
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<tr>
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<td>99</td>
<td>6</td>
<td>15 (15.2)</td>
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<tr>
<td>50</td>
<td>59</td>
<td>4</td>
<td>6 (10.2)</td>
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</table>

GV-stage oocytes at 0 and 20 h of culture and in the maturing oocytes at 45 h. Furthermore, the two bands did not change in signal strength throughout culture, but shifted up at 45 h, showing the phosphorylation form of MAP kinase.

Discussion
In this study we used pFF as an in vitro maturation medium for porcine oocytes to assay MAP kinase activity during meiotic maturation. According to
previous reports (Naito et al., 1988, 1992), porcine oocytes matured in pFF in vitro had normal MPF activity and normal ability to form male pronuclei, and developed after fertilisation almost as well as those matured in vivo (Naito et al., 1989). The results obtained in this experiment, therefore, trace physiological maturation processes.

Fluctuation of MAP kinase activity during meiotic maturation has been reported in sea star (Sanghera et al., 1991), Xenopus (Gotoh et al., 1991a; Posada et al., 1991), clam (Shibuya et al., 1992a) and mouse (Sobajima et al., 1993; Verlhac et al., 1993, 1994) oocytes. According to these studies, high activity was maintained throughout meiosis. In the present study we assayed this kinase activity in porcine oocytes for the first time, and found similar activation of the kinase during the meiotic process. This suggests that MAP kinase is ubiquitous and has important roles in oocyte maturation.

Previous studies showed that MAP kinase was activated before GVBD in Xenopus oocyte extract (Gotoh et al., 1991a) and in clam oocytes (Shibuya et al., 1992a). In Xenopus, synthesis of c-mos proto-oncogene product (Mos), which is upstream of the MAP kinase cascade, is a prerequisite for meiotic resumption and the effect of Mos was expressed through activation of MEK and subsequently MAP kinase. When the activity of MAP kinase was inhibited by microinjection of anti-MEK antibody, meiotic resumption was blocked in Xenopus oocytes (Kosako et al., 1994a). On the basis of these results, we attempted to show that MAP kinase was activated prior to GVBD in porcine oocytes also. However, no dramatic increase in kinase activity was observed even after 25 h of culture, when more than 30% of oocytes had undergone GVBD. In mouse oocytes, its activation definitely occurred after GVBD (Verlhac et al., 1994; Sobajima et al., 1994). Therefore, it is unlikely that MAP kinase activation directly induces meiotic resumption in mammals, though further studies are clearly required before drawing a conclusion.

Two MAP kinases with molecular masses of 42 kDa and 44 kDa (ERK2 and ERK1, respectively) were reported in the mouse (Verlhac et al., 1994; Sobajima et al., 1994). In the present study also, two MAP kinases with the same molecular masses of 42 kDa and 44 kDa were detectable by immunoblotting and in-gel assay, and should be homologous with ERK2 and ERK1, respectively (Fig. 3). The kinase activity assayed in this investigation, therefore, should reflect porcine ERK2 and ERK1 activity, since the other band was detected by the in-gel assay (Fig. 3, left-hand panel). These two kinases were simultaneously activated in the pig, as reported in the mouse (Sobajima et al., 1993). Although the MAP kinase activity increased at 45 h in the in-gel assay, the signal strength of these bands did not increase at 45 h compared with 0 and 20 h by immunoblotting. Furthermore, the mobility of the bands decreased slightly at 45 h. These data indicated that the increase in the activity of MAP kinase detected in our experiments depends not on its own synthesis but on phosphorylation.

According to previous reports, MAP kinase activity persists during the transition from the first metaphase (MI) to the second metaphase (MII) in Xenopus (Posada et al., 1991) and in mice (Kubiak et al., 1992; Verlhac et al., 1993). In contrast, MAP kinase activity decreased transiently at 40 h in porcine oocytes, though the decrease was not significant. Considering that more than 30% of oocytes were extruding the first polar body during this period, this decrease might reflect the low MAP kinase activity of the first anaphase and telophase oocytes. Previously, we reported that the other important protein kinase in oocyte maturation, MPF (maturation promoting factor or metaphase promoting factor), became inactive transiently but significantly at the time of first polar body extrusion in porcine oocytes (Naito & Toyoda, 1991). Gotoh et al. (1991a) reported that the activation of MAP kinase occurs downstream of MPF. Therefore, in porcine oocytes, the transient and precipitous fall in MPF activity at first polar body extrusion might
influence MAP kinase to decrease its activity slightly at this time.

The role of MAP kinase in porcine oocytes during maturation is not clear. Verlhac et al. (1993) reported that MAP kinase was contained in the isolated mouse microtubule-organising centre (MTOC), present at the spindle poles, and regulated its activity to form the metaphase spindle. They also mentioned that the behaviour of microtubules and chromatin followed MAP kinase activity in mouse oocytes. Recently, MAP kinase has been shown to be a component of the cytokinetic factor that arrests mature oocytes at second metaphase before fertilisation (Haccard et al., 1993; Kosako et al., 1994b). The high MAP kinase activity in mature oocytes, therefore, might play these roles in the pig also.

In summary, we have demonstrated for the first time that MAP kinases with molecular masses of 42 kDa and 44 kDa (ERK2 and ERK1, respectively) exist in porcine GV-stage oocytes, and that their amounts do not change during oocyte maturation. MAP kinase activity was low in GV-stage oocytes, its activation might occur after GVBD, and the high activity remained until MI, showing a transient slight decrease at the time of first polar body extrusion. These results indicate that the activation of MAP kinase is involved in the regulation of meiotic maturation in the pig.

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kinase, but not MPF, in cell-free extracts of *Xenopus* oocytes and eggs. *EMBO J.* 12, 1979–86.