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Mitogen-activated protein kinase in human eggs

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Summary

Mitogen-activated protein (MAP) kinase in human eggs has been investigated by using immunoblotting with both anti-Active MAPK and anti-ERK2 antibodies. The results showed that the main form of MAP kinase was p42ERK2. It was in a dephosphorylated form in oocytes at the germinal vesicle stage, but fully phosphorylated in unfertilised mature eggs. MAP kinase phosphorylation was significantly decreased when pronuclei were formed after intracytoplasmic sperm injection. Neither MAP kinase expression nor activity was detected in morphologically degenerated eggs. Although MAP kinase still existed in early embryos arrested at the 8-cell or morula stages, little, if any, activity could be detected. These data suggest that MAP kinase may play an important role in the cell cycle regulation of human eggs, as in other mammalian species.

Keywords: Cell cycle, Egg, Human, MAP kinase, Oocyte

Introduction

Over the past few years, it has become apparent that mitogen-activated protein (MAP) kinases, also referred to as extracellular signal-regulated kinases (ERKs), play important roles in the regulation of the cell cycle. MAP kinases are activated through a cascade of conserved kinases in response to a variety of extracellular signals, including growth factors and hormones (reviewed by Karin & Hunter, 1995; Waskiewicz & Cooper, 1995). Recent studies have shown that MAP kinase activation is involved in regulating oocyte meiosis maturation in several species, including mouse (Sabajima *et al.*, 1993; Verlhac *et al.*, 1993; Sun *et al.*, 1999*a*), rat (Goren *et al.*, 1995; Zernicka-Goetz *et al.*, 1997), goat (Dedieu *et al.* 1996), pig (Inoue *et al.*, 1995, 1996, 1998), cow (Fissore *et al.*, 1996) and horse (Goudet *et al.*, 1998). Furthermore, Verlhac *et al.* (1994) have suggested that microtubule and chromatin behaviour follows MAP kinase activity but not MPF activity during meiosis in mouse oocytes. On the other hand, down-regulation of MAP kinase is essential for pronucleus formation after fertilisation or artificial activation of mouse metaphase II eggs (Moos *et al.*, 1995, 1996*a*, *b*; Sun *et al.*, 1998, 1999*b*). Recently it has been reported that MAP kinase is also required for G_2–M phase transition of the first mitotic cell cycle in both *Xenopus* and starfish eggs (Abrieu *et al.*, 1997). Therefore, MAP kinase appears to be an important enzyme for oocyte/egg cell cycle regulation. However, the role of MAP kinase in human eggs has never been studied.

In the present study we show, for the first time, the expression and activity of MAP kinase in human oocytes and eggs before or after pronucleus formation as well as in embryos arrested at early stages. The possible role of this kinase in human egg cell cycle regulation is discussed.

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Materials and methods

Collection and preparation of oocytes/eggs

The human oocytes/eggs were retrieved by transvaginal follicular aspiration from normal ovulatory women, aged 26–37 years, undergoing *in vitro* fertilisation (IVF) either for mechanical infertility (blocked Fallopian tube) or due to male factor infertility. In all cases a proportion of the eggs were fertilised and cleaved. The pregnancy rate in those women from whom the eggs were taken was similar to that in all the other patients. The controlled ovarian stimulation consisted of intramuscular gonadotrophin-releasing hormone (GnRH) agonist administration (Decapetyl C.R., 3.75 mg, Ferring, Malmö, Sweden) either on day 1–3 of the IVF cycle or in the mid-luteal phase of the previous cycle. After pituitary desensitisation, using GnRH-agonist analogue, human menopausal gonadotrophin (hMG, Metrodin/Pergonal, Teva, Israel) was administered by daily injections (225 IU FSH/day for 5 days), after which the daily FSH or hMG dosage was adjusted on an individualised protocol, according to the daily 17β-oestradiol and progesterone concentrations, as previously described (Dirnfeld *et al.*, 1997). When the leading follicle reached 18–20 mm in diameter and serum 17β-oestradiol concentration was > 600 pg/ml (2200 pmol/l), 10000 IU human chorionic gonadotrophin (hCG, Chorigon, Teva, Netanya, Israel) was administered to trigger final follicular maturation, and eggs were collected from follicular aspirates 35 h after intramuscular injection of hCG.

The cells of cumulus oophorus and corona radita were removed by pipetting for 30 s in HTF-Hepes medium with 40 IU hyaluronidase/ml (type VIII, specific activity 320 IU/ml, Sigma Chemical Co., Tel Aviv, Israel). Oocytes with extruded polar bodies were considered as mature eggs. Some of the retrieved oocytes were still arrested at the germinal vesicle stage at the time of collection. Eggs that were fragmented irregularly, or hyperchromatic with condensed cytoplasm, or those with intracytoplasmic irregular vacuoles were considered to be degenerated. All mature eggs were cultured in HTF medium supplemented with 5% human serum albumin (HSA, Irvine Scientific, Santa Ana, CA) and incubated for 2 h with 5% CO_2 at 37 °C.

Sperm preparation

Spermatozoa were washed with HTF medium (Irvine Scientific, Santa Ana, CA) + 10% HSA and centrifuged at 800 rpm for 10 min, after which the supernatant was discarded and 50 ml medium was added to the pellet for swim-up procedure. After 1–2 h the swim-up spermatozoa were used for microinjection.

Micromanipulation and early embryo culture

Micromanipulation was performed with the aid of two micromanipulators and microsyringes mounted on an inverted microscope (Diaphot 300, Nikon, Japan). The injection technique used was as described by Palermo *et al.* (1995). After intracytoplasmic sperm injection (ICSI) eggs were cultured in IVF-50 medium (Scandinavian IVF Science, Gothenburg, Sweden). Sixteen hours after ICSI, eggs with two pronuclei were judged as fertilised. The absence of pronuclei was considered to be indicative of non-fertilisation. After 72 h, embryos were cultured in S-2 medium (Scandinavian IVF Science, Gothenburg, Sweden), and some of them were arrested at the 8-cell or morula stage.

Electrophoresis and immunoblotting

Oocytes, eggs or embryos were collected in SDS sample buffer and frozen at –20 °C until use. Before electrophoresis, samples were heated to 100 °C for 4 min, cooled on ice for 4 min, and then centrifuged at 14 000 rpm for 5 min. The proteins were separated by SDS-PAGE with a 4% stacking gel and a 10% separating gel for 50 min at 188 volts, and were electrophoretically transferred onto PROTRAN nitrocellulose membrane (Schleicher & Schull, Germany) for 1 h, 200 mA, at 4 °C. After blocking for 30 min in 5% bovine serum albumin (BSA) in 20 mM Tris, 137 mM NaCl (TBS, pH 7.6), which contains 0.1% Tween-20 (TBS-T), the membrane was incubated overnight at 4 °C with polyclonal anti-Active MAPK antibody (Promega, Madison, WI, lot no. 64840) diluted 1:5000 in TBS. After two washes of 15 min each in TBS-T, the membrane was incubated for 1 h at room temperature with donkey-anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted 1:6000 in TBS-T. The membrane was washed twice in TBS-T, and then processed using an enhanced chemiluminescence (ECL) detection system.

For reprobing, the blots were stripped of bound antibodies by washing in a stripping buffer (100 mM -mercaptoethanol, 20% SDS, 62.5 mM Tris, pH 6.7) at 50 °C for 30 min. The membrane was reprobed with polyclonal anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, lot no. 1276) diluted 1:3000, using the same procedure described above.

Data analysis

The intensity of each of the bands was quantified by densitometry, using TINA 2.0 software. Students *t*-test was used to compare the mean optical density (OD) value per egg between different groups. Differences at $p < 0.05$ were considered significant.

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Results and discussion

MAP kinase is a serine/threonine kinase that requires dual phosphorylation on threonine and tyrosine residues to become fully activated (Nishida & Gotoh, 1993). Two isoforms of MAP kinase, p42ERK2 and p44ERK1, have been shown to be present in mammalian eggs, except in the goat (Dedieu *et al.*, 1996). In the present study, MAP kinase activity in human eggs was detected with a very sensitive and highly reliable assay we recently established (Sun *et al.*, 1997, 1998). Phosphorylation/dephosphorylation was studied by using a polyclonal anti-Active MAPK antibody, which selectively recognises the phosphorylated (active) form of ERK2 and ERK1 (Schaefer & Moravec, 1996). MAP kinase expression was detected by immunoblotting with a polyclonal anti-ERK2 antibody, which is reactive with ERK2 and, to a lesser degree, with ERK1.

As shown in Figs. 1 and 2, only a single band with an approximate molecular size of 42 and 44 kDa was detected when oocyte/egg protein extract was probed with anti-ERK2 and anti-Active MAPK antibodies, respectively. Thus, it seems that the dominant form of MAP kinases in human eggs is p42ERK2. While ERK2 had been fully expressed in oocytes at the germinal vesicle stage (Fig. 2, lane 1), it was in a dephosphorylated (inactive) form (Fig. 1, lane 1). By contrast, the mature eggs that were not fertilised, as indicated by the absence of pronucleus formation, exhibited a high level of phosphorylated MAP kinase (Fig. 1, lane 2). The amount of this kinase per egg is lower in comparison with the amount observed in germinal vesicle stage oocytes ($p < 0.01$) (Fig. 2, lane 2), results which are similar to those obtained in other mammalian oocytes (Verlhac *et al.*, 1993; Dedieu *et al.*, 1996; Fissore *et al.*, 1996). Fertilised eggs, in which two pronuclei were observed, showed significantly decreased MAP kinase activity ($p < 0.01$), as indicated by the dephosphorylation of this kinase (Fig. 1, lane 3). It has been shown by us and others that a decrease in MAP kinase activity is temporally well correlated with pronucleus formation induced by sperm or artificial stimuli in mouse eggs (Moos *et al.*, 1995, 1996*a*, *b*; Sun *et al.*, 1998, 1999*a*, *b*). Therefore, we concluded that MAP kinase is a key protein kinase regulating the cell cycle in human eggs.

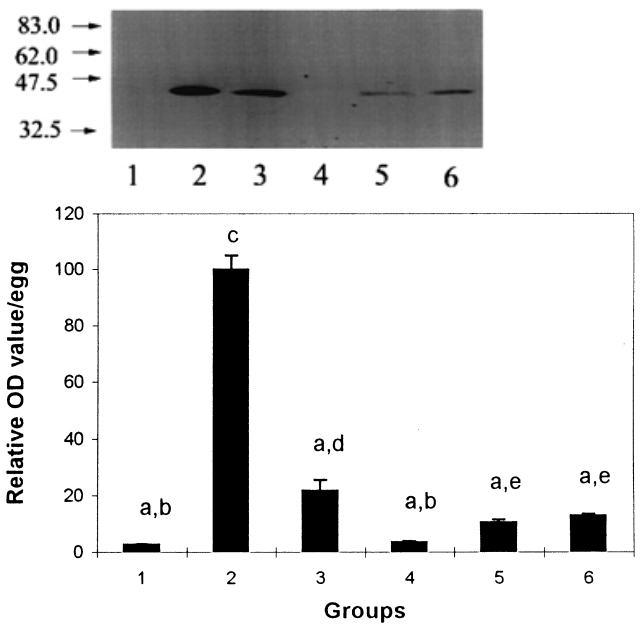


Figure 1 MAP kinase phosphorylation/dephosphorylation in human eggs. (*A*) Results of immunoblotting with anti-Active MAPK antibody. Lane 1, germinal vesicle stage oocytes ($n = 3$); lane 2, unfertilised mature eggs after 16 h of culture ($n = 2$); lane 3, fertilised eggs with two pronuclei ($n = 5$); lane 4, degenerated eggs ($n = 3$); lane 5, embryos arrested at the 8-cell stage ($n = 5$); lane 6, embryos arrested at the morula stage ($n = 4$). (*B*) Relative MAP kinase activity per egg shown by optical density (OD) of the bands in the blot. The OD value per egg in each group was normalised against that of a mature egg without a pronucleus. The value expressed by each bar represents the mean standard deviation of three independent blots. a versus c, b versus d, $p < 0.01$; d versus e, $p < 0.05$.

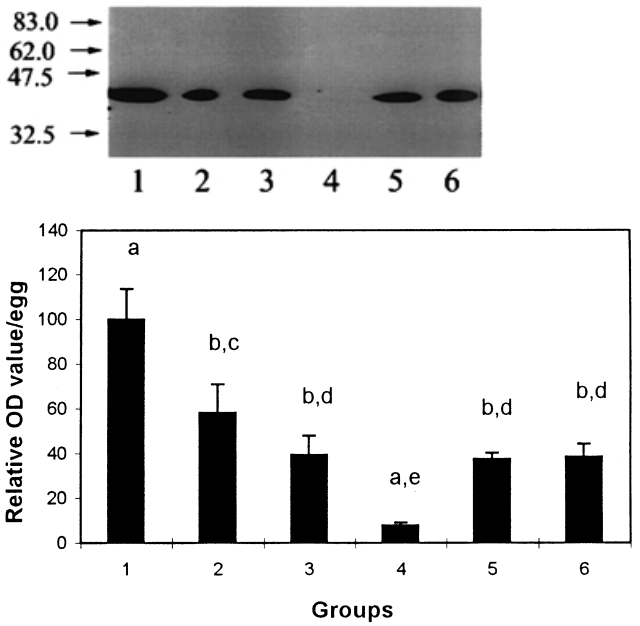


Figure 2 MAP kinase expression in human eggs. (*A*) Results of immunoblotting with anti-ERK2 antibody using the same nitrocellulose membrane after stripping. Lane 1, germinal vesicle stage oocytes ($n = 3$); lane 2, unfertilised mature eggs after 16 h of culture ($n = 2$); lane 3, fertilised eggs with two pronuclei ($n = 5$); lane 4, degenerated eggs ($n = 3$); lane 5, embryos arrested at the 8-cell stage ($n = 5$); lane 6, embryos arrested at the morula stage (n=4). (*B*) Relative MAP kinase amount per egg shown by optical density (OD) of the bands in the blot. The OD value per egg in each group was normalised against that of the germinal vesicle stage oocyte. The value expressed by each bar represents the mean standard deviation (SD) of three independent blots. a versus b, c versus e, d versus e, $p < 0.01$.

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Low MAP kinase activity keeps the oocyte/egg arrested at interphase of the cell cycle.

The present data show that neither ERK2 expression nor its activity was detected in eggs that were judged as being degenerated according to their morphology (Fig. 1, lane 4; Fig. 2, lane 4), although MAP kinase activity is relatively high in unfertilised human eggs that had been cultured *in vitro* for 16 h (Fig. 1, lane 2). Our previous work showed that MAP kinase was completely inactivated by dephosphorylation 48 h after hCG injection (36 h after ovulation) in mouse eggs (Sun *et al.*, 1999*b*). Thus, inactivation and degradation of MAP kinase is an important biochemical criterion for egg degeneration.

Our results also show that MAP kinase exists in early human embryos arrested at the 8-cell or morula stage in a similar quantity as in fertilised eggs (Fig. 2, lanes 5, 6), but only little phosphorylated form could be detected (Fig. 1, lanes 5, 6). It has been reported that MAP kinase activity is low in *Xenopus* cleavage embryos (LaBonne & Whitman, 1997), and the MAP kinase cascade, but not the ERKs, is activated at M-phase during early cleavage of mouse embryos (Haraguchi *et al.*, 1998). Our recent work also indicated that MAP kinase was in a dephosphorylated form in parthenogenic mouse embryos at 2-cell stage (Sun *et al.*, 1998). Thus we concluded that MAP kinase may play little part in human embryo cleavage. However, we should remember that the embryos used in this assay were arrested at the 8-cell or morula stage in culture, since it is difficult to obtain normally developing embryos for biochemical analysis. Therefore, MAP kinase dephosphorylation may also be representative of abnormal conditions.

In summary, our data show for the first time that the main form of MAP kinase in human eggs is p42ERK2. This kinase may play an important role in the cell cycle regulation of human oocytes/eggs. Whether MAP kinase is involved in regulating early embryo cleavage needs further clarification.

Acknowledgements

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