$P34^{cdc2}$ Kinase and MAP Kinase Activities and Parthenogenetic Activation in Porcine Oocytes after Injection of Miniature Pig Sperm Extracts

*Daizou Matsuura[1] and Teruo Maeda[2]**

[1]*Suzuka Kaisei General Hospital, Suzuka 513-8505, Japan*
[2]*Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan*

Abstract: *The aim of the present study was to examine the rate of activation and time-dependent changes in $p34^{cdc2}$ kinase and MAP kinase activities in porcine oocytes after injection of sperm extracts (SE) or treatment with Ca^{2+} ionophore to clarify whether SE injection is useful for porcine oocyte activation. SE was prepared from miniature pig sperm by non-ionic surfactant. Oocytes that were treated with Ca^{2+} ionophore and injected with SE were activated at rates of 41% and 46%, respectively. The activities of $p34^{cdc2}$ kinase and MAP kinase in each culture period (4, 8 and 12 h) in the Ca^{2+} ionophore group and SE injection group were significantly lower than those in the control group. These results suggest that SE injection induces decrease of $p34^{cdc2}$ kinase and MAP kinase activity, and induces oocyte activation, as well as Ca^{2+} ionophore treatment, but the induction was comparatively limited.*

Key words: *Oocyte activation, MAP kinase, $P34^{cdc2}$ kinase, Sperm extracts, Miniature pig*

Introduction

It is well known that mammalian matured oocytes are arrested again at metaphase II of meiosis (MII). These MII oocytes are characterized by the high activity of maturation promoting factor (MPF), a complex of $p34^{cdc2}$ kinase and cyclin B, in mice [1] and pigs [2–4]. The activation of $p34^{cdc2}$ kinase, a catalytic subunit of MPF, depends on binding with cyclin B1 [5]. Mitogen-activated protein (MAP) kinase, a serine/threonine kinase, is also required for arrest at MII [6]. During the fertilization process, sperm release cytosolic substances, called sperm factor (SF), into the oocyte and SE induce Ca^{2+} oscillations [7–9]. The oscillations degrade cyclin B and Mos, that is MAP kinase kinase kinase, which results in decrease of $p34^{cdc2}$ kinase and MAP kinase activities. The inactivation of both kinases at fertilization is considered to be associated with formation of the second polar body and pronuclear formation [10].

In our previous experiments, we demonstrated that injection of sperm extracts (SE) from miniature pig spermatozoa induced comparatively high rates of porcine or bovine oocyte activation, and that oocytes injected with SE exhibited cortical granule exocytosis. These results indicated that SE injection into an oocyte was an effective method of activation, and that SE injection could be used to promote activation in intracytoplasmic sperm injection (ICSI) [11]. Therefore, we examined the effect of SE injection on decrease of $p34^{cdc2}$ kinase and MAP kinase activities, and oocyte activation in comparison with oocytes exposed to Ca^{2+} ionophore.

Materials and Methods

Sperm preparation, extraction of SE by mammalian protein extraction reagent, treatment of the cytosolic fraction with a 2-D Clean Up Kit, porcine oocyte maturation, microinjection of SE into oocytes and evaluation of nuclear status were done according to the procedures previously described by Matsuura and Maeda [12].

Received: September 4, 2007
Accepted: February 27, 2008
*To whom correspondence should be addressed.
e-mail: temaeda@hiroshima-u.ac.jp

Treatment of oocytes with Ca^{2+} ionophore

Ca^{2+} ionophore treatment was performed according to the method described by Ito *et al.* [13]. After 48 h cultivation, cumulus cells were denuded from oocytes by pipetting with flame-drawn pipette tips with an inner diameter slightly larger than the oocyte diameter. Cumulus-free oocytes were washed three times in NCSU37 medium. Ca^{2+} ionophore, A23187 (Sigma, St. Louis, MO, USA), was dissolved in dimethyl sulfoxide (DMSO; Sigma) at 2 mM, and stored at –30°C (stock solution). The stock solution was then diluted with NCSU37 to a final concentration of 50 μM. The matured oocytes were treated with 50 μM of Ca^{2+} ionophore in NCSU37 for 5 min at 39°C, and washed with NCSU37 to quench the action of the ionophore. The treatment was repeated three times at 5 min intervals as described above. After final exposure to Ca^{2+} ionophore, the oocytes were washed three times with TCM-199 (Gibco BRL, Grand Island, NY, USA) containing 10% (v/v) FCS (Gibco BRL), and then cultured in a 48-well multi-dish (NUNC) containing 300 μl of TCM-199 (Gibco BRL) supplemented with 10% (v/v) FCS (Gibco BRL) at 39°C in a humidified atmosphere of 5% CO_2 in air (see Experimental design).

Extract preparation

Oocytes were lysed according to the methods of Shimada and Terada [14]. Oocytes were collected at designated time intervals. Ten oocytes were transferred to a microtube containing 5 μl of lysis buffer consisting of 20 mM Tris HCl (Nacalai Tesque, Kyoto, Japan), 150 mM NaCl (Katayama Kagaku, Osaka, Japan), 1 mM EDTA (Nacalai Tesque), 1 mM EGTA (Nacalai Tesque), 1% (v/w) Triton X-100 (Sigma), 1 μg/ml leupeptin (Sigma), 1 mM PMSF (Sigma), 50 mM 2-mercuptoethanol (Nacalai Tesque), 25 mM β-glycerophosphate (Sigma), and 1 mM Na-orthovanadate (Sigma). After suspending the oocytes in lysis buffer, the samples were frozen in liquid nitrogen, then sonicated three times, 25 sec each time, at 1°C using an ultrasonic disruptor (UD-200; TOMY, Tokyo, Japan) fitted with Cup Horn (CH-0633; TOMY). Cell extracts were frozen and stored at –80°C until just before use.

$P34^{cdc2}$ kinase assay

The $p34^{cdc2}$ kinase assay was performed according to the method of Shimada and Terada [14]. Briefly, a $p34^{cdc2}$ kinase assay was carried out by measuring the activity of its catalytic subunit, $p34^{cdc2}$ kinase, with a MESA-CUP cdc2 kinase assay kit (MBL, Nagoya, Japan). The cdc2 kinase activity in the oocyte lysate was measured according to the manufacturer's protocol. Values were expressed as the fold strength of $p34^{cdc2}$ kinase activity in oocytes matured for 48 h.

MAP kinase assay

The MAP kinase assay was performed according to Shimada and Terada [14, 15]. A p44/42 MAP kinase assay kit (New England Biolabs, Beverly, MA, USA) was used for measuring MAP kinase activity. Briefly, 5 μl of lysated oocytes (containing 20 oocytes) were mixed with 25 μl of kinase assay buffer A [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$], 0.1 mM ATP (Sigma), and 2 μg Elk1 fusion protein. The mixture was incubated for 30 min at 30°C. All chemicals except ATP were purchased from New England Biolabs. The reactions were terminated by the addition of 10 μl of 4 × Laemmli sample buffer. The samples were boiled for 5 min, and then subjected to 12.5% SDS-PAGE. Phosphorylation of Elk1 fusion protein was detected by immunoblotting and chemiluminesence with anti-phospho-specific Elk1 antibody. Relative amounts of MAP kinase activity were determined by scanning densitometry. Values were expressed as the fold strength of MAP kinase activity in oocytes matured for 48 h.

Experimental design

In Experiment 1, parthenogenetic activation was examined in porcine oocytes after SE injection or treatment with Ca^{2+} ionophore. Oocytes that were injected with SE or treated with Ca^{2+} ionophore were cultured in TCM-199 including 10% FCS for 48 h. Rates of activation (pronucleus formation and cleavage) were examined after culture for 48 h. $P34^{cdc2}$ kinase and MAP kinase activities of oocytes were examined 0, 4, 8 and 12 h after SE injection or Ca^{2+} ionophore treatment in experiments 2 and 3, respectively.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA). All percentage data were subjected to arc-sine transformation before statistical analysis. Data were compared using the Tukey-Kramer honesty significant difference test. All experiments were repeated at least three times. Statistical comparisons were performed using JMP IN software (SAS Institute Inc., Cary, NC, USA). Differences were considered significant at $P<0.05$.

Table 1. Porcine oocyte activation after treatment with Ca^{2+} ionophore or injection of sperm extracts (SE)

Treatment	No. of treated oocytes	Number (mean % ± SEM) of oocytes					
		Activated					
		Total	Pronuclear stage			2 to 4-cell stage	Fragmentated
			1PN1PB[1)]	1 PN2PB[2)]	2 PN1PB[3)]		
Buffer injection (Control)	28	0 (0 ± 0.0)[a]	0 (0 ± 0.0)[a]	0 (0 ± 0.0)[a]	0 (0 ± 0.0)[a]	0 (0 ± 0.0)[a]	0 (0 ± 0.0)[a]
Ca^{2+} ionophore	64	26 (41 ± 7.0)[b]	3 (5 ± 5.0)[a]	5 (8 ± 1.3)[a]	2 (3 ± 1.7)[a]	16 (25 ± 5.7)[b]	3 (0 ± 2.9)[a]
Sperm extracts injection	69	32 (46 ± 4.2)[b]	5 (7 ± 1.5)[a]	15 (22 ± 3.8)[b]	2 (3 ± 1.5)[a]	10 (15 ± 1.2)[b]	5 (0 ± 1.7)[a]

[1)]Oocytes with one pronucleus and without a second polar body. [2)]Oocytes with one pronucleus and a second polar body. [3)]Oocytes with two pronuclei and without a second polar body. [a, b]Values within the same column with different letters differ significantly ($P<0.05$).

Results

Experiment 1

Table 1 shows the rates of activation (pronucleus formation and cleavage) after treatment with Ca^{2+} ionophore or SE injection.

The rate of oocytes with one pronucleus and a second polar body (the 1PN2PB) stage in the SE injection group was significantly higher than that in the Ca^{2+} ionophore group ($P<0.05$); however, there was no significant difference between the groups in the rates of the other stages. In the control (buffer injection), the activation rate was 0%. Total activation rates in Ca^{2+} ionophore and SE injection groups were 41% and 46%, respectively. These values were significantly larger than the control ($P<0.05$); however, there was no significant difference between them.

Experiment 2

As shown in Fig. 1, oocyte $p34^{cdc2}$ kinase activities in the control (buffer injection) maintained the level before injection (0 h: 1.00) after culture for 4, 8 and 12 h (1.09, 1.05 and 1.01, respectively).

In oocytes treated with Ca^{2+} ionophore, the activity at 4 h (0.65) was significantly lower than that at 0 h, and the activities were maintained at this level until culture for 12 h (0.61). The activities of the different culture periods (4, 8 and 12 h) in the Ca^{2+} ionophore groups were significantly lower than those of the control group (0.65 vs 1.09, 0.61 vs 1.05, and 0.61 vs 1.01, respectively; $P<0.05$).

In oocytes injected with SE, the activities after 4, 8 and 12 h culture period were 0.65, 0.53 and 0.32, respectively, and they were significantly lower than that at 0 h culture ($P<0.05$). The activities of oocytes at the different culture period (4, 8 and 12 h) oocytes in SE injection group were always significantly lower than those of the control group (0.65 vs 1.09, 0.53 vs 1.05, and 0.32 vs 1.01, respectively; $P<0.05$).

Between the Ca^{2+} ionophore and SE injection groups, there were no significant differences in the activities of the oocytes that were cultured for 4 and 8 h (0.65 vs 0.65, 0.61 vs 0.53, respectively); but the activity at 12 h culture in the SE injection group was significantly lower than that in the Ca^{2+} ionophore treated group (0.32 vs 0.61; $P<0.05$).

Experiment 3

As shown in Fig. 2, control oocytes (buffer injection) also maintained MAP kinase activity at the same level as that of pretreatment (0 h: 1.00) during the culture period (0.88 at 4 h, 1.00 at 8 h and 1.00 at 12 h).

In oocytes that were treated with Ca^{2+} ionophore, MAP kinase activities decreased time-dependently (0.45 at 4 h, 0.26 at 8 h and 0.07 at 12 h), and the activities in the different culture periods (4, 8 and 12 h) in the Ca^{2+} ionophore group were significantly lower than those in the control groups (0.45 vs 0.98, 0.26 vs 1.00, and 0.07 vs 1.00, respectively; $P<0.05$).

In oocytes that were injected SE, the changing pattern of activity was almost the same as that in the Ca^{2+} ionophore-treated group. The activities in the different culture periods (4, 8 and 12 h) in the SE injection group were significantly lower than those in the control group (0.71 vs 0.88, 0.42 vs 1.00, and 0.11 vs 1.00, respectively; $P<0.05$).

The activities in the Ca^{2+} ionophore treated group at 4 h and 8 h were significantly lower than in the SE injection group (0.45 vs 0.71, and 0.26 vs 0.42, respectively; $P<0.05$); however, there were no significant differences in the activity after 12 h culture between the groups (0.07 vs 0.11).

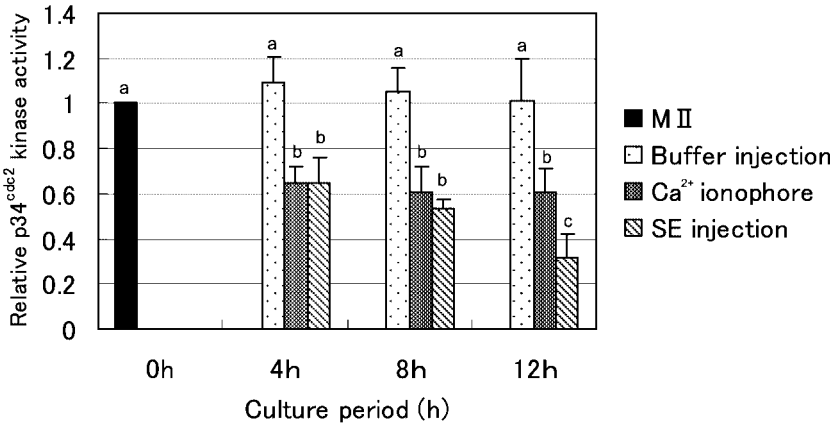

Fig. 1. Time-dependent changes of $p34^{cdc2}$ kinase activity in cultured oocytes after treatment with Ca^{2+} ionophore or injection of SE. Values are the mean ± SEM of three replications. [a–c]Different letters show significant difference among treatments at each culture period ($P<0.05$). Data are shown as values relative to the mean value of three replicates of oocytes matured for 48 h (0 h).

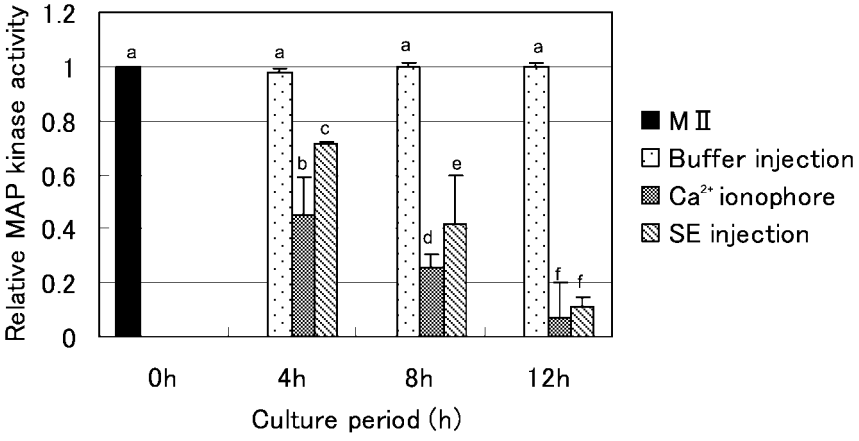

Fig. 2. Time-dependent changes of MAP kinase activity in cultured oocytes after treatment with Ca^{2+} ionophore or injection of SE. Values are the mean ± SEM of three replications. [a–f]Different letters show significant difference among treatments at each culture period ($P<0.05$). Data are shown as values relative to the mean value of three replicates of oocytes matured for 48 h (0 h).

Discussion

Our previous studies showed that SE injection could elicit porcine oocyte activation effectively [11, 12]. The present study examined the effect of SE injection on decrease of $p34^{cdc2}$ kinase and MAP kinase activities, and oocyte activation in comparison with oocytes exposed to Ca^{2+} ionophore.

Oocytes that were treated with the Ca^{2+} ionophore or injected with SE were activated at rates of 41% and 46%, respectively. The activities of $p34^{cdc2}$ kinase in the Ca^{2+} ionophore group and the SE injection group were significantly lower than in the control group at all culture periods (4, 8 and 12 h, $P<0.05$). Similarly, the activities of MAP kinase in the Ca^{2+} ionophore group and the SE injection group were significantly lower than in the control group at all culture periods (4, 8 and 12 h). The inactivation of both $p34^{cdc2}$ kinase and MAP kinase at fertilization is considered to be associated with pronuclear formation [10]. Ito *et al.* [13] reported that lower activities of both MAP kinase and $p34^{cdc2}$ kinase induced normal meiotic completion and pronuclear

formation of porcine oocytes that were parthenogenetically activated by Ca^{2+} ionophore. Our results and these previous reports suggested that SE injection induces normal meiotic completion and pronuclear formation of porcine oocytes, and oocyte activation as well as Ca^{2+} ionophore treatment, though the induction is comparatively limited.

Although there were no significant differences in the $p34^{cdc2}$ kinase activity between Ca^{2+} ionophore-treated and SE-injected oocytes at 4 and 8 h culture, the activity in the SE injection group was significantly lower than in the Ca^{2+} ionophore group at 12 h (0.32 vs 0.61; $P<0.05$), indicating that SE injection is more effective at reducing $p34^{cdc2}$ kinase activity than treatment with Ca^{2+} ionophore. MAP kinase activity after 4 and 8 h culture in the Ca^{2+} ionophore group was significantly lower than in the SE injection group ($P<0.05$); however, there was no significant difference in the activity between the groups at 12 h culture. Therefore, this result means that treatment with Ca^{2+} ionophore more rapidly decreases MAP kinase activity than SE injection, but that the level of the activity at 12 h is substantially the same.

Ito *et al.* [16] reported that the rate of pronuclear formation was about 90% when oocytes were co-cultured with spermatozoa in fertilization medium for 6 h (*in vitro* fertilization, IVF), and that $p34^{cdc2}$ kinase and MAP kinase activities at that time decreased to 20% or less of those in oocytes before IVF (matured oocytes). The present results showed that the oocyte activation rate was 46%, and that $p34^{cdc2}$ kinase and MAP kinase activities in oocytes injected with SE after 8 h culture decreased to approximately 50% and 60% of those in matured oocytes, respectively. It is clear that SE injection induces oocyte activation; however the rate remains low compared to IVF, and the ratio of decrease in both $p34^{cdc2}$ and MAP kinase activities in oocytes injected with SE is also low compared to IVF. Therefore, further study is required to improve the method of porcine oocyte artificial activation by SE injection.

Acknowledgements

The authors thank the staff of the Meat Inspection Office of Hiroshima City for supplying the porcine ovaries.

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