Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged in vitro

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Changes of histone H1 kinase activity before and after electrical stimulation were connected with the ability of cytoplasm of pig oocytes to be activated parthenogenetically when matured and aged in vitro. Cumulus-oocyte complexes were collected from prepubertal gilts and cultured in a modified Waymouth's MB752/1 medium. The first mature oocytes were observed after 30 h of culture. After 36 h of culture, about 65% of oocytes had matured (reached metaphase II stage with the first polar body). When oocytes matured after 36 h of culture were stimulated with an electric pulse and subsequently cultured for 10 h, only 7% became parthenogenetically activated (formation of a female pronucleus). When oocytes matured for 60 h and 72 h underwent the same treatment, significantly more became activated parthenogenetically (46% and 57%, respectively). Oocytes matured for 72 h but not stimulated electrically also exhibited high spontaneous parthenogenetic activation (24%). Activation of oocytes resulted either in the formation of a female pronucleus(ei) or in fragmentation of oocytes. Fragmentation in stimulated and nonstimulated oocytes increased significantly after 72 h of culture (37% and 18%, respectively). Histone H1 kinase activity in immature oocytes at the germinal vesicle stage was low (17.2 fmol h⁻¹ per oocyte). However, when oocytes were cultured for 36 and 48 h, histone H1 kinase activity increased significantly (168.2 and 138.5 fmol h⁻¹ per oocyte, respectively). Prolonged culture (60 h and 72 h) resulted in a significant decrease in histone H1 kinase activity (94.3 and 49.3 fmol h⁻¹ per oocyte, respectively). When oocytes cultured for up to 72 h were electrically stimulated, histone H1 kinase activity in activated oocytes (oocytes that formed a female pronucleus and fragmented oocytes) was significantly lower (24.7 mol h⁻¹ per oocyte) than that in nonactivated oocytes (99.9 mol h⁻¹ per oocyte). The present data clearly indicate that the gradual decrease of histone H1 kinase activity is correlated with ageing of oocytes matured in vitro and with their ability to be parthenogenetically activated.

Introduction

Follicular oocytes from some mammals can be matured in vitro. In oocytes matured in vivo and in vitro, development is arrested at metaphase II until the oocyte is penetrated by a spermatozoon. During meiotic arrest, nuclear status does not change. However, cytoplasmic changes, for example, viability, ability to be fertilized and ability for parthenogenetic activation of an oocyte, occur when the culture period is prolonged. This phenomenon is called 'ageing' in a mammalian oocyte.

The effects of ageing on fertilization rates, the ability for activation and embryonic development have been reported in some mammalian oocytes. In hamsters, spontaneous oocyte activation (Yanagimachi and Chang, 1961), abnormal fertilization (Jedlicki et al., 1986) and cleavage failure (Juetten and Bovier, 1983) have been reported in oocytes aged in vitro. Mouse oocytes, matured in vivo gain the ability for activation during arrest at metaphase II (Kubiak, 1989). In cattle, prolonged maturation in vitro enhances parthenogenetic activation (Nagai, 1987) and ageing results in abnormal cleavage after fertilization in vitro (Chian et al., 1992). In pigs, abnormalities of development have been reported in oocytes aged in vivo (Hunter, 1967) and in vitro (Sato et al., 1979). These reports indicate a decrease in the ability to be fertilized, poor developmental ability and enhanced cytoplasmic ability for oocyte activation in aged mammalian oocytes. To date no

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cytoplasmic changes during ageing, which relate to oocyte activation, have been identified in mammalian oocytes matured either in vivo or in vitro.

Maturation or M-phase promoting factor (MPF), which shows histone H1 kinase (H1K) activity (Arion et al., 1988; Labbe et al., 1988a, b, 1989), induces G2- to M-phase transition in a variety of eukaryotic cells (Kishimoto et al., 1982). MPF plays an important role in oocyte maturation and fertilization in mammals (Parrish et al., 1992). Oocytes matured to metaphase II possess high H1K activity (Choi et al., 1991; Naito and Toyoda, 1991; Fulka et al., 1992). H1K inactivation occurs just after oocyte activation resulting from fertilization (Choi et al., 1991; Fulka et al., 1992; Collas et al., 1993; Kikuchi et al., 1995) or parthenogenetic activation by electrical stimulation (Barnes et al., 1993; Collas et al., 1993). H1K activity in unfertilized pig oocytes is lower than that in matured oocytes before insemination (Naito and Toyoda, 1991; Kikuchi et al., 1995). The ability for oocytes to be activated is affected by H1K activity in matured oocytes (Naito et al., 1992). Increasing susceptibility of oocytes to be activated during ageing may be related to changes in MPF activity. However, changes in the activity of MPF or of H1K or of both enzymes during ageing has not yet been demonstrated in any mammal. In the present study, the relationship between the ability for parthenogenetic activation after stimulation by an electric pulse and H1K activity before and after stimulation was examined in pig follicular oocytes that had been matured and aged in vitro.

Materials and Methods

Collection and maturation of pig follicular oocytes

Ovaries were obtained from prepubertal crossbred gilts (Landrace, Large White and Duroc breeds) at an abattoir. Medium-sized, non-atretic follicles (3–5 mm in diameter) in Medium 199 (M199) modified with Hanks’ salts (GIBCO, Life Technologies Inc., Grand Island, NY) and supplemented with 10% (v/v) fetal bovine serum (GIBCO), 20 mmol Hepes L−1 (Dojinllo Laboratories, Kumamoto), 100 μg penicillin G potassium ml−1 (Sigma Chemical Co., St Louis, MO) and 0.1 mg streptomycin sulfate ml−1 (Sigma) were punctured with a surgical blade. Cumulus–oocyte complexes were collected in M199 within 0.5 h as described by Kikuchi et al. (1993). They were transferred to the maturation medium, a modified Waymouth’s MB752/1 medium (GIBCO) supplemented with 10% (v/v) fetal bovine serum, 10% (v/v) pig follicular fluid (pPF) which had been collected earlier (Naito et al., 1988), 2.5 μg FSH ml−1 (Antrin, Denka Pharmaceutical Co. Ltd, Kanagawa) and antibiotics (penicillin G potassium and streptomycin sulfate).

About ten cumulus–oocyte complexes were cultured in 100 μl of maturation medium covered with paraffin oil (Mineral oil, E. R. Squibb & Sons Inc., Princeton, NJ) in a 35 mm plastic dish (Becton Dickinson and Company, Lincoln Park, NJ) at 39°C under air with 5% CO2 for up to 72 h. After culture for 48 h, the maturation medium was changed. The nuclear changes during culture were examined by a phase contrast microscope (OPTIPHOTO, Nikon, Tokyo) after fixing with acetic alcohol and staining with 1% (w/v) aceto-orcein solution.

Parthenogenetic activation of oocytes matured in vitro

After incubation for 36, 48, 60 or 72 h, oocytes with well expanded cumulus cells were used to determine parthenogenetic activation. Oocytes were denuded with 150 μl hyaluronidase ml−1 and transferred to M199 with high osmotic pressure (350 osmol kg−1). Mature oocytes, that is, having a polar body as determined using a Nomarski differential interference contrast microscope (LMT-2, OLYMPUS, Tokyo) were collected and washed three times in the activation solution, consisting of 0.3 mol L−1 mannitol 1−1 (Wako Pure Chemicals, Tokyo), 0.1 mmol CaCl2 · 2H2O 1−1 (Wako), 0.1 mmol MgCl2 · 2H2O 1−1 (Wako) and 0.2 mg BSA ml−1 (Fraction V; Sigma). They were then transferred to a hybridizing chamber (FTC-22W, Shimadzu Corporation, Tokyo, Japan) containing 50 μl activation solution and stimulated with a 20 μs pulse at 1.0 kV DC cm−1 using a somatic hybridizer (SH1-10; Shimadzu). Oocytes that were not stimulated were used as controls. Stimulated and non-stimulated oocytes were transferred to BMOC-II solution (Brinster, 1965) containing 4 mg BSA ml−1 and cultured for 10 h. Oocytes were then fixed and stained, and examined under a phase contrast microscope. Oocytes with a female pronucleus(ei) (Fig. 1a) or fragmented or segmented cytoplasm (Fig. 1b) were considered activated (Sato et al., 1979). Thirty to 60 oocytes were used in each replicate and the experiment was repeated three times.

H1K assay of oocytes matured in vitro

After incubation in a maturation medium for 36, 48, 60 or 72 h, oocytes with well expanded cells were denuded and centrifuged at 15 000 g for 5 min to localize lipid granules (Wall et al., 1985). They were stained with 10 μg Hoechst 33342 ml−1 (Calbiochem Co., San Diego, CA) and classified according to their nuclear status using a fluorescent microscope (DIAPHOTO-TMD, Nikon, Tokyo). Oocytes at the metaphase stage with a polar body were classified as matured. Oocytes that had not been cultured in the maturation medium (0 h of culture) were classified as immature oocytes. They were washed three times in the assay solution which consisted of 60 mmol β-glycerophosphate 1−1, 30 mmol p-nitro-phenyl phosphate 1−1, 25 mmol Mops (pH 7.2) 1−1, 15 mmol EGTA 1−1, 15 mmol MgCl2 1−1 and 0.1 mmol sodium vanadate 1−1 (Wako). Groups of ten oocytes were collected into plastic tubes in 5 μl solution. They were frozen at −70°C to break the oocyte membrane and stored until the H1K assay was performed. Before the H1K assay, oocyte suspensions were thawed to room temperature and centrifuged at 15 000 g for 5 min at 4°C. The supernatant was used as the oocyte extract.

H1K activity was assayed according to the method described by Naito and Toyoda (1991). Each assay tube contained the following in a final volume of 25 μl assay solution: 500 nmol cAMP-dependent protein kinase inhibitor 1−1 (TIYADFIASGRRTGRRNAHID; Sigma), 50 μmol histone H1 1−1 (type III-S; Sigma), 1 mmol dithiothreitol 1−1 (Wako), 20 μmol [γ-32P]ATP 1−1 (3–10 c.p.m. fmol−1; Amersham, Arlington Heights, IL) and 5 μl oocyte extract. The kinase reaction began upon the addition of [γ-32P]ATP and was
carried out for 1 h at 36°C. Assays were terminated by the addition of 0.4 ml 20% (w/v) trichloroacetic acid solution; 0.1 ml 10 mg BSA ml⁻¹ solution was also added as a carrier protein for precipitation. After centrifugation at 15 000 g for 5 min, the precipitates were washed once with 0.4 ml 20% (w/v) trichloroacetic acid and dissolved in 0.4 ml 1 mol NaOH l⁻¹. The solution was then transferred to scintillation vials containing 5 ml scintillation fluid (ACS II; Amersham) and radioactivity was counted using a liquid scintillation counter (LSC-1000: Aloka, Tokyo). The value of blank tubes containing 5 μl assay solution was subtracted from each value. H1K activities are presented as fmol h⁻¹ per oocyte. This unit shows the total number of molecules of ³²PO₄ incorporated into histone H1 h⁻¹ per oocyte.

H1K assay of oocytes stimulated with an electric pulse

Matured oocytes with the first polar body that had been stimulated with an electric pulse and subsequently cultured in BMOC-II solution for 10 h were classified into three groups as determined by fluorescent microscopy: (1) oocytes at metaphase II (nonactivated oocytes); (2) oocytes having a female pronucleus(ies) with two polar bodies and (3) fragmented oocytes. They were placed in the assay solution and H1K activity was assayed as described previously.

Statistical analyses

All data were analysed by analysis of variance using GLM procedures of Statistical Analysis System (SAS). The changes of nuclear status and parthenogenetic activation were tested using Duncan’s multiple range test after transformation using arcsin of percentages (Snedecor and Cochran, 1967). Least-squares means in H1K activity in cultured and stimulated oocytes were tested using the least significant differences test.

Results

Parthenogenetic activation of oocytes matured in vitro

All of the immature oocytes collected and not cultured were found to be at the germinal vesicle stage. Matured oocytes at metaphase II were first observed after 30 h of culture (Table 1). The percentage of oocytes in expanded cumulus–oocyte complexes maturing increased significantly to 58% (P < 0.01) after 36 h of culture and remained high (64–71%) until 72 h of culture. Both immature and mature pig oocytes survived ageing (36–72 h of culture) without any nuclear changes being observed. When oocytes with the first polar body were stimulated after 36 h of culture, the percentage of activated oocytes was low (7%) (Fig. 2). However, when oocytes were stimulated after 48, 60 and 72 h of culture, the percentages of activated oocytes were 32, 46 and 57%, respectively, significantly higher (P < 0.05) than those in the control groups (0, 4 and 24%, respectively). The percentages of parthenogenetic activation in the stimulated oocytes increased significantly (P < 0.05) after 60 h of culture. Activated oocytes consisted of female pronuclear forming oocytes or fragmented oocytes. Fragmented oocytes first appeared in 7% of oocytes stimulated after 60 h of culture and the percentage increased significantly to 37% (P < 0.05) after 72 h of culture. Parthenogenetic activation was also observed in non-stimulated oocytes. In the control groups, the percentage of activated oocytes was significantly higher after 72 h of culture (P < 0.01, 24%) than after 36, 48 and 60 h of culture (1, 0 and 4%, respectively). In this study, all nonactivated oocytes remained at the metaphase II stage after subsequent culture.

H1K activity in oocytes matured in vitro

H1K activity in matured oocytes cultured for 36–72 h was significantly higher (P < 0.01) than that in immature oocytes at the germinal vesicle stage (17.2 ± 0.5 fmol h⁻¹ per oocyte) (Fig. 3). The activities in oocytes matured for 36 and 48 h were
Table 1. Maturation of pig follicular oocytes in vitro

<table>
<thead>
<tr>
<th>Duration of culture (h)</th>
<th>Number of oocytes examined*</th>
<th>Number (%) of immature oocytes</th>
<th>Number (%) of mature oocytes**</th>
<th>Number (%) of degenerated oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>130</td>
<td>123 (95)*</td>
<td>0 (0)*</td>
<td>7 (5)</td>
</tr>
<tr>
<td>30</td>
<td>133</td>
<td>101 (76)*</td>
<td>19 (14)*</td>
<td>13 (10)</td>
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<tr>
<td>36</td>
<td>135</td>
<td>43 (32)*</td>
<td>78 (58)*</td>
<td>14 (10)</td>
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<td>48</td>
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<td>72</td>
<td>117</td>
<td>32 (27)*</td>
<td>79 (68)*</td>
<td>6 (5)</td>
</tr>
</tbody>
</table>

*Cumulus-oocyte complexes were examined after culture.

1Oocytes at the germinal vesicle metaphase I or telophase I stages were classified as immature.

**Oocytes at the metaphase II stage were classified as mature.

Values with different superscripts in each column are significantly different (P < 0.01).

Fig. 2. Parthenogenetic activation of pig follicular oocytes matured and aged in vitro. After culture for 36, 48, 60 or 72 h, matured oocytes (those with a polar body) were collected. After culture for a further 10 h, oocytes were classed as (a) nonactivated, (b) activated or (c) degenerated oocytes. (a, c) The percentages of (■) stimulated and (□) nonstimulated oocytes are shown. (b) Activated oocytes include (■) stimulated and (□) nonstimulated oocytes that formed a female pronucleus and (■) stimulated and (□) nonstimulated oocytes that fragmented. a~d Bars with different letters are significantly different (P < 0.05) for each category.

H1K activity in nonactivated oocytes decreased (P = 0.08) when the duration of maturation was prolonged. Irrespective of the duration of maturation (36–72 h), H1K activity in activated oocytes was significantly lower (P < 0.01; 24.7 ± 7.2 mol h⁻¹ per oocyte) than that in nonactivated oocytes (99.9 ± 6.7 mol h⁻¹ per oocyte).

Discussion

H1K activity in oocytes stimulated with an electric pulse

H1K activity in the stimulated oocytes is shown (Fig. 4). Samples of stimulated fragmented oocytes after 36 and 48 h of culture could not be assayed because the numbers of oocytes in these groups were too small (Fig. 2). H1K activity in electrically stimulated, nonactivated oocytes after 36 h of culture was higher (P < 0.01; 131.1 ± 11.7 mol h⁻¹ per oocyte) than that in oocytes after 48, 60 and 72 h of culture (97.2 ± 16.5, 88.0 ± 11.7 and 66.8 ± 16.5 mol h⁻¹ per oocyte, respectively). Nuclear maturation of pig follicular oocytes was first observed after 30 h of culture. The percentage of oocytes maturing ranged from 64% to 71% between 48 and 72 h of culture. These results are in agreement with the results reported for pig oocytes cultured in modified Medium 199 by Takahashi and Nagai (1990). Thus, oocytes matured in modified Waymouth’s medium are suitable for parthenogenetic activation and for use in H1K assay.

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Fig. 3. Activity of histone H1 kinase (H1K) in pig follicular oocytes. Immature oocytes at the germinal vesicle stage were collected before culture. After culture for 36, 48, 60 or 72 h, oocytes at metaphase II were collected using fluorescence microscopy observations. H1K activity is presented as least squares means ± SEM (fmol h⁻¹ per oocyte). Numbers in parentheses above the bars are the number of samples used for the H1K assay. Bars with different letters are significantly different (P < 0.01).

Fig. 4. Histone H1 kinase (H1K) activity in stimulated pig oocytes. After culture for 36, 48, 60 or 72 h, matured oocytes were stimulated with an electric pulse followed by culture for a further 10 h. H1K activities in [■] nonactivated, [□] female-pronuclear forming and [■] fragmented oocytes were assayed. H1K activity is presented as least squares mean ± SEM (fmol h⁻¹ per oocyte). Numbers in parentheses above the bars are the numbers of samples used for H1K assay. Bars with different letters are significantly different (P < 0.01).

When pig oocytes were electrically stimulated, parthenogenetic activation was low during the early stages of maturation (36 h of culture); however, activation increased after 60 h of culture. A similar result was observed in the parthenogenetic activation of mouse (Kubiak, 1989) and cattle (Nagai, 1987) oocytes aged in vitro. When oocytes cultured for 72 h were not stimulated and subsequently cultured for a further 10 h, the percentage of oocytes undergoing activation was significantly higher than that for oocytes in which the initial culture period was 60 h. Spontaneously activated oocytes consisted of oocytes with female pronuclei and also fragmented oocytes. Sato et al. (1994) also reported spontaneous fragmentation in pig oocytes aged in vitro. Spontaneous fragmentation is considered as activation because the nucleus formed is similar in morphology to a female pronucleus. These results confirm that ageing in pig oocytes enhances the ability for parthenogenetic activation (Hagen et al., 1991; Porczak et al., 1992). However, cytoplasmic changes during ageing have not yet been reported in mammalian oocytes.

H1K activity in oocytes at the germinal vesicle stage was low whereas activity in metaphase II oocytes, matured for 36–48 h was high. These results are in agreement with our previous studies (Naito and Toyoda, 1991; Naito et al., 1992; Kikuchi et al., 1995). We also reported that H1K activity in pig oocytes is lower after sperm penetration and is maintained at basal values equivalent to those seen in oocytes at the germinal vesicle stage (Kikuchi et al., 1995). Basal values of H1K activity were also found in pig oocytes activated by electrical stimulation after culture for 36–72 h. Basal H1K activities were also observed in other mammalian oocytes after fertilization (Choi et al., 1991; Fulka et al., 1992; Collas et al., 1993) or parthenogenetic activation (Barnes et al., 1993; Collas et al., 1993). In the present study, a gradual impairment of H1K activity during ageing in pig follicular oocytes matured in vitro was observed. It appears that MPF/H1K activity in matured oocytes reflects the ability for mammalian oocyte activation. Naito et al. (1992) showed a close relationship between spontaneous activation and H1K activity in pig oocytes matured in different kinds of media. Spontaneous activation was more frequent when oocytes were matured in a modified Krebs–Ringer bicarbonate solution (mKRB) (Toyoda et al., 1971) than in pFF, and H1K activity in oocytes matured in mKRB showed lower H1K activity than that in pFF. It is probable that in aged pig oocytes which already have low H1K activity, electrical stimulation results in basal MPF/H1K activity falling below the threshold value and thus oocyte activation occurs. MPF has been shown to be a complex of cyclin B and p34cdc2 (Arion et al., 1988; Lohka et al., 1988). Kubiak et al. (1993) reported vigorous biosynthesis of cyclin B in matured mouse oocytes and indicated the importance of the equilibrium between the degradation and synthesis of cyclin B in the maintenance of metaphase. Inhibition of protein synthesis resulted in the disappearance of cyclin B and a fall in H1K activity, followed by oocyte activation (Kubiak et al., 1993; Fulka et al., 1994). Naito et al. (1995) also found active biosynthesis of cyclin B1 in matured pig oocytes. Thus, a gradual fall in cyclin B synthesis may occur in aged oocytes and H1K activity decreases as a consequence.

When oocytes were cultured for up to 60 h and then electrically stimulated, most of the activated oocytes had female pronuclei. These seemed to result from a normal oocyte activation process as when fertilization occurs, since the female pronucleus(ies) was well developed. However, abnormal activation, resulting in fragmentation, after electrical stimulation increased when the duration of maturation was prolonged. In
the present study, the percentage of fragmented oocytes increased to 25% after stimulation in oocytes that had undergone maturation for 72 h and the subsequent culture. The mechanism of fragmentation in aged oocytes is not clear at present. Oocytes that produced female pronuclei and fragmented oocytes both showed the same basal H1K activity after stimulation with an electric pulse; thus, there seems to be no difference in the mechanism of MPF/H1K inactivation between both types of oocyte activation. Sato et al. (1994) suggested that CAMP in pig oocytes, matured in vitro, is involved in the induction of the fragmentation. It is also possible that fragmentation is related to decreasing H1K activity because oocytes with high MPF/H1K activity can form a normal pronucleus(ei) after activation of the oocyte (Borsuk, 1991; Naito et al., 1992; Funahashi et al., 1993).

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