Intracellular Calcium Oscillations Signal Apoptosis Rather than Activation in In Vitro Aged Mouse Eggs

Ana Carla Gordo, Patricia Rodrigues, Manabu Kurokawa, Teru Jellerette, Ginger E. Exley, Carol Warner, and Rafael Fissore

Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts 01003
Instituto de Ciencias Biomedicas de Abel Salazar, Universidade Do Porto, Porto 4050, Portugal
Northeastern University, Boston, Massachusetts 02115

ABSTRACT
We have previously demonstrated that initiation of intracellular calcium ([Ca$^{2+}$]) oscillations in mouse eggs signals activation or apoptotic death depending on the age of the eggs in which the oscillations are induced. To extend these studies, mouse eggs were aged in vitro to 24, 32, and 40 h post-hCG and injected with sperm cytosolic factor (SF), adenophostin A, or sperm (intracytoplasmic sperm injection), and the times at which signs of apoptosis first appeared were examined. These treatments, which induced [Ca$^{2+}$]i oscillations, caused fragmentation and other signs of programmed cell death in eggs as early as 32 h post-hCG. The susceptibility of aged eggs to apoptosis appeared to be due to cytoplasmic deficiencies, because fusion of recently ovulated eggs with aged, SF-injected eggs prevented fragmentation. Evaluation of mRNA and protein levels of the apoptotic regulatory proteins Bcl-2 and Bax showed a prominent decrease in the amounts of Bcl-2 mRNA and protein in aged eggs, whereas Bax mRNA levels did not appear to be changed. Lastly, the Ca$^{2+}$i responses induced by the aforementioned Ca$^{2+}$i oscillations terminated or progressed as advanced in aged eggs. Together, these results suggest that one or several critical cytosolic molecules involved in the regulation of Ca$^{2+}$i homeostasis, and in maintaining the equilibrium between anti- and proapoptotic proteins, is either lost or inactivated during postovulatory egg aging, rendering the fertilizing Ca$^{2+}$i signal into an apoptosis-inducing signal.

INTRODUCTION
Ovulated mammalian eggs are arrested at the metaphase stage of the second meiotic division (MII), and they remain arrested until fertilization. Egg activation, which comprises several events, including cortical granule exocytosis, exit from MII and extrusion of the second polar body, pronuclear formation, and the first mitotic division, is driven by long-lasting intracellular calcium ([Ca$^{2+}$]) oscillations initiated by the sperm [1-3]. To trigger and to maintain these oscillations, the sperm is thought to induce hydrolysis of the phosphatidylinositol-(4,5)-bisphosphate that results in oscillations, the sperm is thought to induce hydrolysis of the phosphatidylinositol-(4,5)-bisphosphate that results in the production of inositol 1,4,5-trisphosphate (IP3). IP3, a widespread Ca$^{2+}$i-release agonist, binds to IP3 receptors (IP3R) localized in the endoplasmic reticulum (ER), the Ca$^{2+}$i store of the cell, and induces Ca$^{2+}$i release [3]. Egg activation can also be accomplished by injection of whole sperm (i.e., intracytoplasmic sperm injection [ICSI]) or sperm cytosolic fractions (i.e., sperm factor [SF]) into the egg’s cytoplasm [4-6]. Both ICSI and SF are thought to stimulate the same signal transduction mechanisms as natural fertilization [4, 7].

The time after ovulation during which mammalian eggs can give rise to developmentally competent embryos is short. Under in vivo conditions, ovulated mouse eggs exhibit maximum ability to fertilize for only 4-6 h [8]. After this time, signs of deterioration can be observed that lead to decreased fertilization rates and, more importantly, to reduced embryo developmental rates [8-10]. As time post-ovulation progresses, aged unfertilized eggs as well as aged fertilized embryos exhibit variable degrees of fragmentation and cell death [11, 12]. Recently, it has been demonstrated that these fragmenting eggs/embryos undergo molecular changes characteristic of apoptosis or programmed cell death, a process of cell selection that is observed in many cell types [13-15].

Apoptosis requires activation of specific genes involved in execution of the cell suicide program [16, 17]. Characteristic morphological changes observed in cells undergoing this program have been widely described [16, 18], and two apoptotic pathways have been elucidated so far. The first pathway is stimulated by cell surface “death receptors” that recognize ligands, such as FasL and tumor necrosis factor α. After activation, these receptors undergo oligomerization and stimulate activation of caspases, which are proteases that reside mainly in the cytosol and are responsible for carrying out the dismantling of the cell [19, 20]. The second cell death pathway involves the mitochondria. Many triggers, including DNA damage, hypoxia, growth factor withdrawal, glucocorticoids, and mitogenic oncogenes, activate this pathway [21-23]. These stimuli promote the release of cytochrome c, a component of the respiratory chain, into the cytosol that, in turn, results in activation of the caspase cascade [24]. Caspase activity is regulated by the Bcl-2 family of proteins [25, 26]. The Bcl-2 family is comprised of proapoptotic (e.g., Bax and Bak) and antiapoptotic (e.g., Bcl-2 and Bcl-XL) regulators that block or stimulate cytochrome c release in response to multiple death-inducing stimuli [26].

Although mammalian eggs express the majority of genes that encode known members of the Bcl-2 family of proteins and caspase proteases [13, 27, 28], the signaling mechanisms that trigger apoptosis in these cells remain to be elucidated. It has been demonstrated that exposure of mammalian eggs to increasing concentrations of hydrogen peroxide, staurosporine, or doxorubicin, an antioncogenic drug, results in fragmentation and apoptosis [29-31]. In addition, our own studies have shown that [Ca$^{2+}$i] oscillations...
tions can also signal cell death when initiated in aged eggs [32], and the role of Ca\(^{2+}\) and its regulation in programmed cell death has long been recognized [33–35]. Furthermore, Bcl-2 has been demonstrated to have an important role in the regulation of Ca\(^{2+}\) homeostasis [36, 37]. Thus, to further elucidate the role of [Ca\(^{2+}\)]\(_i\) oscillations in inducing cell death in mouse eggs, we investigated the time post-ovulation at which [Ca\(^{2+}\)]\(_i\) oscillations first signal apoptosis. We then evaluated whether important cytosolic survival factors in the egg are lost during aging, and we tested whether the levels of Bax and Bcl-2 mRNAs and Bcl-2 protein changed during aging. Finally, we studied whether the Ca\(^{2+}\) responses induced in aged eggs by several agonists were different from those elicited by these same agonists in recently ovulated eggs.

**MATERIALS AND METHODS**

**Egg Collection and Handling**

The MII eggs were collected from the oviducts of CD-1 female mice stimulated with 5 IU of eCG (Sigma, St. Louis, MO) followed 48 h later by 5 IU of hCG (Sigma) to induce ovulation. Eggs were collected 14–15 h post-hCG into a Heps-buffered solution (Tyrode lactate [TL]-Heps supplemented with 10% (v/v) heat-treated fetal calf serum (FCS; Gibco BRL, Grand Island, NY)). Cumulus cells were removed by incubation for 3–7 min in bovine testis hyaluronidase (Sigma). For this study, only eggs that had their first polar body and showed no signs of degeneration were chosen. These eggs were cultured before and after activation in 50-μl drops of potassium simplex optimized medium (KSOM; Specialty Media, Lavallette, NJ) under paraffin oil at 36.5°C in a humidified atmosphere containing 5.5% CO\(_2\) until the time of injection. Aging of eggs in our studies was accomplished exclusively in vitro by extending the culture time of eggs after collection as indicated in each experiment. Whether in vitro aging replicates the molecular events that occur during in vivo aging is not known, although, if anything, in vitro culture of eggs appears to delay the quick deterioration that occurs in eggs within the oviduct after ovulation [38, 39].

**Microinjection Techniques and Parthenogenetic Activation**

Microinjection procedures were as previously described [5, 32]. In brief, eggs were microinjected under a Nikon Diaphot microscope (Nikon, Inc., Garden City, NY) using Narishige manipulators (Medical Systems Corp., Great Neck, NY). Injection pipettes containing 0.5 mM fura-2 dex-tran (fura-2D; Molecular Probes, Eugene, OR), 1 mg/ml of SF, 10 μM adenophostin A (a powerful IP\(_3\) R agonist; a generous gift of Dr. K. Tanzawa, Sankyo Co., Ltd., Tokyo, Japan), or 500 μM MI P\(_3\) (Molecular Probes) were injected into the cytoplasm of eggs by pneumatic pressure (PLI-100 picoinjector; Harvard Apparatus, Cambridge, MA). All reagents were diluted in an injection buffer (IB) containing 75 mM KCl and 20 mM Hepes, pH 7.0. The injection volume was approximately 5–10 pl and resulted in final intracellular concentrations of the injected compounds of approximately 1.5%–3% of the concentration in the injection pipette. Injections of SF or adenophostin A were performed in eggs aged in vitro to 24, 32, and 40 h post-hCG, and eggs were observed for signs of activation and/or apoptosis at 8 and 16 h postinjection. The [Ca\(^{2+}\)]\(_i\) levels were measured in recently ovulated eggs (15 h post-hCG) and in aged eggs (40 h post-hCG). Control eggs were either left uninjectected or injected with buffer.

**Fluorescence Recordings and Ca\(^{2+}\) Determination**

Fura-2D fluorescence was monitored as previously described [4, 32]. Briefly, illumination was provided by a 75-W xenon arc lamp on a Nikon Diaphot microscope equipped with a 40× ultraviolet oil-immersion objective (Nikon). Excitation wavelengths were at 340 and 380 nm, and the emitted light, attenuated 32-fold by neutral-density filters, was quantified by a photomultiplier tube, which averaged the fluorescence signal of the whole egg. The rotation of a filter wheel and a shutter apparatus was controlled by a modified Phoscan 3.0 software program (Nikon) on a 486-processor IBM-compatible computer to alternate wavelengths. The [Ca\(^{2+}\)]\(_i\) concentrations, \(R_{1/2}\), and \(R_{max}\) were calculated according to the methods of Grynkiewicz et al. [40] and Poenie [41] and as described previously [4]. Eggs were individually monitored for [Ca\(^{2+}\)]\(_i\) levels in a 50-μl drop of medium placed on a glass coverslip sealed over an opening in the bottom of a culture dish and then covered with mineral oil. Before the injection of IP\(_3\), adenophostin A, or SF; fluorescence recordings were taken to establish baseline values. Fluorescence ratios were obtained every 4 sec, after a 1-s reading at each wavelength, for 15–35 min. All [Ca\(^{2+}\)]\(_i\) data are presented as the mean ± SEM.

**SF Preparation**

The SF was prepared from boar semen as previously described [4, 5, 42]. Briefly, semen samples were washed twice with TL-Heps medium, and the sperm pellet was resuspended in a solution containing 75 mM KCl, 20 mM Heps, 1 mM EDTA, 10 mM glycophosphate, 1 mM dithiothreitol (DDT), 200 μM PMSE 10 μg/ml of pepstatin, and 10 μg/ml of leupeptin; pH 7.0. The resulting suspension was lysed by sonication for 30–35 min at 4°C (XL2020; Heat Systems, Inc., Farmingdale, NY). The lysate was then centrifuged twice at 10,000 × g for 15 min at 4°C. The precipitates were collected and stored at −80°C until use. Protein concentrations were determined using a protein determination kit (Sigma).

**Intracytoplasmic Sperm Injection**

The ICSI was performed as previously described [43]. Sperm were obtained from 7- to 11-wk-old CD-1 male mice. The epididymides were isolated and placed in TL-Heps with BSA, and the large tubules were cut in several places to allow the spermatozoa to escape into the medium. A drop of concentrated sperm was transferred into 0.5 ml of TL-Heps with BSA and incubated for 5–15 min at 37°C. Then, 1 part of the sperm suspension was mixed with 2 parts of IB supplemented with 10% (w/v) polyvinylpyrrolidone (PVP; 360 kDa; Sigma), and a drop of the diluted sperm sample was placed on a plastic Petri dish (dimension, 100 × 15 mm; Falcon Plastics, Oxnard, CA), which served as a microinjection chamber. Manipulations were carried out on an inverted Nikon Diaphot microscope fitted with a cooling stage (18–19°C; BC-100 Bionomic Controller; Research Instrument Limited, Cornell, England, U.K.). A method using a piezo-micropipette-driving unit (PMM-01 Piezo Micromanipulator; Tsuchiura-City, Ibaraki-ken, Japan) was used to expel the spermatozoon head into the cytoplasm of eggs. The spermatozoon was aspirated tail-first into the injection pipette, and the head was separated from the tail by 2 or 3 piezo-pulses. Sperm-injected eggs were transferred into 50-μl drops of KSOM under paraffin oil and incubated at 36.5°C in a humidified atmosphere containing 5.5% CO\(_2\). Eggs undergoing the same procedure, but without introduction of the sperm, were used as controls. Observations were made at 8 and 16 h post-sperm injection.

**Western Blot Analysis**

Microsomes from mouse ovaries were prepared as previously described [44]. In brief, ovaries were homogenized with a glass silicone-coated homogenizer in 0.3 M sucrose, 1 mM EDTA, 1 mM 2-mercaptoethanol, 200 μM PMSE, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, 50 mM Tris-HCl (pH 8.0; all chemicals from Sigma) and then centrifuged at 2500 × g for 15 min to remove the heavy-particulate fraction. The supernatant was recentrifuged at 100,000 × g for 30 min, and the microsomal precipitates were resuspended in 0.3 M sucrose, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 50 mM Tris-HCl (pH 8.0). Crude lysates from oocytes were prepared from 200 oocytes collected in Dulbecco phosphate-buffered saline (DPBS) supplemented with 3 mg/ml of PVP (Sigma).

Microsomal preparations or crude egg lysates were combined with double-strength electrophoresis sample buffer [45]. Samples were boiled for 5 min and loaded into 12% SDS-polyacrylamide gels. The separated proteins were then transferred onto nitrocellulose membranes (Micron Separations, Inc., New Jersey, CA). The membranes were first washed in PBS-0.005% (v/v) Tween 20 (PBS-T) and then blocked in PBS-T supplemented with 6% (v/v) nonfat dry milk. After several washes in PBS-T, the membranes were incubated overnight at 4°C with a 1:500 dilution of an anti-Bcl-2 rabbit polyclonal antibody (Calbiochem, La Jolla, CA) in PBS-T. The membranes were washed again in PBS-T and incubated in a 1:5000 dilution of anti-rabbit monoclonal horseradish peroxidase-conjugated antibody (Bio-Rad, Hercules, CA). The membranes were then washed 3 times in PBS-T and developed using the enhanced chemiluminescence detection.
system according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Semiquantification of immunoreactive Bcl-2 was done using Adobe Photoshop (Mountain View, CA). The intensity of the band in recently ovulated eggs was used as the baseline value and arbitrarily assigned a value of 1; the intensity of the Bcl-2 band in aged eggs was calculated relative to this value. Western blot analysis for quantification of Bcl-2 was repeated at least 2 times.

**Caspase Activity**

Assays for caspase activity were performed as previously described [13, 32] using the PhiPhiLux kit (OncoImmunin, Inc., College Park, MD), a rhodamine-conjugated DEVD (Asp-Glu-Val-Asp)-specific caspase substrate at a final concentration of 5 μM for 1 h at 37°C in 5.5% CO₂. After several washes in cold TL-Hepes containing the synthetic caspase substrate at a final concentration of 5 μM for 1 cycle; 96°C for 2 cycles; 94°C for 2 min, 72°C for 15 ± 25 sec (depending on the length of the product) for 20 cycles. The PCR primers were designated using Oligo 5.0 software from National Biosciences, Inc. (Plymouth, MN), based on published sequences obtained from GenBank. One-tenth of each sample was amplified using primers for the control RNA, pAW109. For assessment of the expression of Bcl-2, the cDNA equivalent of 16 cells was used in each reaction.

**Isolation of RNA**

Eggs were collected and washed 3 times in PBS plus 1% BSA, and then transferred into a 0.6-ml tube in as small a volume as possible. Eggs were immediately lysed by adding 100 μl of denaturing solution (prepared according to the protocol for the Micro-RNA Isolation Kit; Stratagene, La Jolla, CA) and vortexing. The samples were spun down briefly, then frozen at -80°C before RNA purification. Just before RNA isolation, 1 × 10⁶ copies of the synthetic RNA pAW109 (Perkin-Elmer, Foster City, CA) were added to each sample to control for differences in the efficiency of RNA purification and reverse transcription (RT). Total RNA was purified using the Micro-RNA Isolation Kit according to the manufacturer’s protocol and using glycogen as a carrier during precipitation of the RNA. Purified RNAs were immediately converted to cDNA as described below.

**Reverse Transcription**

Each precipitated and dried RNA sample was resuspended in a 12-μl solution consisting of 10.75 μl of nuclease free water (Promega, Madison, WI), 0.2 μl of 0.1 M DTT (Gibco BRL, Gaithersburg, MD), 1 μl of 50 μM random hexamers (Gibco BRL), and 0.05 μl of 40 U/μl RNAsin (Promega) and prepared as a master mix for all samples. Each sample was vortexed and spun down briefly, then overlaid with 50 μl of sterile mineral oil. Hexamers were annealed to the RNA by heating the samples to 70°C for 5 min, followed by a 1-min incubation at 25°C. Eight microliters of a solution consisting of 4 μl of 25 mM MgCl₂ (Perkin-Elmer), 2 μl of 10× polymerase chain reaction (PCR) buffer (Perkin-Elmer), 0.4 μl of 100 mM dNTPs (Gibco BRL), 0.5 μl of 40 U/μl of RNasin, 0.6 μl of nuclelease-free water, and 0.5 μl of 200 U/μl of Moloney murine leukemia virus reverse transcriptase (Gibco BRL), prepared as a master mix, were then added to each sample, and the sample was mixed by pipetting up and down. The tubes were incubated at 37°C for 3 h, then heated to 99°C for 5 min to terminate the reaction.

**Polymerase Chain Reaction**

The PCR primers were designated using Oligo 5.0 software from National Biosciences, Inc. (Plymouth, MN), based on published sequences obtained from GenBank. One-tenth of each sample was amplified using primers for the control RNA, pAW109. For assessment of the expression of Bcl-2, the cDNA equivalent of 16 cells was used in each reaction.

**Statistics**

The proportion data in Tables 1 and 2 were compared using a one-way ANOVA following arcsine transformation [47]. Comparison of data in Table 3 was performed using the chi-square test. Frequency and amplitude of [Ca²⁺], responses were compared using one-way ANOVA. In all cases, statistical significance was considered to be at P < 0.05. All analyses were performed using the JMP IN software (SAS Institute, Cary, NC).

**TABLE 1. Effect of age on egg activation by SF or adenophostin A.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>16 h post-hCG</th>
<th>24 h post-hCG</th>
<th>32 h post-hCG</th>
<th>40 h post-hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eggs (n)</td>
<td>Activated (%)</td>
<td>Fragmented (%)</td>
<td>Eggs (n)</td>
</tr>
<tr>
<td>Control</td>
<td>34</td>
<td>0 (0)</td>
<td>59 (2)</td>
<td>44</td>
</tr>
<tr>
<td>Buffer</td>
<td>——</td>
<td>——</td>
<td>——</td>
<td>14</td>
</tr>
<tr>
<td>Adenophostin</td>
<td>83</td>
<td>78 (94)</td>
<td>5 (6)</td>
<td>62</td>
</tr>
<tr>
<td>SF</td>
<td>42</td>
<td>40 (95)</td>
<td>0 (0)</td>
<td>36</td>
</tr>
</tbody>
</table>

* The rest of the eggs were nonfragmented.

bc Treatments with different letters are significantly different from each other within a column (ANOVA, P < 0.05).

d—f Treatments with different letters are significantly different from each other within a row (ANOVA, P < 0.05).

**TABLE 2. [Ca²⁺], oscillations induced by SF or adenophostin A first signal fragmentation in eggs aged in vitro to 24 h post-hCG.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>16 h post-injection</th>
<th>24 h post-injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eggs (n)</td>
<td>Activated (%)</td>
</tr>
<tr>
<td>Control</td>
<td>59</td>
<td>7 (21)</td>
</tr>
<tr>
<td>Buffer</td>
<td>34</td>
<td>41 (51)</td>
</tr>
<tr>
<td>Adenophostin</td>
<td>81</td>
<td>26 (59)</td>
</tr>
</tbody>
</table>

* The rest of the eggs remained unchanged.

bc Treatments with different letters are significantly different from each other within a column (ANOVA, P < 0.05).
TABLE 3. Effect of egg age on activation by ICSI.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>7 h post-injection</th>
<th>16 h post-injection</th>
<th>32 h post-hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eggs (n)</td>
<td>Activated (n [%])</td>
<td>Fragmented (n [%])</td>
</tr>
<tr>
<td>Buffer</td>
<td>4</td>
<td>3 (75)a</td>
<td>0 (0)b</td>
</tr>
<tr>
<td>ICSI</td>
<td>20</td>
<td>11 (55)</td>
<td>9 (45)c</td>
</tr>
</tbody>
</table>

a The rest of the eggs remained unchanged.
bc Treatments with different letters are significantly different from each other within a column (chi-square, P < 0.05).

RESULTS

Time Course of \([Ca^{2+}]_i\), Oscillation-Induced Apoptosis in Mouse Eggs

Our previous studies have shown that fertilization-like \([Ca^{2+}]_i\) oscillations induced by injection of SF trigger activation and embryonic development in freshly ovulated eggs, but that if the same \([Ca^{2+}]_i\) oscillations are initiated in eggs aged in vitro to 40 h post-hCG, they induce fragmentation and cell death. To determine the time at which \([Ca^{2+}]_i\) oscillations first signal apoptosis, mouse eggs were collected at 16 h post-hCG; used immediately or aged in vitro to 24, 32, and 40 h post-hCG; and injected at all these time points with 1 mg/ml of SF or 10 \(\mu\)M adenophostin A, a nonhydrolyzable agonist of the IP\(_3\)R, which is the receptor thought to be activated by the sperm during fertilization [3]. These eggs were then observed within 7 h of injection for signs of activation, such as extrusion of the second polar body and pronuclear formation, and for signs of cytoplasmic fragmentation. Forty of 42 (95%) and 78 of 83 (94%) eggs aged to 24 h post-hCG and injected with SF or adenophostin A, respectively, exhibited normal activation by 7 h postinjection as evidenced by pronuclear formation (Table 1). Freshly ovulated eggs showed similar activation rates following SF or adenophostin A injections (Table 1).

When injections were done at 32 h post-hCG, 26 of 36 (72%) SF-injected and 37 of 62 (60%) adenophostin A-injected eggs showed fragmentation within 5 h of injection, and as expected, severe fragmentation and cell death were observed in 28 of 30 (93%) and 47 of 55 (85%) eggs aged to 40 h post-hCG injected with the same agonists, respectively. Control uninjected and buffer-injected eggs (injection of buffer does not induce \(Ca^{2+}\) release; data not shown) at 24 and 40 h post-hCG showed very low rates of fragmentation (Table 1). We then determined whether the observed fragmentation in SF-injected eggs at 32 h post-hCG was accompanied by activation of caspases. As shown in Figure 1, all SF-injected eggs that showed fragmentation exhibited widespread cytoplasmic caspase activity (Fig. 1, C and D). Uninjected eggs aged in vitro for the same period of time (Fig. 1, A and B) did not exhibit cytoplasmic caspase activity, although some activity was associated with the polar body, as previously reported [13, 15, 32].

Although eggs aged to 24 h post-hCG did not exhibit signs of fragmentation within 7 h post-Ca\(^{2+}\) stimulation, a significant number of eggs aged to and injected at 32 h post-hCG exhibited fragmentation, suggesting that, during this interval, the activating Ca\(^{2+}\) signal becomes an apoptotic-inducing signal. To evaluate this possibility, eggs aged to 24 h post-hCG were injected with SF or adenophostin A and evaluated for signs of fragmentation 16 h postinjection. In these experiments, 18 of 44 (41%) SF-injected eggs and 40 of 81 (49%) adenophostin A-injected eggs showed signs of fragmentation; meanwhile, control uninjected and buffer-injected eggs exhibited low rates of fragmentation.
Fertilization by ICSI Induces Apoptosis in Aged Eggs

To examine whether fertilization could also trigger fragmentation in aging eggs, we performed ICSI in recently ovulated eggs and in eggs aged in vitro to 24 and 32 h post-hCG. ICSI was chosen rather than regular fertilization, both because aged eggs exhibit zona pellucida hardening, which significantly decreases the rate of fertilization [48], and because zona-free in vitro fertilization was not attempted to avoid polyspermy [49]. All 15 h post-hCG eggs fertilized by ICSI exhibited pronuclear formation and initiated embryonic development (Fig. 2B). Conversely, the majority of eggs subjected to ICSI at 24 h post-hCG initiated normal activation, but within 16 h posttreatment, 12 of 20 (60%) of these eggs underwent fragmentation. Furthermore, although these eggs cleaved to the 2-cell stage, none of them developed to the blastocyst stage (data not shown). When ICSI was performed at 32 h post-hCG, signs of fragmentation were observed earlier, because 15 of 26 (58%) eggs showed signs of fragmentation within 7 h of injection, and 17 of 26 (65%) eggs exhibited severe fragmentation by 16 h postinjection (Fig. 2C and Table 3). Conversely, none, or very few, of similarly aged eggs that were injected with a comparable pipette containing buffer showed signs of fragmentation at 16 h postinjection (Fig. 2A and Table 3). Aged, ICSI-activated eggs also exhibited widespread stimulation of caspase activity (Fig. 2D).

Protective Cytoplasmic Components Are Degraded/Inactivated During Aging

The \([Ca^{2+}]_i\) oscillations are the normal egg activating signal; however, in aged eggs, this signal can trigger apoptosis. Thus, during aging, it is possible that critical egg components are lost, rendering eggs developmentally incompetent and susceptible to cell death. This led us to test whether the fusion of a recently ovulated egg with an aged, SF-injected egg could prevent/rescue these eggs from undergoing apoptosis. To accomplish this, recently ovulated (15 h post-hCG) or aged (40 h post-hCG) eggs were fused by an electrical pulse with aged eggs injected with 1 mg/ml of SF within 30 min of the injection. Fusion of recently ovulated eggs delayed or inhibited the fragmentation/death induced by SF in aged eggs, and only 3 of 16 (19%) fused eggs exhibited signs of fragmentation. The majority (13 of 16 [81%]) of these eggs did not fragment and either exhibited pronuclear formation (3 of 16 [19%]) (Fig. 3A) or cleaved to the 2-cell stage 24 h after fusion (10 of 16 [63%]) (Fig. 3B). In contrast, 9 of 9 (100%) of the aged eggs fused to aged, SF-injected eggs underwent rapid fragmentation and cell death (\(P < 0.05\)) (Fig. 3, C and D).

Aged Eggs Exhibit Decreased Levels of Bcl-2 mRNA and Bcl-2 Protein

A model initially proposed by Oltvai et al. [50] postulates that the ratio of the total amount of antiapoptotic Bcl-2 to proapoptotic Bax determines whether a cell will live or die. This model has been suggested to operate in eggs, because Bcl-2 and Bax proteins are expressed in recently ovulated eggs [13, 27]. Mouse embryos undergoing fragmentation were shown to exhibit increased expression of proapoptotic members of the Bcl-2 family of proteins (Bad, Bcl-xS, and Bax) and decreased presence of antiapoptotic members (Bcl-2) [13, 27]. It is possible, then, that the sensitivity of aged eggs to the induction of apoptosis by \([Ca^{2+}]_i\) oscillations correlates with decreased levels of antiapoptotic proteins and increased levels of proapoptotic proteins. Thus, the levels of Bcl-2 and Bax mRNAs and of Bcl-2 protein in recently ovulated and in vitro-aged eggs were evaluated. RT-PCR analysis showed that Bcl-2 and Bax mRNA levels are easily detectable in recently ovulated eggs (18 h post-hCG), but Bcl-2 mRNA levels rapidly decrease whereas Bax mRNA levels remain unchanged during the same period of time (Fig. 4A). Similarly, as shown in Figure 4, B and C, the levels of Bcl-2 protein are reduced in aged eggs (40 h post-hCG) as compared to recently ovulated eggs.

Fertilization-Like \([Ca^{2+}]_i\) Oscillations Triggered by Injection of IP₃, Adenophostin A, or SF Stop Prematurely in Aged Eggs

Previous studies have demonstrated that the IP₃-R-mediated Ca²⁺ release is adversely affected in aged mouse fragments, at 5 of 59 (8%) and 4 of 34 (12%) eggs, respectively (Table 2).
FIG. 3. Phase-contrast images indicate that fusion of recently ovulated eggs (15 h post-hCG) with aged, SF-injected eggs (40 h post-hCG) rescued these eggs from undergoing apoptosis. Pronuclear formation and cleavage to the 2-cell stage was observed at 7 and 24 h postfusion, respectively (A and B, respectively). Aged eggs (40 h post-hCG) fused with aged, SF-injected eggs (40 h post-hCG) exhibited full fragmentation 3 h postfusion (C and D, respectively).

FIG. 4. RT-PCR analysis of Bcl-2 and Bax in MII eggs at 18, 23, and 27 h post-hCG. A synthetic RNA, pAW109, was added as an internal control (A). Western blot analysis and quantification of Bcl-2 protein in recently ovulated (15 h post-hCG) and aged (40 h post-hCG) MII mouse eggs was performed, and ovary supernatant was used as a positive control (10 μg). Two-hundred eggs were used per lane (B). Values are the means of 2 Western blot experiments, performed on different batches of eggs (C).

eggs [51, 52]. However, to our knowledge, the effect of this defect on the persistence and amplitude of \([Ca^{2+}]_i\) oscillations has not been investigated. Thus, we tested whether the \(Ca^{2+}\) responses induced by IP\(_3\), SF, or adenophostin A were different in aged eggs versus recently ovulated eggs. As shown in Figure 5, these agonists initiated persistent oscillations in recently ovulated eggs, but in aged eggs, the initiated oscillations ceased prematurely \((P < 0.05)\). Furthermore, the amplitudes of the first and subsequent spikes were significantly lower in aged eggs. For example, the amplitude of the first rise induced by adenophostin A in young eggs was 890 ± 80 nM, compared with 350 ± 60
FIG. 5. Aged eggs injected with IP$_3$, adenophostin A, or SF exhibited [Ca$^{2+}$]$_i$ oscillations that abruptly stopped 10–15 min postinjection. Injection of IP$_3$, adenophostin A, or SF into recently ovulated eggs induced persistent [Ca$^{2+}$]$_i$ oscillations that lasted for more than 30 min (A, D, and G, respectively), but in aged eggs, these [Ca$^{2+}$]$_i$ oscillations stopped within 15 min of injection (B, E, and H, respectively). Total numbers of recently ovulated and aged eggs injected with the above agonists and that persistently oscillated, that exhibited a single [Ca$^{2+}$]$_i$ rise, or that started to oscillate and then abruptly stopped are also shown (C, F, and I, respectively).

DISCUSSION

The results of the present study show the following: 1) that [Ca$^{2+}$]$_i$ oscillations initiated by injection of SF or adenophostin A induce activation in recently ovulated eggs, but that, as eggs age in vitro, these oscillations signal egg fragmentation with activation of caspases, both of which are signs of apoptosis; 2) that fertilization of eggs aged in vitro by ICSI also induces fragmentation rather than activation; 3) that aged eggs may be deficient in cytoplasmic components that are required for preventing apoptosis; 4) that presence of the antiapoptotic Bcl-2 protein is reduced in aged eggs; and 5) that injection of Ca$^{2+}$ agonists into aged eggs induce abnormal Ca$^{2+}$ responses characterized by oscillations of low amplitude and abrupt cessation. These results suggest that as eggs age, their ability to mount [Ca$^{2+}$]$_i$ oscillations decreases, and that these changes render the fertilizing [Ca$^{2+}$]$_i$ signal into an apoptosis-inducing signal.

The fertilizable life of ovulated mammalian eggs is limited, and soon after ovulation, biochemical and cytoskeletal changes ensue that result in spontaneous cell cycle progression and increased susceptibility to parthenogenesis [9, 53, 54]. Ovulated aged eggs also lose their ability to be fertilized normally, and their capacity to give rise to fully viable embryos is reduced [11, 12], which may result in developmentally compromised offspring, as recently reported [10]. Eggs of older females also exhibit lower rates of fertilization and a greater degree of fragmentation [55]. In a previous report, we hypothesized that sperm-induced
[Ca\(^{2+}\)], oscillations were responsible for inducing fragmentation and apoptosis of fertilized aged eggs [32]. In the present study, we confirm and extend those results by demonstrating that initiation of oscillations by injection of SF or adenophostin A or by the sperm after ICSI induce fragmentation as early as 32 h post-hCG. Approximately 30 h post-hCG appears to be the critical time after which CD1 mouse eggs are unable to respond to [Ca\(^{2+}\)], oscillations with normal activation and, instead, undergo cell death.

To our knowledge, whether the susceptibility of in vitro-aged mouse eggs to cell death and fragmentation resides in their nuclear and/or cytoplasmic components has not been previously demonstrated. In previous studies, it was shown that mouse eggs subjected to oxidative stress by exposure to H\(_2\)O\(_2\) underwent mitochondrial and developmental changes consistent with apoptosis [31, 56]. The apoptosis-inducing factors appeared to reside in the cytoplasm, because zygotes reconstituted by combining the nuclei of untreated eggs and cytoplasm from H\(_2\)O\(_2\)-treated eggs underwent apoptosis whereas zygotes reconstituted from untreated cytoplasm and treated nuclei exhibited normal developmental capacity [56]. Interestingly, cytoplasmic deficiencies have been suggested to be the cause of many cases of human infertility, especially those related to advanced age, and several techniques have been suggested to transfer/replace the presumed lack of essential cytoplasmic factors in these eggs [57, 58]. Our finding that fusion of recently ovulated eggs with aged, SF-injected eggs is able to prevent fragmentation and to allow normal activation of the fused eggs suggests that aging of mouse eggs has a significant impact on cytoplasmic factors that are required for normal activation and early development.

Several organelles could be adversely affected during postovulatory aging of mouse eggs, leading to enhanced susceptibility to fragmentation and cell death. The mitochondria have been shown to be central regulators of apoptosis in numerous cell types [21, 59]. Cytochrome c is stored in the mitochondria, and its release from the intermembrane space, as well as the release of other, less characterized apoptosis-inducing factors, has been noted to be a significant early event in the activation of caspases [60, 61]. Furthermore, Bcl-2 and other critical antiapoptotic proteins are also located in the mitochondria and are known to inhibit the release of cytochrome c into the cytoplasm [26, 62, 63]. During aging of mouse oocytes/eggs, therefore, it is possible that the function of the mitochondria may be compromised, and several recent reports support this possibility. For instance, oocytes of older women were shown to be more likely to contain deletions of mitochondrial DNA than were oocytes from younger women [64], and oocytes undergoing oxidative stress exhibited a rapid decrease of the mitochondrial membrane potential and loss of mitochondrial membrane integrity [31]. In addition, injection of fresh mitochondria from granulosa cells was able to abrogate the rate of spontaneous apoptosis in in vitro-cultured oocytes [65]. Collectively, these data suggest that the mitochondria may be compromised in aging mouse eggs. In addition, our results show that the levels of Bcl-2 mRNA and protein are decreased in aged mouse eggs, whereas the levels of Bax mRNA do not undergo dramatic changes during postovulatory aging. These results are in agreement with previous findings showing that eggs and embryos undergoing fragmentation and apoptosis exhibit decreased gene expression of antiapoptotic Bcl-2 family members, whereas the expression of genes favoring cell death appears to be increased [13, 27].

The reduced amounts of Bcl-2 observed in aged eggs may be associated with lower levels of the mitotic kinases maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK). MPF and MAPK, which are responsible for the MII arrest, are down-regulated in aged eggs [38]. Notably, recent studies in endothelial cells have shown that reduction of MAPK activity leads to ubiquitination and degradation of the Bcl-2 protein [66]. In addition, in human pancreatic cells, inhibition of MAPK activity induces down-regulation of the expression levels of the antiapoptotic homologues Bcl-2, Mcl-1, and Bcl-xL, but without affecting the levels of Bax and Bak [67]. Furthermore, treatment of p53-mutated pancreatic cells with butyrolactone I, a specific inhibitor of MPF, resulted in up-regulation of Bax expression and down-regulation of Bcl-2 expression [68]. Consistent with these findings, it was recently reported that expression and activity of MAPK were absent in degenerated human eggs [69]. Therefore, reduced levels of MPF and MAPK activities may be responsible for the altered balance of Bcl-2 and Bax in aged eggs. The significance of the balance of these proteins on the survival of mouse eggs can be deduced from studies demonstrating the resistance to spontaneous or chemically induced apoptosis of eggs that overexpress the Bcl-2 protein [70] or that lack Bax or caspase-2 proteins [71, 72].

Besides serving as the host of many proteins involved in apoptosis, the mitochondria participate in the regulation of [Ca\(^{2+}\)], oscillations [73] and, consequently, may play a role in apoptosis induced by [Ca\(^{2+}\)], oscillations. The mitochondria have been demonstrated to serve as a Ca\(^{2+}\) storage organelle, although the amount of Ca\(^{2+}\) stored is significantly less than that in the ER [74]. Furthermore, a privileged Ca\(^{2+}\) transfer via IP\(_3\)R to the mitochondria has been shown that stimulates mitochondrial energy metabolism [75]. Interestingly, IP\(_3\)-mediated rises in [Ca\(^{2+}\)], can also induce cell death accompanied by cytochrome c release and a substantial drop in the mitochondrial membrane potential if, before the initiation of oscillations, cells are exposed to proapoptotic stimuli [76]. Therefore, we propose that postovulatory aging of mouse eggs may serve as a proapoptotic stimulus, and that the generation of fertilization/SF-initiated oscillations, which is mediated thorough the IP\(_3\)R, leads to cell death of aged eggs. Interestingly, it was recently shown that cadium exposure induces cell death in human lymphoma cells by causing a [Ca\(^{2+}\)], increase and up-regulation of the type I IP\(_3\)R [77].

The precise molecular pathway by which Ca\(^{2+}\) release may induce egg fragmentation is not known, although several proteases and nucleases involved in apoptosis are Ca\(^{2+}\)-dependent [33, 78–80]. For example, exposure of lymphoid cells to glucocorticoids or addition of Ca\(^{2+}\) ionophores to other cell types induces apoptosis by triggering a Ca\(^{2+}\) influx [81]. Importantly, emptying of the cell’s Ca\(^{2+}\) stores appears to be an equally effective stimulus to induce cell death [82]. For instance, induction of Ca\(^{2+}\) release and emptying of the Ca\(^{2+}\) stores by exposure to thapsigargin, a sequesterlactone that specifically inhibits the Ca\(^{2+}\)-ATPase pump in the ER (SERCA), induced apoptosis [83] even when the increase in [Ca\(^{2+}\)], was blocked by the addition of a Ca\(^{2+}\) chelator [84]. Our findings that the [Ca\(^{2+}\)], oscillations initiated in aged eggs by injection of agonists known to stimulate the IP\(_3\)R are of smaller amplitude and cease abruptly suggest that the ER Ca\(^{2+}\) concentration and, possibly, the ER function may be severely compromised in these eggs, as suggested by others [52]. An altered ER lumen has been shown to stimulate the release
of cytochrome c from the mitochondria, as previously demonstrated in calreticulin-expressing HeLa cell lines [85], and this may be the cause of cell death in aged mouse eggs. The molecular deficiency responsible for the depletion of Ca\(^{2+}\) stores in aged mouse eggs is not known, although the decreased levels of Bcl-2 may negatively affect Ca\(^{2+}\) homeostasis in these cells. Bcl-2 has been shown to affect Ca\(^{2+}\)-dependent events at multiple sites, including regulation of the ER Ca\(^{2+}\) contents and function of the SERCA pumps [86–88]. Alternatively, the levels and/or function of SERCA proteins or of molecules involved in their regulation may be affected independently of the Bcl-2 concentrations in aged eggs. Nevertheless, the demonstration that uptake of Ca\(^{2+}\) is delayed in aged eggs confirms that Ca\(^{2+}\) homeostasis is severely compromised in these eggs [89].

In conclusion, our results show that in vitro-aged CD1 mouse eggs respond to [Ca\(^{2+}\)]\(_i\) oscillations by initiating programmed cell death rather than by normal activation, and that they are unable to sustain long-lasting [Ca\(^{2+}\)]\(_i\) oscillations. Therefore, we suggest that one or several critical molecules involved in the regulation of Ca\(^{2+}\) homeostasis, and in establishing the equilibrium between anti- and proapoptotic proteins, is lost/inactivated during postovulatory egg aging. Elucidating the sequence of events responsible for the loss/inactivation of these proteins may prolong the fertilizable life span of mouse eggs and result in higher rates of in vitro development of mouse embryos.

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