Akt expression in mouse oocytes matured in vivo and in vitro

Sandra Cecconi a,*, Gianna Rossi a, Adriana Santilli a, Leonardo Di Stefano b, Yumi Hoshino c, Eimei Sato c, Maria Grazia Palmerini a, Guido Macchiarelli a

Abstract To improve developmental competence of in vitro matured oocytes, culture medium can be supplemented with hypoxanthine (Hx) and FSH or epidermal growth factor (EGF) to trigger the activation of essential signalling pathways regulating meiotic resumption and progression. Since the serine/threonine kinase, Akt, contributes to the regulation of the meiotic cell cycle, this study analysed its expression level and localization at the meiotic spindle in oocytes matured in vivo or in vitro in the presence of Hx-FSH or Hx-EGF. Independently of culture conditions adopted, Akt mRNA concentration did not vary from germinal vesicle to metaphase I (MI), while at MI a significant decrease in Akt1 mRNA concentration was recorded in oocytes matured in vivo and in those stimulated by Hx-EGF (P < 0.05). Phoshorylated Akt protein content was similar in the different groups of MI oocytes, but it decreased at MII in oocytes matured either in vivo or in vitro with Hx-EGF. Ser-473-phosphorylated Akt was localized uniformly to the meiotic spindle in more than 90% of oocytes. These results indicate that, in mouse oocytes, Akt expression is differentially regulated during in vivo and in vitro maturation and suggest that EGF could be a positive modulator, even stronger than FSH, of oocyte meiotic maturation.

Introduction

To obtain developmentally competent oocytes from in vitro maturation (IVM) procedures, it is fundamental to identify culture systems capable of reproducing during culture the physiological processes occurring in vivo, as oocyte maturation and cumulus expansion. In vivo, the processes of ovulation, luteinization and oocyte maturation are stimulated by...
the LH surge, that triggers an elaborate signalling cascade leading to resumption of meiosis (Conti et al., 2006; Richards, 1994; Tsafiri and Dekel, 1994) through activation of specific protein kinases (Farin et al., 2007; McGinnis et al., 2009; Su et al., 2002). In vitro, oocytes can resume and complete meiotic maturation either spontaneously or under the stimulation of specific hormones. In the first case, meiosis is triggered as soon as oocytes, either surrounded by cumulus cells or denuded, are removed from follicular microenvironment (Tsafiri and Pomerantz, 1986). In the second, the maturation of cumulus-enclosed oocytes can be induced by adding FSH (Cecconi et al., 2008; Coticchio et al., 2004; Downs et al., 1988; Eppig and Downs, 1987; Fan et al., 2003; Rodriguez and Farin, 2004; Rossi et al., 2006; Su et al., 2002), or EGF (Coticchio et al., 2004; De La Fuente et al., 1999; Downs et al., 1988; Lonergan et al., 1996; Merriman et al., 1998; Rossi et al., 2006), as well as EGF-like growth factors (Downs and Chen, 2008; Park et al., 2004). These hormones can be added either alone (Fan and Sun, 2004), together with cAMP-elevating agents such as hydropoxanthine (Hx; Byskov et al., 1997; Coticchio et al., 2004) or with phosphodiesterase inhibitors (Coticchio et al., 2004; Gilchrist and Thompson, 2007; Nogueira et al., 2006; Sirard and Coenen, 2006; Thomas et al., 2004). The beneficial effect of hormonal supplementation in vitro is shown by the significant improvement of oocyte developmental potential (Farin et al., 2007; Sirard et al., 2007;) and by the activation of the Mos/MAP-erk kinase/mitogen-activated protein kinase and EGF-dependent pathways (Cecconi et al., 2008; Conti et al., 2006; Downs and Chen, 2008; Fan et al., 2003; Farin et al., 2007; Panigone et al., 2008; Richards, 2001; Su et al., 2002). Recently, several studies have pointed out to the role played by the oncogenic serine/threonine kinase, Akt or protein kinase B, in the regulation of meiotic resumption in mammalian oocytes. Akt protein is phosphorylated at two sites, a threonine residue (Thr-308) in the catalytic domain and a serine residue (Ser-473) at the C-terminus (Alessi et al., 1996) and it transduces intracellular signals by phosphorylating target proteins (Yang et al., 2004). In the mammalian ovary, FSH-dependent activation of Akt in granulosa cells (Gonzalez-Robayna et al., 2000) contributes to the regulation of apoptotic/survival signalling (Fan et al., 2008; Johnson et al., 2001; Ryan et al., 2007) and to cell differentiation (Wayne et al., 2007). Also, in cumulus cells, active kinase mediates survival (Takahashi et al., 2006) and the synthesis/retention of hyaluronic acid (Nemcova et al., 2007). Although the exact mechanism by which full Akt activation is achieved needs to be definitively clarified (Yang et al., 2004), its involvement in the process of oocyte meiotic resumption has been demonstrated in vivo and in vitro (Han et al., 2006; Hoshino and Sato, 2008; Hoshino et al., 2004; Kalous et al., 2006; Panigone et al., 2008; Shimada and Terada, 2001; Shimada et al., 2003; Tomek and Smiljakovic, 2005).

Previous work has demonstrated that oocytes matured in vitro up to metaphase II (MII) in the presence of FSH showed significant differences in spindle morphology with respect to oocytes matured in vivo or in the presence of EGF (Rossi et al., 2006). Since these results suggest that selection of culture conditions can affect specific processes occurring during meiotic maturation, the present study compared Akt expression profile between in vivo and in vitro matured oocytes, either cultured in the presence of FSH or EGF. To this end, we determined mRNA content for the three highly homologous members known as Akt1, Akt2 and Akt3 (Song et al., 2005), phosphorylated and total protein content as well as the localization of the kinase at the MII spindle.

**Materials and methods**

**Chemicals**

All the chemicals were of the purest analytical grade and were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated.

**Collection of oocytes**

Immature (23- to 25-day-old) female Swiss CD1 mice (Harlan, Udine, Italy; n = 250) were injected i.p. with PMSG (5 IU) and cumulus cell-enclosed oocytes were released by puncturing the large pre-ovulatory follicles 48 h later. In order to maintain the oocytes arrested at the germinal vesicle (GV) stage, cumulus-enclosed oocytes were collected in HEPES-buffered Eagle’s minimal essential medium (Invitrogen, Paisley, UK) supplemented with 0.23 mmol/l pyruvic acid, 2 mmol/l l-glutamine, 0.3% bovine serum albumin and 4 mmol/l hyoxanthine (Hx). Cumulus cells were removed by gentle pipetting and GV-arrested oocytes were immediately stored for reverse transcriptase polymerase chain reaction (RT-PCR; 20 oocytes/sample) and immunoblotting (100 oocytes/sample). For IVM experiments, cumulus-enclosed oocytes were cultured as previously described by Coticchio et al. (2004) and Rossi et al. (2006). Briefly, cumulus-enclosed oocytes were cultured in alpha minimal essential medium (Invitrogen, Paisley, UK) supplemented with 0.23 mmol/l pyruvic acid, 2 mmol/l l-glutamine, 0.3% bovine serum albumin, 4 mmol/l Hx and, depending on the maturation model, with 100 mIU/ml FSH (Dr. Parlow, National Hormone and Pituitary Program, USA) or 10 ng/ml EGF, at 37°C, 5% CO2 in air and saturated humidity. MI (450–500 oocytes for each experimental condition) and MII oocytes (450–500 oocytes for each experimental condition) were recovered after about 9 h and 18 h of culture, respectively; expanded cumulus cells were removed by a brief treatment with hyaluronidase (1 mg/ml). Samples were stored refrigerated at −80°C for further analyses. In vivo matured MI and MII oocytes were recovered from PMSG-primed mice by puncturing follicles about 9 h or from the ampullae 14–16 h after human chorionic gonadotrophin (HCG) injection, respectively. Expanded cumulus cells were removed by brief hyaluronidase treatment. Animal procedures were approved by the Animal Care Committee of the University of L’Aquila and compliant with the "Guide for the care and use of laboratory animals".

**RT-PCR**

Total RNA was extracted and DNA synthesis was performed using Cells-to-cDNA™ II kit (Ambion, Austin, USA) as described by Hoshino and Sato (2008). RT-PCR was performed using Red Hot polymerase (ABgene). For Akt1, the sense
Akt expression and oocyte maturation

(5-GCCAAATCCACGAAAGG-3\') and antisense (5'-CTGAACCGCATGGACACAG-3') primers generated a 182-base pair (bp) fragment. For Akt2, the sense (5'-CGCCCTGAGCTCACCAAG-3') and antisense (5'-CGGGCTTCTCTTATACC-3') primers generated a 176-bp fragment. For Akt3, the sense (5'-CCCACACTGCACCATGG-A-3') and antisense (5'-GAGGGAGATGCTCAGTGTTG-3') primers generated a 226-bp fragment. The amplification conditions were here reported: 94°C for 10 min; 35 cycles of 30 s each of denaturation at 94°C, annealing (60°C for Akt2, 57.3°C for Akt3 and 58°C for Akt1 and G3PDH), extension at 72°C, and a final extension for 5 min at 72°C. As regular PCR is not a true quantitative method, 35 cycles were used because preliminary results demonstrated that PCR at 35 cycles gave a linear dose—response result.

Immunoblotting

Oocytes were lysed in sample buffer containing protease inhibitors (2 mmol/l phenylmethyl sulphonyl fluoride, 10 μg/ml aprotinin, 0.1 mmol/l sodium pyrophosphate, 10 mmol/l sodium fluoride and 1 mmol/l sodium orthovanadate). Lysates were separated by electrophoresis and transferred to nitrocellulose membranes (Hybond C Extra, Amersham, Little Chalfont, UK). Membranes were incubated with antibodies (1:1000) against total Akt and Ser-473-phosphorylated Akt (pAkt) (No. 9272 and 9271, respectively; Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. Membranes were then washed three times with 0.1% Tween 20 and 1X Tris-buffered saline, 0.1% Tween 20 (TBS-T) for 30 min at room temperature, and incubated for 2 h at room temperature. Then, the horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) (1:1000) was used as secondary antibody and peroxidase activity was detected using chemiluminescence reagent (ECL, Pierce, Rockfort, USA).

Immunostaining of oocytes

To detect Akt distribution to microtubules of the meiotic spindle, oocytes (50–60 for each experimental condition) were labelled according to the protocol described by Rossi et al. (2006). Briefly, oocytes were fixed and incubated with anti-α-tubulin (1:100) to detect microtubules and with anti-pAkt (1:100) (No. 9277; Cell Signaling Technology), followed by Texas Red (1:100) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and anti-rabbit IgG-fluorescein isothiocyanate (1:50), respectively. All antibodies were added for 1 h at 37°C. Chromosomes were labelled with Hoechst 33342 (1 μg/ml). Labelled oocytes were analysed using a fluorescence microscope (40x objective; Axiosplan 2; Zeiss) with digital images collected with Leica DFC350 FX camera interfaced with IMS500 Leica software.

Statistical analysis

All experiments were performed at least three times and data were expressed as the mean ± SEM. Differences between groups were analysed for statistical significance using ANOVA with Tukey–Kramer multiple comparison test as a post test. Results were considered significantly different if \( P < 0.05 \).

Results

Akt mRNA expression

Akt1, 2 and 3 mRNA concentrations were determined in oocytes during in vivo and in vitro (IVM) maturation, from GV to MII. To this end, IVM was conducted in the presence of Hx-FSH (100 μIU/ml) or Hx-EGF (10 ng/ml). According to previous results (Coticchio et al., 2004; Rossi et al., 2006), more than 95% of cultured as well in vivo matured oocytes resumed meiosis and reached the MII stage. As shown in Figure 2A, oocytes at GV stage expressed mRNA for the three Akt isoforms. When collected at MI, oocytes matured in vivo and in vitro expressed mRNA at similar levels and no significant difference was recorded between FSH and EGF treatments in vitro (Figure 2A). By contrast, a significant decrease of Akt1 mRNA was evident in MI oocytes matured in vivo and in those cultured in the presence of Hx-EGF in comparison with FSH (Figure 2B; \( P < 0.05 \)). Akt2 and 3 were expressed at comparable levels in all the experimental groups.

Detection of Ser-473-phosphorylated and total Akt

The signals for pAkt and total Akt were determined by immunoblotting in oocytes at GV stage and in those matured up to MI and MII, either in vivo or in vitro. The pAkt signal was comparable between GV and MI-arrested oocytes and was not influenced by the maturation protocols utilized (Figure 2A). At MI stage, pAkt content was lower in oocytes matured in vivo with respect to those matured in vitro (\( P < 0.05 \); Figure 2). At this meiotic stage, oocytes undergoing IVM in the presence of Hx-EGF displayed pAkt content lower than that of FSH-stimulated oocytes (\( P < 0.05 \); Figure 2B). Total Akt content was equally abundant at any time and in all the classes examined.

Localization of Ser-473-phosphorylated Akt by immunofluorescence analysis

The analysis of pAkt localization in the MI (not shown) and MI meiotic spindle revealed that the kinase was present along microtubules in more than 90% of analysed oocytes, independently of culture conditions and hormonal supplementation (Figure 3).

Discussion

The present study has been designed to determine and compare Akt expression level in mouse oocytes undergoing in vivo and in vitro maturation. The results showed a significant decrease of Akt1 mRNA concentration in MI-arrested oocytes matured either in vivo or in vitro in the presence of Hx-EGF in comparison with Hx-FSH. Hoshino and Sato (2008) reported that oocytes, collected at GV and at specific stages (prometaphase I, MI, MII) during FSH-stimulated IVM, expressed Akt1 and 3 but not Akt2 mRNA. The fact that all the three isoforms of the kinase are expressed could be attributed to the different strains of mice utilized in the two studies (CD1 in this study versus ICR in Hoshino and Sato’s...
The presence of Akt2 mRNA also suggests that this isoform should have a function during meiosis. A possibility is that, in mouse oocytes, Akt2 plays a redundant role, thereby participating in the regulation of cytoskeleton rear-

Figure 1  Expression pattern of Akt isoforms in oocytes matured in vivo and in vitro in the presence of hypoxanthine-FSH or hypoxanthine-epidermal growth factor (EGF). (A) Germinal vesicle (GV) and metaphase I (MI), (B) metaphase II (MII). Representative pictures of reverse-transcriptase polymerase chain reaction experiments are shown. Statistical analysis was made on three different experiments. Data are represented as mean ± SEM of the relative ratio percentage between each Akt isoform and G3PDH. Significant differences (P < 0.05) are represented with different letters (a–c). bp, base pairs.

Figure 2  Representative Western blot of Ser-473-phosphorylated Akt (pAkt) content in (A) metaphase I (MI) and (B) MII oocytes (100 oocytes/lane), matured in vivo and in vitro, either with hypoxanthine-FSH or hypoxanthine-epidermal growth factor (EGF). The ratio of pAkt to total Akt was determined for each condition. Results are expressed as the mean ± SEM of three separate experiments. Significant differences (P < 0.05) are represented with different letters (a–c).
rangements at meiosis as proposed for Akt1 and 3 (Hoshino and Sato, 2008). Alternatively, Akt2 could participate in the regulation of the insulin signal transduction pathway in collaboration with Akt1 (Acevedo et al., 2007). This last function has been recently confirmed in other cell systems (Garofalo et al., 2003; Illario et al., 2009; Wang et al., 2008).

Several reports demonstrate that Akt phosphorylation, triggered by its activating kinases (Song et al., 2005), is necessary to stimulate resumption of meiosis in vivo and in vitro (Conti et al., 2006; Han et al., 2006; Kalous et al., 2006; Newhall et al., 2006). Under the in vitro conditions, MI oocytes show comparable concentrations of pAkt and a similar localization of the kinase to microtubules of the first meiotic spindle, thus confirming previous results (Hoshino et al., 2004; Kalous et al., 2006). Indeed, it has been demonstrated that Akt needs to be expressed at MI because its inhibition induces failure of MI itself (Hoshino and Sato, 2008). However, maturation conditions have different effects on Akt protein activity at MI stage. This conclusion is supported by the observation that pAkt content in oocytes matured in vivo is significantly lower than in vitro. In addition, EGF supplementation caused a decrease of active kinase content with respect to FSH, even if not at levels comparable to those in vivo. These differences are not related to total protein content. The fact that pAkt level is higher in FSH-stimulated oocytes with respect to the other classes analysed could be due to prolonged gonadotrophin-independent stimulation of the molecular pathway(s) controlling kinase phosphorylation (Wayne et al., 2007) and/or to the stimulation of additional pathways, such as the phosphoinositide 3-kinase-independent pathway (Cohen and Rokhiin, 2009). Another possibility is that FSH could interfere with the mechanisms controlling the activity of phosphatases such as phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (Carracedo and Pandolfi, 2008; Fan et al., 2008) and/or those belonging to the PH domain leucine-rich repeat protein phosphatase (PHLPP) family, that specifically dephosphorylate distinct Akt isoforms (Brognard et al., 2007).

The differences in Akt expression level recorded between in vivo and in vitro maturation confirm that culture per se affects the synergy of the molecular mechanisms controlling nuclear and cytoplasmic maturation. Indeed, mammalian oocytes undergoing IVM have a lower developmental competence with respect to their in vivo matured counterparts, even in the presence of a high nuclear maturation rate (Coticchio et al., 2004; Downs and Mastropolo, 1997; Farin et al., 2007; Rossi et al., 2006). More recently, it has been demonstrated that conditions of meiotic maturation influence MII spindle positioning and shape as well as distribution of specific microtubule-associated proteins (Rossi et al., 2006; Sanfins et al., 2003). This is not the case with Akt, as its distribution along MII spindle microtubules is identical under both in vivo and in vitro conditions. This observation strongly supports the idea that Akt contributes to spindle formation/stabilization, as also suggested by others (Hoshino and Sato, 2008; Hoshino et al., 2004; Kalous et al., 2006).

The determination of Akt expression level could contribute to explain the reduced developmental potential of embryos derived from IVM-IVF procedures. In fact, Hoshino and Sato (2008) reported that when FSH-stimulated oocytes were fertilized in vitro, both Akt activities (Thr-308 and Ser-473) were extruded with the polar body 2 from the ooplasm and derived embryos expressed total Akt protein and mRNA at very low or undetectable levels, at least until the blastocyst stage. This is in contrast with the finding that pAkt has a significant effect on normal blastocyst physiology (Riley et al., 2005) and plays a key role in the first round of mitosis (Feng et al., 2007). Also, in Drosophila melanogaster embryos, reduced concentrations of Akt cause altered mitotic spindle orientation and centrosome separation (Buttrick et al., 2008). Work is in progress in the study laboratory to verify Akt expression level in embryos derived from EGF-matured oocytes and kinase localization after fertilization of in vivo matured oocytes.

Altogether, these results indicate that Akt expression, in terms of Akt1 mRNA and pAkt content, undergoes a physiological decrease in oocytes matured in vivo, probably with the aim of triggering/collaborating with the degeneration of unfertilized eggs. In vitro, only EGF seems to be able to mimic the trend recorded in vivo. This observation supports the hypothesis that EGF plays a key role in the complex regulation of meiotic maturation and that supplementation of culture media with this growth factor (Downs, 1989; Goud et al., 1998), as well as EGF-like peptides (Downs and Chen, 2008), could contribute to increase IVM efficiency, in terms of steroidogenesis, oocyte maturation and cumulus expansion. In vivo, EGF-like growth factors are potent stimulators.
of oocyte maturation and cumulus expansion and perturba-
tion of this EGF network dramatically impairs ovulation (Hsieh et al., 2009).

Acknowledgements

The authors thank Professor Rita Canipari (University 'La Sapienza' Rome), for critical reading of the manuscript. This work was supported by MIUR (ex 60%, PRIN project) to S.C. This paper is dedicated to the victims of the earthquake of L'Aquila in April 2009.

References


Declaration: The authors report no financial or commercial conflicts of interest.

Received 24 April 2009; refereed 21 May 2009; accepted 7 October 2009.