Activation of Akt (protein kinase B) stimulates metaphase I to metaphase II transition in bovine oocytes

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Abstract

In somatic cells, the serine/threonine kinase Akt (or protein kinase B) was shown to contribute to processes linked to cellular growth, cell survival and cell cycle regulation. In contrast to these findings, the function of Akt during the meiosis of mammalian oocytes remains to be investigated. We analysed the phosphorylation pattern and the activity of Akt during meiotic maturation (transition from prophase I to metaphase II) of bovine oocytes. The oocytes were matured in vitro (IVM) for 0, 10 and 24 h to reach the germinal vesicle (GV), metaphase I (M I) and metaphase II (M II) stages respectively. The abundance and phosphorylation pattern of Akt was revealed by Western blotting using total Akt or phospho-Akt-specific antibodies. The activity of this particular kinase was determined by an in vitro kinase assay. Furthermore, functional properties were analysed by cultivating oocytes in the presence of the Akt inhibitor SH6. The results showed that the overall abundance of Akt did not change significantly during IVM. On the other hand, Akt became phosphorylated at Thr 308 and Ser 473, reaching its maximum at the M I phase. In the GV and M II stages, only low basal phosphorylation levels were observed on both sides. This phosphorylation profile corresponded strictly to the activity of the kinase. The cultivation of oocytes in the presence of the phosphatidylinositol analogue SH6 for 24 h showed that, with higher concentrations, up to 65% of the oocytes were arrested in the M I stage. This result indicated that Akt is involved in the M I/M II transition during the meiotic maturation of bovine oocytes. The physiological aspects of the Akt function will be discussed.

Introduction

Mammalian oocytes are arrested in their follicles at the diplotene (dictyate) stage of the first meiotic prophase, the so-called germinal vesicle (GV) stage. When fully grown oocytes are released from their follicles, they can resume meiosis spontaneously if cultured in a suitable culture medium. This process (transition from the GV stage to metaphase II (M II)) is referred to as meiotic maturation of oocytes. During meiotic maturation, characteristic morphological changes occur. These include condensation of the chromatin, germinal vesicle breakdown (GVBD) and formation of the meiotic spindle.

It is generally known that the processes of cellular proliferation, differentiation or maturation are triggered by signalling cascades involving the action of protein kinases which specifically phosphorylate distinct targets. In this context, phosphorylation activates or deactivates proteins which are responsible for the progression of the cell cycle and, especially in oocytes, for the resumption of meiotic maturation from the arrested GV stage (Chian et al. 2003, Kishimoto 2003).

Several years ago, a factor was postulated to affect the onset of the meiotic maturation of oocytes. This factor was named maturation-promoting factor (MPF). With the discovery of cyclins (Evans et al. 1983, Gerhard et al. 1984, for review see Doreé & Hunt 2002) it became evident that the MPF is a cyclin-dependent kinase (CDK) and consists of a catalytic subunit Cdc2 (cell division cycle kinase) and a regulatory subunit cyclin B (Jessus & Beach 1992, Claude et al. 2002, Jones 2004). The MPF is thought to be a universal G2/M-phase regulator for both meiosis and mitosis (Ye et al. 2003) in all eukaryotic cells (the GV stage of meiosis is usually assumed to be equivalent to the G2/M border in mitosis (Moor et al. 1990)). An evaluation of the activation profiles of distinct kinases during the transition from the GV to the M II stage in oocytes revealed that several kinases are present during this period, but not all of them are in an activated state. These kinases include, besides the MPF, the proto-oncogene c-Mos (which is an MAP kinase (MAPK) kinase), MAPK-extracellular activating kinase (MEK), which is a substrate of c-Mos and phosphorylates and activates MAPK) and MAPK itself (Nebreda &
Hunt 1993, Shibuya & Ruderman 1993). The activity of Cdc2 kinase, which is said to initiate events leading to nuclear progression, is high during GVBD and M II (Tian et al. 2002), low after the M I phase, and the kinase is inactive during the GV stage. MAPK, on the other hand, which was shown to be involved in translational regulation in bovine oocytes by its stimulation of phosphorylation of the initiation factor eIF4E (eucaryotic initiation factor 4E; Tomek et al. 2002a) is inactive only in the GV stage, after which it becomes phosphorylated; its activity steadily increases, reaching its maximum in M II. The upstream kinase MEK also shows an activity pattern corresponding to that of MAPK (Liu & Yang 1999).

It was previously shown that another kinase named Akt (protein kinase B; PKB) is also abundant in mouse and bovine oocytes (Hoshino et al. 2004, Vigneron et al. 2004a, 2004b). The results reported indicate that Akt is likely to participate in the maturation of oocytes, probably independent of MPF activation.

In somatic cells, Akt was shown to contribute to networks integrating growth, survival and proliferation of cells (Massague 2004). In this context, Akt is involved, for instance, in CDK activation, inhibition of caspase activation and the stimulation of translation. In contrast to these observations taken from somatic cells, much less is known about the molecular function of Akt during oocyte maturation. Recently published results obtained from starfish oocytes indicate that Akt stimulates the meiotic G2/M-phase transition by down-regulating the activity of Myt 1 (Okumura et al. 2002). In this way, the inhibitory phosphorylation of the MPF is impaired and this factor can be activated by cdc25 phosphatase. These observations identify Akt as an M-phase initiator, but do not necessarily reflect the situation in mammalian oocytes.

The activation of Akt depends on the phosphorylation at Thr 308 and Ser 473. It was shown previously that the Thr 308 residue is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and that membrane localization is a necessary criterion for Ser 473 phosphorylation (Scheid et al. 2002, Troussard et al. 2003). New results have shown that in Drosophila and human somatic cells the target of rapamycin kinase and its associated protein rictor are necessary for the phosphorylation at Ser 473 (Sarbassov et al. 2005). Furthermore, results obtained from lung cancer cell lines indicate that phosphatidylinositol analogues can prevent Ser 473 phosphorylation and specifically impair Akt activation. In this context it is of interest that SH6 was reported to block Akt activation without affecting either the upstream kinase PDK1 or other kinases downstream of Ras, such as MAPK (Kozikowski et al. 2003).

With regard to the facts described above, we investigated the phosphorylation pattern of Akt during in vitro maturation (IVM) of bovine oocytes. Furthermore, we elucidated the activity of this kinase, using the novel phosphatidylinositol analogue SH6 to investigate the influence of Akt inhibition on the meiotic maturation of bovine oocytes. This approach can contribute to the acquisition of more detailed information on molecular events during the meiotic maturation of oocytes.

Materials and Methods

Materials
All chemicals were from Sigma (Taufenkirchen, Germany) unless otherwise indicated.

Source and collection of oocytes
Bovine ovaries, collected with aseptic precautions from a local slaughter house, were transported to the laboratory in 0.9% physiological saline (at 20–25°C) in a Thermos container within 2 h of slaughtering and were immediately washed after arrival in freshly made phosphate-buffered saline (PBS). To collect the cumulus cell–oocyte complexes (COCs), the surfaces of the ovaries were slit open with a blade, according to the ovarian-slicing method (Eckert & Niemann 1995) and washed with PBS solution (100 ml PBS per ovary). The flushing fluid so collected was sieved through a micro-pore sieve, with a pore size of 50 μm. The COCs were rinsed with PBS and collected in a Petri dish. The COCs were then picked up, sorted out and only COCs with compact layers of cumulus cells and evenly granulated ooplasm were selected for further investigations.

Oocyte maturation and treatment of oocytes with the phosphatidylinositol analogue SH6

The COCs collected were matured according to the method of Torner et al. (2001) in TCM 199, supplemented with 3 mg/ml bovine serum albumin (BSA), covered with a layer of mineral oil, at 38.5°C, 5% CO2 and saturated humidity for different time intervals (0, 10 and 24 h). The IVM was performed in four-well dishes with 50 COCs in 500 μl medium. To assess the influence of the phosphatidylinositol analogue SH6 (α,β,γ,δ-tetrahydroxy-sphingosine-1-phosphate; Alexis, Grünberg, Germany), the oocytes were matured in a maturation medium containing SH6 (final concentration of 10, 25, 50 or 75 μM, prepared from a 50 mM stock solution) dissolved in H2O. H2O without SH6 was added to the control groups. After maturation, cumulus cells were removed by treatment with hyaluronidase (2.5 mg/ml) for 2 min and repeated pipetting. Then the oocytes were evaluated for the stage of meiosis by staining with aceto-orcein, or used for biochemical analysis. The analysis of samples was carried out in well-defined time periods (Torner et al. 2001) of 0, 10 and 24 h of IVM (GV, M I and M II stages) respectively.

Morphological evaluation

In order to establish that the oocytes collected at the defined time points of maturation were actually at the...
aforementioned stages of maturation, some of them were randomly picked and fixed with acetic alcohol (acetic acid:ethanol = 1:3) and stained with the aceto-orcein dye to enable their classification into the stages of GV, GVBD, M I, anaphase I, telophase I and M II with extruded polar body (M II).

**One-dimensional (1D) gel electrophoresis (SDS-PAGE)**

To separate the proteins according to their apparent molecular masses, SDS-PAGE was performed in 10% gels, according to Laemmli (1970). The ratio of acrylamide to bisacrylamide was 30:0.8 to analyse Akt or 29.7:0.3 to analyse MAPK.

After maturation, the oocytes were washed five times in protein-free PBS, lysed in 5 µl double-concentrated SDS sample buffer, kept at 95°C for 2 min and immediately thereafter subjected to electrophoresis. Extracts of 50 oocytes per time point were analysed on the gels. Electrophoresis was performed from anode to cathode at 65 mA per gel, until bromophenol blue reached the bottom of the gel. For all 1D SDS-PAGE, the Mighty Small SE 250 system (Hoefer, Amer- sham Biosciences, Freiburg, Germany) was used.

**Western blotting and immunodetection**

The proteins separated by 1D gel electrophoresis (from 50 oocytes per time point) were transferred from the gels onto PVDF membranes (Millipore, Schwalbach, Germany) according to the method of Towbin & Staehlin (1979) using a semidyry electroblotting apparatus (PEQLab, Erlangen, Germany). The transfer was effected at 1 mA/cm² for 1 h. After maturation, the oocytes were washed five times in protein-free PBS, lysed in 5 µl double-concentrated SDS sample buffer, kept at 95°C for 2 min and immediately thereafter subjected to electrophoresis. Extracts of 50 oocytes per time point were analysed on the gels. Electrophoresis was performed from anode to cathode at 65 mA per gel, until bromophenol blue reached the bottom of the gel. For all 1D SDS-PAGE, the Mighty Small SE 250 system (Hoefer, Ame- sham Biosciences, Freiburg, Germany) was used.

**Histone H 1 kinase assay**

The activity of histone H 1 kinase reflecting maturation-promoting factor (Cdc2) kinase was measured in oocytes via the capacity to phosphorylate the external substrate-histone H 1 protein. At each time interval, ten oocytes per sample were collected. The histone H 1 phosphorylation was measured as described by Motlik et al. (1996). Briefly, 5 µl buffer A (40 mM MOPS, pH 7.2, 20 mM para-nitrophenyl phosphate, 40 mM β-glycerophosphate, 10 mM EGTA, 0.2 mM EDTA, 2 mM dithiothreitol, 0.2 mM Na₂VO₃, 2 mM benzamidine, 40 µg/ml leupeptin and 40 µg/ml aprotinin) was added to each sample. The tubes were briefly vortexed and centrifuged at 10 000 g for 15 s. The kinase reaction was initiated by the addition of 5 µl buffer B (100 mM MOPS, pH 7.2, 20 mM para-nitrophenylphosphate, 40 mM β-glycerophosphate, 20 mM MgCl₂, 10 mM EGTA, 0.2 mM EDTA, 5 µM cAMP-dependent protein kinase inhibitor, 2 mM benzamidine, 40 µg/ml leupeptin, 40 µg/ml...
aprotinin, 600 μM ATP and 2 mg histone H 1/ml) with 500 μCi/ml [13P]-γ-ATP (10 mCi/ml; Amersham Pharmacia Biotech). The reaction was carried out for exactly 30 min at 30°C and stopped instantaneously by the addition of 10 μl double-concentrated SDS-PAGE sample buffer and boiling for 3 min. After electrophoresis on 15% SDS-PAGE gel (Laemmli 1970), the gels were stained with Coomassie Blue R250, destained overnight, dried and autoradiographed.

**Statistical analysis**

In all cases, the data from three independent experiments were evaluated. The data of the Western blots, morphological analysis of oocytes during IVM and in response to SH6 treatment are expressed as means ± s.d. of triplicate measurements. All data were analysed by one-way ANOVA (Sigma Stat, version 1.0; Jandel Corporation, San Rafael, CA, USA). If the effects of the main treatment were significant, a t-test was performed (P < 0.05).

**Results**

**Analysis of Akt abundance, phosphorylation and activity during IVM**

In Fig. 1, a typical profile of the evaluation of the chromatin configuration during IVM of bovine oocytes is presented. It can be seen that at 0 h of IVM approximately 90% of the oocytes persisted in the GV stage. After 10 or 24 h of IVM the majority of oocytes had reached M I and M II respectively. These observations are in accordance with previous analysis revealed that concentrations lower than 20 μM had no pronounced effect whereas concentrations higher than 75 μM were cytotoxic (authors’ unpublished results). For this reason these concentrations were not used in further experiments. The results obtained by Western blotting are presented in Fig. 4. It is apparent that SH6 had no significant effect on the abundance of Akt in comparison with the loading control actin (Fig. 4A, B and E). On the contrary, concentrations of 25, 50 and 75 μM SH6 significantly reduced the phosphorylation on both sides, Thr 308 and Ser 473 during IVM, an enhanced phosphorylation of GSK3 could be observed as compared with the GV and M II stages.

Taken together, the results indicated that the protein kinase Akt becomes activated around the GVBD and M I stages and that this activation corresponds with the phosphorylation of this particular kinase. In the M II stage, only basal activity comparable with that found in the GV stage could be detected. It should be mentioned in this context that the antibody used did not discriminate between different isoforms of Akt. Therefore the activation profile reflects the sum of the activity of Akt 1, 2 and 3 potentially present in the oocytes.

**Influence of the phosphatidylinositol analogue SH6 on Akt phosphorylation at Thr 308 and Ser 473 during IVM**

As described before, Akt phosphorylation and activity was highest in M I-stage oocytes (10 h of IVM). To analyse the influence of SH6 on the maturational phosphorylation of this kinase, we cultivated oocytes for 10 h in the presence of different concentrations of SH6 (20–75 μM). Our previous analysis revealed that concentrations lower than 20 μM had no pronounced effect whereas concentrations higher than 75 μM were cytotoxic (authors’ unpublished results). For this reason these concentrations were not used in further experiments. The results obtained by Western blotting are presented in Fig. 4. It is apparent that SH6 had no significant effect on the abundance of Akt in comparison with the loading control actin (Fig. 4A, B and E). On the contrary, concentrations of 25, 50 and 75 μM SH6 significantly reduced the phosphorylation on both sides, Thr 308 and Ser 473 (Fig. 4C and D, lanes 2–4 and E), though this process was reversible. This reversibility was
demonstrated by the additional cultivation of the oocytes for 10 h without the inhibitor (Fig. 4C and D, lane 5).

**Morphological evaluation of SH6-treated oocytes**

An experiment was designed in which oocytes were maturated for 24 h in the presence of SH6. Under control conditions, oocytes reach the M II phase in this time period; thus with this experiment, morphological alterations which were caused by impaired Akt activation could be analysed. The results from three independent experiments are presented in Fig. 5. Besides the controls (in lane 1, 24 IVM without SH6; in lane 6, 24 h IVM with 50 μM SH6 followed by an additional 24 h of cultivation without the inhibitor), different concentrations of SH6 were applied (10, 25, 50 and 75 μM in lanes 2–5). Fifty oocytes were evaluated in each group per experiment. The results showed that under control conditions 75–80% of the oocytes reached the M II stage after 24 h of IVM. The lowest concentration of SH6 (10 μM) had no obvious effect on the progression of the maturation process. On the other hand, increasing concentrations of SH6 also increased the number of oocytes which persisted in M I even after 24 h of maturation (up to 60% compared with 20% in the control group). Thus, the results showed that the inhibition observed is far from being complete (see Discussion). The reason for this effect is not clear, but even longer cultivations (up to 30 h) did not alter the values observed (data not shown). Obviously the concentration used had no cytotoxic effect nor did it induce apoptosis, since the inhibition was for the most part reversible (Fig. 3, lane 6).

**Evaluation of the side effects of SH6 on MAPK and MPF**

In order to exclude unspecific side effects of SH6 on MAPK and MPF activation during IVM, oocytes were matured for 10 or 24 h in the presence of different concentrations of this particular inhibitor. In Fig. 6A the phosphorylation of the MAPK ERK 2 analysed by band shift...
assay on Western blots is depicted. It can be seen that ERK 2 was unphosphorylated at 0 h of IVM. At 10 h approximately half of the kinase was shifted to lower electrophoretic mobility even in the presence of different concentrations of SH6, indicating its partial phosphorylation, comparable with the extent obtained in the control group. Finally, ERK 2 was fully phosphorylated at 24 h IVM which was not influenced by 50 μM SH6.

Corresponding results were obtained by histone H 1 kinase assay (Fig. 4B). The phosphorylation of the external substrate histone H 1 reflects the activity of the MPF in the oocyte extract. These extracts were derived at the same maturation times as from the MAPK analysis. It can be seen that SH6 had apparently no influence on MPF activation.

Taken together, these results indicated that the inhibitory effect of SH6 was not caused by alterations of MAPK and/or MPF activation in the groups treated and must considered to be caused exclusively by Akt inhibition.

Discussion

In the present study we have investigated the abundance and phosphorylation state of the serine/threonine kinase Akt (which is also referred as PKB) during IVM of bovine oocytes. We used the phosphatidylinositol analogue SH6...
as a tool to investigate Akt function during meiotic maturation of bovine oocytes.

The phosphatidylinositol analogue SH6 is a novel inhibitor which was shown to prevent Akt activation specifically in somatic cells (Kozikowski et al. 2003, Chrysis et al. 2005) but it had not been used before to investigate the maturation processes of oocytes. One advantage of this particular inhibitor is its potential selectivity for Akt. In contrast, other substances such as wortmannin or LY294002 which have been shown to influence the Akt-dependent signalling cascade, were shown to act on the upstream phosphatidylinositol-3-OH kinase and therefore influence the activity of the PDK1 (Vlahos et al. 1994, Burgering & Coffer 1995, Franke et al. 1995). Therefore unspecific side effects of wortmannin or LY294002 on, for instance, p70S6K and protein kinase C have to be considered (Kozikowski et al. 2003, Scheid & Woodgett 2003, Tang & McLeod 2004).

Like MAPK (Kubelka et al. 2000, Tomek et al. 2002a, present study) Akt could be detected during the whole maturation process without any significant alteration in abundance at the GV, M I and M II stages. It should be mentioned here that this result is not in agreement with observations made by Vigneron et al. (2004b). These authors reported no or only very low abundance of Akt in the GV stage (time 0) of bovine oocytes. This discrepancy is probably the result of the use of different antibodies. The antibodies used in our study (one against total Akt, and two against pAkt Ser 308 or Thr 473) recognize all three isoforms of Akt, which all show an apparent molecular mass of 63 kDa on SDS gels. In the study cited, no information about the specificity of the antibody used is given.

In contrast to MAPK, which becomes activated at the time of GVBD and shows a maximum of activity in the M II stage (Kubelka et al. 2000), the kinase Akt is mainly active in the M I stage, as is confirmed by the analysis of the phosphorylation pattern and by in vitro kinase assay. When MPF and/or MAPK activation is blocked, e.g. by the CDK inhibitor bryorolactone I or by roscovitine, oocytes persist in the GV stage (e.g. Kubelka et al. 2000, Lonergan et al. 2003, Vigneron et al. 2004a). On the other hand, the present study revealed that the majority of the oocytes persist in the M I stage when they were matured in the presence of SH6. Our observations have indicated that this is an effect exclusively of Akt inhibition, since no side effects of SH6 on MAPK or MPF activation could be observed.

Although we obtained a strong inhibition of Akt activation during IVM by SH6, surprisingly a considerable number of oocytes reached the M II stage (approximately 40% at a concentration of 50 μM SH6). The reason for this observation is not clear at the moment. The most straightforward explanation for this effect would be to assume that a sufficient portion of activated Akt is already present in several GV-stage oocytes which allows them to overcome the inhibitory effect of SH6. But at the moment we do not have any convincing direct proof of this hypothesis.

Detailed analysis of somatic cells has revealed that Akt is involved in the regulation of cap-dependent translation (Sonenberg & Gringas 1998, Gingras et al. 2001). This function is mediated by the ability of the Akt signalling cascade to phosphorylate (and inactivate) a specific inhibitor of the function of the mRNA–cap binding protein eIF4E, namely its repressor protein 4E-BP1. As recently revealed by our group, eIF4E subsequently becomes phosphorylated during IVM (which should result in stimulation of translation) under the influence of MAPK; there is also a concurrent presence of 4E-BP1 in bovine oocytes (Tomek et al. 2002a, Smiljakovic et al. 2003). Furthermore, we have shown that protein synthesis during the IVM of bovine oocytes is at its maximum at the time of GVBD and during the M I stage (Tomek et al. 2002a,b) which are precisely the two stages of maturation in which Akt is active. Therefore, an attractive hypothesis concerning a particular Akt function during meiotic maturation of bovine oocytes would be to assume that Akt is involved in balancing protein synthesis by phosphorylating 4E-BP1. An inhibition of this regulatory process, for example by SH6, would result in M I arrest. We will try to verify this hypothesis in further experiments by investigating the effects of SH6 treatment on 4E-BP1 phosphorylation and on translation rates during the IVM of bovine oocytes.

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