AKT (protein kinase B) is implicated in meiotic maturation of porcine oocytes

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Abstract

The aim of this study was to investigate the involvement of the serine/threonine protein kinase AKT (also called protein kinase B) in the control of meiosis of porcine denuded oocytes (DOs) matured *in vitro*. Western blot analysis revealed that the two principal AKT phosphorylation sites, Ser473 and Thr308, are phosphorylated at different stages of meiosis. In freshly isolated germinal vesicle (GV)-stage DOs, Ser473 was already phosphorylated. After the onset of oocyte maturation, the intensity of the Ser473 phosphorylation increased, however, which declined sharply when DOs underwent GV breakdown (GVBD) and remained at low levels in metaphase I- and II-stage (MI- and MII-stage). In contrast, phosphorylation of Thr308 was increased by the time of GVBD and reached maximum at MI-stage. A peak of AKT activity was noticed around GVBD and activity of AKT declined at MI-stage. To assess the role of AKT during meiosis, porcine DOs were cultured in 50 μ M SH-6, a specific inhibitor of AKT. In SH-6-treated DOs, GVBD was not inhibited; on the contrary, a significant acceleration of meiosis resumption was observed. The dynamics of the Ser473 phosphorylation was not affected; however, phosphorylation of Thr308 was reduced, AKT activity was diminished at the time of GVBD, and meiotic progression was arrested in early MI-stage. Moreover, the activity of the cyclin-dependent kinase 1 (CDK1) and MAP kinase declined when SH-6-treated DOs underwent GVBD, indicating that AKT activity is involved in the regulation of CDK1 and MAP kinase. These results suggest that activity of AKT is not essential for induction of GVBD in porcine oocytes but plays a substantial role during progression of meiosis to MI/MII-stage.

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Introduction

In mammalian ovaries, the oocytes are naturally arrested in prophase of the first meiotic division, at the so-called germinal vesicle (GV) stage. The fully grown oocytes resume meiosis *in vivo* after LH surge or *in vitro* when released from antral follicles and further cultured under suitable conditions.

cAMP is one of the main regulators of the oocyte entry into meiosis. In mammalian oocytes, the concentration of cAMP is regulated by gonadotropins such as FSH and LH acting through the follicular cells (Bornslaeger & Schultz 1985). In mouse oocytes, elevated levels of cAMP and activated protein kinase A (PKA) are involved in maintaining oocytes in GV stage (Tsafiri *et al.* 1996, Conti *et al.* 1998). PKA activity is maintained at high levels by the increased cAMP, thus preventing maturation *in vitro* and *in vivo* (Masciarelli *et al.* 2004). Degradation of cAMP by a phosphodiesterase type IIIA (PDE3A) leads to a decrease of PKA activity and initiates resumption of meiosis in rodent oocytes (Conti 2002, Kovo *et al.* 2006). Similarly, in porcine oocytes, resumption of meiosis is also associated with a decrease in cAMP concentration and subsequent inactivation of PKA (Kim & Menino 1995).

The activity of maturation promoting factor (MPF) is crucial for meiotic maturation of oocytes. MPF complex consists of the catalytic subunit p34^{cdc2} kinase (also termed cyclin-dependent kinase 1 (CDK1)) and regulatory subunit cyclin B (Gautier & Maller 1991, Jessup & Beach 1992). The activity of CDK1 is controlled by phosphorylation at three residues; phosphorylation on Thr161 is necessary for CDK1 kinase activation, whereas phosphorylation of CDK1 by WEE1 and MYT1 kinases on either Thr14 or Tyr15 residues dominantly inhibits activation of CDK1 (Coleman & Dunphy 1994). This inhibition is reversed when CDK1 is dephosphorylated by activated CDC25C phosphatase (Nilsson & Hoffmann 2000). It has been reported that PKA phosphorylates and inactivates CDC25C in frog oocytes (Duckworth *et al.* 2002, Shibuya 2003). Also in mouse oocytes, the WEE1 kinase is a substrate for PKA *in vitro* (Han *et al.* 2005). These findings support the concept that PKA maintains meiotic arrest via dual regulation of the kinase that phosphorylates CDK1 and the phosphatase that dephosphorylates the same residues of CDK1 (Han & Conti 2006).

Another kinase implicated in MPF regulation is a serine/threonine protein kinase B (PKB/AKT), a key downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway.

The PI3K–AKT pathway is activated by insulin, growth factors, and adhesion to extracellular matrix or other cells. Activated PI3K converts its lipid substrate phosphatidylinositol 4,5 biphosphate (PtdIns (4,5)P₂) to phosphatidylinositol 3,4,5 triphosphate (PtdIns (3,4,5)P₃). Consequently, AKT is recruited to the cell membrane after binding PtdIns (3,4,5)P₃ through its PH domain, and membrane-bound AKT is activated by phosphorylation at two sites. PDK1 phosphorylates Thr308 residue located in the activation loop of the kinase (Stokoe *et al.* 1997, Kim *et al.* 2001), whereas mTORC2 was identified as the kinase responsible for phosphorylation of the Ser473 residue situated in the hydrophobic site (Sarbasov *et al.* 2005). The dual-specificity lipid phosphatase phosphatase and tensin homolog deleted on chromosome 10 (PTEN) dephosphorylates PI (3,4,5)P₃ and in such a way negatively regulates AKT kinase activity (Maehama *et al.* 2001).

It has been reported that PI3K/AKT pathway contributes to cyclin B1 expression and CDK1 activation (Roberts *et al.* 2002). Data obtained on somatic cells indicate that AKT promotes cell survival and participates in the regulation of the G2/M transition. In epithelial cells, the activity of AKT is increased at the G2/M transition of the cell cycle, and it is necessary for the timely progression through mitosis (Shtivelman *et al.* 2002). The cell cycle arrest imposed by inhibitors of PI3K could be reversed by the expression of constitutively active AKT (Shtivelman 2003). A G2/M cell cycle checkpoint induced by DNA damage is surpassed by constitutively activated AKT, and this effect has also been observed in cells lacking PTEN that limits activation of AKT (Kandel *et al.* 2002). AKT was also reported to promote cell cycle progression at the G2/M transition through WEE1 inactivation in HEK 293 cells (Katayama *et al.* 2005). In HeLa cells, AKT phosphorylates the CDC25B phosphatase and regulates its intracellular localization (Baldin *et al.* 2003).

The glycogen synthase kinase-3 (GSK3), an important substrate of AKT, is involved in numerous cellular processes, including regulation of the activity of microtubule-associated proteins affecting formation and function of mitotic spindle (Moreno & Avila 1998). In mouse oocytes, pharmacological inhibition of GSK3 causes abnormal meiotic spindle configuration, chromatin organization, and bivalent chromatin segregation (Wang *et al.* 2003).

Experiments performed on starfish oocytes provided the evidence of AKT as an initiator of the meiotic M phase. It has been documented that AKT activates meiotic G2/M transition by phosphorylation and inactivation of protein kinase MYT1, which has been shown to phosphorylate and inactivate MPF

(Okumura *et al.* 2002). Study on *Xenopus* oocytes revealed that insulin-like growth factor 1 (IGF1)- or insulin-induced meiotic maturation is mediated by activation of PI3K and AKT (Liu *et al.* 1995, DeuterReinhardt *et al.* 1997). It has been shown that expression of constitutive active PI3K can activate MAPK and induce GV breakdown (GVBD) in *Xenopus* oocytes, and this effect is mediated through its lipid kinase activity and activation of AKT (Hehl *et al.* 2001). Furthermore, it was documented in *Xenopus* oocytes that phosphorylation of PDE3A by AKT plays a role in meiotic maturation induced by growth factors (Andersen *et al.* 2003). Also the data obtained in mouse oocytes indicate that activation of PDE3A by PKB-mediated phosphorylation is implicated in the control of PDE3A activity and resumption of meiosis (Han *et al.* 2006). Suppression of AKT activity was accompanied with a decrease of CDK1 activity and postponed resumption of meiosis in mouse oocytes (Kalous *et al.* 2006). Similarly, in cattle oocytes, AKT is also involved in the regulation of meiotic metaphase I and II (MI/MII) transition (Tomek & Smiljakovic 2005).

The objectives of this study were to evaluate the dynamics of AKT phosphorylation and activation in porcine oocytes matured *in vitro* and to analyze possible role of AKT in the regulation of meiosis.

Results

Over 80% of porcine denuded oocytes reached MII-stage during control culture

In experiment 1, fresh porcine oocyte–cumulus complexes (OCCs) were deprived of cumulus, and denuded oocytes (DOs) were cultured in control medium subsequently. During 44 h of control culture, 84% of DOs reached MII-stage (Fig. 1). However, at 24 h culture interval, the time course of meiotic maturation was not synchronous as 18% of DOs retained GV, 27% exhibited late diakinesis stage (LD), 36% advanced to MI, and 19% to MII-stage (Fig. 1).

Figure 1 Progression of meiosis in porcine DOs cultured in control conditions. Isolated porcine OCCs were deprived of cumulus cells, and DOs oocytes were subsequently cultured *in vitro* in control medium. GV, germinal vesicle; LD, late diakinesis; MI, metaphase-I; MII, metaphase-II. A total of 977 oocytes were scored.

Figure 2 Phosphorylation of AKT in porcine DOs cultured in control conditions. Isolated porcine OCCs were deprived of cumulus cells, and DOs were subsequently cultured *in vitro* in control medium for indicated times. (A) Western blot with anti-pSer473 antibody. Lysates of 50 DOs/sample were evaluated. Extracts of PDGF-treated NIH 3T3 cells were used as a positive control (+). (B) Western blot with anti-pThr308 antibody. Lysates of 100 DOs/sample were evaluated. Extracts of PDGF-treated NIH 3T3 cells were used as a positive control (+). (C) Bar graph of pSer473 and pThr308 levels. Each bar represents the mean $\pm$ S.D. ($n=3$ for each time interval).

AKT is phosphorylated and activated during *in vitro* control culture of porcine DOs

In experiment 1, Ser473 was already phosphorylated when freshly isolated OCCs were stripped of cumulus, and noncultured DOs were subjected to immunoblot analysis (Fig. 2A and C). When DOs were further cultured in control conditions, the intensity of Ser473 phosphorylation increased initially, dropped around GVBD at 15 h of culture, and remained at low levels at 24 and 44 h culture interval (Fig. 2A and C). Phosphorylation of Thr308 was not detected in fresh DOs, but emerged when DOs were cultured in control and peaked

Figure 3 AKT kinase activity in porcine DOs cultured in control conditions. (A) AKT kinase assay (immunoprecipitates of 200 oocytes/sample were assayed). (B) Bar graph of relative AKT activity. Each bar represents the mean $\pm$ S.D. ($n=3$ for each time interval).

Figure 4 CDK1 and MAP kinase activities in porcine DOs cultured in control conditions. Kinase assay was performed on lysates of ten oocytes/sample.

at 24 h culture interval (Fig. 2B and C). The activity of AKT, being low in fresh uncultured DOs, increased during GVBD occurring at 10 and 15 h of culture and declined subsequently (Fig. 3A and B). The CDK1 and MAP kinase activities reached its maximum at 15 h of culture, and the activities of both kinases remained elevated until the 44 h culture interval (Fig. 4).

Dibutyryl cAMP pretreatment resulted in a synchronized progression of meiosis

In experiment 2, OCCs were initially pretreated with 1 mM dibutyryl cAMP (dbcAMP) for 20 h and stripped of cumulus subsequently. When DOs were further cultured in control medium, 44% of DOs progressed to MI-stage and 40% to MII-stage at 24 h culture interval (Fig. 5). These data indicate that the dbcAMP pretreatment resulted in a more synchronous progression of meiosis comparing with the dbcAMP-nontreated oocytes in experiment 1 (Fig. 1).

Figure 5 Progression of meiosis in dbcAMP-pretreated porcine oocytes cultured either in control or SH-6-supplemented medium. Isolated porcine OCCs were pretreated with 1 mM dbcAMP for 20 h, deprived of cumulus subsequently, and DOs were further cultured *in vitro* either in control or SH-6 (50 μ M)-supplemented medium for indicated time intervals. Bars with identical superscripts represent significantly different data ($P<0.05$). GV, germinal vesicle; LD, late diakinesis; MI, metaphase-I; MII, metaphase-II. A total of 1657 oocytes were scored.

Inhibition of AKT impairs progression of meiosis

The data from experiment 2 reveal that GVBD was not inhibited in DOs cultured in 50 μ M SH-6. On the contrary, SH-6 treatment significantly ($P < 0.05$) promoted resumption of meiosis as 50% of SH-6-treated DOs and 39% of control DOs underwent GVBD within 10 h of culture (Fig. 5). These data suggest that AKT inhibitor SH-6 has possible side effect upon regulatory mechanism of meiotic resumption. Moreover, in SH-6-treated DOs, progression of meiosis was arrested in early MI-stage. At 24 h of culture, 44% of control dbcAMP-pretreated oocytes reached MI-stage and exhibited regular MI-spindle (Figs 5 and 6A). At this time interval, in all of the 69% of MI-stage SH-6-treated oocytes, an aberrant MI-spindle was observed (Figs 5 and 6B). These data indicate that inhibition of AKT interfered with formation of the regular MI-spindle and progression of meiosis to MII-stage. There was no difference in MI-spindle conformation when both dbcAMP-pretreated and nontreated oocytes were cultured in control conditions.

Kinase activity is restored when dbcAMP-pretreated oocytes are released to control culture

In experiment 2, phosphorylation of Ser473 decreased (Fig. 7), phosphorylation of Thr308 was low (Fig. 8), and AKT activity declined (Fig. 9) in oocytes pretreated with dbcAMP for 20 h. Activity of CDK1 and MAP kinase did not increase as well (Fig. 10). After the dbcAMP-pretreated OCCs were deprived of cumulus and DOs were further cultured in control medium, meiosis resumed and phosphorylation of Ser473 increased rapidly and remained elevated until 44 h of culture

Figure 6 Representative confocal images of porcine oocytes. Porcine OCCs were precultured in 1 mM dbcAMP for 20 h, stripped of cumulus, and DOs were further cultured either in control or SH-6 (50 μ M)-supplemented medium for 24 h. (A) Normal barrel-shaped MI-spindle with regularly aligned chromosome bivalents in control oocytes cultured for 24 h. All MI-stage control oocytes exhibited normal barrel-shaped MI-spindle. (B) Disorganized MI-spindle with aberrantly organized chromosomes in oocytes cultured in SH-6 (50 μ M)-supplemented medium for 24 h. All SH-6-treated oocytes reaching MI-stage exhibited aberrant spindle configuration. Scale bar, 10 μ m.

Figure 7 Phosphorylation of pSer473 AKT in dbcAMP-pretreated porcine oocytes cultured either in control or SH-6-supplemented medium. Isolated porcine OCCs were pretreated with 1 mM dbcAMP for 20 h, deprived of cumulus cells, and DOs were further cultured either in control or SH-6 (50 μ M)-supplemented medium for indicated time intervals. (A) A representative western blot with anti-pSer473 antibody. Lysates of 50 oocytes/sample were evaluated. Extracts of PDGF-treated NIH 3T3 cells were used as a positive control (+). (B) Bar graph of pSer473 levels. Each bar represents the mean $\pm$ s.d. ($n = 3$ for each time interval).

(Fig. 7). Also the Thr308 residue became intensively phosphorylated reaching the maximum at 24 h of culture (Fig. 8). However, the quantity of AKT protein remained stable during the culture (Fig. 11). The activity of AKT increased at 10 h control culture interval and declined at 24 h (Fig. 9); the CDK1 and MAP kinase activities raised and remained elevated till the end of control culture (Fig. 10). The uniformity of protein load in analyzed samples was documented by silver staining of western blot membranes used for pSer473 and pThr308 detection (Supplementary Figure 1, which can be viewed online at www.reproduction-online.org/supplemental/).

Figure 8 Phosphorylation of pThr308 AKT in dbcAMP-pretreated porcine oocytes cultured either in control or SH-6-supplemented medium. Isolated porcine OCCs were pretreated with 1 mM dbcAMP for 20 h, deprived of cumulus cells, and DOs were further cultured either in control or SH-6 (50 μ M)-supplemented medium for indicated time intervals. (A) A representative western blot with anti-pThr308 antibody. Lysates of 100 oocytes/sample were evaluated. Extracts of platelet-derived growth factor-treated NIH 3T3 cells were used as a positive control (+). (B) Bar graph of pThr308 levels. Each bar represents the mean $\pm$ s.d. ($n = 3$ for each time interval).

Figure 9 AKT kinase activity in dbcAMP-pretreated porcine oocytes cultured either in control or SH-6-supplemented medium. The OCCs were precultured in 1 mM dbcAMP for 20 h, stripped of cumulus subsequently, and DOs were further cultured either in control or SH-6 (50 μ M)-supplemented medium for indicated time intervals. (A) AKT kinase assay (immunoprecipitates of 200 oocytes/sample). (B) Bar graph of relative AKT activity. Each bar represents the mean $\pm$ S.D. ($n=3$ for each time interval).

Inhibitor SH-6 affects phosphorylation of AKT and attenuates kinase activity

In experiment 2, when DOs were cultured in SH-6-supplemented medium, AKT became phosphorylated at Thr308 with lower intensity comparing with control DOs (Fig. 8). In SH-6-treated DOs, a reduction in Thr308 phosphorylation was more prominent at 15 h culture interval (Fig. 8), whereas Ser473 phosphorylation was not affected (Fig. 7). AKT kinase assay revealed that SH-6 treatment resulted in a decrease in AKT activity at 10 h of culture (Fig. 9). Although the initial activation of histone H1 kinase and MAP kinase was not prevented in SH-6-treated DOs, suppression of activity of these kinases was apparent at 24 and 44 h of culture (Fig. 10).

Figure 10 Activities of CDK1 and MAP kinase. Porcine OCCs were precultured in 1 mM dbcAMP for 20 h, stripped of cumulus, and DOs were further cultured either in control or SH-6 (50 μ M)-supplemented medium. CDK1 and MAP kinase assay was performed on lysates of ten oocytes/sample.

Figure 11 The levels of AKT in dbcAMP-pretreated porcine oocytes cultured in control conditions. Isolated porcine OCCs were pretreated with 1 mM dbcAMP for 20 h, deprived of cumulus cells subsequently, and DOs were further cultured in control conditions for indicated time intervals. (A) Western blot with total PKB antibody. Lysates of 100 oocytes/sample were evaluated. Extracts of PDGF-treated NIH 3T3 cells were used as a positive control (+), whereas extracts of starved NIH 3T3 cells were used as a negative control (-). (B) Bar graph of total AKT levels. Each bar represents the mean $\pm$ S.D. ($n=3$ for each time interval).

Discussion

As we previously reported, AKT is involved in CDK1 activation and resumption of meiosis in mouse oocytes (Kalous *et al.* 2006). In porcine OCCs, the activity of AKT is involved in cumulus expansion by promoting the FSH-stimulated synthesis of hyaluronic acid (Nemcova *et al.* 2007). However, when porcine OCCs are cultured in gonadotropin-free medium, expansion of cumulus layer does not occur, AKT in oocytes is dephosphorylated, and GV in oocytes persists for 24 h (J Kalous, M Kubelka & J Motlík 2007, unpublished results). In order to achieve meiotic resumption of porcine oocytes *in vitro* without affecting AKT pathway by treatment of OCCs by gonadotropins, the present study was performed on oocytes deprived of cumulus. In experiment 1, we documented that AKT is phosphorylated and activated during progression of meiosis in porcine dbcAMP-nontreated oocytes. In experiment 2, porcine OCCs were pretreated with dbcAMP to achieve more consistent progression of meiosis, similarly as already reported in mouse and porcine oocytes (Funahashi *et al.* 1997, Vanhoutte *et al.* 2008). After the dbcAMP-pretreated porcine OCCs were deprived of cumulus and DOs were further cultured in control conditions, phosphorylation and activity of AKT increased at the time of GVBD, similarly as revealed in experiment 1 on dbcAMP-nontreated oocytes. These data are in accordance with those obtained in mouse and cattle oocytes, suggesting the importance of AKT activity during meiotic G2/M transition (Tomek & Smiljakovic 2005, Kalous *et al.* 2006). Similarly, enhanced activity of AKT has been reported at the G2/M transition of somatic cells (Shtivelman *et al.* 2002). In experiment 2, after the

dbcAMP-pretreated oocytes were subjected to AKT inhibitor SH-6, a reduction in Thr308 phosphorylation occurred and kinase activities of both AKT and CDK1 decreased. Moreover, meiosis in SH-6-treated DOs was arrested in early MI-stage exhibiting defective MI-spindles. During *in vitro* maturation of porcine DOs, the abundance of AKT protein remained stable, similarly as reported in mouse and cattle oocytes (Tomek & Smiljakovic 2005, Kalous *et al.* 2006, Hoshino & Sato 2008).

In fresh GV-stage porcine DOs, a low activity of AKT was detected; however, Ser473 was phosphorylated in these oocytes. Likewise, phosphorylation of AKT at Ser473 has also been reported in mouse GV-intact oocytes (Smith & Ding 2005). It has been documented that AKT-mediated phosphorylation of PDE3A plays a role in the control of PDE3A activity in mouse oocytes (Han *et al.* 2006). Therefore, it is possible to deduce that also in porcine GV-stage oocytes, AKT is involved in the regulation of cAMP levels. However, it has been revealed that PKB is stimulated by various stress factors including H₂O₂ (Konishi *et al.* 1997). This suggests that Ser473 phosphorylation and partial activation of AKT in fresh GV-stage porcine oocytes could be initiated by oxidative stress occurring in the process of oocytes isolation. This is supported by the fact that initial phosphorylation of Ser473 in oocytes declined when fresh porcine OCCs were cultured for 5 h in gonadotropin-free medium and deprived of cumulus before western blot analysis. Ser473 was rephosphorylated in 3 h when OCCs were cultured in FSH/LH-supplemented medium subsequently (J Kalous, M Kubelka & J Motlík 2007, unpublished data). After porcine oocytes were pretreated with dbcAMP for 20 h, both Ser473 and Thr308 residues were non-phosphorylated. This is in accordance with the data on somatic cells, indicating that enhanced level of cAMP and elevated activity of PKA have inhibitory effects on the PI3K/PDK/AKT signaling and suppress phosphorylation of AKT on both Thr308 and Ser473 (Kim *et al.* 2001, Nijholt *et al.* 2008).

When DOs were cultured in control conditions, phosphorylation of the Ser473 residue and activity of AKT were elevated at the time of GVBD, indicating that AKT is possibly implicated in the regulation of events leading to GVBD. It has been published that separate phosphorylation of AKT on either Thr308 or Ser473 was sufficient to induce AKT activation (Lengyel *et al.* 2004, Viniegra *et al.* 2005). Nevertheless, phosphorylation of AKT on both Thr308 and Ser473 is a prerequisite for full activation of AKT (Alessi *et al.* 1996). Moreover, phosphorylation of AKT on Ser473 represents an essential prerequisite for autophosphorylation of Thr308 (Goren *et al.* 2008). Also, our data on porcine DOs reveal that increase in Thr308 phosphorylation occurred later than Ser473 phosphorylation. In cultured porcine DOs, phosphorylation of Thr308 was postponed until GVBD and reached maximal intensity at the

LD/MI-stage, implying that phosphorylation of Thr308 is important to support the activity of AKT during the MI-spindle formation. However, the ratio of Ser473 and Thr308 phosphorylation necessary for full AKT activation is not known. The results presented reveal that the time course of AKT phosphorylation and activation was somewhat different in samples of dbcAMP-nontreated and dbcAMP-pretreated oocytes, as the dbcAMP pretreatment resulted in a more synchronous progression of meiosis during subsequent control culture. It can be proposed that phosphorylated Ser473 and Thr308 act individually in the regulation of meiosis in porcine oocytes. Also, the data on mouse oocytes suggest that AKT phosphorylated on either Ser473 or Thr308 residue is separately distributed in oocytes and plays different roles in the regulation of meiosis (Kalous *et al.* 2006, Hoshino & Sato 2008).

The phosphatidylinositol analog SH-6 has been characterized as an effective AKT inhibitor that decreases AKT activity and induces apoptosis in cancer cells (Kozikowski *et al.* 2003). GVBD was not prevented when mouse and cattle oocytes were treated with SH-6; however, progression of meiosis was affected in treated oocytes (Tomek & Smiljakovic 2005, Hoshino & Sato 2008). Also our experiments revealed that GVBD was not inhibited in SH-6-treated porcine DOs. Surprisingly, we observed that resumption of meiosis was accelerated during the SH-6 treatment. It has been shown that PKA activation leads to inhibition of the PI3K/PDK/AKT signaling and reduction in AKT phosphorylation (Kim *et al.* 2001, Nijholt *et al.* 2008). However, phosphatidylinositol analogs like SH-6, employed to inhibit AKT, also negatively affect PKA activity (Gills *et al.* 2007). It is possible to deduce that during SH-6 treatment of porcine DOs, a reduction in PKA activity was accelerated, the activity of PKA substrates including PI3K/AKT and WEE1 kinase was affected, and hence GVBD promoted. Upon these conditions, the direct inhibitory effect of SH-6 on AKT was counteracted by the SH-6-induced suppression of PKA. Consequently, suppressed PKA activity promoted phosphorylation of AKT during resumption of meiosis and hence interfered with AKT inhibition in SH-6-treated DOs. Suppression of AKT by SH-6 became apparent after GVBD, when Thr308 phosphorylation and activity of AKT decreased; however, phosphorylation of Ser473 was not affected. The data reporting that phosphorylation of AKT on either Ser473 or Thr308 is regulated by different upstream mechanisms (Stokoe *et al.* 1997, Sarbassov *et al.* 2005) give explanation of the fact that phosphorylation of Ser473 in porcine DOs was not influenced by SH-6.

Our earlier data indicate that resumption of meiosis in porcine oocytes is accompanied by a substantial increase in CDK1 and MAP kinase activities (Kalous *et al.* 1993, Kubelka *et al.* 1995). The data presented here reveal that reduction in AKT activity occurring during SH-6 treatment did not prevent initial activation of CDK1

and MAP kinase essential for induction of GVBD. However, when GVBD was accomplished, both CDK1 and MAP kinase were inhibited in SH-6-treated porcine DOs. In starfish oocytes, AKT downregulates MYT1, a regulatory kinase of CDK1 and hence induces the initial activation of cyclin B/CDK1 at the meiotic G2/M-phase transition (Okumura *et al.* 2002). Our results indicate that in SH-6-treated porcine DOs, induction of GVBD and MPF activation is either AKT-independent or reduced activity of AKT is sufficient to support resumption of meiosis. We observed that in control culture, AKT became phosphorylated at Thr308 around GVBD. In contrast, in SH-6-treated DOs, phosphorylation of Thr308 was reduced, and progression of meiosis was arrested in early MI-stage. These data indicate that phosphorylation of AKT at Thr308 is not implicated in the events leading to the resumption of meiosis in porcine DOs; however, a full phosphorylation of the Thr308 is likely to be essential for meiosis to progress to the MI- and MII-stage. Similarly, inhibition of AKT in cattle oocytes did not prevent GVBD; however, meiosis was arrested in the MI-stage (Tomek & Smiljakovic 2005).

In mouse MI- and MII-stage oocytes, AKT phosphorylated at Ser473 was localized with microtubules, while Thr308-phosphorylated AKT was present in pericentriolar material (Hoshino *et al.* 2004). Activated AKT phosphorylated at Thr308 is able to phosphorylate and inhibit GSK3 during mitosis (Cross *et al.* 1995, Cohen & Frame 2001). Such AKT-mediated phosphorylation and activation of GSK3 occur on the centrosome and spindle poles and is involved in stabilization of microtubules in this region (Wakefield *et al.* 2002). We suggest that suppression of activity of AKT in porcine DOs induced by SH-6 led to an increase of GSK3 activity in centrosomal region. Consequently, progress of meiosis was interrupted, as assembly of MI-spindles was disturbed and aberrant MI-spindles were formed. In conclusion, our results on porcine oocytes indicate that AKT is involved in the regulation of CDK1 activity during meiosis, and activated AKT is necessary for successful course of meiotic maturation.

Materials and Methods

Experimental design

The aim of the present study was to investigate the role of AKT in meiotic events occurring during *in vitro* maturation of porcine oocytes. To avoid the necessity of inducing meiotic *in vitro* progression in OCCs by gonadotropins and thus influence AKT pathway in oocytes by hormonal stimulus, the study was performed on DOs maturing in gonadotropin-free medium.

In experiment 1, phosphorylation and activation of AKT during control culture of dbcAMP-nontreated porcine DOs were investigated. Fresh OCCs were stripped of cumulus before culture, and DOs were further cultured in control medium for 3, 5, 10, 15, 24, and 44 h. After culture, DOs were proceeded for western blot analysis, kinase assays, and assessment of nuclear maturation. In experiment 2, the effect of AKT inhibitor SH-6

upon AKT activity and meiotic progression in DOs were analyzed. To achieve more synchronous progression of meiosis, porcine OCCs were pretreated with 1 mM dbcAMP for 20 h, and OCCs were deprived of cumulus subsequently. The DOs were further cultured either in control or SH-6 (50 μ M)-supplemented medium for 5, 10, 15, 24, and 44 h. After culture, DOs were subjected to phase-contrast and immunofluorescent microscopy, western blot analysis, and kinase assays.

Oocyte collection, culture, and evaluation

Ovaries, collected from slaughtered noncycling gilts, were transported to the laboratory in physiological saline at 20 °C. The ovaries were briefly washed in physiological saline, and OCCs were aspirated from 2- to 5-mm follicles using a 21-gauge needle attached to a syringe. M-199 medium (Sigma) supplemented with 5% BSA (Sigma), 0.68 mM glutamine, 100 mM sodium pyruvate, 200 mM cysteamine, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Sigma) was used as a control medium. In experiment 1, the OCCs were deprived of cumulus before culture, and DOs were further cultured in nonsupplemented control medium. In experiment 2, the OCCs were precultured for 20 h in control medium supplemented with dbcAMP (1 mM final concentration; Calbiochem, San Diego, CA, USA). OCCs were stripped of cumulus after preculture, and DOs were further cultured either in nonsupplemented control medium or in control medium supplemented with SH-6 (50 μ M final concentration; Calbiochem) for defined time intervals. All cultures were performed in a humidified atmosphere of 5% CO₂ at 38.5 °C. At the end of culture, the DOs were washed in physiological saline, and after the last wash, the oocytes were fixed for microscopy or stored at –80 °C until western blot or kinase assay analysis.

Western blotting

Oocytes were lysed in 10 μ l SDS/PAGE sample buffer (62.5 mM Tris/HCl, pH 6.8, at 25 °C, 2% w/v SDS, 10% v/v glycerol, 0.01% w/v bromophenol blue, and 5% v/v 2-mercaptoethanol). Samples were heated at 100 °C for 3 min and subjected to SDS/PAGE. After electrophoresis, proteins were electrically transferred onto PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA) using a semi-dry blotting system. Subsequently, blotted membranes were blocked for 1 h in 5% (v/v) nonfat milk (Sigma) in T-TBS (Tris/HCl-buffered saline (pH 7.6) with 0.05% Tween 20). T-TBS was used for all following incubations and washes. Blocked membranes were incubated overnight at 4 °C with a PKB-specific primary antibody diluted 1:1000 in T-TBS with 5% nonfat milk (pAKT Ser473 (SC-7985), pAKT Thr308 (SC-16646), or AKT1 antibody (SC-5298)); all primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The blots were then washed (thrice for 5 min) in T-TBS and incubated for 1 h at room temperature (18–25 °C) with HRP-linked donkey anti-rabbit IgG (Amersham; 1:10 000, phospho-AKT detection) or rabbit anti-mouse IgG (Amersham; 1:15 000, total AKT protein detection), both diluted in T-TBS with 5% nonfat milk. After extensive washing (three to five times for 5 min), the immunoreactive signal was detected using ECL (Amersham International).

Histone H1 assay

At each time interval, ten oocytes/sample were collected, washed (4×) in PBS, and transferred into 3 µl PBS in Eppendorf tubes. Samples were immediately frozen on solid CO₂ and stored at −80 °C until assays were performed. The histone H1 and myelin basic protein (MBP) kinase activities were measured as previously described (Motlik *et al.* 1998); H1 and MBP are substrates for CDK1 and MAPK respectively. Data from autoradiograms were analyzed using Scion Image software (Scion Corporation, Frederick, MD, USA).

AKT kinase assay

Eppendorf tubes with frozen oocytes (200 DOs in each tube) were placed on ice, and 200 µl 1× ice-cold cell lysis buffer (catalog no. 9803; Cell Signaling Technology, Beverly, MA, USA) containing 1 mM phenylmethylsulphonyl fluoride was added to each tube; oocytes were lysed by several cycles of freezing and thawing. After centrifugation (14 000 *g* for 10 min at 4 °C), the supernatant was removed and 3 µl AKT1 MAB (SC-5298; Santa Cruz Biotechnology) was added, and then the samples were incubated for 1 h at 4 °C. Protein A Sepharose (Sigma) (200 µl of the suspension) was added, and the mixture was incubated at 4 °C overnight. The pellet was collected by low-speed centrifugation and washed twice with ice-cold cell lysis buffer (catalog no. 9803; Cell Signaling Technology) and ice-cold kinase buffer (catalog no. 9802; Cell Signaling Technology). The washed pellet was assayed for PKB activity as follows: the reaction was started by adding 30 µl kinase buffer (25 mM Tris/HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 mM MgCl₂, and 200 µM ATP) to each sample together with 2 µg GSK3 fusion protein (Cell Signaling Technology) as an external substrate for AKT and 500 µCi/ml [γ -³²P]ATP (10 mCi/ml; Amersham). The reaction was performed for 30 min at 30 °C and terminated by adding 30 µl 4× concentrated SDS/PAGE sample buffer and boiled for 3 min. After SDS/PAGE (15% w/v PAGE) (Laemmli 1970), the gels were stained with Coomassie Blue R250 (Serva, Heidelberg, Germany), destained overnight, dried, and autoradiographed. Subsequently, the gel was scanned with a Fujifilm BAS-2500 Phosphorimager, and the data were analyzed by Scion Image software.

Morphological analyses of oocytes

To check the current stage of maturation at the time of sample collection, an aliquot of 30–40 DOs was mounted on microscope slides with Vaseline (Sigma) strips, covered with cover glass, and fixed in solution ethanol:acetic acid (3:1 v/v) for 48 h. Staining was performed with 2% orcein in 50% aqueous acetic acid and 1% sodium citrate. The slides were then placed in 40% acetic acid and observed with a phase-contrast NU Zeiss microscope.

Immunocytochemistry

Denuded zona-intact oocytes were washed in PBS, fixed for 1 h in 3.7% paraformaldehyde (16005, Aldrich), washed in PBS, and stored in PBS with 1% sodium azide. Such prepared oocytes

were washed for 30 min in PBS containing 1% Triton-X (T9284, Sigma–Aldrich) and subsequently blocked in 2% BSA fraction V (Sigma) for 2 h in PBS. The oocytes were incubated overnight at 4 °C with mouse monoclonal anti-acetylated tubulin antibody (T7451, Sigma, diluted 1:500). The excess primary antibody was removed by extensive washing in PBS containing 0.2% BSA/0.05% saponin (16109, Sigma). Oocytes were then incubated with donkey anti-mouse antibody conjugated with Alexa Flour-594 (Invitrogen, diluted 1:800) in PBS/0.2% BSA/0.05% saponin for 60 min at room temperature. As a control, oocytes were incubated only with secondary antibody. The well-washed oocytes were mounted in mounting medium with DAPI (H-1500; Vector Laboratories, Peterborough, UK) and examined using confocal microscopy (Leica).

Statistical analyses

Statistical analysis of all data was performed using NCSS 2000 computer software (NCSS, Kaysville, UT, USA). Error sections in graphs express confidence limits. The frequencies of meiosis progression were pooled from three replicate experiments, and cumulative results were subjected to χ^2 analysis. Differences were considered significant at $P < 0.05$. A total of 2634 oocytes were subjected to morphological analyses. The results of the western blotting and AKT kinase assays were quantified by Scion Image software and expressed as the means $\pm$ s.d. of three independent experiments. The data were normalized to the mean value by mean centering.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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