Identification and regulation of glycogen synthase kinase-3 during bovine embryo development

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Abstract

The aim of this study was to examine the presence and regulation of glycogen synthase kinase-3 α (GSK3A) and GSK-3 β (GSK3B) in bovine embryos and their possible roles in embryo development. Our results show that GSK3A and GSK3B are present in bovine embryos at the two-cell stage to the hatched blastocyst stage. Bovine embryo development was associated with an increase in the phosphorylation of both isoforms, being statistically significant at blastocyst and hatched blastocyst stages, compared with earlier stages. Inhibition of GSK3 with CT99021 (3 μ M) resulted in a significant increase in the percentage and quality of blastocysts, while inhibition of GSK3 with lithium chloride (LiCl; 20 mM) significantly reduced at the proportion of eight-cell embryos on day 3 and inhibited blastocyst formation. The use of LY294002 (10 μ M), a specific inhibitor of phosphatidylinositol-3 kinase, also produced a significant decrease in embryo development. In addition, treatment with LiCl and LY294002 produced a significant decrease in the serine phosphorylation of both isoforms of GSK3. Finally, CT99021 and LiCl reduced the phosphorylation of β -catenin on Ser45 in two-cell embryos, while LY294002 increased it. Despite the fact that LiCl inhibited GSK3 activity, as demonstrated by β -catenin phosphorylation, its effects on the bovine embryo could be mediated through other signaling pathways leading finally to a decrease in the phosphorylation of GSK3 and a reduction in embryo development. Therefore, in conclusion, GSK3A/B serine phosphorylation was positively correlated with embryo development, indicating the importance of an accurate regulation of GSK3 activity during developmental stages to achieve normal bovine embryo development.

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Introduction

Glycogen synthase kinase-3 (GSK3) is a highly evolutionary conserved intracellular serine–threonine kinase which exists as two isoforms, GSK-3 α (GSK3A) and GSK-3 β (GSK3B), ubiquitously expressed in mammalian tissues (Woodgett 1990). The isoforms share 97% sequence similarity within their kinase catalytic domain, but differ significantly outside this region, with GSK3A possessing an extended N-terminal glycine-rich tail (Woodgett 1990). GSK3 is constitutively activated in mammals, but its activity is significantly reduced by the phosphorylation of an N-terminal serine, Ser9 in GSK3B and Ser21 in GSK3A (Frame *et al.* 2001). Phosphorylation, and therefore inactivation of GSK3, can be catalyzed by insulin, growth factors, and amino acids throughout phosphatidylinositol-3 kinase (PI3K)/AKT, MAPK cascade, protein kinase C (PKC), or by cAMP-dependent protein kinase/protein kinase A (PKA; Goode *et al.* 1992, Fang *et al.* 2000, Frame & Cohen 2001).

Originally identified as a regulator of glycogen metabolism throughout the classical PI3K/AKT signaling pathway (Frame & Cohen 2001), GSK3 regulates a diverse array of cell functions including protein

synthesis, cell proliferation, cell differentiation, apoptosis, microtubule dynamics, and cell motility. It has been recently described that GSK3B may regulate oocyte meiosis, in particular the metaphase-I/II transition, being part of the MAPK3/1 and MAPK14 pathways in oocytes and cumulus cells in cattle (Uzbekova *et al.* 2009). GSK3 has been demonstrated to be a key regulator of cellular fate and a participant in the differentiation events during embryonic development through its participation in the *Wnt* signal transduction pathway (reviewed in Forde & Dale (2007)). GSK3 phosphorylates β -catenin, the central component in *Wnt* signaling which is responsible for the transmission of *Wnt* signals to the nucleus. Phosphorylation of β -catenin by GSK3B leads to ubiquitination of β -catenin and its subsequent degradation in proteasomes. However, when GSK3B is inactivated by phosphorylation, β -catenin translocates into the nucleus and stimulates the transcription of *Wnt* genes (Aberle *et al.* 1997). It has been shown that there is a correlation between a proper regulation of *Wnt* signaling and normal embryo development. For example, bovine embryos which develop past the 16-cell stage showed a proper distribution of β -catenin in all blastomeres and an appropriate morphology

(Modina *et al.* 2007). However, the deletion of certain *Wnt* genes in the mouse, *Caenorhabditis elegans*, and *Drosophila* results in strong changes in the phenotypes (Cadigan & Nusse 1997).

Lithium, one of the most effective drugs for the treatment of bipolar disorder, exerts its effects through the inhibition of GSK3 (Klein & Melton 1996) by two mechanisms that act in concert. First, there is a direct inhibitory effect by lithium on GSK3 through competition with magnesium ions for binding to GSK3. Secondly, lithium causes indirect inhibition of GSK3 by increasing the inhibitory serine phosphorylation of GSK3 (reviewed in Jope (2003)). Lithium can mimic the actions of *Wnt/Wingless* on β -catenin/Armadillo in mammalian and *Drosophila* cells (Stambolic *et al.* 1996). Treatment with lithium has dramatic effects on morphogenesis during early development of diverse organisms. In zebrafish, lithium exposure produces excessive shield formation and extreme hyper-dorsal development (Stachel *et al.* 1993). In *Xenopus*, it causes an expansion of dorsal mesoderm, leading to duplication of the dorsal axis or, in extreme cases, entirely dorsalized embryos (Kao *et al.* 1986). A short treatment with lithium chloride (LiCl) at the two- or eight-cell stage causes mouse embryos to develop axial defects similar to those observed in some mutations that adversely affect gastrulation (Rogers & Varmuza 1996).

Repeated mitosis during embryonic cleavage requires a careful regulation of microtubule dynamics for assembling a spindle apparatus that accurately segregates chromosomes. In somatic cells, Wakefield *et al.* (2003) reported that GSK3 is present along the length of spindle microtubules, being phospho-GSK3 abundant at the centrosome and spindle poles. Furthermore, inhibition of GSK3 leads to an increase in the length of mitotic microtubules and defective chromosome alignment, suggesting that GSK3 activity is involved in regulating the balance of microtubule dynamics during mitosis (Wakefield *et al.* 2003). In *C. elegans* embryos, GSK3 has been shown to act positively to promote both endoderm specification and proper mitotic spindle orientation via the *Wnt* pathway (Schlesinger *et al.* 1999).

Therefore, given the importance of GSK3 during embryo development through the *Wnt* pathway and the lack of information on the role of GSK3 in mammalian embryos, the aims of this study were to examine the presence and regulation of both isoforms of GSK3 during early bovine preimplantation development and to study the role of GSK3 in embryo development by its inhibition using two inhibitors: LiCl and CHIR99021 (CT99021). The mechanism of lithium action on GSK3 activity is well studied (Jope 2003). The aminopyrimidine CHIR99021 is a cell-permeable compound that acts as potent, ATP-competitor, and is one the most selective inhibitors of GSK3 reported to date (Ring *et al.* 2003, Meijer *et al.* 2004). Finally, we aimed to

study the relationship of GSK3 to the PI3K and *Wnt* signaling pathways using LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), which is a potent and specific cell-permeable inhibitor of PI3K (Vlahos *et al.* 1994). LY294002 competitively inhibits ATP binding to the catalytic subunit of PI3K.

Results

GSK3 identification and regulation during early embryo development

Anti-GSK3A and anti-GSK3B antibodies detected two bands of ~51 and 46 kDa respectively in bovine cumulus cells, used as positive samples (Uzbekova *et al.* 2009), and in two-cell embryos (Fig. 1A). The same antibodies detected the total form of GSK3A and GSK3B in two- and eight-cell embryos, morulae, and blastocysts, demonstrating the presence of GSK3 throughout early bovine embryo development (Fig. 1B). Serine phosphorylation of GSK3A and GSK3B was also studied during embryo development using two specific antibodies. Antibodies detected two protein bands of 51 and 46 kDa corresponding to the phosphorylated form of GSK3A and GSK3B respectively in bovine oocytes before and after *in vitro* maturation (IVM), used as positive samples (Uzbekova *et al.* 2009), and in two-cell embryos (Fig. 2A). Results showed an increase in the phosphorylated form of both isoforms, indicative of an inactivation, as embryo development progressed, being statistically significant at the blastocyst and hatched blastocyst stages compared with earlier stages of development (Fig. 2).

Figure 1 GSK3 identification in bovine embryos. (A) Bovine cumulus cells were used as a positive control to study the specificity of the antibodies against the total form GSK3A and GSK3B. (B) Proteins extracted from cumulus cells and proteins from embryos (35 per lane) at the two-cell (lane 1), eight-cell (lane 2), morula (lane 3), and blastocyst (lane 4) stages were loaded and resolved by SDS-PAGE in a 10% acrylamide gel. Immunoblotting was performed using two specific antibodies against the total forms of GSK3A or GSK3B ($n=3$). MW, molecular weight.

Figure 2 GSK3 phosphorylation during early bovine embryo development. (A) Bovine immature and mature oocytes were used as a positive control to study the specificity of two antibodies against the phosphorylated form of GSK3A or GSK3B. (B) Proteins extracted from oocytes (40 per lane) and embryos (35 per lane) at the two-cell (lane 1), eight-cell (lane 2), morula (lane 3), blastocyst (lane 4), and hatched blastocyst (lane 5) stages were loaded and resolved by SDS-PAGE in a 10% acrylamide gel. Immunoblotting was performed using two specific antibodies against the phosphorylated form of GSK3A or GSK3B. Densitometry of protein bands was measured and represented as ratios of phosphoprotein kinase/total protein kinase ($n=4$). Columns marked with different letters indicate significant differences ($P<0.05$). MW, molecular weight.

Effect of GSK3 inhibition on embryo development and quality

Given that GSK3 activity is regulated during embryo development, we aimed to study the effect of GSK3 inhibition using two inhibitors, LiCl (20 mM) and CT99021 (3 μ M). Concentrations chosen for each inhibitor were based on previous studies (Li *et al.* 2008, Uzbekova *et al.* 2009). Treatment of presumptive zygotes with CT99021, a well-characterized highly selective small molecule inhibitor of GSK3 (Murray *et al.* 2004), produced a significant increase in the proportion of embryos reaching the blastocyst stage at days 7 (31 ± 1.58) and 8 (38 ± 4.71), compared with control embryos (23 ± 2.93 , 24 ± 4.38 respectively), in which only the vehicle (DMSO) was added (Table 1). Moreover, the number of cells observed in blastocysts

and hatched blastocysts at day 8 were higher after treatment with CT99021 than in control embryos (Fig. 3). In contrast to the observations with CT99021, incubation of presumptive zygotes with 20 mM LiCl resulted in a lower cleavage rate, a significant decrease in the number of five- to eight-cell embryos at day 3 (29 ± 5.70) compared with control embryos (53 ± 3.98) and complete failure of embryos to reach the blastocyst stage (Table 2).

Effect of PI3K inhibition on embryo development and quality

To study the effect of PI3K inhibition on embryo development, presumptive zygotes were incubated with 10 μ M LY294002, a specific inhibitor of PI3K.

Table 1 Effect of glycogen synthase kinase-3 inhibition by CT99021 (3 μ M) on bovine embryo development *in vitro*. Results are expressed as a percentage of the total number of zygotes ($n=5$).

Treatment	N zygotes	% Cleaved at day 2	% Five- to eight-cell embryos at day 3	% Blastocyst stage		
				Day 6	Day 7	Day 8
Control	232	83 ± 4.35	52 ± 4.30	15 ± 3.23	23 ± 2.93^a	24 ± 4.38^a
CT99021 (3 μ M)	227	88 ± 2.44	59 ± 3.70	18 ± 4.11	31 ± 1.58^b	38 ± 4.71^b

Data marked with different letters indicate significant differences ($P<0.05$) compared with the control.

Figure 3 Effect of GSK3 inhibition on bovine embryo quality. To assess embryo quality, day 8 blastocysts produced in the absence or in the presence of 3 μM CT99021 were washed in PBS, fixed in ethanol overnight at 4 °C, and stained with Hoechst 33342 at 25 μg/ml ($n=5$). Columns marked with an asterisk (*) indicate significant differences ($P<0.05$) compared with controls.

Treatment with LY294002 leads to a decrease in cleavage rate (56 ± 5.23) at 48 h post insemination, a significant decrease in the proportion of five- to eight-cell embryos at day 3 (20 ± 2.17), and an almost complete inhibition of blastocyst development. The cell numbers in the few blastocysts that did form in the presence of the inhibitor were significantly lower than in control blastocysts (Fig. 4).

Regulation of serine phosphorylation of GSK3A/B after GSK3 and PI3K inhibition

Culture of embryos in the presence of LiCl (20 mM) resulted in a significant decrease in phosphorylated form of GSK3A and GSK3B compared with control embryos. In contrast, CT99021 (3 μM) had no effect on GSK3 phosphorylation. However, when two-cell embryos were treated for 3 h with LY294002 (10 mM), a significant decrease in the phosphorylated GSK3 form of both isoforms was observed (Fig. 5).

β-Catenin phosphorylation: detection in bovine embryos and regulation of Ser45 phosphorylation by LiCl, CT99021, and LY294002

Because β-catenin can be regulated by phosphorylation at different residues, we aimed to study all of them using specific antibodies that recognize β-catenin phosphorylated at Threonine 41, Ser33 and Ser37, Ser45, Ser552, and Ser675. β-Catenin was phosphorylated in day 8 bovine blastocysts on all residues mentioned above except those which are directly phosphorylated by GSK3 (Thr41 and Ser33/37; Fig. 6). Despite the differences in the degree of phosphorylation detected by the antibodies, we aimed to study the phosphorylation at Ser45 because it is necessary for subsequent phosphorylation of β-catenin by GSK3. Results showed a decrease in the quantity of β-catenin phosphorylated on Ser45 after inhibition of GSK3 with LiCl and CT99021, but an increase after inhibition of PI3K (Table 3).

Discussion

We have demonstrated for the first time that bovine embryos express both GSK3A and GSK3B isoforms from the two-cell stage to the blastocyst stage. The phosphorylation of both isoforms increased as development progressed, suggesting that the inhibition of GSK3 and the signaling pathway mediated by this protein are associated with normal embryo development. The presence of GSK3 has been recently described in bovine oocytes and cumulus cells (Uzbekova *et al.* 2009). The same authors showed that GSK3B may regulate oocyte meiosis, in particular the metaphase-I/II transition, being part of MAPK3/1 and MAPK14 pathways in oocytes and cumulus cells in cattle (Uzbekova *et al.* 2009). Therefore, GSK3 present before the maternal to embryonic transition is likely to be of maternal origin (i.e. stored in the oocyte; Vigneault *et al.* 2004).

Most of the roles described for GSK3 in embryonic development are through its participation in the Wnt signal transduction pathway (reviewed in Forde & Dale (2007)) phosphorylating β-catenin. Phosphorylation of β-catenin is regulated by different kinases: GSK3 which phosphorylates at residues Threonine 41, Ser33, and Ser37 (Yost *et al.* 1996); CK1 which phosphorylates at Ser45, priming β-catenin for subsequent phosphorylation

Table 2 Effect of glycogen synthase kinase-3 inhibition by lithium chloride (LiCl; 20 mM) on bovine embryo development *in vitro*. Results are expressed as a percentage of the total number of zygotes ($n=4$).

Treatment	N zygotes	% Cleaved at day 2	% Five- to eight-cell embryos at day 3	% Blastocyst stage		
				Day 6	Day 7	Day 8
Control	194	81 ± 3.31	53 ± 3.98^a	9 ± 1.53^a	17 ± 1.66^a	24 ± 2.07^a
LiCl (20 mM)	193	69 ± 7.24	29 ± 5.70^b	0 ± 0.00^b	0 ± 0.00^b	0 ± 0.00^b

Data marked with different letters indicate significant differences ($P<0.05$) compared with the control.

Figure 4 Effect of PI3K inhibition on bovine embryo quality. To assess embryo quality, day 8 blastocysts produced in the absence or in the presence of 10 μM LY294002 were washed in PBS, fixed in ethanol overnight at 4 °C, and stained with Hoechst 33342 at 25 μg/ml ($n=4$). Columns marked with an asterisk (*) indicate significant differences ($P<0.05$) compared with controls.

by GSK3 (Amit *et al.* 2002, Yanagawa *et al.* 2002); AKT and PKA which phosphorylate at Ser552 and Ser675 (Hino *et al.* 2005, Taurin *et al.* 2006, Fang *et al.* 2007). β -Catenin was phosphorylated in bovine embryos on all residues mentioned above except those which are directly phosphorylated by GSK3 (Thr41 and Ser33/37), indicating that phosphorylation of β -catenin on Ser45 in bovine embryos precedes, and is required by, subsequent phosphorylation by GSK3. Despite the fact that β -catenin is phosphorylated on different residues, we

focused this study on the phosphorylation on Ser45 as it is specific to GSK3 (Fig. 7).

It has been previously reported that lithium inhibits GSK3B activity and mimics the biochemical effect of *Wnt* signaling by leading to a decrease in the phosphorylation of β -catenin protein and its stabilization (Hedgepeth *et al.* 1997, Rao *et al.* 2005), which is consistent with the results of the present study. Also, a decrease in β -catenin phosphorylation was observed after treatment with CT9921, indicating that GSK3 activity was also inhibited. However, despite the fact that both GSK3 inhibitors inhibited GSK3 activity, the effects on embryo development were divergent; LiCl decreased the proportion of zygotes reaching the blastocyst stage, while CT99021 increased development. One of the mechanisms proposed for the actions of lithium in *Xenopus* embryos and bovine and mouse oocytes is through the direct inhibition of GSK3B (Klein & Melton 1996, Hedgepeth *et al.* 1997, Wang *et al.* 2003, Uzbekova *et al.* 2009). However, lithium also caused a significant decrease in the phosphorylation of GSK3A and GSK3B, indicating activation of the protein. GSK3 has been described as being present in the cytosol, nucleus, and mitochondria (Bijur & Jope 2003), and is capable of processing more than one stimulus and delivering distinct outcomes due to compartmentalization of its action within the cell (Harwood 2001). One plausible explanation for the results obtained here is that lithium is affecting different pools of GSK3 (Bijur & Jope 2003), producing an inactivation of GSK3, which is reflected in the phosphorylation of β -catenin, and an activation of GSK3 through an inhibition of its phosphorylation and decreasing bovine embryo development. The decrease in GSK3 phosphorylation observed

Figure 5 Effect of LiCl, CT99021 and LY294002 on GSK3 serine phosphorylation. To study the effect of GSK3 and PI3K inhibitors on GSK3 phosphorylation, two-cell embryos were treated with LiCl (lane 2), CT99021 (lane 3), or LY294002 (lane 4) for 3 h. Subsequently, 35 embryos were loaded and resolved by SDS-PAGE in a 10% acrylamide gel. Immunoblotting was performed using two specific antibodies against the phosphorylated form of GSK3A or GSK3B. Densitometry of protein bands was measured and represented as ratios of phosphoprotein kinase/total protein kinase ($n=4$). Columns marked with an asterisk(s) indicate significant differences compared with controls. * $P<0.05$; ** $P<0.01$.

Figure 6 β-Catenin phosphorylation and regulation by GSK3 and PI3K inhibition. (A) To study which residues were phosphorylated by β-catenin, 30 day 8 blastocysts were loaded and resolved by SDS-PAGE in a 10% acrylamide gel. Immunoblotting was performed using specific antibodies against β-catenin phosphorylated at Thr41, Ser33/37 (gel 1)–Thr41, Ser45 (gel 2)–Ser552 (gel 3) and Ser675 (gel 4). (B) To investigate the phosphorylation of β-catenin on Ser45, two-cell embryos after being treated with LY294002 (10 μM; lane 2), LiCl (20 mM; lane 3), CT99021 (3 μM; lane 4) for 3 h were loaded and resolved by SDS-PAGE in a 10% acrylamide gel. Immunoblotting was performed using the specific antibody against β-catenin phosphorylated at Thr41/Ser45. Densitometry of protein bands was measured and represented as percentage of maximum ($n=4$ replicates). Columns marked with an asterisk(s) indicate significant differences compared with controls. * $P<0.05$; ** $P<0.01$.

after lithium treatment may be due to lithium action not only inactivating GSK3, but also inhibiting the actions of forskolin and dbcAMP (Bagger *et al.* 1993) and interacting directly with the catalytic unit of the adenylate cyclase system (Mork & Geisler 1987, Manji *et al.* 1995) decreasing the concentration of cAMP.

Moreover, in germinal cells and in the bovine corpus luteum, an increase in the phosphorylation of GSK3 in response to agonists that elevate intracellular concentrations of cAMP has been demonstrated (Aparicio *et al.* 2007, Roy *et al.* 2009), showing the interaction of cAMP and GSK3 (Fang *et al.* 2000). Lithium also interferes with another second messenger system, the inositol pathway causing selective reductions of PKC (Manji & Lenox 1999), which has been shown to phosphorylate and inactivate GSK3 mediating acentromeric spindle stabilization in mouse oocytes (Baluch & Capco 2008). This reduction in cAMP concentration or PKC by lithium in bovine embryos would lead to a decrease in the phosphorylation of GSK3, as observed here, and may explain the detrimental effect on embryo development as previously shown in mouse, rabbit, and *Xenopus* embryos (Kao *et al.* 1986, Fahy & Kane 1994, Rogers & Varmuza 1996). In the present study, because both inhibitors reduced β-catenin phosphorylation, the detrimental effect of lithium on bovine embryo is mainly mediated through other signaling pathways leading finally to a decrease in the phosphorylation of GSK3 and a reduction in embryo development.

One of the most studied and best characterized intracellular pathway that produces the phosphorylation and activity downregulation of GSK3 is the PI3K/AKT pathway. Jousan & Hansen (2007) and Jousan *et al.* (2008) demonstrated the presence of PI3K/AKT and its role in mediating the antiapoptotic effects of insulin-like growth factor 1 (IGF1) in bovine embryos. In the present work, treatment of presumptive zygotes with LY294002 produced a significant reduction in the phosphorylation of GSK3 together with a decrease in embryo development and quality. This decrease observed in bovine embryo development could be produced by an increase in apoptosis, as mentioned earlier, or by a G₂/M arrest as showed previously in mouse embryos after silencing the catalytic subunit of PI3K (Xu *et al.* 2009). Although it is well known that the *Wnt* signal transduction pathway is activated by wnts, a family of secreted proteins that act on target cells in a paracrine fashion through members of frizzled receptor family (Seidensticker & Behrens 2000), in the present study, inhibition of PI3K resulted in an increase in phosphorylation of β-catenin, suggesting a crosstalk between PI3K and *Wnt* signaling pathway. The increase in the phosphorylation of β-catenin would lead

Table 3 Effect of phosphatidylinositol-3 kinase inhibition with 10 μM of LY294002 on bovine embryo development *in vitro*. Results are expressed as a percentage of the total number of zygotes ($n=4$).

Treatment	N zygotes	% Cleaved at day 2	% Five- to eight-cell embryos at day 3	% Blastocyst stage		
				Day 6	Day 7	Day 8
Control	178	74 ± 2.87 ^a	40 ± 0.32 ^a	7 ± 0.94 ^a	22 ± 2.12 ^a	28 ± 1.58 ^a
LY294002 (10 μM)	174	56 ± 5.23 ^b	20 ± 2.17 ^b	0 ± 0.00 ^b	4 ± 1.54 ^b	9 ± 2.17 ^b

Data marked with different letters indicate significant differences ($P<0.05$) compared with the control.

Figure 7 Proposed model of involvement of GSK3 in bovine embryo development. The inhibition of PI3K with LY294002 induced a decrease in GSK3 phosphorylation, activating it, which, subsequently phosphorylated β -catenin. Phosphorylation of β -catenin by GSK3 would lead to ubiquitination of the protein and its subsequent degradation in proteasomes, inhibiting bovine embryo development. However, when GSK3 is inactivated by LiCl and CT99021, β -catenin would translocate into the nucleus stimulating the transcription of *Wnt* genes, promoting bovine embryo development. However, LiCl, apart from its effects on *Wnt* signaling pathway, simultaneously reduces the phosphorylation of GSK3, which could be mediated by a reduction of cAMP or inactivation of PKC, promoting the activation of GSK3 and inhibiting bovine embryo development. Continuous arrows, signaling in basal conditions; discontinuous arrows, signaling in the presence of inhibitors.

to ubiquitination of β -catenin and its subsequent degradation in proteasomes, blocking the transcription of *Wnt* genes (Aberle *et al.* 1997) which are important for a normal embryo development (Cadigan & Nusse 1997).

In summary, the results of the current study indicate a positive correlation between bovine embryo development and blastocyst quality and phosphorylation of GSK3A/B. Despite the fact that lithium inhibited GSK3 activity, as demonstrated by β -catenin phosphorylation, its effects on the bovine embryo are mainly mediated through other signaling pathways leading finally to a decrease in the phosphorylation of GSK3 and a reduction in embryo development. Specific inhibition of GSK3 by CT99021 resulted in a decrease in β -catenin phosphorylation and an increase in embryo development and quality. Finally, the inhibition of PI3K resulted in an activation of GSK3 and an increase in the phosphorylation of β -catenin together with a decrease in the proportion of embryos reaching the blastocyst stage and blastocyst cell number, suggesting an important cross-talk between *Wnt* and PI3K pathways in the regulation of bovine embryo development. In conclusion, the results show the importance of the correct regulation of both isoforms of GSK3 phosphorylation and activity to achieve a proper bovine embryo development.

Materials and Methods

Materials

TCM-199 culture medium, FCS, epidermal growth factor (EGF), and LiCl were purchased from Sigma; CT99021 (CHIR 99021, #1386) from Axon Medchem BV (Groningen,

The Netherlands); LY294002 (#440204) from Calbiochem (Darmstadt, Germany); GSK3A antibody (#9338), GSK3B (27C10) rabbit mAb (#9315), phospho-GSK3B (Ser9) (#9323), phospho-GSK3A (Ser21) (#9316), phospho- β -catenin (Ser33/37/Thr41) (#9561), phospho- β -catenin (Thr41/Ser45) (#9565), phospho- β -catenin (Ser552) (#9566), phospho- β -catenin (Ser675) (#9567), anti-rabbit IgG, HRP-linked antibody (#7074) were purchased from Cell Signaling (Beverly, MA, USA).

In vitro embryo production

Cumulus-oocyte complex collection and IVM

Immature cumulus-oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of cattle killed at a local abattoir. Good quality COCs were selected and washed two times in PBS containing 36 mg/ml pyruvate, 50 mg/ml gentamycin, and 0.5 mg/ml BSA, followed by a final wash in maturation medium. Groups of up to 50 COCs were placed in 500-ml maturation medium (TCM-199 supplemented with 10% (v/v) FCS and 10 ng/ml EGF) in a four-well dish and cultured at 39 °C for 24 h in a humidified atmosphere containing 5% CO₂.

IVF and in vitro culture

Matured COCs were washed four times in PBS, and then placed in wells containing 250 μ l of fertilization medium. The fertilization medium consisted of Tyrode's medium with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/ml fatty acid-free BSA, and 10 μ g/ml heparin-sodium salt (184 units/mg heparin; Calbiochem, San Diego, CA, USA) per well. Each well was inseminated with frozen-thawed Percoll-separated bull sperm (GE Healthcare Bio-Sciences, Uppsala, Sweden) at a concentration of 1×10^6 spermatozoa/ml.

Plates were incubated for 24 h at 39 °C under an atmosphere of 5% CO₂ in air with maximum humidity.

At ~20-h post fertilization, presumptive zygotes were denuded by gentle vortexing, washed three times in PBS and twice in culture medium before being transferred to 500 µl of synthetic oviduct fluid +5% FCS (Holm *et al.* 1999). Dishes were incubated at 39 °C under an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ with maximum humidity. Cleavage rate was recorded on day 2 (48-h post insemination), the proportion of eight-cell embryos was recorded on day 3, and the proportion of embryos reaching the blastocyst stage was recorded on days 6–8 (day 0=day of IVF).

Western blotting

To separate the proteins according to their apparent molecular mass, SDS-PAGE was performed according to Laemmli (1970). Immature ($n=40$) and mature oocytes ($n=40$) were denuded of their cumulus investments by gentle pipetting and repeated washing in PBS, and the cumulus cells were recovered for analysis by centrifugation. Cumulus cells, oocytes, and embryos were resuspended in lysis buffer (M-PER Mammalian Protein Extraction Reagent; #78503; Thermo Scientific, Rockford, IL, USA), supplemented with protease and phosphatase inhibitors (Halt protease inhibitor cocktail EDTA-free and Halt phosphatase inhibitor cocktail, #78415, #78420; Pierce, Rockford, IL, USA), frozen and thawed three times to extract the proteins, and centrifuged for 1 min at 10 000 *g* at 4 °C. Proteins were denatured by boiling for 5 min at 95 °C in loading buffer (Laemmli sample buffer, Bio-Rad Laboratories, Inc., #161-0737) supplemented with 5% mercaptoethanol. Proteins extracted were loaded and resolved by SDS-PAGE on a 10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane which was blocked with blocking buffer (5% nonfat dry milk in a Tris-buffered saline–Tween-20 (TBST) containing 10 mM Trizma base, 100 mM NaCl, and 0.5% Tween-20) for 1 h at room temperature. Immunoblotting was performed by incubating the membranes in blocking buffer overnight at 4 °C with primary antibodies. Membranes were subsequently washed in TBST and incubated with the appropriate species-specific HRP-conjugated secondary antibodies. Following three washes for 10 min each with TBST, the signal was visualized using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce) according to the manufacturer's instructions.

Experiment 1: GSK3 identification and regulation during early embryo development

To study the specificity of the antibodies against the total and the phosphorylated form of GSK3A and GSK3B, bovine cumulus cells and oocytes (40 per lane) before and after IVM were used as positive samples (Uzbekova *et al.* 2009). Embryos (35 per lane) at the two-cell, five- to eight-cell, morula, blastocyst, and hatched blastocyst stages were loaded and resolved by SDS-PAGE in a 10% acrylamide gel. To identify GSK3, immunoblotting was performed using two specific

antibodies against the total form of GSK3A and GSK3B (1/1000 dilution, incubation overnight at 4 °C). To study the regulation of both GSK3 during bovine embryo development, immunoblotting was performed using two specific antibodies that recognized phosphorylation at Ser21 and Ser9 of GSK3A and GSK3B respectively (1/1000 dilution, incubation overnight at 4 °C).

Experiment 2: effect of GSK3 inhibition on embryo development and quality

To test the effect of GSK3 inhibition on embryo development and quality, *in vitro*-produced zygotes were cultured in the absence or in the presence of 20 mM of LiCl ($n=387$, four replicates) or 3 µM of CT99021 ($n=459$, five replicates) added to the culture medium. Embryo cleavage rate was recorded at 48 h post insemination, the proportion of five- to eight-cell embryos was recorded on day 3, and the proportion of blastocysts was recorded from day 6 to 8. To assess embryo quality, day 8 blastocysts and hatched blastocyst produced in the absence or in the presence of GSK3 inhibitors were washed in PBS, fixed in ethanol overnight at 4 °C, and stained with Hoechst 33342 at 25 µg/ml. The number of cells was counted under a fluorescence microscope.

Experiment 3: effect of PI3K inhibition on embryo development and quality

To test the effect of PI3K inhibition on embryo development and quality, *in vitro*-produced zygotes ($n=352$, four replicates) were cultured in the absence or in the presence of 10 µM LY294002. Embryo cleavage and development and blastocyst cell number were recorded as described above.

Experiment 4: regulation of serine phosphorylation of GSK3A/B after GSK3 and PI3K inhibition

To study the effect of GSK3 and PI3K inhibitors on GSK3 phosphorylation, two-cell embryos were treated with LiCl (20 mM), CT99021 (3 µM), or LY294002 (10 µM) for 3 h ($n=4$ replicates). Subsequently, 35 embryos were loaded and resolved by SDS-PAGE in a 10% acrylamide gel. Immunoblotting was performed using specific antibodies that recognized phosphorylation at Ser21 and Ser9 of GSK3A and GSK3B respectively (1/1000 dilution, incubation overnight at 4 °C).

Experiment 5: β -catenin phosphorylation: detection in bovine embryos and regulation of Ser45 phosphorylation by LiCl, CT99021, and LY294002

To study β -catenin phosphorylation, day 8 blastocysts ($n=30$) were loaded and resolved by SDS-PAGE in a 10% acrylamide gel. Immunoblotting was performed using specific antibodies against β -catenin phosphorylated at Thr41, Ser33/37–Thr41/Ser45–Ser552 and Ser675 (1/1000 dilution, incubation overnight at 4 °C). To investigate the

phosphorylation of β -catenin on Ser45, two-cell embryos, after being treated with LY294002 (10 μ M), LiCl (20 mM), or CT99021 (3 μ M) for 3 h, were loaded and resolved by SDS-PAGE in a 10% acrylamide gel. Immunoblotting was performed using the specific antibody against β -catenin phosphorylated at Thr41/Ser45 (1/1000 dilution, incubation overnight at 4 °C; $n=4$ replicates).

Statistical analysis

Data obtained from densitometry of protein bands and comparison between different treatments of bovine embryo development were analyzed by ANOVA. All statistical analyses were carried out using SPSS versus 15.0 software package for Windows (SPSS Inc., Chicago, IL, USA). Differences between groups were considered significant when P values were <0.05 .

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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