Short-Term Culture of In Vitro Produced Bovine Preimplantation Embryos With Insulin-Like Growth Factor-I Prevents Heat Shock-Induced Apoptosis Through Activation of the Phosphatidylinositol 3-Kinase/Akt Pathway

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ABSTRACT
Insulin-like growth factor (IGF-I) has been implicated as a thermoprotective molecule for the preimplantation bovine embryo. Here, it was shown that effects of heat shock (41°C for 15 hr) on induction of apoptosis and reduction in cell number in bovine embryos collected at Day 5 after fertilization were blocked by addition of 100 ng/ml IGF-I at the initiation of heat shock. This action of IGF-I to block heat shock-induced apoptosis was eliminated if embryos were cultured with either a phosphatidylinositol 3-kinase (PI3K) inhibitor (wortmannin) or an Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-o-methyl-3-o-octadecylcarbonate). Immunofluorescence microscopy confirmed the expression of phosphorylated Akt for IGF-I and control embryos. Immunoblotting using an antibody to Akt (phospho S473) indicated increased phosphorylation of Akt in IGF-I-treated embryos. In conclusion, short-term treatment of embryos with IGF-I can block induction of apoptosis caused by heat shock through signaling events requiring PI3K and Akt.


Key Words: insulin-like growth factor-I; bovine; embryo; heat shock; apoptosis

INTRODUCTION
One of the best characterized regulatory molecules for the preimplantation bovine embryo is insulin-like growth factor-I (IGF-I), which in the cow is secreted by the oviduct (Pushpakumara et al., 2002), endometrium (Geisert et al., 1991), and embryo (Lonergan et al., 2000). One of the functions of IGF-I is to act as a cytoprotective molecule for the embryo. Thus, IGF-I reduced effects of hydrogen peroxide on the development of mouse embryos (Kurzawa et al., 2002) and blocked apoptosis in rabbit embryos exposed to UV radiation (Herrler et al., 1998) and in mouse embryos treated with tumor necrosis factor-α, camptothecin, and actinomycin D (Kurzawa et al., 2001; Byrne et al., 2002; Fabian et al., 2004). In the preimplantation bovine embryo, continuous culture with IGF-I beginning after fertilization blocked the detrimental effects of heat shock at Day 5 after fertilization on induction of apoptosis and inhibition of development (Jousan and Hansen, 2004, 2007).

The pathway by which IGF-I regulates apoptosis in preimplantation embryos is not known. Anti-apoptotic actions of IGF-I in many cells are mediated through activation of the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), which in turn leads to the phosphorylation and activation of Akt/protein kinase B (PKB) (Vincent and Feldman, 2002; Mauro and Surrnazc, 2004; Foulstone et al., 2005). This latter kinase plays a major role in the prevention of apoptosis through phosphorylation and subsequent inactivation of pro-apoptotic proteins involved in apoptosis, including Bad, caspase-9, and forkhead transcription factors (Brazil and Hemmings, 2001; Vara et al., 2004; Song et al., 2005). Additionally, Akt activates the transcription factor cyclic AMP response element-binding protein and IκB kinase, which phosphorylates IκB and targets it for degradation to lead in turn to the activation of nuclear factor-kB and the transcription of anti-apoptotic genes such as Bcl-2 and Bcl-xL (Mitsiades et al., 2002; Osaki et al., 2004; Thompson and Thompson, 2004). IGF-I also activates the mitogen-activated protein kinase kinase (MAPKK) pathway to increase cell proliferation and contribute to inhibition of apoptosis (Jones and Clemmons, 1995; Rubinfeld and Seger, 2005).

As stated previously, continuous culture with IGF-I beginning after fertilization blocked the detrimental effects of heat shock on bovine embryos at Day 5 after
fertilization on induction of apoptosis and inhibition of development (Jousan and Hansen, 2004, 2007). Studies with PI3K (LY 294002) and MAPK inhibitors (PD 98059) indicated that the anti-apoptotic actions of IGF-I required the PI3K pathway whereas the action of IGF-I to promote proliferation and blastocyst development required the MAPK pathway (Jousan and Hansen, 2007). In these experiments in which embryos were chronically exposed to IGF-I, it is possible that some of the anti-apoptotic actions of IGF-I represent differentiation events mediated by IGF-I rather than acute activation of signaling pathways. The objective of the present series of studies was to test whether short-term addition of IGF-I to culture medium of bovine preimplantation embryos would block induction of apoptosis caused by heat shock and, if so, to test whether these anti-apoptotic actions of IGF-I involve the PI3K/Akt pathway.

MATERIALS AND METHODS

Materials

Unless otherwise mentioned, reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA). HEPES-Tyrode Lactate (TL), IVF-TL, and Sperm-TL solutions were purchased from Caissong (Sugar City, ID) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP as previously described (Parrish et al., 1986). Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hank’s salts without phenol red (Atlanta Biologicals, Norcross, GA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was TCM-199 (Gibco, Grand Island, NY) with Earle’s salts supplemented with 10% (v/v) bovine steer serum, 2 µg/ml estradiol-17β, 20 µg/ml bovine FSH (Folltrropin-V; Vetrepharm Canada, London, ON), 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, and 1 mM glutamine. Percoll was from Amer sham Pharmacia Biotech (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL).

Potassium simplex optimized medium (KSOM) that contained 1 mg/ml BSA was obtained from Cell and Molecular Technologies or Caisson. Essentially fatty-acid free (EFAF) BSA was from Sigma (St. Louis, MO). Recombinant human IGF-I was purchased from Upstate Biotechnology (Lake Placid, NY). On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto et al., 2003). For the addition of IGF-I to KSOM-BE2, a vial containing 25 µg of lyophilized IGF-I was rehydrated with 100 µl of 0.1 M acetic acid. This solution was then stored at −20°C in 5 µaliquots until use, when a single aliquot of IGF-I was diluted with KSOM-BE2 to a concentration of 100 ng/ml. Control media contained an equivalent volume of acetic acid. Dimethyl sulfoxide (DMSO) and wortmannin (PI3K inhibitor) were obtained from Sigma and the Akt inhibitor (1L-6-hydroxymethylchiro-inositol 2-(R)-2-o-methyl-3-octadecylcarbonate; HIMO) was from Calbiochem (San Diego, CA). For the addition of the PI3K inhibitor, a vial containing 1 mg of wortmannin was rehydrated with 23.34 µl of 100% DMSO and stored at −20°C in 1.25 µl aliquots until use when a single aliquot was diluted in KSOM-BE2 ± IGF-I to a concentration of 100 nM. For the addition of the Akt inhibitor, a vial containing 1 mg of HIMO was rehydrated with 172 µl DMSO and stored at −20°C in 1.25 µl aliquots until use when a single aliquot was diluted in KSOM-BE2 ± IGF-I to a concentration of 10 µM. The final concentration of DMSO in all treatments was 0.1% (v/v). A 10× solution of phosphate-buffered saline (PBS; 0.1 M KPO₄, 9% NaCl, and 0.2% NaNO₃ at pH 7.4) was diluted 1:10 with double distilled water to make a 1× solution of PBS.

The In Situ Cell Death Detection Kit (rhodamine) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). The Zenon Alexa Fluor 488 rabbit IgG labeling kit was purchased from Molecular Probes (Eugene, OR) while Hoechst 33342 was purchased from Sigma. The rabbit polyclonal antibody to phosphorylated Akt1 (phospho S473) was obtained from Abcam, Inc. (Cambridge, MA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was from GE Healthcare (Piscataway, NJ). Rabbit IgG was purchased from Sigma. Polyvinylpyrrolidone (PVP) was from Eastman Kodak (Rochester, NY) and RQ1 RNase-free DNase was from Promega (Madison, WI). Paraformaldehyde (8%) was purchased from Electron Microscopy Sciences (Fort Washington, PA).

In Vitro Production of Embryos

Embryo production was performed using in vitro maturation of oocytes and in vitro fertilization as previously described (Jousan and Hansen, 2004, 2007). Putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 ml of 1,000 U/ml hyaluronidase in HEPES-TALP, placed in groups of 30 in 50 µl microdrops of KSOM-BE2, overlaid with mineral oil, and cultured at 38.5°C in an atmosphere of 5% (v/v) CO₂ in humidified air until selected for treatment at Day 5 after insemination. At this time, embryos ≥16 cells were harvested from culture drops according to the specific experimental design.

TUNEL and Hoechst 33342 Nucleic Acid Labeling

The TUNEL assay was performed as described previously (Jousan and Hansen, 2004, 2007) using TMR red-conjugated dUTPs and the enzyme terminal deoxynucleotidyl transferase to label nuclei of apoptotic cells and Hoechst 33342 to label all nuclei. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Labeling was observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for TUNEL-positive blastomeres (red nuclei) and
total cell number (blue nuclei) with rhodamine and DAPI filters, respectively, using a 20× objective. Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera.

Experiments

Effectiveness of acute exposure of preimplantation bovine embryos to IGF-I in blocking apoptosis caused by heat shock. Embryos ≥16 cells were collected on Day 5 after insemination. Approximately half of the harvested embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml IGF-I while the other half were cultured in a fresh drop of KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I-treated embryos. The experiment was designed as a 2 × 2 factorial arrangement of treatments to determine if addition of IGF-I starting coincidently with heat shock was effective in blocking the induction of apoptosis. Embryos were maintained at 38.5°C for 24 hr or were heat shocked at 41°C for 15 hr. All harvested embryos were fixed in 4% (v/v) paraformaldehyde (by diluting 8% paraformaldehyde 1:1 with PBS) on Day 6. Embryos were then stored in PBS containing PVP (1 mg/ml; PBS-PVP) at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated five times using 68–74 embryos per treatment.

Inhibition of PI3K. Embryos ≥16 cells were collected on Day 5 after insemination. Approximately half of the harvested embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml of IGF-I while the other half were cultured in a fresh drop of KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I-treated embryos. The experiment was designed as a 2 × 2 × 2 factorial arrangement of treatments to determine the role of IGF-I in preventing the induction of apoptosis caused by heat shock. Embryos were maintained at 38.5°C or were heat shocked at 41°C for 15 hr followed by culture at 38.5°C for 9 hr. All harvested embryos were fixed in 4% (v/v) paraformaldehyde and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated five times using 68–74 embryos per treatment.

Inhibition of Akt. Embryos ≥16 cells were collected on Day 5 after insemination. Approximately half of the harvested embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml of IGF-I while the other half were cultured in a fresh drop of KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I-treated embryos. The experiment was designed as a 2 × 2 × 2 factorial arrangement of treatments to determine the role of IGF-I in preventing the induction of apoptosis caused by heat shock. Embryos were maintained at 38.5°C or were heat shocked at 41°C for 15 hr followed by culture at 38.5°C while being cultured with 0.1% (v/v) DMSO (vehicle) or 10 μM HIMO reconstituted in 0.1% (v/v) DMSO. Heat shock, IGF-I, and inhibitor treatments were initiated nearly simultaneously. After culture, embryos were fixed in 4% (v/v) paraformaldehyde on Day 6 and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated four times using 24–31 embryos per treatment.

Immunofluorescent analysis of phosphorylated Akt. Embryos ≥16-cells were collected on Day 5 after insemination. Approximately half of the harvested embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml of IGF-I while the other half were cultured in a fresh drop of KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I-treated embryos. Embryos were cultured at 38.5°C for 10 min or 1 hr in humidified air, fixed in 4% (v/v) paraformaldehyde, and stored in PBS-PVP at 4°C until immunofluorescent analysis using an antibody to phosphorylated Akt1. The staining was replicated three times using approximately 20 embryos per treatment.

The procedure for immunofluorescence was as follows. A rabbit antibody against phosphorylated Akt1 (at amino acid S473; initial concentration of 900 μg/ml) was diluted to ~135 μg/ml and then labeled with Fab fragments against rabbit IgG conjugated to Alexa Flour 488 using the Zenon Alexa Fluor 488 rabbit IgG labeling reagent as per manufacturer’s instructions. The labeled antibody complex was then diluted in antibody buffer at a final concentration of 45 μg/ml. All immunostaining steps were performed using microdrops covered with mineral oil in a humidified chamber in the dark. The blocking buffer consisted of PBS containing 20% (v/v) normal goat serum and the antibody buffer contained PBS + 2% (w/v) bovine serum albumin. Fixed embryos were permeabilized [0.5% (v/v) Triton-X, 0.1% (w/v) sodium citrate] for 15 min at room temperature and then washed two times in antibody buffer. Embryos were blocked for 2 hr in 50 μl drops of the blocking buffer and then incubated for 2 hr in 45 μg/ml of the Zenon-labeled Akt1 antibody. As a negative control, rabbit IgG labeled similarly to the anti-Akt1 was used. Embryos were washed two times in antibody buffer and fixed a second time in 4% (v/v) paraformaldehyde for 15 min at room temperature. Embryos were washed two times in antibody buffer and mounted on 10% (w/v) poly-I-lysine-coated slides using 4–5 μl drops of glycerol, and coverslips were placed on the slides. Labeling of embryos was observed using a Zeiss Axioplan 2 epifluorescence microscope. Each embryo was analyzed for phosphorylated Akt1 (green staining) with a FITC filter using a 40× objective. Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera.

Analysis of phosphorylated Akt1 by Western blotting. Embryos ≥16-cells were collected on Day 5 after insemination. Approximately half of the harvested embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml of IGF-I while the
other half were cultured in a fresh drop of KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I-treated embryos. Embryos were cultured at 38.5°C for 10 min. Immediately afterwards, embryos were frozen at −80°C in groups of 25 in 4 μl PBS until analysis by Western blotting. For sample preparation, four groups containing 100 embryos total from each treatment were thawed and lysed by two cycles of freeze thawing. Embryos were solubilized by adding an equal volume of 125 mM Tris-HCl buffer (pH 6.8) containing 10% (w/v) sodium dodecyl sulfate (SDS), 20% (w/v) sucrose, and 5% (v/v) 2-mercaptoethanol. The lysed embryos (100 per lane) were separated under reducing conditions using one-dimensional, discontinuous SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with 4–15% (w/v) gradient polyacrylamide gels and Tris-HCl buffer and transferred electrophoretically to Hybond ECL 0.2 mm nitrocellulose membranes. Membranes were blocked overnight in TBS-T [10 mM Tris pH 7.6, 0.9% (w/v) NaCl, and 0.3% (v/v) Tween-20] that also contained 1% (w/v) gelatin (TBS-TG). After washing, membranes were incubated overnight at room temperature with a rabbit antibody recognizing phosphorylated Akt1 (S473, 1:200 in TBS-TG), washed, and then incubated for 2 hr at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:1,000 dilution in TBS-TG). After additional washing, blots were developed using the ECL Plus Western blotting detection reagents (GE Healthcare). The membrane was exposed to X-ray film for 5 min.

**Statistical Analysis**

Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, Version 8, 1999–2001, Cary, NC). Percentage data were transformed by arcsin transformation before analysis. Independent variables included IGF-I, temperature, inhibitor (when present in the experiment), and replicate. The mathematical model included main effects and all interactions. Tests of significance were made using errors terms based on replicate being a random effect and other main effects being fixed. When more than one inhibitor was used in an experiment, additional analyses were performed for each inhibitor subset. Unless otherwise mentioned, P-values reported for these experiments are based on analysis of these subsets. All values reported are least-squares means ± SEM. Probability values for percentage data are based on the analysis of arcsine-transformed data while least-squares means are from the analysis of untransformed data.

**RESULTS**

**Acute Exposure to IGF-I Blocks Apoptosis Caused by Heat Shock**

Culture of embryos with IGF-I added at the initiation of heat shock blocked the induction of apoptosis caused by heat shock (Fig. 1A). In particular, heat shock increased the number of apoptotic blastomeres and IGF-I treatment prevented this increase (IGF-I × heat shock, \( P < 0.001 \)). Heat shock reduced total cell number (\( P < 0.01 \)) but there was no effect of IGF-I or IGF-I × heat shock (Fig. 1B).

**Inhibition of PI3K Eliminates the Anti-Apoptotic Actions of IGF-I**

For embryos cultured in the DMSO vehicle, heat shock increased the percentage of blastomeres that were TUNEL-positive and IGF-I blocked induction of apoptosis caused by heat shock (Fig. 2A; IGF-I × heat shock, \( P < 0.01 \)). For embryos cultured in wortmannin, heat shock increased the number of TUNEL-positive nuclei (\( P < 0.05 \)) but IGF-I was no longer effective in blocking the induction of apoptosis caused by heat shock (IGF-I × heat shock, \( P = 0.73 \)). Analysis of the entire dataset revealed that the treatment with wortmannin increased the number of TUNEL-positive nuclei (\( P < 0.001 \)).

The other endpoint determined was total cell number at Day 6. Analysis of the combined dataset revealed that IGF-I blocked the reduction in embryo total cell number caused by heat shock in the presence and absence of...
wortmannin (Fig. 2B; IGF-I × heat shock, \( P = 0.07 \)) although culture of embryos with wortmannin decreased total cell number (\( P < 0.01 \)).

**Inhibition of Akt Eliminates the Anti-Apoptotic Actions of IGF-I**

For embryos cultured in the DMSO vehicle, heat shock increased the percentage of blastomeres that were TUNEL-positive and IGF-I was effective in blocking induction of apoptosis caused by heat shock (Fig. 3A; IGF-I × heat shock, \( P < 0.001 \)). For embryos cultured with HIMO, heat shock increased the number of TUNEL-positive nuclei (\( P < 0.05 \)) but IGF-I did not block this effect (IGF-I × heat shock, \( P = 0.71 \)). For the combined dataset, heat shock (\( P < 0.05 \)) and culture of embryos with HIMO (\( P < 0.05 \)) increased the number of TUNEL-positive nuclei but IGF-I was not able to block the induction of TUNEL-positive nuclei in the presence of HIMO (IGF-I × heat shock × inhibitor, \( P < 0.01 \)).

For the subset of embryos cultured in the DMSO vehicle, IGF-I blocked the reduction in total cell number at Day 6 caused by heat shock (Fig. 3B; IGF-I × heat shock, \( P < 0.05 \)). There were no effects of heat shock, IGF-I, or IGF-I × heat shock for embryos cultured in the presence of HIMO. Analysis of the entire dataset revealed that IGF-I blocked the reduction in total cell number caused by heat shock (IGF-I × heat shock; \( P < 0.05 \)).

**Immunolocalization of Phosphorylated Akt1**

Specific immunolabeling was detected for all embryos labeled with an antibody that recognized Akt1 phosphorylated at S473 (compare Fig. 4B, D labeled with antibody to Fig. 4A, C labeled with rabbit IgG). Differences between control and IGF-I-treated embryos were not apparent (Fig. 4A, B for control embryos and Fig. 4C, D for IGF-I-treated embryos) at either 10 min or 1 hr after IGF-I treatment.
Immunoblotting of Phosphorylated Akt1

Phosphorylated Akt1 was detected by Western blotting as a major band of 61 kDa (Fig. 5). In addition, several immunoreactive bands of lower molecular weight were also detected. Phosphorylated Akt1 was present in control embryos and embryos treated with IGF-I and the amount of immunoreactive product was greater for embryos treated with IGF-I.

**DISCUSSION**

Results of the current study confirm the thermoprotective actions of IGF-I in the preimplantation bovine embryo seen earlier (Jousan and Hansen, 2004, 2007). In particular, IGF-I blocked the increase in apoptosis caused by heat shock. Moreover, this thermoprotective effect, which in earlier studies was tested in embryos exposed to IGF-I for several days, was initiated upon short-term exposure to IGF-I. Short-term IGF-I treatment also prevented the reduction in embryo total cell number caused by heat shock. Thus, the thermoprotective effects of IGF-I in the current study were not the result of some differentiation or other long-term effect of IGF-I (e.g., increased cell number) but were rather the result of activation of signal transduction systems acting within minutes after IGF-I treatment.

Embryos at Day 5 clearly contain activated Akt as revealed by immunofluorescence and Western blotting using the antibody against phosphorylated Akt1. The major immunoreactive band found by Western blotting controls were rabbit IgG (panels A and C). Examples of control embryos are depicted in panels A and B, while IGF-I-treated embryos are depicted in panels C and D. In general, images appeared similar for control embryos and embryos treated with IGF-I. Bars depict 50 μM.

**Fig. 4.** Representative fluorescent images of control and IGF-I-treated embryos that were immunolabeled with antibody against phosphorylated Akt1. Embryos were cultured for 1 hr before labeling. While not shown, images appeared similar for embryos cultured for 10 min before labeling. The rabbit antibody used recognized Akt1 phosphorylated at Ser 473 (panels B and D) while labeling controls were rabbit IgG (panels A and C). Examples of control embryos are depicted in panels A and B, while IGF-I-treated embryos are depicted in panels C and D. In general, images appeared similar for control embryos and embryos treated with IGF-I. Bars depict 50 μM.

**Fig. 5.** Increased phosphorylation of Akt1 by IGF-I. Embryos were cultured with or without 100 ng/ml IGF-I and collected for analysis after 10 min. Solubilized and reduced embryo proteins were separated by SDS–PAGE and analyzed by immunoblotting with an antibody that recognizes Akt1 phosphorylated at Ser 473. Note the presence of a major immunoreactive band at 61 kDa. Numbers and lines on the left of the blot represent migration distances of molecular weight standards (kDa). Each lane represents a pool of 100 embryos.
had an apparent molecular of 61 kDa that is similar to results for other cells (Cross et al., 1995; Kim et al., 2007). The immunoreactive bands of lower molecular weight are characteristically seen with the antibody used (manufacturer data sheet) and probably represent proteolytic products of Akt1. In addition, inhibition of PI3K with wortmannin or Akt with HIMO increased the number of TUNEL-positive cells even in the absence of IGF-I.

Results also clearly demonstrate that the anti-apoptotic effects of IGF-I resulted from activation of the PI3K/Akt pathway because anti-apoptotic actions of IGF-I were eliminated when either wortmannin or HIMO was added. While immunofluorescence was not precise enough to identify differences in amounts of phosphorylated Akt1 between control and IGF-I-treated embryos, increased phosphorylation of Akt1 caused by IGF-I was apparent from Western blotting. Thus, the bovine preimplantation embryo uses the same pathway for anti-apoptotic effects of IGF-I as other cell types (Nitta et al., 2004; Zheng and Quirion, 2004; Bridgewater et al., 2005; Scott et al., 2005).

Akt can phosphorylate and inhibit pro-apoptotic proteins such as Bad (del Peso et al., 1997) and caspase-9 (Cardone et al., 1998) and phosphorylate and stabilize X-linked inhibitor of apoptosis proteins involved in cell survival (Cheng et al., 2002; Dan et al., 2004). In addition, Akt can phosphorylate and inhibit the forkhead transcription factors, thereby preventing the induction of genes involved in pro-apoptotic processes such as Bim and Bax (Brunet et al., 2001; Nitta et al., 2004). Due to the inhibition of pro-apoptotic proteins by Akt, levels of anti-apoptotic proteins such as Bcl-2 and Bcl-x are increased (Leverrier et al., 1999; Chrysis et al., 2001).

IGF-I also prevented the reduction in embryo total cell number caused by heat shock. This effect, which has been reported earlier, appears to require signaling through the MAPKK pathway because addition of the MAPKK inhibitor PD 98059 abolished the ability of IGF-I to block the reduction in total cell number caused by heat shock (Jousan and Hansen, 2007). Present results are consistent with the idea that effects of IGF-I on proliferation involve MAPKK rather than the PI3K pathway because IGF-I was still effective in blocking the reduction in total cell number caused by heat shock in the presence of wortmannin.

An unanswered question is whether IGF-I is effective at blocking effects of heat shock at all stages of preimplantation development. At least in mice, the PI3K/Akt pathway seems to be intact from the earliest stages of development because expression of the p85 and p110 subunits of PI3K and Akt occurs at all stages from the one-cell through blastocyst stage (Riley et al., 2005). Moreover, PI3K activity is important for glucose metabolism and embryo survival (Riley et al., 2006).

The cytoprotective actions of IGF-I may be beneficial to the bovine embryo during early development. Maternal hyperthermia experienced by pregnant females as a consequence of elevated temperatures can impede continued embryonic development (Ealy et al., 1993). Concentration of IGF-I is high in peripheral blood and is also produced by the oviduct (Pushpakumara et al., 2002), endometrium (Geisert et al., 1991) and embryo (Lonergan et al., 2000). It is possible that IGF-I in the reproductive tract, from local and systemic sources, creates a thermoprotective environment that limits deleterious effects of heat shock. Given that IGF-I protects embryos from various inducers of apoptosis, such as hydrogen peroxide (Kurzawa et al., 2002), UV radiation (Herrler et al., 1998), camptothecin and actinomycin D (Fabian et al., 2004), and tumor necrosis factor-α (Kurzawa et al., 2001; Byrne et al., 2002), it is likely that IGF-I has protective effects against a variety of adverse environmental agents.

In conclusion, short-term treatment of the bovine preimplantation embryo with IGF-I can block induction of heat shock-induced apoptosis through the PI3K/Akt pathway and also prevent the reduction in total cell number caused by heat shock through a pathway that is likely independent of PI3K/Akt.

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Molecular Reproduction and Development

688  F.D. JOUSAN ET AL.


