The block to apoptosis in bovine two-cell embryos involves inhibition of caspase-9 activation and caspase-mediated DNA damage

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Abstract

The capacity of the preimplantation embryo to undergo apoptosis in response to external stimuli is developmentally regulated. Acquisition of apoptosis does not occur in the cow embryo until between the 8- and 16-cell stages. The purpose of the present experiments was to determine the mechanism by which apoptosis is blocked in the bovine two-cell embryo. Heat shock (41 °C for 15 h) did not increase activity of caspase-9 or group II caspases (caspase-2, -3, and -7) in two-cell embryos but did in day 5 embryos. Exposure of embryos to carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to depolarize mitochondria resulted in activation of caspase-9 and group II caspases at both stages of development. For day 5 embryos, CCCP also increased the proportion of blastomeres that underwent DNA fragmentation as determined by the TUNEL assay. In contrast, CCCP did not increase TUNEL labeling when applied at the two-cell stage. In conclusion, failure of heat shock to increase caspase-9 and group II caspase activity in the two-cell embryo indicates that the signaling pathway leading to mitochondrial depolarization and caspase activation is inhibited at this stage of development. The fact that CCCP treatment of two-cell embryos induced caspase-9 and group II-caspase activity indicates that caspase activation is possible following mitochondrial depolarization. However, since CCCP did not increase TUNEL labeling of two-cell embryos, actions of group II-caspases to activate DNases is inhibited.

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Introduction

For the preimplantation mammalian embryo, apoptosis can play an important role in determining survival following cellular stress. The best studied example is the bovine embryo produced *in vitro*. Among the inducers of apoptosis in this organism are elevated temperature (i.e., heat shock; Paula-Lopes & Hansen 2002a, 2002b, Jousan & Hansen 2004), arsenic (Kringer *et al.* 2002), tumor necrosis factor- α (TNF- α ; Soto *et al.* 2003, Loureiro *et al.* 2007), and pro-oxidants (Feugang *et al.* 2004). The consequences of apoptosis for embryonic survival depend upon its extent. A massive increase in the proportion of blastomeres that become apoptotic is detrimental to embryonic survival. Indeed, use of RNA interference to reduce amounts of the anti-apoptotic protein, survivin, decreased blastocyst development in bovine embryos (Park *et al.* 2006). However, signals for apoptosis such as exposure to heat shock of 41 °C or TNF- α cause only about 15–25% of blastomeres to become apoptotic (Kringer *et al.* 2002, Paula-Lopes & Hansen 2002a, 2002b, Soto *et al.* 2003, Jousan & Hansen 2004, Loureiro *et al.* 2007), and this degree of apoptosis is not necessarily deleterious to sustained

embryonic development. In fact, TNF- α does not reduce the proportion of embryos that become blastocysts (Soto *et al.* 2003). For heat shock, limited apoptosis can be an adaptive response that facilitates survival of the embryo after stress. This conclusion is based on observations that inhibition of apoptosis responses using the group II caspase inhibitor z-DEVD-fmk exacerbated the deleterious effects of elevated temperature on development of bovine preimplantation embryos (Paula-Lopes & Hansen 2002b, Jousan & Hansen 2007).

Like for other cells, heat shock induces apoptosis in the preimplantation bovine embryo through activation of the mitochondrial or intrinsic pathway. Culture at 41 °C causes activation of caspase-9 and caspase-3 activity (Kringer *et al.* 2002, Paula-Lopes & Hansen 2002a, 2002b, Loureiro *et al.* 2007). Moreover, induction of TUNEL-positive cells by culture at 41 °C can be blocked by inhibitors of caspase-9 or caspase-3 activity (Paula-Lopes & Hansen 2002a, Loureiro *et al.* 2007). TNF- α also utilizes the mitochondrial pathway in the bovine embryo, probably through caspase-8-dependent activation of mitochondrial depolarization (Loureiro *et al.* 2007).

Induction of apoptosis is a developmentally regulated event. TUNEL-positive cells are first seen in embryos cultured *in vitro* between the six- and eight-cell stages of development (Matwee *et al.* 2000, Gjørret *et al.* 2003). Acquisition of apoptosis responses in response to heat shock first develops around day 4 after insemination, when the embryo is between the 8- and 16-cell stages (Paula-Lopes & Hansen 2002a). Induction of apoptosis by TNF- α also first occurs in embryos after the eight-cell stage (Soto *et al.* 2003). The mechanism by which apoptosis is blocked before this stage is not known. Addition of the protein kinase inhibitor staurosporine caused apoptosis in 1- to 16-cell embryos (Matwee *et al.* 2000), so the biochemical machinery for apoptosis is present in the two-cell embryo. Here, we report results of experiments that indicate that apoptosis in response to heat shock is inhibited in the two-cell embryo at two points in the mitochondrial pathway – caspase-9 activation and caspase-mediated DNA damage.

Results

Caspase-9 activity following heat shock

In the first experiment, two-cell and day 5 embryos were cultured at 38.5 or 41.0 °C for 15 h and then assayed for caspase-9 activity. Representative fluorescent images of caspase-9 activity from groups of embryos are presented in Fig. 1A–D and the proportion of embryos classified as having high caspase-9 activity is presented in Fig. 1E. There was little detectable caspase-9 activity in two-cell embryos cultured at either 38.5 or 41 °C (Fig. 1A and C). In contrast, caspase-9 activity was present in a fraction of day 5 embryos cultured at both temperatures (Fig. 1B and D) and the proportion of embryos classified as having high caspase-9 activity was increased ($P<0.05$) for embryos cultured at 41 °C as compared with those cultured at 38.5 °C (Fig. 1E).

Caspase-9 activity following heat shock and treatment with carbonyl cyanide 3-chlorophenylhydrazone (CCCP)

Two-cell and day 5 embryos were cultured with and without CCCP for 15 h at either 38.5 or 41.0 °C. Following culture, embryos were assessed for caspase-9 activity. Data are presented in Fig. 2. For embryos cultured with vehicle, results were similar to the previous experiment. There was little caspase activity in two-cell embryos and heat shock did not increase amounts of active caspase-9 (compare Fig. 2A with B). No embryos were classified as having high caspase-9 activity (Fig. 2I). For day 5 embryos, caspase-9 could be detected in some embryos and the amount of caspase-9 was greater for embryos at 41 °C than for embryos at 38.5 °C (compare Fig. 2C with D). Treatment with CCCP

Figure 1 Differential effects of heat shock on caspase-9 activity in two-cell and day 5 bovine embryos. Representative images of caspase-9 activity are shown for two-cell (A and C) and day 5 (B and D) embryos cultured at 38.5 °C (A and B) or 41.0 °C (C and D) for 15 h before caspase assay. The lower panel (E) indicates the percentage of embryos classified as exhibiting high caspase-9 activity. Temperature affected the proportion of embryos classified as having high caspase-9 activity for day 5 embryos only ($P<0.05$).

to chemically depolarize mitochondria caused a massive change in caspase activity (Fig. 2E–H). In particular, CCCP caused an increase in the proportion of embryos exhibiting caspase-9 activity as well as in the intensity of caspase-9-dependent fluorescence in individual embryos. The effect of CCCP occurred for both two-cell embryos (Fig. 2E and F) and day 5 embryos (Fig. 2G and H) and for embryos cultured at both 38.5 (Fig. 2E and G) and 41.0 °C (Fig. 2F and H).

Data on the proportion of embryos classified as having high caspase-9 activity are shown in Fig. 2I for two-cell embryos and Fig. 2J for day 5 embryos. There was a temperature $\times$ treatment interaction ($P<0.001$) affecting the proportion of embryos with high caspase activity for both two-cell and day 5 embryos. For two-cell embryos, no embryos were classified with high caspase activity in vehicle treatments. Following culture with CCCP, the proportion of embryos with high caspase activity increased

Figure 2 Caspase-9 activity in two-cell and day 5 (≥ 16 cell) embryos following mitochondrial depolarization with carbonyl cyanide 3-chloro-phenylhydrazone (CCCP). Representative images of caspase-9 activity are shown in the upper panel. Embryos were cultured at 38.5 °C (A, C, E, and G) or 41.0 °C (B, D, F, and H) for 15 h in either dimethyl sulfoxide (DMSO; A–D) or CCCP (E–H). Data on the percentage of embryos classified with high caspase-9 activity following culture are shown in I (two-cell embryos) and J (day 5 embryos). The percentage was affected by the temperature $\times$ treatment interaction ($P < 0.001$) at both stages of development.

to 30% for embryos at 38.5 °C and to 93% for embryos at 41 °C (Fig. 2I). Thus, CCCP activated caspase-9, and the activation was greater for embryos at 41 °C. For day 5 embryos treated with vehicle, 41 °C increased the proportion of embryos with high caspase-9 activity as compared with embryos at 38.5 °C (Fig. 2J). Again, CCCP increased the percentage of embryos classified with high caspase activity at 38.5 °C and, to a greater extent, at 41 °C.

Group II caspase activity following heat shock and treatment with CCCP

Two-cell and day 5 embryos were cultured with and without CCCP for 15 h at 38.5 or 41.0 °C and then assessed for group II caspase activity using a fluorescent probe that is cleaved by active caspase-2, -3, and -7 (Fig. 3). Caspase activity was low in two-cell embryos and heat shock did not increase activity (compare Fig. 3A with B). For day 5 embryos, more embryos had detected group II caspase, especially for embryos cultured at 41 °C (Fig. 3C and D). As for caspase-9, CCCP treatment increased group II caspase activity in both two-cell embryos and day 5 embryos (Fig. 3E–H).

Quantitative analysis is presented in Fig. 3I and J. There was a temperature $\times$ treatment interaction ($P < 0.001$) affecting the proportion of embryos with high caspase activity. This was true for both two-cell embryos (Fig. 3I) and day 5 embryos (Fig. 3J). For two-cell embryos, few embryos treated with vehicle had high group II caspase activity, and heat shock at 41 °C did not increase that proportion. Treatment with CCCP

caused a large increase in the proportion of embryos with high group II caspase activity at both 38.5 and 41 °C. For day 5 embryos, culture of vehicle-treated embryos at 41 °C caused an increase in the proportion of embryos that were classified as having high caspase activity. This increase was smaller, however, than the increase caused by treatment with CCCP. The CCCP-induced increase in caspase II activity was greater for embryos at 41 °C than for embryos at 38.5 °C.

TUNEL labeling in embryos treated with heat shock and CCCP

Representative images of TUNEL analysis are shown in Fig. 4A–H and least squares means $\pm$ S.E.M. for the percentage of nuclei positive for the TUNEL reaction and for total cell number are shown in Fig. 4I–K.

Few two-cell embryos contained TUNEL-positive nuclei regardless of treatment group (see Fig. 4A, B, E, and F for representative images and Fig. 4I for least squares means). Thus, even though CCCP induced caspase-9 and group II caspase activation, it did not result in DNA fragmentation of nuclei in two-cell embryos. Treatment with CCCP did inhibit further development of two-cell embryos as indicated by a reduction in total cell number caused by CCCP ($P < 0.001$; Fig. 4J).

Day 5 embryos responded to both heat shock and CCCP by experiencing an increase in the percentage of nuclei positive for the TUNEL reaction (see Fig. 4C, D, G, and H for representative images). The percentage of

Figure 3 Group II caspase activity in two-cell and day 5 (≥ 16 cell) embryos following mitochondrial depolarization with carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Panels A–H display representative images of group II caspase activity in embryos following culture for 15 h at 38.5 °C (A, C, E, and G) or 41.0 °C (B, D, F, and H) in either dimethyl sulfoxide vehicle (A–D) or CCCP (E–H). Data on the percentage of embryos classified with high group II caspase activity are shown in I (two-cell embryos) and J (day 5 embryos). The percentage was affected by the temperature $\times$ treatment interaction ($P < 0.001$) at both stages of development. The bars on each figure represent 100 μ m.

nuclei positive for the TUNEL reaction was affected by a temperature $\times$ treatment interaction ($P < 0.01$). An increase in the percentage of nuclei positive for the TUNEL reaction was caused by culture at 41 °C and CCCP treatment and the increase caused by 41 °C was greater for embryos treated with CCCP (Fig. 4K). There was no effect of heat shock or CCCP on total cell number (Fig. 4L).

Discussion

As for other cells, the pathway for induction of apoptosis by heat shock in the preimplantation bovine embryo involves the mitochondrial pathway. Heat shock increases the activity of caspase-9 and group II caspases (Krinner *et al.* 2002, Paula-Lopes & Hansen 2002a, 2002b, Loureiro *et al.* 2007), and inhibitors of caspase-9 and caspase-3 block induction of TUNEL-positive cells by 41 °C (Paula-Lopes & Hansen 2002b, Loureiro *et al.* 2007). This pathway is inactivated in the earliest stages of development: heat shock first causes increased TUNEL labeling around day 4 after insemination, when the embryo is between the 8- and 16-cell stages (Paula-Lopes & Hansen 2002a). Using the two-cell embryo as a model, experiments described here indicate that the block to apoptosis occurs at two points in the apoptotic cascade: activation of caspase-9 activity and cleavage of DNA by caspase-3 and other execution caspases. A model illustrating the points in the mitochondrial pathway for apoptosis that are blocked in the two-cell embryo is shown in Fig. 5.

Although heat shock increased caspase-9 activity in day 5 embryos, it had no effect on caspase-9 activity in the two-cell embryo. This lack of enzyme activity at the two-cell stage does not reflect an absence of the components of the apoptosome in the two-cell embryo. Indeed, artificial depolarization of mitochondria with CCCP resulted in a large increase in caspase-9 activity. It is possible, however, that mitochondria from the two-cell embryo are deficient in one or more components of the apoptosome and more mitochondrial depolarization is required to activate procaspase-9.

Mitochondria may also be resistant to depolarization at the two-cell stage. Some mitochondrial depolarization does occur in the two-cell embryo in response to heat shock. Exposure of two-cell embryos to 41 °C for 12 h results in about 7% of the mitochondria exhibiting a swollen phenotype versus 0.7% of mitochondria from embryos at 38.5 °C (Rivera *et al.* 2003). This degree of depolarization may be too low to lead to caspase-9 activation, especially if some apoptosome components are present in reduced quantity. There is a report using polarity-sensitive dyes that mitochondria are more polarized from the two- to eight-cell stages than afterwards (Tarazona *et al.* 2006). The drop in polarity between the 8- and 16-cell stages is coincident with acquisition of capacity for heat shock-induced apoptosis (Paula-Lopes & Hansen 2002a).

Little is known about the developmental changes in the relative abundance of pro-apoptotic and anti-apoptotic members of the *bcl-2* family in the preimplantation bovine embryo; these changes, if occurring, could

Figure 4 TUNEL labeling of nuclei in two-cell and day 5 (≥ 16 cell) embryos following mitochondrial depolarization with carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Representative images of embryos following the TUNEL procedure are shown in the panels A–H. Nuclei were labeled with Hoechst 33342 and those that are TUNEL-positive nuclei are additionally labeled with green fluorescence. Bars in each figure represent 100 μ m. Panels I and J represent least squares means $\pm$ S.E.M. for the percentage of blastomeres that are TUNEL positive (panel I) and cell number (panel J) for embryos treated at the two-cell stage. The percentage of TUNEL-positive cells was not significantly affected by temperature, CCCP, or the interaction. Cell number was reduced by CCCP treatment ($P < 0.001$). Panels K and L represent least squares means $\pm$ S.E.M. for the percentage of blastomeres that are TUNEL positive (panel I) and cell number (panel J) for embryos treated at day 5. The percentage of TUNEL-positive cells was affected by the temperature $\times$ CCCP interaction ($P < 0.01$) and the cell number was not significantly affected by any treatment.

control resistance of mitochondria to depolarizing signals. Transcripts for the pro-apoptotic protein, Bax, were undetectable until the eight-cell stage (Lonergan *et al.* 2003). In contrast, transcripts for Bcl-2 were abundant in pool of embryos from the two- to eight-cell stages (Yang & Rajamahendran 2002). These results, based on transcript abundance and not amounts of protein, are consistent with the idea that the membrane of the Bax/Bcl-2 ratio is less favorable to mitochondrial depolarization early in development.

A key signal for mitochondrial depolarization in response to heat shock is ceramide generated by sphingomyelinase (Chung *et al.* 2003, Jenkins 2003). Almost nothing is known about the functionality of this signaling system in the preimplantation embryo. It is possible, however, that the two-cell embryo is deficient in the molecules leading to ceramide biosynthesis or in

other signaling systems involved in heat shock-induced apoptosis like c-Jun N-terminal kinase (Chung *et al.* 2003, Hayashi *et al.* 2004).

Another possibility is that the two-cell embryo has increased amounts of molecules that block caspase-9 activation. One of these, survivin, is present in the preimplantation bovine embryo. However, amounts of survivin are reduced at the two-cell stage (Park *et al.* 2006) and are unlikely to be a determining factor in the failure of the embryo at this stage of development from undergoing apoptosis. Another inhibitor of caspase-9 activation, X-linked inhibitor of apoptosis, is present at the blastocyst stage of development (Knijn *et al.* 2005), but it is not known whether it is present in increased amounts early in development.

Failure of caspase-9 activation is not the only cause for the resistance of the two-cell embryo to undergo

Figure 5 Model describing points in the mitochondrial pathway for apoptosis that are inhibited at the two-cell stage of development in the bovine embryo. Induction of apoptosis by heat shock involves depolarization of the mitochondria. The signal for depolarization is likely to involve the activation of sphingomyelinase to produce the signaling molecule ceramide. Mitochondrial depolarization leads to release of various pro-apoptotic molecules into the cytoplasm. One of these, cytochrome c, forms the apoptosome complex with pro-caspase-9 and APAF-1. Cleavage of procaspase-9 to form active caspase-9 results and is followed by activation of caspase-3 and other execution caspases. Among its actions, caspase-3 cleaves caspase-activated DNase and DNA fragmentation results. This pathway is blocked at two points in the two-cell embryo. First, the embryo's capacity for caspase-9 activation is blocked, probably because the signal for depolarization is blocked or the mitochondrial membrane is resistant to depolarization. Secondly, activation of caspase-3 does not lead to DNA degradation, either because caspase-activated DNase is limited or inhibited.

apoptosis. Indeed, the apoptosis pathway is also inhibited at a point downstream from group II caspase activation. Depolarization of mitochondria with CCCP would be expected to lead to DNA fragmentation because of activation of caspase-9 and caspase-3 as well as release of apoptosis-activating factor and endonuclease G from mitochondria (Widlak & Garrard 2005). Indeed, CCCP caused caspase activation and increased TUNEL labeling in day 5 embryos. In the two-cell embryo, in contrast, there was no increased DNA fragmentation in response to CCCP treatment even though caspase-3 was activated. The concentration of CCCP used was adequate to depolarize mitochondria because caspase-9 was activated at concentrations of CCCP as low as 1 μ M. The observation that caspase-9 and caspase-3 were activated by CCCP in the two-cell embryos leads to the speculation that the two-cell embryo may have a deficiency in caspase-activated DNase (CAD), endonuclease G, or the co-factors required for activation of these DNases. One of the co-factors for CAD, histone H1 (Widlak & Garrard 2005), is present in higher quantities in two-cell embryos than later in development (McGraw *et al.* 2006). Another co-factor, topoisomerase II, is present throughout early preimplantation development in the mouse (St Pierre *et al.* 2002). Alternatively, CAD or other components of the process leading to DNA hydrolysis are present in the two-cell embryo but are being actively inhibited by one or more regulatory molecules. This explanation is consistent with the observation that addition of the

protein kinase inhibitor staurosporine can induce apoptosis in two-cell embryos (Matwee *et al.* 2000).

One surprising result was that CCCP was more effective at inducing caspase activation and DNA fragmentation when embryos were cultured at 41 $^{\circ}$ C than at 38.5 $^{\circ}$ C. The most likely explanation for this phenomenon was that integration of CCCP into the cell and mitochondria was amplified at elevated temperature. It is also possible that heat shock-induced changes in mitochondrial polarity enhanced effectiveness of CCCP.

The importance of inhibition of apoptosis responses in the early preimplantation period has not been delineated experimentally. At later stages of development, i.e., at days 4 and 5 after insemination, the capacity for apoptosis can enhance embryonic survival to stress because inhibition of apoptosis responses makes embryos more susceptible to effects of heat shock on development (Paula-Lopes & Hansen 2002b, Jousan & Hansen 2007). Perhaps embryos with only a few cells, such as the two- and four-cell embryos, are less able to survive the loss of a cell to apoptosis than larger embryos later in development. Two-cell bovine embryos can develop to the blastocyst stage after bisection (Loskutoff *et al.* 1993), but loss of both cells by apoptosis would be incompatible with sustained development. Present results indicate that the block to apoptosis that prevents such loss involves inhibition of caspase-9 activation as well as a loss of capacity for function of apoptosis-associated endonucleases.

Materials and Methods

Materials

Solutions of HEPES-Tyrodes lactate (TL), *in vitro* fertilization (IVF)-TL, and sperm-TL were purchased from Caisson Laboratories (Sugar City, ID, USA) 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 was tissue culture medium-199 with Hanks' salts without phenol red (Hyclone, Logan, UT, USA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR, USA) containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine on the day of use. Oocyte maturation medium was tissue culture medium-199 (Invitrogen) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 µg/ml estradiol 17-β, 20 µg/ml bovine follicle-stimulating hormone (Folltropin-V; Vetrephearm Canada, London, ON, Canada), 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, and 1 mM glutamine. Percoll was purchased from GE Healthcare (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL, USA). Potassium simplex optimized medium (KSOM) containing 1 mg/ml BSA was obtained from Caisson Laboratories. Essentially fatty acid-free BSA was purchased from Sigma. On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto *et al.* 2003). CCCP (Sigma) was dissolved in dimethyl sulfoxide (DMSO) to make a 100 mM stock solution. Aliquots were stored at -20 °C until the day of use. Stock solutions of CCCP were diluted in KSOM-BE2 for a final concentration of 100 µM CCCP in 0.1% DMSO. An equivalent amount of DMSO was added to KSOM-BE2 for control media.

The *In Situ* Cell Death Detection Kit (fluorescein) was obtained from Roche Diagnostics Corporation. The Hoechst 33342 dye (Sigma) was used for staining DNA of embryonic cells. The Prolong Antifade Gold Kit was purchased from Promega. Polyvinylpyrrolidone (PVP) was from Eastman Kodak and RQ1 RNase-Free DNase was from Promega. The CaspaLux 9-M₁D₂ and PhiPhiLux-G₁D₂ assay kits were obtained from Oncolmmunin Inc. (Gaithersburg, MD, USA).

In vitro production of embryos

In vitro embryo production was performed as previously described (Soto *et al.* 2003). Briefly, beef and dairy cattle ovaries were obtained from Central Beef Packing Co. (Center Hill, FL, USA). Cumulus-oocyte complexes (COCs) were obtained by slicing 2–10 mm follicles on the surface of ovaries. Complexes with at least three complete layers of compact, intact cumulus cells were washed two times in oocyte collection medium and then matured in groups of ten in 50 µl drops of oocyte maturation medium overlaid with mineral oil (Sigma). Oocytes were matured for 20–22 h at 38.5 °C in an atmosphere of 5% (v/v) CO₂ in humidified air. Following maturation, COCs were washed once in HEPES-TALP and transferred in groups of 30–40 to four-well plates containing 600 µl IVF-TALP and 25 µl of a mixture of 0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% (w/v) NaCl per well. Oocytes were fertilized by addition of ~1 × 10⁶ Percoll-purified spermatozoa from a pool of frozen-thawed semen from three bulls (a different pool of three bulls was

used for each replicate). Following co-culture at 38.5 °C in an atmosphere of 5% (v/v) CO₂ in humidified air for 20–22 h, presumptive zygotes were removed from fertilization wells and denuded of cumulus cells by vortex mixing in 1 ml of 1000 U/ml hyaluronidase in HEPES-TALP. Putative zygotes were placed in groups of 30 in 50 µl drops of KSOM-BE2 and cultured at 38.5 °C in an atmosphere of 5% (v/v) CO₂ in humidified air until embryos were selected for treatment at the two-cell stage (30–32 h post-insemination) or at day 5 after insemination. At day 5, only embryos ≥ 16 cells (i.e., the most advanced embryos in the dish where individual cells could not be visualized) were selected.

Caspase assays

Embryos were washed three times in 50 µl drops of pre-warmed HEPES-TALP and then placed in 25 µl drops of HEPES-TALP containing 5 µM of either CaspaLux 9-M₁D₂ (caspase-9 substrate) or PhiPhiLux-G₁D₂ (group II caspase specific for caspase-2, -3, and -7) at 38.5 °C for 40 min in the dark. Negative control embryos were incubated only in HEPES-TALP. Following incubation, embryos were washed four times in 50 µl HEPES-TALP and placed onto two-well slides containing HEPES-TALP. Caspase activity was examined using a Zeiss Axioplan microscope (Zeiss, Göttingen, Germany). AxioVision software and an AxioCam MRm digital camera (Zeiss) were used to acquire images of embryos. Embryos were classified based on the fluorescence intensity as low (none or a few fluorescent cells), medium (less than half of the cells fluorescent), or high (more than half of the cells fluorescent) caspase activity.

TUNEL labeling

DNA fragmentation was determined by means of terminal deoxynucleotidyl TUNEL. Immediately following the caspase assay, embryos were washed in 50 µl of 10 mM KPO₄ (pH 7.4) containing 0.9% (w/v) NaCl (PBS) and 1 mg/ml PVP (PBS-PVP). Embryos were fixed in 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature. Embryos were stored in PBS-PVP and stored at 4 °C until the time of TUNEL assay. The procedure for performing the TUNEL assay was described elsewhere (Roth & Hansen 2004).

Experiments

Caspase-9 activity in heat shocked two-cell and day 5 (≥ 16-cell) embryos

Two-cell embryos were selected 30–32 h after insemination and embryos that were ≥ 16 cells were selected at day 5 after insemination. Embryos were placed into fresh drops of KSOM-BE2 (up to 30 embryos per drop) and cultured at 38.5 or 41.0 °C for 15 h. A heat shock of 41.0 °C for 15 h was chosen because this stress consistently induces apoptosis in a fraction of the blastomeres of day 5 embryos (Jousan & Hansen 2007, Loureiro *et al.* 2007). Immediately following culture, the caspase-9 assay was performed on embryos as previously described. The experiment was replicated three times using a total of 134 two-cell embryos (67 embryos/group) and a total of 151, day 5 embryos (70–81 embryos/group).

Caspase-9 activity in two-cell and day 5 embryos following mitochondrial depolarization with CCCP

Two-cell and day 5 embryos (≥ 16 -cells) were harvested and placed into fresh drops of KSOM-BE2 containing either 0.1% DMSO (as vehicle) or 100 μ M CCCP (a protonophore that depolarizes mitochondria; Terada 1981). In a pilot experiment, a similar degree of caspase-9 activation in two-cell embryos occurred at 1, 10, 100, and 200 μ M. Embryos were cultured at 38.5 or 41.0 °C for 15 h and then subjected to the caspase-9 assay immediately afterwards. A total of four replicates were completed for two-cell embryos using 170 embryos (41–44 embryos/group). For day 5 embryos, the experiment was replicated two times using 139 embryos (33–36 embryos/group).

Group II caspase activity in two-cell and day 5 embryos following mitochondrial depolarization with CCCP

This experiment was conducted as described in the previous paragraph except that group II caspase activity was measured following 15 h culture at 38.5 or 41.0 °C. Immediately following culture, the group II caspase assay was performed as previously described. For caspase assay, the experiment was replicated five times for two-cell embryos using a total of 255 embryos (63–65 embryos/group). The experiment was replicated four times for day 5 embryos using a total of 217 embryos (54–55 embryos/group).

TUNEL labeling in two-cell and day 5 embryos following mitochondrial depolarization with CCCP

Following caspase assay, embryos described above were fixed in 4% (w/v) paraformaldehyde and analyzed using the TUNEL assay to determine the number of apoptotic nuclei. For two-cell embryos, the TUNEL assay was performed using five replicates for a total of 182 embryos ($n=43$ –48 embryos/treatment). For day 5 embryos, the TUNEL assay was performed using six replicates and 315 total embryos ($n=78$ –79 embryos per treatment).

Statistical analysis

Data on the percentage of cells that were TUNEL positive were analyzed by least squares ANOVA using the general linear models procedure of SAS (SAS for Windows, Version 9.0, Cary, NC, USA). Percentage data were transformed by arcsin transformation before analysis. The mathematical model included main effects and all interactions. For example, an experiment with main effects of replicate, temperature, and CCCP ($\pm$) used a mathematical model with effects of temperature, CCCP, temperature $\times$ CCCP, replicate, replicate $\times$ temperature, and replicate $\times$ CCCP. Replicate was considered as a random effect and other main effects were considered fixed. Tests of significance were made using error terms determined by the calculation of expected mean squares. All values reported are least squares means $\pm$ S.E.M. Probability values for the percentage data are based on analysis of arcsin-transformed data, while least squares means are from analysis of untransformed data. Categorical data regarding the percentage of embryos classified as having low, medium, or high

caspase activity were analyzed by the CATMOD procedure of SAS using the effects of temperature, CCCP treatment, and the interaction.

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