The Effects of Calcitonin on the Development of and Ca\textsuperscript{2+} Levels in Heat-shocked Bovine Preimplantation Embryos In Vitro

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Abstract. Intracellular calcium homeostasis is essential for proper cell function. We investigated the effects of heat shock on the development of and the intracellular Ca\textsuperscript{2+} levels in bovine preimplantation embryos in vitro and the effects of calcitonin (CT), a receptor-mediated Ca\textsuperscript{2+} regulator, on heat shock-induced events. Heat shock (40.5 C for 10 h between 20 and 30 h postinsemination) of in vitro-produced bovine embryos did not affect the cleavage rate; however, it significantly decreased the rates of development to the 5- to 8-cell and blastocyst stages as compared with those of the control cultured for the entire period at 38.5 C (P < 0.05). The relative intracellular Ca\textsuperscript{2+} levels at the 1-cell stage (5 h after the start of heat shock), as assessed by Fluo-8 AM, a fluorescent probe for Ca\textsuperscript{2+}, indicated that heat shock significantly lowered the Ca\textsuperscript{2+} level as compared with the control level. Semiquantitative reverse transcription PCR and western blot analyses revealed the expression of CT receptor in bovine preimplantation embryos. The addition of CT (10 nM) to the culture medium ameliorated the heat shock-induced impairment of embryonic development beyond the 5- to 8-cell stage. The Ca\textsuperscript{2+} level in the heat-shocked embryos cultured with CT was similar to that of the control embryos, suggesting that heat shock lowers the Ca\textsuperscript{2+} level in fertilized embryos in vitro and that a lower Ca\textsuperscript{2+} level is implicated in heat shock-induced impairment of embryonic development. Intracellular Ca\textsuperscript{2+}-mobilizing agents, e.g., CT, may effectively circumvent the detrimental effects of heat shock on early embryonic development.

Key words: Calcitonin, Calcitonin receptor, Calcium, Heat shock, Preimplantation embryo

Materials and Methods

Ethics statement

This study was carried out in accordance with the Regulation on Animal Experimentation at Kyoto University.
Chemicals
All chemicals used were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan), unless otherwise specified.

Culture media for in vitro production of bovine embryos
Culture media for in vitro maturation (IVM) of immature oocytes recovered from ovaries, in vitro fertilization (IVF) of matured oocytes, and in vitro culture (IVC) of zygotes up to the blastocyst stage were prepared based on synthetic oviduct fluid (SOF) containing amino acids [27] with some modifications: the concentration of sodium pyruvate was increased to 0.5 mM, and the media were modified for each of the applications. In brief, the medium for IVM (IVMM) was supplemented with 5.6 mM glucose, 10% (v/v) fetal calf serum (FCS), and 0.2 IU/ml follicular-stimulating hormone (Kyoritsu Seiyaku, Kawasaki, Japan), the medium for IVF (IVFM) was not supplemented with glucose, and the medium for IVC used from day 1 to day 3 (day of IVF = day 0) was supplemented with 1.5 mM pyruvate was increased to 0.5 mM, and the media were modified to avoid possible bias, measurement of Ca²⁺ levels during the first mitosis [31, 32].

Heat shock of bovine embryos
From 20 hpi, 1-cell embryos were cultured in IVC1M basically at 38.5 °C (Normal). The heat-shocked embryos (Heat) were incubated at 40.5 °C between 20 and 30 hpi and afterward at 38.5 °C. All the embryos were at the 1-cell stage at the start (20 hpi) and midpoint (25 hpi) of heat shock, and 63 (152/241) and 37% (89/241) of embryos were at the 1-cell and 2-cell stages, respectively, at the end of the treatment (30 hpi). The heat shock condition roughly mimicked the daily change in the rectal temperature of cows exposed to high temperatures in the hot season [2, 30]. The cleavage rate and rate of development to the 5- to 8-cell stage for the Normal and Heat groups were compared at 72 hpi (the fastest developing embryos were at the 8- to 16-cell stage), and their progression to blastocysts was compared at 192 hpi. The cultures were replicated five times, and the number of embryos allocated to each treatment group was 31–42 per replicate.

Measurements of intracellular Ca²⁺ levels in bovine embryos
Intracellular cytosolic free Ca²⁺ levels were measured using Fluo-8 AM (AAT Bioquest, Sunnyvale, CA, USA). At 19 hpi, the 1-cell embryos were denuded from cumulus cells as described above and cultured for 1 h in IVC1M supplemented with 5 μM Fluo-8 AM and 0.02% (w/v) Pluronic F-127 (AnaSpec, Fremont, CA, USA). The Fluo-8-loaded embryos were then washed twice with IVCM and allocated to each culture condition. As described above, 1-cell and 2-cell embryos coexisted at the end of heat shock (30 hpi). In addition, the 2-cell embryos exhibited higher fluorescence along with the cleavage plane compared with the cytosol, which hampered unbiased measurement of the fluorescence intensity due to the unequal orientation of the cleavage plane to the focal plane. Therefore, in order to equalize the developmental stage of the examined embryos (1-cell stage) and to avoid possible bias, measurement of Ca²⁺ levels was implemented at 25 hpi (5 h after the start of heat shock). At this time point, embryos were transferred into a 1 μl IVC1M drop covered with mineral oil in a well of a 4-well dish. Fluo-8 fluorescence was captured using a fluorescence microscope (FSX100, Olympus, Tokyo, Japan), and fluorescence intensities were measured using the ImageJ software (National Institute of Health, Bethesda, MD, USA). Embryos that fluoresced highly intensely (greater than three interquartile ranges, which were 4% of the total) were not used to evaluate the resting (basal) Ca²⁺ levels, because fertilized embryos generate Ca²⁺ transients at nuclear envelope breakdown and cleavage during the first mitosis [31, 32].

Semiquantitative RT-PCR of CTR transcripts
Bovine in vitro-produced embryos at the 1-cell, 2-cell, 8-cell, morula, and blastocyst stages (20, 36, 72, 144, and 192 hpi, respectively; n = 15 for each stage) were stored in small volumes of RNAlater (Sigma) at −20°C until RNA extraction. In addition, immature oocytes at the germinal vesicle stage immediately after recovery from the...
ovaries and mature oocytes with first polar bodies after IVM were also acquired. Total RNA was extracted from each sample using an RNeasy Micro Kit (Qiagen, Hilden, Germany) and then subjected to first-strand cDNA synthesis in a 31.5 µl reaction volume using SuperScript III Reverse Transcriptase and Oligo-(dT)20 primers (Invitrogen, Carlsbad, CA, USA). Transcripts of CTR and β-actin (ACTB) as an internal control were PCR amplified in a 25 µl reaction volume using Platinum PCR SuperMix (Invitrogen) from 1 µl of the cDNA solution with one of the following primer sets: AGCACTGCCCAGGCTATTTT (forward) and AGCTAAGGCTCCTAACACGC (reverse) for CTR and CCAAGGCCAACCGTGAGAAGAT (forward) and CCACGTTCCGTGAGGATCTTCA (reverse) for ACTB [33]. After an initial denaturation at 94 C for 1.5 min, 35 PCR cycles for CTR and 30 cycles for ACTB, which had been experimentally confirmed as the amplification stages, were performed as follows: 94 C for 30 sec, 58 C for 30 sec, and 72 C for 30 sec. The 10 µl PCR products of CTR (255 bp) and ACTB (256 bp) were subjected to electrophoresis through 2% (w/v) agarose gels and then stained with 0.5 µg/ml ethidium bromide. The stained gels were photographed by using an InGenius3 gel documentation system (Syngene, Cambridge, UK).

Western blot analysis of CTR protein

Bovine in vitro-produced embryos at the 1-cell (n=400) and blastocyst (n=10) stages were lysed in NuPAGE LDS Sample Buffer with Reducing Agent (Invitrogen) in a 20 µl volume and boiled
for 5 min. The samples were subjected to SDS-PAGE through a 4–12% Bis-Tris gel and transferred to a PVDF membrane using an iBlot Dry Blotting System (Invitrogen). The blotted membrane corresponding to 30–60 kDa was blocked with 10% (v/v) FCS in PBS containing 0.05% (v/v) Tween 20 (PBST) for 30 min. A rabbit polyclonal antibody to CTR (ab103422, Abcam, Cambridge, UK) was diluted 200 times with PBST containing 5% (v/v) FCS and mounted onto the membrane for 1 h. After extensive washing with PBST, the membrane was treated for 30 min with 2,000 times-diluted alkaline phosphatase-conjugated bovine anti-rabbit IgG (sc-2372, Santa Cruz Biotechnology, Dallas, TX, USA). After washing, the signal was developed for 5 min with Novex AP Chemiluminescent Substrate (Invitrogen) and exposed to FP-3000B film (Fujifilm, Tokyo, Japan). The same procedure except for the use of an anti-Histone H2A antibody (1,000-times diluted, ab88770, Abcam) was applied to the membrane corresponding to 10-30 kDa to assess the expression levels of Histone H2A as a loading control.

Exposure of heat-shocked bovine embryos to CT

Synthetic human CT was purchased from Peptide Institute, Inc. (Minoh, Japan), and reconstituted in IVC1M to obtain a 10 µM solution. The 10µM solution was further diluted with IVC1M (1:1000) to yield a final concentration of 10 nM. The preparation was performed 1 day before use. The concentration of CT (10 nM) in the culture medium was set according to an effective dose for the promotion of Ca²⁺ mobilization and preimplantation development of murine embryos [24, 25]. In addition, 10 nM CT was confirmed to be more effective compared with 100 nM in preliminary experiments (data not shown).

Beginning at 20 hpi, 1-cell embryos were cultured in 500 µl of IVC1M in the presence or absence of 10 nM CT until 72 hpi. Embryos were also allocated to the Normal and Heat thermal conditions as described above. Thus, the embryos were divided into four groups: (i) Normal in the absence of CT (Normal-None), (ii) Normal in the presence of CT (Normal-CT), (iii) Heat in the absence of CT (Heat-None), and (iv) Heat in the presence of CT (Heat-CT). The four groups were compared with respect to their cleavage rates and rates of development to the 5- to 8-cell stage at 72 hpi and with respect to their rates of development to the blastocyst stage at 192 hpi. The cultures were replicated seven times with 30-45 embryos allocated to each group per replicate.

Statistical analyses

The embryonic development expressed as a percentage and logarithmic-transformed Fluo-8 fluorescence intensities were subjected to a general linear model in which treatments and replicates were taken as fixed variables. All analyses were performed using SAS (SAS Institute, Cary, NC, USA). When multiple comparisons were made, Tukey’s (for all pairwise comparisons) or Dunnett’s (for pairwise comparisons with the control) test was used. Significance was accepted at P<0.05.

Results

Effects of heat shock on the development of and intracellular Ca²⁺ levels in bovine embryos

We investigated the effects of exposure to heat shock (40.5 C for 10 h) on day 1 post-IVF on postfertilization development of bovine embryos in vitro (Table 1). The heat shock did not affect the cleavage rate; however, it significantly decreased the rates of development to the 5- to 8-cell and blastocyst stages as compared with those of the Normal group (P < 0.05). On the other hand, heat shock for only 5 h (between 20 and 25 h postinsemination) did not affect these developmental rates (Supplementary Table 1). The relative intracellular Ca²⁺ level for the two groups was determined using the fluorescence Ca²⁺ probe, Fluo-8 AM, at the midpoint (5 h) during heat shock, which corresponded to 25 hpi (Fig. 1). The heat shock significantly decreased the Ca²⁺ level as compared with that of the Normal group. In addition, embryos that remained at the 1-cell stage at the end point (10 h) of heat shock (30 hpi) also exhibited a lower Ca²⁺ level compared with 1-cell embryos of the Normal group (Supplementary Fig. 1).

Expression of CTR in bovine oocytes and preimplantation embryos

The results for the semiquantitative RT-PCR of CTR are shown in Fig. 2A. CTR transcripts were relatively more abundant in oocytes and the 1- and 2-cell embryos and decreased thereafter to undetectable levels in the morula and blastocyst stages. Western blot analysis of CTR protein in 1-cell and blastocyst stage embryos showed a band of approximately over 50 kDa corresponding to the published molecular weight of CTR on SDS-PAGE [34]. The signal of the band relative to that of the loading control (Histone H2A) was higher in the 1-cell
stage compared with the blastocyst stage (Fig. 2B).

**Effects of CT on the development of and intracellular Ca\(^{2+}\) levels in the heat-shocked bovine embryos in vitro**

The effects of CT treatment on embryonic development after heat shock were investigated (Table 2). As described above, heat shock decreased the rates of development to the 5- to 8-cell and blastocyst stages as compared with those of the Normal-None group. However, the rates of development to the 5- to 8-cell and blastocyst stages in the heat-shocked and CT-treated group (Heat-CT) were not significantly different from those of the Normal-None group. Figure 3 shows the relative intracellular Ca\(^{2+}\) levels in bovine embryos cultured in the presence or absence of CT and with or without heat shock. When the normal temperature group was cultured in the presence of CT, the Ca\(^{2+}\) level tended to increase (P=0.065) in comparison with that of the normal temperature group without CT (Normal-None). The Ca\(^{2+}\) level at 25 hpi (5 h after the start of heat shock) in heat-shocked embryos cultured in the absence of CT was significantly lower (P=0.0035) than that in the Normal-None group. However, the heat-shocked embryos cultured in the presence of CT exhibited a Ca\(^{2+}\) level that was statistically indistinguishable from that of the Normal-None group (P=0.37).

**Discussion**

We examined the effects of heat shock (40.5 C for 10 h) on bovine embryos because this condition roughly mimics the body temperature of cattle exposed to high temperatures in the hot season [2, 30]. Heat shock on day 1 of embryo culture did not affect the cleavage rate; however, it significantly decreased the rate of development to the 5- to 8-cell and blastocyst stages.

Many studies have found direct inhibitory effects of heat shock on bovine preimplantation development in vitro [2, 8, 9]. The conditions for heat shock used in the present study can be considered to be slightly higher than the threshold necessary to induce impaired development: Rivera and Hansen reported that exposure of bovine 1-cell embryos to 40 C for 12 h had no effect on embryonic development; in contrast, exposure of embryos to 41 C for 9 h decreased their development to the blastocyst stage (similar to our finding), and heat shock at 41 C for 12 h decreased both cleavage and blastocyst rates [2]. Given our findings and previous results concerning the heat shock conditions under which embryonic development is affected, we wondered if heat shock affects intracellular Ca\(^{2+}\) levels of bovine embryos. As a result, heat shock significantly lowered the Ca\(^{2+}\) level as compared with the control level. Although 5 h of heat shock was not sufficient to impair development (Supplementary Table 1), this time point was chosen for the assessment of Ca\(^{2+}\) levels on account of the experimental reasonability described in the Materials and Method. In addition, 1-cell embryos after 10 h of heat shock also exhibited the lower Ca\(^{2+}\) levels compared with the control embryos (Supplementary Fig. 1). These results suggest that the persistent lower Ca\(^{2+}\) level (that persists more than 5 h) was implicated in the impaired development of heat-shocked embryos.

Deleterious stress can either increase [20] or decrease [18, 19, 35, 36] intracellular Ca\(^{2+}\) levels, and both types of Ca\(^{2+}\) changes are associated with the impairment of cellular functions and/or the promotion of cell death. In addition, certain stresses attenuate Ca\(^{2+}\) mobilization that is mediated by the G protein-coupled receptor [37, 38]. The stress-induced increase in the intracellular Ca\(^{2+}\) level has been attributed to the opening of various plasma membrane-localized channels [20]. The present result that heat shock decreased the intracellular Ca\(^{2+}\) level suggests that the used heat shock did not activate these plasma membrane-localized channels involved in the stress-induced rise in intracellular Ca\(^{2+}\) and/or that the embryos at the examined stage (1-cell) did not possess such stress-sensitive channels. For example, we could not detect the mRNA expression of a representative heat-sensitive plasma membrane-localized Ca\(^{2+}\) channel, transient receptor potential vanilloid type 1 (TRPV1), in bovine 1-cell embryos (unpublished result).

On the other hand, mechanisms by which stress decreases intracellular Ca\(^{2+}\) include dysfunction of the endoplasmic reticulum (ER)-resident Ca\(^{2+}\) channel, i.e., the inositol 1,4,5-trisphosphate receptor (IP3R) [37], and altered expression of genes involved in calcium homeostasis (e.g., those encoding calcium channels, calcium-binding proteins, and the plasma membrane calcium ATPase) [39, 40]. We measured Ca\(^{2+}\) levels at 25 hpi, which is long after the period of sperm-induced frequent Ca\(^{2+}\) oscillation [17, 32], and embryos that exhibited extremely high fluorescence (4% of total) possibly due to the Ca\(^{2+}\) transient during this period [31, 32] were discarded from the statistical analysis as outliers. Therefore, the presented data is considered to more reflect resting (basal) levels of Ca\(^{2+}\) rather than the transient Ca\(^{2+}\) increase. The resting intracellular Ca\(^{2+}\) could be lowered by the alteration of IP3R-dependent Ca\(^{2+}\) mobilization from intracellular stores [39, 41].

The finding that heat shock decreased the Ca\(^{2+}\) level in preimplantation embryos prompted us to explore the possibility that modulation of the intracellular Ca\(^{2+}\) level would ameliorate the detrimental effects of heat shock on preimplantation development. Although the physiological importance of CT in systemic Ca\(^{2+}\) homeostasis in mammals is controversial [42], the function of CT as a paracrine factor in the female reproductive tract has been suggested [24–26]. The expression of CTR mRNA in murine preimplantation embryos has been reported [24], and CT can increase intracellular Ca\(^{2+}\) levels by activating CTR [24, 25, 43, 44]. Therefore, we examined the expression of CTR mRNA in bovine preimplantation embryos and the effects of CT treatment on the in vitro development of embryos that had been heat shocked. Our RT-PCR study revealed a unique pattern of CTR expression in bovine embryos that is distinct from the pattern found for murine embryos. In the bovine embryos, CTR mRNA was relatively highly expressed in oocytes through the 2-cell stage and decreased afterward to undetectable levels in the morula and blastocyst stages (Fig. 2A), whereas murine oocytes and embryos exhibit relatively lower expression in oocytes and the early cleavage stage and higher expression in the later stage of preimplantation development [24]. Consistent with the transcript expression pattern, the western blot analysis showed that 1-cell embryos had higher expression of CTR than blastocysts on a per-cell basis (Fig. 2B), suggesting possible CT sensitivity in 1-cell stage embryos.

In accordance with the expression of CTR during the early cleavage stages, CT treatment during this time period tended to increase (P = 0.065) the intracellular Ca\(^{2+}\) levels in the bovine embryos (Fig. 3). Heat shock significantly decreased the Ca\(^{2+}\) levels in the embryos,
although CT treatment during heat shock resulted in a Ca\textsuperscript{2+} level similar to that of the Normal-None embryos. Interestingly, CT treatment also ameliorated the heat shock-induced developmental impairment (Table 2).

The rise in intracellular free Ca\textsuperscript{2+} caused by CT results from the release of Ca\textsuperscript{2+} from intracellular stores and the influx of extracellular Ca\textsuperscript{2+} [45]. Activation of phospholipase C pathways, which results in IP3-mediated mobilization of Ca\textsuperscript{2+} from the ER is a well-known mechanism by which CT enhances the intracellular Ca\textsuperscript{2+} level [46, 47]. Constitutive IP3-mediated release of Ca\textsuperscript{2+} from the ER and uptake of the released Ca\textsuperscript{2+} by mitochondria are required for efficient mitochondrial respiration [48, 49]. The proper mitochondrial bioenergetics is emerging as a correlation to developmental outcome in oocyte maturation, fertilization, and postfertilization development of the embryo [50–52]. Thus, CT may enhance embryonic development when an embryo is heat shocked through the ER-directed regulation of mitochondrial bioenergetics. When compared within the respective thermal conditions (38.5 and 40.5 C), CT treatment increased the Ca\textsuperscript{2+} levels (P = 0.02, t-test) compared with the no additive control at 38.5 C, while it did not (P = 0.06) at 40.5 C. This result also suggests that CT sensitivity differs between the thermal conditions; consequently, it suggests that IP3-mediated mobilization of Ca\textsuperscript{2+} from the ER is one of the targets by which heat shock decreases intracellular Ca\textsuperscript{2+} in preimplantation embryos.

In addition, CT can induce a sustained Ca\textsuperscript{2+} elevation due to a Ca\textsuperscript{2+} release-activated capacitative Ca\textsuperscript{2+} influx from the extracellular environment [43]. Importantly, Wang et al. [25] reported that the P38MAPK pathway is partially involved in the enhancing effects of CT on murine preimplantation development in vitro. Therefore, it cannot be excluded that the heat shock-ameliorating effect of CT on bovine preimplantation embryos is also mediated by signal transduction pathways like this that are independent of Ca\textsuperscript{2+} signaling.

In summary, heat shock, which impaired the postfertilization development of bovine preimplantation embryos in vitro, decreased the intracellular Ca\textsuperscript{2+} level in the embryos. CT treatment ameliorated this heat shock-induced developmental impairment and recovered the normal intracellular Ca\textsuperscript{2+} level. Consequently, intracellular Ca\textsuperscript{2+} may be a new target for circumventing the detrimental effects of heat shock on preimplantation development.

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