REGULATION OF HEAT SHOCK PROTEIN 70 SYNTHESIS BY HEAT SHOCK IN THE PREIMPLANTATION MURINE EMBRYO

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Received for publication: January 31, 1995
Accepted: March 10, 1995

ABSTRACT

Induced thermotolerance in murine embryos occurs at the 8-cell stage when embryos are maintained in vitro but not until the blastocyst stage if development proceeds in vivo. Present results indicate that ability of embryos to undergo induced thermotolerance is not limited by heat shock protein 70 (HSP70) synthesis. Exposure of 8-cell embryos to 40°C enhanced synthesis of 2 constitutive HSP70 proteins (HSC70 and HSC72) and induced another protein, HSP68; exposure of 43°C was required to induce similar responses in expanded blastocysts. Unlike induced thermotolerance, increased synthesis of HSP70 molecules did not depend on whether embryos were cultured or developed in vivo. Thus, other biochemical mechanisms in addition to HSP70 confer thermotolerance in the preimplantation-stage murine embryo. The observation that the temperature threshold for induction of HSP70 synthesis increased from the 8-cell to the blastocyst stage is indicative of these other biochemical processes.

Key words: heat shock, heat shock protein 70, thermotolerance, murine embryo

INTRODUCTION

In many domestic animals, exposure to heat stress during early pregnancy causes embryonic mortality. The effects of heat stress decline as pregnancy progresses so that the effects are minimal by Days 3 to 5 in the ewe (5) and cow (6), and by Day 5 in the pig (22). One possible explanation for this phenomenon is that embryos become more resistant to the deleterious effects of elevated temperatures as they advance in development. There is evidence for this in the cow (6). The biochemical processes by which embryos develop resistance to elevated temperatures are not known; their identification could result in novel

Acknowledgments

The authors thank J.A. Davidson for computer assistance and M. E. Hissem for help with the preparation of the manuscript. This is Journal series No. R-03944 of the Florida Agricultural Experiment Station. Research was supported by grants from the Florida Dairy Checkoff Program and USDA (CBAG 9204572).

1 Supported by a USDA Food and Agricultural Sciences Fellowship (Grant 92-38420-7331).
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methods for protecting embryos from elevated temperatures. Tolerance to transient periods of elevated temperatures in many cells has been correlated with the synthesis of a small subset of intracellular proteins known as heat shock proteins (HSP; 13,18,19). Proteins of the HSP70 family include a constitutively expressed form (HSC70; 9) and a heat-inducible form (HSP68; 14). These proteins are produced in increased amounts in response to heat shock and have been demonstrated to play a protective role within the cell, presumably through their ability to refold damaged proteins and protect ribosomal RNA (4,18). Neutralization of HSP70 with antibodies increased thermal sensitivity of fibroblasts (19) while microinjection of HSP70 mRNA conferred thermal resistance in murine oocytes exposed to 42 to 43°C (13).

We have used the mouse as a model to study how embryos acquire thermotolerance during development. Preimplantation-stage murine embryos first undergo induced thermotolerance (a phenomenon whereby prior exposure to a mild heat shock makes cells more resistant to a subsequent, more severe heat shock) at the 8-cell stage if developed in vitro, but not until the blastocyst stage of development if the embryos are developed in vivo. Induction of HSP70 synthesis by heat shock can occur by the late morula or blastocyst stage of development (10,12,16,17,23), but it is not known whether the acquisition of induced thermotolerance in the embryo is caused by developmental changes in HSP70 synthesis in response to heat.

The objectives of this study were to determine whether developmental patterns of heat-induced HSP70 synthesis are coincident with ontogeny of induced thermotolerance. The results indicate that heat-induced HSP70 synthesis occurs earlier in development than had previously been reported (10,12,16,17,23), that the heat shock threshold for induction of HSP70 synthesis increases during development, and that HSP70 synthesis occurs under conditions in which induced thermotolerance does not occur. These results also indicate that other developmental changes in addition to HSP70 molecules are required to produce induced thermotolerance.

**MATERIALS AND METHODS**

**Embryo Collection and Culture**

Mice (ICR strain; Harlan Sprague Dawley Inc., Indianapolis, IN, USA) were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Procedures for collection and culture of embryos were carried out as previously described (7). Briefly, embryos developed in vitro were collected at the 2- to 4-cell stage and cultured to the desired stage of development at 37°C. Cultures were performed in 5- to 7-μl microdrops of M16 medium containing 0.4% (w/v) bovine serum albumin that were covered with mineral oil. Embryos developed in vivo were obtained by sacrificing females and collecting embryos at appropriate times following injection of hCG (i.e., 8-cell embryos were obtained 63 h post-hCG and blastocysts at 96 to 100 h post hCG). To reduce experimental variation, embryos that were developed in vitro and in vivo were synchronized for a given replicate so that both groups were at the same stage of development on the same day to which treatment was applied.
Heat Shock and Radiolabeling of Embryos

Embryos were randomly assigned within replicate to treatment and transferred to 50 μl of M16 medium ± 10% (v/v) fetal calf serum (FCS) and containing 50 μCi of a 70:30 [35S]methionine and cysteine mixture (Amersham, Arlington Heights, IL; spec. act. > 1000 Ci/mmol; 12-24 8-cell embryos/microdrop; 6-21 blastocysts/microdrop). Control embryos were radiolabeled at 37°C for a total of 5 h and 20 min; heat-shocked embryos were radiolabeled at 40 or 43°C for 80 min and then at 37°C for 4 h. After labeling, embryos were washed 3 times in M16 medium containing 0.1% (w/v) polyvinyl alcohol and transferred in the smallest volume possible to 50 μl of 5 mM K2CO3 containing 9.4 M urea, 2% (v/v) Nonidet P-40, and 0.5% (w/v) dithiothreitol and frozen at -80°C until analysis.

Analysis of Radiolabeled Proteins

Incorporation of radiolabel into intracellular proteins was determined by trichloroacetic acid (TCA) precipitation (15). Proteins from solubilized embryos were analyzed using 2-D SDS-PAGE, with isoelectric focusing in the first dimension [1% (v/v) each of 2 preblended ampholines (pH 3.5-9.5 and pH 5.0-8.0) from Pharmacia, Uppsala, Sweden] and SDS-PAGE [10% (v/v) polyacrylamide] in the second dimension (20). Radiolabeled proteins were detected by fluorography. Each gel was loaded with 25,000 to 50,000 dpm of TCA-precipitable protein and exposed to X-Ray film (Fuji; Tokyo, Japan); within each replicate, equal amounts of radioactivity were loaded and films were exposed for the same amount of time.

Statistical Analysis

Trichloroacetic acid-precipitable radioactivity for control and heat-shocked embryos was expressed on a per embryo basis and analyzed by least squares analysis of variance using the General Linear Models procedure of SAS (21). When heterogeneity of variance was present, data were ranked and then the ranks were analyzed by least squares analysis of variance. For data characterized by heterogeneity of variance, actual means ± SEM were reported.

RESULTS

Exposure of embryos to a heat shock of 40°C did not affect overall synthesis of proteins during the course of treatment; a reduction in TCA-precipitable proteins was noted only when embryos were exposed to a heat shock of 43°C (Table 1). A representative fluorograph of proteins synthesized de novo for embryos radiolabeled at 37°C or while heat shocked is presented in Figure 1. Three different proteins corresponding to HSP70 were noted. Two proteins, designated HSC70 (Mw = 69,906 ± 1016) and a slightly more basic HSC72 (Mw = 72,432 ± 1271) were present in all embryos. The third protein, called HSP68, was of lower Mw (67,733 ± 1199) and more basic than HSC70 or HSC72 (Figure 2). Although HSP68 was generally absent in embryos cultured at 37°C (Figure 2), it was present in embryos exposed to heat shock.
Table 1. Effect of heat shock on $[^{35}\text{S}]$labeled intracellular TCA-precipitable protein

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Temperature</th>
<th>n$^a$</th>
<th>TCA-precipitable protein (dpm/embryo)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-cell</td>
<td>37°C</td>
<td>4</td>
<td>$6141 \pm 2454^b$</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>4</td>
<td>$4737 \pm 2454$</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expanded blastocyst</td>
<td>37°C</td>
<td>3</td>
<td>$67255 \pm 11325^b$</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>3</td>
<td>$66354 \pm 11325$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>3</td>
<td>$40142 \pm 19353^c$</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>43°C</td>
<td>3</td>
<td>$4373 \pm 2146$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$n=number of replicates.

$^b$Least squares means $\pm$ SEM.

$^c$Means $\pm$ SEM.

Figure 1. Representative profiles of $[^{35}\text{S}]$labeled proteins synthesized by 8-cell murine embryos that developed in vivo and were cultured at 37 or 40°C. De novo synthesis was assessed using 2-D SDS-PAGE and fluorography. For each gel, 25,000 dpm of TCA-precipitable radioactivity was loaded. Location of HSP70 molecules is indicated by arrows.
Eight-cell embryos were examined for de novo synthesis of proteins as affected by temperature and type of development (in vitro versus in vivo). Both HSC70 and HSC72 were noted in all 8-cell embryos radiolabeled at 37 or 40°C; HSP68 was not present at 37°C with the exception that slight amounts were present in embryos developed in vivo (Figure 2). Exposure of embryos to 40°C generally increased synthesis of HSC70 and HSC72 and induced synthesis of HSP68 (Table 2). No differences in responses to heat shock were noted for embryos developed in vitro versus those that developed in vivo (Table 2, Figure 2).

![Figure 2](image)

Figure 2. Representative profiles of [35S]labeled HSP70 proteins synthesized by murine 8-cell embryos and expanded blastocysts cultured at 37, 40 or 43°C. Proteins were assessed using 2-D SDS-PAGE and fluorography. Each treatment (37 or 40°C) applied to 8-cell embryos was replicated with 3 sets of embryos (in vitro developed) or performed with one set of embryos (in vivo developed); treatments (37, 40 or 43°C) applied to expanded blastocysts were replicated 3 times. Within a replicate, equal amounts of TCA-precipitable radioactivity (25,000 to 50,000 dpm) were loaded per gel. Shown is the region of the gel corresponding to HSC72 (a), HSC70 (b) and HSP68 (c).

Like the 8-cell embryo, HSC72 and HSC70 were present in expanded blastocysts at 37°C. HSP68 was not present at 37°C if embryos had developed in vitro; however, HSP68 was noted in expanded blastocysts cultured at 37°C and developed in vivo (data not shown). In contrast to the 8-cell embryo, exposure of expanded blastocysts to 40°C caused only slight or no change in synthesis of HSP70 molecules (Table 2, Figure 2). This was true for embryos that developed in vitro and in vivo and for embryos cultured with or without fetal calf serum (Table 2). However, exposure to 43°C caused appearance of large amounts of radiolabeled
Table 2. Effect of heat shock on synthesis of heat shock protein 70: summary of results from individual replicates

<table>
<thead>
<tr>
<th>Stage</th>
<th>Type of development</th>
<th># embryos (37°C / heat shock)</th>
<th>Heat shock (°C)</th>
<th>Intensity HSC70</th>
<th>HSC72</th>
<th>HSP68</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>37 HS</td>
<td>37 HS</td>
<td>37 HS</td>
</tr>
<tr>
<td>8-cell</td>
<td>In vitro +</td>
<td>19/16</td>
<td>40</td>
<td>+ ++ ++ ++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>8-cell</td>
<td>In vitro +</td>
<td>20/24</td>
<td>40</td>
<td>+ ++ ++ ++</td>
<td>+</td>
<td>++</td>
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<tr>
<td>8-cell</td>
<td>In vitro +</td>
<td>24/23</td>
<td>40</td>
<td>++ ++ ++ ++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8-cell</td>
<td>In vivo +</td>
<td>12/15</td>
<td>40</td>
<td>+ +++ ++ ++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>EB</td>
<td>In vitro -</td>
<td>12/14</td>
<td>40</td>
<td>++ +++ ++ +</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>EB</td>
<td>In vitro -</td>
<td>18/20</td>
<td>40</td>
<td>+ + +</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>EB</td>
<td>In vivo +</td>
<td>8/11</td>
<td>40</td>
<td>+++ +++ ++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>EB</td>
<td>In vitro +</td>
<td>6/6</td>
<td>43</td>
<td>+ +++ ++ ++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>EB</td>
<td>In vitro +</td>
<td>22/21</td>
<td>43</td>
<td>++ +++ ++ +</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>EB</td>
<td>In vitro +</td>
<td>10/15</td>
<td>43</td>
<td>++ +++ + +</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

*In vivo developed embryos were synchronized with 1 replicate of embryos developed in vitro so that both groups were at the same stage of development on the same day to which treatment was applied.

bExpanded Blastocyst
- Protein absent
++ Very intense
+++ Intense
++ Moderate intensity
+ Faint spot

HSW on fluorographs to produce a pattern similar to 8-cell embryos at 40°C (Table 2). In particular, 43°C caused appearance of HSP68; amounts of HSC70 and HSC72 were also enhanced as compared to 37°C (Figure 2, Table 2).

DISCUSSION

The M, isoelectric point and thermal induction of the proteins characterized in the present study are similar to murine HSP70 molecules previously reported (3,18). The present finding that
the 8-cell embryo can produce increased amounts of HSC72, HSC70 and HSP68 in response to heat shock in contrast with previous reports that embryos are incapable of synthesizing HSP70 in response to heat shock until the late morula or blastocyst stage of development (10,12,16,17,23). These earlier studies utilized heat shocks ≥ 43°C and this may have impaired the ability of cleavage-stage embryos to mount a heat shock response. Accordingly, heat shock of less severity, such as 40°C, may be required to adequately characterize the ontogeny of induced HSP70 synthesis in the murine embryo. Other evidence also exists that 8-cell embryos can undergo biochemical changes in response to 40°C because 8-cell embryos developed in vitro can undergo induced thermotolerance (7).

Within each replicate, a similar amount of TCA-precipitable radioactivity was analyzed by 2-D SDS-PAGE for control (37°C) and heat-shocked embryos. This was done to allow equivalent visualization of proteins at all temperatures. It is clear that changes in patterns of HSP70 in 8-cell embryos caused by exposure to 40°C represent alterations in synthesis rather than in the number of embryos loaded per gel because overall rates of protein synthesis were similar at 37 and 40°C. However, exposure of blastocysts to 43°C resulted in an 89% reduction in total TCA-precipitable protein. Accordingly, more embryos were used to prepare an electrophoretogram at 43°C than at 37°C. Thus, it is possible that the increase in the relative amounts of HSP70 molecules at 43°C may represent either 1) increased synthesis of the proteins by heat shock, as reported in several cellular systems (7,9,18) or 2) a less-severe reduction in synthesis of HSP70 molecules in comparison with other intracellular proteins.

Molecules of the HSP70 family play a thermoprotective role since neutralization with antibodies increases thermal sensitivity of fibroblasts (19) and microinjection of HSP70 mRNA confers thermal resistance in murine oocytes exposed to 42-43°C (13). Nonetheless, present results indicate that induced thermotolerance in embryos requires more than HSP70 synthesis. Ealy and Hansen (7) demonstrated that induction of thermotolerance in murine embryos was first noted at the 8-cell stage of development for embryos developed in vitro but not until the blastocyst stage for embryos developed in vivo. In the present study, 8-cell embryos synthesized increased amounts of HSC72, HSC70 and HSP68 in response to heat shock regardless of the type of development. It is possible that there are subtle differences in the amounts of individual HSP70 molecules that could explain effects of type of development on induced thermotolerance responses. It is more likely, however, that these effects on embryonic capacity to undergo induced thermotolerance reflect changes in other biochemical systems. In this regards, DL-buthionine-[S,R]-sulfoximine (BSO) an inhibitor of glutathione synthesis, prevented induced thermotolerance in murine morula (2). Moreover, Harris et al. (11) demonstrated that BSO attenuated the thermotolerance response in postimplantation rat embryos without decreasing HSP70 mRNA or HSP70 synthesis.

While exposure of 8-cell embryos to 40°C increased HSP70 synthesis, this temperature was ineffective for expanded blastocysts; 43°C was required to cause large appearances of HSP70 on the electrophoretogram. The signal for heat-shock induced HSP70 synthesis involves increased accumulation of denatured proteins (1). Thus, present results indicate that embryonic proteins may be more thermosensitive at the 8-cell stage than at the blastocyst stage of development. This developmental difference in protein stability to elevated temperature, possibly caused by the association of intracellular proteins with heat shock proteins or protection via antioxidants, could be one of the causes for the increase in resistance to elevated temperature exhibited as embryos
progress through development (6,7,17). The biochemical basis for this increased thermostability may also determine in part the developmental timing of induced thermotolerance responses.

REFERENCES


