doi: http://dx.doi.org/10.5016/1806-8774.1999v1p5

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Possible Roles for Heat Shock Protein 70 and Glutathione in Protection of the Mammalian Preimplantation Embryo from Heat Shock

Contents

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The author's research cited in this review was supported by USDA CBAG Grants 92-34135-7432 and 95-34135-1860 and USDA NRICGP Grant No. 96-35205-3728. This is Journal Series nº R-O 7092 of the Florida Agricultural Expt. Sta.

Abstract

Embryonic mortality is increased in females exposed to heat stress during early pregnancy. This phenomenon likely reflects, at least in part, disruption of the preimplantation embryo by exposure to elevated temperature. The thermoprotective mechanisms that embryos use to maintain cellular function in the face of heat shock include the molecular chaperone, heat shock protein 70 (HSP70), and the antioxidant, glutathione. Embryonic resistance to heat shock appears to increase as embryos become advanced in development. The evidence that changes in HSP70 synthesis are involved in developmental acquisition of thermal resistance is incomplete and weak. Indeed, changes in resistance of embryos to heat shock can sometimes be divorced from changes in embryonic capacity to synthesize HSP70. In contrast, glutathione has been shown to be important for induced thermotolerance (i.e., where exposure to a mild heat shock makes embryos resistant to a subsequent, more severe heat shock) in mouse embryos. Also, embryonic capacity for glutathione synthesis appears to increase as embryos progress in development.

Key words: heat shock, thermotolerance, preimplantation embryo, heat shock protein 70, glutathione, free radicals.

Introduction

Embryogenesis represents a series of temporally and spatially organized progressions in gene expression, cell division and morphogenesis. For successful development, these events must unfold despite unpredictable and adverse changes in the embryo's microenvironment. One change that is very deleterious to embryonic survival is an elevation in temperature (Alliston et al., 1965; Ulberg & Sheean, 1973; Arechíga et al., 1995; Edwards & Hansen, 1997; Arechíga & Hansen, 1998). Mammals exposed to heat stress[1] frequently develop hyperthermia and this elevation in body temperature is probably a major reason why fertility is reduced during periods of hot weather (Thatcher & Hansen, 1993). The preimplantation embryo does possess some ability to cope with alterations in its thermal environment, particularly as it becomes more advanced in development. Indeed, like other cells, the embryo possesses biochemical systems that mitigate adverse perturbations in the thermal environment including antioxidants (Gardiner and Reed, 1995[a,b]; Aréchiga et al., 1995; Harvey et al., 1995) and heat shock proteins (Edwards et al., 1995, 1997).

Identifying mechanisms embryos use to stabilize cellular function during heat stress may lead to development of methods for improving early embryonic survival in heat-stressed females. Also, some of the molecules involved in protection of cells

[1]In this review, the term heat stress will be used to refer to the forces external to the animal that act to disrupt body temperature regulation and cause hyperthermia. Features of the environment contributing to heat stress include high air temperatures, intense solar radiation and high humidity. The term heat shock, which deals with the direct effects of elevated temperature on cellular function, refers to exposure of cells to elevated temperature.

from thermal stress can also protect against other stresses (Anderson et al., 1990; Dix et al., 1998). Therefore, understanding the function of thermoprotective molecules in the preimplantation embryo may lead to development of procedures for reducing embryonic loss caused by a variety of perturbations in the embryo's microenvironment. Development in culture is itself potentially stressful and can lead to changes in embryonic gene expression and abnormal development This has been demonstrated by the observation that embryos produced or manipulated in culture can give rise to offspring that are of unusual birth weight, have physical defects or increased mortality (Rieger, 1998). Enhancing the efficiency and effectiveness of reproductive technologies based on embryo culture may depend in part on understanding the control and actions of stress responses in the embryo.

The purpose of this review is to describe what is known about thermoprotective systems in the preimplantation embryo. The focus will be on the mouse and cow because these species are the ones that have been most closely studied. Moreover, the discussion will be limited to the role of two thermoprotectants, heat shock protein 70 (HSP70) and glutathione, because these have been the only two such molecules studied in any depth in the preimplantation embryo. It is likely that other embryonic thermoprotective molecules are also important. Mammalian cells contain many molecules that stabilize cellular structure and function in the presence of heat shock and other stresses and it is probable that the preimplantation embryo is comparable to other cells in this regard.

Heat Shock Protein 70

General Properties

The molecular chaperone HSP70 has been implicated as a key molecule in protection of cells from heat shock and other stresses. There are several members of the HSP70 family in mammals (Georgopoulos & Welch, 1993). These include constitutively-produced proteins called heat shock cognate 70 (HSC70) that are produced in the absence of heat shock and whose synthesis is increased slightly by heat shock; highly heat-inducible proteins present in low amounts in unstressed cells (HSP70i); and proteins whose genes are regulated by glucose rather than heat shock (glucose-regulated protein 75 and 78). For this review, the term HSP70 will be used to generically describe members of the heat shock protein 70 family while more specific terms (HSC70, HSP70i) will be used to describe particular HSP70 proteins. The lower case in italics (*hsc70*) will be used to denote genes.

Members of the HSP70 family are weak ATPases that are classified as molecular chaperones because they are involved in post-translational modification of proteins especially as relates to changes in protein conformation (Georgopoulos & Welch, 1993; Lund, 1995). Heat shock protein 70 participates in many aspects of cellular metabolism in unstressed cells including protein folding after translation, transport of proteins across mitochondrial membranes and interaction with steroid receptors. Heat shock protein 70 binds hydrophobic amino acid motifs exposed on

unfolded proteins - binding of HSP70 to protein substrate increases after the protein's denaturation and binding is followed by increased HSP70 ATPase activity (Palleros et al., 1991). It is believed that the ATP provides the energy required for HSP70 to properly fold the bound protein; dissociation of HSP70-protein complex requires ATPase activity (Flaherty et al., 1991).

Synthesis of HSP70 can be induced by several factors that disrupt cellular function. In addition to heat shock, these include cadmium and arsenite (Opanashuk & Finklestein, 1995; Cigliano et al., 1996), agents increasing free radical oxidation such as nitric oxide (Kim et al., 1997), various drugs [for example, geranylgeranyl acetone (Hirakawa et al., 1996)], ethanol (Su et al., 1998), and low pH (Narasihman et al., 1996). Synthesis of HSP70 molecules can also be increased by growth factors (Wu et al., 1987; Teshima et al., 1996).

Induction of *hsp70* gene expression by heat shock involves binding of transcription factor to a heat shock element (HSE) in the 5' flanking region. There are two transcription factors for HSE, heat shock factors 1 and 2 (HSF1 and HSF2). HSF1 is activated by heat shock and other stresses (Sistonen et al., 1994; Liu et al., 1994; Huang et al., 1995; Morimoto et al., 1996) while HSF2 is involved with regulation of gene expression under non-stressed conditions (Sistonen et al., 1994). In a non-stressed cell, HSF1 is present as a monomer bound to HSP70. Protein denaturation caused by heat shock or other stimuli leads to utilization of HSP70 and its removal from HSF1. As a result, HSF1 trimerizes, binds to HSE and leads to transcriptional activation (Morimoto et al., 1996).

Role in Heat Shock

Heat shock protein 70 is important in protection from heat shock - cells can be made resistant to heat by increasing intracellular HSP70 concentrations (Hendrey & Kola, 1991; Angelidis et al., 1991) and made more susceptible to heat shock by decreasing HSP70 concentrations (Riabowol et al., 1988; Johnson & Kucey, 1988). Elevations in HSP70 also protect from other shocks including nitric oxide (Bellmann et al., 1996), endotoxin (Chi & Mestral, 1996), ultraviolet light (Simon et al., 1995), tumor necrosis factor (Jaattela et al., 1992) and various anticancer drugs (Sliutz et al., 1996). It is believed that HSP70 protects cells during heat shock by maintaining protein structure, refolding denatured proteins, stabilizing the cytoskeleton and preserving ribosomal function (Georgopoulos & Welch, 1993; Mizzen & Welch, 1988; Welch & Mizzen, 1988; Liang & MacRae, 1997). The ATP-binding domain is required for the thermoprotective effect of HSP70 (Li et al., 1995). HSP70 also blocks induction of apoptosis in cells through inhibition of CED-3 related caspases

(Mosser et al., 1997) and can inhibit translation through reduced phosphorylation of eukaryotic-initiation factor-2α (Chang et al., 1994; Thulasiraman et al., 1998). This latter action likely is important for reducing protein synthesis during heat shock when the potential for protein misfolding is high.

Regulation of HSP70 Synthesis in the Preimplantation Embryo

The Mouse Embryo

Like many other messages in the oocyte, mRNA for HSP70i decreases rapidly after oocyte maturation (Manejwala et al., 1991). However, amounts of HSP70i mRNA and protein increase at the 2-cell stage in an α-amanitin sensitive manner (Bensaude et al., 1983; Manejwala et al., 1991; Christians et al., 1995). The embryonic genome first undergoes transcription at the late 1-cell and 2-cell stage (Schultz et al., 1995); thus, *hsp70i* is one of the first genes to be transcribed by the mouse embryo. Administration of butyrate to hyperacetylate histones causes premature transcription of HSP70i mRNA (Thompson et al., 1995), implicating nucleosomal structure in the activation of *hsp70i* gene expression at the time of embryonic genome activation. Moreover, cAMP-dependent phosphorylation events are implicated in activation of *hsp70i* gene expression since H-8, the protein kinase A inhibitor, blocks the increase in HSP70 mRNA seen at the 2-cell stage (Manejwala et al., 1991).

The initiation of HSP70i synthesis at embryonic genome activation does not require heat shock but the degree of activation does depend upon the environment. In particular, the increase in HSP70i was greater for embryos in culture than embryos that developed in utero; antioxidants reduced slightly the magnitude of the increase seen in cultured embryos (Christians et al., 1995). The increase at the time of embryonic genome activation likely involves HSF-1 activation of gene expression because HSF-1 is already concentrated in the nucleus by the 1-cell stage (Christians et al., 1997[a]). Mutagenesis studies have indicated that HSEs are involved in the activation (Christians et al., 1997[a]).

It is not known how early in development heat shock induces HSP70i synthesis. Originally, it was thought that heat shock does not induce HSP70i synthesis until the morula or blastocyst stage. However, early studies were performed with very severe heat shock temperatures ≥ 43°C (Morange et al., 1984; Muller et al., 1985; Hahnel et al., 1996). In a more recent study using a heat shock of 40°C, induction of HSC70 and HSP70i synthesis could be detected at the 8-cell stage, the earliest stage examined (Edwards et al., 1995). Furthermore, expression of a HSP70 transgene could be induced by heat shock as early as the 2-cell stage, although maximal levels of transgene activity were reached sooner following heat shock for blastocysts than for embryos at other stages (Christians et al., 1997[b]). Binding of transcription factors to the HSE in response to heat has been reported to occur for oocytes and embryos at all stages of development except for the 4-cell stage (Metzger et al., 1994).

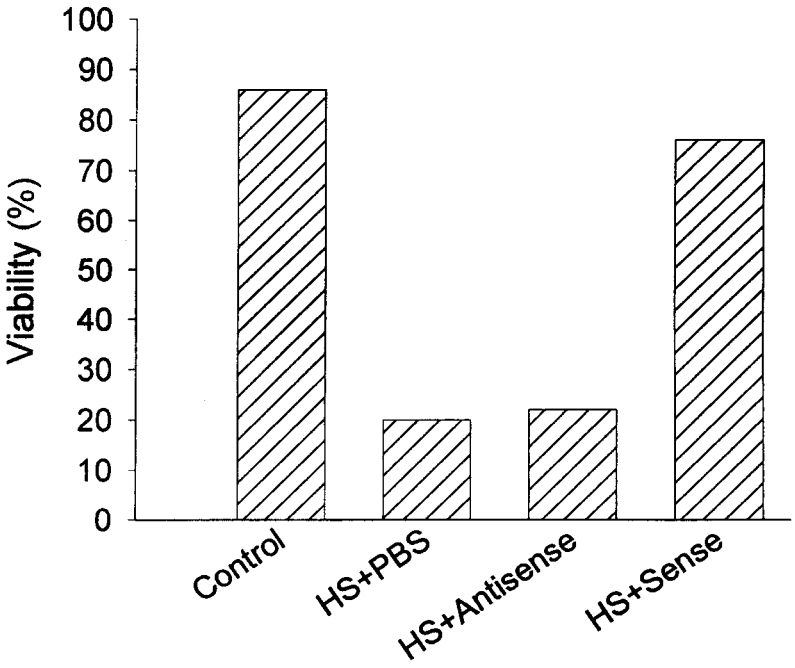

Figure 1. Thermoprotective effects of heat shock protein 70 in mouse oocytes. Mouse oocytes were injected with PBS vehicle, HSP70 mRNA (Sense) or HSP70 antisense mRNA (Antisense). Embryos were cultured at 37°C for 6 h after injection and then were kept at 37°C (Control) or exposed to 42-43°C for 1 h (HS). After an additional 4 h at 37°C, oocyte viability was determined by staining with flourescein diacetate. Data are redrawn from Hendrey and Kola (1991).

To date, the importance of HSP70 for the maintenance of embryonic function can be inferred only indirectly. As illustrated in Figure 1, injection of mRNA for HSP70 into oocytes increased oocyte survival following exposure to heat shock (Hendrey & Kola, 1991). Also, HSP70 is important for protection from other stresses as indicated by the finding that provision of antisense oligonucleotides to two HSP70 molecules (HSP70-1 and HSP70-3) made mouse embryos more susceptible to arsenic toxicity (Dix et al., 1998). Indeed, HSP70 is important for development in the absence of heat shock since both antisense treatment (Dix et al., 1998) and provision of HSP70 antibody to culture medium (Neuer et al., 1998) decreased development in the absence of stress. Similar effects were seen when antibodies to HSP90 or HSP60 were added to culture. The results of Neuer et al. (1998), besides implicating heat shock proteins in embryonic development, also indicate that these heat shock proteins are accessible to antibody, either because the antibody penetrates the cell or the heat shock proteins are on the cell surface.

The Bovine Embryo

Based on the fact that the embryonic genome of cattle is not believed to undergo transcription until the late 4-cell or 8-cell stage (Frei et al., 1989; Kopecny et al., 1989; Barnes & First, 1991; Jones & First, 1995; de Sousa et al.,1998), it was originally hypothesized that 2-cell embryos would be unable to synthesize increased amounts of HSP70 in response to heat shock. It is now known, however, that some transcription occurs as early as the 1-cell stage (Saeki et al., 1999) and the transcriptional inhibitor a-amanitin reduces protein synthesis by 2-cell embryos and cleavage rate (Edwards et al., 1997; Memili & First, 1998; Chandolia et al., 1999).

Moreover, as shown by Edwards and Hansen (1996), the embryo can undergo increased synthesis of HSP70i in response to heat shock as early as the 2-cell stage.

The pattern of synthesis of HSP70 proteins in the bovine oocyte and 2-cell embryo is shown in Figure 2 (Edwards & Hansen, 1996). The immature bovine oocyte can synthesize two HSC70 molecules and a protein corresponding to HSP70i - however, the rate of synthesis of these proteins is not altered by heat shock (Edwards & Hansen, 1996). Following oocyte maturation, synthesis of one of the HSC70 proteins ceases and rates of synthesis of the other two HSP70s remain unaffected by heat shock. By the 2-cell stage, there is little or no detectable synthesis of HSP70i in control embryos. However, synthesis of this protein increases greatly following heat shock. This same pattern of HSP70 synthesis continues for embryos at all stages of development through the blastocyst stage (Edwards et al., 1997).

The increase in synthesis of HSP70i by 2-cell embryos in response to heat could be due to new transcription or to post-transcriptional regulation of protein synthesis. In some cellular systems, HSP70 synthesis can increase in the absence of transcription, for example by increased translational efficiency of HSP70 mRNA (Theodorakis & Morimoto, 1987; Theodorakis et al., 1988). Also, α-amanitin, an inhibitor of transcription, did not block the increase in synthesis of HSP70i caused by heat shock at the 2-cell stage although it did at later stages (Edwards et al., 1997). However, more recent studies using RT-PCR support the idea that the heat-induced

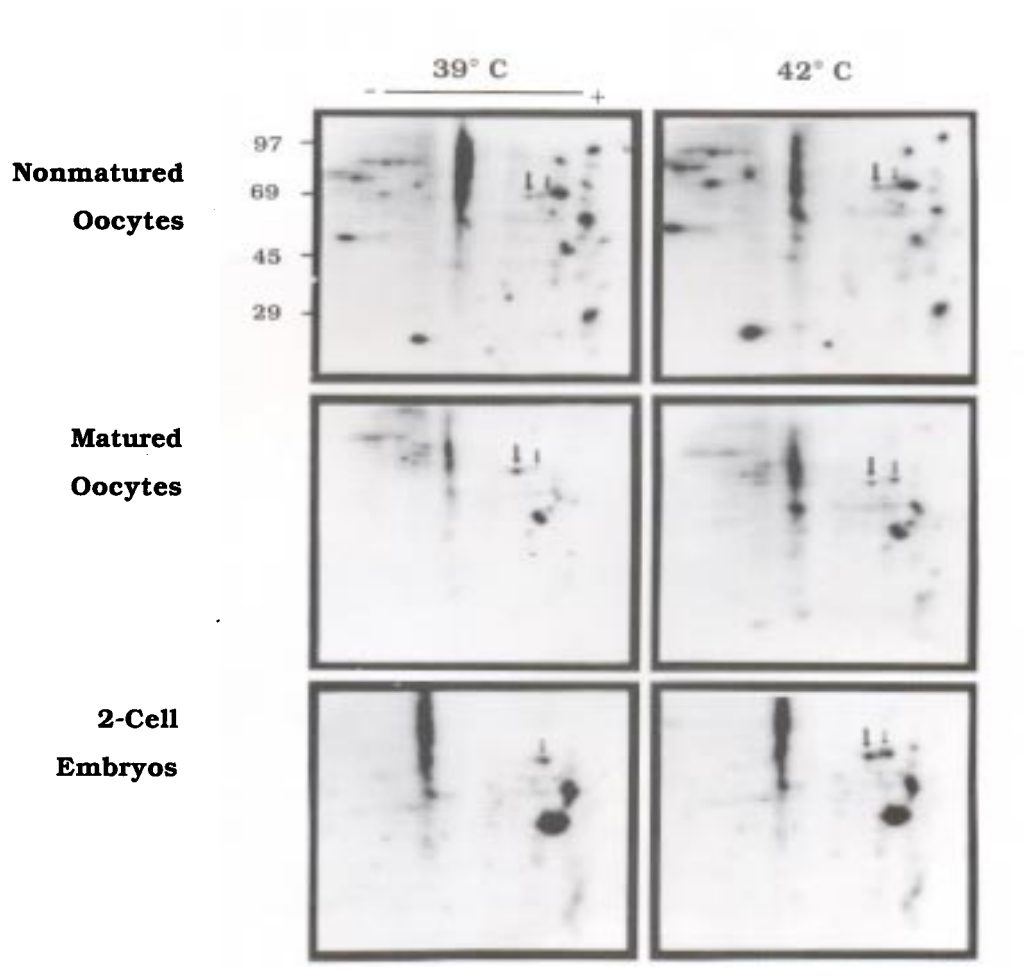

Figure 2. Synthesis of heat shock protein 70 variants by bovine oocytes and 2-cell embryos. Protein synthesis was determined by culturing oocytes or embryos with [^{35}S]-amino acids for 4.33 h at 39°C or 42°C for 1.33 h and 39°C for 3 h. Radiolabeled proteins were identified by two-dimensional gel electrophoresis and fluorography. The HSC70 is indicated by the small arrow and the HSP70i by the large arrow. Data are from Edwards and Hansen (1996) and are reproduced with permission from *Biology of Reproduction*.

increase in HSP70i synthesis at the 2-cell stage is due in large part to transcription (Chandolia et al., 1999). In particular, heat shock increased amounts of HSP70i mRNA in 2-cell embryos and this increase was reduced by co-culture with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole or actinomyin-D (Figure 3), two transcriptional inhibitors that act through separate mechanisms from that of α-amanitin. There was some indication that, when large numbers of embryos were extracted for RT-PCR, HSP70i mRNA increased slightly in the presence of actinomycin D. Thus, heat shock may also cause some increased mRNA stability.

The regulation of the *hsc70* gene by heat shock has not been well studied. However, transcripts for a constitutive *hsc70* gene have been detected in oocytes and embryos through the expanded blastocyst stage (Wrenzycki et al., 1998).

Glutathione

Free Radicals and Antioxidant Mechanisms

Approximately 1-2% of metabolized oxygen is converted to a reactive oxygen species (Fulbert & Cals, 1992). Free radicals and other reactive oxygen molecules are very toxic: their strong affinity for electrons leads to oxidation of most molecules in the cell including DNA, proteins, and lipids. Given that metabolic rate of cells can increase with temperature, it is likely that free radical production is increased by heat shock. Indeed, Loven (1988) and others have proposed that reduced oxygen species generated during heat shock play a key role in the disruption of cellular function by heat. There are several lines of evidence to support this thesis. First, there is increased activity of enzyme systems that produce superoxide during heat shock including cyclooxygenase (Malayer et al., 1990), and xanthine oxidase (Skibba et al., 1989a). Secondly, peroxidation products have been shown to increase after heat shock of perfused liver (Skibba et al., 1989[b]). Similarly, heat stress in vivo

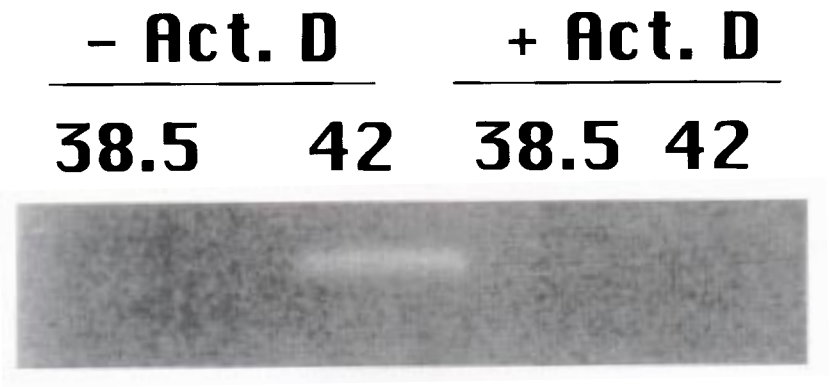

Figure 3. Changes in transcripts for HSP70i in bovine 2-cell embryos as affected by temperature and actinomycin D. Embryos were cultured with or without actinomycin-D (Act. D) beginning at 8 h after insemination, collected at 32-34 h post-insemination, and then exposed to either 38.5°C for 4.33 h or 42°C for 1.33 h followed by 38.5°C for 3 h. RNA was extracted from pools of embryos and subjected to RT-PCR using primers specific for the inducible form of HSP70. Data are modified from Chandolia et al. (1999) and are reproduced with permission of *Biology of Reproduction*.

increased lipid peroxidation in the liver of rats (Ando et al., 1997) and decreased the total antioxidant activity in blood of cows (Harmon et al., 1997) [note, however, there was no effect of heat stress on muscle lipid peroxidation or circulating concentrations of β-carotene or vitamin E in cows (Trout et al., 1998)]. Also, heat shock increased oxidation of the antioxidant glutathione (Zou et al., 1998) and experimental depletion of glutathione increased sensitivity of cells to heat shock (Mitchell et al., 1983; Russo et al., 1984; Shrieve et al., 1986; Roison-Towle et al., 1986; Will et al., 1999). Moreover, administration of extracellular antioxidants conferred thermoprotection to CHO cells (Kapiszewska & Hopwood, 1988) and lymphocytes (Malayer et al., 1992).

Given the potentially harmful effects of free radicals, several biochemical systems exist in cells and extracellular fluid to remove these molecules. Antioxidant systems include molecules such as β-carotene and vitamin E which react with free radicals in cell membranes to maintain the integrity of phospholipids against oxidative damage and peroxidation. Glutathione plays a similar role as a free-radical sink in the cytoplasm by donating a electron to reduce reactive oxygen species. Organisms also contain various enzymes in the cellular and extracellular compartments that catalyze the removal of free radicals. Among these are superoxide dismutase, which converts superoxide to hydrogen peroxide, and glutathione peroxidase, which is a selenium-dependent enzyme that utilizes electrons from glutathione and other thiols to convert peroxides to water.

Like other cells, the embryo possesses many of these antioxidant defenses. In the preimplantation cow embryo, for example, transcripts for cupper-zinc superoxide dismutase, catalase, and glutathione peroxidase can be identified (Harvey et al., 1995). Of the antioxidant systems present in the embryo, glutathione is the only one studied in any detail with regards to resistance to heat shock.

General Properties of Glutathione

Glutathione is a tripeptide with the sequence γ-glu-cys-gly. Existing at intracellular concentrations of 0.1-10 mM (Kosower, 1976), glutathione is one of the major antioxidants of the cell. Glutathione is synthesized in a two-step process whereby γ-glutamylcysteine is formed by the actions of γ-glutamylcysteinyl synthetase and then converted by glutathione synthetase to reduced glutathione (GSH) by the addition of a glycine. Oxidized glutathione exists as glutathione disulfide (GSSG), formed from a disulfide bond between two γ-glu-cys-gly chains. Much GSSG is formed during oxidative stress. While some GSH is regenerated from GSSG by the actions of glutathione reductase, it is probable that much of the oxidized form of glutathione is lost from the cell because GSSG can readily cross cellular membranes (Meister, 1985). In contrast, GSH does not easily enter the cell: transport across the membrane is achieved through breakdown of GSH at the membrane by γ-glutamyl transpeptidase and resynthesis of GSH inside the cell. Consequently, GSH administration causes only a small rise in intracellular GSH content (Jensen & Meister, 1983).

Because of its abundance in the cell and the strong nucleophilic character of its thiol group, GSH is a major antioxidant in cells. It reacts directly with free radicals and peroxides (Kosower, 1976) and also is oxidized in several enzyme-catalyzed reactions (Flohé and Günzler, 1976). The major enzymes through which GSH is involved in minimizing oxidative stress are glutathione peroxidase (a Se-containing enzyme), several isozymes of glutathione transferase, and various transhydrogenases. Glutathione peroxidase and glutathione transferase function as peroxidases by using GSH as a hydrogen donor to reduce hydroperoxides to alcohols according to the formula: $2\ GSH + ROOH \rightarrow ROH + H_2O + GSSG$. Substrates included both H_2O_2 and lipid peroxides. Glutathione dehydrogenases are important because they regenerate protein sulfhydryl groups from S-S-groups formed during oxidative stress (Di Mascio et al., 1991). Glutathione reduces the magnitude of the depletion of vitamin E that occurs during peroxidation because GSH-mediated reactions spare vitamin E (Di Mascio et al., 1991). Because of these effects of GSH, depletion of glutathione from cells results in decreased cellular concentrations of vitamin E and protein thiols and increased lipid peroxidation (Pascoe et al., 1987).

Role of Glutathione in Protection of Cells from Heat Shock

Several lines of evidence suggest that glutathione plays an important role in protecting cells from heat shock. Inhibition of GSH synthesis with the γ-glutamylcysteinyl synthetase inhibitor, D,L-buthionine-S-R-sulfoximine (BSO), increased sensitivity of tumor cells to heat shock (Steels et al., 1992). Also, BSO prevented or reduced the induction of thermotolerance by sublethal heat shock that ordinarily makes cells resistant to a subsequent, more lethal heat shock (Mitchell et al., 1983; Russo et al., 1984; Shrieve et al., 1986; Harris et al., 1991). Secondly, intracellular GSH concentrations have been reported to increase after heat shock in hamster fibroblasts (Mitchell et al., 1983), perfusates of rat liver (Skibba et al., 1989[a]) rat postimplantation embryos (Harris et al., 1991), mouse fibroblasts (Konings & Peninga, 1985) and Ehrlich ascites tumor cells (Konings & Penninga, 1985). The intracellular content of GSH increases following heat shock at least in part due to the regulation of GSH synthesis by HSP27 and other members of the small heat shock protein family since these proteins can increase glutathione synthesis in L929 and NIH 3T3 cell lines (Mehlen et al., 1996). Finally, administration of extracellular GSH prevented blebbing of plasma membranes of CHO cells caused by heat shock (Kapiszewska & Hopwood, 1988) while CHO cells could be made thermotolerant by intracellular injection of GSSG (Lumpkin et al., 1988).

Taken together, there is strong evidence that glutathione plays an important role in the events leading up to thermotolerance. It should be pointed out, however, that the relative importance of glutathione in preventing heat damage of cells is likely to vary with cell type. For example, Roizin-Towle et al. (1986) found that glutathione depletion increased thermosensitivity of lung carcinoma cells while not affecting thermal resistance of hamster fibroblasts.

Glutathione in the Preimplantation Embryo

In the mouse, GSH content declines as a result of fertilization and continues to decline as embryos develop to the blastocyst stage (Gardiner & Reed, 1994; see Figure 4). Cultured embryos had lower GSH content than embryos that developed in vivo (Gardiner & Reed, 1994), reflecting the higher oxidative stress associated with culture. According to Gardiner and Reed (1995 [a,b]), who studied embryonic capacity for synthesis of GSH by measuring restoration of GSH content after depletion with tertiary-butyl hydroperoxide or diethyl maleate, the mouse embryo can synthesize GSH as early as the 2-cell stage (the earliest stage examined) although the blastocyst has greater capacity for synthesis than the 2-cell embryo. Transcripts for γ-glutamylcysteinyl synthetase were present throughout development to the blastocyst stage in the mouse (Harvey et al., 1995).

In the cow, transcripts for γ-glutamylcysteinyl synthetase were detected through the morula stage but not in blastocysts (Harvey et al., 1995). Moreover, the developmental pattern in GSH content in embryos is somewhat different than in mice (Figure 4). As in mice, levels of GSH decreased from the 1-cell to the 2-8 cell stage. However, unlike in mice, concentrations in bovine embryos became elevated again at later stages of development (Lim et al., 1996).

Glutathione is important for embryonic development. Inhibition of synthesis of GSH by BSO reduced development of mouse (Gardiner & Reed, 1995[b]; Aréchiga & Hansen, 1998) and bovine (Takahashi et al., 1993) embryos and blocked maturation (de Matos et al., 1996) and developmental competence (Edwards & Hansen, 1997) of bovine oocytes. Addition of GSH to culture media improved development rate in mice (Legge & Sellens, 1991; Gardiner & Reed, 1994) and cattle (Luvoni et al., 1996).

Role of Glutathione in Protection of Embryos from Heat Shock

Generation of free radicals plays some role in mediating damaging effects of heat shock on embryonic development because addition of antioxidants to culture medium, including GSH, vitamin E and taurine, reduced the inhibitory effects of heat shock on bovine (Ealy et al., 1992) and mouse embryos (Malayer, et al., 1992; Aréchiga et al., 1994, 1995). Moreover, stimulation of GSH synthesis by addition of S-adenosyl methionine to culture medium increased resistance of mouse morulae to heat shock (Aréchiga et al., 1995). As illustrated in Figure 4, amounts of GSH in mouse morulae tend to decrease following heat shock (Aréchiga et al., 1995), suggesting that mechanisms involving GSH are involved in elimination of free radicals generated by heat shock. Somewhat surprisingly, however, sensitivity of mouse embryos (Aréchiga & Hansen, 1998) and bovine oocytes (Edwards & Hansen, 1997) to heat shock was not increased by addition of BSO to culture medium. As described in the next section, however, there are indications that Glutathione is involved in embryonic adaptation to heat shock.

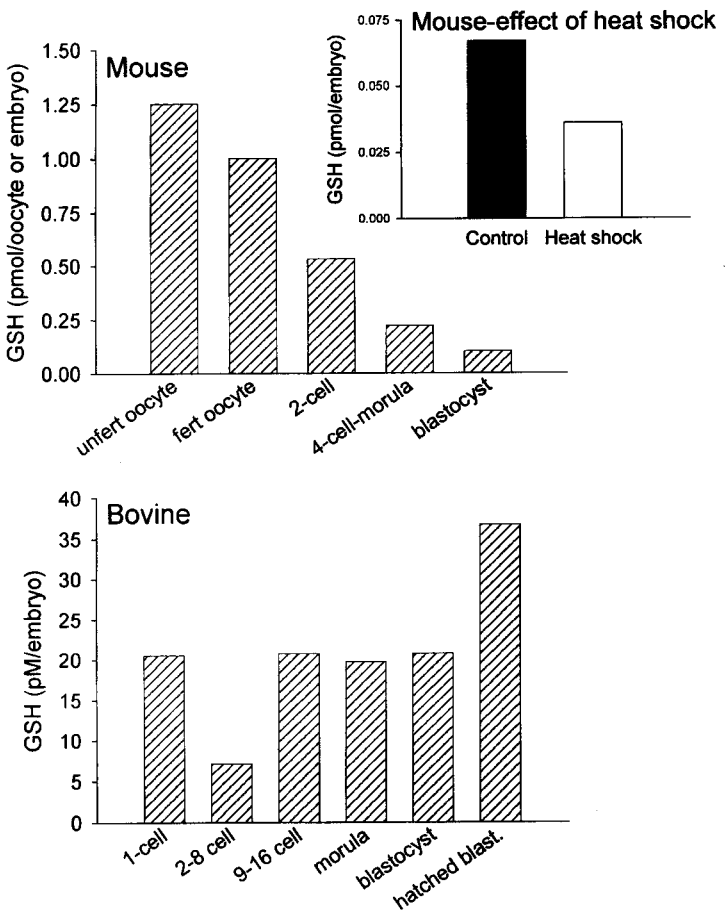

Figure 4. Changes in embryonic content of reduced glutathione with stage of development and heat shock. The main graphs are redrawn from Gardiner and Reed (1994) (mouse) and Lim et al. (1996) (bovine). The inset panel represents data from mouse morula-stage embryos cultured at 37°C for 3 h (Control) or 41°C for 1 h followed by 37°C for 2 h (Heat shock). Results are redrawn from Aréchiga et al. (1995).

Induced Thermotolerance in Preimplantation Embryos

The literature is replete with examples where cells that are briefly exposed to a sublethal heat shock become resistant to a subsequent, more severe heat shock that would otherwise be lethal (see Nover, 1991). This phenomenon, called induced thermotolerance, is a manifestation of adaptive biochemical mechanisms utilized by cells to maintain structure and function in the face of elevated temperature. Mild heat shock can also confer protection to other stresses, for example, arsenic, cadmium, ethanol, nitric oxide and various free radicals (Kapron-Bras & Hales, 1991; Kampinga et al., 1995; Bellmann et al., 1995; Wang et al., 1996). Both heat shock proteins (Koishi et al., 1992; Sato et al., 1996; Kim et al., 1997) and glutathione (Mitchell et al., 1983; Russo et al., 1984) have been implicated in induced thermotolerance.

That preimplantation embryos can undergo induced thermotolerance has been shown for both mouse (Muller et al., 1985; Ealy & Hansen, 1994; Arechíga et al., 1995) and bovine embryos (Ealy & Hansen, 1994). At least in the mouse, induced thermotolerance is a developmentally-acquired process. Exposure to mild heat shock did not increase resistance of 1-cell (Muller et al., 1985) or 2-cell embryos (Aréchiga & Hansen, 1998) to a more severe heat shock. Induced thermotolerance was possible for embryos at the 8-cell, morula and blastocyst stages of development (Muller et al., 1985; Ealy & Hansen, 1994; Aréchiga et al., 1995; Aréchiga & Hansen, 1998).

While heat-induced synthesis of HSP70 is a logical explanation for the process by which thermal resistance is induced by exposure to a mild heat shock, the phenomenon of induced thermotolerance can be divorced from HSP70 synthesis.

Ealy and Hansen (1994) noted that induced thermotolerance in mouse morula and blastocyst-stage embryos occurred if embryos were cultured in medium containing serum but did not occur if embryos were cultured in medium where serum was replaced by bovine serum albumin. However, heat-induced synthesis of HSP70 can occur in embryos cultured in serum-free medium (Edwards et al., 1995). Moreover, exposure of blastocysts to 40°C was sufficient to induce thermotolerance in blastocysts (Ealy & Hansen, 1994) even though this temperature did not increase HSP70 synthesis (Edwards et al., 1995). Taken together, it seems that the amount of embryonic HSP70 synthesis is not a critical determinant of embryonic thermal resistance following mild heat shock. A conclusive determination of the role of HSP70 must await studies whereby HSP70 is removed from embryos (for example, by using knockout mice or antisense oligonucleotide experiments).

In contrast to the situation with HSP70, glutathione has been implicated in the induced thermotolerance phenomenon in mouse embryos. Culture of mouse morulae with BSO blocked the ability of a brief exposure of 40°C to make embryos resistant to a subsequent exposure to 43°C (Figure 5). Perhaps, increased synthesis of GSH following mild heat shock provides the embryo with higher amounts of intracellular GSH to counteract free radical generation during the subsequent, more severe heat shock. This explanation is consistent with findings that embryonic resistance to heat shock was increased by administration of GSH or S-adenosyl methionine to culture medium (Aréchiga et al., 1994, 1995). Glutathione may also be playing a more permissive role. In some (Russo et al., 1984; Rokutan et al., 1996) but not all (Harris et al., 1991) cellular systems, synthesis of HSP90, HSP70, and HSP60 was dependent upon cellular capacity for GSH synthesis. In pig gastric mucosal cells, BSO blocked ability of HSF1 to translocate to the nucleus following stress (Rokutan et al., 1996). Thus, BSO may indirectly inhibit synthesis of other molecules required for thermotolerance.

Changes in Embryonic Resistance to Heat Shock during Early Embryonic Development

The fact that induced thermotolerance is a developmentally acquired characteristic, at least in mouse embryos (Ealy & Hansen, 1994), implies that as embryos advance in development, thermoprotective mechanisms become more effective or new thermoprotective mechanisms are acquired. While additional studies are warranted, other data also point to the embryo becoming more resistant to heat shock as it proceeds through development.

One of the characteristic features of heat stress on embryonic survival, at least in the sheep (Dutt, 1963), pig (Tompkins et al., 1967) and cow (Ealy et al., 1983), is that the magnitude of the depression in fertility is less when heat stress is applied later in the preimplantation period than when applied earlier in development. In cows, for example, exposure of superovulated females to heat stress at day 1 after breeding (i.e., when embryos were at the 1 or 2-cell stage) reduced the proportion of

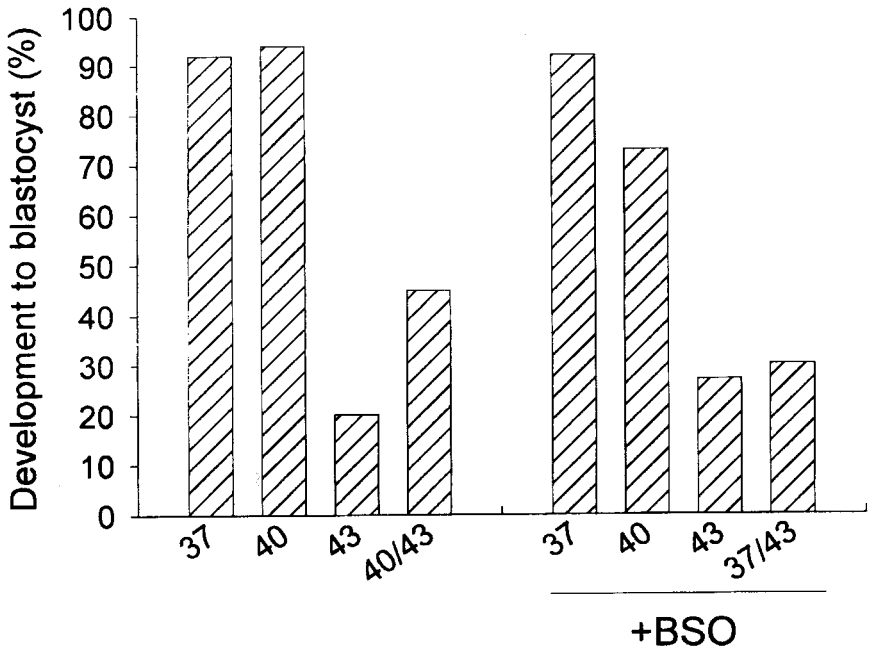

Figure 5. Inhibition of induced thermotolerance in mouse morulae by buthionine sulfoximine (BSO). Embryos were cultured at 37° continuously (37), 40°C for 1 h and then 37°C (40), 43°C for 2 h and 37°C thereafter (43) or 40°C for 1 h (to induce thermotolerance), 37°C for 2 h, 43°C for 2 h and then 37°C thereafter (40/43). Development to the blastocyst stage was assessed at 23 h after start of culture. Note that exposure of embryos to 40°C had no effect on embryonic development (compare 37 vs 40) but pre-exposure to 40°C reduced the deleterious effects of 43°C on development (compare 43 with 40/43 groups). This thermoprotective effect of exposure to 40°C was blocked for embryos cultured with BSO. Results are taken from Aréchiga et al. (1995).

embryos at day 8 of pregnancy that were at the blastocyst stage of development (Ealy et al., 1994). However, heat stress had no effect on the proportion of embryos at day 8 classified as blastocysts when heat stress was applied at day 3 (4-8 cell), 5 (16 cell-morula) or day 7 (morula-blastocyst stage).

Using reciprocal embryo transfer between heat-stressed donor or recipient ewes, Alliston and Ulberg (1961) demonstrated that both the embryo and reproductive tract are compromised by heat stress, with greater effects being exerted on the embryo itself. This result implies that the cause for the reduction in severity of deleterious effects of heat stress as pregnancy proceeds lies in the acquisition of thermal resistance by the preimplantation embryo as it progresses through development. There is evidence to support this explanation in cattle and mice. For cattle embryos, there is a biphasic pattern in developmental changes in resistance to heat shock (Edwards & Hansen, 1997). Heat shock (a 12-h exposure to 41°C) caused a greater reduction in ability to develop to the blastocyst stage when applied at the 2-cell stage than when applied to oocytes before fertilization. Thereafter, heat shock at the 4-8 cell stage disrupted development but less so than when applied to 2-cell embryos. Morulae were unaffected by heat shock. In the mouse, a heat shock of 40°C for 6 h reduced subsequent development when applied at the 1-cell stage but not when applied at the 2-cell stage (Alliston et al., 1965). In another study, a heat shock of 41°C for 1 h reduced the proportion of 2-cell embryos becoming blastocysts, had a slight effect on development

of 4-cell embryos and had no effect on development of morulae to the blastocyst stage (Aréchiga & Hansen, 1998; Figure 6). Two-cell embryos that were delayed in development (i.e., became 2-cell embryos at 30-34 h after detection of a vaginal plug) were more affected by heat shock than embryos that reached the 2-cell stage faster (i.e., were found at 24-27 h after detection of a vaginal plug). This result implies that embryos with reduced developmental competence may also be deficient in biochemical mechanisms for establishing thermal resistance. Differences in thermal resistance between embryos of different stages were not apparent when a more severe heat shock (41°C for 2 h) was applied. Rather, the reduction in percent development to blastocyst was similar for all stages.

Caution must be exercised when interpreting studies of Edwards and Hansen (1997) and Arechíga and Hansen (1998) because of the endpoint used to determine effects of heat stress (development to the blastocyst stage). It is possible that earlier embryos could appear more sensitive to heat shock than later stages only because they have more developmental stages to pass through than older embryos before becoming blastocysts. Interestingly, while the effect of 41°C for 1 h on the percentage of mouse embryos becoming blastocysts depended on stage of development at which heat shock was applied, there was no similar effect of stage of development on the magnitude of heat-induced disruption of the proportion of embryos that hatched from the zona pellucida (Aréchiga & Hansen, 1998; Figure 6). Also, little work has been done to date to examined whether the size and functional properties of blastocysts formed after heat shock is altered.

There are, however, two additional pieces of evidence using other measurements of embryonic function that support the idea that embryos become more resistant to heat shock as development proceeds. First, the reduction in embryonic protein synthesis caused by heat shock is muted in more advanced embryos. Exposure of mouse embryos to 40°C for 80 min reduced protein synthesis in 8-cell embryos but not in expanded blastocysts (Edwards et al., 1995). Similarly, in cattle, a heat shock of 42°C for 80 min reduced protein synthesis in embryos at the 4-cell, 16-32 cell, morula and blastocyst stages of development but not at the 8-cell, expanded blastocyst or hatched blastocyst stages (Edwards et al., 1997). Secondly, the temperature needed to induce synthesis of HSP70 in mouse embryos increases as development proceeds (Edwards et al., 1995). In particular, a heat shock of 40°C for 80 min, which induced synthesis of three HSP70 proteins in 8-cell embryos, had no effect on HSP70 synthesis in expanded blastocysts. The blastocysts were capable of HSP70 synthesis since heat shock of 43°C for 80 min induced synthesis of the HSP70s. Since one major signal for HSP70 synthesis in heat shocked cells is the presence of denatured protein (Ananthan et al., 1986), it is likely that the expanded blastocyst possesses biochemical mechanisms to stabilize protein structure that are absent or reduced in the 8-cell embryo.

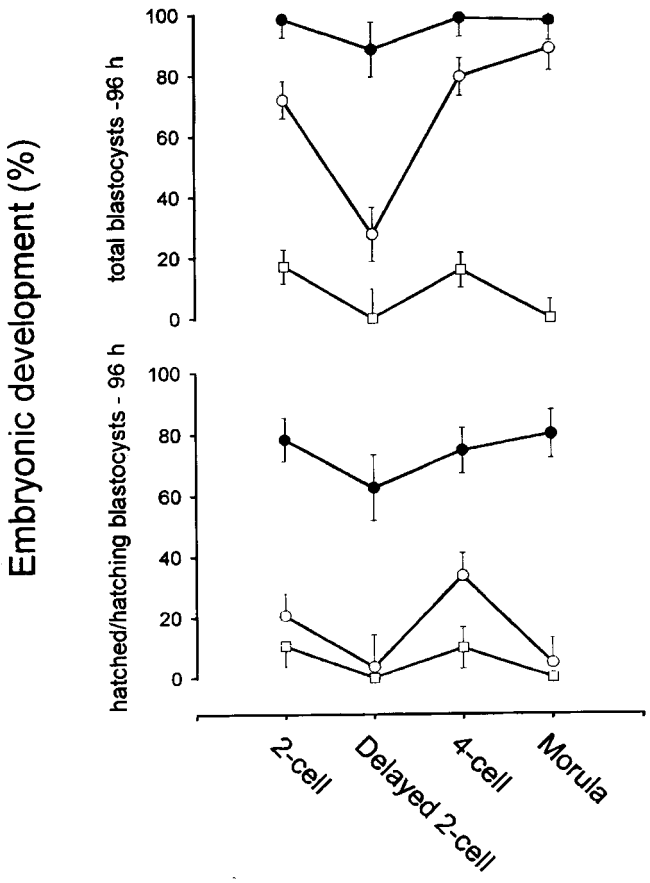

Figure 6. Development of murine embryos exposed to 37°C continuously (●) or to a heat shock of 41°C for 1 h (0) or 41°C for 2 h (□) at the developmental stages indicated on the x-axis. The figure is reproduced from Aréchiga and Hansen (1998) with permission from *In Vitro Cellular and Developmental Biology-Animal.*

Summation - The Role of HSP70 and Glutathione in Ontogeny of Thermal Resistance

When seeking to explain the phenomenon by which embryos become more resistant to heat shock with advancing developmental stage, the question arises as to whether development of thermal resistance is due to changes in the HSP70 or glutathione systems or to other biochemical systems not yet explored. The answer to this question is not known with certainty but available evidence would indicate that the biochemical systems that change in development to allow for embryonic resistance to heat shock are complex and involve more than just HSP70 and glutathione.

For a molecule to be responsible for stage-specific differences in embryonic resistance to heat shock, it is likely that changes in synthesis or intracellular content of the molecule would parallel differences in resistance to heat shock between embryos of different stages. Furthermore, experimental reduction in the synthesis of the molecule would increase sensitivity of embryos at resistant stages of development to heat shock. To date, no studies have been performed to test whether inhibition of HSP70 synthesis makes embryos more susceptible to heat shock and therefore the importance of this molecular chaperone for embryonic resistance to heat shock is unknown. However, the case has already been made in Section 4 that conditions for the mouse embryo that are suitable for increased synthesis of HSP70 in response to heat shock are not necessarily sufficient or required induced for thermotolerance and may not be required. Thus, HSP70 alone is probably insufficient for induced thermotolerance. There is evidence that failure of 1-cell embryos to undergo induced thermotolerance (Muller

et al., 1985) is due to failure of these early embryos to experience increased HSP70 synthesis following heat shock. Such an explanation is plausible because the 1-cell embryo has not yet undergone embryonic genome activation and relies on maternal mRNA for directing protein synthesis (Schultz et al., 1995). However, these studies should be repeated with less severe heat shocks than the 43°C used by Muller et al (1995) because of observations that 40°C can induce HSP70 synthesis in mouse 8-cell embryos previously thought not capable of a HSP70 response to heat shock

In the bovine embryo, there is no qualitative difference in heat-shock induced HSP70 synthesis between embryos at different stages from the 2-cell to blastocyst stage (Edwards et al., 1997) despite the fact that heat shock causes progressively smaller reduction in the percentage of embryos developing to blastocysts as development precedes (Edwards & Hansen, 1997). Thus, qualitative capacity for HSP70 synthesis may not be a determinant of developmental changes in resistance to heat shock. It is possible, however, that the amount or rate of maximal synthesis of HSP70 following heat shock increases as embryos proceed in development (Christians et al., 1997[b]). There may also be maturation in the biochemical pathways that HSP70 interacts with so that HSP70 is more effective at stabilizing cellular function in the face of heat shock as embryos become advanced in development.

A stronger case can be made for the role of glutathione in ontogeny of embryonic resistance to heat shock than for HSP70. While the amounts of GSH in the mouse embryo decline as the embryo advances in development (Gardiner & Reed, 1994), embryonic capacity for GSH biosynthesis increases from the 2-cell stage to the blastocyst stage (Gardiner & Reed, 1995 [a,b]). In the cow, intracellular content of GSH is lowest at the 2-8 cell stage and increases thereafter (Lim et al., 1996). Furthermore, inhibition of GSH synthesis by administration of BSO, while having no effect on embryonic survival following heat shock of mouse embryos (Aréchiga & Hansen, 1998) or bovine oocytes (Edwards & Hansen, 1997), blocked the ability of mouse morulae to undergo induced thermotolerance (Aréchiga et al., 1995). One interpretation of these findings is that intrinsic resistance to heat shock (i.e., in the absence of heat-induced biochemical protective responses) does not depend upon cellular GSH content but that induced thermotolerance reflecting cellular adaptations to heat shock is dependent upon GSH biosynthesis.

The choice of glutathione and HSP70 as subjects of study into thermoprotective mechanisms has been somewhat arbitrary and undoubtedly other molecules are also involved in embryonic resistance to heat shock. These include heat shock proteins such as HSP90, HSP60 and HSP47 as well as proteins such as ubiquitin (Nowak et al., 1990) and heme oxygenase-1 (Ewing et al., 1992; also called HSP32) whose synthesis can be increased by heat shock and which play roles in maintenance of cellular function following stress or damage. There are also many antioxidant systems that could be important for embryonic resistance to heat shock including not only molecules like glutathione, vitamin E and vitamin C, which react directly with free radicals, but

enzymes such as glutathione peroxidase, catalase and superoxide dismutase that participate in antioxidant defense. Glutathione itself is a cytosolic antioxidant and, given the importance of lipid oxidation in free radical damage, attention should be paid to the role of lipid-soluble antioxidants such as vitamin E and vitamin E-sparing molecules such as vitamin C (Buettner, 1993). Finally, it is possible that later embryos are more resistant to heat shock than earlier embryos not because they have better thermoprotective systems than earlier embryos but because specific molecules in the early embryo are more susceptible to heat. The fact that a greater heat shock is required to induce HSP70 synthesis in mouse blastocysts than mouse 8-cell embryos implies less protein denaturation in the blastocyst. This could reflect more effective thermoprotective molecules in the blastocyst or changes in the proportion of intracellular protein that is thermolabile. Similarly, changes in lipid membrane composition could play a role in embryonic resistance to heat shock if there is a change in the degree of saturation of membrane phospholipids to reduce heat-induced lipid peroxidation.

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