Published in final edited form as:

Comp Biochem Physiol A Mol Integr Physiol. 2008 July ; 150(3): 307–314.

Heat stress-induced heat shock protein 70 expression is dependent on ERK activation in zebrafish (*Danio rerio*) cells

Jill M. Keller, June F. Escara-Wilke, and Evan T. Keller

Departments of Urology and Pathology, School of Medicine, University of Michigan, Ann Arbor, MI, USA 48109-0940

Abstract

Heat shock response is a common event that occurs in many species. Despite its evolutionary conservation, comparative studies of heat shock response have been largely unexplored. In mammals, heat shock response decreases with age through unclear mechanisms. Understanding how the age-related decline in heat shock response occurs may provide information to understanding the biology of aging. We have previously shown that heat shock response similarly declines with age in zebrafish. However, signaling pathways that regulate the heat shock response in zebrafish are unknown. In mammals there is evidence that mitogen-activated protein kinases (MAPKs) of the ERK family alter Hsp70 transcription, serving as a potential regulator of the heat shock response. We explored if heat stress-induced Hsp70 expression is altered by activation of ERK in the zebrafish Pac2 fibroblast cell line as occurs in mammalian cells. Heat stress induced both Hsp70 mRNA expression and phosphorylation of both ERK1 and ERK2 (ERK1/2) in Pac2 cells. ERK inhibitors PD98059 and U0126 blocked both heat stress-induced and plated-derived growth factor (PDGF)-induced ERK1/2 phosphorylation, and also diminished heat-induced Hsp70 expression. Pac2 cell viability was not affected by either the ERK inhibitors or heat stress. These results demonstrate that induction of Hsp70 in response to heat stress is dependent on ERK activation in Pac2 cells. This suggests that the heat shock response in zebrafish utilizes a similar signaling pathway to that of mammals and that zebrafish are a good model for comparative studies of heat shock response.

Keywords

Aging; ERK; Heat shock response; Hsp70; MAPKs; Zebrafish

Introduction

Mitogen-activated protein kinases (MAPKs) compose a superfamily of protein kinases whose function and regulation has been highly conserved during evolution from simple unicellular organisms such as yeast to complex organisms including mammals and fish (Widmann et al. 1999). Similarly, the heat shock proteins (Hsps) are ubiquitous, highly conserved proteins that have been found in the cells of all organisms studied thus far, including plants, bacteria, yeast, flies, and vertebrates (Jaattela 1999). Both the MAPK and Hsp families of proteins function in stress-related pathophysiological states of the cell and play critical roles in maintaining normal

Address correspondence to: Evan T. Keller DVM, PhD, Rm 5308 CCGCB, 1500 E. Medical Center Dr., Ann Arbor, MI 48109-0940, USA, Tel: 734-615-0280, Fax: 734-764-3013, Email: etkeller@umich.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cellular homeostasis (Lee et al. 1996; Kiang et al. 1998; Schafer et al. 2000). It is well documented that expression of Hsps is induced in response to various types of metabolic or environmental stresses such as heat, ischemia, heavy metal ions, ethanol, nicotine, viral agents, surgical stress, and reactive oxygen species (Welch 1992; Whitley et al. 1999).

Multicellular organisms have three well-characterized subfamilies of MAPKs, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase, and p38 (Hommes et al. 2003). Though the JNK and p38 MAPKs are often collectively referred to as stress-activated protein kinases (SAPKs) due to their activation in response to extracellular stress stimuli, it is now clear that the ERK MAPKs can be activated by various stress stimuli as well, including heat stress (Aikawa et al. 1997; Chen et al. 1998; Ng et al. 2000). Several studies have demonstrated interactions between members of the Hsp family and ERK MAPKs in mammalian species. Specifically, these studies showed that the ERK signaling pathway induces expression of Hsp70 by acting on the phosphorylation of HSF1 (Hung et al., 1998) and that inhibition of ERK reduces Hsp70 expression in response to various stressors (Chen et al. 2001; Yang et al. 2004; Taylor et al. 2007).

Zebrafish provide a powerful vertebrate system to identify genes, through large scale mutagenesis assays, that play roles in a variety of biological processes. Thus, understanding the biology of pathways of interest is important in order to take advantage of the zebrafish model system. Recently, members of the MAPK gene family, including the ERK, JNK and p38 subfamilies, were cloned in zebrafish (Krens et al. 2006). Studies involving MAPKs in zebrafish are few and have focused on patterns of expression in embryos during development (Pozios et al. 2001; Shinya et al. 2001). More specifically, interactions between MAPKs and Hsps have not been examined in zebrafish. Furthermore, comparative studies of the heat shock response and its downstream signaling pathways have not received much attention, despite the conservation of this response across species. For example the comparative heat shock response between humans and zebrafish, which offer great potential for genetic dissection of signaling pathways, has not been reported. We have previously characterized the heat shock response in the adult zebrafish and demonstrated their usefulness as an animal model for studying this important biological pathway (Murtha et al. 2003; Keller et al. 2004). Therefore, in the current study we explored if heat shock induces Hsp70 expression through ERK activation as occurs in mammals.

Materials and Methods

Cells

Pac2 cells, a fibroblastic cell line derived from zebrafish embryos, were a generous gift from Dr. Shawn Burgess (National Institutes of Health, Bethesda, MD, USA). They were maintained in Leibovitz's L-15 medium supplemented with 15% FBS and 1% penicillin/streptomycin at 32°C without CO₂. Pac2 cells were split at a ratio of 1:10 approximately every 3–4 days by trypsinizing cells with 0.25% trypsin, 1mM EDTA. Primary human foreskin fibroblasts (HFF) were a generous gift from Dr. James Varani (University of Michigan, Ann Arbor, MI, USA). HFFs were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO₂. The murine macrophage cell line RAW 264.7 and human prostate cancer cell line LNCaP were grown in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO₂. All cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA, USA).

PCR

To verify the origin of the Pac2 cells, DNA was isolated from Pac2 cells as well as from a murine macrophage cell line (RAW 264.7) and human prostate cancer cell line (LNCaP) using

the DNeasy® kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). DNA was then analyzed by PCR for Hsp70 expression using species-specific primers and the GeneAmp® PCR machine (Perkin Elmer, Norwalk, CT, USA) and Taq PCR Core kit according to the manufacturer's protocol (Qiagen Inc.). Primers were designed based on sequences obtained through GenBank (Table 1). Conditions for PCR were as follows: 3 min at 94°C followed by 35 cycles of PCR consisting of 30 s at 94°C, 30 s at 60°C, and 1 minute at 72°C; 10 minutes at 72°C for final extension; and a final holding temperature of 4°C. PCR products were separated on 1.5% agarose gels containing ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) and visualized with ultraviolet light. In addition, all 3 cell lines were mixed and grown together and DNA collected from the combined cells and subjected to PCR as described above.

Heat stress

Pac2 and HFF cells were grown to 80–90% confluency in 25 cm² tissue culture flasks. For timecourse of ERK activation in Pac2 cells, existing medium was removed and replaced with 10 mL of fresh medium that had been pre-heated to 40°C. Cells were then incubated at 40°C for various time points (5 to 60 min) by floating in a water bath. For ERK inhibition studies in Pac2 and HFF cells, 18 mL of fresh medium that was pre-heated to 32°C or 40°C (for Pac2) or to 37°C or 45°C (for HFF) was added to the existing 2 mL containing ERK inhibitors. Cells were incubated for 30 min at the above temperatures by floating in a water bath. Medium was then removed and replaced with 5 mL of fresh medium at 32°C (for Pac2) or 37°C (for HFF) and cells were incubated at indicated temperatures in an incubator for 4 h (recovery phase).

Protein isolation

Following heat stress, protein was isolated according to a protocol from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Briefly, cells were rinsed once with room temperature phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) and 0.6 mL of ice cold radioimmunoprecipitation assay (RIPA) buffer (1xPBS, 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS) with freshly added inhibitors (10 µL/mL RIPA of 10 mg/mL PMSF in isopropanol, 30 µL/mL RIPA of aprotinin (Sigma), 10 µL/mL RIPA of 100mM sodium orthovanadate) was added to each flask. Flasks were then scraped with a cell scraper and lysates transferred to 1.5 mL microcentrifuge tubes using a syringe with a 22-gauge needle. Flasks were rinsed once with an additional 0.3 mL of RIPA buffer and that was combined with the first lysate. Lysates were passed through the 22-gauge needle 20–30 times to shear the DNA. 10 µL of 10 mg/mL PMSF was added to each tube and lysates were incubated on ice for 60 min. Lysates were then centrifuged at 10,000 g for 10 min at 4°C and supernatant fluid containing the total cell lysate was transferred to fresh 1.5 mL microcentrifuge tubes. Samples were quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) and stored at –20°C until used for Western analysis.

Western analysis

Total cell lysates were analyzed for ERK activation by Western analysis using antibodies for phospho-ERK1/2 (Anti-Active® MAPK pAb, Promega Corp., Madison, WI, USA) and total ERK1/2 (p44/42 MAP Kinase Antibody, Cell Signaling Technology, Beverly, MA, USA). The manufacturers' recommended protocols were followed for each antibody. Briefly, 20 µg of each sample plus 6x sample buffer (0.5M TrisCl, pH 6.8, plus 0.4% SDS, 30% glycerol, 10% SDS, 0.6M DTT, 0.012% bromophenol blue) was separated by electrophoresis on a 12% Tris-glycine polyacrylamide gel (Cambrex, Rockland, ME, USA) in 1x running buffer (1x Tris/glycine/SDS buffer, Bio-Rad Laboratories, Hercules, CA, USA) at 100 volts for approximately 1.5 h at room temperature. Protein was then transferred to a PVDF membrane (Bio-Rad) overnight in 1x transfer buffer (1x Tris/glycine buffer (Bio-Rad) plus 20% methanol) at 18

volts and 4°C. Membranes were then probed using phospho-ERK1/2 antibody as follows. The membranes were blocked for 1 h at 37°C in PVDF buffer (1x TBS (Tris-buffered saline (Bio-Rad), 0.1% Tween@20, 0.2% I-Block™ (Tropix Inc., Bedford, MA, USA)). Then membranes were incubated with phospho-ERK1/2 primary antibody (diluted 1:5000 in PVDF buffer) for 2 hours at room temperature with agitation. This was followed by three 15-min washes in PVDF buffer. HRP-linked donkey anti-rabbit secondary antibody (Promega) was then added (diluted 1:5000 in PVDF buffer) and membranes were incubated for 1 h at room temperature with agitation, followed by three 15-min washes in PVDF buffer. Membranes were then soaked in ECL™ Detection Reagent (Amersham Biosciences, Piscataway, NJ, USA) for 1 min and the exposed to X-ray film.

Membranes were stored wet-wrapped in 1xTBS until they were stripped and re-probed for total ERK1/2. To strip the membranes, each membrane was submerged in stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH 6.7) for 30 min at 50°C and then washed twice in 1xTBST (1xTBS plus 0.1% Tween@20) for 10 min each at room temperature. To probe for total ERK1/2, membranes were blocked in blocking buffer (1xTBST plus 5% nonfat dry milk) for 1 h at room temperature with agitation and then washed three times for 5 min each in 1xTBST. Membranes were then incubated with total ERK1/2 primary antibody, diluted 1:1000 in primary antibody dilution buffer (1xTBST plus 5% BSA) overnight at 4°C with agitation. This was followed by 3 washes for 5 min each in 1xTBST and then incubation in HRP-linked secondary antibody (Cell Signaling Technology, Beverly, MA, USA), diluted 1:2000 in blocking buffer, for 1 h at room temperature with gentle agitation. Membranes were washed again 3 times for 5 min each in 1xTBST and then soaked for 1 min in LumiGLO™ detection reagent (Cell Signaling Technology) and exposed to X-ray film.

Band density was quantified using Bioquant Nova (R&M Biometrics Inc., Nashville, TN, USA) image analysis software program.

ERK inhibition

25 cm² flasks of Pac2 or HFF cells that were 80–90% confluent were incubated for 1 h at 32°C (for Pac2) or 37°C (for HFF) with 2 mL of medium containing vehicle (DMSO), PD98059, or U0126 at doses indicated. Inhibitors were purchased from Calbiochem (San Diego, CA, USA). This was followed by a 30-min heat stress and 4 hour recovery phase as described above or treatment with 50 ng/mL PDGF for 10 min. To analyze for Hsp70 expression, total RNA was isolated using RNeasy® Mini Kit and QIAshredder® (Qiagen Inc.) according to the manufacturer's instructions and analyzed by RT-PCR as detailed below. To determine whether ERK activation was inhibited, protein was isolated and analyzed by Western analysis for phospho-ERK1/2 and total ERK1/2 as described above.

RT-PCR

Total RNA (50 ng) was analyzed by RT-PCR for expression of Hsp70 and beta-actin using the Lightcycler real-time PCR system and Lightcycler RNA Amplification Kit SYBR Green I (Roche, Indianapolis, IN, USA) according to the manufacturer's recommendations. Primers for Hsp70 and zebrafish beta-actin were designed based on sequences obtained through GenBank (Table 1). Primers for human beta-actin were obtained from Qiagen Inc. RT-PCR conditions were as follows: 10 min at 55°C followed by 30 s at 94°C for reverse transcription; 45 cycles of PCR consisting of 5 s at 94°C, 10 s at 60°C, and 13 s at 72°C. Hsp70 expression was quantified using a standard curve and the Lightcycler software program and was normalized for beta-actin levels.

Cell Viability

To determine the effect of inhibitors on Pac2 cell viability, confluent 10 cm tissue culture plates of Pac2 cells were treated with vehicle (DMSO), 250 μ M PD98059, or 100 μ M U0126 at 32°C for 1 h. To determine the effect of heat stress on Pac2 cell viability, cells were incubated at 40°C for 1 h. Following treatment, cell culture supernatant (to account for detached cells) and trypsinized cells were combined and centrifuged at 146 g for 10 min, and then resuspended and counted using trypan blue to determine the percentage of viable cells. Cells were then replated and incubated for an additional 24 h at 32°C, and then counted again using trypan blue to determine the percentage of viable cells as well as the total cell number per plate.

MTS Assay

Pac2 cells were plated at 2×10^4 cells/well in a 96-well tissue culture plate at a volume of 100 μ L. Cells were treated with PDGF (50ng/mL) or vehicle (PBS) plus either PD98059 (25 μ M), U0126 (10 μ M), or inhibitor vehicle (DMSO) as indicated. Cells were then incubated at 32°C without CO₂ for 48 h. Following incubation, 20 μ L of MTS reagent (CellTiter 96® AQueous Assay, Promega Corp.) was added to each well and plates were incubated for an additional 1 h at 32°C without CO₂. Absorbance was then measured at 490nm to determine relative cell proliferation.

Statistical analysis

Multiple comparisons were analyzed using analysis of variance (ANOVA) and Fisher's protected least significant difference for post-hoc analysis using Statview® software program (SAS Institute Inc., Cary, NC, USA). A $p \leq 0.05$ was considered significant. Each experiment was repeated 2 or 3 times.

Results

Verification of Pac2 cell line purity

Because Pac2 cells have not been well characterized, and also because interpretation of our data and comparison with other species requires the assurance that Pac2 cells are indeed zebrafish in origin, we sought to verify the purity of the Pac2 cell line. Accordingly, DNA from Pac2 cells as well from a murine (RAW 264.7) and human (LNCaP) cell line was analyzed by PCR for Hsp70 expression using species-specific primers. The results revealed that zebrafish-specific Hsp70 primers amplified only the Pac2 templates, while the mouse-specific and human-specific Hsp70 primers amplified only RAW 264.7 and LNCaP templates, respectively (Figure 1a), demonstrating that Pac2 cells are zebrafish in origin. In addition, DNA collected from all 3 cell lines mixed together and subjected to PCR showed amplification with all 3 species-specific primers, demonstrating that we would be able to detect contamination of Pac2 cells if it occurred (Figure 1b).

ERK activation in response to heat stress

To examine the effect of heat stress on ERK activation, Pac2 cells were exposed to a heat stress of 40°C for various lengths of time (5 to 60 min) and then protein was isolated and subjected to Western analysis for phospho-specific and total ERK expression. Heat stress activated both ERK1 (p44) and ERK2 (p42) in a time dependent manner in Pac2 cells, with a gradual increase in the phospho/total (P/T) ratio over time (Figure 2a). The activation peaked at 30 min and was sustained for up to 60 min (Figure 2b).

Effect of ERK inhibition on heat stress-induced Hsp70 expression

To evaluate the effect of ERK inhibition on heat stress-induced Hsp70 expression, Pac2 and HFF cells were treated with the ERK pathway-specific inhibitors PD98059 and U0126, which

block MEK1/2 phosphorylation and the subsequent activation of ERK1/2, then followed by exposure to heat stress. Total RNA was isolated following heat treatment and a recovery period and analyzed by real time RT-PCR for Hsp70 mRNA and beta-actin mRNA expression. Heat stress induced Hsp70 mRNA expression in both cell lines. Treatment with ERK inhibitors diminished heat stress-induced Hsp70 mRNA response in both cell lines (Figure 3a and 3b). These results suggest that heat stress-induced Hsp70 expression is dependent on ERK activation in Pac2 cells, and that this stress response mechanism is similar to that in human cells, as demonstrated by our results in HFF cells.

To verify that treatment with ERK inhibitors or heat stress was not adversely affecting cell viability, cells were treated with a high dose of each inhibitor (250 μ M PD98059 or 100 μ M U0126) or with heat stress and the percentage of viable cells was determined immediately post-treatment as well as 24 h after replating the cells post-treatment. Total cell numbers were also determined 24 h after replating the cells. The results showed that neither treatment with ERK inhibitors nor heat stress had any effect on cell viability (data not shown), indicating that the decreased Hsp70 response seen upon treatment with ERK inhibitors was not due to changes in cell number or viability.

Numerous studies have shown that these ERK inhibitors are functional in mammalian cells. To verify that the ERK inhibitors were also functional in Pac2 cells, cells were treated with each inhibitor plus either heat stress or PDGF, known activators of the ERK pathway. Total cell lysates were analyzed for phospho-specific and total ERK1/2 expression via Western analysis. The results revealed that both PD98059 and U0126 were able to inhibit ERK1/2 activation in response to both heat stress (Figure 4) and PDGF (Figure 5). This inhibition was complete and reduced ERK activity back down to basal levels. In addition, to show that these inhibitors are able to block a biologic response that is associated with ERK activation in Pac2 cells, we also looked at whether the inhibitors were able to block PDGF-induced cell proliferation. We found that cell proliferation was significantly induced in Pac2 cells in response to PDGF treatment and that treatment with ERK inhibitors PD98059 and U0126 blocked that proliferation in response to PDGF (Figure 6), demonstrating that ERK inhibitors are able to block a biologic response that is associated with ERK activation in Pac2 cells. These data confirm that the ERK inhibitors were functional in Pac2 cells.

Discussion

Our results demonstrate that heat stress activates ERK1/2 and induces Hsp70 expression in the zebrafish cell line Pac2, and that inhibiting ERK activation attenuates heat stress-induced Hsp70 expression. These results demonstrate that heat stress induced-Hsp70 expression is dependent on ERK activation in zebrafish cells. These findings are similar to those reported in mammals, in which ERK activation is required for stress-induced activation of Hsp70 expression. These results provide (1) a mechanistic link between ERK signaling and HSP response in zebrafish and (2) indicate that information gleaned from mutagenesis studies to identify genes that impact ERK and HSP activity in zebrafish will have relevance to humans.

There are very limited cell lines available from zebrafish. Cell lines that are available are fibroblast in origin, and so we used one of these zebrafish fibroblast cell lines. The human cell line we used (HFFs) was chosen because it is a similarly derived fibroblast cell line, and thus we felt it an appropriate choice for comparison with our zebrafish fibroblast cell line. For our human cell line, the temperature used for heat stress (45°C) was chosen because it is the most commonly used temperature in literature involving heat stress experiments in human and mammalian cell lines. We chose to use a heat stress temperature of 40°C for our zebrafish cells so that the amount of temperature increase would be equivalent in both cell lines (i.e. an 8°C increase for both human and zebrafish cells). Furthermore, in our previous work we found that

this degree of temperature increase resulted in maximal induction of Hsp70 without adversely affecting cell viability. It should be noted that zebrafish cells were derived from an ectothermic organism in which regulation of body temperature is dependent upon its environment, while human cells were derived from an endothermic organism which maintains body temperature independent of its surroundings. It is unknown whether different mechanisms of whole body temperature regulation would translate into different mechanisms in cell culture. Therefore, our results should be interpreted with the caveat that a change in temperature of 8°C could have different implications at the cellular level in different species. However, we feel this is unlikely given that our results were similar in both zebrafish and human cells.

The heat shock response is a highly conserved pathway in which heat shock proteins (Hsps) are upregulated in response to various types of metabolic or environmental stresses, which plays a critical role in maintaining normal cellular homeostasis (Welch 1992; Whitley et al. 1999). Several members of the Hsp family, including Hsp47, Hsp60, Hsp70, Hsc70, Hsp90, Hsp27, alpha-crystallins, and several other small Hsps (sHsps) have been identified and cloned in the zebrafish (Krone et al. 1994; Graser et al. 1996; Pearson et al. 1996; Krone et al. 1997; Fujii et al. 2000; Rabergh et al. 2000b; Makino et al. 2005; Mao et al. 2005; Krens et al. 2006; Elicker et al. 2007). The heat shock transcription factors Hsf1 and Hsf2 have also been cloned in zebrafish (Rabergh et al. 2000a; Yeh et al. 2006). These genes are highly conserved across species and have been shown to be orthologous to their mammalian counterparts, with similar protein structures, expression patterns, and physiologic functions. We previously reported that zebrafish respond to heat shock with both nuclear translocation of Hsf1 and production of several Hsps in a variety of tissues (Murtha et al. 2003), similar to what has been reported in other species. More specifically, we found that Hsp70 and Hsp47 were induced in response to heat stress in a tissue-specific manner, while Hsp90 α , Hsp90 β , and Hsf1 were expressed constitutively and not induced by heat stress. Though Hsf1 RNA levels did not change with heat stress, Hsf1 protein was translocated to the nucleus following heat stress, indicating activation of this transcription factor. Mammalian species show a similar pattern of Hsp and Hsf expression and Hsf activation in response to heat stress (Santoro 2000).

The heat shock response and the expression of heat shock proteins has been found to decrease with age in a variety of species (Liu et al. 1989; Heydari et al. 1994; Kregel et al. 1995; Pahlavani et al. 1995; Lee et al. 1996). For example, cultures of primary human peripheral lymphocytes exposed to heat stress revealed an age-related attenuation in the expression of many heat shock proteins, including Hsp70, Hsp90, and Hsp60 (Rao et al. 1999). Murine and human fibroblasts also showed a decline in heat shock protein expression with age upon heat treatment (Miyaiishi et al. 1995). The expression of Hsp70 protein and mRNA levels in response to heat shock were found to be decreased with age in rat neurons (Pardue et al. 1992), hepatocytes (Wu et al. 1993), and myocardium (Locke et al. 1996). This decline in Hsp70 transcription correlated with a decrease in the binding of Hsf1 to the heat shock element. We have previously shown that aging modulates heat shock responsiveness and Hsp70 expression in zebrafish in a similar manner (Murtha et al. 2003). Specifically, we found that both basal and induced levels of Hsp70 were less in mature versus young zebrafish, which is consistent with previous reports in other species (Heydari et al. 1994; Kregel et al. 1995; Lee et al. 1996; Liu et al. 1996; Pahlavani et al. 1996). We also observed that Hsf1 mRNA levels were elevated in mature compared to young zebrafish and that heat shock did not induce Hsf1 mRNA expression in either age group but did induce nuclear translocation. This is consistent with previous reports on Hsf1 expression and induction in other species (Locke et al. 1996; Heydari et al. 2000; Locke 2000), in which Hsp70 levels are diminished with age in the face of increased Hsf1 levels, which is thought to be due to a decreased ability of Hsf1 to bind DNA in aged animals.

Members of the MAPK family, including ERK, p38, and JNK have recently been identified and cloned in zebrafish (Krens et al. 2006). In mammals, it is well documented that ERK MAPKs are not only activated in response to proliferation-inducing agents (Page et al. 2000; Peyssonnaud et al. 2001; Xaus et al. 2001; Lee et al. 2002; Mishima et al. 2002), but also in response to various stresses, including oxidative stress, arsenite, and heat shock (Aikawa et al. 1997; Chen et al. 1998; Ng et al. 2000). There have also been numerous studies demonstrating a relationship between ERK and HSP response in mammals. Interestingly, these studies have examined mechanisms of stress induction other than heat stress and not heat stress itself. For example, treatment of human colorectal carcinoma cells with sodium arsenite induces significant expression of Hsp70 and activation of ERK (Chen et al. 2001). ERK blockade by the specific inhibitor PD98059 prevents this phosphorylation and activation of ERK and attenuates the sodium arsenite-induced increase in Hsp70 expression. In the same study, it was shown that overexpression of a dominant negative mutant of an upstream regulator of ERK also prevented arsenite-induced ERK activation and Hsp70 expression. Additionally, it has been reported that ERK activation plays a role in cadmium stress-induced Hsp70 expression through the phosphorylation of Hsf1, the transcription factor for Hsp70 (Hung et al. 1998). Hsp70 induction and Hsf1 activation were eliminated in the presence of PD98059. Thus, in mammals, ERK may participate in the activation of Hsf1, which leads to the induction of the heat shock genes. ERK inhibitors PD98059 and U0126 or expression of a dominant negative ERK1/2 abolished sodium salicylate-induced Hsp72 synthesis in another study (Seo et al. 2005). The ERK inhibitor PD98059 has also been shown to significantly lower levels of benzo (a) pyrene-induced Hsp70 expression (Yang et al., 2004).

Given the evidence in mammalian species that ERK MAPKs participate in the regulation of the Hsp response, and that there is a high degree of evolutionary conservation of both of these families of proteins, we hypothesized that ERK MAPKs may be involved in the regulation of the heat shock response in the zebrafish as well. Our results indicate that this appears to be the case. We have shown that similar to mammals, ERK is activated in response to heat stress in a zebrafish-derived cell line. Furthermore, we have also shown that ERK activation appears to regulate heat shock-induced Hsp70 expression in both zebrafish Pac2 cells and in human HFF cells. Similar to those mammalian studies which examined other mechanisms of stress induction, our results demonstrate that ERK activation also plays a role specifically in heat stress-induced Hsp70 expression in human cells. Thus, the heat shock response in zebrafish appears to be dependent on ERK pathways, similar to what we and other have reported in mammals.

It is important to note that we have only examined the ERK pathway and therefore additional interactions between the other MAPKs, JNK and p38, and the heat shock response in zebrafish cannot be ruled out. We have chosen to focus on the ERK pathway in particular for several reasons, including an inability to find phosphor-specific antibodies that work for JNK in the zebrafish and the lack of a positive p38 activation response following heat stress in our cell line (data not shown). As more reagents become available for use in zebrafish systems and as knowledge concerning MAPK pathways in zebrafish is advanced, we may further examine these other pathways for possible interactions with the heat shock response.

As the results of our current study suggest that in the zebrafish regulation of heat stress-induced Hsp70 expression occurs via a similar mechanism to that of mammals, namely by a pathway that is dependent on ERK activation, the zebrafish is a valuable model in which to study the heat shock response. Zebrafish respond to heat shock with the activation of Hsf1 and the induced expression of several heat shock proteins, especially Hsp70, which is the same response that is seen in mammals. In addition, zebrafish show an age-related attenuation in their heat shock response similar to what has been reported in mammals and other species. Thus, there appears to be many similarities in how the heat shock response of zebrafish and

mammals is regulated. This could serve as a valuable tool for carrying out comparative studies and examining how various regulatory mechanisms correspond with response to environmental stresses. Therefore, we feel that zebrafish could prove to be an excellent model for studying the heat shock response and its relationship to aging.

Acknowledgements

This work was supported by NIH grants T32-RR-07008 and R01-AG-21866. We thank Dr. Shawn Burgess for providing the Pac2 cells, and Dr. James Varani for providing HFF cells.

The abbreviations used are

ERK	extracellular regulated kinase
Hsf	heat shock transcription factor
Hsp	heat shock protein
MAPK	mitogen-activated protein kinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium, inner salt
PDGF	platelet-derived growth factor
SAPK	stress-activated protein kinase

References

- Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Tanaka M, Shiojima I, Hiroi Y, Yazaki Y. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. *J Clin Invest* 1997;100:1813–1821. [PubMed: 9312182]
- Chen W, Martindale JL, Holbrook NJ, Liu Y. Tumor promoter arsenite activates extracellular signal-regulated kinase through a signaling pathway mediated by epidermal growth factor receptor and Shc. *Mol Cell Biol* 1998;18:5178–5188. [PubMed: 9710602]
- Chen YC, Tsai SH, Shen SC, Lin JK, Lee WR. Alternative activation of extracellular signal-regulated protein kinases in curcumin and arsenite-induced HSP70 gene expression in human colorectal carcinoma cells. *Eur J Cell Biol* 2001;80:213–221. [PubMed: 11322385]
- Elicker KS, Hutson LD. Genome-wide analysis and expression profiling of the small heat shock proteins in zebrafish. *Gene* 2007;403:60–69. [PubMed: 17888590]
- Fujii R, Yamashita S, Hibi M, Hirano T. Asymmetric p38 activation in zebrafish: its possible role in symmetric and synchronous cleavage. *J Cell Biol* 2000;150:1335–1348. [PubMed: 10995439]
- Graser RT, Malnar-Dragojevic D, Vincek V. Cloning and characterization of a 70 kd heat shock cognate (hsc70) gene from the zebrafish (*Danio rerio*). *Genetica* 1996;98:273–276. [PubMed: 9204550]
- Heydari AR, Takahashi R, Gutschmann A, You S, Richardson A. Hsp70 and aging. *Experientia* 1994;50:1092–1098. [PubMed: 7988669]
- Heydari AR, You S, Takahashi R, Gutschmann-Conrad A, Sarge KD, Richardson A. Age-related alterations in the activation of heat shock transcription factor 1 in rat hepatocytes. *Exp Cell Res* 2000;256:83–93. [PubMed: 10739655]

- Hommel DW, Peppelenbosch MP, van Deventer SJ. Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut* 2003;52:144–151. [PubMed: 12477778]
- Hung JJ, Cheng TJ, Lai YK, Chang MD. Differential activation of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinases confers cadmium-induced HSP70 expression in 9L rat brain tumor cells. *J Biol Chem* 1998;273:31924–31931. [PubMed: 9822662]
- Jaattela M. Heat shock proteins as cellular lifeguards. *Ann Med* 1999;31:261–271. [PubMed: 10480757]
- Keller ET, Murtha JM. The use of mature zebrafish (*Danio rerio*) as a model for human aging and disease. *Comp Biochem Physiol C Toxicol Pharmacol* 2004;138:335–341. [PubMed: 15533791]
- Kiang JG, Tsokos GC. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol Ther* 1998;80:183–201. [PubMed: 9839771]
- Kregel KC, Moseley PL, Skidmore R, Gutierrez JA, Guerriero V Jr. HSP70 accumulation in tissues of heat-stressed rats is blunted with advancing age. *J Appl Physiol* 1995;79:1673–1678. [PubMed: 8594028]
- Krens SF, He S, Spaink HP, Snaar-Jagalska BE. Characterization and expression patterns of the MAPK family in zebrafish. *Gene Expr Patterns* 2006;6:1019–1026. [PubMed: 16774848]
- Krone PH, Lele Z, Sass JB. Heat shock genes and the heat shock response in zebrafish embryos. *Biochem Cell Biol* 1997;75:487–497. [PubMed: 9551174]
- Krone PH, Sass JB. HSP 90 alpha and HSP 90 beta genes are present in the zebrafish and are differentially regulated in developing embryos. *Biochem Biophys Res Commun* 1994;204:746–752. [PubMed: 7980538]
- Lee JT Jr, McCubrey JA. The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. *Leukemia* 2002;16:486–507. [PubMed: 11960326]
- Lee YK, Manalo D, Liu AY. Heat shock response, heat shock transcription factor and cell aging. *Biol Signals* 1996;5:180–191. [PubMed: 8864063]
- Liu AY, Lee YK, Manalo D, Huang LE. Attenuated heat shock transcriptional response in aging: molecular mechanism and implication in the biology of aging. *Exs* 1996;77:393–408. [PubMed: 8856987]
- Liu AY, Lin Z, Choi HS, Sorhage F, Li B. Attenuated induction of heat shock gene expression in aging diploid fibroblasts. *J Biol Chem* 1989;264:12037–12045. [PubMed: 2745427]
- Locke M. Heat shock transcription factor activation and hsp72 accumulation in aged skeletal muscle. *Cell Stress Chaperones* 2000;5:45–51. [PubMed: 10701839]
- Locke M, Tanguay RM. Diminished heat shock response in the aged myocardium. *Cell Stress Chaperones* 1996;1:251–260. [PubMed: 9222610]
- Makino S, Whitehead GG, Lien CL, Kim S, Jhavar P, Kono A, Kawata Y, Keating MT. Heat-shock protein 60 is required for blastema formation and maintenance during regeneration. *Proc Natl Acad Sci USA* 2005;102:14599–14604. [PubMed: 16204379]
- Mao L, Bryantsev AL, Chechenova MB, Shelden EA. Cloning, characterization, and heat stress-induced redistribution of a protein homologous to human hsp27 in the zebrafish *Danio rerio*. *Exp Cell Res* 2005;306:230–241. [PubMed: 15878347]
- Mishima K, Inoue K, Hayashi Y. Overexpression of extracellular-signal regulated kinases on oral squamous cell carcinoma. *Oral Oncol* 2002;38:468–474. [PubMed: 12110341]
- Miyaishi O, Ito Y, Kozaki K, Sato T, Takechi H, Nagata K, Saga S. Age-related attenuation of HSP47 heat response in fibroblasts. *Mech Ageing Dev* 1995;77:213–226. [PubMed: 7739268]
- Murtha JM, Keller ET. Characterization of the heat shock response in mature zebrafish (*Danio rerio*). *Exp Gerontol* 2003;38:683–691. [PubMed: 12814804]
- Ng DC, Bogoyevitch MA. The mechanism of heat shock activation of ERK mitogen-activated protein kinases in the interleukin 3-dependent ProB cell line BaF3. *J Biol Chem* 2000;275:40856–40866. [PubMed: 11005808]
- Page K, Hershenson MB. Mitogen-activated signaling and cell cycle regulation in airway smooth muscle. *Front Biosci* 2000;5:D258–267. [PubMed: 10704154]

- Pahlavani MA, Harris MD, Moore SA, Richardson A. Expression of heat shock protein 70 in rat spleen lymphocytes is affected by age but not by food restriction. *J Nutr* 1996;126:2069–2075. [PubMed: 8814193]
- Pahlavani MA, Harris MD, Moore SA, Weindruch R, Richardson A. The expression of heat shock protein 70 decreases with age in lymphocytes from rats and rhesus monkeys. *Exp Cell Res* 1995;218:310–318. [PubMed: 7737368]
- Pardue S, Groshan K, Raese JD, Morrison-Bogorad M. Hsp70 mRNA induction is reduced in neurons of aged rat hippocampus after thermal stress. *Neurobiol Aging* 1992;13:661–672. [PubMed: 1491731]
- Pearson DS, Kulyk WM, Kelly GM, Krone PH. Cloning and characterization of a cDNA encoding the collagen-binding stress protein hsp47 in zebrafish. *DNA Cell Biol* 1996;15:263–272. [PubMed: 8634155]
- Peyssonaux C, Eychene A. The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell* 2001;93:53–62. [PubMed: 11730323]
- Pozios KC, Ding J, Degger B, Upton Z, Duan C. IGFs stimulate zebrafish cell proliferation by activating MAP kinase and PI3-kinase-signaling pathways. *Am J Physiol Regul Integr Comp Physiol* 2001;280:R1230–1239. [PubMed: 11247849]
- Rabergh CM, Airaksinen S, Soitamo A, Bjorklund HV, Johansson T, Nikinmaa M, Sistonen L. Tissue-specific expression of zebrafish (*Danio rerio*) heat shock factor 1 mRNAs in response to heat stress. *J Exp Biol* 2000a;203:1817–1824. [PubMed: 10821739]
- Rabergh CM, Airaksinen S, Soitamo A, Bjorklund HV, Johansson T, Nikinmaa M, Sistonen L. Tissue-specific expression of zebrafish (*Danio rerio*) heat shock factor 1 mRNAs in response to heat stress [In Process Citation]. *J Exp Biol* 2003 Pt 2000b;12:1817–1824.
- Rao DV, Watson K, Jones GL. Age-related attenuation in the expression of the major heat shock proteins in human peripheral lymphocytes. *Mech Ageing Dev* 1999;107:105–118. [PubMed: 10197792]
- Santoro MG. Heat shock factors and the control of the stress response. *Biochem Pharmacol* 2000;59:55–63. [PubMed: 10605935]
- Schafer C, Williams JA. Stress kinases and heat shock proteins in the pancreas: possible roles in normal function and disease. *J Gastroenterol* 2000;35:1–9. [PubMed: 10632533]
- Seo MS, Oh SY, Park MJ, Kim SM, Kim MY, Han SI, Park HG, Kang HS. Implication of reactive oxygen species, ERK1/2, and p38MAPK in sodium salicylate-induced heat shock protein 72 expression in C6 glioma cells. *Int J Mol Med* 2005;16:841–849. [PubMed: 16211253]
- Shinya M, Koshida S, Sawada A, Kuroiwa A, Takeda H. Fgf signalling through MAPK cascade is required for development of the subpallial telencephalon in zebrafish embryos. *Development* 2001;128:4153–4164. [PubMed: 11684653]
- Taylor AR, Robinson MB, Gifondorwa DJ, Tytell M, Milligan CE. Regulation of heat shock protein 70 release in astrocytes: role of signaling kinases. *Dev Neurobiol* 2007;67:1815–1829. [PubMed: 17701989]
- Welch WJ. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol Rev* 1992;72:1063–1081. [PubMed: 1438579]
- Whitley D, Goldberg SP, Jordan WD. Heat shock proteins: a review of the molecular chaperones. *J Vasc Surg* 1999;29:748–751. [PubMed: 10194511]
- Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 1999;79:143–180. [PubMed: 9922370]
- Wu B, Gu MJ, Heydari AR, Richardson A. The effect of age on the synthesis of two heat shock proteins in the hsp70 family. *J Gerontol* 1993;48:B50–56. [PubMed: 8473688]
- Xaus J, Comalada M, Valledor AF, Cardo M, Herrero C, Soler C, Lloberas J, Celada A. Molecular mechanisms involved in macrophage survival, proliferation, activation or apoptosis. *Immunobiology* 2001;204:543–550. [PubMed: 11846217]
- Yang JB, Ke L, Jiang CZ, Xu Q, He HZ, Hu BC, Wu TC. [Effects of mitogen activated protein kinase signal transduction pathways on heat shock protein 70 gene expression in endothelial cells exposed to benzo(a)pyrene]. *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* 2004;22:100–103. [PubMed: 15130437]

Yeh FL, Hsu LY, Lin BA, Chen CF, Li IC, Tsai SH, Hsu T. Cloning of zebrafish (*Danio rerio*) heat shock factor 2 (HSF2) and similar patterns of HSF2 and HSF1 mRNA expression in brain tissues. *Biochimie* 2006;88:1983–1988. [PubMed: 16938384]

Figure 1.

Pac2 cells are zebrafish in origin. **(a)** DNA from a zebrafish (Z), mouse (M), and human (H) cell line was analyzed by PCR for expression of species-specific Hsp70. Zebrafish-specific primers amplified only Pac2 template, while mouse- and human-specific primers amplified only mouse and human template, respectively. **(b)** DNA from all 3 cell lines grown together was amplified by all 3 species-specific primer pairs. (-) represents the negative control with no template added. (L) represents 100 bp ladder.

Figure 2.

Heat stress activates ERK1/2 in Pac2 cells. Cells were exposed to a 40°C heat stress for times indicated and proteins analyzed for phospho-specific (P) and total (T) ERK1/2 (p44/42) expression via Western analysis (**a**). The P/T ratio increased with heat treatment in a time dependent manner and activation was sustained for up to 60 minutes of heat exposure (**b**). Each time point was evaluated 3 times. Data are presented as mean $\pm$ SD and represent the relative increase in the P/T ERK ratio compared to 0 time point. * $p \leq 0.05$ compared to 0-minute time point.

Figure 3.

Treatment with ERK inhibitors diminishes heat stress-induced Hsp70 expression in Pac2 cells (a) and in HFF cells (b). Cells were treated with the ERK inhibitors PD98059 or U0126 at doses indicated for 1 hour followed by a 30 minute heat stress and a 4 hour recovery period. Total RNA was isolated and analyzed for Hsp70 expression by real-time quantitative RT-PCR. Hsp70 expression was dramatically induced in response to heat stress, and this response was attenuated by pre-treatment with ERK inhibitors. Hsp70 expression was normalized for beta-actin levels. Each treatment was evaluated twice. Data are presented as mean $\pm$ SD and represent the fold increase in Hsp70 expression compared to control cells (32°C, without inhibitors). * $p \leq 0.05$ compared to control cells (32°C).

Figure 4.

ERK inhibitors block heat-induced ERK activation in Pac2 cells. Cells were treated with vehicle (DMSO), 250 μ M PD980559, or 100 μ M U0126 for 1 hour at 32°C and then heat stressed for 30 minutes at 40°C (control cells were kept at 32°C). Total cell lysates were analyzed for ERK activation by Western analysis using phospho-specific and total ERK1/2 antibodies. Both inhibitors significantly suppressed heat-induced ERK activation. Each treatment was evaluated twice. Data are presented as mean $\pm$ SD and represent the relative increase in the P/T ERK ratio compared to control cells (without heat stress or inhibitors). * $p \leq 0.05$ compared to control cells.

Figure 5.

ERK inhibitors block PDGF-induced ERK activation in Pac2 cells. Cells were treated with vehicle (PBS), 250 μ M PD980559, or 100 μ M U0126 for 1 hour at 32°C and then with PDGF (50 ng/ml) for 10 minutes at 32°C (control cells received PBS). Total cell lysates were analyzed for ERK activation by Western analysis using phospho-specific and total ERK1/2 antibodies. Both inhibitors significantly suppressed PDGF-induced ERK activation. Each treatment was evaluated twice. Data are presented as mean $\pm$ SD and represent the relative increase in the P/T ERK ratio compared to control cells (without PDGF or inhibitors). * $p \leq 0.05$ compared to control cells.

Figure 6.

ERK inhibitors block PDGF-induced proliferation in Pac2 cells. Cells were treated with PDGF (50ng/ml), PD98059 (PD; 25 μ M), and U0126 (U0; 10 μ M) as indicated and incubated at 32° C without CO₂ for 48 hours. MTS reagent was then added for an additional 1 hour and plates read at 490nm to assess proliferation. Results represent relative proliferation compared to control based on O.D. values. Experiment was done twice. * $p \leq 0.05$ compared to control cells.

Table 1

PCR primer design

Primer	Sequences (5'-3')	Expected Product Size	GenBank Accession Number
Hsp70 (zebrafish)	ATCCTCATGGGCGACACCTCTG (Upper) TGTCGGCCTCTTGACCACTCTC (Lower)	436 bp	AF210640
Hsp70 (mouse)	ACTTTCTGCGTCCGCCATCCTGTAG (Upper) CGCCGCTCGCTCTGCTTCTCTT (Lower)	499 bp	M35021
Hsp70 (human)	CTTCCCACCGCCACTCCCCCTTCCTCTCA (Upper) GCTTTGGCCATGCGGTTCCCTGCTCTC (Lower)	480 bp	M11717
β -actin (zebrafish)	CCCCATTGAGCACGGTATT (Upper) AGCGGTTCCCATCTCCTG (Lower)	484 bp	AF057040