Hyperthermia in the febrile range induces HSP72 expression proportional to exposure temperature but not to HSF-1 DNA-binding activity in human lung epithelial A549 cells

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Abstract Expression of heat shock proteins (HSPs) is classically activated at temperatures above the physiologic range ($\geq 42^{\circ}\text{C}$) via activation of the stress-activated transcription factor, heat shock factor-1 (HSF-1). Several studies suggest that less extreme hyperthermia, especially within the febrile range, as occurs during fever and exertional/environmental hyperthermia, can also activate HSF-1 and enhance HSP expression. We compared HSP72 protein and mRNA expression in human A549 lung epithelial cells continuously exposed to 38.5°C , 39.5°C , or 41°C or exposed to a classic

heat shock (42°C for 2 h). We found that expression of HSP72 protein and mRNA increased linearly as incubation temperature was increased from 37°C to 41°C , but increased abruptly when the incubation temperature was raised to 42°C . A similar response in luciferase activity was observed using A549 cells stably transfected with an HSF-1-responsive luciferase reporter plasmid. However, activation of intranuclear HSF-1 DNA-binding activity was comparable at 38.5°C , 39.5°C , and 41°C and only modestly greater at 42°C but the mobility of HSF1 protein on a denaturing gel was altered with increasing exposure temperature and was distinctly different at 42°C . These findings indicate that the proportional changes in HSF-1-dependent HSP72 expression at febrile-range temperatures are dependent upon exposure time and temperature but not on the degree of HSF-1 DNA-binding activity. Instead, HSF-1-mediated HSP expression following hyperthermia and heat shock appears to be mediated, in addition to HSF-1 activation, by posttranslational modifications of HSF-1 protein.

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Abbreviations

GAPDH	glyceraldehydes-3 phosphate dehydrogenase
HS	heat shock
HSF-1	heat shock factor-1
HSP	heat shock protein

Introduction

The heat shock (HS) response is a phylogenetically ancient cellular response to exogenous stress, including high

temperature, that shifts gene expression to a set of evolutionarily conserved HS proteins (HSPs; Feder and Hofmann 1999). HSPs can preserve cell viability in the face of adverse conditions by sequestering denatured cellular proteins and facilitating their refolding or elimination. In eukaryotes, HSP genes are regulated by HS-activated transcription factors (HSFs), which bind *cis*-acting HS response elements (HSE) comprising inverted dyad nGAAn repeats (Perisic et al. 1989). Of the three mammalian HSFs, HSF-1 is activated by exposure to HS (Sarge et al. 1993) and is required for HS-induced HSP expression (McMillan et al. 1998). In the face of thermal or chemical stress, HSF-1 undergoes a stepwise activation comprising trimerization, nuclear translocation, and independent acquisition of DNA-binding and transactivating capacities. Mutational analysis and DNA-binding assays indicate that trimerization directly confers DNA-binding capacity, but that transactivating activity requires subsequent modifications that include phosphorylation and sumoylation (Hietakangas et al. 2006).

The thermal threshold for activating HSF-1 and inducing the HS response not only differs across species (Tomanek and Somero 2002) but also differs across cell types (Gothard et al. 2003; Sarge 1998). For example, lymphoid tissues, including spleen, exhibit a low thermal threshold for induction of heat shock protein expression (Gothard et al. 2003; Ostberg et al. 2002), which appears to derive from T lymphocyte rather than B lymphocyte behavior (Gothard et al. 2003). Furthermore, the thermal threshold for induction of HSF-1 activation and HSP expression may be modified by exposure to certain agents, such as arachidonic acid (Jurivich et al. 1994) and type I interferons (Morange et al. 1986). Collectively, these studies suggest that regulation of the HS response is complex and may be modified by multiple factors in addition to the magnitude of hyperthermia to which the organism is exposed.

We have previously shown that exposing RAW 264.7 mouse macrophages to temperatures in the febrile rather than classic HS range activates HSF-1 DNA binding, but not transactivating activity (Singh et al. 2000). In mice, raising core temperature to 39.5°C for 3 h was sufficient to activate HSP72 expression in liver and kidney, but at much lower levels than mice exposed to 42°C for only 20 min (Jiang et al. 1999). We also found that in human, exertional hyperthermia in the febrile range could also enhance HSP expression (McClung et al. 2008). These observations led us to hypothesize that induction of the HS response and activation of HSP gene transcription could be achieved at temperatures within the febrile range and that the response might be dependent upon the magnitude of hyperthermia and the duration of exposure.

We utilized the A549 human respiratory epithelial cell line to analyze the time and temperature dependence of HS

response activation by measuring generation of HSP72 protein and activation of a reporter construct driven by an artificial HSF-1-responsive promoter. We found HSP72 mRNA and protein accumulated at rates that increased as incubation temperature increased between 37°C and 41°C, but increased much more dramatically with a further increase in temperature to 42°C. While intranuclear levels of HSF-1, DNA-binding activity were similar in cells exposed to each of the temperatures between 38.5°C and 42°C, immunoblotting showed a progress temperature-dependent increase in electrophoretic mobility of HSF-1.

Materials and methods

Reagents and cell culture Rabbit antihuman antibodies against human HSP25, HSP40, HSP60, HSP72, and HSP90 were purchased from Assay Designs (Ann Arbor, MI, USA). Rat antihuman HSF-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β -tubulin was purchased from Chemicon/Millipore. Cell Culture Lysis buffer was purchased from Promega (Madison, WI, USA). A synthetic luciferase reporter plasmid regulated by a synthetic promoter composed of multiple HSEs and the TATA-like promoter region from the Herpes simplex virus thymidine kinase gene (pHSE-Luc) was purchased from Becton Dickinson (San Diego, CA, USA). A blasticidin resistance plasmid, pcDNA6/TR, blasticidin, and DNase I were purchased from Invitrogen (Carlsbad, CA, USA). Fugene-6 was purchased from Roche Applied Science (Indianapolis, IN, USA). T4 polynucleotide kinase was purchased from Promega (Madison, WI, USA). SYBR-Green reaction mix was purchased from Biorad (Mountainview, CA, USA). A549 cells were purchased from the American Type Cell Collection (Manassas, VA, USA) and maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 50 U/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (Gibco/Invitrogen, Carlsbad, CA, USA), pH 7.3 (CRPMI), and containing 10% defined fetal bovine serum (Hyclone, Logan, UT, USA) at 37°C in 5% CO₂-enriched air.

Immunoblotting A549 cells were plated at 4×10^5 cells/well in 60 mm plates and, 24 h later, were incubated in prewarmed humidification chambers at 38.5°C, 39.5°C, or 41°C for the indicated time then switched to 37°C for the remainder of a 24-h incubation or at 42°C for 2 h and switched to 37°C for an additional 4 h. The cells were lysed in Cell Culture Lysis buffer, resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (PAGE), and transferred to polyvinylidene fluoride membrane. The

membranes were blocked for 1 h at room temperature in blocking buffer (TBS-T (10 mM Tris-HCl, pH 7.5, 136 mM NaCl, 2.0 mM KCl, 0.1% Tween 20) containing 5% nonfat dry milk). Following blocking, the membranes were washed with TBS-T and incubated with antibodies against HSP72, β -tubulin, or HSF-1 in blocking buffer for 2 h. After primary antibody reactions, the membrane was washed with TBS-T, incubated for 1 h with horseradish peroxidase-conjugated secondary antibody and developed with a chemiluminescence detection system (Renaissance™; New England Nuclear; Boston, MA, USA), quantified by direct imaging (Fuji gel documentation system and ImageGauge software), and subsequently exposed to X-ray film. When all samples from an experiment could not fit on a single gel, the samples were analyzed on gels each containing the same 37°C baseline sample and all gels were run, electrostatically transferred, and exposed to antibodies simultaneously.

HSE reporter analysis For stable transfections, A549 cells were cotransfected with a luciferase reporter construct driven by a synthetic promoter composed of multiple HSEs and the TATA-like promoter region from the Herpes simplex virus thymidine kinase gene (pHSE-Luc) along with the blasticidin resistance plasmid, pcDNA6/TR, using Eugene-6 transfection agent. Transfectants were selected using 4 μ g/ml blasticidin and maintained in media containing 2 μ g/ml blasticidin and cloned by limiting dilution. Single colonies were isolated and analyzed for HS-induced activation of the HSE-containing promoter and a single clone was used for further studies. For reporter assays, 1×10^5 cells/ml were plated in blasticidin-free medium for 24 h, then were exposed to 38.5°C, 39.5°C, 41°C, or 42°C for 2 h, switched to 37°C for 4 h, and were lysed in 1X Cell culture lysis buffer (Promega) and luciferase activity was analyzed using a commercial luciferase assay kit (Promega).

Electrophoretic mobility shift assays Nuclear extracts from A549 cells were prepared according to the method of Schreiber et al. (1989) as described earlier (Singh et al. 2000) and total protein concentration was measured using a commercial reagent (Biorad) against a bovine serum albumin standard curve. Double-stranded oligonucleotide containing the HSE corresponding to -107/-83 of the human HSPA1A promoter (Mosser et al. 1988; 5'-GATCTCGGCTGGAA TATCCCCGACCTGGCAGCCGA-3') were generated by annealing complementary strands and radiolabeling using T4 polynucleotide kinase (Promega) and [γ - 32 P] dATP according to the manufacturer's protocol. Where indicated, excess unlabeled competitor oligonucleotide or 1:20 dilution of anti-HSF-1 antibody were incubated with

the nuclear extracts for 30 min at room temperature before the addition of the radiolabeled probe. The DNA-protein complexes were then electrophoretically resolved on 4% nondenaturing polyacrylamide gels. The dried gels were analyzed by phosphorimaging (Molecular Dynamics) and exposed to X-ray film. Band intensities were expressed as percent of the maximal band intensity for each experiment.

Quantitative reverse transcription/polymerase chain reaction assay Total RNA from 2×10^6 A549 cells was isolated using Trizol (Invitrogen) and contaminating DNA was eliminated using DNase I digestion. RNA was reverse-transcribed using 1 μ g total RNA and oligo-dT primer using a cDNA synthesis kit according to the manufacturer's protocol (Promega). Duplicate 25- μ l real-time polymerase chain reaction (PCR) reactions were performed in 96-well plates using a SYBR-Green reaction mix and a Biorad iCycler according to the supplier's protocol with the following forward and reverse primers: glyceraldehydes-3 phosphate dehydrogenase (GAPDH), 5'-agcctcgtcccgtaga caaat and 5'-tggcaacaatctccacttgc and HSP72, 5'-accaag cagacgcagatcttc and 5'-agcctcaagatcatcagcaatg. Data were quantified using the gene expression Ct difference method described by Schefe et al. (2006) and standardized to levels of the housekeeping gene, GAPDH using Ct values automatically determined by the thermocycler. Briefly, the efficiency of amplification for each primer pair was calculated using the equation:

$$\text{Efficiency} = 10^{(1/\text{slope})} - 1$$

where slope is measured from the linear portion of the log fluorescence vs. cycle number curve. The expression of the gene of interest (GOI) in each sample relative to a reference, untreated 37°C cells, was calculated using the equation:

Relative GOI expression

$$= \frac{(1 + \text{efficiency}_{\text{GOI}})^{-\Delta\text{Ct}(\text{GOI})}}{(1 + \text{efficiency}_{\text{GAPDH}})^{-\Delta\text{Ct}(\text{GAPDH})}}$$

where ΔCt (GOI) and ΔCt (GAPDH) is the difference in threshold cycle between sample and reference for the gene of interest and GAPDH, respectively.

Statistical analysis Data are presented as mean $\pm$ standard error (SE). Differences among more than two groups were analyzed by applying a Tukey honestly significant difference test to a one-way analysis of variance. Differences with $p < 0.05$ were considered significant.

Results

HSP72 protein expression is proportional to temperature in the febrile range

A549 cells were incubated at 38.5°C, 39.5°C, or 41°C for up to 24 h or were exposed to 42°C for 2 h and then transferred to 37°C for an additional 4 h. Cells were sequentially lysed and immunoblotted for HSP72 and β -tubulin and HSP72 band intensities were standardized to β -tubulin levels and expressed as fold-change relative to cells at 37°C (Fig. 1A, B). Maximal HSP72 protein accumulation occurred within the first 6 h of exposure at 38.5°C to 41°C at rates that were proportional to incubation temperature. The duration of exposure required for HSP72 protein

levels to significantly exceed levels in 37°C cells was 1 h at 41°C, 6 h at 39.5°C, and 24 h at 38.5°C (Fig. 1B). The maximal HSP72 protein levels reached at each temperature was also proportional to incubation temperature between 37°C and 42°C, but the increase in HSP72 expression was much greater when incubation temperature exceeded 41°C (Fig. 1C). HSP72 expression increased by approximately 0.5-fold for every 1°C increase in temperature between 37°C and 41°C. However, an additional 1°C increase in incubation temperature from 41°C to 42°C stimulated a further 2.4-fold increase in HSP72 levels, suggesting distinct activation kinetics following exposure to classic HS temperatures.

Since each HSP gene has distinct promoter architecture with contributions of transcription factors other than HSF-

Fig. 1 HSP72 protein expression is temperature dependent. Subconfluent A549 monolayers were exposed to the indicated temperature for the indicated time and then were switched to 37°C for the remainder of a 24-h incubation. Cells were lysed and analyzed for HSP72 levels by immunoblotting. **a** A representative immunoblot is shown. All times from each temperature were run on the same gel. Aliquots of the same 37°C time 0 sample were also run on each of the three gels. **b** Band intensities were analyzed by direct imaging of the chemiluminescent signal, corrected for loading by normalizing to β -tubulin levels, and standardized to 37°C baseline levels (0). **c** The HSP72 protein levels after 6 h exposure to the indicated temperature between 38.5°C and 41°C or to 42°C for 2 h followed by 4 h recovery at 37°C were compared. **d** A549 cells were exposed to 41°C for 1 h, 39.5°C for 6 h, or 38.5°C for 24 h and a 24-h incubation was completed at 37°C. Each of five heat shock protein family members and β -tubulin were measured by Western blotting. Data are mean $\pm$ SE of six experiments. * p <0.05 vs. time 0. † p <0.05 and ‡ p <0.05 vs. 38.5°C and 39.5°C, respectively, values at the same exposure time

1, we analyzed the HSP expression profile activated by each level of hyperthermia (Fig. 1D). We selected conditions that stimulated similar HSP72 expression, 38.5°C for 24 h, 39.5°C for 6 h, and 41°C for 1 h. With the exception of HSP60, which was not increased above baseline under any of the experimental conditions, each of the HSPs was activated to similar levels with each of the warming protocols.

HSP72 mRNA expression is proportional to temperature in the febrile range

A549 cells were incubated at 42°C for up to 2 h or at 38.5°C to 41°C for up to 24 h and cells were sequentially lysed. HSP72 and GAPDH mRNA levels were analyzed by quantitative reverse transcription (RT)-PCR and HSP72/GAPDH ratios were expressed as fold-change compared with time 0. HSP72 mRNA peaked after 3 h of warming at levels that were proportional to exposure temperature between 38.5°C and 41°C (Fig. 2A). HSP72 mRNA levels were 1.8 ± 0.61 -, 4.3 ± 1.0 -, and 15 ± 3.2 -fold higher than the 37°C baseline after 3 h exposure to 38.5°C, 39.5°C, and 41°C. Similar to HSP72 protein levels, the rate of HSP72 mRNA generation rose sharply to 60 ± 23 -fold above levels in 37°C cells when temperature was increased from 41°C and 42° (Fig. 2B).

HSP72 promoter activity is proportional to temperature in the febrile range

Transcription of HSP72 is regulated by the heat-activated transcription factor, HSF-1. (Trinklein et al. 2004). To study

the time and temperature dependence of HSF-1-dependent gene expression, we analyzed the effect of various levels of hyperthermia on activity of a reporter construct driven by a synthetic HSF-1-responsive reporter plasmid (Fig. 3). A549 cells stably transfected with the reporter plasmid were incubated at 37°C for 6 h or at 38.5°C, 39.5°C, 41°C, or 42°C for 2 h and switched to 37°C for an additional 4-h incubation. The relationship between reporter plasmid activity and incubation temperature was similar to that found between HSP72 protein (Fig. 1C) and mRNA (Fig. 2B) levels and temperature. Luciferase activity increased by twofold with an increase in incubation temperature from 37°C to 41°C and an additional 14-fold with a temperature increase from 41°C and 42°C.

Levels of intranuclear HSE-binding activity was comparable in 38.5°C, 39.5°C, and 41°C cells

To further analyze the relationship between temperature and activation of HSF-1, we analyzed the activation of HSF-1 to its intranuclear DNA-binding state by electrophoretic mobility shift assays (EMSA) using the well-characterized HSE sequence from the human HSP72 promoter as probe (Singh et al. 2002, 2000). HSF-1-binding activities in nuclear extracts prepared from A549 cells maintained at 37°C or exposed to 42°C for 1 h, 39.5°C or 41°C for 1 or 6 h, or to 38.5°C for 1, 6, or 24 h were compared (Fig. 4). Interestingly, despite proportional increases in HSP72 expression and HSE reporter plasmid activity with increasing incubation temperature between 38.5°C to 41°C, HSF-1 HSE-binding was comparable at the three temperatures. Moreover, while HSF-1-dependent reporter plasmid activity

Fig. 2 HSP72 mRNA expression is temperature dependent. **a** Subconfluent A549 monolayers were exposed to the indicated temperature for the indicated time, lysed, total RNA collected, and analyzed for HSP72 and GAPDH mRNA levels by RT-PCR. Data are expressed as fold-change in HSP72/GAPDH levels compared with time 0 levels. **b** Subconfluent A549 monolayers were exposed to 38.5°C,

39.5°C, or 41°C for 3 h or to 42°C for 2 h and HSP72 and GAPDH mRNA levels were measured by RT-PCR. Data are mean $\pm$ SE of six separate experiments each for **a** and **b**. * $p < 0.05$ vs. time 0. † $p < 0.05$ and ‡ $p < 0.05$ vs. 38.5°C and 39.5°C, respectively, values at the same exposure time

Fig. 3 HSF-1-dependent gene expression is temperature dependent. A549 cells were stably transfected with an HSF-1-responsive reporter plasmid (pHRE), cloned by limiting dilution, and subconfluent monolayers of one clone were incubated at 37°C for 6 h or at 38.5°C, 39.5°C, 41°C, or 42°C for 2 h, then switched to 37°C for an additional 4 h. Cells were lysed and luciferase activity assayed and expressed as fold-change vs. 37°C controls. Data are mean $\pm$ SE of six experiments. * p < 0.05 vs. time 0

was 14-fold higher in cells exposed to 42°C compared with cells exposed to 41°C (Fig. 3), levels of activated HSF-1 detected by EMSA were only marginally higher in 42°C cells compared with 38.5°C cells (Fig. 4B), indicating that HSF-1 DNA-binding activity at HS and febrile temperatures are comparable and might not account for the enhanced HSP72 activation observed.

HS and hyperthermia in the febrile range posttranslationally modify HSF1 protein in a differential manner

Since posttranslation modifications, especially phosphorylation, play a major role in HSF-1 transactivation, we analyzed the effects of increasing temperature exposure on HSF-1 protein. It is well documented that HSF-1 protein is heavily modified by HS resulting in an apparent mobility shift toward a higher molecular weight in denaturing SDS-polyacrylamide gels (Chu et al. 1996; Guettouche et al. 2005; Holmberg et al. 2001; Park and Liu 2001; Xavier et al. 2000; Xia et al. 1998; Xia and Voellmy 1997). In fact in our earlier studies with RAW 264.7 murine macrophages, we have shown that HSF-1 mobility shift was markedly greater in 42°C-exposed cells in comparison to 39.5°- or 37°C-exposed cells (Singh et al. 2000). Unfractionated cell lysates and nuclear extracts from A549 cells exposed to hyperthermic temperatures between 38.5°C and 42°C for 1 h were resolved by 7% SDS-PAGE and immunoblotted using a rat monoclonal anti-HSF-1 antibody (Fig. 5). As expected, HSF-1 was

undetectable in nuclear extracts from 37°C cells, but increased to similar levels in nuclear extracts from cells exposed to 38.5°C to 42°C. However, HSF-1 in both nuclear extracts and cell lysates exhibited electrophoretic mobility that increased proportionally to incubation temperature with the most profound increases occurring between 39.5°C and 42°C. In fact, unmodified HSF-1 is undetectable following exposure to 42°C with all HSF-1 shifted to forms with higher apparent molecular weight. Multiple discrete bands are evident in the cells exposed to $\geq 41^\circ\text{C}$, suggesting multiple posttranslational modifications occur at these temperatures.

Discussion

We have shown that temperatures within the usual febrile range and below the classic HS range are capable of stimulating expression of HSP72, although the exposure time required for activation is longer and the magnitude of HSP72 gene expression is lower compared with classic HS temperatures. We measured expression of HSP72 protein by immunoblotting using an antibody that recognizes the nearly identical proteins encoded by the HSPA1A and HSPA1B genes (Daugaard et al. 2007). These two genes have similar promoter architecture and are activated in parallel in response to HS (Daugaard et al. 2007; Milner and Campbell 1990). To avoid the contribution of hyperosmolar stress, a potential costimulator of HSP72 expression (Schliess et al. 1999), we warmed cells in humidification chambers and confirmed that the volume of culture medium did not change during incubation.

Previous studies have shown that the temperature threshold for induction of the HS response differs across species (Tomanek and Somero 2002) and cell types (Sarge 1998) at temperatures that are generally several degrees centigrade above normal basal temperature. Moreover, the thermal threshold for induction of the HS response in ectothermic species, including goby fish, snails, and mussels, can be modified by chronic exposure to new baseline temperatures (Buckley et al. 2001; Lund et al. 2006; Tomanek and Somero 2002). We have now demonstrated that (1) *in vitro* HSF-1 DNA-binding activity and HSP72 expression are activated by exposure to temperatures $\geq 38.5^\circ\text{C}$ in human cells, (2) the rate of HSP72 expression increases proportionally as temperature increases from 38.5°C to 42°C, (3) the temperature-dependent increase in HSP72 expression is much greater when exposure temperature exceeds 41°C, and (4) the temperature-dependent increase in HSP72 expression occurs with little or no further increases in HSF-1 DNA-binding activity but is accompanied by a temperature-dependent increase in the apparent molecular weight of

Fig. 4 HSE-binding activity is comparable in cells incubated at 38.5°C to 41°C. **a** Subconfluent A549 monolayers were exposed to the indicated temperature for the indicated time, cells were lysed, and nuclear extracts prepared. Cells maintained at 37°C were harvested at time 0. Extracts were analyzed for HSF-1 DNA-binding activity by EMSA using the HSE-containing sequence of the HSPA1A promoter as probe. Lane 1 contained no probe. Lane 12–15 utilized the same nuclear extract from A549 cells exposed to 42°C for 1 h (lane 10). In

lane 13, the complex was supershifted with anti-HSF-1 antibody. In lane 14, the complex was performed in the presence of 30-fold excess unlabeled HSPA1A oligonucleotide. The positions of the HSF-1-specific band, a nonspecific band, and the supershifted band (lane 13) are indicated by the *thick arrow*, *arrowhead*, and *thin arrow*, respectively. **b** Band densities were quantified by phosphorimaging and the results of three experiments. Mean $\pm$ SE. * p < 0.05 vs. 37°C and † p < 0.05 vs. 39.5°C and 41°C

HSF-1. These data confirm that exposure to febrile-range temperatures activate HSF-1-dependent gene expression but at a rate much lower than classic HS temperature (Jiang et al. 1999).

Fig. 5 The electrophoretic mobility of HSF-1 increases in proportion to exposure temperature. Subconfluent A549 monolayers were exposed to the indicated temperature for 1 h. Total cell lysates (*upper panel*) and nuclear extracts (*lower panel*) were analyzed for HSF-1 by immunoblotting. The *arrow* indicates a nonspecific (NS) band. A representative of three similar experiments is shown

We analyzed HSP72 protein and mRNA levels generated in the human respiratory epithelial-like A549 adenocarcinoma cell line in response to warming to 38.5°C, 39.5°C, and 41°C and those stimulated by a classic HS inducing protocol, 42°C for 2 h with 4 h recovery at 37°C. Whereas A549 cells were exposed to hyperthermic temperatures between 38.5°C and 41°C for up to 24 h to simulate temperature exposures that occur during fever, we did not subject cells to 42°C for more than 2 h because such exposure causes cell death in the absence of prior heat adaptation. Peak HSP72 protein and mRNA levels exhibited a similar pattern of temperature dependence. HSP72 protein levels increased approximately 0.5-fold for every 1°C above 37°C. However, an additional 1°C increase in temperature from 41°C to 42°C stimulated an additional 2.4-fold increase. Peak levels of mRNA doubled as incubation temperature was increased from 37°C to 38.5°C and between 38.5°C and 39.5°C and increased fourfold as incubation temperature increased between 39.5°C and 41°C and between 41°C and 42°C. To further analyze the temperature dependence of HSF-1-dependent gene expres-

sion, we studied activity of an HSF-1-responsive reporter plasmid in stably transfected A549 cells. Stable transfectants were exposed to 38.5°C, 39.5°C, 41°C, or 42°C for 2 h and then returned to 37°C for 4 h, and luciferase activity was measured. The pattern of temperature dependence of plasmid activity paralleled that of HSP72 protein generation, exhibiting a relatively modest increase in activity as temperature increased from 37°C to 41°C and a much greater increase in activity as exposure temperature was increased from 41°C to 42°C. These results suggest that there may be fundamental differences in regulation of HSP72 gene activation at temperatures below and above 41°C. That 41°C is the upper limit of the normal human febrile range underscores the biological significance of this relationship (Roth et al. 2006). We have previously suggested that fever, which evolved long after emergence of the HS response, co-opted elements of the HS response to generate a distinct, partially overlapping process (Hasday and Singh 2000). The pattern of temperature dependence of HSP72 expression and HSF-1-dependent gene expression supports this hypothesis.

We found that HSP27, HSP40, and HSP90 expression paralleled HSP72 expression between 37°C and 41°C (Fig. 1D). These genes share heat-inducibility and heat-activated recruitment of HSF-1 to their promoter sequences (Trinklein et al. 2004), suggesting the differential effects of the various heating protocols derives from variable temperature-dependent activation of HSF-1. HSF-1 is activated through a stepwise process comprising trimerization, nuclear translocation, and phosphorylation of serines in the transactivation domains (Cotto and Morimoto 1999). Regulation of this process is incompletely understood, but involves phosphorylation and dephosphorylation of certain serine residues (Chu et al. 1996; Guettouche et al. 2005; Holmberg et al. 2001; Park and Liu 2001; Xavier et al. 2000; Xia et al. 1998; Xia and Voellmy 1997) and heterologous oligomerization of HSF-1 (Ali et al. 1998; Bharadwaj et al. 1999; Duina et al. 1998). We have previously demonstrated in the mouse RAW 264.7 macrophage cell line that HSF-1 is activated to a hypophosphorylated DNA-binding form without transactivating activity by exposure to 39.5°C for 1 h (Singh et al. 2000). In the present study, we confirmed that HSF-1 activation to a DNA-binding form and attainment of transactivating activity are independently regulated. That HSP72 gene expression increased as temperature increased from 38.5°C to 41°C while HSF-1 capacity to bind HSEs was similar at all temperatures between 38.5°C and 41°C suggests that one or more posttranslational events such as phosphorylation or sumoylation (Hong et al. 2001) may be differentially activated when temperature exceeds the usual febrile range. Furthermore, the 2.6-fold and fourfold increases in HSP72 protein and mRNA expression and 14-fold increase in HSF-1-responsive reporter plasmid activity stimulated by an increase in temperature from

41°C to 42°C was accompanied by only a 73% increase in levels of the DNA-binding form of HSF-1. These data confirm the findings of Laszlo et al. (2005) that even modest levels of hyperthermia, 38°C for 15 min in their study of Chinese hamster ovarian cells, induce near-maximal activation of HSF-1 DNA-binding activity and suggest that the proportional increases in HSF-1-dependent gene expression at temperatures within and above the normal febrile range are regulated by additional signaling events. This was further evidenced by the mobility shift of HSF-1 protein in immunoblots of cell and nuclear extracts resolved by 7% SDS-PAGE. The progressive temperature-dependent increase in electrophoretic mobility of HSF-1 that paralleled HSP72 gene expression suggests the posttranslational modifications of HSF-1 that regulate its HSP72 transactivating potential are temperature dependent within and above the usual febrile range.

We and others have demonstrated that elements of the HS response are activated at febrile-range temperatures and regulate genes involved in inflammation and the innate immune response, including tumor necrosis factor α (Ostberg et al. 2000; Singh et al. 2002, 2000), interleukin (IL)-1 β (Cahill et al. 1996; Fairchild et al. 2000), IL-8, and granulocyte macrophage colony-stimulating factor (Rice et al. 2005; Singh et al. 2008), as well as HSPs. A computer-assisted analysis of CXC chemokine promoters showed that almost every member of this family of neutrophil chemotaxins contains HSEs within their promoter sequences, suggesting these genes may be a newly recognized class of HSF-1-regulated genes (Nagarsekar et al. 2005). In further support of the link between febrile-range temperatures and genes-regulating neutrophil recruitment, we found that exposure to febrile-range hyperthermia (core temperature 39.5°C) increases expression of CXC chemokines, augments neutrophil recruitment, and increases tissue injury in mouse models of lung injury (Hasday et al. 2003; Rice et al. 2005); however, augmentation of CXC chemokine expression did not occur in HSF-1-null mice (Singh et al. 2008). These data demonstrate how activation of HS pathways at febrile-range temperatures has effects beyond HSP expression that may have important consequences in the febrile host.

In summary, we have shown that exposure to temperatures within the usual febrile range are capable of activating the heat shock response, but at submaximal levels that are proportional to the exposure temperature. The profound difference in the relationship between temperature and HSF-1-dependent gene expression below and above 41°C, the upper limit of the human febrile range, suggests that HSF-1 activation may occur via distinct mechanisms and have different consequences at febrile and HS temperatures, which may involve the posttranslational modification of HSF-1.

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References

- Ali A, Bharadwaj S, O'Carroll R, Ovsenek N (1998) HSP90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. *Mol Cell Biol* 18:4949–4960
- Bharadwaj S, Ali A, Ovsenek N (1999) Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 in vivo. *Mol Cell Biol* 19:8033–8041
- Buckley BA, Owen ME, Hofmann GE (2001) Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. *J Exp Biol* 204:3571–3579
- Cahill CM, Wareman WR, Xie Y, Auron PE, Calderwood SK (1996) Transcriptional repression of the prointerleukin 1 β gene by heat shock factor 1. *J Biol Chem* 271:24874–24879
- Chu B, Soncin F, Price BD, Stevenson MA, Calderwood SK (1996) Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J Biol Chem* 271:30847–30857. doi:10.1074/jbc.271.48.30847
- Cotto JJ, Morimoto RI (1999) Stress-induced activation of the heat-shock response: cell and molecular biology of heat-shock factors. *Biochem Soc Symp* 64:105–118
- Daugaard M, Rohde M, Jaattela M (2007) The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions. *FEBS Lett* 581:3702–3710. doi:10.1016/j.febslet.2007.05.039
- Duina AA, Kalton HM, Gaber RF (1998) Requirement for Hsp90 and a Cyp-40-type cyclophilin in negative regulation of the heat shock response. *J Biol Chem* 273:18974–18978. doi:10.1074/jbc.273.30.18974
- Fairchild KD, Viscardi RM, Hester L, Singh IS, Hasday JD (2000) Effects of hypothermia and hyperthermia on cytokine production by cultured human mononuclear phagocytes from adults and newborns. *J Interferon Cytokine Res* 20:1049–1055. doi:10.1089/107999000750053708
- Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* 61:243–282. doi:10.1146/annurev.physiol.61.1.243
- Gothard LQ, Ruffner ME, Woodward JG, Park-Sarge OK, Sarge KD (2003) Lowered temperature set point for activation of the cellular stress response in T-lymphocytes. *J Biol Chem* 278:9322–9326. doi:10.1074/jbc.M209412200
- Guettouche T, Boellmann F, Lane WS, Voellmy R (2005) Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC Biochem* 6:4. doi:10.1186/1471-2091-6-4
- Hasday J, Garrison A, Singh I, Standiford T, Ellis G, Rao S, He J-R, Rice P, Frank M, Goldblum S, Viscardi R (2003) Febrile-range hyperthermia augments pulmonary neutrophil recruitment and amplifies pulmonary oxygen toxicity. *Am J Pathol* 162:2005–2017
- Hasday JD, Singh IS (2000) Fever and the heat shock response: distinct, partially overlapping processes. *Cell Stress Chaperones* 5:471–480. doi:10.1379/1466-1268(2000)005<0471:FATHSR>2.0.CO;2
- Hietakangas V, Anckar J, Blomster HA, Fujimoto M, Palvimo JJ, Nakai A, Sistonen L (2006) PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc Natl Acad Sci U S A* 103:45–50. doi:10.1073/pnas.0503698102
- Holmberg CI, Hietakangas V, Mikhailov A, Rantanen JO, Kallio M, Meinander A, Hellman J, Morrice N, MacKintosh C, Morimoto RI, Eriksson JE, Sistonen L (2001) Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *Embo J* 20:3800–3810. doi:10.1093/emboj/20.14.3800
- Hong Y, Rogers R, Matunis MJ, Mayhew CN, Goodson ML, Park-Sarge OK, Sarge KD, Goodson M (2001) Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification. *J Biol Chem* 276:40263–40267
- Jiang Q, DeTolla L, Kalvakolanu I, Van Rooijen N, Singh IS, Fitzgerald B, Cross AS, Hasday JD (1999) Febrile range temperature modifies early systemic TNF α expression in mice challenged with bacterial endotoxin. *Infect Immun* 67:1539–1546
- Jurivich DA, Sistonen L, Sarge KD, Morimoto RI (1994) Arachidonate is a potent modulator of human heat shock gene transcription. *Proc Natl Acad Sci U S A* 91:2280–2284. doi:10.1073/pnas.91.6.2280
- Laszlo A, Moros EG, Davidson T, Bradbury M, Straube W, Roti Roti J (2005) The heat-shock factor is not activated in mammalian cells exposed to cellular phone frequency microwaves. *Radiat Res* 164:163–172. doi:10.1667/RR3406
- Lund SG, Ruberte MR, Hofmann GE (2006) Turning up the heat: the effects of thermal acclimation on the kinetics of hsp70 gene expression in the eurythermal goby, *Gillichthys mirabilis*. *Comp Biochem Physiol A Mol Integr Physiol* 143:435–446. doi:10.1016/j.cbpa.2005.12.026
- McClung JP, Hasday JD, He JR, Montain SJ, Chevront SN, Sawka MN, Singh IS (2008) Exercise-heat acclimation in humans alters baseline levels and ex vivo heat inducibility of hsp72 and hsp90 in peripheral blood mononuclear cells. *Am J Physiol Regul Integr Comp Physiol* 294:R185–191. doi:10.1152/ajpregu.00532.2007
- McMillan DR, Xiao X, Shao L, Graves K, Benjamin IJ (1998) Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *J Biol Chem* 273:7523–7528. doi:10.1074/jbc.273.13.7523
- Milner CM, Campbell RD (1990) Structure and expression of the three MHC-linked HSP70 genes. *Immunogenetics* 32:242–251. doi:10.1007/BF00187095
- Morange M, Dubois MF, Bensaude O, Lebon P (1986) Interferon pretreatment lowers the threshold for maximal heat-shock response in mouse cells. *J Cell Physiol* 127:417–422. doi:10.1002/jcp.1041270310
- Mosser DD, Theodorakis NG, Morimoto RI (1988) Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. *Mol Cell Biol* 8:4736–4744
- Nagarsekar A, Hasday J, Singh I (2005) CXC chemokines: a new family of heat-shock proteins. *Immunol Invest* 34:381–398. doi:10.1081/IMM-200067648
- Ostberg JR, Kaplan KC, Repasky EA (2002) Induction of stress proteins in a panel of mouse tissues by fever-range whole body hyperthermia. *Int J Hyperthermia* 18:552–562. doi:10.1080/02656730210166168
- Ostberg JR, Taylor SL, Baumann H, Repasky EA (2000) Regulatory effects of fever-range whole-body hyperthermia on the LPS-induced acute inflammatory response. *J Leukoc Biol* 68:815–820
- Park J, Liu AY (2001) JNK phosphorylates the HSF1 transcriptional activation domain: role of JNK in the regulation of the heat shock response. *J Cell Biochem* 82:326–33. doi:10.1002/jcb.1163
- Perisic O, Xiao H, Lis JT (1989) Stable binding of *Drosophila* heat shock factor to head-to-head and tail-tail repeats of a conserved 5 bp recognition unit. *Cell* 59:797–806. doi:10.1016/0092-8674(89)90603-X
- Rice P, Martin E, He J-R, Frank M, DeTolla L, Hester L, O'Neill T, Manka C, Singh I, Hasday J (2005) Febrile-range hyperthermia

- augments neutrophil accumulation and enhances lung injury in experimental Gram-negative bacterial pneumonia. *J Immunol* 174:3676–3685
- Roth J, Rummel C, Barth SW, Gerstberger R, Hubschle T (2006) Molecular aspects of fever and hyperthermia. *Neurol Clin* 24:421–439. doi:10.1016/j.ncl.2006.03.004
- Sarge KD (1998) Regulation of HSF1 activation and Hsp expression in mouse tissues under physiological stress conditions. *Ann N Y Acad Sci* 851:112–116. doi:10.1111/j.1749-6632.1998.tb08983.x
- Sarge KD, Murphy SP, Morimoto RI (1993) Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress [published errata appear in *Mol Cell Biol* 1993 May;13(5):3122–3 and 1993 Jun;13(6):3838–9]. *Mol Cell Biol* 13:1392–1407
- Scheffe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H (2006) Quantitative real-time RT-PCR data analysis: current concepts and the novel “gene expression’s CT difference” formula. *J Mol Med* 84:901–910. doi:10.1007/s00109-006-0097-6
- Schliess F, Wiese S, Haussinger D (1999) Osmotic regulation of the heat shock response in H4IIE rat hepatoma cells. *Faseb J* 13:1557–1564
- Schreiber E, Matthias P, Muller MM, Schaffner W (1989) Rapid detection of octamer binding protein with mini extracts prepared from a small number of cells. *Nucl Acids Res* 17:6419. doi:10.1093/nar/17.15.6419
- Singh I, He J-R, Calderwood S, Hasday J (2002) A high affinity hsf-1 binding site in the 5′-untranslated region of the murine tumor necrosis factor- α gene is a transcriptional repressor. *J Biol Chem* 277:4981–4988. doi:10.1074/jbc.M108154200
- Singh IS, Calderwood S, Kalvakolanu I, Viscardi R, Hasday JD (2000) Inhibition of tumor necrosis factor- α transcription in macrophages exposed to febrile range temperature: a possible role for heat shock factor-1. *J Biol Chem* 275:9841–9848. doi:10.1074/jbc.275.13.9841
- Singh IS, Gupta A, Nagarsekar A, Cooper Z, Manka C, Hester L, Benjamin IJ, He J-E, Hasday JD (2008) Heat shock co-activates interleukin-8 transcription. *Am J Respir Cell Molec Biol* 39: 235–242
- Tomanek L, Somero GN (2002) Interspecific- and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*): implications for regulation of hsp gene expression. *J Exp Biol* 205:677–685
- Trinklein ND, Chen WC, Kingston RE, Myers RM (2004) Transcriptional regulation and binding of heat shock factor 1 and heat shock factor 2 to 32 human heat shock genes during thermal stress and differentiation. *Cell Stress Chaperones* 9:21–28
- Xavier IJ, Mercier PA, McLoughlin CM, Ali A, Woodgett JR, Ovsenek N (2000) Glycogen synthase kinase 3 β negatively regulates both DNA-binding and transcriptional activities of heat shock factor 1. *J Biol Chem* 275:29147–29152. doi:10.1074/jbc.M002169200
- Xia W, Guo Y, Vilaboa N, Zuo J, Voellmy R (1998) Transcriptional activation of heat shock factor HSF1 probed by phosphopeptide analysis of factor 32P-labeled in vivo. *J Biol Chem* 273:8749–8755. doi:10.1074/jbc.273.15.8749
- Xia W, Voellmy R (1997) Hyperphosphorylation of heat shock transcription factor 1 is correlated with transcriptional competence and slow dissociation of active factor trimers. *J Biol Chem* 272:4094–4102. doi:10.1074/jbc.272.7.4094