International Journal of Hyperthermia

ISSN: 0265-6736 (Print) 1464-5157 (Online) Journal homepage: informahealthcare.com/journals/ihyt20

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To cite this article: Ishwar S. Singh & Jeffrey D. Hasday (2013) Fever, hyperthermia and the heat shock response, International Journal of Hyperthermia, 29:5, 423-435, DOI: 10.3109/02656736.2013.808766

To link to this article: https://doi.org/10.3109/02656736.2013.808766

International Journal of Hyperthermia

http://informahealthcare.com/hth
ISSN: 0265-6736 (print), 1464-5157 (electronic)

Int J Hyperthermia, 2013; 29(5): 423–435
 DOI: 10.3109/02656736.2013.808766

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REVIEW ARTICLE

Fever, hyperthermia and the heat shock response

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Abstract

The heat shock response is a highly conserved primitive response that is essential for survival against a wide range of stresses, including extremes of temperature. Fever is a more recently evolved response, during which organisms raise their core body temperature and temporarily subject themselves to thermal stress in the face of infections. The present review documents studies showing the potential overlap between the febrile response and the heat shock response and how both activate the same common transcriptional programme (although with different magnitudes) including the stress-activated transcription factor, heat shock factor-1, to modify host defences in the context of infection, inflammation and injury. The review focuses primarily on how hyperthermia within the febrile range that often accompanies infections and inflammation acts as a biological response modifier and modifies innate immune responses. The characteristic 2–3 °C increase in core body temperature during fever activates and utilises elements of the heat shock response pathway to modify cytokine and chemokine gene expression, cellular signalling and immune cell mobilisation to sites of inflammation, infection and injury. Interestingly, typical proinflammatory agonists such as Toll-like receptor agonists modify the heat shock-induced transcriptional programme and expression of HSP genes following co-exposure to febrile range hyperthermia or heat shock, suggesting a complex reciprocal regulation between the inflammatory pathway and the heat shock response pathway.

Keywords

Fever, HSF1, HSP, immunity, inflammation

History

Received 1 April 2013
Revised 13 May 2013
Accepted 21 May 2013
Published online 17 July 2013

Introduction

Fever is a complex physiological response to infection and injury, the key feature of which is a temporary resetting of the body's thermostatic set point resulting in an increase in core temperature. Although fever is recognised as a component of the acute-phase response to infection and perceived to be a response limited to mammals and birds, many poikilothermic animals, including lower vertebrates, arthropods, and annelids, also increase their core temperature in response to infection or injury [1]. The prevalence of fever in such diverse modern animals suggests that it first appeared over 600 million years ago. This evolutionary persistence of fever is even more remarkable when one considers its substantial metabolic cost. In humans, generating fever through thermogenic shivering requires up to a 6-fold increase in metabolic rate [2], and maintaining a physiological core temperature at febrile levels requires an approximately 12% increase in metabolic rate per 1 °C increase in core temperature [3,4]. In poikilothermic animals with infections, moving to warmer environs not only requires increased energy expenditure, but may also expose vulnerable individuals to attack by predators. Therefore, fever must confer benefit that generally outweighs these costs in the infected or injured host. Furthermore, given the phylogenetic age of fever, the immunological processes that are active during febrile illnesses have had ample opportunity to evolve for optimal function at febrile temperatures.

This review will focus on how the characteristic 2–3 °C increase in body temperature that often accompanies infections and inflammation acts as a biological response modifier by regulating signalling pathways and gene expression involved in immune defence, inflammation, and cell death and survival. We discuss how elements of the heat shock (HS) response pathway have been co-opted as immune response modifiers and how the knowledge of how the temperature responsiveness of elements of the immune response can be translated to the care of the acutely ill patient.

Heat shock response

While fever is a systemic response to infection and injury, the HS response acts as a defence mechanism against cellular stress. The HS response, a highly conserved ancient biological process, is essential for survival against a myriad of environmental stresses, including extremes of temperature, chemicals and radiations, each of which can cause denaturation of essential cellular proteins. Also referred to as the 'cellular stress response' the HS response is accompanied with reprogramming of the cellular transcriptional and

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translational machinery to preferentially express a set of stress-inducible proteins namely the heat shock proteins (HSPs). During stress these HSPs act as chaperones and bind to denatured proteins to either preserve them until the stress has abated or to target the denatured proteins for degradation [5–7]. Genes encoding the five families of HSPs are highly conserved. Their presence in all species studied to date including archaebacteria, eubacteria, and eukaryotes, suggests that they first arose at least 2.5 billion years ago. While prokaryotic and eukaryotic HSP genes exhibit striking cross-domain homology, they use different mechanisms of transcriptional regulation. In eukaryotes, HSP expression is regulated at the transcriptional level by the stress-activated transcription factor heat shock factor (HSF). Mammals, including humans have three HSF orthologues of which HSF1 is the heat inducible orthologue [8–10]. Human HSF1 is a complex protein with an N-terminal DNA binding domain, three hydrophobic regions that regulate trimerisation, a serine-rich regulatory domain that regulates trasnscriptional activation, and two independent C-terminal transactivation domains [11]. HSF1 is retained as inactive monomers by intramolecular interactions between the first two hydrophobic regions and the third hydrophobic region [12,13]. During HSF1 trimerisation, the intramolecular interactions are replaced by intermolecular interactions between the first two hydrophobic repeats of each of the three trimerising HSF1 molecules. Heat-inducible HSFs, including mammalian HSF1 exists in dynamic equilibrium between a transcriptionally inactive, cytosolic, hetero-oligomeric pool and a transcriptionally active intranuclear homotrimeric pool. Spontaneous trimerisation of HSF has been reported to be concentration-dependent, spontaneously forming DNA-binding trimers when present at sufficiently high concentrations in cell-free reactions [12] and within intact cells [13]. Zhong et al. [14] demonstrated that dilution of trimerised *Drosophila* HSF in crude cell lysates from Schneider line-2 (SL-2) cells caused reversible dissociation of HSF trimers to monomers. They used this model system to calculate the equilibrium constant, K_d, for the HSF trimer dissociation reaction and showed that the K_d decreased, thereby favouring trimerisation, as the reaction temperature increased or upon exposure to oxidant stress. This analysis provides a useful conceptual model to interpret studies in which the expression levels of HSF may vary widely by describing the mathematical relationship among HSF concentration, temperature, and the extent of HSF trimerisation. Importantly, this study also demonstrates that HSF trimerisation may occur as a continuous temperature-dependent process that is activated over a temperature range rather than as a binary process activated when a distinct thermal threshold is exceeded.

Induction of HSF1 trimerisation is a hierarchical process. HSF is directly activated by heat in cell-free reactions, but the temperature range at which the reaction occurs is a species-specific intrinsic property of HSF, and related to the normal temperature range of the organism. For example, cell-free *Drosophila* HSF undergoes trimerisation between 28° and 38 °C while mouse HSF1 trimerises between 37° and 39 °C [14,15]. While cell-free HSF can be directly activated by heat, Zhong et al. showed that trimerisation of intracellular HSF is heat-activated at lower temperatures than cell-free HSF or by chemical stresses that have no effect on cell-free HSF, such as salicylate, dinitrophenol, arsenite, and ethanol [14]. When human HSF1 is expressed in *Drosophila* SL-2 cells, it trimerises at 32–37 °C, the *Drosophila* HS range, rather than the usual human temperature threshold [16]. In addition, the temperature threshold for mammalian HSF1 can differ between different tissues in the same organism [17] and change over time such as after prolonged exposure to hyperthermia [18] or in response to soluble mediators like arachidonic acid or type I interferon [19,20].

Activation of HSF1 and its transcriptional activity is greatly dependent upon its post-transcriptional modifications. HSF1 has 60 serines and threonines, at least 12 of which have been shown to be phosphorylated [21] by various kinases including members of the MAP kinase pathway. Most of the phosphorylation events modify trans-activation domain function but phosphorylation of threonine 142 increases [22] and phosphorylation of serine-121 reduces [23] activation of human HSF1 to its DNA binding trimeric form and phosphorylation of serine-419 is required for its heat-induced nuclear translocation independent of trimerisation [24]. HSF1 undergoes additional covalent modifications including sumoylation that are critical for its transcriptional activation of HSP genes [25,26]. Collectively, these studies demonstrate that HSF1 trimerisation and its transcriptional competency is regulated through multiple steps, each affected by temperatures, soluble mediators, and protein modifying signalling events that are encountered during febrile illnesses.

Fever and the HS response

Over twelve years ago we proposed a partial overlap between fever and the HS response [27] based on data showing partial activation of HS signalling pathways at febrile temperatures, the participation of HSF1 in the regulation of several inflammatory mediator genes, and the cytoprotective effects of intracellular HSPs generated at febrile temperatures. In the subsequent decade additional experimental evidence has been generated that supports a functional overlap between fever and the HS response and identifies HSF1 as central to the relationship between these two distinct, evolutionarily conserved host defence mechanisms.

HSF1 activation has not only been shown to occur at temperatures in the febrile range but the temperature required for HSF1 activation and HSP gene expression has been shown to differ across species [28] and across different cell types and tissues in the same organism [17,29], and to be lowered by exposure to certain inflammatory mediators [19,20]. For example, mouse lymphoid tissues, including spleen, exhibit a low thermal threshold for induction of HSP expression, which appears to derive from T lymphocyte rather than B lymphocyte behaviour [29,30], suggesting variable cell- and tissue-specific activation of HS response at febrile temperatures (Table I).

As discussed in the previous section, HSF1 trimerisation and nuclear translocation is required but not sufficient for gene transcription [12,13] and is dissociable from HSP gene transcription [31,32]. We found that exposing the RAW 264.7 mouse macrophage cell line to hyperthermia in the febrile

Table I. HSF1 activation and HSP induction in various tissue and tissue culture cells at febrile range temperatures.

Cell/tissue	Exposure	Effect	Reference
RAW 264.7 mouse macrophages	39.5 °C for 30–60 min	HSF1 nuclear translocation and DNA-binding activity in vitro and in vivo	[33,91]
	39.5 °C for 2 h	Hsp70 mRNA induction	[40]
	39.5 °C for 6 h	Hsp70 protein induction	[40]
A549 human lung epithelial cells	38.5, 39.5 or 41 °C for 1 h	HSF1 DNA-binding activity	[35]
	38.5 °C for 24 h	Hsp70 protein induction	
	39.5 °C for 6 h		
Purified $CD4^+$ T cells	39.5 °C for 6 h	Hsp70 protein induction	[154]
Mouse C3H 10 T and hamster ovary HA-1 fibroblasts	38.5, 39.5 or 41 °C for 15 min	HSF1 DNA-binding activity	[34]
Isolated primary cells from mouse testis	34–44 °C for 1 h	HSF1 DNA-binding activity detected at 36 °C onwards	[155]
	38 °C for 1 h	HSF1 DNA-binding activity 36 °C onwards	[156]
	38 °C for 1 h followed by 2 h recovery at 32 °C	Hsp70 protein induction	
Mouse spleen cells and T-lymphocytes	Whole-body hyperthermia exposure at 38–42 °C for 1 h	HSF1 DNA-binding activity detected at 38–39 °C onwards	[29]
		Hsp70 mRNA induction detected at 39 °C onwards	
Mouse heart, lung, kidney, lymph nodes and thymus	Whole-body hyperthermia exposure at 39–40 °C for 6 h	Hsp70 protein induction	[30]
Mouse liver and kidney	Whole-body hyperthermia, core body temperature 39.5 °C for 3 h	Hsp70 protein induction	[38]
Mouse lung	Whole-body hyperthermia, core body temperature 39.5 °C for 24 h	Hsp70 protein induction	[39,40]

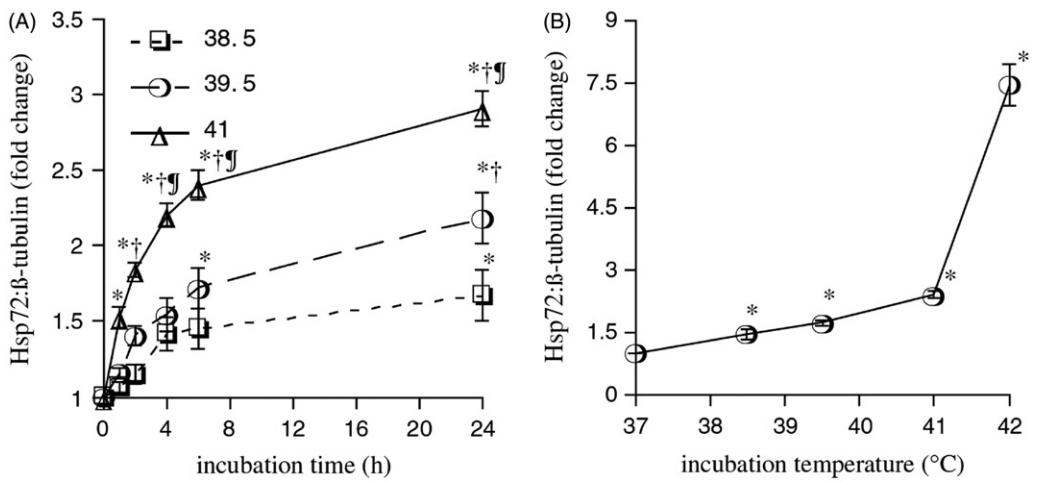

Figure 1. Hsp72 protein expression is temperature and time dependent. Subconfluent A549 monolayers were exposed to the indicated temperature for the indicated time and then were switched to 37 °C for the remainder of a 24-h incubation. Cells were lysed and analysed for Hsp72 levels by immunoblotting. (A) Band intensities were analysed by direct imaging of the chemiluminescent signal, corrected for loading by normalising to β-tubulin levels, and standardised to 37 °C baseline levels (0). (B) 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. Data are mean ± SE of six experiments. *$p < 0.05$ versus time 0. †$p < 0.05$ and ¶$p < 0.05$ versus 38.5 °C and 39.5 °C, respectively, values at the same exposure time. This research was originally published in *Cell Stress and Chaperones* [35]. Reprinted with kind permission from Springer Science and Business Media.

range (39.5 °C) for a brief period activates HSF1 trimerisation and DNA binding activity, but is insufficient to induce expression of HSP genes, while exposing the cells to classic HS temperatures (≥42 °C) induces high levels of Hsp70 [33]. Similarly, Laszlo et al. [34] showed that 15 min exposure to 38 °C was sufficient to activate HSF1 to a DNA binding form in HA-1 hamster fibroblasts and C3H10T1/2 mouse fibroblast-like cells. We recently confirmed that HSF1 activation to its DNA-binding trimeric state is dissociable from Hsp70 expression and showed that the thermal threshold for Hsp70 expression is both temperature- and time-dependent in the A549 human pulmonary epithelial-like adenocarcinoma cell line [35]. Similar to the results in the Laszlo study, exposing A549 cells to 38.5 °C, 39.5 °C, and 41 °C for 1 h each caused similar nuclear translocation and DNA binding activity of HSF1. However, detectable Hsp70 protein expression required 24 h exposure at 38.5 °C, 6 h exposure at 39.5 °C, and only 1 h exposure at 41 °C (Figure 1). The relationship between the exposure temperature and maximal Hsp70 protein levels was linear between 37 °C and 41 °C, increasing approximately 50%/ °C. However, a further 1 °C increase in temperature to 42 °C stimulated an additional 2.6-fold

increase in Hsp70 expression with little additional activation of HSF1 binding activity (Figure 1) [35]. These results suggest that 41–42 °C may represent a key temperature threshold in human cells above which the relationship between Hsp70 gene activation and temperature shifts. That 41 °C is the upper limit of the normal human febrile range underscores the biological significance of this relationship [36,37].

In anaesthetised mice, raising core temperature to febrile-range levels (rectal temperature 39.5 °C) by partial immersion in a heated water bath for 3 h was sufficient to activate Hsp72 expression in liver and kidney, albeit at much lower levels than mice exposed to HS temperature (rectal temperature 42 °C) for only 20 min followed by 160 min normothermic recovery [38]. More recently, we showed that maintaining core temperature at 39.5 °C for 24 h in conscious mice activates expression of Hsp70 in lung parenchyma [39,40]. The relatively low temperature-dependent expression of Hsp70 at temperatures within the normal febrile temperature range and the profound increase in Hsp70 expression at temperatures $\geq$42 °C supports our proposition that fever and HS responses are distinct but partially overlapping processes [27].

Fever, inflammation and immune responses

As expected based on the evolutionary conservation of the febrile response [1,27,41], fever and hyperthermia in the febrile-range (febrile range hyperthermia (FRH), core temperature ~39.5 °C) confers protection in infection by improving pathogen clearance in vivo [39,42–44] although the change in temperature have little effect on the growth rate of the pathogens [39,44]. Increasing body temperature of fish and lizards by ~4 °C greatly increased clearance of the same Gram-negative pathogen, *Aeromonas hydrophila*, despite a 10 °C lower temperature range in the fish [42,43]. These data demonstrate that FRH enhances pathogen clearance *in vivo* through effects on host defence rather than on the pathogen. This effect may also explain the association of fever with improved survival in retrospective clinical studies of bacterial infections [45–47].

We developed a mouse model of FRH in which mice exposed to an ambient temperature of 36–37 °C increase their core temperature by 2–3 °C but maintain normal circadian patterns and appear otherwise healthy and active [48,49]. Using this model we showed that FRH accelerated pathogen clearance in experimental *Klebsiella pneumoniae* peritonitis [44] and pneumonia [39]. Focusing on the lung, we found that FRH, despite reducing pathogen load, tended to reduce survival in the *K. pneumoniae* model while it greatly improved survival in the peritonitis model [39,44] and severe lung injury was found in mice co-exposed to FRH in the pneumonia model [39,44]. Lipke and Martin et al. confirmed these results in the intratracheal bacterial lipopolysaccharide (LPS)-challenged mouse model [50,51]. Co-exposure to FRH and LPS exerted similar effects in a model of lethal pulmonary oxygen toxicity [52]. Considering these results it appears that FRH augmented innate immune processes, which accelerated pathogen clearance but also enhanced collateral tissue injury, and the net effect on survival depended on the balance between the two effects. Although the above studies focused primarily on the host's inflammatory responses and neutrophil-mediated vascular injury, the contribution of other factors cannot be ignored. For example, using a similar mouse model of lung injury, D'Alessio et al. [53] identified a critical role for regulatory T cells (Tregs) in resolution of lung injury and showed that depletion/absence of Tregs prolonged LPS-induced proinflammatory responses, reduced neutrophil apoptosis and severely delayed recovery. Considering that hyperthermia and HSPs both greatly modify T cell behaviour [54–57], the contribution of dysregulated Treg mechanisms in lung injury at FRH could not be negated. However, few studies have focused on this aspect of hyperthermia and inflammation.

FRH exposure greatly increased neutrophil infiltration in both the pulmonary oxygen toxicity and intratracheal LPS instillation models [39,52]. In fact, the studies showed that exposure to FRH augmented multiple steps required for neutrophil delivery to sites of infection and injury, including induction of G-CSF expression and expansion of the circulating neutrophil pool [58], increased generation of the CXC chemokine family of endogenous chemotaxins [39,49], and increased capacity for chemokine-directed transendothelial migration (TEM) of neutrophils [59,60]. Furthermore, adoptive transfer of fluorescently labelled neutrophils between normothermic and hyperthermic neutrophil donors and recipients demonstrated that enhanced neutrophil migration capacity required FRH exposure of both the donors and recipients indicating that FRH augments neutrophil transmigration capacity through interdependent effects on both the neutrophils and the vascular endothelia [59].

In addition to augmented neutrophil accumulation in lung, FRH co-exposure had two additional effects in the LPS-challenged mouse lung that are also characteristic of human acute respiratory distress syndrome (ARDS), endothelial hyperpermeability and epithelial injury. In the mouse intratracheal LPS instillation model, exposure to FRH caused extensive epithelial injury [39], and Lipke et al. [50,51] showed it to be caused by augmented TNFα- and fas-dependent apoptosis. Using human neutrophils and the mouse MLE15 lung epithelial cell line, we found that exposure to 39.5 °C greatly accelerates activation of all three initiator caspases, caspase-2, 8, and 10, with evidence of activation as early as 60 min after stimulation with TNFα or agonistic anti-fas antibody (and within 30 min if treated at 42 °C) [61,62]. Accelerated and augmented apoptosis in the FRH-exposed and TNFα- or anti-fas-treated cells was partially blocked by inhibition of all three initiator caspases, did not require HSF1, and still occurred even when NFκB activation was independently blocked by expression of the IκBα super-repressor [62].

The Evans and Repasky laboratories have shown many of the same effects of FRH on cytokine gene expression [63,64], but have extended their studies of FRH effects to lymphocyte trafficking [65–67]. Utilising intravital microscopy to analyse lymphocyte trafficking in high endothelial venules in mice, the Evans laboratory has shown that exposing lymphocytes to FRH enhances their L-selectin- and alpha4beta7 integrin-dependent binding to high endothelial venules that increased their trafficking to secondary lymphoid tissue [65,66] and the enhanced migration is mediated through the IL-6-dependent

endothelial expression of ICAM-1 [57]. The consequences for the FRH-enhanced lymphocyte recruitment in infections and inflammatory disease have not yet been demonstrated experimentally. Recently, Lee et al. [63] showed a unique effect of FRH on activation and reprogramming of macrophages whereby FRH exposure causes a transitory reduction in endotoxin tolerance behaviour in vivo, increases inflammatory macrophage recruitment and maintains a sustained responsiveness to LPS.

Collectively, these studies demonstrate that exposure to hyperthermia achieved during febrile illness has many effects on gene expression, cell signalling, and cell behaviour that includes leucocyte and macrophage recruitment, opening of endothelial paracellular pathways to macromolecules and enhance extrinsic apoptosis in epithelium. These effects can be both beneficial and harmful, and the consequence for host survival and recovery depend on the nature of the pathological process.

HSF1: the central mediator

Heat-inducible HSF, including mammalian HSF1, was originally identified as a stress-activated transcriptional activator of HSP genes. However, evidence for the participation of HSF in more diverse processes such as innate immunity in *Caenorhabditis elegans* [68] and extra-embryonic development [69] suggest a much broader range of biological functions than previously thought.

The concept that HSF1 might have additional functions was initially suggested by Westwood et al. [70] who used *in situ* hybridisation analysis to show that HS stimulated the recruitment of HSF to 150 distinct chromosomal loci in *Drosophila* salivary gland polytene chromosomes, far more than could be accounted for by the known HSP genes. These observations were subsequently complemented by Trinklein et al. [71] who used a combination of chromatin immunoprecipitation and human promoter microarray analyses to show recruitment of HSF1 to multiple non-HSP genes in human K562 cells. Our own *in silico* analysis of CXC chemokine genes showed that the promoter regions of almost all mouse and human CXC chemokine genes contained multiple potential HSE consensus sequences [72]. We subsequently showed that some of the putative HSEs recruited HSF1 *in vivo* and that some of these functioned as a transcriptional activator, some as a repressor and some were functionally silent [73,74]. Additional studies, using cDNA microarrays to analyse the gene expression pattern activated by HS confirmed that exposure to HS also modifies expression of several non-*hsp* genes, including those involved in regulation of transcription, growth, DNA repair, apoptosis, signalling, and cytoskeletal function [75–77]. More recently, Mendillo et al. [78] showed that HSF1 was activated under basal conditions in cancers with high tumorigenic and metastatic potential but not in other cancers. Using high throughput ChIP-sequencing, they showed that HSF1 was recruited to about 500 genes many of which are distinct from those induced by HS and some of which are down-regulated by HSF1.

Studies with the HSF1 knock-out mouse also confirmed HSF1 as the major regulator of the heat/stress response [9], but also demonstrated its participation in the regulation of extra-embryonic development, growth, and endotoxaemia-induced systemic inflammation [69], female [69,79] and male [80] reproductive potential, the ubiquitin proteolytic pathway [81], post-natal brain development [82], in the maintenance of olfactory epithelium and in ciliary beating in the respiratory epithelium, ependymal cells, oviduct, and the trachea [83,84], and a potent promoter of tumorigenesis [85].

Gene-specific studies by our laboratory and by Stuart Calderwood's laboratory have shown that HSF1 can modify the expression of various cytokines, chemokines and acute response genes. The Calderwood group showed that following HS, HSF1 mediates transcriptional repression of human pro-interleukin-1β, c-fms, and c-fos genes [86–89] through quenching of participating trans-activating factors, most notably NF-IL6/c/EBPβ. In our studies we found that HSF1 was activated at febrile-range temperatures (39.5 °C) and mediated the repression of TNFα gene expression by interacting with a putative HSE sequence present in the mouse TNFα 5'-untranslated region [33,90,91]. Interestingly, we found that exposure to febrile-range temperatures also represses TNFα gene expression by selectively blocking recruitment of NFκB and Sp1 to the TNFα proximal promoter sequence [92,93]. In further support of HSF1 as a negative regulator of TNFα expression, HSF1-null mice exhibit higher circulating levels of TNFα expression after intraperitoneal challenge with LPS [69] and higher levels of TNFα in lung lavage after intratracheal LPS challenge [93]. In addition, activated HSF1 has also been found to repress human CXCL5 [73] and the pro-apoptotic factor, xIAP-associated factor 1 [94].

Regarding HSF1-mediated induction of non-HSP genes, we analysed the effect of HS on the expression of interleukin IL-8 [74]. HS enhanced TNFα-induced IL-8 secretion in human A549 epithelial cells but unlike classic HSPs, HS alone was not sufficient to activate IL-8 expression. Using EMSA and ChIP, we identified two IL-8 promoter regions, 800 and 1200 nt upstream of the transcription start site, that bound active HSF1 and, using a 5'-deletion mapping strategy and siRNA knockdown of HSF1, we showed that the interaction of HSF1 with both promoter regions contributed to the increased IL-8 expression in cells co-treated with TNFα and HS. Goldring et al. [95] found that activated HSF1 has a similar co-activator function for the murine iNOS gene. Inouye et al. [96] found that HSF1 constitutively bound to IL-6-associated chromatin in unstressed peritoneal macrophages and fibroblasts and enhanced LPS-induced IL-6 expression by modifying the chromatin accessibility of other transcription factors. The Santoro lab reported two new non-classical HS genes, cyclooxygenase-2 and the zinc finger AN1-type domain-2a gene (AIRAP) that exhibit HS-inducible transcription similar to those of canonical HSPs [97,98].

HSF1 may also exert additional biological effects by binding to and modifying function of proteins involved in diverse cellular processes, including HSPs [11], the nuclear pore-forming TPR protein through which Hsp72 is secreted [99], the catalytic subunit of the DNA-dependent protein kinase [100], other transcription factors [86,101,102], components of the TFIIB transcription complex [103], the cell

division cycle protein, Cdc20 [104], the apoptosis modulator DAXX [105], and the multidrug exporter, RalBP [106]. Collectively, these studies illustrate the broad range of important biological functions of HSF1 and underscore the potential importance and impact of altered HSF1 expression levels and genetic variations in the host cell.

HSF1 genetic variations and potential consequences

As discussed in the previous sections, HSF1 regulates expression of a broad range of genes, including those involved in host defence, inflammation and tumorigenesis as well as exerting additional effects by directly binding to proteins critical for cell proliferation, survival, and death. Studies from heterozygous mice suggest that the level of HSF1 expression may affect capacity for expression of some chemokines [74] and modify risk of tumour progression [85]. Considering the central participation of HSF1 in so many important biological functions, it is surprising that so little is known about genetic variations in elements of the human HS response, especially HSF1, and the potential impact on human health and disease.

Although single-nucleotide polymorphisms (SNPs) have been identified in various HSP genes, few studies have focused on the HSF1 molecule. Recently, Li et al. [107] reported two novel SNPs in the HSF1 gene that are disproportionately associated with thermal tolerance in Chinese Holstein cattle, including one 3'UTR SNP that disrupts a potential microRNA binding sequence in HSF1. However, the occurrence of SNPs in the human HSF1 gene has not yet been systematically analysed. To begin to understand the potential biological and clinical importance of SNPs in the HSF1 gene we analysed the human HSF1 gene for SNPs by mining the NCBI dbSNP database and performing exonic sequencing from anonymous genomic DNA samples. DNA was isolated from 30 healthy Caucasians and 30 healthy African American volunteers, exons amplified by PCR, and bidirectional sequencing performed and each sequence was compared with a reference human HSF1 sequence (NT_037704). Mining the dbSNP database revealed six SNPs (three in the 3′UTR and three in the coding sequence). One of the coding SNPs caused a proline-to-threonine missense at amino acid 365 adjacent to LZ3 and one caused a frame-shift replacement of the 26-amino acid C-terminal transactivation domain. Direct sequencing confirmed the P365T SNP and identified two novel 5'UTR and two novel 3′UTR SNPs [108]. Four of the five 3′UTR SNPs alter predicted miRNA target sequences as identified using the MicroSNiPer online program [109] and both of the 5′UTR SNPs alter the 5'UTR secondary structure predicted using the RNAFold online program [110]. The frequency of these and potentially other HSF1 SNPs and their participation in disease pathophysiology are not yet known.

HS response during infection and sepsis

Modifications in HSF1 activation and HSP expression have been demonstrated in clinical studies of human infections and in experimental infections in animals (Table II). The clinical studies of HS response in human sepsis are small and utilise different methods but generally show that HSP expression is higher in patients with sepsis. Hashiguchi et al. [111] analysed Hsp27, 60, 72 and 90 expression levels in blood neutrophils using mean fluorescence intensity from flow cytometry in 21 patients with early sepsis and 14 healthy controls. They found that neutrophils from the patients with sepsis had higher levels of all four HSPs compared with the control subjects. Similarly, Delogu et al. [112] compared the proportion of peripheral blood mononuclear cells expressing Hsp72 by flow cytometry to be almost 4-fold higher in patients with sepsis than in healthy controls. Other studies showed that levels of cell-free Hsp72 in serum were also elevated in patients with sepsis, including children with septic shock [113–115].

Most studies of animal models of infections also demonstrate increased expression of host HSPs associated with the infection. In mice infected with *Francisella tularensis*, the pathogen causing tularaemia, peritoneal macrophages exhibited increased Hsp72 levels but not until day three of the

Table II. HSF1 activation and HSP expression in infections and injury.

Effect	Clinical condition	Reference
Clinical studies		
Increased Hsp27, 60, 72, 90 in neutrophils	Sepsis	[111]
Increased Hsp72 in PBMC	Sepsis	[112]
Increased serum HSP72	Acute infections, septic shock	[113,114]
Increased Hsp32, 72, 90	PBMCs from patients with inflammation	[115]
Activation of HSF1 HSE-binding in PBMC	Human pancreatitis	[124]
Increased Hsp32, 72, 90 in PBMC	Systemic inflammation	[115]
Increased Hsp72 in cardiac tissue	Post-cardiac surgery	[126,127]
Animal models	Stimulus	
Increased Hsp72 in peritoneal macrophages	*F. tularensis*	[116]
Increased Hsp25, 60, 72 expression in spleen, liver, and muscle	*Trichinella*-infected rats	[117]
Reduced Hsp25, 72 levels in lung	Mouse CLP	[118]
No change in Hsp72 levels in lung	Mouse CLP	[119]
Hsp72, 25 expression and activation of HSF1 HSE-binding in pancreas	Mouse model of cerulean-induced pancreatitis	[125]
Increased Hsp72 in lung	Mouse undergoing surgery (sham CLP)	[119]
Cell culture models	Stimulus	
Increased Hsp90, 25	EBV-infected human B lymphocytes	[121]
Intranuclear Hsp60, 72 expression	RSV-infected A549 cells	[122]
Hsp72 expression	Adenoviral-infected B16 melanoma	[123]
Hsp72 expression	*E. coli*, *S. aureus*-infected human neutrophils	[124]

CLP, cecal ligation and puncture; EBV, Epstein-Barr virus; PBMCs, peripheral blood mononuclear cells; RSV, respiratory syncytial virus.

infection [116]. *Trichinella* infection in rats is associated with increased levels of Hsp25, 60, and 72 protein in spleen and brain, increased Hsp25 protein levels in liver, and increased Hsp25 and 60 levels in muscle [117]. On the other hand, Singleton et al. [118] reported reduced Hsp25 and 72 expression in lung 24 h after sepsis induced by cecal ligation and puncture. Weiss et al. [119] reported that Hsp72 mRNA and protein levels in lung did not increase up to 48 h after cecal ligation and puncture in mice. In the colonic epithelium of mice, steady-state levels of Hsp72 and 25 are constitutively maintained by commensal bacteria through MyD88-mediated TLR signalling and these HSPs play a crucial role in the maintenance of intestinal epithelial homeostasis [120]. Collectively, these data suggest the observed effect of infections on HSP expression may depend on timing relative to infection, the type of infection, and the tissue studied.

HSP induction is not limited to bacterial infections as several viruses also induce HSP expression in target cells, including Epstein-Barr virus in human B lymphocytes [121], respiratory syncitial virus in A549 cells [122], and adenovirus in B16 melanoma cells [123]. HS response activation has also been found to occur in non-infectious inflammatory disorders, including human pancreatitis in which HSF1 was found to be activated [124] and a mouse model of cerulean-induced pancreatitis in which elevated pancreatic levels of Hsp72 and 25 protein and activated HSF1 were detected [125]. In their study of HSP expression in the mouse cecal ligation and puncture, Weiss et al. [119] found elevated Hsp72 mRNA and protein levels in lungs of mice undergoing sham cecal ligation and puncture in which an abdominal incision was made. These results indicate that the stress of surgery might be sufficient to activate the HS response in mice as has been shown to occur in human cardiac surgery in which cardiac expression of Hsp72 was detected post-operatively [126,127].

Extracellular HSPs

Within the past decade HSPs have been shown to have additional cellular functions directly related to inflammation and the innate immune response. HSPs, particularly Hsp70, have been detected in the extracellular milieu and have been reported to be pro-inflammatory agonists for TLR2 and TLR4 [128–130]. Although some earlier studies raised concerns that the TLR4 agonist activity of recombinant Hsp70 preparations was caused by LPS contamination [131,132], subsequent studies showing activity in recombinant Hsp70 generated in insect cells and non-recombinant Hsp70 as well as classic LPS controls support proinflammatory TLR4 agonist and macrophage activating activities of Hsp70 protein itself [133–137]. In contrast to these reports, there is also strong evidence suggesting a potent anti-inflammatory role of HSPs that includes regulation of T cell responses, reducing stimulatory capacity of dendritic cells, and inducing development of immunosuppressive Treg cells [138–141]. While the exact role of exogenous/extracellular HSPs is still debated, it is likely that HSPs can stimulate both innate and adaptive immune responses, at least in the context of infection and fever. This might help explain, at least in part, the molecular mechanisms by which fever and hyperthermia modify host responses in the face of severe infection and how dysregulated responses could lead to severe sepsis and multi-organ dysfunction.

Not only the functional role of extracellular HSPs but also its release mechanism is poorly understood. HSPs lack a classical consensus signal required for secretion and HSP secretion is not blocked by typical inhibitors of the endoplasmic reticulum–Golgi pathway, such as brefeldin A [142]. In the initial reports by Hightower and Guidon [143] and later by Hunter-Lavin et al. [142], both groups showed that Hsp70 release occurred from healthy uninjured cells independent of cell death. Basu et al. [144] showed that bioactive Hsp70 was also released from necrotic cells but not from cells undergoing apoptosis, and Mambula et al. [145] showed that Hsp70 was released from prostate cancer cells via both necrosis and active secretion. Collectively, these studies suggest that Hsp70 is released actively by a non-classical secretory pathway and passively as a result of cellular necrosis but not apoptosis. To account for its active release in the absence of a leader sequence, several mechanisms have been proposed, including release by secretory-like granules [146], via membrane export vesicles [135] and via a lysosome–endosome pathway, where Hsp70 translocates into lysosomes via an ATP binding cassette (ABC) transport-like system and is then exported from the cell via the endocytic process [147,148].

HS response and TLR agonists

While HSPs can activate TLR signalling, recent *in vitro* and *in vivo* studies suggest that TLR agonists, particularly LPS, can activate expression of HSPs in mammalian cells. Edelman et al. [149] reported that LPS activated expression of Hsp60 and 70 in isolated rat lung pericytes at 37 °C, but the increase was modest, only 20 to 40%, and required 18-h incubation with LPS. Similarly, Hirsh and coworkers [150] showed that *in vitro* treatment of human neutrophils with LPS at 37 °C stimulated a rapid increase in the percentage of cells with detectable intracellular and surface expression of Hsp60 and 70 as detected by flow cytometry, but the increase in HSP protein expression level was not reported. Administration of LPS to rats *in vivo* caused up-regulation of Hsp72 in splenocytes [151,152] and increased levels of Hsp70 mRNA in lung and liver compared with rats previously subjected to an endotoxin tolerance protocol [153].

The release of Hsp60 and 70 into the circulation during infection, inflammation, and trauma combined with its TLR agonist activity might suggest a positive feedback mechanism that could amplify inflammation. Recently, we found that co-exposure to TLR agonists synergises with exposure to febrile temperatures to greatly augment Hsp70 synthesis and secretion in the RAW 264.7 mouse macrophage cell line [40]. The increase in HSP expression is mediated through a p38 MAP kinase-dependent signalling pathway leading to increased histone H3 phosphorylation and HSF1 recruitment to the Hsp70 chromatin. The mechanism by which co-exposure to TLR agonists and febrile temperatures increases Hsp70 secretion is not yet known, but occurred without evidence of cytotoxicity. Similar synergism between TLR agonists and

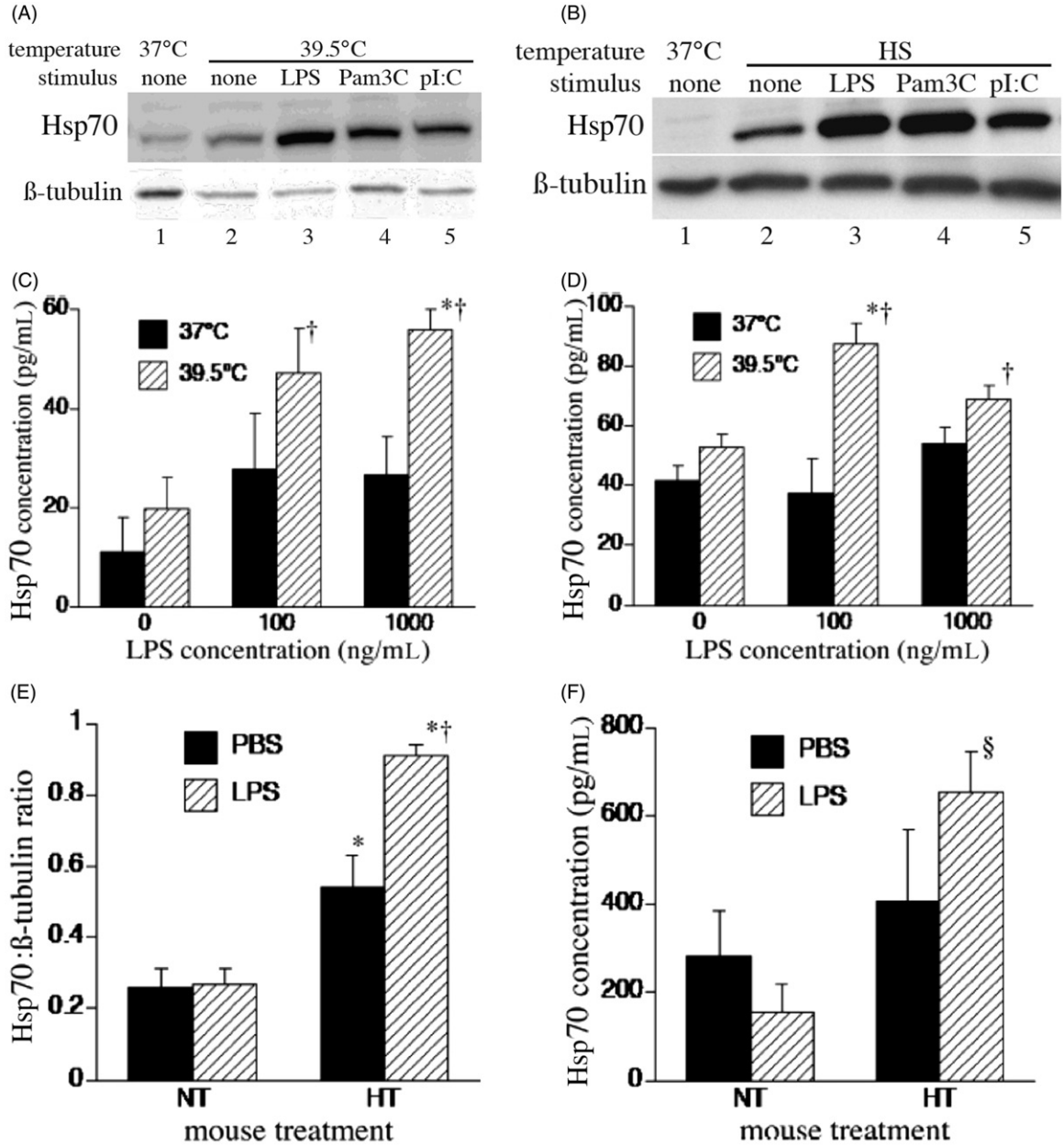

Figure 2. TLR agonists augment Hsp70 expression and release. A and B: RAW cells were incubated with 100 ng/mL LPS, 0.5 μg/mL Pam3CSK4 (Pam3C) or 12.5 μg/mL poly(IC) (pI:C) at 39.5 °C for 6 h (A), or were heat shocked at 42 °C for 2 h, recovered at 37 °C for 4 h (B), lysed, and immunoblotted for Hsp70 and β-tubulin. Lane 1 is the untreated 37 °C control. C and D: RAW cells were incubated with 0, 100, or 1000 ng/mL LPS at 37 or 39.5 °C for 6 h (C) or 24 h (D). Cell culture supernatants were collected and cleared by centrifugation, and Hsp70 was quantified by ELISA and presented as pg/mL. Data presented as the means ± SE ($n = 4$). * and † denote $p < 0.05$ versus similarly treated 37 °C cells and 39.5 °C cells with no LPS or hyperthermia-exposed cells, respectively. E and F: Mice implanted with intraperitoneal thermistors were housed at either 25 °C (normothermic, NT) or 36–37 °C (hyperthermic, HT) ambient temperature. For LPS exposure, mice were intratracheally instilled with LPS or sterile phosphate buffered saline (PBS) (control) and housed under normothermic or hyperthermic conditions for 24 h. The lungs were excised, and the homogenates were immunoblotted for Hsp70 and expressed as a ratio to β-actin (E), or lungs were lavaged and Hsp70 quantified by ELISA in the lavage fluid (F). Data are presented as means ± SE ($n = 4$). *, †, and § denote $p < 0.05$ versus PBS-treated NT controls, PBS-treated HT mice, and LPS-treated NT mice, respectively. This research was originally published in the *Journal of Biological Chemistry* [40]. © The American Society for Biochemistry and Molecular Biology.

febrile temperatures for Hsp70 expression and secretion were seen in IL-1β-stimulated human A549 cells *in vitro* and in an intratracheal LPS-challenge mouse model of acute lung injury *in vivo* (Figure 2) [40]. In the latter model, the combination of FRH and intratracheal LPS stimulated an increase in Hsp70 protein levels in lung homogenates and in cell-free lung lavage fluid. Considering the pyrogenic action of TLR agonists, including Hsp70, we propose that the synergism between fever and TLR agonists for synthesis and release of Hsp70 promotes a vicious proinflammatory cycle that may contribute to the negative consequences of fever in high acuity disease (Figure 3).

Conclusions

In the present review we have discussed how the host's febrile response share components of the HS pathway to generate an

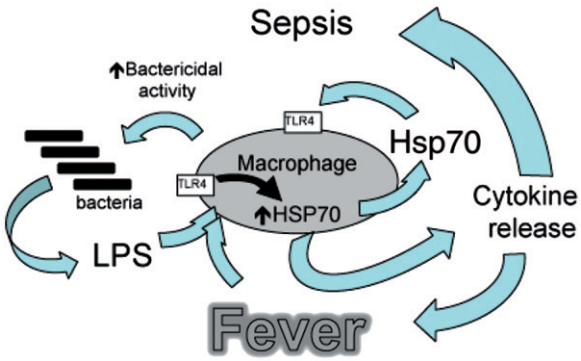

Figure 3. Model of how fever, inflammatory agonists, and Hsp70 interact to cause sepsis. Proposed model of sepsis in which LPS and fever initiate a positive feedback pathway through enhanced Hsp70 expression and release, and subsequent increased TLR activation, Hsp70 expression, and proinflammatory cytokine release. This research was originally published in the *Journal of Biological Chemistry* [40], © the American Society for Biochemistry and Molecular Biology.

optimal host defence during inflammation, infection and injury. It is evident that the three components of the HS response pathway, namely the stressor (temperature), the central activator (HSF1), and the final product, HSPs, have all evolved to perform additional functions beyond the typical cellular stress response. All three components have strong immunmodulatory effects that include mobilisation of immune cells, regulation of proinflammatory cytokine/chemokine gene expression and activation of both pro- and anti-inflammatory pathways. Interestingly, the regulation is mutual between HS response and the inflammatory pathways and reciprocated by proinflammatory agents as well, which either augment the temperature effect or directly activate the HSP gene transcription programme. It is apparent therefore, that a better understanding of the complex interaction between the HS response and the inflammatory pathway is critical not only for conditions where both pathways are activated such as infection, sepsis and multi-organ dysfunction but also for optimal exploitation of thermotolerance and therapeutic hyperthermia, where dysregulated inflammatory signalling could severely compromise the efficiency and final outcome of hyperthermia therapy.

Declaration of interest

This work was supported by US National Institutes of Health grants GM069431 (ISS) and GM066855, HL69057 and HL085256 (J.D.H.), and by Veterans Association Merit Review grants (J.D.H. and I.S.S.). The authors alone are responsible for the content and writing of the paper.

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