Fever, Immunity, and Molecular Adaptations

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ABSTRACT

The heat shock response (HSR) is an ancient and highly conserved process that is essential for coping with environmental stresses, including extremes of temperature. Fever is a more recently evolved response, during which organisms temporarily subject themselves to thermal stress in the face of infections. We review the phylogenetically conserved mechanisms that regulate fever and discuss the effects that febrile-range temperatures have on multiple biological processes involved in host defense and cell death and survival, including the HSR and its implications for patients with severe sepsis, trauma, and other acute systemic inflammatory states. Heat shock factor-1, a heat-induced transcriptional enhancer is not only the central regulator of the HSR but also regulates expression of pivotal cytokines and early response genes. Febrile-range temperatures exert additional immunomodulatory effects by activating mitogen-activated protein kinase cascades and accelerating apoptosis in some cell types. This results in accelerated pathogen clearance, but increased collateral tissue injury, thus the net effect of exposure to febrile range temperature depends in part on the site and nature of the pathologic process and the specific treatment provided. © 2014 American Physiological Society. *Compr Physiol* 4:109-148, 2014.

Introduction

The temperature of every organism is determined by a balance between heat gain and heat loss. Heat is generated by metabolic activity and, depending on ambient temperature, it can be either gained from or lost to the environment. Heat is eliminated through combinations of radiant, evaporative, convective, and conductance heat loss. All animals have a preferred temperature range that is relatively stable during homeostasis. However, in response to infection and injury many animals temporarily increase body temperature 2 to 4°C by altering the balance between heat generation and elimination (136). Some animals also intentionally reduce core temperature during hibernation (110). In addition, most homeothermic animals, including humans, exhibit circadian fluctuations in core temperature associated with sleep-wake cycles (247). These processes are all examples of regulated temperature changes. When heat gain or loss exceeds an organism's capacity to compensate, the resulting temperature change is unregulated, overwhelming its thermoregulatory capacity. Since many of the cell physiologic and immunologic effects of fever are caused by the temperature increase itself (99, 135, 353, 360), unregulated hyperthermia and fever may have similar biological effects and consequences.

Fever is recognized as a component of the mammalian and avian acute-phase response to infection. However, many poikilothermic animals, including lower vertebrates (30, 70, 320), arthropods (35, 45), and annelids (46), increase their core temperature in response to infection or injury, predominantly by seeking a warmer environment (193). The presence of fever in such diverse modern animals suggests that increasing core temperature in response to infection and injury is an evolutionarily conserved strategy for survival. The conserved nature of this strategy not only suggests that the increase in core temperature is generally beneficial but also that components of the host response to infection and injury that coevolved with the febrile response will be regulated by the temperature changes. The evolutionary aspects of fever are discussed later in this review.

The cellular stress response (CSR) is a program that allows cells to cope with endogenous and environmental stress (198). The CSR is comprised of multiple biochemical pathways that are activated by partially overlapping arrays of stresses, including mechanical, chemical, and physical stresses. Each pathway is activated by one or more stresses, is mediated by distinct transcription factors, and results in expression of unique sets of gene products that serve to maintain or reestablish homeostasis (358). The pathways that comprise the CSR are identified by either the nature of the stress or the cellular target of injury and include oxidative stress, DNA damage response, hypoxia, endoplasmic reticulum stress, metal stress, inflammation, osmotic stress, and the heat shock response (HSR) (358). Since fever and other hyperthermic states result in elevations in temperature, these conditions may result in activation of the HSR. The HSR is an ancient and highly conserved biological process that is not only essential for coping

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with extremes of temperature, but is also activated by other chemical and physical stresses, including toxic chemicals, and high levels of radiation, each of which can cause denaturation of essential cellular proteins. Based on the homology in heat shock protein (HSP) genes among modern day eukaryotes, prokaryotes, and archebacteria, the essential elements of the HSR likely emerged around 2.5 billion years ago (94). During exposure to high temperatures, the transcriptional and translational machinery of the cell is reprogrammed to preferentially express a set of stress-inducible HSPs. The HSPs interact with denatured proteins, either preserving them until the stress has ended or targeting the denatured protein for degradation and removal from the cell. While the HSR is a fail-safe mechanism for coping with unavoidable environmental stresses, fever is a complex physiologic response to infection or injury, during which organisms temporarily subject themselves to thermal stress that functions as a global regulator.

This review will focus on the mechanisms of mammalian fever from an evolutionary perspective; the interrelationship between fever and the HSR, host immune defenses, and inflammation; and the clinical consequences of exposure to febrile range temperature (FRT) in critically ill patients with severe infections, trauma, or other overwhelming inflammatory states. A list of abbreviations can be found in Table 1.

Mechanisms of Fever

Evolution of the strategy of fever during infection and injury

From an evolutionary perspective, fever arose as an additional host response to infection in the setting of an already established HSR in higher animals. Classically, fever is defined as a regulated elevation in core temperature that is achieved through the integrated behavioral, physiological, and biochemical processes that determine the balance between heat generation and elimination. This response is generally considered to be limited to homeothermic animals. However, if one broadens the definition of fever to any increase in core temperature stimulated by infection or injury and achieved solely by seeking external sources of heat, the prevalence of fever expands to include many poikilothermic vertebrates, arthropods, and annelids (190). The ability of the same antipyretic drugs that are effective in mammals to block the heat seeking behavior in infected fish (70, 320), reptiles (30), and some invertebrates (46) suggests that the mechanisms of fever in poikilothermic and homeothermic animals might be related. Starks *et al.* (376) made the interesting observation that honeybees increase hive-wide temperature following infection of the hive with the heat-sensitive pathogenic fungus *Ascosphaera apis*. This temperature increase is achieved through a communal increase in wing muscle activity by the adult bees. In this case, the febrile response has been adapted to a hive animal in which survival of the species depends on survival of the hive rather than viability of individuals.

The prevalence of fever in modern day members of the two major animal divisions, Deuterostomia (vertebrates) and Protostomia (arthropods and annelids), suggests that it must have first appeared approximately 600 millions years ago (Fig. 1), before the appearance of adaptive immunity but in the setting of established and well-conserved heat shock and innate immune responses. The evolutionary persistence of fever is even more remarkable when one considers its substantial metabolic cost. During fever in humans, increasing core temperature by shivering results in up to a sixfold increase in metabolic rate (159). Furthermore, for each 1°C increase in core temperature, human metabolic rate increases approximately 12% due to increased activity of most cellular pathways at higher temperature (229, 351). In poikilothermic animals with infections, a move to warmer environs not only requires increased energy expenditure, but may also expose vulnerable animals to attack by predators. Therefore, the increase in core temperature must confer benefits that generally outweigh these costs in the infected or injured host. In addition, as discussed later in this review, fever has incorporated elements of both the HSR and innate immune mechanisms into its regulation and downstream effects, providing further benefits to infected or injured animals.

The system that regulates body temperature comprises a network of temperature sensors, a central controller, and multiple heating and cooling effector mechanisms. Poikilothermic animals rely predominantly on locomotion to regulate temperature by moving to a desired ambient temperature. For many poikilothermic animals, this also includes a febrile response to infection and injury achieved by temporarily increasing preferred ambient temperature. As mentioned previously, much of the early work on fever behavior in poikilothermic animals was performed with the desert iguana, goldfish, the bluegill sunfish, leeches, and grasshoppers (30, 35, 45, 46, 70, 320). Interestingly, the heat seeking behavior in infected desert iguana is blocked by the antipyretic drug, sodium salicylate (416) and in bluefill sunfish and leeches by acetaminophen (46, 320). These drugs exert potent antipyretic effects in mammals by blocking generation of the pyrogenic mediator, prostaglandin (PG)E_2. These studies suggest that despite the limited capacity of thermoregulatory effector mechanisms used by poikilothermic animals, the temperature controller in these animals utilizes the same or similar PGE_2-responsive mechanism as homeothermic animals. Likewise, the thermal sensing molecules that transduce temperature signals in somatosensory afferent nerve endings are evolutionarily conserved (166) as is discussed further below. Thus, the major advance in thermoregulation in homeothermic vertebrates, including humans, appears to be an expansion in the number and sophistication of thermoregulatory effector mechanisms.

Mechanisms of mammalian thermoregulation

In the following sections, we will discuss each of the following components of mammalian thermoregulation: (1) sensory afferent pathways; (2) central integration and the

Table 1 Abbreviations

15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
3′UTR	3′-untranslated region
AIRAP	Arsenite-inducible RNA-associated protein
AP5	DL-2-amino-5- phosphonopentanoic acid
BAT	Brown adipose tissue
CaM	Calmodulin
Cdc	Cell cycle division protein
CHIP	Carboxy-terminus-of-HSC-70-interacting-protein
ChIP	Chromatin immunoprecipitation
ChIPseq	Chromatin immunoprecipitation sequencing
CKII	Casein kinase II
COX	Cyclooxygenase
DAXX	Death-associated protein-6, death-domain associated protein
DMH	Dorsomedial hypothalamus
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal-related kinases
FGF	Fibroblast growth factor
FRH	Febrile-range hyperthermia
FRT	Febrile-range temperature
G-CSF	Granulocyte-colony stimulating factor
GSK-3ß	Glycogen synthase kinase-3ß
GWAS	Genome-wide association study
HMGB1	High mobility group box-1
HSR	Heat shock response
HSE	Heat shock response element
HSF	Heat shock factor
HSP	Heat shock protein
HSR1	Heat shock RNA 1
IAP	Inhibitor of apoptosis protein
IL	Interleukin
IFN	Interferon
iNOS	Inducible nitric oxide synthase (NOS-2)
ICAM	Intercellular adhesion molecule
JNK	c-Jun NH2-terminal kinase
LBPd	Dorsal subnucleus of the LBP
LBPel	Lateral external nucleus of the LBP
LPB	Lateral parabrachial
LPS	lipopolysaccharide
LZ	Leucine zipper
MAP kinase	Mitogen-activated kinase
MAPKAPK2	MAP kinase-activated protein kinase-2
MIF	Macrophage migration inhibitory factor
MnPO	Median preoptic nucleus
mPGES	Microsomal PGE synthase
MPO	Medial preoptic area
NF	Nuclear factor
NRMC	Nucleus reticularis magnocellularis
OAT	Organic anion transporter
PDK-1	PI3K-dependent kinase-1
PG	Prostaglandin
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase-C
PLA_2	Phospholipase-A_2
PLK1	Polo-like kinase-1
POA	Preoptic area
RalBp	Ral-binding protein
rhIL-1ß	Recombinant human IL-1ß
RM	Raphe magnus
rMR	Rostral medullary raphe region
Rp-cAMPS	5′- cyclic monophosphorothioate-Rp isomer
RSK	Ribosomal S6 kinase
SCN	Suprachiasmatic nucleus
SIRS	Systemic inflammatory response syndrome
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
TAD	Transactivation domain
TAK1	TGF-ß-activated kinase
TBP	TATA-binding protein
TEM	Transendothelial migration
TF	Transcription factor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-α
TRP	Transient receptor potential
VMPO	Ventromedial preoptic region
XAF1	xIAP-associated factor-1

hypothalamic set point; (3) command efferent pathways; (4) how the system operates during homeostasis; and (5) the mechanism for generating fever.

Temperature-sensing afferent pathways

Types of thermal sensing: Thermal signal processing serves several purposes. Nociceptive responses to extreme temperatures elicit pain and a withdrawal response that limits injury of the exposed tissue. Conscious perception of more moderate temperatures requires higher order processing of thermal

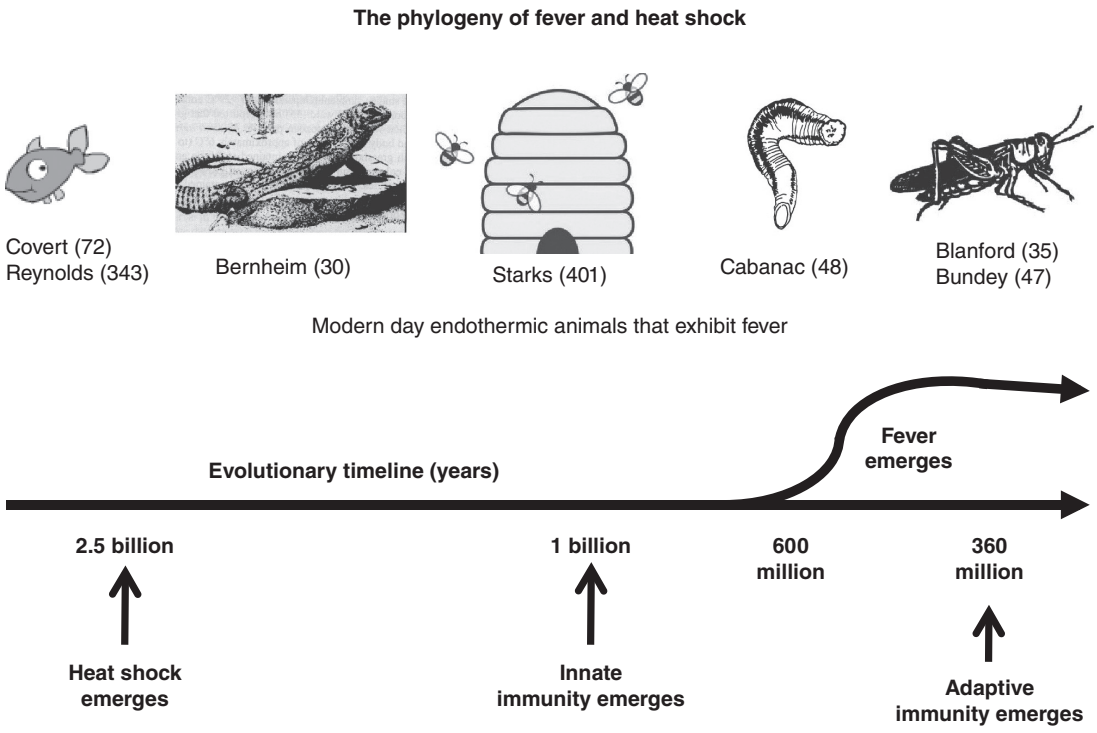

Figure 1 Phylogenetic relationship of fever, heat shock, and innate and adaptive immunity. The modern day animals that utilize fever as a strategy for coping with infection are pictorially displayed with supporting references. The approximate phylogenetic ages of fever (600 million years) and related biological processes are shown.

signals within the cerebral cortex. A third type of thermal processing regulates body temperature through activation of involuntary effector mechanisms of heat generation, conservation, and elimination that confers an overall sensation of being too hot or cold, thereby encouraging relocation to a more suitable ambient temperature. The first two types of sensing are restricted to thermal signals originating from cutaneous and mucus membrane neurons. Thermoregulatory processing integrates thermal signals from the body surface as well as deeper tissues such as intraabdominal viscera, spinal cord, and brain. This review will focus on the neurologic processes involved in thermoregulation and generation of fever.

Thermal sensing neurons: Thermal sensing neurons contain temperature-dependent cation channels that are tuned to specific temperature ranges. The temperature-responsive cation channels expressed on peripheral thermal afferents belong to the transient receptor potential (TRP) superfamily of integral membrane proteins that function as ion channels (295). Members of the TRP superfamily are highly conserved in yeast, invertebrates, and vertebrates highlighting their importance for detecting, avoiding, and surviving environmental stresses. TRP channel subunits consist of six transmembrane spanning segments, a pore-forming loop between fifth and sixth transmembrane segment, and intracellular amino- and carboxy- termini. These molecules homo- or heterotetramerize to form cation-selective channels. The TRP family is divided into seven subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin), and TRPN (NOMPC-like); however, the latter is found only in invertebrates and fish. TRP ion channels are widely expressed in many different tissue and cell types and are involved in diverse physiological processes, including sensing a broad range of environmental stimuli (166). Mammals express 28 TRP channels, about twice as many as are expressed in insects due to a disproportionate increase in the number of mammalian TRPV and TRPM channels (233). Out of the 28 mammalian TRP channels, 9 family members are temperature responsive with distinct thermal thresholds for activation (Table 2). Seven of these TRPs (TRPV1-4; TRPM2, 4, and 5) are heat-responsive, and two, TRPM8 and TRPA1, are cold-responsive (166, 441). Most TRPs, including TRPV1-4, TRPM2 and 8, and TRPA1 are nonselective cation channels, but some, including TRPV5 and 6, are highly Ca^{2+} selective. TRP channels are activated and regulated through strikingly diverse mechanisms, making them suitable candidates for cellular sensors. They respond to environmental stimuli such as temperature, pH, osmolarity, mechanical stress, pheromones, taste, and plant compounds, as well as intracellular stimuli such as Ca^{2+} and phosphatidylinositol signal transduction pathway components. Interestingly, insects utilize an expanded portfolio of TRPA family channels for thermal detection, indicating an evolutionary divergence from other metazoans (233).

This family of ion channels shows a variety of gating mechanisms, with activation triggers ranging from binding of soluble ligands to changes in temperature. Activating TRP

Table 2 Characteristics of the Mammalian Temperature-Responsive Transient Receptor Potential (TRP) Channels

Channel	Temperature range	Soluble agonists	Ion selectivity (P_{Ca}/P_{Na})[1]	Expression site
Warm-sensing				
TRPV1	≥43°C	Capsaicin, arachidonic acid, anandamide, allyl isothiocyanate (wasabi), low pH	4-10	DRG, TG, ND, keratinocytes, smooth muscle, neutrophils, widely in brain
TRPV2	≥52°C[2]	2-aminoethoxydiphenyl borate, cannabidiol	ND[2]	DRG, hypothalamus, lung, heart, macrophages
TRPV3	33-39°C	Bradykinin, histamine, ATP, PGE_2, oregano, thyme, clove, camphor	2-3	DRG, TG, keratinocytes, tongue, nose, testis, brain
TRPV4	27-34°C	Phorbol ester, 5,6 eposyeicosatrienoic acid, arachidonic acid bisandrographolide A	6-10	DRG, TG, hypothalamus, choroid plexus, skin, epithelium, lung enodothelium
TRPM2	34-37°C	Oxidative stress, ADP ribose	~1	Pancreatic ß-cells, brain, neutrophils, monocytes
TRPM4	[3]Relatively temperature-insensitive	None known	Calcium impermeable	Pancreatic ß-cells, heart, prostate, pancreas, placenta, spleen, liver, T cells, skeletal muscle
TRPM5	[3]Relatively temperature-insensitive	None known	Calcium impermeable	Pancreatic ß-cells, taste receptors, intestine, pancreas, pituitary
Cool-sensing				
TRPA1	<10°C	Mustard oil, allicin, diallyl disulfide, cinnamaldehyde, acrolein, chlorine etomidate	~1	
TRPM8	18-25°C	Menthol, icillin, eucalyptol, linalool, geraniol, hydroxyl-citronellal	1-3	DRG, TG, ND, bladder/lung epithelium, heart, vascular smooth muscle

[1]Ratio of calcium to sodium permeability.
[2]ND: Not determined.
[3]TRPV5, 6 and TRPM2 are relatively temperature-insensitive and do not likely participate in thermoregulation.

channels causes depolarization of the cellular membrane, which activates downstream voltage-dependent ion influx and results in a change of intracellular Ca^{2+} and Mg^{2+} concentration (166, 441). Chemical agonists and thermal activation may be additive or synergistic for activation of TRPV channels, thereby providing signal integration at the level of the afferent neuron. For example, topical application of menthol, the soluble agonist for the TRPM8 cold sensor, enhances the sensation of coolness caused by subsequent exposure to cool air. The response of these receptors to soluble agonists as well as physical perturbations is consistent with their likely evolutionary origins as nocioceptors.

Temperature-sensing neurons are distributed throughout surface and deeper tissues and express one or more TRP channels, which determine the temperature range at which they are activated to initiate signaling to the thermoregulatory center in the hypothalamus. The thermoregulatory center integrates thermal signals distributed over space and time to assess the direction and magnitude of the effector response needed to achieve and maintain target body temperature. While the mechanistic details of how thermal information from cutaneous, visceral, and central nervous sources is integrated by the thermoregulatory control center are not well understood, the influence of cutaneous thermal input on thermoregulatory responses is evident clinically (20, 390) and experimentally (269).

Cutaneous cold thermoreception: To maintain core temperature within a narrow range during homeostasis, the thermoregulatory mechanism has developed to primarily prevent rather than reverse core temperature deviations. Environmental temperature has direct and more immediate and profound effects on skin temperature than on the temperatures within the body core (39), thereby providing an advanced warning of impending deviation in core temperature from homeostatic norms. The feed forward information from these cutaneous thermal sensors triggers effector mechanisms that mitigate impending changes in core temperature. The mammalian thermoregulatory effector response to cold includes

shivering thermogenesis, cutaneous vasoconstriction, and a feeling of coolness that compels relocation to a warmer site.

Cutaneous cold sensing has been attributed to two TRP channels, TRPM8 and TRPA1 (166, 441). Of the two, the participation of TRPM8 channels in *in vivo* cold sensing has been more firmly established. Compared with wild-type mice, TRPM8-null mice exhibit reduced cold-avoidance behavior and a profound loss of cold sensitivity in unmyelinated cutaneous C fiber neurons as assessed by patch clamping (28). TRPM8 channels are activated by exposure to temperature below 26°C or by topical application of chemical agonists including menthol and icillin (237, 395). These receptors demonstrate a steep temperature response curve as temperature decreases from 25 to 18°C and a more shallow response curve with further temperature reduction to 10°C (40). TRPM8 activation initiates transmission of electrical impulses from the peripheral nerve endings via the somatosensory neurons that have their cell bodies in the dorsal root and trigeminal ganglia. Activation of these neurons result in a sensation of coolness and triggers warm seeking behavior, shivering thermogenesis and cutaneous vasoconstriction in mice (395). In contrast with TRPM8 channels, TRPA1 channels have a much lower temperature threshold (17°C) for activation than TRPM8 channels (383) and are expressed on only 3.6% of dorsal root ganglion neurons (383). Most importantly, TRPA1-null mice demonstrated no difference in the onset of shivering response to cold compared with wild type mice (200), but did have reduced paw withdrawal from more extreme cold (5-10°C) and from paw pressure (10, 454). These results suggest that TRPM8 is predominant cool sensing receptor involved in mammalian thermoregulation.

Cutaneous warm thermoreception: The pathway leading to cutaneous perception of warmth is initiated primarily by TRPV1-4 channels. These proteins exhibit partially overlapping temperature response thresholds spanning a temperature range from 25 to 53°C (124, 177, 402). TRPV channels are weakly Ca^{2+}-selective cation channels that are also activated by a wide variety of endogenous chemical stimuli including Ca^{2+}, calmodulin (CaM), ATP, and phosphoinositides (295) (see Table 2). TRPV1, also called the capsaicin receptor, forms a voltage-gated weakly Ca^{2+}-selective cation channel that is activated by exposure to temperatures >43°C, low pH (56, 177, 402), or capsaicin as well as by arachidonic acid and its immunomodulatory metabolites (165). TRPV1 is highly expressed in myelinated (Aδ) and unmyelinated (C) nociceptive fibers of dorsal root, trigeminal, and nodose ganglion neurons (55, 143) and may also be expressed in the brain (377). Treatment with TRPV1 antagonists induces hyperthermia in multiple species by increasing metabolism and reducing heat loss from the body surface (109, 389), suggesting that basal TRPV1 activity contributes to thermal homeostasis.

TRPV2 has 50% amino acid similarity with TRPV1, but it appears to only function as a heat nociceptor in some species with an activation threshold >53°C (54, 180, 276), but not in humans or mice (276). TRPV2-null mice exhibit reduced intrauterine growth and survival and deficient macrophage chemotaxis and phagocytosis, which increases susceptibility to intracellular pathogens such as *Listeria monocytogenes.* However, the TRPV2-null mice exhibit nociceptive thermal responses that are indistinguishable from wild-type mice (213, 297). These studies strongly suggest that TRPV2 does not participate significantly in normal thermoregulation.

Unlike TRPV1 and 2, TRPV3 is activated by exposure to temperatures in the clinically significant range (33-39°C) as well as by proinflammatory agents such as bradykinin, histamine, ATP, and PGE_2 and aromatics like oregano, thyme, clove, and camphor (162, 228, 448). TRPV3 activation is unusual in two respects. First, the currents exhibit autosensitization, a pattern of increasing action potential amplitude with sequential activation. Second, the temperature-dependent potentiation of TRPV3 exhibits a marked hysteresis across thermal activation-deactivation cycles so that its activation depends not only on the absolute temperature but also on recent trends in environmental temperature (449). Mice with a truncated mutant TRPV3 maintain normal body temperature but exhibit subtle defects in responsiveness to heat (248). The defects are characterized by a reduced preference for moderate warmth (35°C vs. room temperature) and a delayed avoidance of noxious heat (>50°C) compared with wild-type mice. TRPV3 is also expressed in mouse keratinocytes and its activation may affect skin phenotype. Mice that are hetero- and homozygous for a constitutively active mutant TRPV3 exhibit a mast cell-rich dermatitis and abnormal hair growth (16, 167), while TRPV3-null mice have wavy fur and curly whiskers (62). The consequences of TRPV3 activation in keratinocytes for thermoregulation are unknown.

TRPV4 is activated over a temperature range similar to that for TRPV3, but it is also activated by osmotic and mechanical stimuli. Interestingly, sensitivity of TRPV4 to these stimuli may depend on phospholipase A_2 activation and the subsequent production of arachidonic acid metabolites (98, 210, 384, 420), and therefore, may be a cellular target for antipyretic/analgesic drugs that target the PG synthesis pathway. Unlike TRPV3, TRPV4 is not only expressed on primary sensory neurons, it is widely distributed in the hypothalamus, skin, and osmoreceptive neurosensory cells around the ventricle, and in lung endothelium and may regulate endothelial permeability (124, 127, 210, 300). Despite having similar activation temperatures, TRPV3-null mice and TRPV4-null mice exhibit diametrically different defects in response to moderate warmth. Whereas TRPV3-null mice have reduced preference for a 35°C environment compared with room temperature (204, 248), TRPV4-null mice exhibit an increased preference for 34°C compared with 28°C environment (204, 248). However, healthy TRPV4-deficient mice exhibit a circadian core temperature pattern and ability to defend their core temperature in cold (4°C) and hot (35°C) environments that is indistinguishable from wild-type mice (204, 211). Based on channel responsiveness to temperatures encountered during homeostasis and disease and the effects of gene deletion on temperature preference, TRPV3 and 4 are the TRPV channels are most likely to participate in mammalian thermoregulation.

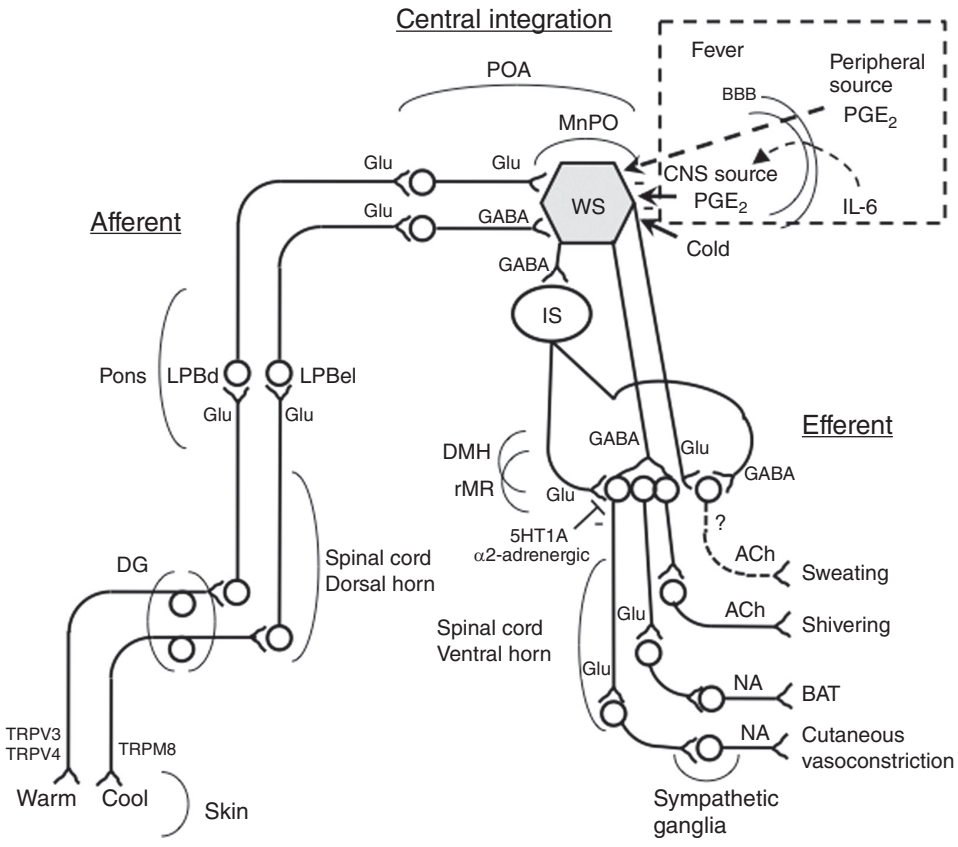

Figure 2 Schematic diagram of thermoregulatory pathway including mechanism of fever regulation. See text for full discussion. To simplify, we have excluded the spinothalamocortical pathway. Abbreviations: Ach, acetycholine; BAT, brown adipose tissue thermogenesis; BBB, blood:brain barrier; DG, dorsal ganglion; DMH, dorsomedial hypothalamus; GABA, gamma-amino butyric acid inhibitory synapse; glu, glutatergic excitatory synapse; IS, temperature-insensitive pacemaker neuron; LPBd, dorsal subnucleus of the lateral parabrachial nucleus; LBPel, lateral external subnucleus of the LBP; MnPO, median preoptic nucleus of the hypothalamus; NA, noradrenaline; POA, preoptic area of the hypothalamus; rMR, rostral medullary raphe region; TRP, transient receptor potential channel; WS, warm-sensing pacemaker neuron.

Pathways from cutaneous afferents to the central nervous system: The structure and function of the thermal sensing afferent pathway have been elucidated largely from nonhuman mammals using retrograde tracing studies and single neuron electrophysiologic monitoring to identify the neurologic pathways activated by different thermal and chemical stimuli (252, 261). Thermal signals are transduced by TRPs in the nonencapsulated nerve endings of cutaneous somatosensory neurons and transmitted through a three-neuron pathway to the brain (see Fig. 2). The primary cutaneous temperature-sensing neurons are unipolar with cell bodies located in the dorsal root and trigeminal ganglia. The primary somatosensory nerve fibers of these cells extend from nerve endings in the skin and synapse with the cell bodies of lamina I neurons located in spinal and trigeminal dorsal horns and can extend up to 1 m in humans. Because these nerve fibers are not fully myelinated they support relatively slow conduction rates. Thinly myelinated Aδ somatosensory fibers transmit signals related to sensations of cool, pressure, and quick shallow pain while unmyelinated C-fibers transmit signals activated by warmth, itch, and noxious heat and cold (344). Each primary somatosensory nerve ending detects the temperature of a discrete surrounding area called the receptive field. A higher density of somatosensory nerve endings increases spatial acuity and possibly thermal sensitivity (183, 382), but the consequences of this for thermoregulation are unclear.

Thermal signals from dorsal horn lamina I neurons reach the brain through one of two pathways. They can synapse with third order neurons in the thalamus, which project to the somatosensory cortex, or they can synapse with third order neurons in the lateral parabrachial (LPB) nucleus of the pons, which project to the thermoregulatory center in the preoptic area (POA) of the hypothalamus. Impulses transmitted by the spinothalamocortical pathway are interpreted as mechanical and thermal pain within the thalamus and perceived as innocuous cooling or warming within the sensory cortex, but these impulses do not contribute significantly to thermoregulation (11, 71) (not depicted in Fig. 2). The LBP pathway transmits thermal signals that are processed by the thermoregulatory center within the POA to regulate body temperature (269).

Experiments in rodents and cats using retrograde tracing studies, single neuron electrophysiologic monitoring, and expression of the neuron activation marker, c-fos, identified the neurons activated by changes in skin temperature. Their contribution to thermoregulation was assessed by stereotactic ablation or nanoinjection of synaptic activators and inhibitors. These studies show that cool-induced signals are relayed through the lateral external subnucleus of the LBP (LBPel) to the median preoptic nucleus (MnPO) in the medial preoptic area (MPO) of the hypothalamic POA through glutamatergic pathways, which are under the inhibitory control of GABAergic neurons (71, 146, 169, 195, 267,269). Warm signals are relayed through distinct, glutamatergic pathways that are relayed through the dorsal subnucleus of the LBP (LBPd) to reach the same MnPO as the cool-responsive neurons (268) (Fig. 2). As discussed later in this article, the signals relayed from warm- and cool-sensing cutaneous afferents accelerate and slow the firing rate of POA warm-sensing neurons, respectively.

Thermoreception in the body core: Compared with cutaneous thermoreceptive afferents, much less is known about the contribution of deep tissue thermal afferents to thermoregulation. Rawson and Quick (318) definitively showed in sheep that intraabominal thermal afferents transmitted via the ipsilateral splanchnic nerves participate in thermoregulation. Similar pathways exist in the cat (126) and rabbit (324). The hepatic branch of the vagus nerve has been shown to respond to liver warming (3), and to transmit signals required for fever induction following intraperitoneal injection of bacterial endotoxin (359), which is discussed later in this review. The vagal nerve may also indirectly modify core temperature through its effects on feeding-dependent effects on energy production (391) but does not appear to transmit thermal input to the hypothalamus.

Temperature changes in the spinal cord affect the activity of thermoregulatory neurons in the POA in rabbits (44) and activate thermoregulatory responses in several avian (131, 317) and mammalian (24, 25, 57, 123, 347) species. These findings support the existence of thermosensitive neurons in the spinal cord that can sense changes in local temperatures. However, it is unclear whether thermal responsiveness of the spinal cord is initiated by intrinsic spinal neurons or by the proximal endings of primary somatosensory fibers in the dorsal horn.

Central integration of thermal input and the hypothalamic set point

The POA of the hypothalamus was identified in animal models as an important thermosensitive area of the brain that responds to direct heating and cooling by activating cool- and heat-defensive actions (4, 129, 130, 144, 222). Case reports demonstrate that hypothalamic lesions and injuries in humans are associated with impaired thermoregulation (315, 437). An analysis of human brain metabolic activity using PET scan during whole body warming and cooling showed that multiple areas of the brain participate in the response to changing temperature including the somatosensory cortex, insula, anterior cingulate, thalamus, and hypothalamus, and demonstrated that the hypothalamus responds differently depending on the direction of temperature change (87). Collectively, these data support a central role for the hypothalamus in human thermoregulation.

Nakayama *et al.* (272,273) identified a population of neurons in the anterior hypothalamus that responded to warming by increasing their action potential firing rate. These neurons exhibit a pattern of preactivation action potential depolarization that is characteristic of pacemaker cells. The intrinsic thermosensitive pacemaker activity of these neurons was supported by electrophysiologic studies of animal brain slices that showed temperature-proportional firing rates of some units that persisted even when input from other neurons was blocked (80, 158, 182). More recently Griffin *et al.* (118) demonstrated that the increased firing rate of the warm-sensing POA pacemaker neurons was caused by a decrease in duration of the postaction potential hyperpolarization duration as temperature increased, which was reflected in a temperature-proportional inactivation of the postaction potential outward potassium A-current.

Based on the early description of warm-sensitive pacemaker neurons by Nakayama, H.T. Hammel (128) proposed a model for thermoregulation in which a simple synaptic network of hypothalamic neurons regulates body temperature around a set-point temperature, much of which has been substantiated by subsequent experimental data (see Fig. 2). Hammel proposed that the output of heat-loss effector neurons is regulated by a balance between tonic inhibition by temperature-insensitive pacemaker neurons and activation by warm-sensitive neurons in which action potential firing rate is proportional to local temperature. As temperature exceeds the threshold at which the inhibitory and stimulatory influences are balanced, output from these effector neurons increases proportionately with increasing temperature. Studies showing that each of the components of the heat-loss response (e.g., vasodilation, panting, and sweating) is activated at slightly different temperature thresholds (1, 129, 130, 144, 222), suggesting that distinct populations of effector neurons control each response. Since Nakayama's (273) initial description of warm-sensitive neurons in the hypothalamus, multiple investigators have confirmed and expanded these findings. Approximately 20% to 30% of neurons in the POA and nearby anterior hypothalamic tissue are warm-sensitive (117-119). Both warm-sensitive and insensitive neurons receive excitatory and inhibitory synaptic inputs from nearby neurons (119), but only the warm-sensing neurons exhibit a proportional increase in firing rate upon skin or spinal cord warming as well as direct warming of the hypothalamus itself (37).

In Hammel's model, regulation of cold-defense effector neurons (e.g., those activating shivering, brown adipose thermogenesis, and cutaneous vasoconstriction) is the reverse of heat-loss effector regulation. He proposed that output from

cold-defense effectors is regulated by a balance between tonic *excitatory* input from temperature-insensitive neurons and temperature-proportional *inhibition* by warm-sensing neurons. Importantly, Hammel's model allowed for regulation of both heat-removal and cold-defense effectors with a single population of temperature-sensitive pacemaker neurons with an action potential firing rate directly proportional to temperature. Hardy and Boulant had previously described a population of hypothalamic cold-responsive pacemaker neurons in which action potential firing rate increased as temperature of the local brain tissue decreased (37). However, Dean and Boulant later showed that, unlike warm-sensing pacemaker neurons, the cold-responsive hypothalamic neurons lose their thermal-responsiveness in the presence of synaptic blockade (79, 81). These findings show that the cold-responsive neurons are not intrinsically thermally responsive and suggest that their action potential firing rate is negatively regulated by temperature-proportional output from warm-sensing neurons. Whether the cold-responsive neurons described by Hardy and Boulant are the cold-effector neurons themselves or interneurons that synapse with cold-effector neurons is not yet known, but the observations are consistent with Hammel's proposal that cold-responsive neurons are intrinsically temperature-insensitive and cold-responsiveness is achieved through their inhibition by warm-sensing neurons.

The Hammel model provides a simple cell physiologic mechanism for a set point. Having the thermostat located in the POA takes advantage of its abundant blood flow from body core sites, which allows hypothalamic temperature to closely approximate blood temperature. It is important to realize that during exposures to hot and cold environments, temperatures in core tissues, including the brain, change much less than skin (39, 102, 144, 216). Thus, the influence of cutaneous thermal sensors predominates under normal conditions, allowing for feed forward activation of effector mechanisms to prevent deviations in core temperature from the established thermal set point. It follows that the contribution of the central thermal sensors, including direct effects of brain temperature on hypothalamic pacemaker neurons, increases when (1) effector mechanisms fail to maintain core temperature at the established set point during exposure to extreme environmental temperatures, (2) an increase in endogenous heat production, such as occurs during vigorous exercise, increases core temperature at moderate environmental temperatures, or (3) the set point changes as occurs during fever.

Mammalian thermoregulatory effector pathways

As mentioned earlier, homeothermic vertebrates have evolved multiple sophisticated effector mechanisms that coordinate endogenous heat generation, heat conservation and dissipation, and behavioral responses. The neural pathways that control these response originate in the dorsomedial hypothalamus (DMH) and the rostral medullary raphe region (rMR) of the brainstem and are tonically suppressed by GABAergic POA neurons that express the EP_3 PGE_2 receptor (264, 270, 271). The principal thermoregulatory effectors are vasomotor tone to direct blood flow to and from the body surface to control heat loss, brown adipose tissue (BAT) thermogenesis, shivering thermogenesis, various mechanisms for evaporative heat loss including sweating (especially in humans), panting, and saliva spreading, and behavioral responses that enhance or prevent heat transfer. There is substantial evidence that the cold-defense effectors are hierarchically activated with different thermal thresholds through parallel but distinct neural pathways. For example, separate populations of EP_3 receptor-expressing MPO/POA neurons project to either the DMH or the rMR (271), but not to both. In rats, the rMR contains sympathetic premotor neurons that innervate BAT and cutaneous blood vessels (49, 263) and mediate BAT thermogenesis and cutaneous vasoconstriction caused by either PGE_2 injection into the POA or by cold challenge (220, 264, 266, 316). The DMH also mediates BAT thermogenic responses (221, 265, 270), but not cutaneous vasoconstriction, which appear to be mediated by effector neurons that are located more distally in the brainstem (316). These observations are consistent with Hammel's model in which tonic inhibition from the dedicated populations of EP_3 receptor-expressing warm-sensing POA neurons suppress neurons in the DMH and rMR that specifically control BAT thermogenesis and cutaneous vasoconstriction.

Thermoregulatory behaviors in animals are stereotypical somatic motor acts that minimize or optimize heat transfer with the environment. In humans, this behavior is different from typical premeditated movement in that the behavior is driven by a strong, often irresistible urge, which Morrison and Nakamura (252) suggest implicates the limbic system in the response. Examples of such behaviors include postural changes that increase or decrease environmental exposure of body surface, relocation to a more preferred ambient temperature, nonshivering movements to increase heat generation in the cold and, in some non-human mammals, spreading saliva to enhance evaporative heat loss. Lesion studies in rats suggest that thermoregulatory behaviors can persist after ablations in the POA that abrogate autonomic and shivering responses (52, 339, 350), demonstrating the reliance on distinct neural pathways for each of these effector responses. However, the exact nature of these pathways has not yet been defined.

Several strategies exist for evaporative heat loss, including panting, saliva spreading, and sweating, the latter being the predominant method used by humans. Since humans are unique among mammals in their exclusive reliance on sweating for evaporative heat loss, our knowledge of the neural pathways is largely restricted to studies in humans and therefore is less completely understood than other thermoregulatory effector responses. However, correlations between direct measurements of postganglionic sympathetic nerve impulses using microelectrodes and sweat gland output (34, 387, 388), human skin biopsies (409), and analysis of how topical agonists affect human sweating rate (340) have provided some information about the neurotransmitters involved. Skin biopsies show that human eccrine sweat glands are innervated

by more cholinergic than adrenergic nerve endings (409). The predominance of cholinergic control of sweating is confirmed by much greater effect of topical cholinergic agonists and antagonists on sweating than adrenergic agonists and antagonists (340). The cholinergic regulation of sweating has important clinical consequences as commonly used drugs with anticholinergic activity can suppress normal sweating and increase the risk of dangerous hyperthermia and heat stroke (95).

Among the thermoregulatory effector responses, shivering deserves special mention. It is usually the last cold-defense response activated (352), but has the most serious consequences in the acutely ill patient. Though it has the greatest capacity for heat generation, the associated high metabolic cost (159) is particularly problematic, especially in critically ill patients (156). Heat generation is usually an unavoidable byproduct of muscle contraction due to inefficiency in converting ATP chemical energy to work. However, with shivering, heat generation is the primary goal and is achieved through a characteristic pattern of rapid, repeated skeletal muscle contractions that serves no other purpose and, in fact, interferes with purposeful movement. Although heat generation through shivering is widely accepted as an important mechanism in cold defense and in fever generation (334), the exact neural pathways that regulate this response are poorly understood. Like the other components of the cold-defense response, shivering effector neurons are under GABAergic suppression by neurons originating in the MPO of the POA (290, 392, 452). Microinjection studies (275) identified the raphe magnus (RM) and the nucleus reticularis magnocellularis (NRMC) of the ventromedial medulla as possible sites of shivering effector neurons. Shivering is activated by rhythmic bursts of activity in spinal α-motor neurons that stimulate rhythmic contractions in extrafusal muscle fibers (145). In humans, progression of shivering activity follows a reproducible recruitment pattern, beginning in the neck and thorax and progressing to the upper and then the lower extremities (19, 159). Tanaka *et al.* (400) found that skin cooling in the rat stimulated activation of γ-motoneurons, which occurred in the absence of α-motoneuron activation and overt shivering, and showed that γ-motoneuron activity increased muscle tension and augmented subsequent shivering. This γ-motoneuron activation was mediated by neurons in the rostral ventral medulla, occurred without a change in core temperature, and, importantly, ceased upon incomplete skin rewarming.

Neurologic basis of fever

As mentioned earlier, PGE_2 has been well-documented as the final common inducer of fever (170). Both intravenous injection of PGE_2 (379) and injection of PGE_2 into the POA (8, 265, 399) stimulate fever. PGE_2-responsiveness has been further localized within the POA to the MPO, MnPO (342), and ventromedial preoptic region (VMPO) (314). PGE_2 is generated from arachidonic acid through a pathway, the key enzyme in which is cyclooxygenase (COX). Arachidonic acid released from phospholipids by phospholipase-A_2 (PLA_2) is converted to PGH_2 by COX-1 and -2 (104). PGH_2 is the common intermediate substrate of several PG synthetic pathways, including generation of PGE_2 by PGE_2 synthase. The most commonly used antipyretic agents target the COX enzymes (150). Interestingly, as mentioned earlier, the COX inhibitor, sodium salicylate, blocks fever behavior in the desert iguana, *Diposaurus dorsalis*, indicating that the COX-dependent pathway for fever induction has been very well conserved during evolution. In mice, COX-2 gene deletion blocks both the early and late phases of fever after i.v. administration of LPS, but COX-1 gene deletion had no effect on either phase of the febrile response (380), identifying COX-2 as the predominant isoform in mediating the febrile response to infection and inflammation. A third COX isoform, originally called COX-3, has since been found to be a splice product of COX-1, now called COX-1b and, importantly, found to not occur in humans (319). Furthermore, since COX-1 gene deletion eliminates both COX-1 and COX-1b, the contribution of either COX-1 splice variant to murine fever is unlikely.

In rats, a single i.v. administration of LPS stimulates a multiphasic fever pattern with brief temperature peaks between 50 and 160 min following LPS administration and a more persistent peak after 3 h (327). In this model, LPS administration stimulated a rapid increase in COX-2 and microsomal PGE Synthase (mPGES) in peripheral tissues but not brain tissue and the increase in these enzymes was temporally associated with increased circulating PGE_2 levels and the first phase of fever (379). Macrophages within the lung and liver appear to be the primary peripheral source of PGE_2 after a single LPS challenge in rodents (327, 379). In the LPS-challenged rat model, hypothalamic expression of genes involved in PGE_2 synthesis, predominantly secretory PLA_2-IIA, COX-2, and mPGES, was delayed compared with peripheral PGE_2 generation, and correlates with the later phases of fever. The first phase of fever after LPS challenge in the rat was blocked by immunoblockade of circulating PGE_2 reduced and reproduced in the absence of LPS by i.v. administration of PGE_2. More recently, Ridder *et al.* (323) showed that the pyrogenic cytokine, IL-1ß, stimulated brain endothelial cell expression of COX-2 and generation of PGE_2 through the TGF-ß-activated kinase (TAK1;MAP3K7)-p38α (MAPK14) signaling pathway *in vitro* and found increased COX-2 expression in POA endothelial cells after treating mice with IL-ß *in vivo*. They found that conditional TAK1 gene deletion in brain endothelial cells, but not astrocytes, oligodendroglial cells or neurons, by Cre expression from the Slco1c1 promoter in mice with floxxed TAK1, interrupted TAK1-p38 signaling in brain endothelial cells and blocked the late phase febrile response to IL-1ß. Collectively, these studies along with other earlier studies show that fever can be caused by PGE_2 generated peripherally as part of the initial inflammatory response or by PGE_2 generated within brain tissue through a TAK1-dependent pathway in brain endothelial cells and possibly through TAK1-independent pathways in other cells within

the brain. As discussed earlier, PGE_2 is also the central driver of behavioral fever in poikilothermic animals (31, 320).

PGE_2 is eliminated from the brain through an active mechanism that includes the organic anion transporter (OAT)-3 (393), but the relationship between rates of PGE_2 elimination from the brain and the duration of fever has not yet been defined. Interestingly, activity of OAT-3 and clearance of PGE_2 from cerebral spinal fluid were inhibited by beta-lactam antibiotics. These antibiotics are frequently used in febrile, infected patients, and have been linked to drug fever. However, the effect of beta-lactam antibiotics on clearance of PGE_2 from the hypothalamus and its implications for length of fever are not known.

PGE_2 exerts its biological effects through a family of four G protein-coupled receptors, $EP_{1\text{-}4}$ (274) of which EP_1, EP_3, and EP_4 mRNA are expressed in the POA (287). EP_3-expressing neurons have been identified in the same location as Hardy's warm-sensing pacemaker neurons that are located within the MnPO, MPO, and parastrial nucleus of the POA and synapse with effector neurons in DMH and rMR (262, 264, 271). Like Hardy's warm-sensing pacemaker neurons, these EP_3-expressing neurons also express a marker for GABAergic function (264) The febrile response to exogenous PGE_2 and LPS is absent in EP_3-deficient mice (412) and in mice with tissue specific EP_3 deletion in MnPO neurons (203) but only partially attenuated in EP_1 receptor-deficient mice (286), further supporting the EP_3 receptor as the predominant PGE_2 receptor in mediating the febrile response. Seven human EP_3 splice variants have been identified and all inhibit adenylate cyclase by coupling with G_i-protein (345). Steiner *et al.* (378) showed injections of PGE_2 into the rat anterior hypothalamus reduced cAMP levels and stimulated an increase in core temperature. Co-injection of the cAMP phosphodiesterase inhibitor, aminophylline, along with PGE_2 restored cAMP levels and attenuated the increase in core temperature. Intra-hypothalamic injection of the cAMP-dependent protein kinase A inhibitor, adenosine-3′, 5′-cyclic monophosphorothioate-Rp isomer (Rp-cAMPS), also stimulated an increase in core temperature while injection of dibutyryl cAMP caused a reduction in core temperature. These results demonstrate that PGE_2 reduces cAMP levels in the anterior hypothalamus and that reduced activity of the cAMP-dependent protein kinase, PKA, is sufficient to cause fever.

Using single unit electrophysiologic recordings of rat brain slices, Ranels and Griffin (314) showed that exogenous administration of PGE_2 in the VMPO of the POA caused reduced firing rates of warm-sensing neurons and increased firing rates in temperature-insensitive neurons that reflected changes in the rate of prepotential depolarization (313). Intra-hypothalamic injection of the stable cyclic GMP analogue, 8-bromo-cGMP exerted similar core temperature-lowering effects in the rat (378) and reduced firing rates in both warm-sensitive and temperature-insensitive hypothalamic neurons (440). Collectively, these studies clearly show that PGE_2 generated either peripherally or within the hypothalamus stimulates fever by reducing the firing rate pacemaker activity of POA neurons. The resultant decrease in GABA-mediated inhibitory activity reduces suppression of cold-defense effector neurons, resulting in activation of cold effector responses.

In addition to stimulating PGE_2 generation, pyrogenic cytokines may induce fever through PGE_2-independent pathways. For example, TNF receptors have been identified in the hypothalamus and intracerebroventricular administration of the TNFα-blocking antibody, infliximab, reduces fever in rat models of sepsis (15). On the other hand, Cao *et al.* (50) found that COX-2 inhibitors blocked TNFα-induced fever whether the TNFα was administered via the intraperitoneal or intracerebroventricular route suggesting that TNFα exerts pyrogenic effects predominantly or exclusively through PGE_2 generation. Nilsberth *et al.* (278) showed that IL-6 knockout mice failed to generate fever in response to LPS administration but did exhibit increased cerebrospinal fluid levels of PGE_2 and were capable of generating fever in response to PGE_2 injected into the central nervous system. Collectively, these studies suggest that fever is generated predominantly through a PGE_2-dependent pathway, but alternative pathways may also contribute to fever, which may explain the therapeutic failure of antipyretic therapy with COX inhibitors in some patients.

The role of the vagal nerve in mediating the fever response is less clear. Much of the investigation of vagal participation in fever has been performed in rats and guinea pigs using surgical transection of the vagal nerve or individual branches or vagal desentization with capsaicin injections (326). The results of these studies must be interpreted carefully considering the untoward effects of complete vagotomy on multiple homeostatic mechanisms that could indirectly affect the capacity for mounting a febrile response. Whether vagal disruption blocks the capacity to generate fever depends on the species studied, the pyrogen administered, and pyrogen dose and route of administration. Most models use i.v. or i.p. injection of LPS or IL-1ß. In a review of the literature prior to 2000, Romanovsky (326) concluded that (1) some of the attenuation of fever caused by vagal disruption was indirectly caused by loss of other critical vagal functions required for homoeostasis and response to stress and (2) an intact vagus nerve, particularly the hepatic branch (359), was more essential for fever induced by lower than higher doses of LPS whether administered i.p. or i.v. Hansen *et al.* (132) subsequently showed that the subdiaphragmatic vagotomy attenuated fever induced by 0.1 μg/kg recombinant human IL-1ß (rhIL-1ß) but not fever induced by a 1 μg/kg dose of IL-1ß. In this study circulating rhIL-1ß was only detectable with the higher dose. Ootsuka *et al.* (288) showed that subdiaphragmatic vagotomy did not affect fever induced by i.v. injection of PGE_2. Collectively, these data suggest that afferents in the hepatic branch of the vagus may transmit signals activated by IL-1ß generated within the liver than can induce fever in response to low levels of pyrogens when circulating IL-1ß levels are not sufficient to induce fever.

In summary, infection and injury cause a temporary increase in core temperature through multiple inflammatory mediators that converge on an evolutionarily conserved PGE_2-dependent final common pathway and activate multiple cold-defense responses. The consequences of the increase in temperature in infected or injured animals are discussed in the next section.

Biological Consequences of Febrile-Range Hyperthermia in the Infected Host

Fever typically results in a 2 to 4°C increase in core temperature and retrospective clinical studies suggest that temperature increases >2.5°C may be harmful (152, 219). The most common treatment modalities for fever either target the most distal mediator, PGE_2 (150) or physically remove heat without resetting the thermal setpoint (348) thereby eliminating the thermal component of fever. Core temperature elevations can also occur when the balance between heat generation and elimination is dysregulated and these can be associated with significant morbidity and mortality (51). This section is focused on the biological consequences of the thermal component of fever. We review studies of the influence of temperature shifts between basal and febrile levels on cell culture and animal models of infections and injury. In most cases, febrile-range hyperthermia (FRH) reflects a 2.5°C change in culture temperature *in vitro* or core temperature *in vivo*.

Studies in diverse animal species support a protective role for fever during infections. In two studies of poikilothermic vertebrates, increases in core temperature from 38 to 40°C in the lizard *Diposaurus dorsalis* (218) and from 28 to 32.7°C in goldfish (70) improves survival from 25% to 67% and from 64% to 100%, respectively during infection with the same Gram-negative pathogen, *Aeromonas hydrophila*. Studies in experimentally infected sockeye salmon, rainbow trout, crickets, and grasshoppers showed similar protective effects of increased body temperature during infection (Reviewed) (192). In a lethal Herpes simplex-infected mouse model, housing infected mice at 38°C for six days increased both their core temperature (by approximately 2°C) and their survival rate (from 0% to 85%) compared with infected mice housed at 23 to 26°C (14). Schmidt *et al.* confirmed these results in a similar model of Herpes simplex-infected mice (346). Bell and Moore (29) reported a survival benefit of warming mice infected with rabies virus. In our own study of mice with experimental *Klebsiella pneumoniae* peritonitis, the survival rate improved from 0% to 50% and the intraperitoneal bacterial load decreased 100,000-fold when the infected mice were coexposed to FRH (core temperature ~39.5°C) by housing mice at 36 to 37°C rather than 24°C ambient temperature (172).

In several other animal models, administration of antipyretic agents blocked fever and decreased survival (31, 89, 199, 414, 417) during bacterial infections. In *A. hydrophilia*-infected lizards, treatment with sodium salicylate blocked fever in 7 of 12 animals, all of which died, while all febrile animals survived (31). In experimental murine *Streptococcus pneumoniae* pneumonia, treatment with aspirin impairs bacterial clearance and reduces the LD_{50} from 6.3×10^6 CFU to 3.3×10^5 CFU (89).

Febrile-range temperatures are directly cytotoxic or cytostatic for certain microbial pathogens, including the human pathogens *Cryptococcus hominis* (197), *Streptococcus pneumonia* (322), *Neisseria gonorrhoeae* (53), and *Mycobacterium leprae* (325). In the study of the *Ascosphaera apis*-infected honeybee hive, the 7 to 8°C increase in hive temperature caused by the communal increase in wing muscle activity directly kills the fungal pathogen, preserves the viability of the larvae, and secures survival of the hive (376). However, as discussed below, many pathogens proliferate equally well at basal and febrile temperatures.

The *Klebsiella pneumoniae* pathogen used in our mouse studies exhibited virtually identical growth rates in 37 and 39.5°C cultures, but pathogen load was reduced several orders of magnitude in mice exposed to FRH *in vivo* (172, 321). In studies of fish and lizards, increasing body temperature by the same 4°C increment greatly increased clearance of the same Gram-negative pathogen, *Aeromonas hydrophila*, in both animals despite a 10°C lower temperature range in the fish (30, 70). These data demonstrate that FRH can enhance pathogen clearance *in vivo* through effects on host defense rather than on the pathogen. This effect may also explain the association of fever with improved survival in retrospective clinical studies of bacterial infections (5, 43, 153, 219, 432).

Several studies have confirmed that FRH is a biological response modifier with potent immunomodulatory effects. Work in our laboratory and others have shown that exposure to FRH can modify cytokine and chemokine expression *in vivo* and *in vitro* in macrophages and epithelial and endothelial cells and increase neutrophil and lymphocyte recruitment and increase cell death through apoptosis (13, 59, 60, 67, 68, 99, 214, 215, 223, 255, 257, 354, 361, 363, 405, 407, 415, 427, 453).

In our mouse FRH model, core temperature increases by 2 to 3°C but normal circadian temperature and sleep-wake patterns were maintained and the animals appear otherwise healthy and active (335). Using this model, we showed that FRH accelerated pathogen clearance in both experimental *Klebsiella pneumoniae* peritonitis (172) and pneumonia (321). Focusing on the lung, we found that FRH tended to reduce survival in the *Klebsiella* pneumonia model while it greatly improved survival in the peritonitis model. Antibiotic treatment prevented death in normothermic mice with *Klebsiella* pneumonia, but when coexposed to FRH, mortality increased to 50% in the mice with antibiotic-treated pneumonia (Fig. 3). FRH also increased mortality in a mouse intratracheal LPS-induced acute lung injury model from 0% to 50%. In both the treated pneumonia and intratracheal LPS instillation models, severe lung injury was found in the FRH-coexposed mice. Lipke and Martin confirmed these results

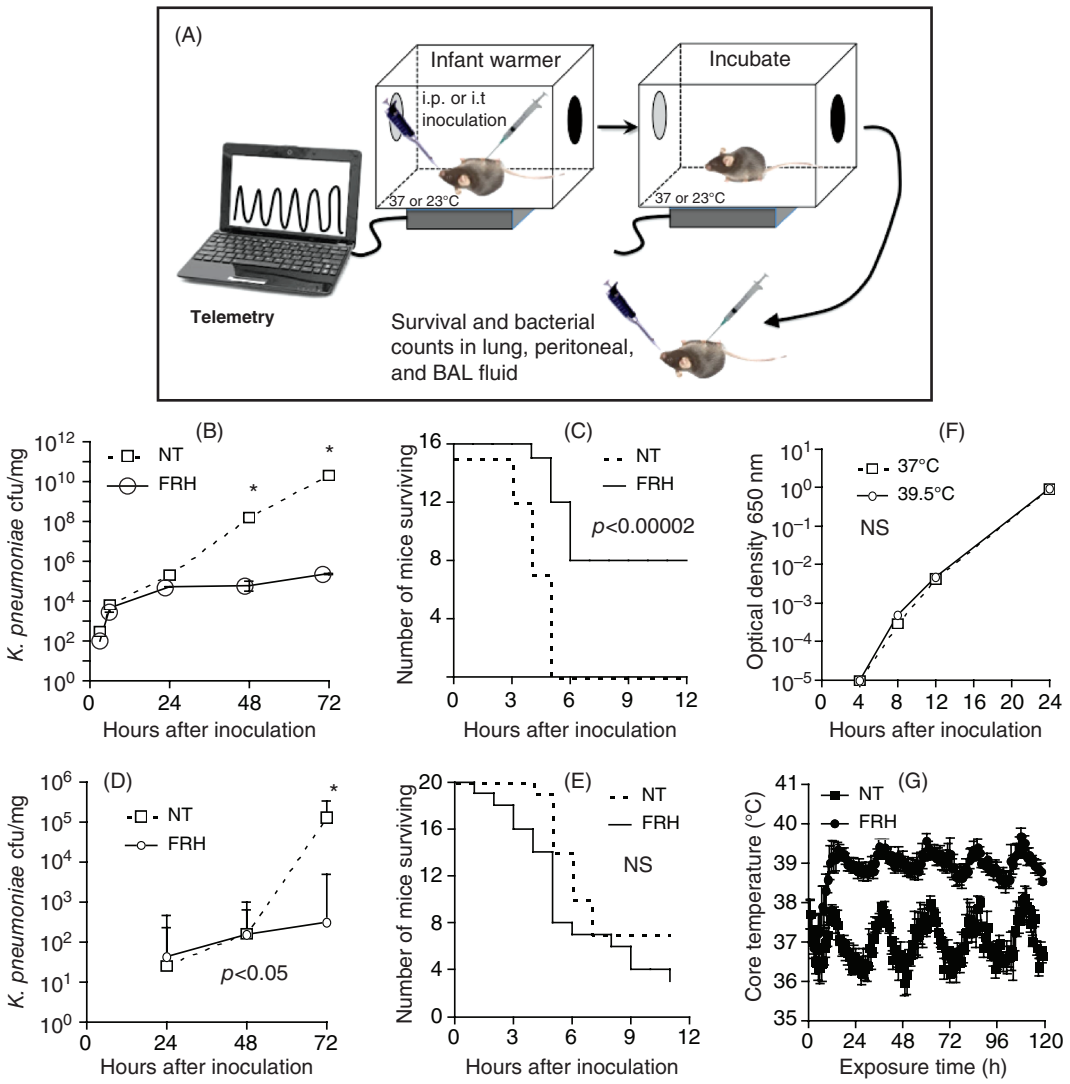

Figure 3 Effect of Febrile-range hyperthermia (FRH) on pathogen clearance and host survival in mouse models of bacterial peritonitis and pneumonia. (A) The mouse FRH model in which mice in standard cages are transferred to infant incubators set at 37°C and mice then are inoculated with *Klebsiella pneumoniae* Caroli strain. Mice were inoculated with 100 cfu via i.p injection (B and C) or 250 cfu via i.t. instillation (D and E) and mice were housed at 23 or 37°C ambient temperature. Survival (C and E) was determined and bacterial colony counts were determined in peritoneal fluid (B) and lung homogenates (D). The *p* value for the survival studies is shown. The bacterial colony counts are mean ± SE of six experiments. * denotes $p < 0.05$ versus FRH. (F) To analyze direct effect of temperature on bacterial growth rate, 100 mL aliquots of LB medium were inoculated with 10 cfu *K. pneumoniae*, incubated at 37 or 39.5°C, and OD_{650} sequentially measured. (G) Core temperature in four mice per group housed at 23°C (NT) or 37°C (FRH). *Coexposure to FRH accelerated pathogen clearance in both the pneumonia and peritonitis models but only improved survival in the peritonitis model.* Reprinted in modified form with permission from references 172 and 321.

in the intratracheal LPS-challenged mouse model (214, 215). Co-exposure to FRH exerted similar effects in a model of lethal pulmonary oxygen toxicity (133). The results of the peritonitis and pneumonia studies underscore an important principle in understanding the consequences of fever and FRH in the infected or injured host. Since augmentation of immune defenses can accelerate pathogen clearance and enhance collateral tissue injury, the ultimate consequence for survival depends on the balance between the two effects. The greater susceptibility of the lung to immune-mediated injury and the importance of pulmonary gas exchange function for survival explain why FRH coexposure is more lethal in the setting of pulmonary than peritoneal infections. Furthermore, when inflammation and injury is caused by a nonreplicating pathogen, as in the case of an antibiotic-treated pneumonia, the major benefit of FRH is lost and mortality increases.

We found that FRH coexposure was associated with greatly increased neutrophil infiltration in both the pulmonary oxygen toxicity and intratracheal LPS instillation models (133, 321). Interestingly, Bernheim *et al.* (30) showed in

the poikilothermic desert lizard, *Diposaurus dorsalis*, that increasing core temperature from 38 to 40°C after intradermal inoculation with *Aeromonas hydrophila* increased survival, reduced bacterial burden, and increased the accumulation of granulocytic leukocytes at the inoculation site. Considering the phylogenetic age of fever and the similarities between the mouse and lizard models, we reasoned that the processes responsible for increased granulocytic leukocyte recruitment upon FRH exposure are phylogenetically old, evolutionarily conserved, and functionally important for survival during infections and injury. As mentioned above, the evolutionary considerations of fever's protective effects do not necessarily apply to modern-day patients in whom pathogen clearance can be pharmacologically aided.

Using the intratracheal LPS instillation model, we showed that exposure to FRH augments 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 (88), increased generation of the CXC chemokine family of endogenous chemotaxins (321, 407), and direct effects on endothelial cells and neutrophils (134, 354, 405). We showed that the promoter regions of the CXC chemokine genes contained binding elements for the heat-activated transcription factor, HSF-1 (223, 256, 364), the significance of which is discussed later in this article. Utilizing both *in vitro* human neutrophil transendothelial migration (TEM) assays and an *in vivo* mouse model of chemokine-directed transalveolar neutrophil recruitment, we have shown that FRH exerts effects on endothelium and neutrophils that increase their capacity for chemokine-directed TEM. In models of formylated-peptide-directed human neutrophil TEM through human pulmonary artery endothelial cells (134) and IL-8-directed TEM through human lung microvascular endothelial cells (405), neutrophil TEM was up to sevenfold greater at 39.5°C than 37°C. We utilized a mouse assay of *in vivo* transalveolar neutrophil migration (72) to analyze the effects of FRH on the capacity for chemokine-directed neutrophil migration (Fig. 4). In this model, a fixed chemokine gradient is established in the lung by the intratracheal instillation of recombinant human IL-8, and the neutrophil content in lung tissue and lung lavage quantified 4 h later. Preexposure to FRH increased subsequent IL-8-directed transalveolar migration of neutrophils by 23.5-fold (405). Adoptive transfer of fluorescently labeled neutrophils between normothermic and FRH-exposed neutrophil donors and recipients demonstrated that enhanced neutrophil migration capacity required FRH exposure of both the donors and recipients (405), which indicates that FRH augments neutrophil transmigration capacity through interdependent effects on neutrophils and endothelium. In exploring potential mechanisms for these effects of FRH, we found that exposure to FRH was sufficient to activate extracellular signal-related kinases (ERK) and p38 mitogen-activated protein (MAP) kinases in human microvascular lung endothelial cells *in vitro* and in mouse lung and circulating neutrophils *in vivo* and inhibitors of these two MAP kinases blocked the effects of FRH on human neutrophil TEM (405).

In addition to augmented neutrophil accumulation in lung, FRH coexposure had two additional effect in the LPS-challenged mouse lung that are also characteristic of human ARDS, endothelial hyperpermeability (Fig. 5) and epithelial injury (Fig. 6) (214, 215, 321). We found similar effects of FRH on the permeability of TNFα-treated human pulmonary artery and microvascular lung endothelial cell monolayers *in vitro* (134, 354). Exposing human lung endothelial cells to a temperature as low as 38.5°C endothelial permeability using a 10 kDa dextran tracer. Permeability increased by up to four-fold, was evident at low and high TNFα concentrations and in some lots of cells without exogenous TNFα, occurred without detectable cytotoxicity, and was reversible upon removal of TNFα and return to 37°C. FRH-augmented permeability was accompanied by formation of actin cytoskeletal stress fibers and intercellular gaps. As was found for FRH-enhanced neutrophil TEM, the effects of FRH on human microvascular lung endothelial cells were blocked by ERK and p38 inhibitors.

In our study of the mouse intratracheal LPS instillation model, we found that co-exposure to FRH caused extensive epithelial injury (321), Lipke and Martin (214) confirmed that FRH caused extensive epithelial injury in this model and showed it to be caused by augmented TNFα- and Fas-dependent apoptosis (215). Using the mouse MLE15 lung epithelial cell line and primary culture lung epithelial cells, Lipke and Martin showed that coexposure to 39.5°C enhanced TNFα-induced apoptosis of epithelial monolayers *in vitro* and suggested that the effect may be caused by repression of Nuclear Factor (NF)-κB activation (214). In our own recent study using the same MLE15 cell model, 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) (257). 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 IκB superrepressor.

The Evans and Repasky laboratories have shown many of the same effects of FRH on cytokine gene expression (292), but have extended their studies of FRH effects to lymphocyte trafficking (91, 99, 427). Utilizing intravital microscopy to analyze lymphocyte trafficking in high endothelial venules in mice, the Evans laboratory has shown that exposing lymphocytes to FRH enhances their L-selectin- and α4β7 integrin-dependent binding to high endothelial venules that increased their trafficking to secondary lymphoid tissue (91, 427) (Fig. 7). The Evans laboratory has subsequently shown the FRH enhances migration through high endothelial venule endothelium through the IL-6-dependent endothelial expression of ICAM-1 (59, 60). The consequences for the FRH-enhanced lymphocyte recruitment in infections and inflammatory disease have not yet been demonstrated experimentally.

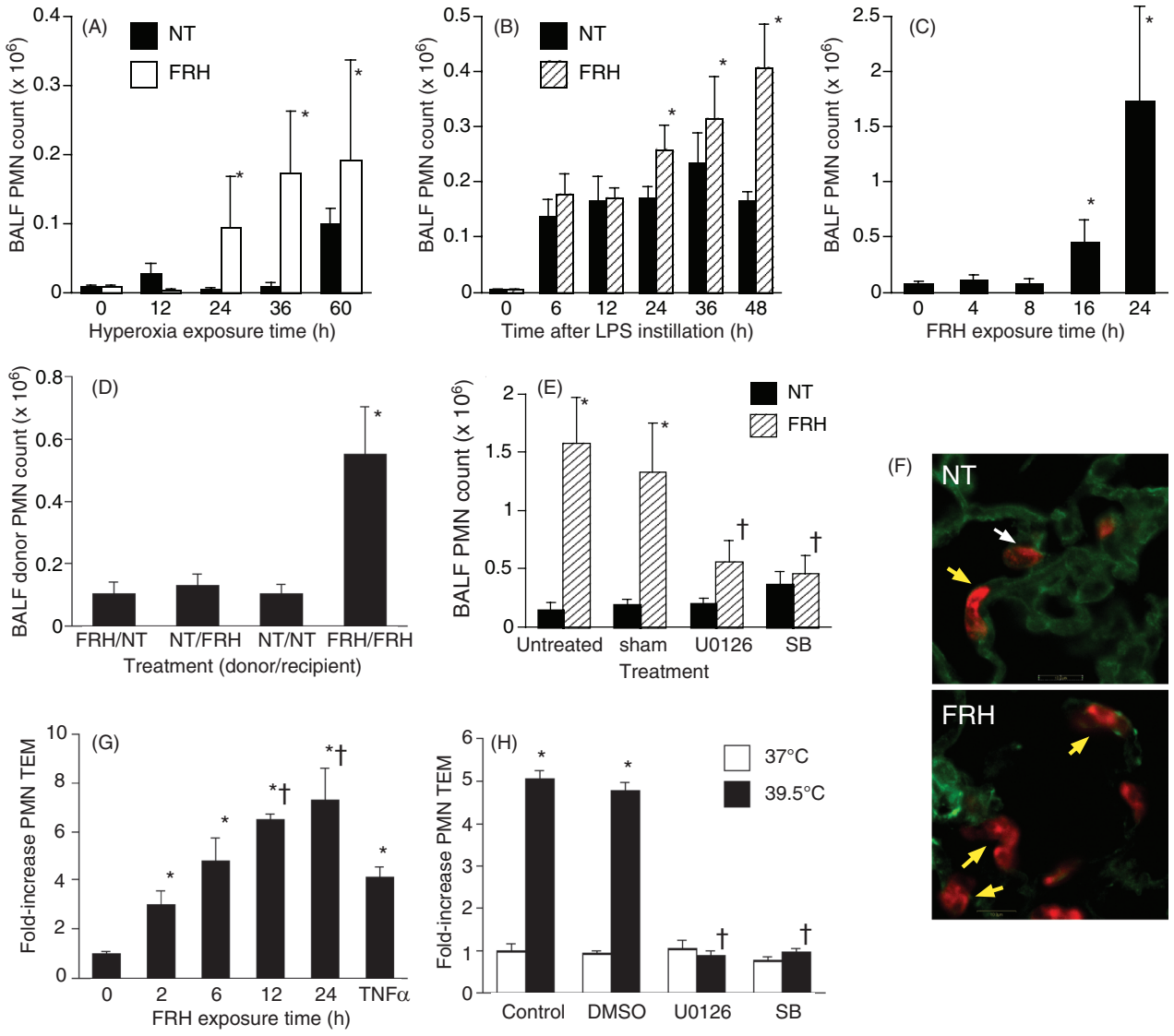

Figure 4 Effect of FRH on neutrophil recruitment. Mice were exposed to >95% oxygen (A) or received 50 μg LPS via i.t. instillation (B) and were housed at 23° (NT) or 37°C (FRH) ambient temperature for the indicated time before euthanasia, lung lavage, and quantitation of lavage neutrophil content. Mean ± SE of eight (hyperoxia) or six mice per time point. * denotes $p < 0.05$ versus NT. (C) IL-8-directed trans-alveolar migration capacity was measured in NT mice and mice exposed to FRH for the indicated time and returned to NT conditions by instilling 1 μg recombinant human IL-8 via i.t. instillation and quantifying lung lavage neutrophil content 4 h later. Mean ± SE eight mice per group. * denotes $p < 0.05$ versus time 0 (prewarming). (D) To determine whether the FRH increased neutrophil migration capacity through effects on neutrophils or endothelium we performed neutrophil transfer experiments between NT and FRH. Recipient or donor mice were exposed to FRH for 24 h, donor PMNs isolated, fluorescently labeled, and injected via tail vein, and IL-8-directed donor PMN TAM determined by flow cytometry and manual neutrophil counts. The donor/recipient treatment is indicated on the *x*-axis. Mean±SE; six mice per group. * denotes $p < 0.05$ versus all other groups. (E) To determine the participation of ERK and p38 MAP kinases in FRH-augmented neutrophil migration, groups of 8 mice were untreated or treated with 2% DMSO (sham), 200 μg U0126 or 1 mg SB203580 (SB) 30 min before 16 h FRH or normothermic exposure and IL-8-directed PMN transalveolar migration was measured. Mean±SE. * and † denotes $p < 0.05$ versus normothermic and untreated mice, respectively. (F) Inflation-fixed lungs from normothermic (NT) and 24 h-FRH-exposed mice 4 h after intratracheal IL-8 instillation were stained for GR-1 (PMNs, red) and VE-cadherin (ECs, green), and analyzed by confocal microscopy. White and yellow arrows denote intravascular and extravasating PMNs, respectively. (G) Human Microvascular Lung Endothelial Cells (HMVEC-Ls) were incubated at 39.5°C for indicated time or treated with 1 ng/ml TNFα for 6 h at 37°C and IL-8 directed transendothelial migration of calcein AM-stained human neutrophils measured over 2 h at 37°C and standardized to untreated HMVEC-Ls. Mean±SE; four experiments. * and † denote $p < 0.05$ versus time-0 and TNFα. (H) HMVEC-Ls were untreated (Control) or pretreated for 30 min with DMSO, U0126, or SB203580 (SB), incubated for 24 h at 37°C or 39.5°C, and neutrophil transendothelial migration measured. Mean±SE; four experiments. * and † denote $p < 0.05$ versus 37°C and untreated 39.5°C. *Coexposure to FRH increases capacity for chemokine-directed neutrophil migration through effects on neutrophils and endothelium.* Reprinted in modified form with permission from references 143 and 343, 430.

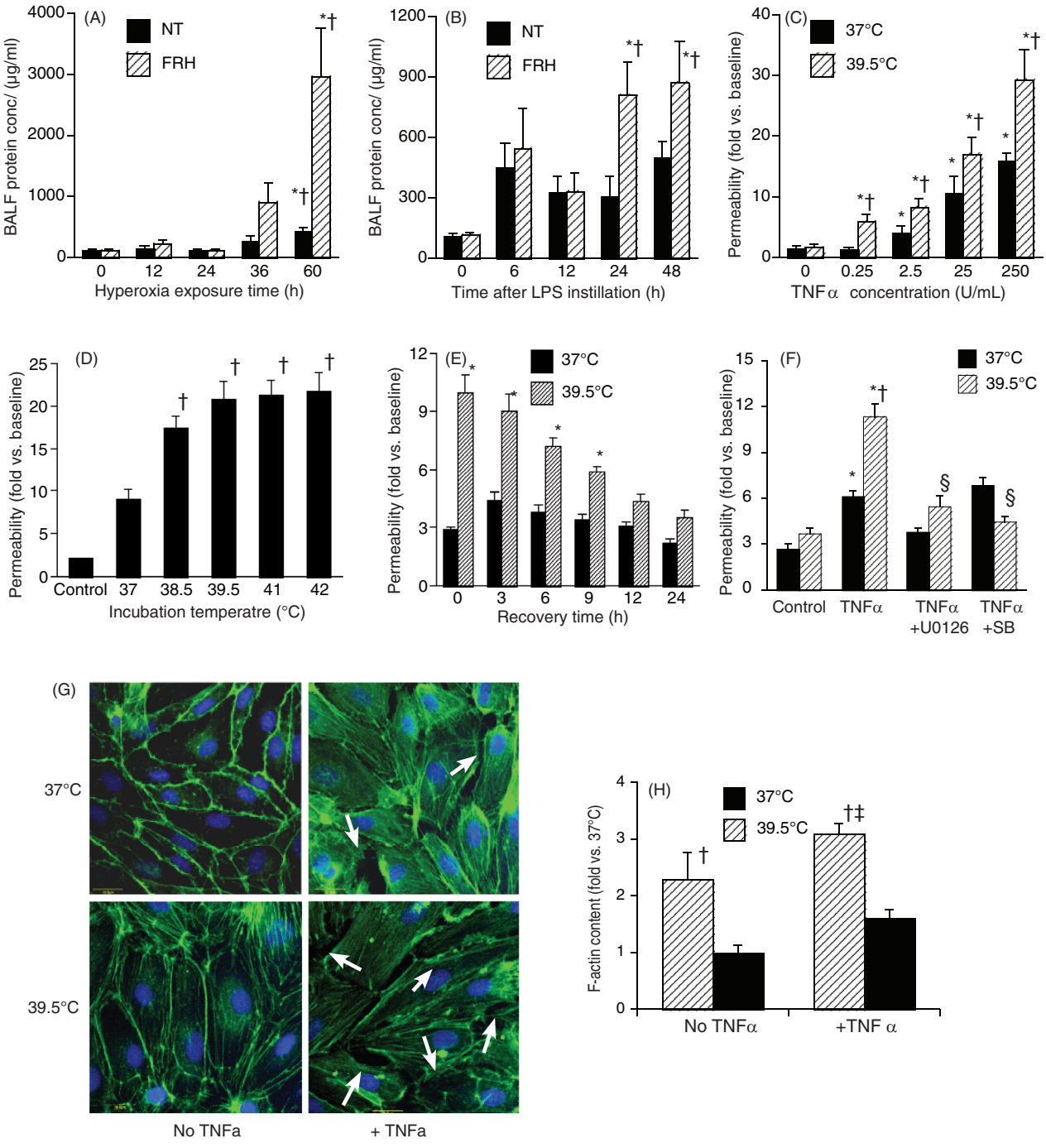

Figure 5 Effect of FRH on pulmonary vascular endothelial permeability. Mice were exposed to >95% oxygen (A) or received 50 μg LPS via i.t. instillation (B) and were housed at 23° (NT) or 37°C (FRH) ambient temperature for the indicated time before euthanasia, lung lavage, and quantitation of lavage neutrophil content. Mean ± SE of eight (hyperoxia) or six mice per time point. * and † denote $p < 0.05$ versus NT and time 0, respectively. (C) HMVECLs were incubated with the indicated concentration of TNFα for 6h at 37 or 39.5°C, the TNFα was removed and transendothelial flux of 10 kDa Cascade blue dextran over 30 min at 37°C was measured. (D) HMVEC-Ls were incubated with 0.25 U/mL TNFα for 6 h at the indicated temperature and 10 kDa Cascade blue flux measured. Mean ± SE, $n = 21$. * and † denote changes with TNFα-free controls and 37°C cells, respectively. (E) HMVECLs were incubated for 6 h with either 2.5 U/mL TNFα at 37°C or 0.25 U/mL TNFα at 39.5°C, the TNFα was removed, all monolayers returned to 37°C and 10 kDa Cascade blue dextran flux measured immediately and then sequentially during recovery. Mean ± SE, $n = 9$. * denotes $p < 0.05$ versus 39.5°C at time 0. (F) HMVECLs were pretreated with 10 μmol/L UO126 or p38 MAPK SB203580 for 30 min at 37°C, then incubated with 0.25 U/mL TNFα for 6 h at the indicated temperature, the TNFα was removed and 10 kDa Cascade blue dextran flux measured. Mean ± SE, $n = 9$. * and † denote $p < 0.05$ versus TNFα-free controls and 37°C cells, respectively. (G) HMVECLs grown on chamber slides were incubated for 6 h without or with 0.25 U/mL TNFα at 37°C or 39.5°C, fixed and stained with phalloidin coupled with Alexafluor488, counterstained with DAPI, and visualized by fluorescent confocal microscopy. Intercellular gaps are noted by the arrows. (H) F-actin staining intensity from panel G quantified and expressed relative to 37°C without TNFα. Mean±SE, $n = 4$. † and ‡ denote $p < 0.05$ versus 37°C with and without TNFα, respectively. *Coexposure to FRH reversibly increases endothelial permeability.* Reprinted in modified form with permission from references 133, 321, 405.

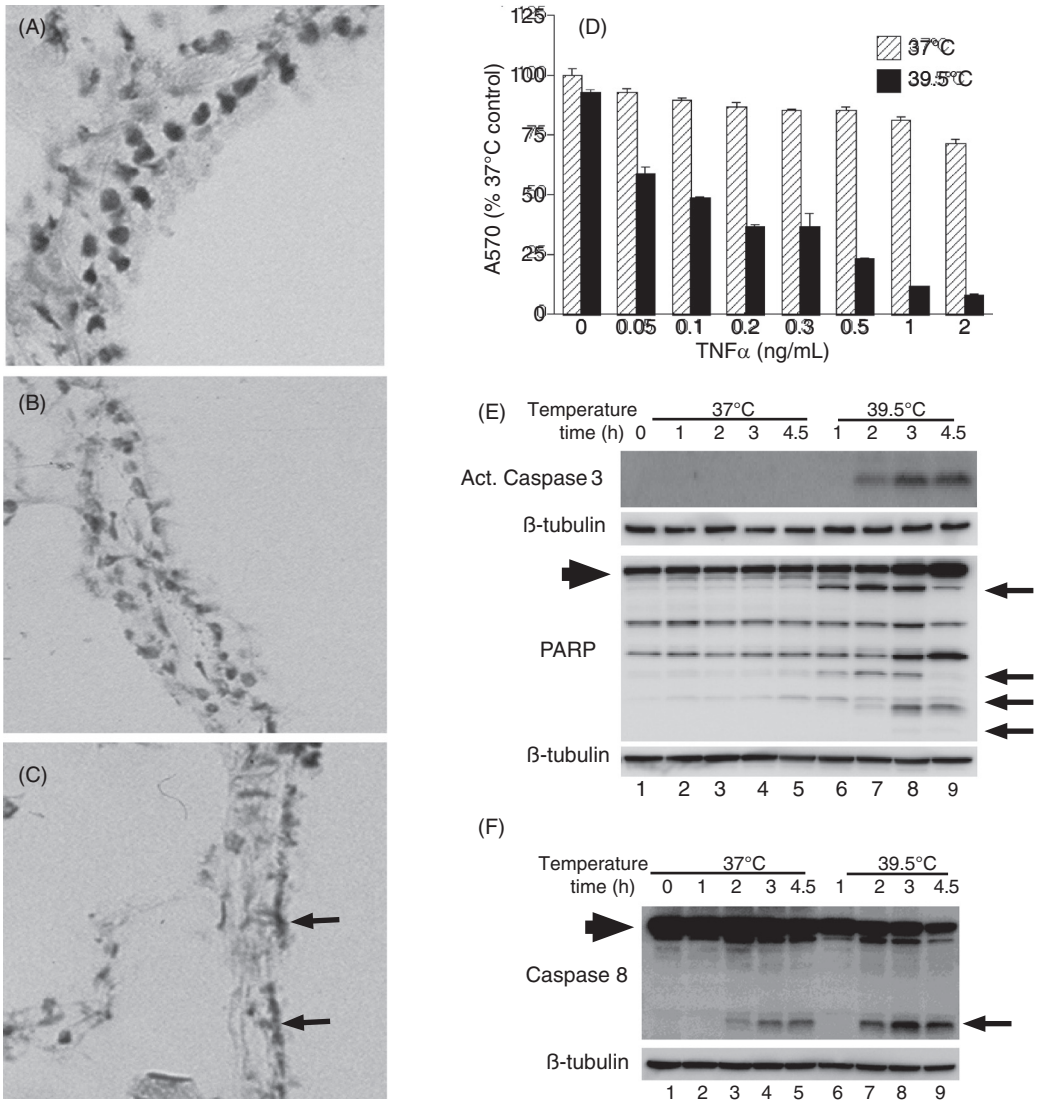

Figure 6 Effect of FRH on epithelial apoptosis. (A-C) Mice received 50 μg LPS via i.t. instillation and were housed at 23 or 37°C ambient temperature for 48 h, the lungs were then inflation fixed and stained with hematoxalyn and eosin. A is untreated control, B and C are normothermic and FRH-exposed mice, respectively. Arrows indicate loss of cilia and distinct nuclei. (D) Mouse MLE15 epithelial cells were incubated with indicated concentration of recombinant mouse TNFα for 24 h at 37 or 39.5°C and survival assessed by crystal violet staining and measuring absorbance at 570 nm. Mean ± SE. Survival was different with $p < 0.05$ by MANOVA. (E and F) MLE15 cells were incubated for indicated time at 37° or 39.5°C with or without 2 ng/mL TNFα, lysed, and immunobloted for active caspase-3 (C3), PARP, and β-tubulin (E) or caspase-8 (F). Thick and thin arrows indicate full-length and cleaved PARP and caspase-8. *Coexposure to FRH enhances LPS-induced lung epithelial injury in vivo and accelerates TNFα-induced apoptosis in lung epithelial cells in vitro.* Reprinted in modified form with permission from references 257, 321.

Collectively, these studies demonstrate that exposure to temperatures achieved during febrile illness have many effects on gene expression, cell signaling, and cell behavior that increase leukocyte recruitment, open 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 pathologic process. Thus, ablating the thermal component of fever using COX inhibiting drugs or physical cooling may have important consequences in patients with infections or injuries. Whether or not fever should be treated will require empiric studies in patients with defined clinical states as was recently done for septic shock (348).

Fever and the Heat Shock Response

Overview of the heat shock response

While fever is a systemic response to infection and injury the HSR acts as a defense mechanism against multiple cellular

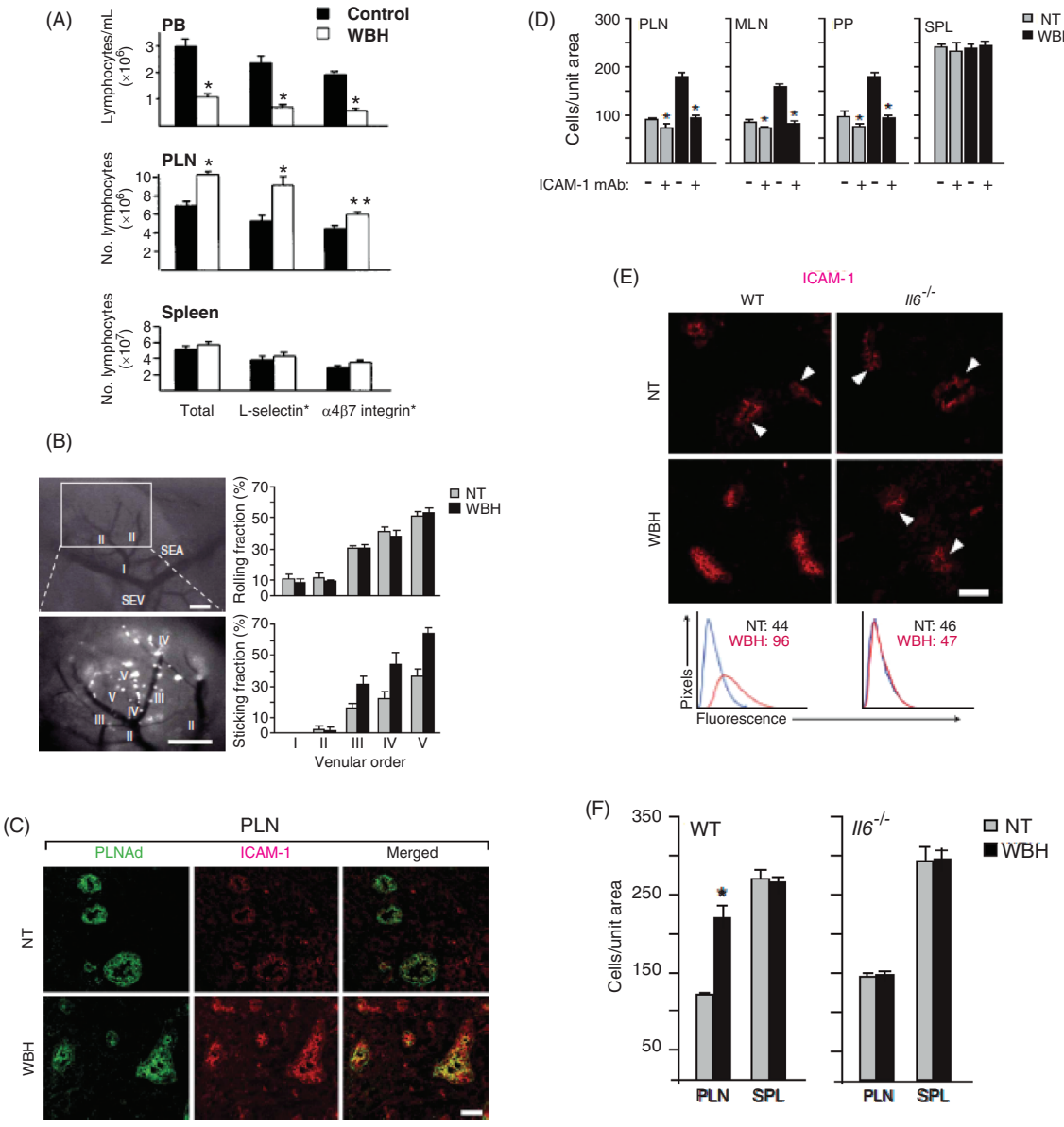

Figure 7 FRH enhances lymphocyte trafficking to the lymph high endothelial venule (HEV). (A) BALB/c mice were exposed to FRH (whole body hyperthermia, WBH) for 6 h and the total number of lymphocytes in peripheral blood (PB), peripheral lymph node (PLN), and spleen was quantified. Lymphocyte expression of L-selectin and $\alpha 4\beta 7$ integrin was analyzed by flow cytometry. Mean ±SE of three experiments. * and ** denote $p < 0.02$ and 0.03 versus control. (B) Intravital microscopy (left) of the interactions of calcein-labeled splenocytes with the lymph node venular tree of an WBH-treated mouse, showing the vascular structure, including the superficial epigastric artery (SEA), superficial epigastric vein (SEV) and venular branches (I-V) in an inguinal lymph node. Right, rolling fractions and sticking fractions in normothermic (NT) and WBH-treated mice. Mean ±SE, three mice per group. * and ** denote $p < 0.0001$, and $p < 0.01$, normothermic versus NT. (C) Expression of trafficking molecules in NT or FRH-treated mice was analyzed by scanning confocal microscopy of PLN cryosections dually stained for ICAM-1 (red) and peripheral node addressin (PNAd; green). (D) To determine whether ICAM-1 is required for the thermal enhancement of trafficking across HEVs, homing of rhodamine-labeled splenocytes was analyzed by fluorescence microscopy 1 h after adoptive transfer to NT or WBH-treated mice with (+) or without (–) pretreatment with anti-ICAM-1 blocking antibodies. Mean ± SE; 10 high-power fields per mouse, three mice per group. * denotes $p < 0.0001$ versus no antibody. (E and F) To determine the role of IL-6 in thermal augmentation of lymphocyte trafficking, the effects of WBH on ICAM-1 expression (E) and lymphocyte homing (F) were compared in wild type and IL-6-deficient mice. (E) PLNs were immunostained for ICAM-1 (red) and CCL21 (green) and analyzed by confocal microscopy. Arrowheads indicate HEVs with weak staining of ICAM-1 or CCL21. (F) Homing of rhodamine-labeled splenocytes in tissue cryosections from individual $Il6^{-/-}$ and wild-type mice with (WBH) or without (NT) WBH treatment were quantified by fluorescence microscopy. Mean ± SW, 10 fields per mouse, three mice per group. * denotes $p < 0.0001$ versus NT. *Coexposure to FRH enhances lymphocyte localization to HEVs through the IL-6-dependent expression of ICAM-1.* Reprinted in modified form with permission from references 60, 91.

stresses. The HSR, 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. This response is accompanied by reprogramming of the cellular transcriptional and translational machinery to preferentially express a set of stress-inducible proteins namely the 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 (94, 212, 251). Genes encoding the five families of HSPs are highly conserved. Their presence in all species studied to date including archebacteria, 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, heat shock protein expression is regulated at the transcriptional level by HSF. Mammals, including humans have three HSF orthologues of which HSF1 is the heat inducible orthologue (6, 238, 304). As will discussed later in this review, HSF1 function is modified by several proinflammatory kinases (64, 65, 77, 163, 189, 296, 429) and both HSPs and HSF1 can exert profound immunomodulatory effects on inflammation and host response.

Basic biology of HSF1

Overview of HSF structure and function

Eukaryotic HSFs are members of the "winged" helix-turn-helix (HNF-3/fork) family of transcription factors (421,422). All Metazoan HSFs have a conserved architecture with an N-terminal DNA binding domain that binds to a conserved nGAAn pentanucleotide (301), and two adjacent leucine zipper regions, LZ 1 and 2 (also referred to as heptad hydrophobic repeats, HR-A and B) in the N-terminal half of the molecule. The C-terminal half of HSF contains a third leucine zipper (LZ3/HR-C) and a transactivation domain (TAD) (116, 302). The LZ domains can homo-oligomerize, most likely by forming intermolecular coiled-coil structures (309, 374, 435). They may also modify the conformation of HSF monomers by forming intramolecular coiled-coil structures (93, 289, 460) that regulate the association between multiple HSF monomers or HSF and other LZ-containing proteins with potential functional consequences for both HSF and its binding partners (2, 7, 22, 32, 36, 205, 253, 299, 343, 357, 381, 445, 446, 451, 459).

Mammals express three HSFs, HSF1, 2, and 4, (260, 308, 349) of which HSF1 is the heat/stress-responsive orthologue of the single heat-inducible HSF expressed by nonvertebrate Metazoans (308). *Xenopus laevis* expresses two distinct HSFs (149, 386). A unique heat-inducible HSF, HSF3, has been found only in birds (259), has a higher thermal threshold for activation than avian HSF1 (398), and may provide a second layer of protection against severe heat stress. A recently discovered mammalian ortholg of avian HSF3 was discovered in mouse but it was only found to regulate atypical HSPs and did not affect the classical HSR or survival during heat stress (103). Plants express more than twenty HSF-like proteins (244), many of which lack a TAD and have functions that have not yet been identified (73). This section of the review will focus on the role of the heat/stress activated HSFs, including the single HSF in nonvertebrate Metazoans and mammalian HSF1 (collectively referred to as HSF for the remainder of this article), in modifying the host response to infection and injury. For additional information about other vertebrate HSFs, the reader is referred other recent reviews (249, 258).

Studies of *Drosophila* HSF and mouse and human HSF1 have shown that activation of HSF to a transcriptionally competent form is a complex, multi-step process that likely involves intrinsic stress-responsive properties of the HSF molecule as well as interactions with other stress-responsive regulatory molecules and signaling pathways (419, 461) (Fig. 8). Under stress-free basal conditions, Metazoan HSF exists largely as an inactive monomer that cannot bind stably to DNA (21, 337, 435). HSF is maintained in this form by both its intrinsic properties and by its association with heterologous proteins (23, 32, 93, 282, 338, 457-461). Earlier studies of Drosophila HSF and vertebrate HSF1 showed it to reside almost exclusively in the cytosol under stress-free conditions (21, 461) and identified an atypical cryptic nuclear localization sequence that is only expressed following exposure to stress (289). However, Mercier *et al.* (242), using immunocytochemistry and gentle cell fractionation methods, showed that a substantial portion of inactive monomeric HSF1 in mammalian cells may be loosely associated with the nucleus rather than cytosolic under stress-free conditions. Jolly *et al.* (175) showed that human HSF1 redistributes to nuclear granular structures following stress and these HSF1-containing granules specifically associate with certain chromosomal loci (174). Although the location of HSF in the unstressed cell may differ among species and cell types, the conversion from monomers that lack stable DNA binding activity to DNA-binding oligomers has been consistently found in all Metazoan cells (21, 93, 337, 338, 435, 457, 458, 460, 461).

Activation of HSF trimerization and heat shock element binding capacity

Studies utilizing native gel electrophoresis and chemical cross-linking to estimate the molecular weight of mouse (337) and human (21). HSF1 in the presence and absence of stress found an increase in apparent molecular weight of HSF1 following exposure to heat, but the results of these studies did not conclusively demonstrate trimerization stoichiometry. Considering the number of heterologous proteins that have been reported to bind to HSF, this is not surprising. However, Westwood and Wu (435), utilizing a classic biochemical approach based on gel filtration and density gradient centrifugation, presented persuasive data supporting homotrimerization of HSF in heat-exposed *Drosophila* cells. Based on these studies, it is generally accepted that HSF exists as a monomer

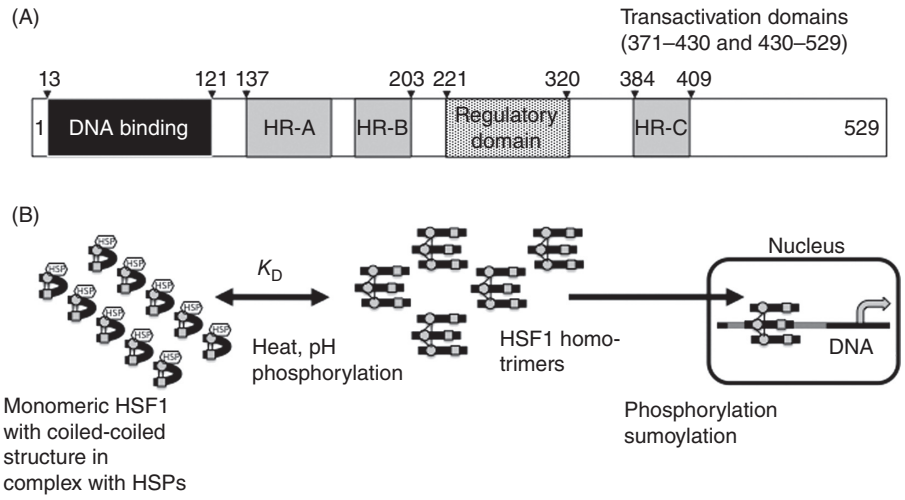

Figure 8 HSF1 structure and mechanism of activation. (A) Molecular organization of human HSF1. HR = hydrophobic region. (B) Schematic of HSF1 organization. Cytosolic HSF1 is maintained in inactive monomeric form by intramolecular interactions between HRA/B and C and heterologous binding to HSPs and other proteins. Stress (including heat) shifts the equilibrium toward trimerization, which unmasks a nuclear localization signal. The three DNA binding domains in the HSF1 trimer are oriented to produce high affinity binding to nGAAn repeats.

and homotrimerizes in response to stress in most Metazoans, including mammals (419).

Mutational analyses of HSF suggests that homotrimerization is sufficient for HSF to attain capacity for high-affinity DNA binding (93, 460, 461), which has been attributed to an orientation of the DNA-binding domains that optimizes their cooperative binding to three contiguous inverted nGAAn dyad repeats, termed the heat shock response element (HSE) in promoter sequences of target genes (301). However, the additional gain of transcriptional activating function requires further posttranslational modification of the HSF trimers (64, 65, 77, 154, 184, 191, 194, 245, 277, 372, 442, 443). Whether partially activated trimerized HSF that lacks transcription-activating function exerts specific cellular functions is not yet known. Since HSF has been shown to act through HSEs to coactivate certain non-HSP genes, such as IL-8 (364) and inducible nitric oxide synthetase (iNOS) (112), it is possible that partially activated HSF may repress expression of these genes. We have previously shown that both fully activated HSF1 in heat-shocked cells and partially activated HSF1 in cells exposed to FRT both repress TNFα expression (361, 363), which is discussed in the context of infections later in this review.

Evidence from multiple studies of HSF in cell-free lysate systems suggests a hierarchical regulation of HSF trimerization/DNA binding. Responsiveness to the most extreme physical and chemical stresses appears to derive from intrinsic properties of HSF itself. Evidence for direct temperature sensing properties of HSF was provided by studies of *Drosophila* HSF (457, 458) and mouse HSF1 (93, 338) that show high temperatures, low pH, and oxidants synergistically activate purified HSF in cell-free reactions. The temperature required to directly activate HSF in cell-free reactions differs among organisms and is typically several degrees centigrade above the normal temperature range for each organism. For example, the temperature required to directly activate mouse HSF1, 39 to 41°C (338), is higher than that required to activate *Drosophila* HSF, 28 to 36°C (458). However, interpretation of HSF behavior in cell-free reactions is complicated by potential artifacts of the experimental systems utilized in these studies. Spontaneous trimerization and activation of DNA-binding function of HSF has been reported to be concentration-dependent in both cell-free reactions (93) and within intact cells (461). Zhong *et al.* (458) demonstrated that dilution of trimerized Drosophila HSF in crude cell lysates from Schneider line-2 (SL-2) cells caused dissociation of HSF trimers to monomers over several hours. They used this model system to calculate the equilibrium constant, K_d, for the HSF trimer dissociation reaction and showed that the K_d decreases (indicating an equilibrium state favoring trimerization) with an increase in temperature (36°C) or exposure to oxidant stress with hydrogen peroxide. 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 trimerization. Importantly, this study also demonstrates that HSF trimerization may occur as a continuous temperature-dependent process that is activated over a temperature range that includes the febrile range.

It may also be possible that heterologous proteins in the eukaryotic or prokaryotic expression systems that are used to generate cell-free HSF can bind to and modify HSF conformation and function. In most cases, demonstrating similar properties of recombinant molecules generated in eukaryotic and bacterial expression systems would be sufficient to eliminate contributions of host-cell factors to the observed

molecular function. However, because the HSR pathway genes are so well-conserved among eukaryotes and prokaryotes, the possibility of residual bacterial HSPs or related proteins contributing to the behavior of recombinant HSF remains a concern. In an attempt to circumvent these potential pitfalls, Farkas *et al*. (93) showed that mouse HSF1 that had been generated in *E. coli* and purified to near homogeneity (with the exception of a 94 kD protein contaminant detectable by silver stain), formed a DNA-binding trimer after exposure to either elevated temperatures or low pH. Collectively, these data suggest that HSF itself is likely to be directly activated by exposure to high temperature and certain chemical stresses.

HSF trimerization is achieved by forming intermolecular α-helical coiled-coil structures between the LZ1/2 domains on adjacent HSF molecules (309, 374, 435). Evidence from mutational analyses of Metazoan HSF, indicates that the hydrophobic heptad repeats in all three leucine zipper domains are required to repress HSF trimerization in unstressed cells (93, 460, 461). These studies suggest that LZ3 might form an intramolecular coiled-coil structure with LZ1 and/or LZ2, thereby competing with intermolecular LZ1/2 binding. Zuo *et al.* (460) argue that the capacity of inactivating mutations in any of the three leucine zipper domains to derepress HSF trimerization suggests a triple-stranded intramolecular structure involving all three leucine zipper domains. These studies, however, did not prove that the intramolecular interaction among the three HSF leucine zipper domains was direct rather than mediated by interposed heterologous proteins. The exact mechanism through which an increase in temperature directly converts intramolecular coiled-coil structures among LZ1, 2, and 3 in monomeric HSF to intermolecular coiled-coil structures of HSF trimers is not completely understood.

Several lines of evidence suggest that the participation of other molecules and signaling pathways are important for regulating HSF activation under physiologically relevant conditions, including infections and injury. First, several activators of HSF trimerization/DNA binding in intact cells fail to activate HSF in cell-free conditions, including salicylate, dinitrophenol, ethanol, and arsenite (458). Second, when expressed in *Drosophila* SL-2 cells, human HSF1 trimerization is activated at 32 to 37°C (66), several degrees lower than the threshold for human HSF1 activation in human HEK293 cells. Interestingly in this study, *Drosophila* HSF, but not human HSF1, trimerized in human HEK293 cells cultured at basal 37°C temperatures. These results indicate that the intrinsic temperature-responsiveness of *Drosophila* HSF, as demonstrated in cell-free lysates (458), is not repressed when expressed in the human cells. Third, the thermal threshold for activation differs among different tissues in the same organism (336). Fourth, the temperature required for activation of cellular HSF can be modified within the same cell by prolonged exposure to a new basal temperature (44, 217, 235, 450) or by exposure to certain soluble factors, such as arachidonic acid or type I interferons (IFNs) (178, 250).

Multiple, nonmutually exclusive mechanisms for intracellular regulation of HSF activation have been proposed. A widely accepted model is based on the capacity of certain HSPs and related proteins, principally HSP70 (23, 282), HSP90 (459), the cochaperone p23 (32), and possibly one or more immunophilins, to bind to and prevent trimerization of monomeric, non-DNA-binding HSF1. The proponents of this model argue that as denatured proteins accumulate in stressed cells, they compete with HSF monomers for binding to HSPs. Once HSF is released from these multi-protein complexes, it spontaneously trimerizes and initiates the HS gene expression program. The data supporting this model have been recently reviewed (419).

The eukaryotic translation elongation factor eEF1A and a novel noncoding RNA, HSR1, has also been implicated in the activation process of HSF1 following exposure to heat shock (356). Both HSR1, a novel, large, noncoding RNA, and eEF1A, a key component regulating the actin cytoskeleton architecture in the cell are highly conserved and abundantly present (120, 355). It is proposed that following stress, both eEF1A and HSR1 interact with HSF1 to either promote the formation of active HSF1 trimers or favor its stabilization (356). This model is very attractive since RNA secondary structure is exquisitely temperature-dependent and easily modified by modest sequence changes. However, additional studies are required to evaluate the participation of these factors in HSF activation in response to infections and inflammation.

HSF transactivating activity

Compared with regulation of HSF trimerization, much less is known about the regulation of HSF transactivating activity. Yeast and Metazoan HSFs share a similar architecture in which a constitutively active C-terminal TAD interacts with upstream repressor domains, but the sequences comprising these domains vary greatly across species. Even in two related yeast, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, HSF TAD sequences are very different. *S. cerevisiae* HSF contains two cooperatively functioning TADs distributed over 180 amino acids whereas *K. lactis* HSF has a single 32 amino acid TAD (61). However the TADs of both yeast species contain hydrophobic heptad repeats with potential for forming coiled-coil structures. Furthermore, inactivating the LZ2 but not the LZ1 domain in yeast HSF confers constitutive transcriptional activating activity (61). Using a GAL4-based chimeric transactivation assay to map the TAD domains in *Drosophila* HSF, Wisniewski *et al.* (439) found TAD activity to reside in the C-terminal 80 amino acids and, like yeast HSF, the isolated TADs were constitutively active in a heterologous GAL4 transactivation assay. Mason and Lis (232) showed that *Drosophila* HSF, like the prototypical acidic transcription factor VP16, binds to the general transcription factor TATA-binding protein (TBP), and competes with binding of RNA polymerase II to TBP. Based on these findings, they proposed that HSF activates transcription by displacing RNA

Table 3 Protein Kinases Shown to Phosphorylate Human/Mouse HSF1, the Amino Acid Targeted and the Functional Consequences of Phosphorylation

Kinase	Amino acid target	Functional consequences	Reference number
ERK, GSK3β	S^{307}, S^{303}	Represses transcriptional transactivating activity and HSE binding	64,65, 191, 194, 442, 443
PKCαζ	S^{363}	Represses transcriptional transactivating activity	65
CaMKII	S^{230}	Activates HSE binding, translocation to stress granules, and transactivating activity	154
Casein kinase II	T^{142}	Induction of transcriptional activating activity	372
PLK1	S^{419}	Heat-induced nuclear translocation	83
JNK	TAD, S^{307}, ?	Prolongs nuclear localization of HSF, increases transactivating activity, may also repress activity in some systems	77, 189, 296, 203, 316
$P38^{MAPK}$	?	Increases HSE binding and HSP72 expression	163
RSK	?	Reduces HSE binding	428
MAPKAPK2	S^{121}	Inhibits HSE binding and increases HSF1 binding to HSP90	429

polymerase II from TBP thereby releasing it from its paused position and allowing transcription to proceed. Mason and Lis (232) also demonstrated that another transcription factor, GAGA, binds to and stabilizes HSF:DNA complex formation on the *Drosophila* HSP70 and HSP26 promoters. Using a similar assay system, Green *et al.* (116) showed that human HSF1 contained two independent TADs with molecular architecture reminiscent of the *S. cerevisiae* HSF. One TAD, comprising the C-terminal 100 amino acids, is proline- and glycine-rich and negatively charged. The other TAD is somewhat less negatively charged and overlaps with heptad repeats in the LZ3 domain. Both TAD sequences were constitutively active when analyzed in the GAL4 transactivator assay. However, inclusion of the sequence between the LZ2 and LZ3 domains with the TAD/GAL4 sequence reduced basal transcription activity and conferred heat-inducibility. Another mutational analysis suggested the presence of a regulatory domain between amino acid 221 and 310 (116). Zuo *et al.* (461) showed that mutations in the human HSF1 LZ2, but not LZ1, domain increased basal transcriptional activity and reduced heat-inducibility by 30-fold, suggesting additional regulatory activity within the LZ2 domain. Collectively, these studies suggest that TAD activity resides in the HSF C-terminus, and that it is repressed by upstream sequences under basal conditions and de-repressed in following heat exposure. Derepression is thought to result from conformational changes that unmask TAD rather than the direct modification of the TAD sequences as occurs with other transcription factors, such as NFκB. The potential role of specific phosphorylation events in mediating these effects is discussed below.

Regulation of HSF by covalent modification and heterologous protein binding

HSF function is regulated by both covalent modifications, including phosphorylation, sumoylation, and possibly oxidation, and noncovalent binding to heterologous proteins, which are discussed below.

Activation/deactivation homeostasis of HSF within cells appears to be predominantly dependent on the phosphorylation state of the molecule. Human HSF1 contains 60 serine residues, many of which are substrates for multiple kinases. By analyzing the effect of mutating potential phosphorylation sites on the behavior of HSF in cells, multiple laboratories have identified the likely consequences of several phosphorylation events (see Table 3). For example, phosphorylation of S^{230} in human HSF1 in HeLa and H1299 cells, K562 erythroleukemia cells, and transfected mouse embryonic fibroblasts enhances (154) and phosphorylation of S^{303}, S^{307}, or S^{363} in human HSF1 in HeLa and H1299 cells, THP1 human promonocytes, 3T3 fibroblasts, and transfected *Xenopus* oocytes inhibits transcriptional transactivating activity (64, 65, 77, 191, 194, 245, 277, 442, 443). Note that three of these four serines are located within the human HSF1 transcription regulatory domain identified by Green *et al.* (116). Sonocin *et al.* reported that phosphorylation of T^{142} by calcium/calmodulin-dependent protein kinase II (CaMKII) is essential for transcriptional activating activity in HeLa cells (372) and Kim *et al.* (188) showed that phosphorylation on S^{419} may contribute to HSF1 nuclear translocation following exposure to heat in HEK293 cells. In a recent study, Guettouche *et al.* (122) showed that exposure to heat induced the phosphorylation of human HSF1 on multiple serines including S^{121}, S^{230}, S^{292}, S^{303}, S^{307}, S^{314}, S^{319}, S^{326}, S^{344}, S^{363}, S^{419}, and S^{444} in HeLa cells; however, only phosphorylation on S^{326} contributed to transcriptional competency of HSF1 in this study. Collectively, these studies indicate that (1) it is the pattern of phosphorylation rather than the absolute number of amino acids phosphorylated that determines HSF functional activity and (2) HSF phosphorylation is extremely complex and the mechanisms, participating sequences, and the functional consequence of each phosphorylation event are

incompletely understood and may differ among different types of cells.

Considering the multitude of phosphorylation sites on the HSF molecule, it is not surprising that HSF is a substrate for multiple signaling kinases, including several that are activated during infections and other inflammatory states. In some studies, the kinase and the target residue have both been identified; whereas in others, use of specific kinase inhibitors and loss/gain of function have identified the participating kinase without identifying the target residue. HSF1 can be phosphorylated by ERK1/2 (64), by glycogen synthase kinase 3β (GSK-3β) (64, 65), and by protein kinases C (PKC)-α and ζ (65) on S^{307}, S^{303}, and S^{363}, respectively, in THP1 and 3T3 cells. These authors have suggested that the quiescent monomeric state of HSF1 is maintained through inhibitory phosphorylation of these specific residues at 37°C (65, 191). Conversely, phosphorylation of T^{142} in mouse HSF1 by casein kinase II (CKII) was found to be essential for induction of transcriptional activating activity in HeLa cells (372) and S^{419} phosphorylation by polo-like kinase-1 (PLK1), a kinase involved in regulation of cell cycle progression, was required for heat-induced nuclear translocation of mouse HSF1 when transfected into HEK293 cells (188). In some cases, the same kinase can exert opposing effects on HSF activation. For example, c-Jun NH2-terminal kinase (JNK) enhanced HSF1 transactivating activity in some experimental systems and inhibited activity in others (77, 189, 296). Another MAP kinase, $p38^{MAPK}$, was required for cadmium-induced HSF1 activation and HSP70 synthesis (163). Kinases that are downstream of ERK and $p38^{MAPK}$ cascades, such as ribosomal S6 kinase (RSK) (428) and mitogen-activated kinase-activated protein kinase-2 (MAPKAPK2) have also been shown to modify HFS1 functional activity by suppressing HSF1-mediated HSP transactivation (429).

The potential role of protein phosphatases in regulating HSF1 function has also been documented in various studies. For example, Mevechi *et al.* (246) reported that okadaic acid, a serine phosphatase inhibitor, increased HSF1 activation and HSP synthesis following exposure to heat, but vanadate, a tyrosine phosphatase inhibitor exerted the opposite effect. Calyculin A, another serine/threonine phosphatase inhibitor, abrogated heat-induced HSP70 expression in one study (184) and our laboratory reported that calyculin A abrogates FRT-induced repression of murine TNFα expression (362) probably by maintaining HSF1 in a hyperphosphorylated form after stimulation with the proinflammatory agonist, bacterial endotoxin lipopolysaccharide (LPS). Furthermore, Ding *et al.* (84) showed that inhibition of HSF phosphorylation by overexpression of HSP70 was mediated by activation of protein phosphatases (in addition to directly binding to HSF as discussed below), suggesting a role for phosphatases in the negative feedback regulation of HSF.

The covalent conjugation of small ubiquitin-like modifier (SUMO) to lysine residues has been shown to alter the function of a number of cellular proteins (9). Hong *et al.* showed that SUMOylation of K^{298} in human HSF1 follows phosphorylation of S^{303} and results in increased HSF1 DNA binding and transcriptional activity (157). In contrast, Hietakangas *et al.* (147) showed that mutation of K^{298} to alanine increased rather than decreased the transactivating activity of human HSF1 in Cos7 cells, suggesting that SUMOylation of this site repressed activity in these cells. Additional studies are required to better understand the full potential of this post-translational modification and its functional consequences, which may vary among different species and/or cell types.

As mentioned earlier, most studies show that oxidative stress activates HSF (293, 457, 458). Manalo *et al.* (227) showed that treating cells and cell lysates with the oxidizing agent, diamide, converted human HSF1 to a compact monomeric form that was resistant to heat-induced trimerization. However, there have been no reports of similar changes in HSF by more physiologically relevant oxidative stresses. Whether the observed activation of HSF trimerization by exposure to hydrogen peroxide (457, 458) or hypoxia/reoxygenation (293) is caused by direct oxidation of HSF itself rather than by the accumulation of oxidized cellular proteins that release HSF from HSP-containing inhibitory complexes has not yet been conclusively determined. However, in the latter case, Ozaki *et al.* (293) showed that the small GTPase, Rac, was required for HSF activation following exposure to hypoxia/reoxygenation.

HSF1 forms hetero-oligomers with several proteins including HSP70 and HSP90, each of which regulates its activity through distinct mechanisms. HSP90 maintains HSF1 in the inactive monomeric state (7, 32, 459) (see Table 4). HSP90, a ubiquitously expressed chaperone, participates in diverse signaling pathways and forms multi-chaperone complexes with client proteins that include steroid hormone receptors, kinases, transcription factors, proteins like HIP, HOP, HSP70/40, p23, immunophilin, and HSF1 (299). Several groups have shown that pharmacologic inhibition of HSP90 with geldanamycin or knockdown of HSP90 with siRNA disrupts the complex, induces HSF1 trimer formation and HSE-binding activity, and activates HSP gene transcription (7, 32, 459). While earlier studies suggested that HSP70 regulated HSF1 by a negative feedback mechanism involving formation of an HSP70/HSP40 complex that binds to and inactivates HSF1 trimers (2, 22, 253, 343, 357), subsequent studies have shown that an interaction between HSF1 and HSP70 occurs in both stressed and unstressed cells and is probably insufficient to repress HSF under normal conditions (310). Using yeast two-hybrid screening, Satyal *et al.* (341) identified a novel 8.5 kDa nuclear protein, HSF-binding protein-1, while Hu and Mivechi (160) showed that Ral-binding protein 1, formed a multimeric complex with HSF1 along with HSP90 and ß-tubulin in the absence of stress. Another protein identified by this method was the apoptosis modulator, Death-associated protein-6 (DAXX) (36). Although DAXX is generally considered to be a repressor of basal transcription, the interaction between DAXX and HSF1 enhanced the transactivation activity of HSF1 in this study (36). Other

Table 4 HSF-binding proteins and effects on HSF function

Proteins that bind to HSF	Functional consequence	Reference number
HSP90	Maintains HSF1 in the inactive monomeric state	7, 32, 459
HSP72	Maintains HSF1 in the inactive monomeric state; may recruit phosphatase leading to HSF1 dephosphorylation	2, 22, 23, 84, 253, 282, 343, 357
Cochaperone p23	Maintains HSF1 in the inactive monomeric state	32
eEF1A/HSR1	Protein/RNA complex promotes or stabilizes HSF1 trimers	120, 355, 356
GAGA	Stabilizes HSF1:HSE binding on HSP72 promoter	232
DAXX	Enhances the transactivation activity	36
STAT-1	STAT1 and HSF1 synergistically activated transcription of HSP72 and HSP90	381
NF-IL-6 (eEBPβ)	HSF1 and NF-IL6 are mutually antagonistic	445, 446
TBP and TFIIB	Participates in formation of transcription preinitiation complex	451
Cell division cycle protein, Cdc20	Blocks exit from mitosis, leading to aneuploidy	205
Nuclear pore TPR protein	Facilitate the export of HSP72 mRNA from the nucleus during stress	106
CHIP	De-represses HSF1 after HS by ubiquitinylating HSP72	76, 307, 371

transcriptional regulators, including signal transducer and activation of transcription (STAT)-1, NF-IL6, and Transcription Factor (TF)-IID, have also been shown to bind to HSF1 (381, 445, 446, 451) with variable effects on HSF1 function. For example, STAT-1 and HSF1 synergistically activated transcription of HSP70 and HSP90 (381), whereas HSF1 and NF-IL6 are mutually antagonistic (445,446). Similarly, Yuan and Gurley (451) reported that HSF1 could bind both TATA-binding protein (TBP) and the TFIIB transcription factor complex, and implied that these interactions could direct the formation of either a dysfunctional or a transcriptionally competent preinitiation complex.

Two recent studies identified new binding partners for HSF1 that suggested expanded functions for HSF. Lee *et al.* (205) reported that the regulatory domain of human HSF1 (amino-acid sequence 212-380) interacted directly with the amino-acids 106-171 of the human cell division cycle protein, Cdc20, and inhibited the interaction between Cdc27 and Cdc20, phosphorylation of Cdc27, and the ubiquitination activity of anaphase-promoting complex. Kevin Sarge's group (371) showed that HSF1 interacts with the nuclear pore-associating TPR protein in a stress-responsive manner to selectively facilitate the efficient export of HSP70 mRNA from the nucleus during stress, while the export of most other mRNAs is depressed in the stressed cell. Cam Patterson's laboratory demonstrated a role for the ubiquitin ligase, carboxy-terminus-of-HSC-70-interacting-protein (CHIP), in derepressing HSF1 following exposure to heat (76, 307). CHIP is recruited to HSF1 following heat exposure (187) and targets HSP70 for degradation via the proteosome pathway (76, 307), thereby providing an alternative mechanism for removal of HSP70 from the HSF1-containing complexes and reversal of HSF1 repression.

Collectively, these studies demonstrate that HSF1 activation is regulated through multiple steps, each of which can be affected by temperatures, soluble mediators, and protein modifying signaling events that are encountered during febrile illnesses.

Overlap between the fever and heat shock responses

Over ten years ago, we proposed that there is a partial overlap between fever and the HSR that may help shape the host response during febrile illnesses (136). We based this proposal on data showing partial activation of heat shock signaling pathways at febrile temperatures, the participation of HSF1 in the regulation of some 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 HSR and identifies HSF1 as central to the relationship between these two distinct, evolutionarily conserved processes.

Activation of HSF1 at febrile-range temperatures

The thermal threshold for activating HSF1 and inducing the HSR not only differs across species (401), but also across different cell types and tissue in the same organism (115, 336), and can be lowered further by exposure to certain inflammatory mediators (178, 250). For example, mouse lymphoid tissues, including spleen, exhibit a low thermal threshold for induction of HSP expression (115, 291), which appears to derive from T lymphocyte rather than B lymphocyte behavior (115), suggesting variable cell- and tissue-specific HSR activation that may occur at FRT.

As discussed in the previous section, HSF1 trimerization and nuclear translocation is required but not sufficient for HSP gene expression (93, 460, 461) and can be dissociated from activation of HSP gene expression (33, 69). We found that exposing RAW 264.7 mouse macrophages to FRT (39.5°C) activates HSF1 trimerization, but not transactivating activity while exposure to classic heat shock temperatures (≥42°C) activates both and HSF1 functions (363). Similarly, Laszlo *et al.* (202) 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 dissociated 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 (406). Similar to the results in the Laszlo study, exposing A549 cells for 1 h to 38.5°C, 39.5°C, or 41°C caused similar nuclear translocation of HSF1 with HSE-binding activity. 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. The relationship between the exposure temperature and maximal HSP70 protein levels was linear between 37°C and 41°C, increasing approximately 50% per degree-Celsius. However, a further one degree-Celsius increase in temperature to 42°C stimulated an additional 2.6-fold increase in HSP70 expression with little additional activation of HSE-binding activity (406). A luciferase reporter controlled by the human HSP70 gene promoter sequence exhibited a similar pattern of temperature dependence. These results suggest that 41 to 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 and its importance in febrile disease states (330, 331).

In anesthetized mice, raising core temperature to febrile-range levels (39.5°C) for 3 h was sufficient to activate HSP70 expression in liver and kidney, albeit at much lower levels than mice exposed to a classic HSR-activating temperature (42°C) for only 20 min (173). 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 (321). We have previously posited that fever, which evolved long after emergence of the HSR, coopted elements of the HSR to generate a distinct, partially overlapping process (136). The dramatic increase in HSP70 gene expression as ambient temperature increases from ≤41°C to ≥42°C supports this hypothesis.

Several studies demonstrate that the thermal threshold for HSR activation may be modified as part of adaptation to new basal temperatures. Two recent studies in humans (235, 450) have demonstrated that physiologic adaptation to recurrent exertional hyperthermia is sufficient to increase baseline HSP70 and 90 expression in peripheral blood mononuclear cells. In our own study, we showed that subjecting human subjects to a 10-day heat acclimation program consisting of daily exposure to exertional hyperthermia that achieved core temperatures up to 39.5°C for ≤ 100 min increased baseline levels of HSP70 and HSP90 and blunted *ex vivo* inducibility of HSP70 (235). Intertidal mussels (44) and goby fish (217) each exhibit a similar pattern of increased thermal threshold for HSP70 and HSP90 expression following chronic exposure to elevated body temperatures. In the case of intertidal mussels, the increase in thermal threshold for HSP70 expression occurred without an increase in the temperature threshold for HSF HSE binding activity, indicating a change in post-trimerization modification of HSF or activation of factors that synergize with HSF for HSP70 gene activation (e.g., STAT-1).

Collectively, these data suggest that HSP gene expression can occur at temperatures within the usual febrile range, that post-HSF-trimerization events play a large role in its regulation, and that the threshold for HSP gene expression may be modified by chronic exposure to elevated temperatures as may occur during prolonged febrile illnesses.

Activation of heat shock signaling by products of infection

Pathogens, pathogen-derived products, and host-derived inflammatory mediators can activate many of the kinases that regulate HSF1 activation and HSP gene expression. Mammalian toll-like receptors (TLRs) are a family of 13 highly conserved type I transmembrane pattern recognition receptors that recognize various pathogen components as well as certain host molecules that are released during tissue injury [reviewed in (42)]. Activation of all TLRs except TLR3 are transduced through a multi-protein signaling complex that includes MyD88, IRAK-1 and -4, and TRAF6, and leads to activation of all three MAP kinase cascades. Moreover, certain TLR agonists like the TLR4 agonist LPS also activate the lipid kinase phosphoinositide-3-kinase (PI3K) and PI3K-dependent kinase-1 (PDK-1). PDK-1 directly activates Akt/Protein kinase B, which leads to activation GSK-3β and RSK-1 and 2, as well as PKC-α and ζ (64, 65, 155, 163, 185, 189). As discussed earlier (Table 3), each of these kinases can phosphorylate HSF1 and modify its activation state and transactivating activity. In addition to the MyD88-dependent pathway, LPS also activates expression of IFN-β via a second, MyD88-independent pathway (100). Type I IFNs not only reduce the thermal threshold for activation (250) but may also enhance the HSR by activating STAT-1, a transcription factor that binds to HSF1 and the HSP70 and HSP90α promoters and enhances transcriptional output of both genes (381).

While the studies of TLR-mediated kinase activation suggest that post-TLR signaling in general and LPS-induced signaling in particular has the capacity to up- and down-regulate HSF transactivating activity, most empiric studies show that LPS causes an increase in expression of HSPs when applied to mammalian cells. Isolated rat lung pericytes increased HSP60 and 72 mRNA and protein levels after 18 h

incubation with LPS (86). Hirsh and co-workers (151) showed that LPS rapidly elicited the expression of HSP70 on the surface of human neutrophils, which may target them for destruction by $\gamma\delta$ T cells. Unoshima *et al.* (410) reported that administration of LPS to rats *in vivo* caused upregulation of HSP70 in splenocytes. Similarly, Ofentsein *et al.* (284) showed that intravenous administration of LPS to rats for 2 h increased levels of HSP70 in splenocytes incubated *ex vivo* at 37°C for an additional 3 h. Flohé and co-workers (101) showed that rats that received intravenous LPS 5 h prior to euthanasia exhibited increased levels of HSP70 mRNA in lung and liver compared with untreated controls and rats made LPS-tolerant prior to LPS challenge.

In addition to its intracellular functions, HSP60 and 72 have been shown to exert TLR4 agonist activity (18, 285) when present in the extracellular milieu as may occur following cellular necrosis or as a result of active HSP secretion (224,225). One can appreciate how this might activate a positive feedback loop that could enhance activation of HSPs and inflammatory mediators. Furthermore, prolonged exposure to low concentrations of extracellular HSP70 induced LPS tolerance in human THP-1 promonocytes (12), thereby demonstrating at least one mechanism through which this positive feedback loop may be interrupted. In addition, as discussed earlier, accumulation of HSP90 and 72 limits further HSP expression by blocking and reversing HSF1 activation.

Exposure to TLR agonists activate a stereotypic acute phase response in mammals comprising fever, release of the proinflammatory cytokines TNFα, IL-1β, IL-6, and IFNγ, an increase in circulating leukocytes and certain proteins (e.g. fibrinogen, serum amyloid A, albumin, and C-reactive protein) and decrease in others (albumin, transferrin, and insulin growth factor I) (106). That IL-1β stimulates the same MyD88-dependent signaling cascade as TLR agonists (209) and TNFα activates many of the same HSF1-targeting kinases as TLR agonists [reviewed in (423)] suggests these cytokines might also enhance the HSR. The few reported studies of cytokine effects on the HSR have confirmed this prediction. D'Souza *et al.* (74) reported that IL-1α, TNFα, and IFNγ each stimulate an increase in HSP70 immunostaining in human oligodendoglial cells incubated without exposure to heat. Maulik *et al.* (234) found that *in vivo* IL-1α administration caused an increase in HSP27 mRNA levels in rat heart. Overexpression of TNFα increased HSF1 HSE-binding activity and HSP70 expression and knockdown of TNFα with an antisense plasmid had the opposite effects in HeLa cells and several human pancreatic cell lines (430,431). IFNγ activates HSP72 expression in the human HepG2 liver cell line via a STAT1-dependent process (381). IL-6 enhanced heat-induced HSP70 expression in the human Huh-7 hepatoma cell line by derepressing HSF1 through the inhibition of ERK and GSK-3ß (438). Collectively, these data demonstrate that pathogens themselves, pathogen-derived products, and multiple components of the host acute phase response, including fever and multiple proinflammatory cytokines, can activate or enhance HSF1 activation and HSP expression.

Heat shock 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 (see Table 5). The clinical studies of HSR in human sepsis are small and utilize different methods but generally show that HSP expression is higher in patients with sepsis. Hashiguchi *et al.* (138) analyzed HSP27, 60, 70, 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.* (83) found the proportion of peripheral blood mononuclear cells (PBMCs) expressing HSP70 as assessed 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 HSP70 in serum were also elevated in patients with sepsis, including children with septic shock (281, 436).

Most studies of animal models of infections also demonstrate that host HSP expression is increased during infections. In mice infected with *Francisella tularensis*, the causative agent of tularemia, peritoneal macrophages exhibited increased HSP70 levels but not until three days after infection (385). *Trichinella* infection in rats is associated with increased levels of HSP25, 60, and 70 protein in spleen and brain, increased HSP25 protein levels in liver, and increased HSP25 and 60 levels in muscle (230, 231). On the other hand, Singleton *et al.* (370) reported reduced HSP25 and 70 expression in lung 24 h after sepsis induced by cecal ligation and puncture. Weiss *et al.* (433) reported that HSP72 mRNA and protein levels in lung did not increase up to 48 h after cecal ligation and puncture in mice. Collectively, these data suggest that the observed effect of infections on HSP expression may depend on the timing relative to infection, the type of infection, and the tissue affected. Zheng et al. (456) showed that exposing cultured human neutrophils to *E. coli* or *S. aureus* induced apoptosis, but unlike neutrophils that were made apoptotic by exposure to ultraviolet irradiation, the pathogen-exposed neutrophils generated increased expression levels of HSP60 and 70. Furthermore, the bacteria-exposed neutrophils induced classic rather than alternative macrophage activation following phagocytosis. As the shift to alternative macrophage activation is important for the resolution of inflammation (113, 114), this effect might prolong the inflammatory phase of infection. Infections also cause elevated HSP expression in lower vertebrates, including in sea bream infected with *Vibrio alginolyticus* (82) and the western painted turtle (*Chrysemays picta bellii*) with septicemic cutaneous ulcerative dermatitis in which HSP72 levels were increased in brain and liver (312).

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 (196), respiratory syncitial virus in A549 cells (38), and adenovirus in B16 melanoma cells (239). HSR activation has also been found

Table 5 Alterations in HSF1 Activation and HSP Expression in Infections and Injury

Effect on HS	Clinical condition	Reference number
Clinical studies		
Increased HSP27, 60, 72, 90 in neutrophils	Sepsis	138
Increased HSP72 in PBMC	Sepsis	83
Increased serum HSP72	Acute infections, septic shock	281, 436
Activation of HSF1 HSE-binding in PBMC	Human pancreatitis	283
Increased HSP72 in neutrophils	Trauma	137
Increased HSP32, 72, 90 in PBMC	Systemic inflammation	279, 280
Increased HSP72 in cardiac tissue	Postcardiac surgery	236, 394
Animal models	**Stimulus**	
Increased HSP72 in peritoneal macrophages	*Francisella tularensis* infection	385
Increased HSP25, 60, 72 expression in spleen, liver, and muscle	*Trichinella*-infected rats	230, 231
Reduced HSP25, 72 levels in lung	Mouse CLP	370
No change in HSP72 levels in lung	Mouse CLP	433
Elevated HSP72 in liver	Vibrio alginolyticus-infected sea bream	82
Elevated HSP72 in brain and liver	Septicemic cutaneous ulcerative dermatitis in Western painted turtle	312
HSP72, 25 expression and activation of HSF1 HSE-binding in pancreas	Mouse model of cerulean-induced pancreatitis	90
Increased HSP72 in lung	Mouse undergoing surgery (sham CLP)	433
Cell culture models	**Stimulus**	
Increased HSP90, 25	EBV infected human B lymphocytes	196
Intranuclear HSP60, 72 expression	RSV infected A549 cells	38
HSP72 expression	Adenoviral-infected B16 melanoma	239
HSP72 expression	E. coli, S. aureus-infected human neutrophils	456

to occur in noninfectious inflammatory disorders, including human pancreatitis in which HSF1 was found to be activated (283) and a mouse model of cerulean-induced pancreatitis in which elevated pancreatic levels of HSP72 and 25 protein and activated HSF1 were detected (90). Hashiguchi *et al.* (137) demonstrated that neutrophils obtained from trauma patients had higher levels of HSP72 than cells from control subjects. Njemini *et al.* (279) reported that PBMCs obtained from patients with inflammation, based on elevated circulating levels of C-reactive protein and IL-6, expressed higher levels of HSP32, 72, and 90 than cells from control patients. In their study of HSP expression in the mouse cecal ligation and puncture, Weiss *et al.* (433) 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 HSR in mice. A similar association of HSP expression and surgery has been demonstrated in human cardiac surgery in which cardiac expression of HSP72 was detected postoperatively (236, 394).

Extended activities of HSF1 and the immune response

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* (368) and extra-embryonic development (444) suggest a much broader range of biological functions than previously thought (Table 6).

The concept that HSF1 might have additional functions was initially suggested by Westwood *et al.* (434) who used *in situ* hybridization analysis to show that exposure to heat 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.* (403) 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

Table 6 Additional Functions of HSF

Biological effect of HSF	Reference number
Information from HSF1 deletion in animal models	
Increases expression of LIX in murine lung	364
Normal extra-embryonic murine development	444
Normal murine reproductive behavior and female fertility	63, 171, 444
Normal postnatal murine brain development	333
Maintenance of murine olfactory epithelium	396
Ciliary beating in murine respiratory epithelium, oviduct	397
Limits LPS-induced TNFα expression	67
Increases respiratory epithelial injury in LPS-challenged mice	321
Resistance to *E. fecaelis* infection in *C. elegans*	368
Information from *in vitro* studies	
Increases expression of IL-8 (and likely other CXC chemokines)	256, 364
Represses expression of human prointerleukin-1, c-fos, and urokinase-type plasminogen activator genes	47, 445
Represses expression of c-fos	447
Represses TNFα expression	361, 363
Enhanced expression of inducible nitric oxide synthase	112
Optimizes IL-6 expression by altering chromatin architecture	168

CXC chemokine genes showed that the promoter regions of almost all mouse and human CXC chemokine genes contained multiple potential HSE consensus sequences (256). 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 (223, 364). Additional studies, using cDNA microarrays to analyze the gene expression pattern activated by heat exposure confirmed that such exposure also modifies expression of several non-HSP genes, including those involved in regulation of transcription, growth, DNA repair, apoptosis, signaling, and cytoskeletal function (85, 254, 373). More recently, Mendillo *et al.* (240) showed that HSF1 was activated under basal conditions in cancers with high tumorigenic and metastatic potential but not in other cancers and, using high throughput chromatin-immunoprecipitation-sequencing (ChIPseq), that HSF1 was recruited to about 500 genes, many of which are distinct from those induced by heat exposure and some of which are downregulated by HSF1.

Studies with the HSF1 knock-out mouse developed by Ivor Benjamin's group confirmed that HSF1 is the major regulator of the HSR (238) and also demonstrated that HSF1 participates in the regulation of extra-embryonic development, growth, and endotoxemia-induced systemic inflammation (444), female (63, 444) and male (171) reproductive potential, the ubiquitin proteolytic pathway (303), postnatal brain development (333), in the maintenance of olfactory epithelium (397) and in ciliary beating in the respiratory epithelium, ependymal cells, oviduct, and the trachea (396), and tumorigenesis (75).

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 exposure to heat, HSF1 mediates transcriptional repression of human prointerleukin-1β, c-fms, and c-fos genes (47, 445-447) through quenching of other transcription factors, most notably NF-IL6/c/EBPβ. In our studies, we found that HSF1 was activated at FRT (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 (361-363). Interestingly, we found that exposure to FRT also represses TNFα gene expression by selectively blocking recruitment of NFκB and Sp1 to the TNFα proximal promoter sequence (67, 68). In further support of HSF1 as a negative regulator of TNFα expression, HSF1-null mice exhibited higher circulating levels of TNFα expression after intraperitoneal challenge with LPS (444) and higher levels of TNFα in lung lavage after intratracheal LPS challenge (67). Activated HSF1 has also been found to repress human CXCL5 (223) and the proapoptotic factor, xIAP-associated factor 1 (XAF1) (424).

As mentioned above, we analyzed the effect of heat exposure on the expression of interleukin IL-8. Exposure to a classic HSR-activating temperature enhanced TNFα-induced IL-8 secretion in human A549 epithelial cells but unlike classic HSPs, exposure to heat alone was not sufficient to activate IL-8 expression (364). Using electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) assays, we identified two IL-8 promoter regions, approximately 800 and 1200 nt upstream of the transcription start site, that bound active HSF1. Using a 5′-deletion mapping strategy and siRNA knockdown of HSF1, we showed that the interaction of HSF1 with both of these promoter regions contributed to the increased IL-8 expression in cells cotreated with IL-8 and heat. Goldring *et al.* (112) found that heat-activated HSF1 has a similar coactivator function for the murine iNOS gene. Inouye *et al.* (168) 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 nonclassical heat shock genes, COX-2 and the zinc finger AN1-type domain-2a gene, Arsenite-inducible RNA-associated protein (AIRAP), that exhibit heat-inducible transcription similar to those of canonical HSPs (328, 329).

HSF1 may also exert additional biological effects by binding to and modifying function of proteins involved in diverse cellular processes, including HSPs (365), the nuclear pore

forming TPR protein through which HSP72 is secreted (371), other transcription factors (367, 446), components of the TFIIB transcription complex (451), the cell division cycle protein, Cdc20 (205), the apoptosis modulator DAXX (58), and the multidrug exporter, Ral-Binding Protein (Ralbp)-1 (369). Collectively, these studies illustrate the broad range of important biological functions of HSF1. The potential impact of alterations in HSF1 expression level and genetic variations in HSF1 are discussed in the following sections.

Overlap between the HSR and TLR signaling

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 proinflammatory agonists for TLR2 and 4 (48, 78, 413). Although some earlier studies raised concerns that the TLR4 agonist activity of recombinant Hsp70 preparations was caused by LPS contamination (108, 404), more recent studies showing TLR4-activity expressed by recombinant Hsp70 prepared in insect cells and by nonrecombinant Hsp70 as well as the inclusion of classic LPS controls demonstrate that HSP70 exerts proinflammatory TLR4 agonist and macrophage activating activities of Hsp70 protein that are intrinsic to the molecule and not caused by artifacts of its preparation (12, 17, 418, 426, 455).

In the initial reports by Hightower and Guidon (148) and later by Hunter-Lavin *et al*. (164), both groups showed that Hsp70 release occurred from healthy uninjured cells independent of cell death. Basu *et al.* (27) showed that bioactive HSP70 was also released from necrotic cells but not from cells undergoing apoptosis. Mambula *et al.* (224) showed that HSP70 was released from prostate cancer cells via both necrosis and active secretion. These studies demonstrate that HSPs can not only reach the extracellular milieu by leaking from necrotic cells, but can also be actively secreted by viable cells. However, HSPs lack a classical consensus signal required for secretion and HSP secretion. Moreover, HSP secretion is not blocked by typical inhibitors of the ER-Golgi pathway, such as brefaldin A (164), suggesting that HSP secretion occurs through nonclassical mechanisms such as those used by IL-1α and -1β, high mobility group box-1 (HMGB1), macrophage migration inhibitory factor (MIF), and fibroblast growth factor 2 (FGF2) (225, 226). There is some evidence that Hsp70 release may occur via a lysosome-endosome pathway in which case Hsp70 translocates into lysomes via an ATP-binding cassette (ABC) transport-like system and is then exported from the cell via the endocytic process (225, 226). Hsp70 has also been proposed to be released by secretory-like granules (92) and via membrane export vesicles (418). Collectively, these studies suggest that Hsp70 is released actively by nonclassical active secretion and passively as a result of cellular necrosis but not apoptosis. These mechanisms may explain the elevated serum levels of HSP72 found in patients with sepsis (111, 281, 436), acute lung injury, (107), chronic inflammatory diseases (280), and trauma (305). As discussed earlier, once HSPs reach the extracellular space, they can exert proinflammatory TLR agonist activity.

While extracellular HSPs can activate TLR signaling, recent *in vitro* and *in vivo* studies suggest that TLR agonists, particularly LPS, can activate expression of HSPs in mammalian cells. Edelman *et al.* (86) 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 (151) 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 upregulation of HSP70 in splenocytes (284, 411) and increased levels of HSP70 mRNA in lung and liver compared with rats previously subjected to an endotoxin tolerance protocol (101).

The release of HSP60 and 70 into the circulation during infection, inflammation, and trauma (107, 111, 280, 281, 305, 436) combined with its TLR agonist activity (12, 17, 48, 78, 413, 418, 426, 455) suggest a positive feedback mechanism that might amplify inflammation. Recently, we found an interaction between TLR agonists and FRT that further enhances this proinflammatory amplification loop. We found that coexposure to TLR agonists or IL-1ß, which shares the same downstream MyD88-dependent signaling pathway as TLRs, synergizes with exposure to FRT to greatly augment Hsp70 synthesis and secretion in the RAW 264.7 mouse macrophage cell line (125). The increase in HSP expression is mediated through a p38 MAP kinase-dependent signaling pathway leading to increased histone H3 phosphorylation and HSF1 recruitment to the Hsp70 chromatin. The mechanism by which coexposure to TLR agonists and febrile temperature increase Hsp70 secretion is not yet known, but occurred without evidence of cytotoxicity. Similar synergism between TLR agonists and febrile temperatures for HSP70 expression and secretion were seen in IL-1ß-stimulated human A549 cells *in vitro* and in an intratracheal LPS-challenge mouse model of acute lung injury *in vivo* (125). 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 (298), we propose that the synergism between fever and TLR agonists for synthesis and release of HSP70 promotes a proinflammatory vicious cycle that may contribute to the negative consequences of fever in high acuity disease (Fig. 9).

Genetic Variations of the Heat Shock Pathway and Potential Biological and Clinical Consequences

As discussed in the previous sections, HSF1 regulates expression of a broad range of genes, including those involved in

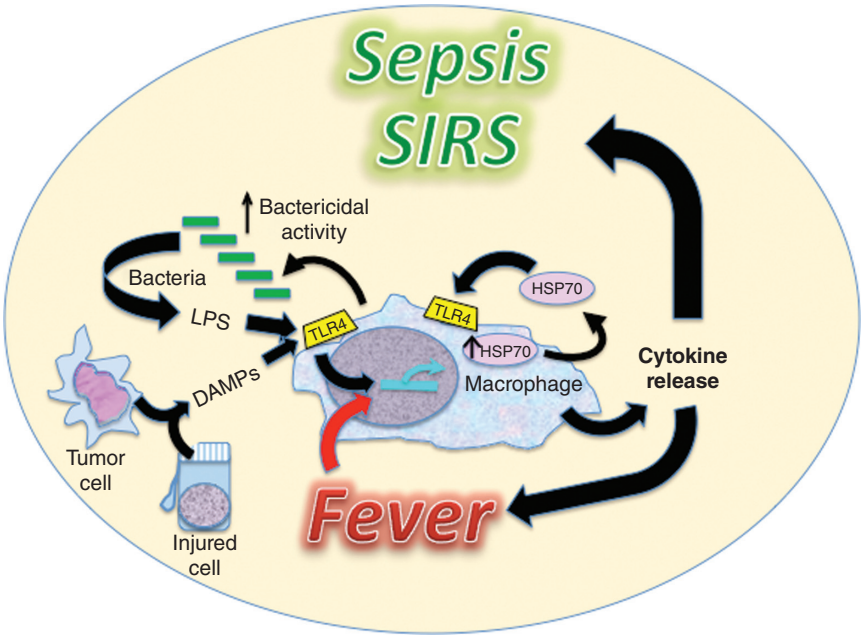

Figure 9 Model of how fever, LPS, and Hsp70 interact to cause sepsis. Proposed model of sepsis in which LPS and fever initiate a positive feedback pathway through enhanced Hsp70 expression and release and subsequent increased TLR4 activation, Hsp70 expression, and proinflammatory cytokine release. Reprinted in modified form with permission from reference 125.

host defense, inflammation, and tumorigenesis (47, 67, 68, 112, 168, 223, 240, 328, 361-364, 424, 445-447) as well as exerting additional effects by directly binding to proteins critical for cell proliferation, survival, and death (365, 371), other transcription factors (58, 205, 367, 369, 446, 451). Studies from heterozygous mice suggest that the level of HSF1 expression may affect capacity for expression of some chemokines (364) and modify risk of tumor progression (75). 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 HSR, especially HSF1, and the potential impact on human health and disease.

Single nucleotide polymorphisms (SNPs) have been identified in human HSP40, HSP60, HSP70, and HSP90 and the following associations have been reported: HSP40 SNPs with hyperandrogenic and metabolic dysfunction in women (176); HSP60 SNPs with coronary heart disease and sudden infant death (142, 311); HSPA1A and B SNPs with urinary tract malformations, systemic lupus erythematosus, childhood acute lymphoblastic leukemia, severity and outcome of diabetic foot ulcer, schizophrenia, high altitude pulmonary edema, coronary heart disease, Meniere's Disease, essential hypertension, longevity, preeclampsia, and septic shock (96, 97, 105, 140, 181, 186, 206, 207, 243, 294, 306, 332, 366, 375, 408); HSPA8 SNP with coronary heart disease (141); and HSP90 SNPs with bipolar disease and male infertility (139, 179). While specific studies of human HSF1 genetic variation have not yet been performed, genome-wide association studies (GWAS) have linked 8q24.3, the location of the HSF1 gene, to schizophrenia, bipolar disorder (425), and attention deficit hyperactivity disorder (201), suggesting HSF1 genetic variation may contribute to neuropsychiatric disease.

Recently, Li *et al*. (208) reported two novel SNPs in the HSF1 gene that are disproportionately associated with thermal tolerance in Chinese Holstein cattle, including one SNP in the 3′-untranslated region (3′UTR) that disrupts a potential microRNA (miRNA)-binding sequence in HSF1 (208) is disproportionately associated with heat tolerance in Chinese Holstein cows. Huang *et al.* (161) found a SNP in a mature microRNA, mIR-608, that is predicted to weaken its interaction with its target sequence in human HSF1 3′UTR, and which is associated with increased Human epithelial growth factor receptor-2 (HER2)-positivity and larger size of human breast cancers. While a reduction in HSF1 protein expression that might be caused by reduced miRNA targeting is consistent with HER2-positivity and larger tumors (241), the effect of this SNP on HSF1 protein level has not been confirmed experimentally.

The occurrence of SNPs in the human HSF1 gene has not yet been systematically analyzed. To begin to understand the potential biological and clinical importance of SNPs in the HSF1 gene, we analyzed the human HSF1 gene for SNPs by mining the NCBI dbSNP database and performing exonic sequencing from anonymous genomic DNA samples. DNA 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 causes a proline-to-threonine missense at amino acid 365 adjacent to LZ3 and one causes a frame-shift replacement of the 26-amino acid C-terminal TAD. Direct sequencing confirmed the P365T SNP and identified two novel 5′UTR and two novel 3′UTR SNPs (41). Four of the five 3′UTR SNPs alter predicted miRNA target sequences as identified using the MicroSNiPer on-line program (26) and both of the 5′UTR SNPs alter the 5′UTR secondary structure predicted using the RNAFOLD on-line program (121). The frequency of these and potentially other HSF1 SNPs and their participation in disease pathophysiology are not yet known.

Conclusion

The strategy and mechanisms of fever in the infected and injured host are evolutionarily old and well-conserved. As fever evolved, it incorporated elements of older biological responses including the HSR, the innate immune response, and signaling pathways including MAP kinases and others. We presented the experimental evidence that the temperature increase during fever is a potent biological response modifier with myriad effects on other elements of the immune response, which have important consequences for survival. We discussed the evidence for multiple overlaps between the heat shock and febrile responses, including overlapping temperature ranges and mutual cross-regulation. We describe the potential of a positive-feedback mechanism in which fever and TLR agonists synergize to augment expression and secretion of HSP70, which through the TLR agonist activity of extracellular HSP70 can potentiate a vicious proinflammatory cycle. We showed how HSF1 plays a central role in heat shock, febrile, and immune responses and discussed the potential importance of HSF1 genetic variations. The biological processes discussed have great clinical significance as fever is a common feature of infectious and inflammatory disease and traumatic injury, the effect of fever depends on the site and nature of the disease process, and little empiric evidence is available to establish the benefit or harm of fever is different disease states.

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