Heat Shock Co-Activates Interleukin-8 Transcription

Ishwar S. Singh^{1,2,4}, Aditi Gupta¹, Ashish Nagarsekar¹, Zachary Cooper¹, Cheu Manka¹, Lisa Hester^{1,3}, Ivor J. Benjamin⁵, Ju-ren He¹, and Jeffrey D. Hasday¹⁻⁴

¹Division of Pulmonary and Critical Care Medicine, Department of Medicine, ²Mucosal Biology Research Center, and ³Cytokine Core Laboratory, University of Maryland School of Medicine; and ⁴Medicine and Research Services of the Baltimore VA Medical Center, Baltimore, Maryland; and ⁵Department of Medicine, University of Utah School of Medicine, Salt Lake City, Utah

The heat shock (HS) response is a phylogenetically ancient cellular response to stress, including heat, that shifts gene expression to a set of conserved HS protein (HSP) genes. In our earlier studies, febrile-range hyperthermia (FRH) not only activated HSP gene expression, but also increased expression of CXC chemokines in mice, leading us to hypothesize that the CXC chemokine family of genes might be HS-responsive. To address this hypothesis we analyzed the effect of HS on the expression of IL-8/CXCL8, a member of the human CXC family of ELR⁺ chemokines. HS markedly enhanced TNF- α -induced IL-8 secretion in human A549 respiratory epithelial-like cells and in primary human small airway epithelial cells. IL-8 mRNA was also up-regulated by HS, but the stability of IL-8 mRNA was not affected. TNF- α -induced reporter activity of an IL-8 promoter construct IL8^{-1471/+44}-luc stably transfected in A549 cells was also enhanced by HS. Electrophoretic mobility and chromatin immunoprecipitation assays showed that the stress-activated transcription factor heat shock factor-1 (HSF-1) binds to at least two putative heat shock response elements (HSE) present in the IL-8 promoter. Deletional reporter constructs lacking either one or both of these sites showed reduced HS responsiveness. Furthermore, depletion of HSF-1 using siRNA also reduced the effects HS on TNF- α -induced IL-8 expression, demonstrating that HSF-1 could also act to regulate IL-8 gene transcription. We speculate that during evolution the CXC chemokine genes may have co-opted elements of the HS response to amplify their expression and enhance neutrophil delivery during febrile illnesses.

Keywords: neutrophil; hyperthermia; IL-8; chemokine; heat shock factor-1

The heat shock (HS) response is a phylogenetically ancient cellular response to exogenous stress, including high temperatures, that shifts gene expression to a set of evolutionarily conserved HS proteins (HSPs) (1). In eukaryotes, HSP genes are regulated by HS-activated transcription factors (HSFs), which bind cis-acting HS response elements (HSEs) comprising inverted dyad nGAAn repeats (2). Of the three mammalian HSFs, HSF-1 is activated by exposure to HS (3) and required for HS-induced HSP72 expression (4). cDNA microarray (5–7) and *in situ* hybridization (8) studies indicate that HS-induced expression of genes is not limited to only the HSP family of genes.

We have previously identified overlaps between the HS response to exogenous thermal stress, and fever, a state of regulated, endogenous hyperthermia (9). We have shown that temperatures within the usual febrile range are sufficient to activate HSF-1 and HSP expression *in vitro* and *in vivo* (10–13). We have also shown that whole body febrile range hyperthermia

CLINICAL RELEVANCE

We have identified IL-8 as a new heat shock-responsive gene, described a novel pattern of heat shock responsiveness, and showed that the IL-8-like chemokine, LIX, exhibits the same pattern of heat shock responsiveness in a mouse lung injury model.

(FRH; core temperature $\sim 39.5^{\circ}\text{C}$) augments neutrophil recruitment, and accelerates pathogen clearance in diverse animal species (13–16), but also increases collateral tissue injury. In mouse models of pneumonia (13) and pulmonary oxygen toxicity (15), FRH increases neutrophil recruitment to the affected lungs by augmenting expression of the glutamic acid-leucine-arginine-containing (ELR⁺) CXC chemokines, which play a central role in neutrophil recruitment and activation.

We have reported that the 5'-flanking sequences of these genes, including the major human ELR⁺ CXC chemokine, IL-8/CXCL8, contain multiple HSE-like sequences, the functional significance of which is unknown (17). In this study we tested the hypothesis that IL-8 is an HS-responsive gene. Using the human respiratory epithelial-like A549 cell line and primary human small airway epithelial cells (SAECs), we provide direct evidence that HS co-activates IL-8 gene expression through a novel mechanism that requires HSF-1, multiple HSE-like sequences in the IL-8 promoter, and co-exposure to a proinflammatory agonist. We show that expression of the IL-8-like chemokine, LPS-induced CXC chemokine (LIX) in the LPS-challenged mouse lung, is enhanced by exposure to FRH in wild-type, but not HSF-1-deficient mice. These results identify an overlap between the HS response and regulation of inflammation that may have important consequences during febrile illnesses.

MATERIALS AND METHODS

Cell Culture

The A549 cell line was purchased from the American Type Cell Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 supplemented with 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (Gibco/Invitrogen, Carlsbad, CA), pH 7.3 (CRPMI) and containing 10% defined fetal bovine serum (FBS; Hyclone, Logan, UT) at 37°C in 5% CO_2 -enriched air. Human SAECs and AEC culture medium were purchased from Cambrex (Gaithersburg, MD) and cultured on plastic as described in the manufacturer's protocol. Rabbit anti-human HSF-1 was obtained from Santa Cruz Biotechnology (H311; Santa Cruz, CA). Nonimmune rabbit IgG was purchased from Sigma-Aldrich (St. Louis, MO).

IL-8 Secretion

For measurement of IL-8 secretion, A549 or SAEC cells were plated in 24-well culture plates at 5.0×10^4 cells/ml/well 24 hours before stimulation with TNF- α . Cells were stimulated with recombinant human TNF- α (R&D Systems, Minneapolis, MN) for 15 minutes at 37°C and then either were incubated at 37°C for an additional 6 hours (control) or

(Received in original form July 31, 2007 and in final form January 19, 2008)

This work was supported by NIH grants GM069431 (I.S.S.), GM066855 and HL69057 (J.D.H.), and VA Merit Review Awards (I.S.S. and J.D.H.).

Correspondence and requests for reprints should be addressed to Jeffrey D. Hasday, M.D., Health Science Facility-II, rm. S347, University of Maryland School of Medicine, 20 Penn St., Baltimore, MD 21201. E-mail: jhasday@umaryland.edu

Am J Respir Cell Mol Biol Vol 39, pp 235–242, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-02940C on March 26, 2008

Internet address: www.atsjournals.org

were heat-shocked by incubating for 2 hours at 42°C then returned to 37°C for an additional 4 hours. IL-8 concentration in culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) using paired antibodies and a recombinant human IL-8 standard from Biosource (Camarillo, CA) in the Cytokine Core Lab at the University of Maryland. The lower detection limit of this assay is 1 pg/ml.

Real-Time RT-PCR

A549 cells were plated at 2×10^5 cells/well in 35-mm plates, and 24 hours later the cells were exposed to TNF- α and HS using the same protocol as was used for the IL-8 secretion studies. Total RNA was isolated using TRIzol (Invitrogen) and 1 μ g of total RNA was reverse transcribed using the Reverse transcription kit from Promega (Madison, WI). Real-time PCR was performed in a LightCycler using LightCycler FastStart DNA Master SYBR Green I kit (Roche, Indianapolis, IN) according to the manufacturer's protocol. Primers for IL-8 and GAPDH were purchased from Real Time PCR Primers.org. The relative changes in IL-8 mRNA level was calculated using the Delta(CT) method described by Livak and Schmittgen (18).

Analysis of IL-8 mRNA Stability

Transcription in TNF- α -stimulated A549 cells exposed to HS (42°C for 2 h) and control cells was arrested by adding 5 μ g/ml actinomycin D (Sigma) dissolved in ethanol. The cells were then incubated at 37°C, total RNA was collected 0.5, 1, 1.5, 2, and 2.5 hours after addition of actinomycin D and IL-8 was mRNA quantified by real-time RT-PCR as described above. IL-8 levels were normalized to levels of GAPDH, a stable housekeeping transcript. Curve fitting, assuming first-order decay, was performed using a commercial software package (SPSS; Deltagraph, Chicago, IL) and the half life, $T_{1/2}$, was calculated from the decay constant, k , using the equation $T_{1/2} = 0.693/k$.

IL-8 Reporter Plasmid Assays

An IL-8 reporter construct IL8^{-1471/+44}-luc was generated by PCR amplification of 5'-flanking sequence comprising nucleotides -1471 to +44 (relative to the transcription start site) from human genomic DNA and cloning into the pGL3 basic vector (Promega). IL8^{-1081/+44}-luc and IL8^{-762/+44}-luc were generated by PCR amplification of the respective fragments from IL8^{-1471/+44}-luc and cloning into the pGL3 basic vector. For transient transfections A549 cells plated at 2×10^5 cells/well in 35-mm plates were transiently transfected with IL-8 reporter constructs (1 μ g plasmid) and pHRL-TK renilla control vector (0.1 μ g plasmid) using FuGene6 (Roche), and 48 hours later the cells were exposed to TNF- α and HS using the same protocol as was used for the IL-8 secretion studies. Cells were lysed in passive lysis buffer (Promega) and assayed for firefly and renilla luciferase activity using the Dual Reporter Assay kit (Promega). For stable transfections, A549 cells were co-transfected with IL8^{-1471/+44}-luc and the blasticidin resistance plasmid, pcDNA6/TR (Invitrogen), using Fugene6, selected using 4 μ g/ml of blasticidin (Invitrogen), and maintained in media containing 2 μ g/ml blasticidin. For reporter assays, 1×10^5 cells/ml were plated in blasticidin-free medium and 24 hours later were exposed to TNF- α and HS and luciferase activity was analyzed using a commercial luciferase assay kit (Promega).

Immunoblotting

A549 cells were plated at 4×10^5 cells/well in 60-mm plates, and 24 hours later the cells were subjected to HS (42°C for 2 h) followed by incubation at 37°C for an additional 4 hours. The cells were lysed in cell culture lysis buffer, resolved on 10% SDS-polyacrylamide gels, and transferred to PVDF membrane. The membranes were blocked for 1 hour at room temperature in blocking buffer (TBS-T [10 mM Tris-HCl, pH7.5, 136 mM NaCl, 2.0 mM KCl, 0.1% Tween 20] containing 5% nonfat dry milk). After blocking, the membranes were washed with TBS-T, and incubated with antibodies against HSP72 (StressGen; Assay Designs, Ann Arbor, MI) or β -tubulin (Chemicon, Billerica, MA) in blocking buffer for 2 hours. After primary antibody reactions, the membrane was washed with TBS-T, incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody and developed with a chemiluminescence detection system (Renaissance; New England Nuclear, Boston, MA), quantified by direct imaging (Fuji gel documentation system and

ImageGauge software; Fuji, Tokyo, Japan) and subsequently exposed to X-ray film.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared according to Schreiber and coworkers (19), and electrophoretic mobility shift assay (EMSA) was performed using double-stranded oligonucleotide probe containing the heat shock response element (HSE) corresponding to -107/-83 of the human HSPA1A (HSP72) promoter 5'-GATCTCGGCTGGAATATTCCTGACCTGGCAGCCGA-3' as we have described earlier (10), or a commercial NF- κ B probe (Promega), or one of three probes each containing one of the putative HSEs in the IL-8 5'-flanking sequence: -1213 to -1182 nt: ccaaGACcTTCaaGAAagGTCTtGGAttctt, -1151 to -1120 nt, tttttatgatTTGttGAAatTTCtactccat, and -813 to -782 nt, ctgcctttgGAAGaTTCtgCTCttatgcctcc (central core of dyad repeats in bolded capital letters). The DNA-protein complexes were then electrophoretically resolved on 4% nondenaturing polyacrylamide gels, and the dried gels were analyzed by phosphorimaging (Molecular Dynamics, Pittsburgh, PA) and subsequently exposed to X-ray film.

siRNA Transfections

Validated siRNA targeted against human HSF-1 was obtained from Ambion (Austin, TX). A549 cells were plated at 2×10^5 cells/well in 35-mm plates 24 hours before transfection. RNA duplexes were transfected using Dharmafect 1 (Dharmacon, Lafayette, CO) according to the manufacturer's protocol, transferred to 24-well plates in CRPMI 24 hours later, and after an additional 24 hours were exposed to TNF- α and HS as described above and IL-8 concentration in culture supernatants was measured by ELISA.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed using a kit from Upstate Biotechnology (Lake Placid, NY) as we have previously described (10). After exposure to 2 ng/ml TNF- α alone, HS (42°C) alone, or the combination of TNF- α + HS for 1 hour, A549 cells were fixed by adding formaldehyde (Sigma) to the medium to a final concentration of 1%. After 15 minutes the cells were washed, resuspended in SDS lysis buffer, and sonicated. Immunoprecipitation was performed at 4°C overnight using 2 μ g anti-HSF-1 antibody or nonimmune rabbit IgG, and immune complexes were washed, eluted, and protein-DNA cross linking was reversed according to the manufacturer's protocol. PCR was performed (36 cycles, denaturing at 94°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s) using primers specific for the human IL-8 sequence between -861/-642 (forward 5'-tcattctctctattgaagcc-3' and reverse 5'-tctgagtaattgtggggatct-3') and for sequences between -1291/-1030 (forward 5'-attatgtactgtccagagc-3' and reverse 5'-aactctgtgtcagcactgtt-3'). As a positive control, immunoprecipitated DNA was also amplified using PCR primers specific for an HSE-containing 180-nt fragment of the murine HSP70 promoter: 5'-aactccgattactcaaggagc-3' (forward) and 5'-gattctgagtagctgtcagc-3' (reverse) using the same PCR conditions as for IL-8 except for a 60°C annealing temperature.

HSF-1-Null Mice

HSF-1-null mice used for the study were from a colony established at the University of Maryland as previously described (13). Homozygous HSF-1-null males were bred with heterozygous females and the genotypes determined from PCR analysis of tail snip DNA. Eight- to 10-week-old male outbred CD-1 mice, weighing 25 to 30 g, were purchased from Harlan Sprague Dawley, housed in the Baltimore Veterans Administration Medical Center animal facility under American Association of Laboratory Animal Care-approved conditions and under the supervision of a full-time veterinarian. All animals were used within 4 weeks of delivery. LPS prepared by trichloroacetic acid extraction from *Escherichia coli* O111:B4 and 2,2,2-tribromoethyl was purchased from Sigma-Aldrich. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore.

Temperature Clamping and Intratracheal Administration of LPS

Mice were adapted to standard plastic cages for at least 4 days before study. To avoid the influence of diurnal cycling, all experiments were

started at approximately the same time each day (between 8:00 and 10:00 A.M.). Mice were placed in either 24°C (euthermia) or 34°C (FRH) infant incubators beginning 2 hours before LPS instillation, exposures that we previously showed maintain core temperatures at approximately 37°C and 39.5°C, respectively (13). Six hours after LPS instillation, the mice were killed by isoflurane inhalation and cervical dislocation, and lung lavage was performed with a total of 2 ml PBS as previously described (13). LIX levels in cell-free supernatants were measured in the University of Maryland Cytokine Core Laboratory by ELISA using a commercial antibody pair and recombinant standard (R&D Systems).

Statistical Analysis

Data are presented as mean $\pm$ SE. Differences between two groups of data were analyzed using the unpaired Student *t* test for *in vitro* experiments and Mann-Whitney for the *in vivo* LPS-challenge experiments. Differences among more than 2 groups were tested by applying the Fisher PLSD test applied to a one-way ANOVA.

RESULTS

HS Augments TNF- α -Induced IL-8 Expression

To determine whether exposure to HS modifies IL-8 expression, we incubated human respiratory epithelial-like A549 cells with medium alone or with 0.5 to 2 ng/ml TNF- α for 15 minutes at 37°C, then exposed these cells to HS (42°C for 2 h) and measured IL-8 in culture supernatants 4 hours later. Euthermic controls without or with TNF- α were incubated in parallel at 37°C. TNF- α stimulated a dose-dependent 7.2- to 13.7-fold increase in IL-8 secretion in euthermic controls. Co-exposing TNF- α -stimulated cells to HS doubled IL-8 secretion at each

TNF- α concentration tested, but failed to stimulate IL-8 secretion in the absence of TNF- α (Figure 1A). Analysis of IL-8 mRNA in these cells using real-time RT-PCR demonstrated a similar pattern of TNF- α - and HS-responsiveness. Treatment with 2 ng/ml TNF- α stimulated a 177-fold increase in IL-8 mRNA in euthermic cells. Co-exposure to HS stimulated a further 1.72, 1.75, and 2.75-fold increase in IL-8 mRNA levels compared with euthermic cells 1, 2, and 4 hours after treatment with TNF- α (Figure 1B). To evaluate the potential contribution of A549 cell injury or death to the measured release of IL-8, we measured plasma membrane integrity by trypan blue dye exclusion. Little or no cell injury was evident under any of the treatment conditions. Cells incubated for 6 hours at 37°C without or with 2 μ g/ml TNF- α or for 2 hours at 42°C and then 4 hours at 37°C without or with TNF- α were 100 $\pm$ 0%, 99.4 $\pm$ 0.6%, 97.2 $\pm$ 0.3%, and 97.6 $\pm$ 0.3% intact.

To further analyze the effect of HS on IL-8 gene activation, we analyzed A549 cells that had been stably transfected with a luciferase reporter construct driven by a 1.5-kb fragment of human IL-8 5' flanking sequence, including 44 nt of 5'-untranslated region (UTR) (IL8-1471/+44-luc) (Figure 1C). Treating the transfectants with 1 ng/ml TNF- α for 6 hours at 37°C stimulated a 5.5-fold increase in luciferase activity. Co-exposing TNF- α -treated cells to HS stimulated an additional increase in luciferase activity that was almost 2-fold greater than TNF- α -treated euthermic cells. However, as we found for IL-8 secretion, exposure to HS in the absence of TNF- α had no detectable effect on activity of the stably transfected reporter gene. Since IL-8 transcription is driven by NF- κ B, we excluded a generalized effect of HS on NF- κ B-dependent transcription by analyzing

Figure 1. Effects of heat shock (HS) on IL-8 expression. (A) A549 cells were stimulated without or with TNF- α for 15 minutes at 37°C and either incubated at 37°C for 6 hours without (euthermic) or with HS (42°C for 2 h). IL-8 released in cell culture media was measured by enzyme-linked immunosorbent assay (ELISA). Mean $\pm$ SE of six experiments, **P* < 0.05 versus euthermic cells. (B) A549 cells were stimulated with 2 ng/ml TNF- α or for 15 minutes at 37°C and incubated for the indicated time at 37°C (euthermic) or 42°C (HS), then IL-8 mRNA was measured by real-time RT-PCR, normalized to levels of GAPDH mRNA, and expressed at each time point as fold-change versus time 0. Mean $\pm$ SE of four experiments, **P* < 0.05 versus euthermic cells at the same time point. (C) A549 cells stably transfected with a reporter plasmid driven by a 1.5-kb IL-8 promoter fragment (IL8-1471/+44-luc) were incubated without (unstimulated) or with 1 ng/ml TNF- α for 15 minutes at 37°C, then incubated at 37°C for 6 hours without (euthermic) or with 2 hours HS at 42°C. Mean $\pm$ SE of six experiments, **P* < 0.05 versus time 37°C cells. (D) Electrophoretic mobility shift assay (EMSA) analysis of NF- κ B activation by HS. A549 cells were incubated with or without 2 ng/ml TNF- α at 37°C or 42°C, and nuclear extracts were collected and analyzed by EMSA using a commercial NF- κ B probe. (E) Small airway epithelial cells were treated and IL-8 measured as described for A549 cells in A. Mean $\pm$ SE of three replicates, **P* < 0.05 versus 37°C cells.

the effect of TNF- α and HS on NF- κ B activation by EMSA in A549 cells (Figure 1D). Treatment with TNF- α induced rapid activation of NF- κ B (Figure 1D, lanes 2–4). Exposure to HS (42°C for 2 h) neither activated NF- κ B in cells incubated without TNF- α (Figure 1D, lanes 5–7) nor enhanced NF- κ B activation in cells treated with TNF- α (Figure 1D, lanes 8–10).

IL-8 secretion in primary cultured SAECs demonstrated a similar pattern of responsiveness to HS and TNF- α as was found in A549 cells (Figure 1E). Treatment with 2 ng/ml TNF- α stimulated a 57.5-fold increase and co-exposure to HS a 1.9-fold further increase in IL-8 secretion. HS also co-activated IL-8 expression in A549 cells treated with either IL-1 β or phorbol myristate acetate (PMA) (data not shown).

While these results indicate that exposure to HS augments IL-8 transcription, stabilization of IL-8 mRNA levels may also have contributed to higher IL-8 mRNA levels. The IL-8 3'UTR contains adenosine uridine-rich elements (AREs) (20) that confer regulated mRNA instability by binding the AUF1 family of ARE-binding factors. Laroia and colleagues have shown that HSP72 stabilizes ARE-containing transcripts by sequestering AUF1 for degradation (21). To evaluate whether HS stabilizes IL-8 mRNA, we measured IL-8 mRNA stability by following IL-8 mRNA levels after transcriptional arrest by actinomycin D (Figure 2). A549 cells were stimulated with TNF- α for 15 minutes, incubated for 2 hours at 42°C or 37°C, then treated with 5 μ g/ml actinomycin D at 37°C and IL-8 mRNA levels were sequentially measured by real time-RT-PCR. The half-life of IL-8 mRNA calculated from the rate of loss of IL-8 mRNA was similar in the absence (60.5 $\pm$ 5.5 min) and presence (69.0 $\pm$ 3.0 min) of HS, indicating that HS does not affect IL-8 mRNA stability in A549 cells. These combined results demonstrate that exposure to HS co-activates IL-8 transcription in TNF- α -stimulated epithelial cells, but unlike classical HSPs, HS alone is not sufficient to activate IL-8 expression.

After Exposure to HS, HSF-1 Binds to HSEs Present in the IL-8 Promoter

To confirm that HSF-1 was activated in HS-exposed A549 cells, we analyzed HS-inducible HSP72 generation and HSF-1 DNA-

binding activity. A549 cells were incubated at 42°C for 2 hours and, after an additional 4 hours of incubation at 37°C, the cells were lysed and immunoblotted for HSP72. As expected, HS caused a marked increase in HSP72 levels (Figure 3). EMSA using the human HSP72 HSE as probe and nuclear extracts from heat shocked A549 cells (Figure 4A, lanes 1–4) demonstrated that HSF-1 was activated to a DNA-binding form in A549 cells exposed to 42°C for 2 hours in the absence or presence of TNF- α .

The IL-8 5' flanking sequence contains three HSE-like sequences (17) comprising slightly imperfect inverted nGAAn triad repeats in the sequence between –1213 and –1182 nt (ccaaGACctTTCaaGAAagGTCttGGAAttctt), –1151 and –1120 nt (tttttatgatTTGttGAAatTTCtactccat), and –813 and –782 nt (ctgcctttgGAagaTTCtgCTCttatgcctcc) (location relative to the transcription start site). EMSA analysis using double-stranded oligonucleotide probes spanning each of these three sequences and nuclear extracts from heat-shocked TNF- α -treated A549 cells showed that HS-induced DNA-protein complexes formed on two (–1213 to –1182 and –813 to –782 nt) of the three putative HSE sequences (Figure 4A), and were supershifted by anti-HSF-1 antibody. The relative affinity of HSF-1 for each of the three IL-8 HSE-like sequences was compared by cold-label competition studies using the classical HSPA1A HSE sequence as a probe (Figure 4B). This analysis confirmed that the HSE-like sequence located between nt –1151 and –1120 does not bind HSF-1 and suggested the following apparent rank order of HSF-1 binding affinity under these assay conditions: HSPA1A > IL-8/–813 to –782 > IL-8/–1213 to –1182.

We extended the EMSA studies by using a ChIP assay to analyze interactions between HSF-1 and the endogenous IL-8 gene *in vivo* (Figure 4C). Cross-linked chromatin from TNF- α -treated, heat-shocked A549 cells was immunoprecipitated using either a rabbit anti-HSF-1 antibody (H311) or nonimmune rabbit IgG. PCR primers were selected to amplify the IL-8 5'-flanking region containing the two upstream HSE-like sequences (–1291 to –1030 nt) and the region containing the proximal HSE-like sequence (–861 to –642 nt). In addition, the HSE-containing region of the HSPA1A promoter was amplified. The HSPA1A primers and both IL-8 promoter primer pairs generated PCR products from H311-immunoprecipitated chromatin from A549 cells exposed to HS without (Figure 4C, lane 8) or with TNF- α (Figure 4C, lane 10), whereas no PCR products were generated from chromatin immunoprecipitated with nonimmune IgG (Figure 4C, lanes 7 and 9). Exposure to TNF- α in the absence of HS failed stimulate recruitment of HSF-1 to the HSPA1A or IL-8 promoters.

Deletion of HSE-Like Sequences Reduces HS Responsiveness of the IL-8 Promoter

To analyze the consequences of HSF-1:IL-8 promoter interactions for transcriptional activation, we measured the effect of deleting HSE-containing regions of the IL-8 promoter on HS responsiveness. Two 5'-deletion IL-8 promoter-driven reporter constructs were generated and transiently transfected into A549 cells, and the effect of HS on TNF- α -activated reporter activity of each was determined 48 hours after transfection. All three constructs (full-length and the two deletion constructs) exhibited a similar fold-increase in reporter gene activity upon stimulation with TNF- α at 37°C, demonstrating that the deletions had no effect on the TNF- α responsiveness (Figure 5A). However, the three constructs exhibited different responsiveness to HS (Figure 5B). In the presence of TNF- α , the activity of the 1.5-kb IL-8 promoter containing all three putative HSEs (IL8^{–1471/+44}-luc) was increased 3.3-fold by HS compared with similarly treated non-heat-shocked cells. A 5'-deletional mutant (IL8^{–1081/+44} luc)

Figure 2. Effect of HS exposure on half-life of IL-8 mRNA. A549 cells were stimulated with 2 ng/ml TNF- α for 15 minutes at 37°C and incubated at either 37°C (euthermic) or 42°C (HS) for 2 hours. All cells were then switched to 37°C, 5 μ g/ml actinomycin D was added to arrest transcription, and IL-8 and GAPDH mRNA was sequentially measured by real-time RT-PCR and the ratio of IL-8/GAPDH calculated. Curve fitting, assuming first-order decay, was performed using the Deltagraph software package (SPSS), and the half-life, $T_{1/2}$, was calculated from the decay constant, k , using the equation $T_{1/2} = 0.693/k$. Mean $\pm$ SE of four experiments.

lacking the two upstream HSE-like sequences (–1213 to –1182 nt and –1151 and –1120 nt) exhibited 28% less HS responsiveness than the 1.5-kb promoter construct. The shortest construct (IL8^{-760/+44} luc), lacking all three HSE-like sequences, demonstrated a further decrease in HS responsiveness, but still retained 45% of the HS responsiveness of the full-length construct.

HSF-1 Is Required for HS-Induced Activation of IL-8 Expression

To further demonstrate that HSF-1 participates in the HS-induced augmentation of IL-8 expression, we analyzed the effects of HSF-1 depletion on IL-8 gene expression in TNF- α -stimulated A549 cells. A549 cells were transfected with siRNA directed against human HSF-1 (Ambion) or control siRNA. When analyzed by immunoblotting 48 hours after transfection, the cells transfected with HSF-1 siRNA contained 70 to 85% less HSF-1 protein than untransfected cells or cells transfected with control siRNA (Figure 6A). While TNF- α inducibility of IL-8 expression was unaffected by HSF-1 knockdown (data not shown), augmentation of IL-8 expression by HS (TNF- α plus HS versus TNF- α alone) was abrogated in the HSF-1-depleted cells (Figure 6B).

We have previously demonstrated that exposure to FRH activates the HS response and augments ELR⁺ CXC chemokine expression in LPS-challenged murine lungs (13). Because the mouse does not have an IL-8 ortholog, we chose to analyze expression of one of the three functional IL-8 analogs expressed by mice, LIX, to determine whether HSF-1 is required for the augmented expression of ELR⁺ CXC genes by FRH *in vivo*. We compared lung lavage levels of LIX/CXCL5 in euthermic

Figure 4. Analysis of HSF-1 interaction with HSE-like sequences in IL-8 5'-flanking sequence. (A) EMSA analysis of nuclear extracts from A549 cells incubated for 1 hour at 37°C (lanes 1, 5, 9, 13) or 42°C (lanes 2–4, 6–8, 10–12, 14–16) using the human HSPA1A HSE sequence (lanes 1–4) or IL-8 HSE sequences –1213/–1183 (lanes 5–8), –1151/–1120 (lanes 9–12), or –813/–782 (lanes 13–16). Complexes were super-shifted with anti-HSF-1 (lanes 3, 7, 11, and 15) or control (IgG; lanes 4, 8, 12, and 16) antibody. Representative autoradiographs of four separate experiments are shown. (B) Cold label competition was performed using the conditions described for Figure 1A and the HSPA1A HSE sequence as probe. Competition with 10-, 30-, and 100-fold excess of the indicated unlabeled double-stranded oligonucleotide. HSF-1–DNA complexes are indicated by the arrow and super-shifted complex by the arrowhead. Representative autoradiographs of four separate experiments are shown. (C) ChIP analysis of A549 cells incubated with or without 2 ng/ml TNF- α at 37°C or 42°C for 1 h. Formaldehyde-cross-linked nuclear extracts were immunoprecipitated with anti-HSF-1 (lanes 4, 6, 8, 10) or control (nonimmune IgG; lanes 3, 5, 7, 9) antibodies. Lane 1 has no template. Lane 2 uses the input material from the 37°C control cells. Two regions of the IL-8 5'-flanking sequence (indicated) and the HSE-containing human HSPA1A promoter region were amplified by PCR and electrophoretically separated through an ethidium bromide-stained agarose gel. Representative autoradiographs of three separate experiments are shown.

and FRH-exposed mice 6 hours after intratracheal instillation of 50 μ g LPS. Like human IL-8, LIX contains multiple HSE-like sequences in its 5' flanking region (17). In homozygous wild-type mice (HSF-1^{+/+}), co-exposure to FRH increased levels of LIX in lung lavage by 4.6-fold compared with euthermic, LPS-challenged mice (Figure 6C). In contrast, exposing HSF-1-null mice to the same FRH treatment failed to augment LIX levels in the LPS-challenged lung. Interestingly, FRH exerted intermediate effects on LIX levels in lung lavage from HSF-1^{+/-} mice, suggesting that HSF-1 may exert a dose-dependent effect on LIX expression. Santos and Saraiva (22) described a similar intermediate phenotype in HSF-1^{+/-} mice with respect to brain development, further suggesting that some phenotypes may exhibit an HSF-1 gene dose effect.

DISCUSSION

In this study, we showed that co-exposure to HS increased the transcriptional activity of the IL-8 promoter and that the effect required both HSF-1 and multiple HSEs in the IL-8 5' flanking sequence. Whereas mice lack an IL-8 ortholog, we have previously reported that *in vivo* expression of other members of the ELR⁺ CXC chemokine family, including LIX, is enhanced when mice were co-exposed to FRH and hyperoxia (15) or intratracheal instillation of LPS (13). In this study we showed that LIX expression in the LPS-challenged mouse lung was enhanced by co-exposure to FRH in wild-type, but not HSF-1-deficient mice. Since almost all mouse and human CXC chemokines contain 5' flanking HSE-like sequences (17), these results suggest these genes may comprise a newly recognized family of HSF-1-regulated genes. However, there is an important difference in the HS responsiveness exhibited by the classical HSP genes and IL-8. While exposure to HS alone is sufficient to activate HSP genes (1), HS exposure alone has no effect on IL-8 expression, but it enhances IL-8 gene activation when accompanied by a co-stimulus (e.g., TNF- α or IL-1 β). EMSA competition studies suggest that HSF-1 binds to the HSEs in IL-8 with lower affinity compared with HSPA1A. While the cell-free EMSA conditions do not mimic the *in vivo* environment for HSF-1-DNA interactions, lower affinity binding of HSF-1 to the

Figure 6. Requirement for HSF-1 for HS-responsiveness of CXC chemokine expression. (A) A549 cells were transfected with the indicated amount of a control scrambled siRNA (lane 5) or siRNA targeted against mouse HSF-1 (lanes 1–4) or were subjected to sham transfection without siRNA (lane 6) and, 48 hours later, cell lysates were analyzed by immunoblotting for HSF-1 and β -tubulin. A representative of three immunoblots is shown. (B) A549 cells were sham-transfected or transfected with 150 pmole scrambled or HSF-1-targeting siRNA and, 48 hours later, were incubated with 1 ng/ml TNF- α at 37°C for 6 hours without (euthermic) or with 2 hours HS at 42°C and IL-8 secretion measured as described in Figure 1. Mean $\pm$ SE of three experiments; * $P < 0.05$ versus non-heat shocked cells for each transfection protocol. (C) HSF-1^{-/-}, HSF-1^{+/-}, and HSF-1^{+/+} mice were housed at 24°C (euthermic) or 34°C (FRH) ambient temperature for 2 hours until core temperature equilibrated. Each mouse then received 50 μ g LPS via intratracheal instillation and, 6 hours later, were killed and lung lavage analyzed for LIX concentration by ELISA. Mean $\pm$ SE; five mice per group; * $P < 0.05$ versus euthermic for each genotype.

IL-8 promoter might require stabilization provided by additional transcription factors activated by co-exposure to proinflammatory agonists.

We have shown that temperatures within the usual febrile range are sufficient to activate HSF-1 and HSP expression *in vitro* and *in vivo* (10–13). However, compared with temperatures in the classic heat shock range ($\geq 42^\circ\text{C}$), febrile temperature incompletely activate HSF-1 *in vitro* (11). Since this study addressed the hypothesis that activated HSF-1 enhanced IL-8 expression, we used a heating protocol, 42°C for 2 hours, that would reliably activate HSF-1 during a well-defined period of time. However, our finding that exposing HSF-1-sufficient, but

not HSF-1-deficient mice to febrile-range hyperthermia (core temperatures of 39.5°C) increased LIX expression indicates that it is activated HSF-1 rather than an absolute temperature threshold that is the link to enhanced CXC chemokine expression. These findings also support our previous work identifying an overlap between febrile and heat shock temperatures (9, 11).

The incremental reduction in HS-responsiveness with deletion of the two upstream (−1213 to −1182 and −1151 to −1120 nt) and the proximal (−813 to −782 nt) HSE-like sequences indicate that the proximal and at least one of the upstream HSE-like sequences in the IL-8 5′ flanking sequence participate in HS-augmented IL-8 gene activation. The retention of partial HS responsiveness of the IL-8 deletion mutant lacking all three HSE-like sequences suggest that HS may also regulate IL-8 transcription through mechanisms that do not require consensus HSE sequences. However, the complete loss of HS-responsiveness in A549 cells treated with HSF-1 siRNA suggests that HS-responsiveness of the IL-8 gene requires HSF-1 protein. Trinklein and colleagues (23) have identified several genes that are activated by HS and bind HSF-1 despite lacking consensus HSE sequences within their 5′ flanking sequences. Shen and coworkers (24) demonstrated that the first intron of the human HSP90β gene contains two HSEs that bind HSF-1 and activate transcription. We performed a computer-assisted sequence analysis (TF Search) of the first IL-8 intron that revealed a potential HSE (aTTCagGAAt) between nt 683 and 692 of the 815 nt first intron. While the functional activity of this IL-8 intron element is not known, it could not have contributed to the residual HS-responsiveness of our shortest IL-8 construct (IL-8^{-1081/+44} luc), since the reporter plasmid did not contain IL-8 intron sequence.

The realization that HSF-1 is a co-activator of IL-8 gene expression explains several published observations about associations between exposure to inducers of the HS response and augmented IL-8 expression. Bowman and colleagues (25) showed that subjecting normal human epidermal keratinocytes to very brief exposure (1–30 s) at extreme hyperthermia (50–60°C) causes marked induction of HSP72 expression and IL-8 release. In another study, Jaspers and coworkers (26) showed that sodium arsenite and vanadyl sulfate each enhanced IL-8 expression in normal human bronchial epithelial cells and increased activity of a −1370/+82 nucleotide IL-8 promoter-luciferase reporter construct. Although metal compounds, especially sodium arsenite, are known to be inducers of the HS response, neither HSF-1 activation nor HSP expression was analyzed in this study. Similarly, Souza and colleagues (27) showed that exposing the human hepatocellular carcinoma cell line HepG2 to the HS inducer, cadmium chloride, causes a marked increase in IL-8 gene expression coincident with an increase in HSP72 expression, but these authors focused on the role of AP-1 activation in the regulation of cytokine gene expression by cadmium.

Some investigators have reported that exposure to HS reduces CXC chemokine expression. Housby and coworkers (28) reported that treating the THP-1 human monocyte cell line with the HSF-1 activator sodium salicylate inhibits LPS-induced IL-8 expression in THP-1 cells. However, sodium salicylate incompletely activates HSF-1 to a form that is not transcriptionally competent (29) and therefore not capable of activating IL-8 transcription. Yoo and colleagues (30) reported that prior induction of HSP72 by either HS exposure or sodium arsenite treatment suppresses subsequent, cytokine-induced IL-8 expression in human bronchial epithelial cells. Since HSP72 protein is a negative regulator of HSF-1 activation (31), prior generation of HSP72, as achieved in the model by Yoo and coworkers, would have reduced the availability of active HSF-1 available to drive IL-8 transcription. Dunsmore and colleagues (32) reported that exposing A549 cells to heat shock reduced rather than enhanced

TNF-α-induced IL-8 secretion. In that study, A549 cells were exposed to 43°C for 1 hour followed by 1 hour of recovery before stimulation with TNF-α. Similar heating protocols have been shown to block activation of NF-κB (33, 34), a transcription factor required for IL-8 transcriptional activation (35). Therefore the findings of Dunsmore and coworkers are consistent with our proposed model in which HSF-1 acts as a transcriptional co-activator of IL-8 that requires coincident exposure to proinflammatory agonists, such as TNF-α or IL-1β, and downstream signaling events, such as activation of NF-κB.

Cooperativity between transcription factors may occur if binding of one factor induces chromatin remodeling that enhances access of the second factor to promoter DNA. To evaluate this possibility, we used ChIP to compare the recruitment of HSF-1 to the putative HSF-1-binding regions of the IL-8 promoter in A549 cells exposed to HS alone or the combination of TNF-α and HS. This analysis showed the two treatments stimulated similar increases in HSF-1 recruitment compared with untreated cells or cells treated with TNF-α without HS. These results argue against TNF-α enhancing access of HSF-1 to the IL-8 promoter. In contrast with our findings, Trinklein and colleagues (36) reported that ChIP analysis failed to detect HSF-1 binding to the IL-8 promoter in the human erythroleukemic K562 cell line exposed to 43°C for 1 hour. However, the IL-8 promoter region that was analyzed in the Trinklein study was not reported. If these investigators did study the same IL-8 promoter regions as we have, their failure to find recruitment of HSF-1 to the IL-8 promoter may reflect a difference in behavior of the K562 leukemia and A549 adenocarcinoma cell lines or a difference in HS protocols used (43°C for 1 h versus 42°C for 1 h in our study).

In summary, we have shown that an important member of the ELR⁺ CXC family of chemokines, IL-8/CXCL8, is an HS-responsive gene that requires HSF-1 and multiple 5′-flanking HSEs for optimal HS-responsiveness. LIX, a related murine gene, exhibited *in vivo* FRH responsiveness that was also HSF-1 dependent. However, unlike the classical HSP genes, HS is not sufficient to activate IL-8 or LIX expression, but requires co-activation with a proinflammatory agonist. We speculate that ELR⁺ CXC chemokines have co-opted elements of the HS response to create a novel regulatory pathway that amplifies their expression and facilitates neutrophil delivery at sites of infection or injury during febrile illnesses.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* 1999;61:243–282.
2. Perisic O, Xiao H, Lis JT. Stable binding of drosophila heat shock factor to head-to-head and tail-tail repeats of a conserved 5 bp recognition unit. *Cell* 1989;59:797–806.
3. Sarge KD, Murphy SP, Morimoto RI. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Mol Cell Biol* 1993;13:1392–1407. (published errata appear in *Mol Cell Biol* 1993;13:3122–3123 and 1993;13:3838–3839).
4. McMillan DR, Xiao X, Shao L, Graves K, Benjamin IJ. Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection against heat-inducible apoptosis. *J Biol Chem* 1998;273:7523–7528.
5. Murray JI, Whitfield ML, Trinklein ND, Myers RM, Brown PO, Botstein D. Diverse and specific gene expression responses to stresses in cultured human cells. *Mol Biol Cell* 2004;15:2361–2374.
6. Sonna LA, Gaffin SL, Pratt RE, Cullivan ML, Angel KC, Lilly CM. Effect of acute heat shock on gene expression by human peripheral blood mononuclear cells. *J Appl Physiol* 2002;92:2208–2220.

7. Hahn JS, Neef DW, Thiele DJ. A stress regulatory network for co-ordinated activation of proteasome expression mediated by yeast heat shock transcription factor. *Mol Microbiol* 2006;60:240-251.
8. Westwood JT, Clos J, Wu C. Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. *Nature* 1991;353:822-827.
9. Hasday JD, Singh IS. Fever and the heat shock response: distinct, partially overlapping processes. *Cell Stress Chaperones* 2000;5:471-480.
10. Singh I, He J-R, Calderwood S, Hasday J. A high affinity HSF-1 binding site in the 5'-untranslated region of the murine tumor necrosis factor- α gene is a transcriptional repressor. *J Biol Chem* 2002;277:4981-4988.
11. Singh IS, Calderwood S, Kalvakolanu I, Viscardi R, Hasday JD. Inhibition of tumor necrosis factor- α transcription in macrophages exposed to febrile range temperature: a possible role for heat shock factor-1. *J Biol Chem* 2000;275:9841-9848.
12. Jiang Q, DeTolla L, Kalvakolanu I, Fitzgerald B, Hasday JD. Fever upregulates expression of pyrogenic cytokines in endotoxin-challenged mice. *Am J Physiol* 1999;276:R1653-R1660.
13. Rice P, Martin E, He J-R, Frank M, DeTolla L, Hester L, O'Neill T, Manka C, Singh I, Hasday J. Febrile-range hyperthermia augments neutrophil accumulation and enhances lung injury in experimental gram-negative bacterial pneumonia. *J Immunol* 2005;174:3676-3685.
14. Jiang Q, Cross AS, Singh IS, Chem TT, Viscardi RM, Hasday JD. Febrile core temperature is essential for optimal host defense in bacterial peritonitis. *Infect Immun* 2000;68:1265-1270.
15. Hasday J, Garrison A, Singh I, Standiford T, Ellis G, Rao S, He J-R, Rice P, Frank M, Goldblum S, et al. Febrile-range hyperthermia augments pulmonary neutrophil recruitment and amplifies pulmonary oxygen toxicity. *Am J Pathol* 2003;162:2005-2017.
16. Bernheim HA, Bodel PT, Askenase PW, Atkins E. Effects of fever on host defence mechanisms after infection in the lizard *Dipsosaurus dorsalis*. *Br J Exp Pathol* 1978;59:76-84.
17. Nagarsekar A, Hasday J, Singh I. CXC chemokines: a new family of heat-shock proteins? *Immunol Invest* 2005;34:381-398.
18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402-408.
19. Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding protein with mini extracts prepared from a small number of cells. *Nucleic Acids Res* 1989;17:6419.
20. Tebo J, Der S, Frevel M, Khabar KS, Williams BR, Hamilton TA. Heterogeneity in control of mRNA stability by AU-rich elements. *J Biol Chem* 2003;278:12085-12093.
21. Laroia G, Cuesta R, Brewer G, Schneider RJ. Control of mRNA decay by heat shock-ubiquitin-proteasome pathway. *Science* 1999;284:499-502.
22. Santos SD, Saraiva MJ. Enlarged ventricles, astrogliosis and neurodegeneration in heat shock factor 1 null mouse brain. *Neuroscience* 2004;126:657-663.
23. Trinklein ND, Murray JI, Hartman SJ, Botstein D, Myers RM. The role of heat shock transcription factor 1 in the genome-wide regulation of the mammalian heat shock response. *Mol Biol Cell* 2004;15:1254-1261.
24. Shen Y, Liu J, Wang X, Cheng X, Wang Y, Wu N. Essential role of the first intron in the transcription of hsp90beta gene. *FEBS Lett* 1997;413:92-98.
25. Bowman PD, Schuschereba ST, Lawlor DF, Gilligan GR, Mata JR, DeBaere DR. Survival of human epidermal keratinocytes after short-duration high temperature: synthesis of HSP70 and IL-8. *Am J Physiol* 1997;272:C1988-C1994.
26. Jaspers I, Samet JM, Reed W. Arsenite exposure of cultured airway epithelial cells activates kappaB- dependent interleukin-8 gene expression in the absence of nuclear factor-kappaB nuclear translocation. *J Biol Chem* 1999;274:31025-31033.
27. Souza V, Escobar Md Mdel C, Gomez-Quiroz L, Bucio L, Hernandez E, Cossio EC, Gutierrez-Ruiz MC. Acute cadmium exposure enhances AP-1 DNA binding and induces cytokines expression and heat shock protein 70 in HepG2 cells. *Toxicology* 2004;197:213-228.
28. Housby JN, Cahill CM, Chu B, Prevelige R, Bickford K, Stevenson MA, Calderwood SK. Non-steroidal anti-inflammatory drugs inhibit the expression of cytokines and induce HSP70 in human monocytes. *Cytokine* 1999;11:347-358.
29. Jurivich DA, Sistonen L, Kroes RA, Morimoto RI. Effect of sodium salicylate on the human heat shock response. *Science* 1992;255:1243-1245.
30. Yoo CG, Lee S, Lee CT, Kim YW, Han SK, Shim YS. Anti-inflammatory effect of heat shock protein induction is related to stabilization of I kappa B alpha through preventing I kappa B kinase activation in respiratory epithelial cells. *J Immunol* 2000;164:5416-5423.
31. Mosser DD, Duchaine J, Massie B. The DNA-binding activity of the human heat shock transcription factor is regulated in vivo by hsp70. *Mol Cell Biol* 1993;13:5427-5438.
32. Dunsmore KE, Denenberg AG, Page K, Wong HR. Mechanism and function of heat shock-dependent IkappaBalpha expression. *Inflamm Res* 2006;55:254-259.
33. Kim WJ, Back SH, Kim V, Ryu I, Jang SK. Sequestration of TRAF2 into stress granules interrupts tumor necrosis factor signaling under stress conditions. *Mol Cell Biol* 2005;25:2450-2462.
34. Pittet JF, Lee H, Pespeni M, O'Mahony A, Roux J, Welch WJ. Stress-induced inhibition of the NF-kappaB signaling pathway results from the insolubilization of the IkappaB kinase complex following its dissociation from heat shock protein 90. *J Immunol* 2005;174:384-394.
35. Kunsch C, Rosen CA. NF-kappa B subunit-specific regulation of the interleukin-8 promoter. *Mol Cell Biol* 1993;13:6137-6146.
36. Trinklein ND, Chen WC, Kingston RE, Myers RM. Transcriptional regulation and binding of heat shock factor 1 and heat shock factor 2 to 32 human heat shock genes during thermal stress and differentiation. *Cell Stress Chaperones* 2004;9:21-28.