

Core temperature correlates with expression of selected stress and immunomodulatory genes in febrile patients with sepsis and noninfectious SIRS

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Abstract Environmental hyperthermia and exercise produce extensive changes in gene expression in human blood cells, but it is unknown whether this also happens during febrile-range hyperthermia. We tested the hypothesis that heat shock protein (HSP) and immunomodulatory stress gene expression correlate with fever in intensive care unit patients. Whole blood messenger RNA was obtained over consecutive days from 100 hospitalized patients suffering from sepsis or noninfectious systemic inflamma-

tory response syndrome (SIRS) as defined by conventional criteria. The most abnormal body temperature in the preceding 24 h was recorded for each sample. Expression analysis was performed using the Affymetrix U133 chip. ANCOVA followed by correlation analysis was performed on a subset of 278 prospectively identified sequences of interest. Temperature affected expression of 60 sequences, either independently or as a function of clinical diagnosis. Forty-eight of these (representing 38 genes) were affected by temperature only, including several HSPs, transcription factors heat shock factor (HSF)-1 and HSF-4, cellular adhesion molecules such as ICAM1/CD54 and JAM3, toll receptors TLR-6 and TLR-7, ribosomal proteins, and a number of molecules involved in inflammatory pathways. Twelve sequences demonstrated temperature-dependent responses that differed significantly between patients with sepsis and noninfectious SIRS: CXCL-13; heat shock proteins DNAJB12 and DNAJC4; the F11 receptor; folate hydrolase 1; HSF-2; HSP 70 proteins HSPA1A, HSPA1B, and HSPA1L; interleukin 8; lipopolysaccharide binding protein; and prostaglandin E synthase. Febrile-range temperatures achieved during sepsis and noninfectious SIRS correlate with detectable changes in stress gene expression *in vivo*, suggesting that fever can activate HSP gene expression and modify innate immune responses. For some genes, it appears that clinical condition can alter temperature-sensitive gene expression. Collectively, these data underscore the potential importance of body temperature in shaping the immune response to infection and injury.

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Introduction

The heat shock response is a phylogenetically ancient cellular response to exogenous stress, including high temperatures, that triggers increased expression of a set of evolutionarily conserved heat shock proteins (HSPs; Feder and Hofmann 1999; Lindquist 1986; Parsell and Lindquist 1993). HSPs are thought to preserve cell viability in the face of physical and chemical stress by sequestering denatured cellular proteins and facilitating their refolding or elimination (Lindquist 1986; Parsell and Lindquist 1993). Experimentally, maximal heat shock responses typically occur at very high temperatures (exposure to temperatures 5–6°C over baseline temperature for 20 to 60 min) and produce changes in gene expression that continue for at least several hours after return to normothermia. Both DNA microarray (Murray et al. 2004; Sonna et al. 2002b) and *in situ* hybridization (Westwood et al. 1991) studies demonstrate that exposure to heat shock affects expression of genes beyond the HSP family, and that the response includes both increases and reductions in expression of non-HSP genes. Furthermore, exposure to heat shock can modify functional pathways involved in inflammatory responses; for example, we have found that expression of the human CXC chemokine, IL-8, is enhanced by exposure to heat shock *in vitro* (Singh et al. 2008).

Clinically, some of the changes that characterize the heat shock response *in vitro* have been detected in individuals who suffered exertional heat injury (Sonna et al. 2004). However, there is evidence to suggest that, even in healthy individuals, modest elevations in body temperature might cause changes in gene expression as well. For example, microarray studies have found that gene expression changes in whole blood and peripheral blood mononuclear cells (PBMCs) can be triggered by acute aerobic exercise at intensities known to produce moderate exertional hyperthermia (Connolly et al. 2004; Sonna et al. 2007; Zieker et al. 2005). We have also recently reported that healthy volunteers subjected to a heat acclimation program comprising ten daily 100-min exposures to exertional/environmental hyperthermia (resulting in core temperatures $\leq 39.5^\circ\text{C}$) was sufficient to increase steady-state PBMC levels of HSP72 and HSP90 (McClung et al. 2008).

By contrast, fever is a complex physiological response to infection or injury in which homeothermic animals temporarily allow body temperature to rise to a moderate level of hyperthermia (typically, 2°C to 4°C above baseline temperature). The known immunomodulatory effects of febrile-range hyperthermia (FRH) include effects on cytokine expression, antigen presentation, and lymphocyte proliferation (Hasday and Singh 2000). We have previously demonstrated that exposure to FRH (39.5°C) *in vitro* attenuates TNF α and IL-1 β expression in human macro-

phages (Fairchild et al. 2004; Fairchild et al. 2000). Exposure to FRH *in vivo* (core temperature $\sim 39.5^\circ\text{C}$) induces G-CSF expression (Ellis et al. 2005), and enhances expression of GM-CSF and CXC chemokines in murine models of pulmonary oxygen toxicity (Hasday et al. 2003) and Gram-negative pneumonia (Rice et al. 2005). Collectively, these studies suggest that febrile-range hyperthermia may modify expression of HSP genes as well as genes that regulate inflammation.

To ascertain whether febrile-range hyperthermia might also influence gene expression in a clinical setting, we performed an additional analysis of microarray data recently reported by Johnson et al. (2007), which were collected to study differences in gene expression between noninfectious systemic inflammatory response syndrome (SIRS) and sepsis. Rather than scan the entire microarray for correlations with fever, we prospectively defined a set of 278 gene sequences that included heat shock proteins, candidate genes in inflammatory pathways that might be temperature-dependent, and control sequences that have previously been reported as showing stable expression *in vivo* after exertional heat injury. Our hypothesis was that several of the HSP and stress gene sequences would show temperature-dependent changes in expression and secondarily, that the temperature-dependent expression responses of at least some of these would be different in patients with sepsis and noninfectious SIRS.

Materials and methods

Patient selection

This study was approved by the University of Maryland Institutional Review Board. All subjects (or their proxies) gave informed consent prior to enrolling.

The criteria for enrollment have previously been reported in detail (Johnson et al. 2007; Lissauer et al. 2007). The study was performed at a large level I, university-affiliated, urban trauma center in Baltimore, Maryland (The R. Adams Cowley Shock Trauma Center at the University of Maryland Medical Center). With the exception of burns (triaged elsewhere), this facility serves as the provider of choice for adult patients with life-threatening trauma within the State of Maryland Emergency Medical System as well as a tertiary care trauma referral center for other area hospitals. For the period 2002–2007, the center admitted between 6,900 and 7,700 patients per year (Wunderlich 2009). The subjects in this study were trauma intensive care unit (ICU) patients who met at least two out of the four conventional criteria for SIRS. Sepsis was said to be present if, at any point during the period of eligibility, a source of infection was identified that could account for the clinical findings.

Typically, this meant a positive culture from a clinically relevant source. Patients for whom no clinically evident source of infection was identified were considered to have noninfectious SIRS.

Sample isolation

Blood samples were obtained daily by venipuncture over a course of up to 14 days, as previously described (Johnson et al. 2007; Lissauer et al. 2007). About 25% of the 352 samples collected were obtained by hospital day 2, 50% by day 4, and 75% by day 7, and the timing of sample collection did not differ significantly between patients with sepsis and patients with noninfectious SIRS ($P=0.243$ by Mann–Whitney U). The most abnormal body temperature recorded in 24 h prior to each blood draw was recorded and taken as the temperature corresponding to that sample. An average of 3.5 ± 0.7 (mean \pm SD; range, 1 to 4) samples per patient was obtained from patients with sepsis and 3.6 ± 0.6 (range, 2 to 4) samples from patients with noninfectious SIRS ($P=0.48$ by t test). RNA was isolated from whole blood using PAXgene (PreAnalytiX, Switzerland) tubes and spin columns, as previously described (Johnson et al. 2007).

Microarray analysis

Microarray analysis was performed by GeneLogic (Gaithersburg, MD, USA), using Affymetrix U133 v2.0 arrays, as previously described (Johnson et al. 2007). Gene expression signal intensities were obtained using MAS 5.

Three of the authors (JDH, LAS, MJC) independently identified genes of interest for analysis, and data from corresponding sequences on the Affymetrix array were selected for analysis. The analysis reported here was limited to these prospectively identified sequences. The final list contained 278 sequences representing about 155 to 170 genes (as defined by NCBI Gene and Unigene identifiers corresponding to the respective Affymetrix probes on the microarray), including HSPs, inflammatory mediators, ribosomal proteins, and control (“housekeeping” sequences) genes.

Statistical analysis

Statistical analysis was performed using SPSS 10.0 and Microsoft Excel. Other data analysis was performed with the assistance of Microsoft Access.

Temperatures below 97.0°F were excluded from analysis, for two reasons: (1) biologically, our interest was in describing gene expression under conditions of hyperthermia; and (2) statistically, the number of data points collected at temperatures below 97.0°F ($N=9$) was too small to draw substantive inferences about gene expression

behavior during hypothermia but large enough to skew the distribution of recorded temperatures away from normality.

To search for statistically significant effects of temperature on gene expression, a general linear model (analysis of covariance—ANCOVA) was created in SPSS. In this model, group (sepsis vs. noninfectious SIRS) was treated as a factor, and temperature was used as a quantitative covariate. Signal intensity for each gene sequence was treated as the dependent variable. The model had the following design: Intercept + temperature + clinical group assignment + interaction of (temperature \times group). A P value of 0.05 or less was taken as statistically significant.

Because samples were drawn on different days during each admission, we also examined a second general linear model (ANCOVA) in SPSS that included the terms described above as well as hospital day (treated as a quantitative covariate). To preserve the ability of this expanded model to detect differences as much as possible, we did not search for interactions between the time the sample was drawn and other terms in the model.

For sequences where the ANCOVA analysis revealed an effect of temperature that was independent of clinical diagnosis, correlation analysis was performed to identify the strength and direction of the association, using Pearson’s correlation for normally distributed expression signals and Spearman’s rho otherwise. We excluded sequences from the final list that had $P>0.05$ in the correlation analysis, even if they showed $P\leq 0.05$ in the ANCOVA. However, this post hoc filter resulted in exclusion of only three sequences out of the 51 identified as significantly affected by temperature alone in the ANCOVA analysis.

For sequences where significant effects were found for the interaction between clinical diagnosis and temperature, Pearson’s correlation between expression signal intensity and temperature was performed on each subgroup (noninfectious SIRS, sepsis) to further characterize the effect of temperature, provided the distribution of gene expression signals within each subgroup was normal as defined by Kolmogorov–Smirnov. For those genes where the gene signal distribution in either of the clinical subgroups deviated from normality, correlation analysis was performed using Spearman’s rho. In this correlation analysis, a $P\leq 0.05$ was considered significant and P between 0.05 and 0.125 was considered a trend.

Results

Characteristics of the enrolled patients and study samples

A total of 361 samples were obtained from 101 patients (180 samples from 51 patients with sepsis, 181 samples

from 50 with noninfectious SIRS) over a course of up to 14 days. Nine samples corresponding to temperatures of less than 97.0°F were excluded from the analysis; this led to exclusion of one patient with sepsis from the analysis, leaving 50 in each group.

The clinical characteristics of the 100 patients included in our analysis are reported in Table 1. The two subgroups of patients (SIRS, sepsis) were closely matched with respect to age, gender, and ethnic background (Table 1). We also examined APACHE II scores in our patients. APACHE II is a widely used scoring system in critical care research that grades severity of illness based on a variety of routinely collected clinical and physiological variables, as well as age and a modifier to account for underlying chronic health conditions (Knaus et al. 1985). The score ranges from 0 to 71; increasing APACHE II scores predict mortality in a variety of ICU populations (Herridge 2003). In our study, there was a small and nonsignificant trend toward slightly higher APACHE II scores in the septic patients at the time of study entry, although this did not translate into a difference in ICU mortality. A greater percentage of patients in the noninfectious SIRS group were discharged directly from the ICU (to home, rehabilitation, chronic care, etc.). Patients with sepsis were more likely to be discharged to an inpatient hospital floor.

The sites of infection and causative organisms in the patients with sepsis were typical of a trauma ICU population. As previously reported (Johnson et al. 2007), about 72% of the infections in the sepsis group were from lung, blood, and

urinary tract sources. Of the organisms isolated, ~42% were Gram-negative bacteria, ~27% were Gram-positive bacteria, and all but one of the remainder were mixed infections (the one exception was a fungal infection; Johnson et al. 2007).

The recorded temperatures ranged from 94.9°F to 104.8°F (34.9°C to 40.4°C) but were slightly higher on average in patients with sepsis. For the 352 data points included in the analysis, the mean temperature recorded was 100.9±1.4°F in the septic patients (±SD, $N=175$) and 100.3±1.1°F (±SD, $N=177$) in the patients with noninfectious SIRS ($P<0.001$ by t test). The number of samples per patient was well-matched after exclusion of the nine hypothermic data points and was 3.5±0.6 (mean ± SD) in each of the two groups ($P=0.75$ by t test).

Genes affected by temperature but not by clinical condition

Of the 278 sequences prospectively chosen for study, the ANCOVA analysis identified 63 (23%) that showed a statistically significant effect of temperature, either independently (51 sequences) of clinical diagnosis or as a function of clinical diagnosis (i.e., had $P\leq 0.05$ for the interaction between clinical diagnosis and temperature, 12 sequences). Of the 51 sequences that were affected by temperature only, three were subsequently excluded because of correlation analysis P values greater than 0.05. Twenty-seven (56%) of the remaining 48 sequences showed increases in expression with increasing temperature; 21 (44%) showed decreases with increasing temperature.

Table 1 Clinical characteristics of the patients

		Sepsis ($N = 50$)		SIRS ($N = 50$)		P value
		N	%	N	%	
Gender	M	35	70%	36	72%	0.826 ^a
	F	15	30%	14	28%	
Race	Caucasian	37	74%	44	88%	0.130 ^a
	Black	11	22%	6	12%	
	Other	2	4%	0	0%	
		Mean	SEM	Mean	SEM	
Age		42.4	2.8	46.2	3.0	0.350 ^b
APACHE II score at study entry		14.6	0.7	12.8	0.7	0.062 ^b
		N	%	N	%	
Outcome	ICU Survivors	47	94%	45	90%	0.593 ^a
	ICU Non-survivors	2	4%	2	4%	
	Unknown	1	2%	3	6%	
Disposition	Discharged directly from ICU	2	4%	15	30%	0.003 ^a
	Transferred to hospital floor	45	90%	30	60%	
	Died	2	4%	2	4%	
	Unknown	1	2%	3	6%	

^aBy Chi-Square test with Yates' correction

^bBy unpaired T-test

The sequences whose expression correlated with temperature independently of clinical diagnosis are listed in Tables 2 and 3. Those which showed increased expression with increasing temperature included several heat shock proteins (including HSP 110, several members of the DNAJ

family of HSPs, HSP 56, and HSP 27-1), superoxide dismutase-1, cell adhesion molecules (including ICAM-1/CD54, CD11b, and JAM3), heat shock transcription factors HSF-1 and HSF-4, and several molecules involved in immune function, such as CXCL5 and -7. Sequences whose expression

Table 2 Sequences whose expression was increased by temperature, independent of clinical diagnosis

Common name(s)	Affymetrix ID	ANCOVA temperature <i>P</i>	Correlation coefficient	Correlation <i>P</i>	Note
Cell adhesion					
Intercellular adhesion molecule 1 (CD54) (ICAM1), human rhinovirus receptor	215485_s_at	0.003	0.160	0.003	
Integrin, alpha M (complement component 3 receptor 3 subunit) (ITGAM); CD11b; CR3A	205786_s_at	<0.001	0.274	<0.001	
Junctional adhesion molecule 3 (JAM3)	231721_at	0.007	0.123	0.021	(1)
	212813_at	0.012	0.152	0.004	
	231720_s_at	0.016	0.142	0.008	
Coagulation					
Platelet factor 4 (chemokine (C-X-C motif) ligand 4) (PF4); CXCL4	206390_x_at	0.018	0.168	0.002	
HSPs, chaperonins, and co-chaperonins					
APG-1; heat shock protein (hsp110 family); HSP 110	205543_at	0.006	0.158	0.003	
DnaJ (Hsp40) homolog, subfamily A, member 1 (DNAJA1); HSPF4	200881_s_at	0.035	0.180	0.001	(2)
	200880_at	0.036	0.170	0.001	
DnaJ (Hsp40) homolog, subfamily A, member 2 (DNAJA2)	209157_at	0.029	0.153	0.004	
DnaJ (Hsp40) homolog, subfamily B, member 1 (DNAJB1)	200664_s_at	0.034	0.122	0.022	(2)
DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4); HLJ-1	203810_at	0.002	0.191	<0.001	(1)
FK506 binding protein 4 (FKBP4), 59 kDa; immunophilin; FKBP52; HSP 56	200894_s_at	<0.001	0.237	<0.001	(1, 2)
	200895_s_at	0.002	0.195	<0.001	
Heat shock 27 kDa protein 1 (HSPB1)	201841_s_at	<0.001	0.270	<0.001	(2)
Suppression of tumorigenicity 13 (colon carcinoma) (ST13); Hsp70 interacting protein; SNC-6; P48	208666_s_at	0.017	0.131	0.014	
	208667_s_at	0.021	0.136	0.011	
Heat shock transcription factors					
Heat shock transcription factor 1 (HSF-1); heat shock factor 1	202344_at	0.010	0.142	0.007	
Heat shock transcription factor 4 (HSF-4)	210977_s_at	0.010	0.128	0.017	
Immune function					
Chemokine (C-X-C motif) ligand 5 (CXCL5)	207852_at	0.007	0.143	0.007	(3)
	214974_x_at	0.039	0.128	0.016	
Phospholipase A2, group IVA (cytosolic, calcium-dependent) (PLA2G4A); cPLA2-alpha	210145_at	0.017	0.162	0.002	
Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7) (PPBP); beta-thromboglobulin (B-TG1); connective tissue-activating peptide III (CTAP3); CXCL7; small inducible cytokine B7; thrombocidin 1; thrombocidin 2	214146_s_at	0.028	0.175	0.001	(1)
Prostaglandin E synthase (PTGES)	207388_s_at	0.037	0.119	0.026	
Toll interacting protein (TOLLIP)	222469_s_at	0.009	0.185	<0.001	
	233881_s_at	0.009	0.143	0.007	
Redox control					
Superoxide dismutase 1 (SOD1), soluble (amyotrophic lateral sclerosis 1 (adult))	200642_at	0.002	0.204	<0.001	

(1) Correlation coefficient and correlation *P* computed by Spearman's rho; (2) previously reported to show increased expression after exertional heat injury (Sonna et al. 2004); (3) also increased after exertional heat injury (Sonna et al. 2004; previously unpublished finding)

Table 3 Sequences whose expression was decreased by temperature, independent of clinical diagnosis

Common name(s)	Affymetrix ID	ANCOVA temperature <i>P</i>	Correlation coefficient	Correlation <i>P</i>	Note
Cell adhesion					
CD47 antigen (CD47) (Rh-related antigen, integrin-associated signal transducer)	242974_at	0.005	-0.166	0.002	
Cell growth, proliferation, and differentiation					
MAL; mal, T-cell differentiation protein	204777_s_at	0.045	-0.143	0.007	(2)
HSPs, chaperonins, and co-chaperonins					
DnaJ (Hsp40) homolog, subfamily A, member 3 (DNAJA3)	205963_s_at	0.009	-0.185	<0.001	
Heat shock 70 kD protein 8 (HSPA8); HSC-70	221891_x_at	0.001	-0.182	0.001	(1, 4)
	208687_x_at	0.006	-0.178	0.001	
	210338_s_at	0.018	-0.144	0.007	
Heat shock 70 kDa protein 9B (HSPA9B) (mortalin 2); 75 kDa glucose-regulated protein (GRP75)	200690_at	0.019	-0.141	0.008	(2)
HSP 70-interacting protein (HSPBP1); HSP 70 binding protein	202415_s_at	0.033	-0.159	0.003	
Spastic ataxia of Charlevoix-Saguenay (sacsin) (SAC)	213262_at	0.038	-0.124	0.020	(1)
TNF receptor-associated protein 1 (TRAP1); HSP75; HSP90L	201391_at	0.016	-0.187	<0.001	(2)
Immune function					
Sterile alpha and TIR motif containing 1 (SARM1); KIAA0524	213257_at	0.021	-0.154	0.004	(3)
Toll-like receptor 6 (TLR-6)	207446_at	0.029	-0.122	0.022	
Toll-like receptor 7 (TLR-7)	220146_at	0.003	-0.209	<0.001	
Signal transduction					
Ring finger protein 216 (RNF216); TRIAD3	218425_at	0.039	-0.122	0.022	
Translation					
Ribosomal protein L13a (RPL13A); 23 kD highly basic protein, 60S ribosomal protein L13a	212790_x_at	0.020	-0.172	0.001	
	210646_x_at	0.026	-0.168	0.002	
Ribosomal protein L37a (RPL37A)	201429_s_at	0.011	-0.192	<0.001	
Ribosomal protein S2 (RPS2)	203107_x_at	<0.001	-0.267	<0.001	
	221798_x_at	<0.001	-0.261	<0.001	
Ribosomal protein S23 (RPS23)	200926_at	0.034	-0.150	0.005	(1)
Ribosomal protein S3 (RPS3)	208692_at	0.013	-0.173	0.001	

(1) Correlation coefficient and correlation *P* computed by Spearman's rho; (2) previously reported to show decreased expression after exertional heat injury (Sonna et al. 2004); (3) also decreased after exertional heat injury (Sonna et al. 2004; previously unpublished finding); (4) previously reported to show *increased* expression after exertional heat injury (Sonna et al. 2004)

decreased with increasing temperature included Toll-like receptors TLR-6 and TLR-7, CD47, T-cell differentiation protein MAL, a handful of HSPs/chaperonins, and several ribosomal proteins.

Several of the sequences whose expression correlated with temperature in this study have previously been shown to be affected by exertional heat injury (EHI) in PBMCs (Sonna et al. 2004).¹ With one exception, the direction of

change with increasing temperature in this study was similar to the direction of change observed after EHI. The sole exception was HSC-70, which showed decreased expression with increasing temperature in sepsis/SIRS but was increased after EHI.

Effect of clinical diagnosis and temperature on gene expression

Twelve genes showed temperature-dependent levels of expression that were affected by clinical diagnosis (i.e., a $P \leq 0.05$ for the interaction of temperature \times clinical diagnosis). These sequences are summarized in Table 4. To better identify the nature of these interactive effects, we performed a correlation analysis of temperature and gene

¹ All but two of these sequences were reported individually in the previous manuscript (Sonna et al. 2004). The other two corresponded to SARM1 and CXCL5, which were in the list of genes found to be affected by EHI but did not meet the 5-fold cutoff required of a non-HSP to be reported in that manuscript's tables.

Table 4 Sequences for which the effect of temperature was dependent on diagnosis in the ANCOVA analysis

Affymetrix ID	Common name(s)	ANCOVA <i>P</i> value for:			Note
		Diagnosis (noninfectious SIRS vs. sepsis)	Diagnosis × temperature	Temperature	
205242_at	Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant) (CXCL13)	0.026	0.025	0.535*	
202865_at	DnaJ (Hsp40) homolog, subfamily B, member 12 (DNAJB12)	0.051*	0.050	0.502*	
206782_s_at	DnaJ (Hsp40) homolog, subfamily C, member 4 (DNAJC4)	0.019	0.019	0.007	
223000_s_at	F11 receptor (F11R)	0.046	0.044	0.527*	
215363_x_at	Folate hydrolase (prostate-specific membrane antigen) 1 (FOLH1)	0.050	0.048	0.875*	
211220_s_at	Heat shock transcription factor 2 (HSF-2)	0.037	0.036	0.589*	
200799_at	Heat shock 70 kD protein 1A (HSPA1A); HSP 70-1A	0.030	0.035	0.106*	(3,4)
202581_at	Heat shock 70 kDa protein 1B (HSPA1B); HSP 70-1B	0.025	0.027	0.285*	(3,4)
210189_at	Heat shock 70 kDa protein 1-like (HSPA1L)	0.042	0.047	0.442*	
202859_x_at	Interleukin 8 (IL-8); CXC chemokine ligand 8	0.003	0.003	0.045	(2)
214461_at	Lipopolysaccharide binding protein (LBP)	0.029	0.028	0.998*	(1)
210367_s_at	Prostaglandin E synthase (PTGES)	0.034	0.036	0.320*	

(1) Serum LBP protein has been reported to be differentially expressed in SIRS vs. sepsis (Prucha et al. 2003); (2) serum IL-8 has been reported to be differentially expressed in SIRS vs. severe sepsis/septic shock (Rodriguez-Gaspar et al. 2001); (3) HSP 70 protein has been reported to be differentially expressed in SIRS vs. sepsis (Adib-Conquy and Cavaillon 2007); (4) HSP 70A1A and A1B mRNA have been reported to show increased expression after exercise (Connolly et al. 2004) and exertional heat injury (Sonna et al. 2004)

* $P > 0.05$, not statistically significant

expression signal for each of these genes, stratified by clinical diagnosis. Correlation analysis was performed using two-tailed Pearson's analysis unless otherwise specified. Four patterns of expression emerged from this analysis (Table 5): (1) A positive correlation of gene expression with temperature (increasing expression with increasing temperature) in sepsis with no effect in noninfectious SIRS; (2) a positive correlation of gene expression with temperature in noninfectious SIRS with no significant effect in sepsis; (3) a negative correlation of gene expression with temperature (decreasing expression with increasing temperature) in sepsis with no effect in noninfectious SIRS; and (4) a negative correlation of gene expression with temperature in noninfectious SIRS with no effect in sepsis. Importantly, sequences corresponding to three members of the HSP 70 family that are tightly co-regulated (HSP 70-1A, 70-1B, and HSPA1-like) all displayed similar expression patterns with respect to temperature and clinical diagnosis (Table 5).

Two sequences corresponding to prostaglandin E synthase (PTGES) showed increasing expression with temperature. For one of the sequences (Table 2), the effect was independent of clinical diagnosis; the other (Table 4) showed an interactive effect between clinical diagnosis and temperature, with a significant relationship between temperature and expression occurring in noninfectious SIRS but not in sepsis.

Effect on control sequences

Table 6 shows the effect of temperature and clinical condition on the expression of sequences corresponding to genes that have previously been reported to display stable expression over time in peripheral blood mononuclear cells obtained from individuals EHI (Sonna et al. 2004). There was no statistically significant effect of temperature or clinical condition on the expression of these control sequences.

Because of their dual potential to stimulate fever and activate expression of many of the sequences analyzed in this study, we also examined the effect of hyperthermia on expression of sequences corresponding to TNF-alpha (one sequence) and interleukin 1-beta (two sequences). None of these sequences were affected by temperature or diagnosis.

Given the increasingly recognized role in the pathophysiology of murine sepsis of heme oxygenase-1 and its product, carbon monoxide (Chung et al. 2008; Kim et al. 2005), we examined the effect of temperature on the expression of a sequence corresponding to human heme oxygenase-1 (HSP 32). There was no effect of temperature or diagnosis on expression of this sequence in our general linear model.

Effect of adjusting for time

To adjust for the possibility that time contributed to the observed changes in gene expression, we performed an

Table 5 Expression patterns produced by the interaction of temperature and clinical diagnosis

Expression pattern (See text)	Gene	Sepsis, N=175				Noninfectious SIRS, N=177				Note	
		P	R	R ²	Significant?	Temperature effect	P	R	R ²		Significant?
1	DNAJC4	<0.001	0.280	0.078	Yes	Up	0.801	0.019	<0.001	No	No effect
2	HSP70-1A	0.211	-0.095	0.009	No	No effect	0.092	0.127	0.016	Trend ^b	Up
	HSP70-1B	0.673	-0.032	0.001	No	No effect	0.024	0.169	0.029	Yes	Up
	HSPA1L	0.387	-0.066	0.004	No	No effect	0.025	0.168	0.028	Yes	Up
	PTGES	0.351	-0.071	0.005	No	No effect	0.063	0.140	0.020	Trend ^b	Up
3	CXCL13	0.402	-0.064	0.004	No	No effect	0.037	0.157	0.025	Yes	Up
	F11R	0.083	-0.131	0.017	Trend ^b	Down	0.598	0.040	0.002	No	No effect
	FOLH1	0.035	-0.159	0.025	Yes	Down	0.376	0.067	0.004	No	No effect
	LBP	0.112	-0.120	0.014	Trend ^b	Down	0.204	0.096	0.009	No	No effect
4	DNAJB12	0.074	-0.135	0.018	Trend ^b	Down	0.165	0.105	0.011	No	No effect
	IL-8	0.300	0.079	0.006	No	No effect	0.093	-0.127	0.016	Trend ^b	Down
		0.840	-0.015	<0.001	No	No effect	0.001	-0.248	0.062	Yes	Down

P and R values were computed by Pearson's correlation unless otherwise specified

^a P and R values calculated by Spearman's rho

^b A trend was defined as $0.05 < P < 0.125$

additional ANCOVA analysis in which the number of days since hospital admission (hospital day number) were included as a quantitative covariate in the model. To preserve the ability of the model to detect significant changes, we did not add interactive terms to this second model. After confirmatory correlation analysis (performed as before), the results of the analysis with adjustment for time overlapped well with those obtained in the original analysis. The adjustment did not remove any genes whose expression was affected by clinical diagnosis (alone or interactively with temperature) from the list of significantly affected genes, though it did add one. After correlation analysis (see "Materials and methods"), the adjustment for time added six sequences to the list of genes affected by temperature and subtracted four.

The adjustment for time added one sequence to the list of genes that showed an interaction between temperature and clinical diagnosis. This sequence corresponded to beta-2 integrin. It displayed a trend toward increased expression with increasing temperature in the patients with sepsis but no significant effect of temperature in SIRS (i.e., it followed pattern 1 as defined in Table 5).

The adjustment for time added six sequences to the list of genes affected by temperature independently of clinical diagnosis (all increased): Toll-like receptor 8 (two sequences), beta-2 integrin (another sequence, separate from the one just mentioned), GAPDH (three sequences).

Four sequences with borderline P values ($0.028 \leq P \leq 0.05$) in the original model (Tables 2 and 3) no longer displayed significant effects of temperature in the model that accounted for time. These corresponded to PPBP/CXCL7, CXCL5, Toll-like receptor 6, and TRIAD3.

Discussion

This microarray study identified 60 sequences, corresponding to about four dozen genes, whose level of messenger RNA (mRNA) expression in whole blood correlates with febrile-range temperatures achieved in ICU patients with sepsis and noninfectious SIRS. Furthermore, for about 1/5 of these genes, the effect of temperature differed between sepsis and noninfectious SIRS.

Based on the present study and other literature findings, temperature itself may serve as a signal for altered gene expression for at least some of the genes reported in this study, such as HSPs. It is well established, both in animal models and in tissue culture systems, that exogenously administered hyperthermic stress triggers gene expression changes that continue for at least several hours even after return to normothermia (for a review, see Sonna et al. 2002a). Furthermore, altered expression of mRNA corresponding to several heat shock proteins and other stress

Table 6 Effect of temperature and clinical condition on control gene expression

Gene	Common name(s)	Affymetrix ID	ANCOVA <i>P</i> value for		
			Diagnosis (noninfectious SIRS vs. sepsis)	Diagnosis × temperature	Temperature
ACTB	Beta actin	200801_x_at	0.220	0.234	0.067
		213867_x_at	0.119	0.128	0.161
		224594_x_at	0.097	0.105	0.107
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	212581_x_at	0.777	0.798	0.071
		213453_x_at	0.949	0.976	0.055
		217398_x_at	0.564	0.580	0.066
PPIA	Peptidylprolyl isomerase A; cyclophilin A	217602_at	0.695	0.686	0.078
		212661_x_at	0.781	0.782	0.070
		211978_x_at	0.865	0.866	0.079
		211765_x_at	0.807	0.808	0.058
		211378_x_at	0.862	0.859	0.122
		201293_x_at	0.855	0.856	0.060
RPL41	Ribosomal protein L41	201492_s_at	0.452	0.468	0.249
RPS29	Ribosomal protein S29	201094_at	0.515	0.531	0.163
RPLP1	Ribosomal protein, large, P1	200763_s_at	0.199	0.212	0.757
RPS12	Ribosomal protein S12	213377_x_at	0.574	0.528	0.116
RPL38	Ribosomal protein L38	221943_x_at	0.164	0.161	0.745

proteins has previously been reported in human peripheral blood mononuclear cells *in vivo* after exertional heat injury (Sonna et al. 2004), and it is noteworthy that about 1/6 of the sequences reported here correspond to genes that also showed strong and significant changes after EHI, and with one exception (HSC-70), showed changes in the same direction as the changes observed after EHI. Nonetheless, other physiological signals that occur during critical illness and which are characteristic of the febrile state (such as production of IL-1beta), as well as the alterations they produce systemically, undoubtedly have the potential to cause gene expression changes in blood cells *in vivo*. Although our microarray data did not show evidence of temperature-related changes in expression of IL-1beta or TNF-alpha mRNA in whole blood, these data cannot address the question of whether or not there were temperature- or diagnosis-related differences in IL-1 and TNF-alpha protein levels in serum or tissues. If present, such differences might themselves be capable of producing some of the changes in mRNA expression that we observed in whole blood. Thus, while our results are consistent with the hypothesis that temperature alone can trigger changes in gene expression in whole blood, we also think it possible (indeed, likely) that at least some of the gene expression changes we detected were the product of tissue signals characteristic of the critically ill state that, in turn, trigger both fever and gene expression changes in whole

blood (i.e., correlation without direct causation). Further mechanistic studies are required to establish the relative contributions of thermal and nonthermal signals to gene expression *in vivo*.

The present study of critically ill patients did not include an unaffected control group (i.e., patients with no evidence of sepsis or SIRS). Thus, the fact that expression of any particular gene (e.g., IL-1beta, or TNF-alpha) failed to show an apparent association with clinical diagnosis or temperature in this particular study does not imply that the level of expression observed was similar to what would have been detected in unaffected controls.

Although microarray data obtained from humans *in vivo* can be notoriously sensitive to experimental conditions (Fehrenbach 2007; Sonna et al. 2007), several of the findings of this study are consistent with previously published observations. As noted previously, about 1/6 of the sequences identified in this study correspond to genes that were previously reported to be affected by EHI (Sonna et al. 2004). Furthermore, of the 12 sequences for which the expression response to temperature was affected by clinical diagnosis in this study, at least three correspond to proteins that have been found by others to display different levels of expression in serum obtained from patients with sepsis vs. noninfectious SIRS: interleukin 8 (Rodriguez-Gaspar et al. 2001), lipopolysaccharide binding protein (Prucha et al. 2003), and HSP 70 (Adib-Conquy

and Cavaillon 2007). Finally, housekeeping genes reported to show stable expression after exertional hyperthermia *in vivo* in a prior work (Sonna et al. 2004) also showed stable expression with respect to temperature and clinical diagnosis in this study. This degree of congruence with prior literature reports increases our confidence that at least some of the novel associations detected in this study will prove to be replicable.

Interestingly, expression of several closely related members of the HSP 70 family of genes showed a correlation with temperature in noninfectious SIRS but not in sepsis. HSP 70 genes are among the most highly heat-inducible mammalian genes known. In animal and cellular models of thermal stress, intracellular expression of these genes correlates with the acquisition of thermotolerance and cross-resistance to a variety of nonthermal stimuli (Wheeler and Wong 2007). Furthermore, release of HSP 70 by stressed cells into the extracellular space may serve as an activating signal to the innate immunity system (Wheeler and Wong 2007). The present findings suggest the interesting possibility that inflammatory mediators or other humoral factors unique to sepsis might somehow blunt the temperature-dependent expression response of HSP 70 family genes. On a mechanistic level, the major transcription factor responsible for heat-responsive HSP 70 expression, HSF-1, has been extensively studied and its activity is known to be affected both by heat and subject to modulation by nonthermal stimuli (Cotto and Morimoto 1999; Mathew et al. 2001; Morimoto 1998). Mechanistic studies of the effect of inflammatory mediators of sepsis on HSF-1 responses to febrile-range hyperthermia would seem warranted.

Several important limitations should be kept in mind when interpreting the results of this study. First, it was an observational clinical study, subject to the substantial sources of variance inherent in this type of work. However, the relatively large number of patients studied and samples obtained should mitigate somewhat the effects of individual random errors. Second, the clinical criteria used in this study to distinguish noninfectious SIRS from sepsis, while widely accepted and based on consensus criteria, are nonetheless still imperfect. Furthermore, this study was performed in a surgical ICU where the primary cause of SIRS was trauma. Accordingly, the list of genes identified as differently affected by noninfectious SIRS and sepsis might be different under a different classification scheme or in a different patient population. Third, the temperatures recorded in this study represent the most abnormal temperature recorded within 24 h of the sample; although it is known from experimental work that the effects of hyperthermia on gene expression continue even after return to normothermia, different (and perhaps higher) correlations might have been obtained had we recorded the temperature

at the time of the blood draw or been able to adjust our statistical model to account for the time interval between the blood draw and the maximum recorded temperature. Fourth, our study was performed in whole blood rather than a more narrowly defined subset of peripheral blood cells, such as peripheral blood mononuclear cells. It is possible that some of the changes in expression observed are the result of temperature-dependent changes in cell-type distribution rather than due to changes in expression within a particular lineage. Fifth, although temperatures were normally distributed in our dataset (after exclusion of the hypothermic temperatures), a few genes did show expression signals whose distribution deviated from normality. We do not believe this to be problematic because ANOVA-based techniques are fairly robust with respect to deviations from normality, and for the few genes whose expression signals deviated from normality, subsequent nonparametric correlation analysis (Spearman's rho) confirmed the effect of temperature on gene expression detected in the parametric ANCOVA analysis. It is nonetheless possible that our use of parametric statistics in the first-pass analysis resulted in a few false negative reports; however, we estimate this error to involve no more than 8 (3%) of all sequences examined.² Sixth, even though APACHE II scores were well-matched, there were a large number of unmeasured variables in our population of trauma ICU patients, such as blood transfusions, use of vasopressor agents, use of sedatives and analgesic agents, all of which could potentially have confounding effects on gene expression. Seventh, although the distinction between noninfectious SIRS and sepsis used in this manuscript reflects common clinical practice, it is imperfect. In particular, patients with sources of infection that were unrecognized due to limitations of available diagnostic technology would have been misclassified as having noninfectious SIRS. Last, though statistically significant and biologically relevant, the observed correlation coefficients were quite low and the observed differences between septic and noninfectious SIRS temperature-dependent signals were generally small. While likely to generate interesting and important mechanistic hypotheses, the results of this study do not suggest a clinically useful marker to discriminate noninfectious SIRS from sepsis.

An additional important limitation of microarray studies is the substantial risk of false-positive reports, given the very large number of genes that are analyzed simultaneous-

² This estimate was derived by looking for sequences that might have been included in the final list of affected genes had we performed Spearman's rho on the nonnormally distributed sequences in the absence of a prior ANCOVA analysis.

ly. To mitigate this risk, we prospectively defined a set of genes as being of interest, rather than data mining the entire array retrospectively for positive findings. Accepting a P value of 0.05 as statistically significant, one would expect only 14 sequences to demonstrate significant changes in expression by random chance alone; in fact, we observed significant effects in almost four times as many. For many of these, the P value for one or more of the effects (temperature, diagnosis, or the interaction diagnosis \times temperature) was substantially below 0.05; indeed, 22 of the 63 sequences identified in the ANCOVA analysis had at least one P value of 0.01 or less. Additionally, our data showed expected correlations between temperature and HSP expression as well as no significant correlation between temperature and several control (“housekeeping”) genes that have previously shown relatively stable expression in vivo (Sonna et al. 2004). Last, all but three of the genes identified as significantly affected by temperature alone in the ANCOVA analysis also showed $P \leq 0.05$ in the correlation analysis. While we cannot entirely exclude the possibility of false-positive results in this study, we think the number is likely to be relatively small. The results of our adjustment for hospital day suggest that our findings are robust, at least for the sequences with P values well below 0.05.

In summary, additional analysis of the microarray data originally reported by Johnson et al. (2007) reveals a significant association between level of fever and expression levels of mRNA corresponding to several HSPs, inflammatory mediators, cytokines/chemokines, signaling molecules, cell adhesion molecules, and ribosomal proteins. Our results are consistent with the possibility that fever in ICU patients can influence expression of certain genes related to heat shock and immune response. For some of these genes, the effect of temperature differed between patients with sepsis and patients with noninfectious SIRS. This suggests the interesting possibility that noninfectious SIRS and sepsis might exert some of their differing effects at the molecular level by altering temperature-dependent expression of a select number of genes in circulating blood cells.

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