Febrile-Range Hyperthermia Modifies Endothelial and Neutrophilic Functions to Promote Extravasation

Mohan E. Tulapurkar^{1,2*}, Eid A. Almutairy^{1*}, Nirav G. Shah¹, Ju-ren He^{1,5}, Adam C. Puche⁴, Paul Shapiro³, Ishwar S. Singh^{1,2,5}, and Jeffrey D. Hasday^{1,2,5}

¹Division of Pulmonary and Critical Care, Department of Medicine, and ²Mucosal Biology Research Center, School of Medicine, ³Department of Pharmaceutical Sciences, School of Pharmacy, and ⁴Department of Anatomy and Neurobiology, University of Maryland, Baltimore, Maryland; and ⁵Research Services, Baltimore Veterans Administration Medical Center, Baltimore, Maryland

Acute respiratory distress syndrome (ARDS) is a neutrophil (polymorphonuclear leukocyte; PMN)-driven lung injury that is associated with fever and heat-stroke, and involves approximately 40% mortality. In murine models of acute lung injury (ALI), febrile-range hyperthermia (FRH) enhanced PMN accumulation, vascular permeability, and epithelial injury, in part by augmenting pulmonary cysteine-x-cysteine (CXC) chemokine expression. To determine whether FRH increases chemokine responsiveness within the lung, we used *in vivo* and *in vitro* models that bypass the endogenous generation of chemokines. We measured PMN transalveolar migration (TAM) in mice after intratracheal instillations of the human CXC chemokine IL-8 *in vivo*, and of IL-8-directed PMN transendothelial migration (TEM) through human lung microvascular endothelial cell (HMVEC-L) monolayers *in vitro*. Pre-exposure to FRH increased *in vivo* IL-8-directed PMN TAM by 23.5-fold and *in vitro* TEM by 7-fold. Adoptive PMN transfer demonstrated that enhanced PMN TAM required both PMN donors and recipients to be exposed to FRH, suggesting interdependent effects on PMNs and endothelium. FRH exposure caused the activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase in lung homogenates and circulating PMNs, with an associated increase in HSP27 phosphorylation and stress-fiber formation. The inhibition of these signaling pathways with U0126 and SB203580 blocked the effects of FRH on PMN extravasation *in vivo* and *in vitro*. Collectively, these results (1) demonstrate that FRH augments chemokine-directed PMN extravasation through direct effects on endothelium and PMNs, (2) identify ERK and p38 signaling pathways in the effect, and (3) underscore the complex effects of physiologic temperature change on innate immune function and its potential consequences for lung injury.

Keywords: ARDS; neutrophil; endothelium; febrile-range hyperthermia; p38 MAP kinase

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) pose frequent complications in critically ill patients, and are responsible for significant morbidity, mortality, and related health-care costs (1). To date, only two interventions, low-volume mechanical ventilation (2) and aggressive fluid management (3), have been

CLINICAL RELEVANCE

This study shows that the temperature increase occurring during fever is sufficient to alter endothelial and neutrophilic function, and to increase neutrophilic transendothelial migration *in vitro* and recruitment *in vivo*. Evidence for a p38-dependent mechanism is provided.

shown to confer benefit in patients with ARDS. However, despite these advances in supportive care, mortality remains at approximately 40% in such patients (4). Fever is common in critically ill patients (5, 6), especially in those with ALI/ARDS (7), and ARDS is a common complication of heat stroke (8). Fever is associated with increased length of stay in the intensive care unit, prolonged mechanical ventilation, and increased mortality (5, 6, 9).

Murine studies in which hyperthermia is induced by raising ambient temperature demonstrated that febrile-range hyperthermia (FRH; an $\sim 2^{\circ}\text{C}$ increase in core temperature) profoundly increases ALI/ARDS (10, 11). In murine models of ALI caused by exposure to hyperoxia (10) or an intratracheal instillation of LPS (11), concurrent exposure to FRH greatly increased pulmonary polymorphonuclear leukocyte (PMN) accumulation, endothelial barrier dysfunction, and epithelial injury, three cardinal manifestations of human ARDS. We showed that gene promoters for neutrophil-attracting cysteine-x-cysteine (CXC) chemokines contain binding sequences for the heat-activated transcription factor HSF-1 (12), that HSF-1 is activated at febrile-range temperatures (13) and augments the expression of some CXC chemokines (14), and that exposure to FRH increases pulmonary expression levels of CXC chemokines in murine models of ALI (10, 11). Immunoblockade of the CXC receptor, CXCR2, blocked neutrophil influx and reduced endothelial barrier dysfunction in mice exposed to FRH and hyperoxia (10), confirming CXC chemokines as the predominant neutrophil chemoattractants in this model. These data implicate the generation of CXC chemokines in the mechanism of FRH-enhanced PMN recruitment to lung. However, in both the hyperoxia-induced and LPS-induced models of ALI, accelerated pulmonary PMN accumulation persisted in FRH-exposed mice for at least 24 hours after CXC chemokines returned to normothermic levels (10, 11), suggesting that FRH enhances PMN recruitment through mechanisms in addition to increased chemokine expression.

Transendothelial migration (TEM) is a complex process that requires coordinated molecular interactions between endothelia and PMNs (15), and can be augmented by exposure to inflammatory mediators such as IL-1 β and TNF- α (16). To determine whether FRH alters the PMN-endothelial interactions required for PMN recruitment, we used an *in vivo* PMN migration assay that measures the transalveolar migration (TAM) of PMNs across a fixed, exogenous chemokine gradient established by the intratracheal instillation of human IL-8, a recognized ligand for murine CXCR2 (17). Exposing mice to FRH for 16 to 24 hours increased IL-8-directed transalveolar migration (TAM) by 10.5- to 23.5-

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E.A.A. is currently at the Department of Medicine at King Faisal Specialist Hospital and Research Center.

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

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fold, compared with normothermic mice. The priming effect of FRH on PMN migration persisted for over 48 hours, and was caused by changes in both PMN and endothelial function, and required the activation of both extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPKs). These profound, previously unappreciated effects of FRH on PMN-endothelial interactions may have important consequences for the management of patients with ALI/ARDS.

MATERIALS AND METHODS

All protocols were approved by the Institutional Animal Care and Use Committee and the Institutional Review Board at the University of Maryland.

Murine Models

Male CD-1 mice were housed under conditions approved by the Association for Assessment and Accreditation of Laboratory Animal Care. To achieve FRH, mice were placed in 36–37°C incubators as described elsewhere (10), and were then either killed for baseline lung lavage, switched to normothermia and the performance of IL-8-directed TAM, or allowed to recover at normothermia before the TAM assay. TAM was measured by instilling 1 µg recombinant human (rh) IL-8 intratracheally, followed 4 hours later by death, lung lavage, and PMN count (18). Some mice received 200 µg U0126, 1 mg SB203580, or 2% DMSO (sham) intraperitoneally, 30 minutes before FRH. Core temperature was monitored using ETA-F10 intraperitoneal thermistors (Data Sciences International, St. Paul, MN), placed 10 days earlier (19).

Immunoblotting

Human lung microvascular endothelial cells (HMVEC-Ls) and snap-frozen lung were homogenized in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors, and were immunoblotted as described (20) for phospho-ERK and total ERK1/2 and p38α/β, intercellular adhesion molecule (ICAM)-1, and ICAM-2, and β-tubulin. Band chemiluminescence was quantified by direct imaging. Phospho-ERK/p38 was normalized to total ERK/p38, and other proteins were normalized to β-tubulin.

Immunofluorescence Confocal Microscopy

Lungs were inflation-fixed and paraffin-embedded as described elsewhere (11), and 25-µm sections were incubated with biotinylated rat anti-mouse granulocyte receptor-1 (GR-1) and rabbit anti-vascular endothelial (VE)-cadherin, anti-ICAM-1, or anti-ICAM-2, blocked with 5% donkey serum and then avidin/biotin blocking reagent, detected with goat anti-rabbit CY5-conjugate and streptavidin CY3-conjugate, and visualized using an Olympus confocal microscope and Olympus Fluo View and Neurolucida software (Neurolucida, Williston, VT). PMN extravasation was analyzed essentially as described previously (21).

Adoptive PMN Transfer

PMNs were isolated from donor blood by dextran sedimentation and anti-Ly-6G micromagnetic bead selection (Miltenyi Biotec, Cologne, DE) and labeled with Cell-Tracker Green (Invitrogen, Carlsbad, CA), 5×10^5 PMNs were injected via tail vein, and TAM was performed. Total lavage PMNs were counted, and the proportion from donors was determined by flow cytometry.

Transendothelial Migration

Postconfluent HMVEC-L monolayers (Lonza, Walkersville, MD) were established on 3-µm-pore Matrigel-coated Transwell inserts (Corning, Corning, NY), and the TEM of freshly isolated, acetomethoxy calcein (calcein AM)-labeled human PMNs was measured as described elsewhere (22), except with 100 ng/ml IL-8 in the lower chamber and PMNs in the upper.

Flow Cytometry

MAPK activation in permeabilized PMNs was analyzed by immunostaining for phosphorylated and total p38/ERK, using allophycocyanin-

conjugated and FITC-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The surface expression of CXCR2 and β2-integrin on murine blood PMNs was analyzed using antibodies from R&D Systems (CXCR2) and BD Biosciences (San Jose, CA). Human PMNs were incubated at 37°C or 39.5°C with 500 U/ml rhG-CSF (R&D Systems, Minneapolis, MN) to maintain viability (20), and β2-integrin expression was analyzed by flow cytometry with antibodies from BD Biosciences, using GR-1 and 7-amino actinomycin labeling to gate viable PMNs.

ICAM-1 binding avidity was analyzed as described elsewhere (23), with modifications. Protein A-coated, 1-µm Fluoresbrite yellow-green carboxylated polystyrene beads (Polysciences, Niles, IL) were derivatized with human ICAM-1/IgG-Fc (R&D Systems). After erythrocyte lysis, leukocytes were incubated for 30 minutes at 37°C with ICAM-1-derivatized microbeads in 1.5-ml microcentrifuge tubes positioned horizontally, and shaken at 150 rpm. GR-1-gated cells were analyzed for green-yellow fluorescence by flow cytometry.

ICAM-1 Cross-Linking

HMVEC-Ls were serum-starved for 12 hours, incubated at 37°C or 39.5°C for 24 hours, and sequentially incubated with mouse anti-human ICAM-1 antibody and goat anti-mouse IgG-Fc, as described elsewhere (24).

Cytoskeletal Changes

HMVEC-L monolayers on multichamber glass slides were incubated with or without 0.25 U/ml TNF-α for 6 hours at 37°C or 39.5°C, fixed, stained with AlexaFluor488-coupled phalloidin and 4',6-diamidino-2-phenylindole, and visualized using confocal microscopy.

Data Analysis

Data are presented as means ± standard errors. Differences among more than two groups were analyzed by one-way ANOVA and the Tukey honestly significant difference test. Core temperatures were compared using MANOVA.

RESULTS

Effect of FRH Exposure on IL-8-Directed PMN Transalveolar Migration

To allow for Circadian rhythm, all FRH exposures were initiated at 8 AM. As we previously demonstrated (19), FRH-exposed mice maintained a normal Circadian pattern with a noon nadir and a peak at 8 PM, but core temperatures diverged, beginning within approximately 4 hours of exposure, and they remained 1.5–2°C higher in FRH-exposed mice during the 24-hour exposure (Figure 1A). In normothermic mice, IL-8 instillation increased the recovery of PMNs in lung lavage from $0.8 \pm 0.3 \times 10^4$ to $7.7 \pm 1.9 \times 10^4$. Pre-exposure to FRH for 16 and 24 hours stimulated additional 10.2-fold and 23.5-fold increases in post-IL-8 PMN recovery (Figure 1B), and the effect persisted for ≥ 48 hours after returning to normothermia (Figure 1C). Without an instillation of IL-8, FRH exposure neither induced TAM (Table 1) nor stimulated the expression of endogenous CXC chemokines (Table 2). Despite profound PMN accumulation, FRH exposure and IL-8 instillation (FRH + IL-8) did not cause an increase in protein extravasation (Table 1).

Confocal microscopy revealed changes in the numbers and locations of pulmonary PMNs after FRH exposure and IL-8 instillation (Figures 1D–1F and E1A and E1B). FRH alone did not alter the number or location of intravascular PMNs, but FRH + IL-8 treatment increased total intravascular PMNs 5-fold versus untreated normothermic mice (Figure 1D), and increased extravasating PMNs 10.9-fold and 3.3-fold versus untreated and IL-8-treated normothermic mice, respectively (Figure 1E). We quantified PMN elongation along endothelial surfaces (Figure 1F) as an indicator of tight binding and migration. FRH alone did not alter PMN shape, but treatment with FRH + IL-8 increased

Figure 1. Effects of exposure to febrile-range hyperthermia (FRH) on IL-8-directed transalveolar polymorphonuclear leukocyte (PMN) migration. (A) Time course of core temperature in normothermic and FRH-exposed mice. Mean $\pm$ SE, $n = 4$ per group. The curves are different ($P < 0.05$ according to MANOVA). (B) Mice were exposed to FRH for the indicated times and returned to normothermia, intratracheal IL-8 was instilled, and 4 hours later, lung lavage PMN and macrophage (MAC) content was measured. BALF, bronchoalveolar lavage fluid. (C) Mice were exposed to FRH for 24 hours, and allowed to recover at normothermia for the indicated times before IL-8-directed transalveolar migration (TAM) assay. Control mice remained normothermic. Mean $\pm$ SEM; eight mice per group. * $P < 0.05$ versus time 0 (B) or control (C). (D and E) Inflation-fixed lungs from normothermic (NT) and 24-hour FRH-exposed mice without intratracheal IL-8 instillation or 4 hours after intratracheal IL-8 instillation were stained for granulocyte receptor-1 (GR-1) and vascular endothelial (VE)-cadherin, and analyzed by confocal microscopy for numbers of total (D) and extravasating (E) PMNs per $\times 60$ high-power field (hpf) and (F) PMN elongation. Mean $\pm$ SEM; five fields per mouse, four mice per group. * $P < 0.05$, versus NT mice without IL-8. $^{\dagger}P < 0.05$, versus FRH mice without IL-8. $^{\ddagger}P < 0.05$, versus NT mice with IL-8, respectively.

PMN elongation 4.7-fold and 1.6-fold versus normothermic mice without and with IL-8, respectively. Although exposure to FRH augmented IL-8-directed PMN TAM, it did not increase PMN expression of the IL-8 receptor, CXCR2 (Figure E2A).

Participation of Endothelia and PMNs

Because PMN extravasation requires active PMN–endothelial interaction (15), we used adoptive PMN transfer to analyze the relative contributions of PMNs and endothelium to enhanced PMN TAM in FRH-exposed mice. When PMNs from donors that had been exposed to FRH for 24 hours were transferred to recipients exposed to FRH for 24 hours, donor PMN TAM was 5.5-fold higher compared with PMNs from normothermic donors transferred to normothermic recipients (Figure 2A). Exposing either donors or recipients alone to 24 hours of FRH did not enhance

PMN migration above normothermic levels, suggesting that FRH modifies interdependent processes in PMNs and endothelium.

Effects on β_2 -Integrin–ICAM Interactions

Because the engagement of PMN β_2 -integrins by pulmonary endothelial ICAM-1/2 is critical to PMN extravasation (15), we analyzed how 24-hour FRH exposure modified PMN β_2 -integrin and lung ICAM-1/2 expression. Flow cytometry of CD18-stained, CD11a-stained, and CD11b-stained PMNs did not detect changes in the proportion of circulating PMNs expressing each β_2 -integrin subunit (Figure 2C) or the total β_2 -integrin expression levels, as indicated by the mean fluorescence intensity of CD18-stained PMNs (data not shown) in FRH-exposed mice. To assess possible β_2 -integrin conformational changes to a high-affinity state (23), we used a bead-based ICAM-1 binding assay (Figure 2D). PMNs from mice exposed to FRH for 24 hours and PMNs from normothermic mice bound similar numbers of ICAM-1-coated beads. The addition of IL-8 to the 30-minute ICAM-1 bead-binding assay to assess potential synergistic effects between FRH exposure and CXC chemokines on β_2 -integrin conformation (23) also failed to detect a difference between normothermic and FRH PMNs (Figure E2B). Finally, neither immunoblotting (Figures 2E and E3A) nor confocal microscopy (Figure E3B) revealed differences in lung ICAM-1/2 expression between normothermic and 24-hour FRH-exposed mice.

Potential Contribution of ERK and p38 MAPKs

Immunoblotting lung homogenates from normothermic control and FRH-exposed mice showed an approximately 2-fold increase in ERK phosphorylation peaking at 9 hours of FRH exposure, and a 30% increase in p38 phosphorylation peaking at 6 hours of FRH exposure (Figure 3A). The phosphorylation of

TABLE 1. EFFECTS OF 24-HOUR FRH EXPOSURE WITH AND WITHOUT INTRATRACHEAL IL-8 INSTILLATION ON IL-8-DIRECTED TAM

Mouse Treatment	Total PMN Numbers in Lung Lavage		P Value [§]
	Without IL-8 Instillation	With IL-8 Instillation [†]	
Normothermia	924 $\pm$ 437 [‡]	0.07 $\pm$ 0.02 $\times 10^6$	0.0001
24-hour FRH exposure*	6,563 $\pm$ 6,257	1.73 $\pm$ 0.85 $\times 10^6$	0.0001

Definition of abbreviations: FRH, febrile-range hyperthermia; PMN, polymorphonuclear leukocyte; TAM, transalveolar migration.

*Core temperature was maintained at approximately 39.5°C.

[†]Lung lavage, 4 hours after instillation of 1 μ g human IL-8.

[‡]Mean $\pm$ SE of lung-lavage PMNs recovered ($n = 8$).

[§]Comparison between mice without and with IL-8 instillation.

TABLE 2. EFFECTS OF 24-HOUR FRH EXPOSURE AND INTRATRACHEAL IL-8 INSTILLATION ON ENDOGENOUS CHEMOKINE GENERATION AND PROTEIN LEAK

Mouse Treatment	IL-8 Instillation*	KC (pg/ml)	MIP-2 (pg/ml)	LIX (pg/ml)	Protein (μ g/ml)
Normothermia	No	634 $\pm$ 33 [†]	187 $\pm$ 2.7	283 $\pm$ 27.3	0.22 $\pm$ 0.004
24-hour FRH exposure [‡]	No	872 $\pm$ 40	216 $\pm$ 11.1	262 $\pm$ 20.7	0.23 $\pm$ 0.019
Normothermia	Yes	814 $\pm$ 40	221 $\pm$ 10.5	223 $\pm$ 8.8	0.31 $\pm$ 0.043
24-hour FRH exposure	Yes	613 $\pm$ 95	173 $\pm$ 19.5	190 $\pm$ 36.8	0.36 $\pm$ 0.059

Definition of abbreviations: KC, keratinocyte-derived chemokine; LIX, LPs-induced CXC chemokine; MIP-2, macrophage inflammatory protein-2.

* Lung lavage, 4 hours after instillation of 1 μ g human IL-8.

[†] Mean $\pm$ SE concentration of indicated cytokine or total protein in lung lavage. Cytokines measured by 2-antibody ELISA, $n = 5$.

[‡] Core temperature was maintained at approximately 39.5°C.

heat shock protein-27 (HSP27), a known consequence of p38 signaling that has been implicated in lung injury (25) and PMN TEM (26), peaked after 9-hour FRH exposure at 2.5-fold above baseline levels (Figure 3B). Flow cytometry demonstrated an approximately 4.5-fold increase in the proportion of circulating PMNs containing phosphorylated ERK and p38 after 9-hour FRH exposure (Figures 3C and 3D), but no change in total ERK or p38 expression. Pretreatment with a single intraperitoneal injection of 200 μ g U0126 or 1 mg SB203580, 30 minutes before the 16-hour FRH exposure, greatly reduced IL-8-directed PMN TAM compared with sham-treated control mice, but the inhibitors exerted no effect in normothermic mice (Figure 3E).

Direct Effects of FRH on Human Lung Microvascular Endothelium and PMNs

Postconfluent HMVEC-L monolayers were incubated at 37°C or 39.5°C for 2 to 24 hours, and returned to 37°C before measuring

the TEM of freshly isolated human PMNs toward IL-8 in a 2-hour TEM assay (Figure 4A). Pre-exposing HMVEC-Ls to 39.5°C stimulated time-dependent increases in PMN TEM capacity, peaking after 12 to 24 hours at concentrations approximately 7-fold higher than in normothermic cells, and almost 2-fold higher than in monolayers treated with TNF- α , a potent endothelial activator (16). As found in murine lung tissue *in vivo*, the HMVEC-L expression of ICAM-1/2, as assessed by immunoblotting, was unaffected by 24-hour incubation at 39.5°C (Figures 4B and E4).

To extend the *in vivo* analysis of ERK and p38, we treated HMVEC-Ls with 10 μ M U0126 or SB203580, 30 minutes before and during 24-hour FRH exposure, and removed the inhibitors before performing the TEM assay (Figure 4C). Both inhibitors reduced PMN TEM in 39.5°C monolayers, but had no effect on TEM through normothermic HMVEC-Ls, suggesting that ERK and p38 pathways are required for FRH priming effects. Immunoblotting revealed an approximately 3-fold increase in phosphorylated p38, 20 to 60 minutes after switching the HMVEC-L

Figure 2. Effects of FRH exposure on PMNs and endothelia. (A and B) Recipient or donor mice were exposed to FRH for 24 hours, and donor PMNs were isolated, fluorescently labeled, and injected via the tail vein, and IL-8-directed donor PMN TAM was determined by flow cytometry. The total donor PMN content in lung lavage (A) and the proportion of lung-lavage PMNs of donor origin (B) are shown for the indicated transfers between FRH-exposed and normothermic (NT) mice. Mean $\pm$ SE; six mice per group. * $P < 0.05$, versus all other groups. (C) Blood was collected from normothermic or 24-hour FRH-exposed mice, and the percentages of PMNs expressing CD11a, CD11b, or CD18 were analyzed according to flow cytometry by gating on GR-1-stained cells. Mean $\pm$ SE; four mice per group. * $P < 0.05$ versus normothermic mice. (D) After erythrocyte lysis, the binding of intercellular adhesion molecule-1 (ICAM-1)-coated fluorescent microbeads during 30-minute incubation at 37°C was analyzed by flow cytometry and expressed as mean number of beads per GR-1-stained PMN. Mean $\pm$ SE; four replicates. (E) Lung homogenates from six normothermic and six 24-hour FRH-exposed mice were analyzed for ICAM-1 and ICAM-2 expression by immunoblotting, normalized to β -tubulin levels, as expressed relative to normothermic levels; mean $\pm$ SE.

Figure 3. Effects of FRH on activation of extracellular regulated kinase (ERK) and p38 *in vivo*. (A) Lung homogenates from normothermic mice (lanes 1–3) and mice exposed to FRH for 9 hours (ERK) or 6 hours (p38) (lanes 4–7) were analyzed for phosphorylated (p) and total (t) ERK and p38 by immunoblotting. (B) Lung homogenates from normothermic mice and mice exposed to FRH for the indicated times were analyzed for phosphorylated and total HSP27 by immunoblotting. (C and D) Blood was collected from four normothermic mice and four mice exposed to FRH for the indicated times, their erythrocytes were lysed, cells were stained for GR-1 and total or phosphorylated ERK (C) or p38 (D), and percentages of labeled PMNs were analyzed by flow cytometry. Mean $\pm$ SE. * P < 0.05 versus time 0. (E) Groups of eight mice were untreated or treated with 2% DMSO (sham), 200 μ g U0126, or 1 mg SB203580 (SB), 30 minutes before 16-hour FRH or normothermic exposure, and their IL-8-directed PMN TAM was measured. Mean $\pm$ SE. * P < 0.05, versus normothermic mice. † P < 0.05, versus untreated mice.

incubation temperature to 39.5°C and biphasic ERK activation, with modest peaks occurring 10 and 120 minutes after the temperature increase (Figure 4D), but no detectable c-Jun N-terminal kinase (JNK) activation (not shown). We analyzed HMVEC-Ls incubated for 6 hours at 37°C and 39.5°C for stress fiber formation, one of the classic morphologic consequences of p38–HSP27 signaling by staining with AlexaFluor488-coupled phalloidin (Figure 4E). Stress fibers were not detectable in the 37°C monolayers, but the 39.5°C monolayers exhibited stress fiber formation, most notably in the perinuclear region. Because ICAM-1 activation by engagement with β 2-integrin or a crosslinking antibody triggers endothelial signaling, including ERK and p38 activation, HSP27 phosphorylation, and cytoskeletal rearrangement (24), we asked whether exposing HMVEC-Ls to 39.5°C would enhance ICAM-1-triggered ERK and p38 activation. As expected, the antibody crosslinking of ICAM-1 caused a rapid activation of ERK and p38 in normothermic HMVEC-Ls. Pre-warming at 39.5°C for 24 hours did not alter this response (Figure 4F).

Immunofluorescence confocal microscopy demonstrated different subcellular distributions of activated p38 in HMVEC-Ls exposed to FRH for 4 hours compared with cells exposed to TNF- α at 37°C for 30 minutes (Figures E5A–E5C). Cells exposed to TNF- α at 37°C for 30 minutes exhibited a plasma membrane-predominant pattern, and HMVEC-Ls exposed to FRH for 4 hours exhibited a cytoplasmic distribution, suggesting that p38 activation in response to FRH qualitatively differs from p38 activation stimulated by TNF- α .

The accelerated PMN apoptosis at 39.5°C (20) precluded an analysis of *in vitro* FRH exposure on PMN TEM capacity, but a flow cytometric analysis of PMNs incubated with 500 U/ml

G-CSF, added to delay apoptosis (21), demonstrated similar surface expression of lymphocyte function-associated antigen-1 (LFA-1) and macrophage-1 antigen (MAC-1) in normothermic PMNs and PMNs incubated at 39.5°C for 6 hours (Figure E6).

DISCUSSION

We previously showed that concurrent exposure to FRH increases PMN recruitment, pulmonary vascular endothelial dysfunction, and epithelial injury in murine ALI models, and we identified the increased expression of CXC chemokines as one of the mechanisms (10, 11). However, because FRH exposure augments chemokine generation in these lung injury models, they cannot be used to identify additional mechanisms by which FRH increases PMN recruitment and lung injury.

To bypass the effects of FRH on endogenous chemokine generation, we modified an *in vivo* PMN transmigration assay (18) in which PMNs migrate across a fixed transalveolar chemokine gradient generated by an intratracheal instillation of human IL-8, an agonist for CXCR2 receptors in murine PMNs (17). Exposure to FRH for 16 to 24 hours increased subsequent IL-8-directed TAM by a remarkable 10.2- to 23.5-fold, compared with IL-8-challenged normothermic control mice. Although the conscious FRH-exposure model used in this study avoids the potential confounding effects of anesthesia, the increase in core temperature is gradual, and the mice are potentially exposed to psychological stress from the high ambient temperature. To control for the stress of manipulations, normothermic and FRH-exposed mice were treated identically, except for different ambient temperatures, but this does not duplicate the additional psychological stress of the exposure to heat. To prove further that the increased

Figure 4. Effects of FRH on PMN transendothelial migration (TEM) through human lung microvascular endothelial cell (HMVEC-L) monolayers. (A) HMVEC-Ls were incubated at 39.5°C for the indicated times or treated with 1 ng/ml TNF- α for 6 hours at 37°C, and the IL-8-directed TEM of acetomethoxy calcein (calcein AM)-stained human PMNs was measured for 2 hours at 37°C and standardized to untreated HMVEC-Ls. Mean $\pm$ SE; four experiments. $^*P < 0.05$ versus time 0. $^{\dagger}P < 0.05$ versus TNF- α . (B) HMVEC-L monolayers on Matrigel-coated plastic were incubated at 37°C or 39.5°C for 6 hours, lysed and immunoblotted for ICAM-1 and ICAM-2, normalized to β -tubulin, and expressed relative to levels in 37°C cells; mean $\pm$ SE. (C) HMVEC-Ls were untreated (Control) or pretreated for 30 minutes with DMSO, U0126, or SB203580, and incubated for 24 hours at 37°C or 39.5°C, and the PMN TEM was measured. Mean $\pm$ SE; four experiments. $^*P < 0.05$, versus 37°C. $^{\dagger}P < 0.05$, versus untreated at 39.5°C. (D) Postconfluent HMVEC-L monolayers were serum-starved for 12 hours, incubated at 39.5°C for 1, 2, 3, 6, or 24 hours under serum-free conditions, and immunoblotted for phosphorylated (p) and total (t) p38 and ERK. Controls include 24 hours at 37°C, and 30 minutes with 1 ng/ml TNF- α at 37°C. Representatives of four similar blots are shown. (E) Postconfluent HMVEC-L monolayers

were incubated at 37°C or 39.5°C for 6 hours, stained with AlexaFluor488-coupled phalloidin, and imaged by confocal microscopy. Representatives of four similar sets of coverslips are shown. (F) HMVEC-Ls were serum-starved for 12 hours, incubated at 37°C or 39.5°C for 24 hours, and subjected to ICAM-1 crosslinking at 37°C by incubating with mouse anti-ICAM-1 for 30 minutes (time 0) and anti-mouse IgG for the indicated times. Lysed cells were immunoblotted for p-p38 and t-p38 and p-ERK and t-ERK. Representatives of four similar immunoblots are shown.

temperature itself augments PMN extravasation potential, we showed that exposing cultured endothelial cells to 39.5°C increased their capacity for PMN transmigration. Because the mice retained their normal Circadian rhythm during FRH exposure (19) and FRH exposures were initiated within 4 hours of the normal noon temperature nadir, the core temperature in FRH-exposed mice did not exceed normal peak levels until at least 10 hours of FRH exposure, just 6 hours before enhanced PMN TAM capacity was detectable. However, because we did not study mice exposed to FRH for durations between 8 and 16 hours, we have not yet defined the minimum FRH duration required for a detectable increase in TAM capacity *in vivo*. However, we did show that exposing HMVEC-Ls to 39.5°C for as little as 2 hours was sufficient to increase their capacity for PMN TEM *in vitro*.

We emphasize that the FRH model used in this study is not a model of fever in which a regulated, rapid increase in core temperature occurs as part of the acute-phase response. The increase in core temperature achieved in our FRH model was more gradual and sustained than in typical fever, and occurred without a proinflammatory signal or other components of the acute-phase response. This model was developed to answer the question of how a temperature increase itself modifies PMN delivery, and so it better represents exertional/environmental hyperthermia than fever. In fact, the FRH-exposed mice in this study did not increase their pulmonary expression of endogenous CXC chemokines (Table 1), and did not exhibit increased TAM in the absence of IL-8. These results also confirm that FRH augments chemokine-dependent PMN recruitment independent of its effects on chemokine

expression, and demonstrate that FRH increases PMN extravasation through mechanisms different from those of proinflammatory cytokines such as TNF- α and IL-1 β (27), which activate the expression of endothelial chemokines.

PMN adoptive transfer studies that showed increased TAM required both the donor and recipient to be exposed to FRH (Figure 2A), which suggests that FRH exerts interdependent effects on PMNs and structural lung cells. To define further the lung cells targeted by FRH, we analyzed lung tissue from normothermic and FRH-exposed mice, using immunofluorescence confocal microscopy. This analysis demonstrated the synergistic effects of FRH and IL-8 on PMN retention in the pulmonary vasculature, PMN shape change consistent with tight PMN-endothelial binding, and PMN extravasation (Figures 1D–1H). We also showed that HMVEC-Ls, the endothelial cells representative of the microvascular bed from which intravascular PMNs emigrate, increase their capacity for IL-8-directed PMN transmigration when incubated at 39.5°C *in vitro* (Figure 4A). Collectively, these results suggest that FRH exerts interdependent effects on PMNs and endothelia that increase IL-8-directed PMN extravasation, but these results do not exclude additional effects of FRH on other potential barriers to TAM, such as extracellular matrix and epithelia.

Unlike other vascular beds, PMN recruitment from the pulmonary microvasculature does not require selectins (28), but does require the engagement of PMN β 2-integrins by endothelial ICAM-1 and ICAM-2 (15, 29). We could not detect any differences between normothermic and 24-hour FRH-exposed mice in the PMN surface expression of CD18, CD11a, and CD11b

according to flow cytometry (Figure 2D), or concentrations of ICAM-1 and ICAM-2 in lung tissue according to immunoblotting, immunostaining, and confocal microscopy (Figures 2E–2G). Immunoblotting also failed to detect differences in ICAM-1 and ICAM-2 concentrations between HMVEC-L monolayers incubated at 37°C and 39.5°C for 6 hours (Figures 4B and 4C) and in the surface expression of CD18, CD11a, and CD11b in human PMNs incubated at 37°C and 39.5°C for 6 hours (Figure 4H). Collectively, these results demonstrate that the changes in endothelial and PMN function that support accelerated PMN TEM occur without a change in total β_2 -integrin and ICAM-1 and ICAM-2 expression. Transition to tight PMN–endothelial binding requires the clustering of β_2 -integrins on PMNs, ICAM-1 dimerization (30) and the activation of intracellular signaling (24), and the transition of β_2 -integrins from low and intermediate affinity states to a high-affinity state (31). These events initiate bidirectional signaling, with functional consequences for both cells that affect PMN extravasation (24, 32). Because antibodies specific for the high-affinity conformation of murine β_2 -integrins were not available, we used an ICAM-1 binding avidity assay (23) to compare β_2 -integrin function in PMNs from normothermic and 24-hour FRH-exposed mice. After a 30-minute incubation with fluorescent ICAM-1–derivatized microbeads at 37°C, with shear stress imposed by shaking the horizontal reaction tubes at 150 rpm, PMNs from normothermic and 24-hour FRH-exposed mice exhibited similar microbead binding (Figure 2D). Although this assay cannot distinguish between contributions from β_2 -integrin clustering and alterations in conformation, the similar surface expression and binding avidity of β_2 -integrins in PMNs from normothermic and 24-hour FRH-exposed mice strongly suggest that the enhanced extravasation potential of PMNs from warmer mice is not caused by changes in β_2 -integrin concentration or function. Because we found synergy between FRH and IL-8 for stimulating PMN–endothelial binding and PMN extravasation *in vivo* (Figures 1D–1H), and because chemokine expression is known to stimulate the β_2 -integrin conformational transition (42), we looked for similar synergy *in vitro* by including IL-8 in 30-minute ICAM-1 binding, but found no additional increase in PMN ICAM-1-binding avidity in either group of PMNs (Figure E2).

The binding of PMNs to endothelia stimulates ERK and p38 signaling pathways in both cells (33–35), and the two signaling pathways are required for PMN transmigration (33). In particular, the p38-dependent phosphorylation of HSP27 and subsequent cytoskeletal rearrangement appear to be important in both PMNs and endothelia (24, 26, 36). Wang and colleagues showed that crosslinking ICAM-1 on human umbilical vein endothelial cells stimulated p38 activation, HSP27 phosphorylation, F-actin rearrangement, ICAM-1 aggregation, endothelial cell stiffening, and the enhanced migration of PMNs to endothelial cell junctions, all of which was blocked by the p38 inhibitor, SB203580 (24, 26). Szczur and colleagues showed that integrin ligation on PMNs caused a rapid activation of ERK and p38 that activated PMN chemokinesis and chemotaxis, respectively (37). In human PMNs, stimulation with IL-8 activates ERK via phosphatidylinositol 3-kinase, and increases adherence to immobilized ICAM-1. Treatment with the inhibitors of either PI3K or ERK reduced PMN adherence to ICAM-1 via effects on β_2 -integrin conformation (38), although, as already mentioned, we found no difference in the ICAM-1-binding avidity of PMNs obtained from normothermic and FRH-exposed mice. MAPKs are also activated by the stimulation of CXC chemokine receptors on PMNs. Cara and colleagues (35) showed that keratinocyte-derived chemokine activates p38, and that the inhibition of p38 reduces PMN emigration in a murine cremasteric muscle model. Damarla and colleagues (39) identified the p38-induced activation of MAPK-activated

protein kinase-2, the phosphorylation of HSP25/27, and cytoskeletal rearrangement as critical to endothelial paracellular pathway opening in response to cyclic stretch *in vitro* and in a murine model of ventilator-induced lung injury.

We found that exposing mice to FRH induced p38 and ERK activation in PMNs and lung tissue (Figures 3A–3D). To evaluate the potential participation of ERK and p38 in the effects of FRH on PMN TAM, we took advantage of the relatively short *in vivo* half-life of the ERK (U0126) and p38 (SB203580) inhibitors (40, 41). Pretreating mice with SB203580 administered 30 minutes before a 16-hour exposure to FRH reduced IL-8–directed PMN TAM by about 75% (Figure 3E). The lack of effect by either SB203580 or U0126 on IL-8–directed PMN TAM in normothermic control mice indicates that the 16-hour delay between inhibitor dosing and IL-8 instillation was sufficient for the clearance of inhibitor activity. Exposing HMVEC-Ls to FRH *in vitro* activated ERK and p38 and increased the capacity of HMVEC-Ls for subsequent PMN TEM, whereas pretreating HMVEC-Ls with U0126 and SB203580 abrogated the effects of FRH on the capacity of HMVEC-Ls for PMN transmigration *in vitro*. We also demonstrated increased HSP25 phosphorylation in the lungs of FRH-exposed mice *in vivo* (Figure 3B) and cytoskeletal stress fiber formation in HMVEC-Ls exposed to FRH *in vitro* (Figure 4F). Collectively, these results suggest that FRH induces the activation of p38 and downstream cytoskeletal alterations in pulmonary vascular endothelia, known to increase the potential for PMN transmigration.

Although we found that exposing mice to FRH stimulates the activation of p38 in circulating PMNs (Figure 3D), we have not definitively established whether p38 activation in PMNs contributes to the effects of FRH on PMN transmigration potential. Heit and colleagues (42) demonstrated that the activation of p38 inhibits rather than enhances PMN chemotaxis toward CXC chemokines, which appears to be inconsistent with our finding of associated increases in PMN p38 activation and TAM potential. However, our *in vivo* model measures extravasation, a complex response that includes chemotaxis as one of its components.

PMN accumulation in the bronchoalveolar compartment was not accompanied by an increase in lung lavage protein in either normothermic or FRH-exposed mice. Although this result suggests a dissociation of the effects by FRH and IL-8 on PMN migration and cytotoxic functions, protein was only measured at a single time point, 4 hours after IL-8 instillation. Therefore, we cannot exclude a delayed increase in alveolar permeability.

In conclusion, we have shown that FRH exerts complementary effects on PMNs and endothelia that profoundly increase the capacity for chemokine-directed PMN influx in the lung. Priming appears to be independent of changes in β_2 -integrin and ICAM-1/2 expression and function, but requires ERK and p38 activation and is associated with HSP25/27 phosphorylation and cytoskeletal changes that mediate changes in endothelial barrier functions. A more complete understanding of these mechanisms may help manage patients with febrile and heat-related disorders, and may be exploited to modify inflammation and innate immunity.

Author disclosures are available with the text of this article at www.atsjournals.org.

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