Febrile-Range Hyperthermia Augments Pulmonary Neutrophil Recruitment and Amplifies Pulmonary Oxygen Toxicity

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Febrile-range hyperthermia (FRH) improves survival in experimental infections by accelerating pathogen clearance, but may also increase collateral tissue injury. We hypothesized that FRH would worsen the outcome of inflammation stimulated by a non-replicating agonist and tested this hypothesis in a murine model of pulmonary oxygen toxicity. Using a conscious, temperature-controlled mouse model, we showed that maintaining a core temperature at FRH (39°C to 40°C) rather than at euthermic levels (36.5°C to 37°C) during hyperoxia exposure accelerated lethal pulmonary vascular endothelial injury, reduced the inspired oxygen threshold for lethality, induced expression of granulocyte-colony stimulating factor, and expanded the circulating neutrophil pool. In these same mice, FRH augmented pulmonary expression of the ELR⁺ CXC chemokines, KC and LPS-induced CXC chemokine, enhanced recruitment of neutrophils, and changed the histological pattern of lung injury to a neutrophilic interstitial pneumonitis. Immunoblockade of CXC receptor-2 abrogated neutrophil recruitment, reduced pulmonary vascular injury, and delayed death. These combined data demonstrate that FRH may enlist distinct mediators and effector cells to profoundly shift the host response to a defined injurious stimulus, in part by augmenting delivery of neutrophils to sites of inflammation, such as may occur in infections. In certain conditions, such as in the hyperoxic lung, this process may be deleterious. (*Am J Pathol* 2003, 162:2005–2017)

Fever is a phylogenetically ancient, evolutionarily conserved response, the key feature of which is a temporary, regulated increase in core temperature. While mammals and birds generate fever through both heat-seeking behavior and augmented heat generation and retention, ectothermic animals increase their body temperature by seeking an external heat source. In both cases, stricken animals expend substantial amounts of energy to achieve an increase in body temperature,¹ suggesting that the increase in temperature itself is beneficial during infections and injury. This conclusion is supported by retrospective clinical studies showing an association between fever and improved survival during bacterial infections² and the beneficial effects of febrile range hyperthermia (FRH) in experimental infections in animals. We previously reported that exposing mice to FRH during experimental *Klebsiella pneumoniae* peritonitis increased survival rate from 0% to 50% and decreased the intraperitoneal bacterial load by 100,000-fold compared with euthermic mice.³ However, FRH did not directly influence pathogen proliferation or survival,³ suggesting that the salutary effects in the infected host were the result of augmented immune defense.

In the retrospective studies of human infections, the association of fever with survival was lost as the acuity of the patient population increased.⁴ Furthermore, in patients with *Escherichia coli* and *Pseudomonas aeruginosa* sepsis, administration of the antipyretic acetaminophen was associated with increased survival.^{5,6} Collectively, these studies suggest that fever might be harmful when it occurs in certain clinical settings. In our experimental murine *Klebsiella* peritonitis model,³ bacterial burden at time of death was much lower in the mice exposed to FRH than the euthermic mice, suggesting that mechanisms other than the infection contributed to death in the warmer mice. A similar effect of FRH on the relationship between survival and pathogen burden occurs in rabbits with experimental *S. pneumoniae* peritonitis in which FRH (41°C) shortens survival time despite reducing levels of

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bacteremia compared with infected euthermic (39°C) animals.⁷ Based on these studies, we reasoned that the ultimate effect of fever is determined by a dynamic balance between accelerated pathogen clearance and augmented collateral host tissue injury. Specifically, fever will be beneficial if it sufficiently shortens the course of an infection, thereby reducing the risk for tissue injury.

The evolutionary persistence of fever indicates that this strategy is usually effective. However, until twentieth-century man, increases in body temperature occurred almost exclusively during active infections or in situations associated with enhanced risk of infection, including traumatic injury, exertion, or flight or fight responses. In modern man, the acute phase response comprising fever and inflammation, is often activated by stimuli other than replicating pathogens, including antibiotic-treated infections and noninfectious causes.⁸ We hypothesized that in such situations, the increased rate of host tissue injury stimulated by fever would not be counterbalanced by accelerated pathogen clearance, thereby causing harm rather than conferring protection.

We chose pulmonary oxygen toxicity, a highly clinically relevant, noninfectious form of injury that is unique to modern medicine to test this hypothesis. Exposure to artificially high concentrations of oxygen is often essential for patient survival during severe illnesses.⁹ Unfortunately, such exposure is associated with severe, often lethal lung injury in man,¹⁰ other primates,¹¹ and rodents.^{12–22} The mechanisms of acute hyperoxic lung injury have not been definitively elucidated, but it is widely believed that generation of reactive oxygen species (ROS) plays a key role.²³ The contribution of neutrophils (PMN) to pulmonary oxygen toxicity is less clear. PMN recruitment to the hyperoxic lung occurs late in the course of lung injury.^{24,25} Furthermore, PMN depletion produces inconsistent effects on hyperoxic lung injury with as many studies showing protection^{19,26,27} as showing no effect^{24,28–30} or even worsening of pulmonary oxygen toxicity.²⁴ The cytokines, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-3, and IL-6, as well as the chemokines monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , MIP-2, and γ -interferon-inducible protein (IP)-10^{18,20,31} each accumulate, and expression of both adhesion molecules, intercellular adhesion molecule (ICAM)-1 and E-selectin increase³² in the hyperoxic lung. Collectively, these studies suggest that despite its non-biological nature, hyperoxia appears to cause lung injury in part by activating the innate immune response.

We used a conscious, temperature-controlled mouse model to determine how co-exposure to FRH modifies pulmonary oxygen toxicity. We showed that maintaining core temperature between 39°C and 40°C (by increasing ambient temperature from 24°C to 34°C) rather than at normal basal levels (36.5°C to 37°C) in mice exposed to hyperoxia accelerates lethal pulmonary vascular endothelial injury, reduces the threshold of inspired oxygen level for lethality, induces expression of the PMN growth factor granulocyte-colony stimulating factor (G-CSF), and expands the circulating PMN pool. Furthermore, FRH was shown to augment pulmonary expression of the glutamic

acid-leucine-arginine motif-positive (ELR⁺) CXC chemokine genes KC and LPS-induced CXC chemokine (LIX), enhance CXC receptor-2 (CXCR2)-dependent recruitment of PMN, and change the histological pattern of lung injury to a neutrophilic interstitial pneumonitis.

Materials and Methods

Animal Exposure and Temperature Monitoring

Eight- to ten-week-old male outbred CD-1 mice, weighing 25 to 30 g were purchased from Harlan-Sprague Co. (Indianapolis, IN), housed in the Baltimore Veterans Administration Medical Center animal facility under the supervision of a full-time veterinarian, and used within 4 weeks of delivery. 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 a.m. and 10:00 a.m.). One week before each experiment, sentinel mice were implanted with telemetric intraperitoneal temperature sensors (Mini Mitter 1.05g *in vivo* temperature sensor model 100-0035; Bend, OR).

Experimental mice were housed in modified plastic cages to allow continuous inflow of air/oxygen mixtures and outflow through a 1 cm² outflow port. The oxygen level in each cage was measured twice daily using an oxygen analyzer (Vascular Technology; Chelmsford, MA). Groups of three to four mice, including one thermometer-implanted sentinel mouse per group, were placed in each cage. The cages were placed in modified infant isolettes with temperature set to 24°C to maintain euthermia or 34°C to 34.5°C to maintain FRH and core temperatures of the sentinel mice were continuously monitored using the Mini Mitter Automated Data Acquisition System. Except for the ambient temperature, handling of the euthermic and hyperthermic mice was identical. Exposures to hyperthermia and hyperoxia were initiated simultaneously. All procedures were approved by the University of Maryland Baltimore Animal Care and Use Committee.

Lung Lavage and Blood Processing

At selected times, groups of mice were anesthetized by 10 to 30 second exposure to isoflurane, euthanized by cervical dislocation, and lung lavage was performed *in situ* through an 18g blunt-end needle secured in the trachea, using 1 ml phosphate-buffered saline (PBS) instilled and withdrawn twice, followed by instillation and recovery of a second 1 ml of PBS. The two aliquots of lung lavage were pooled, cells were collected by centrifugation at 1000 $\times$ g for 3 minutes, and cell-free supernatants were stored at –80°C for analysis of total protein and cytokine concentrations. Total cell counts were performed manually using a hemacytometer and differential cell counts of Diff-Quick-stained cytopreparations were performed by two blinded observers (PR and RMV) using morphological criteria. Heparinized blood was collected via cardiac puncture. Total blood PMN count was determined by manually counting total leukocytes after lysis of

erythrocytes with Tris-buffered ammonium chloride and manually analyzing the leukocyte composition in Diff Quik-stained blood smears. Plasma was stored at -80°C for analysis of cytokine concentrations.

CXCR2 Blockade and G-CSF Neutralization

In experiments to analyze the requirement for CXCR2 for PMN recruitment, mice received a single i.p. injection of 0.5 ml goat anti-mouse CXCR2 antiserum, as previously described,³³ 2 hours before exposure to hyperoxia and FRH and were either sacrificed 40 hours later for analysis of bronchoalveolar lavage fluid (BALF) and lung tissue or were monitored for survival. Control animals received an equal volume of pre-immune goat serum. To determine whether G-CSF was required for FRH-induced neutrophilia, mice were treated with 0.5 ml rabbit anti-G-CSF (T. Hartung; University of Konstanz; Konstanz, Germany) diluted 1:5 in PBS and administered i.p. 2 hours prior to 24-hour exposure to FRH or euthermia. Control mice received an equal volume of diluted rabbit serum. Mice were euthanized, blood was collected by cardiac puncture, and the PMN were manually counted.

Measurement of Cytokine Concentration

Cytokines were measured in the University of Maryland Baltimore Cytokine Core Laboratory using standard two-antibody ELISAs with commercial antibody pairs and recombinant standards from R&D; Minneapolis, MN as previously described.⁹ The IL-6, KC, MIP-2, LIX, G-CSF, and GM-CSF assays had lower detection limits of 3, 15, 62, 31, 15, and 12.5 pg/ml, respectively.

Measurement of Wet:Dry Weight

Lungs were excised, washed of surface blood, blotted dry, and the mediastinum and connective tissue removed. After weighing both lungs, the tissue was dried at 100°C until the final stable dry weight was measured.

Histological Analysis

After euthanasia, the anterior chest wall was removed, the trachea cannulated with an 18g blunt needle, the lungs inflated *in situ* with 10% buffered formalin at 20 cm H_2O pressure, and the lungs and mediastinum removed *en bloc* and fixed in 10% buffered formaldehyde. Gross sections of the trachea, esophagus, and lung parenchyma at the level of mid-bronchial bifurcation were paraffin-embedded and 5- μm sections were processed routinely for hematoxylin and eosin staining. A veterinary pathologist (S.R.) evaluated the sections in a blinded fashion for morphological signs of injury and inflammation. For immunohistologic analysis, lungs were harvested for frozen sectioning exactly as described except lung inflation was accomplished by inflating with a fixed 0.7 ml volume of 50% OCT (v/v in PBS). The inflated lungs were embedded in neat OCT and 8- μm cryosections

were acetone-fixed for 3 minutes at -20°C and stored at -80°C for immunostaining. Slides were washed in PBS at 25°C for 20 minutes. Endogenous peroxidase was inactivated by incubating for 10 minutes with 30% hydrogen peroxide in methanol at 25°C . Non-specific signal was reduced by sequentially blocking with 5% serum (v/v) in PBS for 30 minutes, and then with a commercial avidin/biotin blocking kit (Vector; Burlingame, CA) according to the manufacturer's instructions. The blocked slides were sequentially incubated with primary antibody for 1 hour, a secondary antibody (where indicated) for 30 minutes, a commercial avidin/biotin peroxidase detection system (Vector), and 1 mg/ml DAB (Sigma) in 0.02% hydrogen peroxide in PBS. All blocking and subsequent incubations were at 25°C . Blocking serum, primary, and secondary antibodies used for each cell type were as follows. For macrophages, mouse serum, 1 $\mu\text{g/ml}$ rat anti-mouse Mac-3, and 2.5 $\mu\text{g/ml}$ biotinylated goat anti-rat IgG1/2a (both from BD-Pharmingen; San Diego, CA) were used. For PMN, bovine serum and the 2.5 $\mu\text{g/ml}$ biotinylated rat anti-mouse Gr-1 (BD-Pharmingen) were used. For T lymphocytes, goat serum, 2.5 $\mu\text{g/ml}$ rat anti-mouse CD3, and 2.5 $\mu\text{g/ml}$ biotinylated goat anti-rat IgG2b (both from BD-Pharmingen) were used. For NK cells, goat serum, 1:100 (v/v) dilution of rabbit anti-rat/mouse Aialo-GM, and 1:800 (v/v) dilution of peroxidase-conjugated goat anti-rabbit IgG (both from Accurate Chemical; Westbury, NY) were used. Immunostained slides were counterstained with hematoxylin (BD Sciences) and were scored by two blinded observers (P.R. and J.D.H.). The number of positively stained cells per high power field was manually counted in comparable regions of each section located equidistant from the pleural surface and lung hilum so as to avoid bronchovascular structures. For each mouse, the mean of four fields per section and three sections was calculated.

In Vitro Cell Culture Systems

RAW 264.7 murine macrophages and NIH 3T3 murine lung fibroblasts were obtained from the American Type Culture Collection (Manassas, VA). SVEC-4-10, an SV40-transformed murine endothelial cell line was provided by Dr. A. Passaniti (University of Maryland School of Medicine, Baltimore, MD). RAW 264.7 cells were cultured in RPMI 1640 supplemented with 50 units/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.3 and containing 10% fetal bovine serum (FBS; Life Technologies; Gaithersburg, MD) at 37°C in 5% CO_2 -enriched air. NIH 3T3 cells and SVEC4-10 cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 50 units/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES buffer, pH 7.3 and containing 10% FBS at 37°C in 5% CO_2 -enriched air. MLE12 cells, a cell line derived from murine distal respiratory epithelial cells were obtained from Jeffrey Whitsett (University of Cincinnati School of Medicine, Cincinnati, OH) and were cultured in 5 $\mu\text{g/ml}$ insulin, 5

$\mu\text{g/ml}$ transferrin, 10 pM hydrocortisone, 10 pM β -estradiol 10 mmol/L HEPES, pH 7.3, 2 mmol/L L-glutamine, 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 2% FBS. To determine the capacity of different cell types to increase cytokine generation in response to hyperoxia and hyperthermia, the cells were seeded 2.5×10^5 per ml in 24-well culture plates and cultured in either 95% air/5% CO_2 or 95% oxygen/5% CO_2 and at either 37°C or 39.5°C for 24 hours and culture supernatants were analyzed for concentration of KC, LIX, and G-CSF by ELISA.

Statistical Analysis

All data are presented as mean $\pm$ SE. Differences between two groups were tested using an unpaired Student's *t*-test. Differences among more than two groups were tested by a Fisher protected least-squares difference (PLSD) test applied to a one-way analysis of variance. Survival was analyzed using a log-rank test.

Results

Febrile-Range Hyperthermia Reduced Survival Time and Lowered the Lethal Oxygen Threshold in Mice Exposed to Hyperoxia

Mice housed at usual laboratory temperatures of 24°C and exposed to $\text{F}_1\text{O}_2 > 0.95$ maintained a core temperature within the usual basal range (36.5°C to 37°C). As we previously reported,³ because mice have a limited capacity to eliminate heat, increasing the murine environment from 24°C to only 34°C is sufficient to cause a rapid increase in core temperature that was maintained within the febrile range (39°C to 40°C) (Figure 1A). For the remainder of this report, we will refer to mice housed at 24°C as euthermic and those housed at 34°C as hyperthermic. Air-breathing control mice tolerated FRH for 2 weeks without detectable distress. While exposure to $\text{F}_1\text{O}_2 > 0.95$ was uniformly lethal in both groups (Figure 1B), survival time in hyperthermic mice was reduced by 50% relative to euthermic, hyperoxia-exposed mice (median survival 2.2 versus 4.5 days; $P < 0.001$). To determine whether FRH lowered the threshold for oxygen-induced lethality, euthermic and hyperthermic mice were exposed to F_1O_2 between 0.50 and 0.95 for up to 2 weeks. The LD_{50} for oxygen was reduced from 0.8 to 0.9 in euthermic mice to 0.6 to 0.7 in hyperthermic mice (Figure 2). At necropsy, the lungs in both groups of mice exposed to lethal levels of hyperoxia were grossly edematous and hyperemic (data not shown).

FRH Accelerates Pulmonary Vascular Endothelial Injury in Hyperoxic Mice

Reduced survival time in the hyperthermic mice exposed to $\text{F}_1\text{O}_2 > 0.95$ was associated with an earlier increase in both BALF protein concentration (Figure 3A) and lung wet:dry weight ratios (Figure 3B) compared with euthermic mice, indicating that pulmonary vascular endothelial

Figure 1. Effect of febrile range hyperthermia on survival time in mice exposed to hyperoxia. Mice were exposed to $> 0.95 \text{ F}_1\text{O}_2$ (hyperthermic) or air (normoxic) and housed at either 24°C (euthermic) or 34.5°C (hyperthermic). **A:** Mean ($\pm$ SE) core temperature was measured telemetrically in euthermic (squares; $n = 4$) and hyperthermic (circles; $n = 4$) mice. **Open symbols** are normoxic and **closed symbols** hyperoxic mice. * and $\dagger$ denote $P < 0.05$ versus euthermic-hyperoxic and euthermic-normoxic mice, respectively. **B:** Survival was determined in euthermic ($n = 16$) and hyperthermic ($n = 16$) mice.

injury was accelerated in the warmer animals. The FRH mice also exhibited a more rapid rise in BALF concentration of IL-6 (Figure 3C), a ubiquitously expressed marker of inflammation and tissue injury. In air-breathing mice exposed to FRH for 36 or 60 hours, neither BALF IL-6 nor total protein concentrations were increased compared with baseline levels. Mice exposed to hyperoxia and FRH did not survive beyond 60 hours.

As a first test as to whether the mechanism of hyperoxic lung injury might differ in euthermic and hyperthermic mice, the histological patterns of hyperoxic lung injury in the two groups of mice were compared. As anticipated,

Figure 2. Effect of febrile range hyperthermia on lethal F_{IO_2} threshold. Groups of 10 mice each were exposed to the indicated F_{IO_2} while housed at either 24°C (squares) or 34°C to 34.5°C (circles) and were monitored for survival for 2 weeks.

evidence of hyperoxic lung injury appeared earlier in the hyperthermic mice (24 to 36 hours) than in the euthermic animals (108 hours), but, surprisingly, the histological pattern of lung injury was profoundly different in the two groups. In euthermic mice, exposure to $F_{IO_2} > 0.95$ for 36 hours caused little detectable changes in tissue architecture (Figure 4A). After 108 hours of exposure to hyperoxia, the lungs from euthermic mice showed multifocal hyaline membrane accumulation, alveolar hemorrhage, and perivascular edema (Figure 4B). The pulmonary vasculature was diffusely expanded by accumulation of PMN and lesser numbers of mononuclear cells. The bronchial mucosa demonstrated multiple foci of denuded epithelium and accumulation of PMN, mononuclear cells, and cell debris within the bronchial lumen. In striking contrast to the findings in euthermic mice, hyperthermic mice exposed to hyperoxia for only 36 hours (Figure 4C) demonstrated marked PMN-rich interstitial pneumonitis. The interstitium was two to three times normal thickness and contained primarily PMN and large mononuclear cells. There were multiple foci of bronchial epithelial hyperplasia with intraluminal accumulation of PAS-positive material, areas of type II pneumocyte hyperplasia, and other areas of bullous emphysema.

The infiltrating cells were identified by immunostaining (Figure 5). The number of GR-1-positive PMN in lung tissue increased 4- to 5-fold over the first 24 to 36 hours of hyperoxic exposure in the hyperthermic mice compared with no increase in euthermic mice exposed to hyperoxia for 108 hours (Figure 5, A, C, and D). The number of Mac-3-positive macrophages remained unchanged during hyperoxic exposure in both euthermic and hyperthermic mice (Figure 5B). The large mononuclear cells within the alveolar septal walls in the hyperthermic mice did not immunostain for Mac-3 and based on their location, were most likely type II pneumocytes. Few normal type I pneumocytes were seen in the hyper-

thermic lung. NK cells and T cells were undetectable in the lungs of all of the animals studied, but were detectable in associated mediastinal lymph nodes (data not shown).

Exposure to hyperoxia induced a comparable 23-fold increase in the peak number of PMN recovered in BALF in hyperthermic and euthermic mice, but the increase was first detectable 24 hours earlier in the hyperthermic mice (Figure 6A). BALF macrophage content increased 2.5-fold after 36 to 60 hours of hyperoxic exposure in the hyperthermic mice (Figure 6B), but never exceeded baseline levels in the euthermic mice.

FRH Stimulates Expansion of the Circulating PMN Pool

To determine whether FRH augmented PMN recruitment to the lung, in part through expansion of the circulating PMN pool, the effects of FRH on PMN levels in circulating blood were analyzed. Twenty-four hour exposure to FRH in air-breathing mice increased the mean circulating leukocyte count 1.8-fold (Figure 7A) and the mean PMN count 1.9-fold (Figure 7B). In mice exposed to hyperoxia for 24 hours, co-exposure to FRH increased mean circulating PMN count 3.9-fold compared with euthermic hyperoxic mice. In the same mice, 24 hours of exposure to hyperoxia in the absence or presence of FRH or to FRH alone stimulated 7.1-, 8.1-, and 7.1-fold increases, re-

Figure 3. Effect of febrile range hyperthermia on hyperoxic lung injury. Mice were exposed to $F_{IO_2} > 0.95$ and housed at either 24°C (euthermia) or 34.5°C (hyperthermia). **A:** BALF protein concentration: groups of eight mice were sacrificed after the indicated exposure time, lung lavage was performed, and total protein was measured. **B:** Wet:dry weight: groups of four mice were sacrificed after 36 hours, the lungs were excised, weighed before and after drying, and the wet:dry weight ratio was calculated. **C:** BALF IL-6 concentration: groups of eight mice were sacrificed after the indicated exposure time to $F_{IO_2} > 0.95$ (hyperoxia) or $F_{IO_2} 0.21$ (normoxia), lung lavage was performed, and IL-6 concentration was measured by ELISA. Mean $\pm$ SE; * denotes $P < 0.05$ versus euthermic normoxic controls. ** denotes $P < 0.05$ versus euthermic mice at the same exposure time. Because hyperthermic mice did not survive beyond 60 hours of exposure to hyperthermia, analysis of later time points could not be done (ND).

Figure 4. Influence of core temperature on the pattern of lung injury in mice exposed to hyperoxia. Representative hematoxylin and eosin-stained sections from mice exposed to $F_iO_2 > 0.95$ while euthermic for 36 hours (A) or 108 hours (B) or while hyperthermic for 36 hours (C). **Arrowhead** in B indicates hyaline membranes; **arrows** in C indicate intraseptal PMN. Magnification, $\times 400$.

spectively, in mean plasma G-CSF concentration compared with euthermic, air-breathing mice (Figure 7C). Plasma GM-CSF was not detectable in any of the four groups of mice. To determine whether G-CSF was required for the FRH-induced neutrophilia, the effect of neutralizing G-CSF was measured in normoxic mice exposed to FRH for 24 hours (Figure 7D). Mice were treated with rabbit anti-mouse G-CSF antibody or sham-treated with pre-immune rabbit antiserum 2 hours before initiation of exposure to FRH. Compared with sham-treated controls, treatment with anti-G-CSF antibody reduced the circulating PMN count by 70% in FRH-exposed mice to levels below those in sham-treated euthermic mice. Treatment with anti-G-CSF also tended to reduce the number of circulating PMNs in euthermic mice, likely reflecting the short half-life of circulating PMN.

Hyperthermia and Hyperoxia Modify Cytokine Levels in BALF

To begin to determine how FRH enhances accumulation of PMN in the hyperoxic lung, the concentration of the three known ELR⁺ CXC chemokines, KC, LIX, and MIP-2, as well as two PMN survival factors, G-CSF and GM-CSF in BALF were measured (Figure 8). The concentration of KC peaked after a 24-hour co-exposure to FRH and hyperoxia ($F_iO_2 > 0.95$) at levels 10-fold higher than in air-breathing euthermic mice (Figure 8A) and coincided with the appearance of PMN in lung (see Figure 6). In comparison, BALF KC levels did not increase in the euthermic mice until 60 hours of exposure to hyperoxia, and increased only 2.2-fold relative to levels in normoxic, euthermic mice. BALF in mice exposed for 24 hours to hyperoxia in the presence or absence of FRH or to FRH alone was further analyzed for additional cytokine composition (Figure 8, B and C). Like KC, the BALF concentration of LIX increased 4.2-fold in mice co-exposed to hyperoxia and FRH compared with air-breathing euther-

mic mice; whereas exposure to either stimulus alone failed to stimulate an increase in LIX concentration in BALF (Figure 8B). The mean BALF levels of MIP-2 were modestly increased by approximately 1.7- to 2-fold in mice exposed for 24 hours to either stimulus alone, but, in contrast with the BALF levels of KC and LIX, exposure to FRH and hyperoxia failed to exert additive or synergistic effects on BALF levels of MIP-2 (Figure 8C). Neither G-CSF nor GM-CSF was detectable in the BALF from any of the four groups of mice.

Since KC and LIX were increased during co-exposure to hyperoxia and FRH and they act via a single receptor CXCR2, we asked whether PMN recruitment to the hyperthermic, hyperoxic lung is CXCR2-dependent by analyzing the effect of CXCR2 immunoblockade with anti-CXCR2 antibody on PMN accumulation in BALF (Figure 9A) and in lung tissue (Figure 9, B and C). Goat anti-mouse CXCR2 antiserum³³ or preimmune goat serum was administered 2 hours before a 40-hour co-exposure to FRH and $F_iO_2 > 0.95$. Based on the kinetics of pulmonary vascular endothelial injury (Figure 3) and accumulation of PMN in BALF (Figure 6), we chose a 40-hour co-exposure to ensure detectable lung injury in the sham-treated mice. Seven of 14 control animals died during the 40-hour exposure to FRH and hyperoxia compared with only one death in 14 mice pre-infused with anti-CXCR2 antiserum ($P = 0.02$; Fisher exact test). Necropsy revealed the lungs to be edematous and hyperemic in all mice exposed to lethal hyperoxia. In those mice surviving a 40-hour exposure to hyperoxia and hyperthermia, mean PMN content in BALF (Figure 9A) and in immunostained lung tissue (Figure 9, B and C) was reduced by 98% and 83%, respectively, in the anti-CXCR2 antibody-treated mice compared with the sham-treated mice and were similar to levels in euthermic air-breathing controls. The BALF protein concentration in the CXCR2-treated mice was reduced by 72% (196 ± 65 versus 691 ± 165 $\mu\text{g/ml}$; $P < 0.05$) compared with the sham-treated controls, but

Figure 5. Effect of febrile range hyperthermia on inflammatory cell accumulation in hyperthermic lung. Euthermic and hyperthermic mice were exposed to $F_1O_2 > 0.95$. Groups of four mice were sacrificed at the indicated time, lung sections were immunostained for either PMN (anti-Gr-1) or macrophages (anti-Mac-3) and the number of immunostained cells per high power field were quantified (**A** and **B**). Mean $\pm$ SE; * denotes $P < 0.05$ versus euthermic mice at the same time point. Because hyperthermic mice did not survive beyond 60 hours of exposure to hyperthermia, analysis of later time points could not be done (ND). **Panels C** and **D** show representative PMN-immunostained lung sections from hyperthermic (**C**) and euthermic (**D**) mice exposed to $F_1O_2 > 0.95$ for 36 hours. **Arrows** indicate PMN within the alveolar septal wall. Magnification, $\times 400$.

the number of macrophages in BALF or lung interstitium was not different between the anti-CXCR2-treated and control mice (data not shown). Within groups of nine mice exposed to hyperoxia and FRH, median survival time increased to 60 hours in the CXCR2 antibody-treated

mice compared to 36 hours in the sham-treated mice (Figure 9D).

To begin to determine which cells might contribute to augmented cytokine generation in response to FRH, we analyzed the effect of *in vitro* exposure to hyperthermia

Figure 6. Effect of febrile range hyperthermia on inflammatory cell accumulation in BALF. Euthermic and hyperthermic mice were exposed to $F_{I}O_2 > 0.95$. Groups of eight mice were sacrificed at the indicated time, lungs were lavaged, and the numbers of PMN (A) and macrophages (B) in BALF determined by manual counting. Mean $\pm$ SE; * denotes $P < 0.05$ versus euthermic normoxic controls (time 0). ** denotes $P < 0.05$ versus euthermic mice at the same exposure time. Because hyperthermic mice did not survive beyond 60 hours of exposure to hyperthermia, analysis of later time points could not be done (ND).

(39.5°C) and hyperoxia (95% oxygen) on cytokine generation in four murine cell culture systems, including RAW 264.7 macrophages, NIH 3T3 fibroblasts, MLE12 type II pneumocytes, and SVEC-4-10 endothelial cells. For each cell line, we compared concentrations of KC, LIX, and G-CSF in culture supernatants after 24 hours of incubation at 37°C or 39.5°C in either 95% air/5% CO_2 (normoxia) or 95% oxygen/5% CO_2 (hyperoxia). Cytokines were not detectable in culture supernatants of RAW 264.7 macrophages or MLE12 cells under any of these culture conditions. Generation of LIX in normoxic SVEC-4-10 cell culture tended to be higher at 39.5°C ($P = 0.14$), but cells co-exposed to hyperoxia and hyperthermia failed to generate detectable LIX (Table 1). Hyperoxia alone and in combination with hyperthermia approximately doubled generation of G-CSF compared with 37°C cell culture, but hyperthermia failed to increase G-CSF expression in normoxic SVEC-4-10 cell culture. In NIH 3T3 fibroblast cultures, hyperthermia alone increased KC generation 10% and co-exposure to hyperoxia and hyperthermia increased KC generation by 27%, but hyperoxia alone did not alter KC generation compared with normoxic, euthermic cells. In the same cells, hyperoxia increased G-CSF generation by 35% and co-exposure to hyperoxia and hyperthermia augmented G-CSF generation by 65%, but exposure to hyperthermia

Figure 7. Effect of FRH on numbers of circulating leukocytes and plasma concentration of G-CSF. Groups of five mice were exposed to $F_{I}O_2$ 0.21 (normoxic) or > 0.95 (hyperoxic) and housed at either 24°C (euthermic) or 34.5°C (hyperthermic). Mice were sacrificed after 24 hours of exposure and heparinized blood was collected via cardiac puncture and analyzed for number of leukocytes (A) and PMN (B) by manual counting. C: Plasma concentration of G-CSF was measured by ELISA. Mean $\pm$ SE; * denotes $P < 0.05$ versus euthermic, normoxic mice; † denotes $P < 0.05$ versus euthermic, hyperoxic mice. D: Effect of G-CSF immunoblockade on circulating PMN count in mice exposed to hyperthermia for 24 hours. Mice were pretreated with anti-G-CSF or control rabbit serum (sham) 2 hours before beginning 24 hours of euthermic or hyperthermic exposure; ** denotes $P < 0.05$ versus sham-treated/euthermic; ‡ denotes $P < 0.05$ versus sham-treated/hyperthermic mice.

alone did not alter G-CSF generation compared with normoxic, euthermic cells. LIX generation by these cells was not modified by exposure to hyperoxia or hyperthermia, alone or in combination.

Discussion

We used a conscious hyperthermic mouse model in which core temperature is increased by reducing heat elimination rather than by increasing heat production³ to determine whether FRH modifies pulmonary oxygen toxicity. This animal model is not intended to duplicate the physiology of the febrile state, rather it is intended to isolate the effects of the temperature increase itself on hyperoxic lung injury. Since the temperature of inspired air:oxygen in the hyperthermic mice (34°C) was lower than the core temperature in euthermic mice (36.5°C to 37°C), we attributed the changes in lung injury to the increase in core temperature rather than to direct airway warming. The core temperature achieved in the hyperthermic animals in the present study is similar to the temperature that was protective in mice with experimental *Herpes simplex*,^{34,35} rabies,³⁶ and *Klebsiella pneumoniae*³ infections. This temperature range overlaps the

Figure 8. Effect of febrile range hyperthermia on ELR⁺ CXC expression in the hyperoxic lung. **A:** Groups of eight mice were exposed to F_iO₂ 0.21 or > 0.95 and housed at either 24°C (euthermic) or 34.5°C (hyperthermic) for the indicated time. Lung lavage was performed and KC was quantified by ELISA. * denotes *P* < 0.05 versus euthermic, normoxic controls. ** denotes *P* < 0.05 versus euthermic, hyperoxic mice at the same exposure time. Because hyperthermic mice did not survive beyond 60 hours of exposure to hyperthermia, analysis of later time points was not done (ND). **B** and **C:** BALF from groups of five mice exposed to F_iO₂ 0.21 or > 0.95 and housed at either 24°C (euthermic) or 34.5°C (hyperthermic) for 24 hours were analyzed for concentrations of LIX (**B**) and MIP-2 (**C**) using ELISA. Mean ± SE; * denotes *P* < 0.05 versus euthermic normoxic mice.

upper end of the febrile range in mice^{37,38} and is below the established thermal threshold for induction of the heat shock response.³⁹ To avoid other possible effects of the warming protocol, both groups of mice were handled in exactly the same manner except for the modest increase in ambient temperature. In the absence of hyperoxia, FRH was well tolerated. Mice housed at 34°C for 2 weeks maintained core temperatures at 39°C to 40°C without visible signs of distress, changes in behavior, measurable lung injury, or an increase in circulating IL-6 levels (data not shown).

Using this model, we showed that FRH accelerated hyperoxic lung injury, shortened survival time, and reduced the threshold fractional index of oxygen required for lethality. We used three measures of lung injury. In mice exposed to lethal levels of hyperoxia, both the lung wet:dry weight ratio and BALF protein concentration increased earlier in the hyperthermic mice than in the euthermic mice, indicating an accelerated loss of pulmonary vascular endothelial barrier integrity and pulmonary edema formation in the warmer animals. This was accompanied by an earlier increase in BALF levels of IL-6, a pleiotropic cytokine that is expressed at sites of inflammation and tissue injury and is a predictor of mortality in sepsis.⁴⁰

Histological evidence of lung injury during exposure to hyperoxia was detectable 2 to 3 days earlier in hyperthermic than in the euthermic mice and was temporally associated with the increase in BALF IL-6 concentration and death in both groups of mice. Once injury commenced, the histological patterns of injury were profoundly different in the euthermic and hyperthermic mice, suggesting that co-exposure to FRH might augment pulmonary oxygen toxicity by activating distinct cytotoxic pathways that are not activated by hyperoxia alone. Specifically, co-exposure to FRH altered the kinetics of PMN recruitment, the distribution of PMN within the hyperoxic lung, and the apparent contribution of PMN to lung injury. The histological changes in the euthermic mice after 108 hours of exposure to hyperoxia were similar to those reported by others^{19,41} and characterized by diffuse hyaline membranes, perivascular edema, intra-alveolar hemorrhage, and attenuation of bronchial epithelium. In striking contrast, after only 36 hours of exposure to hyperoxia, the hyperthermic mice developed a marked PMN-rich interstitial pneumonitis. Total lung PMN content as determined by counting GR-1-immunostaining cells peaked more rapidly (36 hours versus 108 hours) and at 2.5-fold higher levels in FRH than euthermic mice. In studies of fever in a lizard model of gram-negative bacterial infection, the accelerated pathogen clearance and improved host survival found in the febrile animals was associated with augmented accumulation of granulocytes at the subcutaneous inoculation site.⁴² Collectively, these results suggest that enhanced PMN recruitment might be a common and evolutionarily conserved pathway through which fever augments host defenses. In contrast, FRH and hyperoxia exerted a more modest effect on pulmonary macrophages. Although co-exposure to FRH and hyperoxia was associated with a moderate increase in macrophages content in BALF, the total number of macrophages detected by analyzing anti-Mac-3-stained lung tissue was not altered in these mice. While the majority of intrapulmonary macrophages are located within the bronchoalveolar compartment, the efficiency of macrophage recovery in BALF is variable.⁴³ It is possible that co-exposure to FRH and hyperoxia might stimulate a redistribution of interstitial macrophages to the bronchoalveolar compartment or reduce macrophage adherence to the bronchoalveolar epithelial surface, thereby increasing the efficiency of cell recovery in BALF.

The ELR⁺ CXC chemokines comprise one of five distinct classes of PMN chemoattractants that activate PMN via distinct G-protein-coupled, 7-transmembrane domain receptors. Mice lack an analogue of the major human ELR⁺ CXC chemokine, IL-8, but they express three other members of this chemokine family, notably KC/Gro α , MIP-2/Gro β , and LIX. Mean BALF concentrations of two of the three murine ELR⁺ CXC chemokines, KC and LIX, increased 10- and 4.2-fold, respectively, during co-exposure to hyperoxia and FRH and BALF levels of MIP-2 were modestly increased by FRH alone, suggesting that FRH might enhance PMN recruitment to the hyperoxic lung, in part, through augmented expression of all three ELR⁺ CXC chemokines. In mice, the three ELR⁺ CXC

Figure 9. Effect of treatment with anti-CXCR2 antiserum. Mice received goat anti-mouse CXCR2 antiserum, preimmune goat serum (control), or no pretreatment (untreated) 2 hours before transfer into a 34.5°C chamber and exposure to F_IO₂ > 0.95 and sacrificed 40 hours later. **A:** The lungs were lavaged and the number of PMN in BALF was determined in groups of 10 mice. **B:** Lungs were fixed at 20 cm H₂O, immunostained with anti-GR-1 antibody, and the number of PMN per HPF were counted in groups of four mice. Untreated, euthermic, air-breathing (F_IO₂ > 0.95) mice were analyzed as a comparison (hatched bar). * denotes *P* < 0.05 versus untreated and pre-immune serum-treated control mice. **C:** Representative micrographs from sham-treated and anti-CXCR2-treated mice. Arrows indicate PMN. **D:** Median survival was determined in groups of nine mice.

chemokines act through a single receptor, CXCR2.⁴⁴ Immunoblockade of this receptor completely blocked the increase in PMN accumulation in lung interstitium and in BALF, in mice exposed to hyperoxia and FRH. Collectively, these results indicate that the ELR⁺ CXC chemokines are required for the early recruitment of PMN to the hyperoxic, hyperthermic lung, and suggest that the increased expression of KC and LIX might directly contribute to the augmented PMN recruitment.

While co-exposure to FRH and hyperoxia stimulated KC and LIX expression within the bronchoalveolar compartment, exposure to FRH alone failed to increase the mean BALF level of either chemokine. KC secretion in 3T3 fibroblast cultures during *in vitro* exposure to hyperoxia and hyperthermia showed a similar pattern in which

co-exposure to 95% oxygen and 39.5°C incubation temperature stimulated a supra-additive increase in KC expression. In contrast, constitutive LIX secretion in 3T3 cell cultures was not augmented by hyperoxia or hyperthermia alone or in combination, demonstrating that the cytokine modifying effects of hyperthermia are gene-specific, even in the same cell. Similarly, constitutive LIX secretion in SVEC-10⁻⁴ EC cultures was also unaltered by exposure to hyperoxia or hyperthermia, but LIX generation was blocked by co-exposure to hyperoxia and hyperthermia and these cells failed to secrete detectable KC during *in vitro* exposure to hyperoxia. We previously reported that incubating human pulmonary artery endothelial cells at 39.5°C augmented TNF- α -induced expression of IL-8,⁴⁵ another member of the ELR⁺ CXC chemo-

Table 1. Effect of Hyperthermia (39.5°C) on Cytokine Generation *in Vitro*

Cytokine/cell type	Cytokine concentration (pg/ml) ^a			
	37°C/air	39.5°C/air	37°C/95% O ₂	39.5°C/95% O ₂
LIX				
SVEC-4-10	946 ± 128	1735 ± 694	1120 ± 30	UD ^b
NIH-3T3	27,230 ± 682	27,033 ± 255	22,483 ± 1107	25,570 ± 583
KC				
SVEC-4-10	UD	UD	UD	UD
NIH-3T3	4902 ± 175	5442 ± 167 ^c	5183 ± 158	6219 ± 168 ^d
G-CSF				
SVEC-4-10	14.3 ± 2.8	14.9 ± 2.9	25.4 ± 4.1 ^e	31.4 ± 2.2 ^f
NIH-3T3	95.9 ± 3.4	87.9 ± 2.8	130 ± 4.9 ^g	158 ± 3.6 ^h

^aThe indicated cells were cultured for 24 hours at the indicated temperature and oxygen level and cytokines were measured by ELISA.

^bUndetectable.

^c $p < 0.03$ versus 37°C/air.

^d $p < 0.0002$ versus 39.5°C/air; $p < 0.0001$ versus 37°C/95% O₂.

^e $p < 0.02$ versus 37°C/air; $p < 0.03$ versus 39.5°C/air.

^f $p < 0.0004$ versus 37°C/air; $p < 0.006$ versus 39.5°C/air.

^g $p < 0.01$ versus 37°C/air and 39.5°C/air.

^h $p < 0.001$ versus 37°C/air, 39.5°C/air, and 37°C/95% O₂.

kine family; however, exposing the same cells to FRH in the absence of another stimulus (exogenous TNF- α) failed to stimulate IL-8 expression. These results identify limitations in extrapolating behavior of *in vitro* cell cultures to the *in vivo* environment. For example, hyperoxia and hyperthermia may indirectly activate chemokine gene expression *in vivo* by activating proximal autocrine/paracrine mediators. Furthermore, cell lines may not faithfully recapitulate *in vivo* gene regulation. Identification of the cellular source of the augmented cytokine generation in the hyperoxic, hyperthermic mouse and the intermediate mediators required for cytokine expression is presently under investigation in our laboratory. However, the differences in responsiveness of KC, MIP-2, and LIX to hyperoxia and FRH in the present study, as well as the previously reported differences in induction kinetics, tissue distribution, and responsiveness to corticosteroids among these three chemokines,⁴⁶ suggest that KC, MIP-2, and LIX serve distinct functions and might differentially contribute to PMN recruitment in the presence and absence of fever.

We showed that FRH expands the circulating PMN pool in air-breathing and hyperoxic mice. Such an increase might result from increased release of PMN from the bone marrow and/or through PMN demargination into the circulating pool. Since the lung is a major site of PMN margination,⁴⁷ the coincidence of increased circulating and intrapulmonary PMN in the hyperoxic hyperthermic mice suggests that the marrow rather than pulmonary demargination is the major source of excess circulating PMN in these mice. We found that exposing mice to FRH for 24 hours increases circulating G-CSF levels 7.1-fold. Uchida et al⁴⁸ reported that administration of exogenous G-CSF to mice increases proliferation of PMN precursors and reduces marrow transit time of PMN, leading to elevated circulating PMN levels detectable within 1 to 2 days. In fact, we showed that neutralization of G-CSF with anti-G-CSF antibodies abrogated FRH-induced neutrophilia. These combined data suggest that G-CSF-induced expansion of granulopoiesis is a major source of the additional circulating PMN present after 24 hours of

exposure to FRH. However, we have not excluded contributions to the expanded circulating pool from other sources. Brenner et al⁴⁹ report that exercise-induced hyperthermia stimulates catecholamine and corticosteroid release and PMN demargination, suggesting that FRH might induce both a rapid G-CSF-independent and a prolonged G-CSF-dependent expansion of the circulating PMN pool. G-CSF also has been reported to increase PMN degranulation, secretory vesicle mobilization, and surface CD11b expression⁵⁰ and augment superoxide generation⁵¹ and chemotaxis.⁵² The capacity of FRH to simultaneously expand the circulating PMN pool and to augment generation of chemotactic gradients at sites of inflammation might serve to rapidly mobilize PMN to sites of infection or injury. Interestingly, Rosenspire et al⁵³ recently reported that release of reactive oxygen intermediates and nitric oxide are greatly augmented in human PMN on exposure to FRH *in vitro*. Thus, FRH might provide both delivery and activation of PMN to sites of inflammation. While this process might be essential to contain infection, it may lead to tissue injury and dysfunction, multiorgan failure, and even death, such as occurs in hyperoxic lung injury. In fact, inhibition of PMN recruitment through prior infusion of hyperthermic, hyperoxic mice with anti-CXCR2 antibody improved survival time. However, median survival time in these animals remained shorter compared with euthermic, hyperoxic mice, suggesting that hyperthermia itself might exert additional injurious effects within the hyperoxic lung. We have previously demonstrated that *in vitro* exposure to hyperthermia (39.5°C) synergizes with TNF- α treatment to disrupt barrier function in cultured human pulmonary EC in the absence of PMN,⁴⁵ suggesting that hyperoxia and hyperthermia might also cause lung injury by directly disrupting endothelial barrier integrity.

Although the temperature used in this study is below the generally accepted threshold for the heat shock response,³⁹ more recent studies have demonstrated that temperatures in the febrile range can stimulate expression of heat shock proteins *in vivo*.^{54,55} Furthermore, inflammatory mediators, including arachidonic acid⁵⁶ and

interferon- γ ,⁵⁷ lower the thermal threshold for induction of the heat shock response. Thus, it is possible that heat shock proteins were generated in the hyperthermic, hyperoxic mouse lung. However, heat shock proteins usually exert tissue protective⁵⁸ and anti-inflammatory effects,⁵⁹ suggesting that if the heat shock response did contribute to the augmented pulmonary oxygen toxicity in the hyperthermic mice, it did so through an atypical mechanism.

In summary, we have shown that exposure to FRH accelerates and enhances pulmonary oxygen toxicity and have provided evidence that this is caused by enhanced recruitment and retention of PMN within the hyperoxic lung. We have also shown that FRH might enhance PMN accumulation by inducing expression of G-CSF, expanding the circulating PMN pool, and augmenting generation of ELR⁺ CXC chemokines within the hyperoxic lung. The capacity of FRH to augment hyperoxic lung injury in light of its protective effects during life-threatening infections underscores the importance of understanding the context in which biological processes function. The core temperature increase that occurs during febrile illnesses is a powerful immune modulator, the net effects of which depend on the clinical setting. The results of the present study underscore the importance of directly analyzing the effects of core temperature manipulation in critically ill patients.

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