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Cell-free hemoglobin: a novel mediator of acute lung injury

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Shaver CM, Upchurch CP, Janz DR, Grove BS, Putz ND, Wickersham NE, Dikalov SI, Ware LB, Bastarache JA. Cell-free hemoglobin: a novel mediator of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 310: L532–L541, 2016. First published January 15, 2016; doi:10.1152/ajplung.00155.2015.—Patients with the acute respiratory distress syndrome (ARDS) have elevated levels of cell-free hemoglobin (CFH) in the air space, but the contribution of CFH to the pathogenesis of acute lung injury is unknown. In the present study, we demonstrate that levels of CFH in the air space correlate with measures of alveolar-capillary barrier dysfunction in humans with ARDS ($r = 0.89$, $P < 0.001$) and in mice with ventilator-induced acute lung injury ($r = 0.89$, $P < 0.001$). To investigate the specific contribution of CFH to ARDS, we studied the impact of purified CFH in the mouse lung and on cultured mouse lung epithelial (MLE-12) cells. Intratracheal delivery of CFH in mice causes acute lung injury with air space inflammation and alveolar-capillary barrier disruption. Similarly, in MLE-12 cells, CFH increases proinflammatory cytokine expression and increases paracellular permeability as measured by electrical cell-substrate impedance sensing. Next, to determine whether these effects are mediated by the iron-containing heme moiety of CFH, we treated mice with intratracheal hemin, the chloride salt of heme, and found that hemin was sufficient to increase alveolar permeability but failed to induce proinflammatory cytokine expression or epithelial cell injury. Together, these data identify CFH in the air space as a previously unrecognized driver of lung epithelial injury in human and experimental ARDS and suggest that CFH and hemin may contribute to ARDS through different mechanisms. Interventions targeting CFH and heme in the air space could provide a new therapeutic approach for ARDS.

acute respiratory distress syndrome; air space inflammation; permeability; lung epithelium; hemoglobin; hemin

HEMOLYSIS LEADS TO ELEVATED plasma (PL) levels of cell-free hemoglobin (CFH) in several clinical conditions including sepsis, hemodialysis, sickle cell crisis, and extracorporeal circulation, and after red blood cell transfusion (9, 26, 32, 43, 46, 50, 64, 65). The effects of extracellular hemoglobin on the vascular endothelium and macrophages are particularly well studied (2, 13, 17, 18, 35, 40, 48, 59). In the circulation, hemoglobin-mediated nitric oxide scavenging precipitates vasoconstriction and pulmonary hypertension during sickle cell crisis (50) and accounts for some of the adverse effects of red blood cell transfusion (15, 26). In macrophages, hemoglobin acts as a damage-associated molecular pattern (DAMP) and results in activation of proinflammatory immune responses (8, 18). Many of the pathological effects of hemolysis are mediated by release of iron-carrying heme. For example, heme infusion increases mortality and organ damage in a mouse model of sepsis (39) and potentiates LPS-mediated cytokine production in macrophages and during endotoxemia (19). CFH also acts as a potent oxidant (9, 26, 47, 49, 54, 55), and elevated levels of CFH have been associated with markers of oxidative injury and with acute kidney injury in critically ill patients with sepsis and in postoperative patients (9, 33). Although these data demonstrate that CFH has numerous detrimental effects on macrophages and endothelial cells, its effects on epithelial cells are unknown. Furthermore, although the central pathogenic role for circulating CFH in systemic disease states has been well studied, there is little known about potential effects of CFH in the lung.

Alveolar hemorrhage is a common feature of the acute respiratory distress syndrome (ARDS) (3). Genetic variation in iron metabolism genes such as ferritin light chain and constitutive heme oxygenase 2 has been shown to affect risk of developing ARDS (38). In addition, levels of iron and iron-processing proteins are elevated in bronchoalveolar lavage (BAL) fluid of patients with ARDS (23, 27, 45). We previously reported that high levels of extracellular CFH were detected in pulmonary edema fluid (EF) samples from patients with ARDS and in BAL fluid from mice with experimental acute lung injury (6). Based on the known effects of CFH on endothelial cells and macrophages and the high levels of CFH in the air space during ARDS, we hypothesize that CFH is a driver of lung permeability and inflammation. The goal of the present study was to test the effects of CFH on the lung epithelium and to determine whether CFH in the air space can increase alveolar capillary barrier permeability during acute lung injury. We further aimed to test whether free heme in the air space, which can be released from CFH during degradation, could also mediate lung injury.

METHODS

Human sample collection and cell-free hemoglobin measurement. Methods for collection of undiluted pulmonary EF and simultaneous PL samples from critically ill mechanically ventilated patients with ARDS have been previously described (7). All samples were centrifuged immediately after collection to remove the cellular fraction and

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the supernatant was stored at −80°C until thawed for analysis. Cell-free hemoglobin and total protein in EF and PL samples were measured spectrophtometrically by using HemoCue (Cypress, CA) and BCA assay (Pierce, Rockford, IL), respectively (6). Use of HemoCue for measurement in EF and PL was validated prior to experimental measurements (data not shown). The EF-to-PL ratio of total protein was calculated as a measurement of alveolar-capillary barrier permeability (66). Human studies were approved by the Vanderbilt Institutional Review Board (IRB no. 020260).

Experimental murine acute lung injury. For ventilator-induced lung injury (VILI), adult C57Bl/6 mice were anesthetized with pentobarbital (5 mg/g mouse) and mechanically ventilated (MV) via tracheostomy with either low (6 ml/kg) or high (12 ml/kg) tidal volumes for 2 h. For some experiments, mice were treated with intratracheal (IT) instillation (6, 58) of 1 μg of lipopolysaccharide (LPS) in 100 μl PBS given 30 min prior to MV. Experimental pneumonia was induced by intranasal instillation of 2,000 CFU *Klebsiella pneumoniae* in 50 μl PBS to mice anesthetized with isoflurane (16). For CFH-induced or hemin-induced acute lung injury, CFH, hemin, and albumin (control) were prepared daily and filtered prior to use. Endotoxin-free CFH (Cell Sciences, Canton, MA) was diluted in PBS to a final concentration of 1 mg/ml (15 μM). This dose of CFH was based on the median levels of CFH measured in undiluted pulmonary edema fluid from patients with ARDS (6). Hemin (the chloride salt of ferric heme) (Sigma) was dissolved in 0.1 N NaOH and diluted in PBS to a final concentration of 60 μM (40 μg/ml), based on the molar equivalent of the dose of CFH since CFH contains four heme moieties. Albumin was diluted in PBS to 1 mg/ml as a protein control for CFH. Mice were anesthetized with isoflurane prior to direct IT instillation (53) of 100 μl of CFH, hemin, or albumin control. Samples were collected after 2 h for VILI, 24 h for pneumonia, and 24 h for IT CFH-mediated injury. Murine BAL fluid, flash frozen lungs, and formalin-fixed paraffin-embedded histological samples were collected as previously described (6) and stored at −80°C until analysis. If overt hemorrhage due to procedural complications occurred during sample collection, the BAL results from that animal were removed from analysis. All experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Measurement of indicators of acute lung injury in BAL. Cell counts and differentials were quantified by microscopic examination. After this, BAL fluid was centrifuged to remove leukocytes and red blood cells prior to quantification of CFH and other injury biomarkers. Cell-free hemoglobin was quantified by HemoCue and protein was measured in duplicate by BCA assay (Pierce) (6). Receptor for advanced glycation end products (RAGE) and cytokines were measured in duplicate by ELISA (R&D Systems, Minneapolis, MN) or a murine 7-plex proinflammatory cytokine assay that we have previously validated for use in mouse BAL (5) (Meso Scale Discovery, Gaithersburg, MD).

Histological assessment of lung injury. Ten nonoverlapping ×20 fields of hematoxylin and eosin-stained lung tissue were assessed in a blinded manner as previously described (6, 21). Histological lung injury of each field was scored for inflammation, septal thickening, edema, and hemorrhage, and a composite total lung injury score was calculated.

Cultured epithelial cell model system. Mouse lung epithelial cells (MLE-12) were maintained in HITES medium and then incubated with 15 μM CFH or 60 μM hemin in medium for 24 h. Conditioned medium was collected, centrifuged, and stored at −80°C until analysis. RNA was extracted from treated cells by use of the PureLink RNA extraction kit (Ambion, Grand Island, NY) and cytokine gene expression was measured by quantitative PCR. Protein, RAGE, and cytokine protein expression were measured in conditioned media by ELISA (R&D Systems). For resistance measurements, MLE-12 cells were grown for 24 h on 8W10E+ PC plates (Applied BioPhysics, Troy, NY) prior to treatment with CFH, hemin, or media control for an additional 24 h. Epithelial permeability was measured by resistance at 4,000 Hz via electrical cell-substrate impedance sensing (ECIS) (Applied BioPhysics). Data were normalized to the baseline resistance prior to addition of treatments. Viability of cells after CFH or hemin treatments was confirmed by manual assessment of Trypan blue exclusion and by MTS/PMS assay (Promega, Madison, WI) (data not shown).

Statistical analysis. All statistical analysis was done with SPSS version 23 for Macintosh. Comparisons between three groups were performed by one-way ANOVA with post hoc Tukey's comparisons. Pairwise comparisons were performed by Student's *t*-tests. Correlations were tested by using Spearman's correlation coefficients. Nonnormally distributed data were natural log transformed prior to analysis. A P value <0.05 was considered statistically significant.

RESULTS

CFH is elevated in the air space in human ARDS and in murine experimental lung injury and correlates with increased alveolar-capillary barrier permeability. Although we previously reported that CFH levels were increased in pulmonary EF of patients with ARDS compared with control patients with hydrostatic pulmonary edema (6), it was not clear from that study what the relationship was between CFH levels in the air space compared with the circulation. To address this question, we compared simultaneous PL CFH levels to previously published levels of CFH in EF in 18 patients with ARDS (6). CFH levels in undiluted EF were significantly greater than simultaneous levels in undiluted PL (Fig. 1*A*). This finding suggests that air space CFH is generated from red blood cell breakdown within the alveolar space rather than by passive leakage from the circulation. In ARDS patients, EF CFH levels were tightly correlated with the EF-to-PL protein ratio, an index of alveolar-capillary barrier permeability (rho = 0.89, $P < 0.001$) (Fig. 1*B*). Total EF protein levels were not simply reflective of CFH levels in patients with ARDS; the mean EF total protein level was 4,314 ± 2,043 mg/dl and the mean CFH level was 227 ± 214 mg/dl, demonstrating that CFH accounted for 5–6% of the total protein present in EF.

To determine whether release of CFH into the air space is also a feature of experimental acute lung injury, we measured CFH in BAL from mice with two types of experimental acute lung injury (42, 56). Mice with ventilator-induced acute lung injury using low (6 ml/kg) or high (12 ml/kg) tidal volume ventilation with or without the addition of IT LPS 30 min prior to mechanical ventilation had increased CFH levels in BAL fluid compared with control mice treated with IT PBS (Fig. 1*C*). Similar to findings in humans with ARDS, levels of CFH in the BAL correlated strongly with BAL protein, an index of alveolar-capillary barrier permeability (rho = 0.89, $P < 0.001$) (Fig. 1*D*). In addition, BAL CFH was elevated in a clinically relevant model of gram-negative pneumonia caused by *Klebsiella pneumoniae* (16) (Fig. 1*E*). These data demonstrate that elevated intra-alveolar CFH is a feature of both human ARDS and experimental acute lung injury.

CFH is sufficient to cause acute lung injury. To define the specific contribution of intra-alveolar CFH to acute lung injury, wild-type C57Bl/6 mice were treated IT with 100 μl of 1 mg/ml CFH (total dose 100 μg per mouse) or albumin control (100 μg per mouse) and studied at 24 h. This dose of CFH was chosen based on the median levels of CFH measured in EF from patients with ARDS. Compared with controls, mice treated with CFH had increased lung inflammation (Fig. 2*A*)

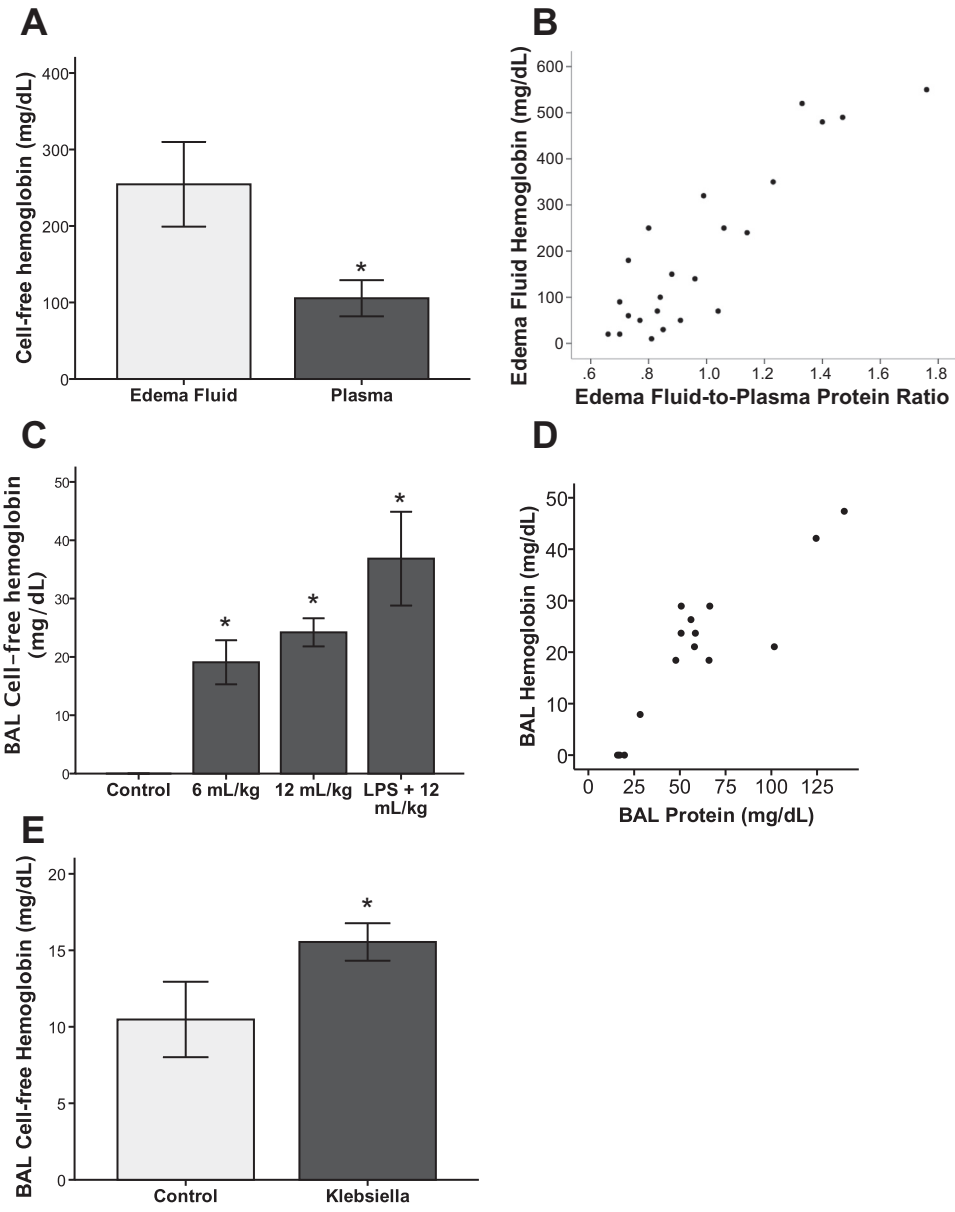


Fig. 1. Release of cell-free hemoglobin into the air space is a feature of human and experimental acute lung injury. *A*: cell-free hemoglobin (CFH) levels are significantly higher in pulmonary edema fluid (EF) compared with plasma (PL) of patients with acute respiratory distress syndrome (ARDS), $n = 18$; *P = 0.011 vs. control. The levels of CFH in EF from these patients was previously published and are reported here for direct comparison to PL CFH levels (6). *B*: in patients with ARDS, EF CFH correlates strongly with EF-to-PL protein ratio, an index of alveolar-capillary barrier permeability, rho = 0.89, $P < 0.001$ by Spearman's correlation coefficient. *C*: CFH is elevated in bronchoalveolar lavage (BAL) fluid of mice treated with mechanical ventilation ± intratracheal LPS; *P < 0.029 for each group vs. control, $n = 3$–5 per group. *D*: BAL CFH correlates strongly with BAL protein in mice with ventilator induced lung injury, rho = 0.89, $P < 0.001$ by Spearman's correlation. *E*: BAL CFH is increased during experimental bacterial pneumonia compared with control animals, *$P = 0.05$, $n = 5$–9 per group.

with neutrophil predominance (Fig. 2*B*). In addition, BAL levels of the proinflammatory cytokines TNF-α, IL-1β, IL-6, CXCL-1/KC, and IL12p70 were elevated in response to CFH (Fig. 2*C*).

Assessment of acute lung injury by histological analysis demonstrated that CFH-treated mice had increased edema and total lung injury scores (Fig. 3, *A*–*C*). To further examine whether lung permeability was altered by CFH, BAL levels of total protein were measured. CFH-treated mice had significantly increased BAL protein (Fig. 3*D*), consistent with disruption of the alveolar-capillary barrier. The mean BAL protein concentration in CFH treated mice was 399 ± 126 μg/ml, exceeding the total amount of CFH administered. In addition, CFH-treated mice had elevated BAL levels of RAGE, a marker of lung epithelial cell injury (62) (Fig. 3*E*). Together, these data demonstrate that IT CFH recapitulates the key features of ARDS even in the absence of other injurious stimuli, causing significant air space inflammation, damage to the lung epithelium, and disruption of the alveolar-capillary barrier.

CFH induces cytokine production and increases permeability in cultured lung epithelial cells. Because the lung epithelium is known to be a critical regulator of permeability and inflammation in the air space (28, 41) and because of evidence of lung epithelial injury after IT CFH, we next tested the direct effects of CFH on cultured lung epithelial cells to better define the cellular mechanisms of CFH effects in the lung. MLE-12 cells treated with increasing doses of CFH for 24 h had dose-dependent increases in expression of the inflammatory mediators IL-6 (Fig. 4, *A* and *C*) and KC (Fig. 4, *B* and *D*) at both the protein (Fig. 4, *A* and *B*) and mRNA (Fig. 4, *C* and *D*) level. MLE-12 cells did not increase TNF-α production in response to CFH (data not shown). In addition, CFH (1 mg/ml) reduced transepithelial resistance of MLE-12 cell monolayers as measured by ECIS over 24 h (Fig. 4*E*), suggesting increased

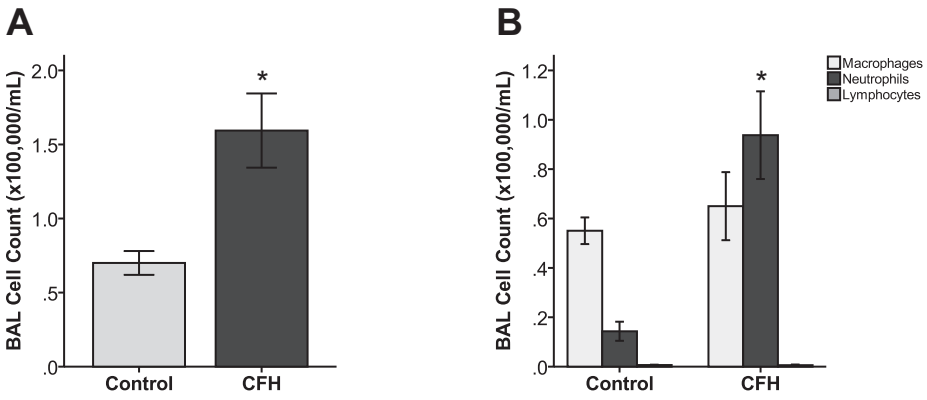


Fig. 2. Cell-free hemoglobin induces acute air space inflammation in mice. Mice were treated with 100 μg endotoxin-free CFH or 100 μg albumin control and sampled after 24 h. CFH increases total BAL fluid cell counts (*$P = 0.032$ vs. albumin control) (*A*) and trends toward increased neutrophils in the air space (*$P = 0.09$ vs. albumin control) (*B*) compared with control mice. There is no significant difference in numbers of macrophages or lymphocytes between treatments. *C*: CFH increases proinflammatory cytokine concentrations in BAL fluid; $n = 6–8$ per group.

C

Cytokine (pg/mL)	Control (n=6)	CFH (n=8)	P value
TNF-α	6.1 (3.1-10.2)	78.1 (39.2-91.0)	0.009
IFN-γ	0.1 (0.1-0.2)	1.2 (1.2-3.9)	<0.001
IL-10	2.8 (2.5-3.2)	1.2 (1.2-19.0)	0.964
IL-12p70	26.3 (14.2-38.6)	19.3 (1.2-63.1)	0.341
IL-1β	0.8 (0.4-1.5)	14.9 (10.9-16.3)	<0.001
IL-6	20.5 (13.1-27.3)	175.2 (22.8-428.8)	0.259
KC	1.1 (0.8-2.5)	122.2 (120.0-139.6)	<0.001

Values are median (interquartile range).

lung epithelial paracellular permeability. There were no effects of CFH on cell viability over 24 h at any of the doses utilized in the in vitro experiments (not shown). These data show that the lung epithelium is directly affected by CFH in vitro and increases proinflammatory cytokines and disrupts permeability.

The heme moiety of CFH increases permeability, but does not affect air space inflammation in vivo. Heme dissociates from CFH in the extracellular space and may mediate the effects of CFH in the lung. We tested whether the isolated heme moiety of CFH could induce acute lung injury by treating C57Bl/6 mice with IT hemin (60 μM in 100 μl PBS), which is the chloride salt of heme, compared with equimolar CFH (15 μM). After 24 h, mice treated with hemin had no evidence of inflammatory cell recruitment into the air space (Fig. 5*A*), no increase in air space neutrophils (Fig. 5*B*), and no significant elevations in a panel of proinflammatory cytokines in BAL (Fig. 5*C*). Compared with CFH, IT hemin resulted in similar increases in alveolar-capillary permeability (Fig. 5*D*) as measured by BAL protein levels. However, in contrast to after CFH, there was no evidence of hemin-dependent epithelial cell injury in the lung as measured by RAGE release into BAL (Fig. 5*E*). Overall, these data suggest that isolated free heme is sufficient to disrupt the alveolar-capillary barrier but insufficient to cause alveolar inflammation.

The heme moiety of CFH increases permeability but fails to induce inflammation in cultured lung epithelial cells. To test whether the heme moiety of CFH had distinct effects on lung epithelial cells in vitro compared with intact CFH, we incubated MLE-12 cells with hemin (60 μM) or CFH (15 μM) for 24 h. Hemin did not increase protein expression of IL-6 or KC in vitro (Fig. 6, *A* and *B*). Hemin reduced the resistance of MLE-12 cells as measured by ECIS (Fig. 6*C*), consistent with the in vivo finding of increased permeability in response to hemin. In vitro, hemin disrupted paracellular permeability more rapidly and to a greater degree than CFH. These data indicate that the CFH and the heme moiety have distinct effects on cultured lung epithelial cells.

DISCUSSION

In this study, we show for the first time that instillation of CFH into the air space is sufficient to recapitulate the main features of human ARDS in an experimental murine acute lung injury model. When delivered directly to the air space, CFH induces inflammation, causes lung epithelial cell injury, and increases alveolar-capillary permeability. These changes are also seen in cultured lung epithelial cells, suggesting that the lung epithelium is a major target for the pathological effects of CFH in vivo. Furthermore, the instillation of the heme moiety of CFH induces similar permeability changes but fails to induce inflammation or release of RAGE. These findings identify CFH and its heme moiety as novel mediators during acute lung injury.

The potential pathogenic consequences of extracellular CFH in the air space have not been well studied, despite intra-alveolar hemorrhage being a frequent feature of ARDS (3). One possible reason for this may be that prior studies showed that delivery of intact red blood cells into the air space was protective in a hyperoxia model of ARDS (63) through a mechanism requiring red blood cell glutathione, a potent antioxidant. In addition, whereas alveolar hemorrhage is a well-described feature of ARDS, release of CFH into the air space in the setting of alveolar hemorrhage was only recently recognized (6). The present studies demonstrate that the injurious effects of CFH in the air space are distinct from the protective effects of intact red blood cells.

Our study identifies the alveolar epithelium as a crucial target for the injurious effects of CFH during acute lung injury. In the presence of CFH, MLE-12 lung epithelial cells have

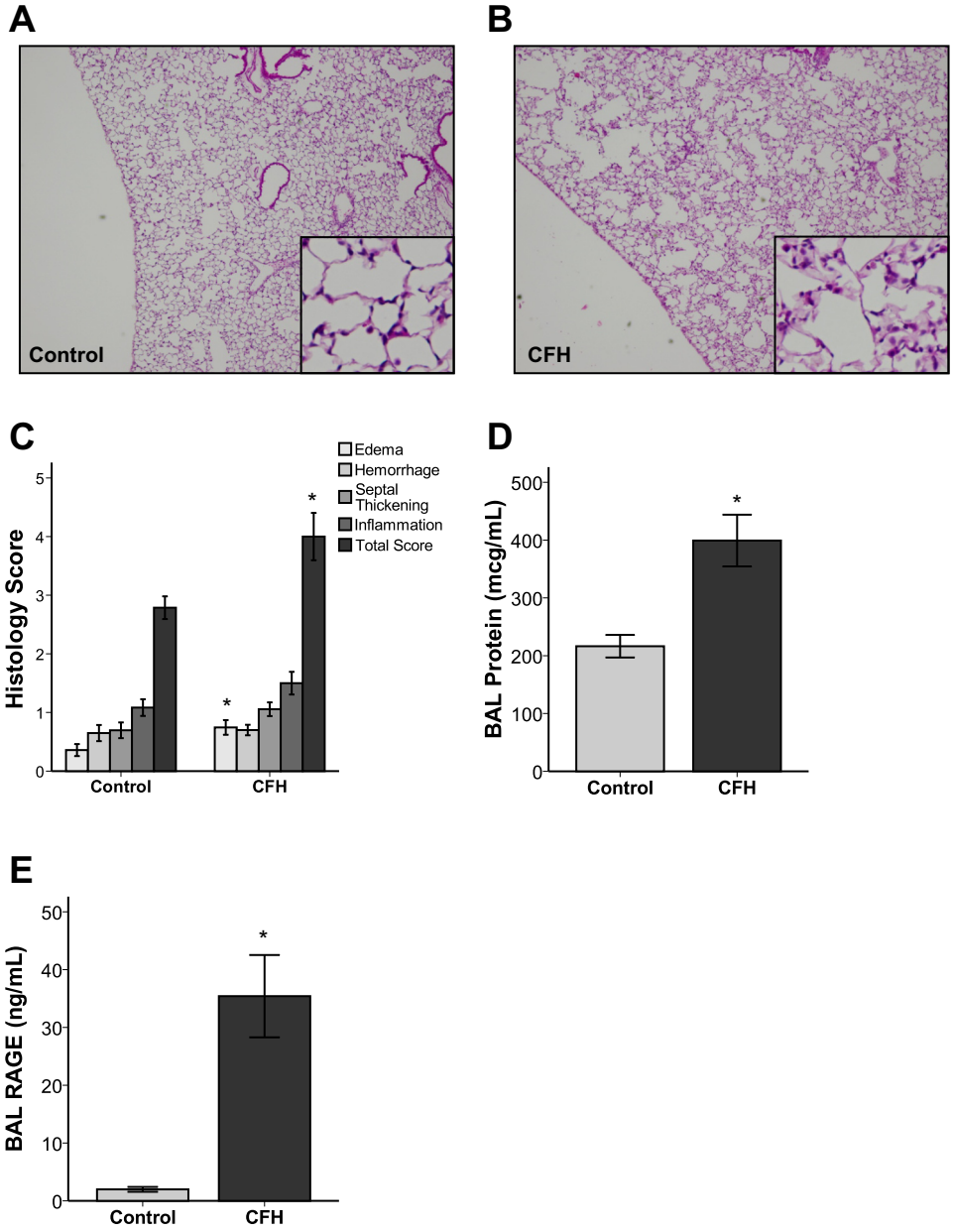


Fig. 3. Cell-free hemoglobin causes histological lung injury and disrupts the alveolar-capillary barrier. *A–C*: CFH increases the total lung injury score and edema score as assessed by histological examination (*P = 0.033 for edema score and P = 0.002 for total lung injury score compared with PBS control). *D*: CFH increases BAL protein, consistent with disruption of the alveolar-capillary barrier (*P = 0.013). *E*: CFH induced injury to epithelial cells as measured by concentration of receptor of advanced glycation end products (RAGE) in BAL fluid (*P = 0.001 vs. control); n = 6–8 in each group.

increased paracellular permeability, a previously unrecognized consequence of CFH exposure. These data are confirmed during acute lung injury in vivo with evidence of alveolar-capillary barrier disruption measured by increased BAL protein and with type I epithelial cell damage as evidenced by increased BAL RAGE (62). In addition to effects on epithelial permeability, CFH at concentrations found in the air space during human ARDS also induces proinflammatory cytokine expression in lung epithelial cells both in vivo and in vitro. These results are consistent with and expand upon one previous study of the inflammatory effects of CFH on lung epithelial cells in which Mumby et al. (44) showed that CFH induced expression of IL-8 and MCP-1 through activation of NF-κB in the A549 human lung epithelial cell line. Additional studies are needed to determine the specific molecular mechanisms of CFH-mediated effects on inflammation and epithelial cell damage in the lung. Specifically, whether NF-κB or Toll-like receptor 4 (TLR4), a key mediator of the proinflammatory effects of heme on macrophages (20), contributes to CFH-mediated acute lung injury needs to be investigated. Whether the impact of purified hemoglobin differs in the presence of other injurious stimuli such as LPS also warrants further study. Furthermore, that CFH can induce epithelial permeability in vitro in the absence of other cell types suggests that CFH has direct effects on the lung epithelium that are not dependent on the presence of inflammatory cells in the air space.

Our studies highlight important distinctions between the effects of intact CFH and the heme component of CFH in the lung. Instillation of the heme component of CFH into the air spaces was sufficient to increase alveolar permeability, but lung epithelial injury and increased inflammation required the intact CFH molecule. Previous work has demonstrated that administration of intravenous hemin resulted in acute lung

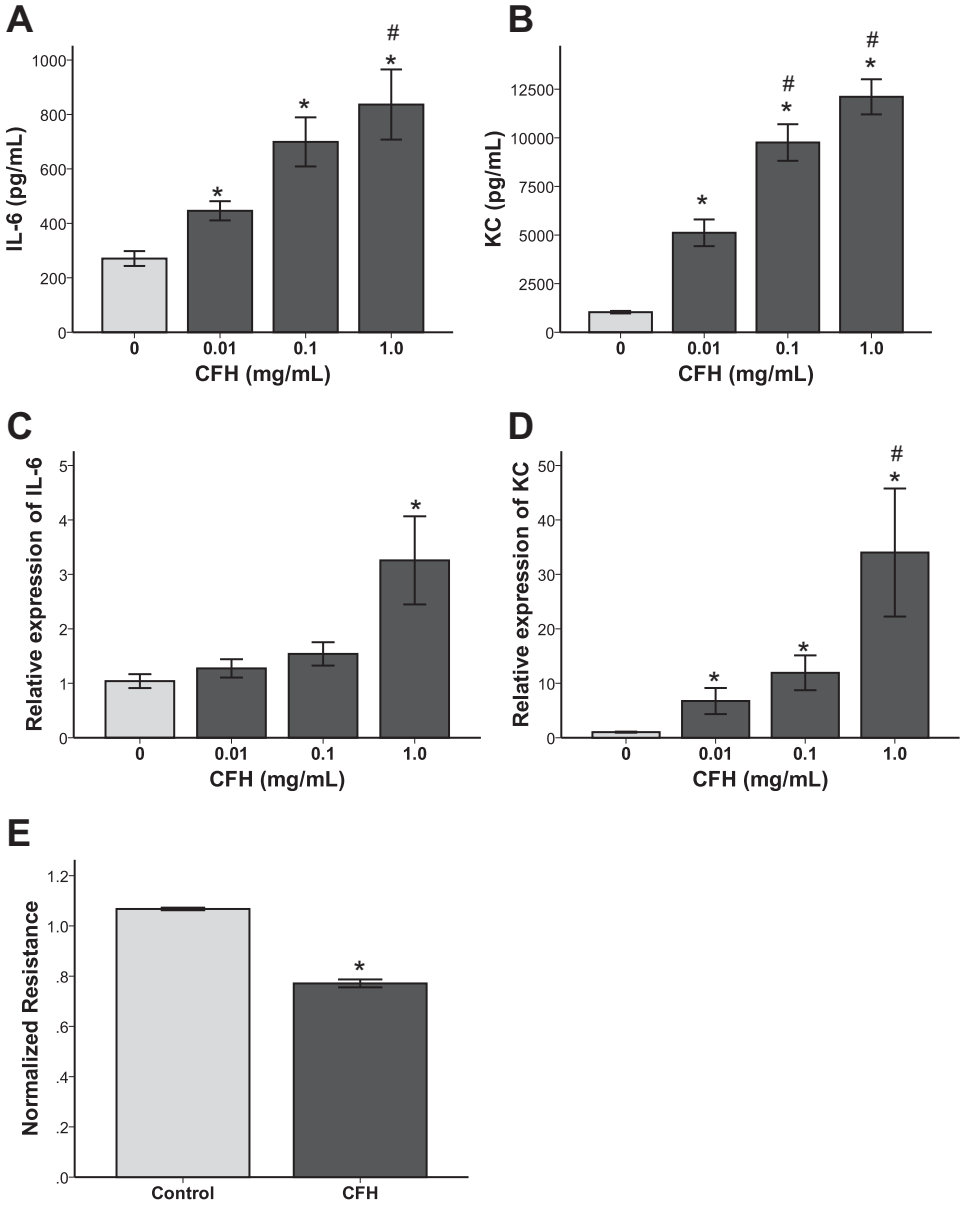
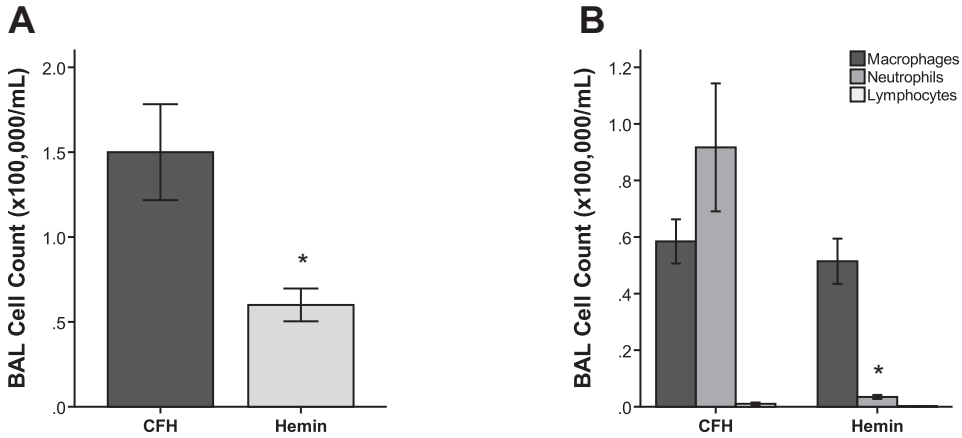


Fig. 4. Cell-free hemoglobin induces cytokine production and permeability defects in cultured mouse lung epithelial cells. MLE-12 lung epithelial cells produce higher levels of IL-6 (*A*) and CXCL-1/KC (*B*) in response to increasing doses of CFH in vitro (*$P < 0.05$ vs. control, #$P < 0.05$ vs. 0.01 mg/ml dose). MLE-12 cells increase IL-6 (*C*) and KC (*D*) transcription in response to CFH exposure (*$P < 0.05$ vs. control, #$P < 0.05$ vs. 0.01 mg/ml). *E*: CFH reduces epithelial permeability as measured by electric cell-substrate impedance sensing (ECIS) over 24 h ($P < 0.001$ vs. control); $n = 4–8$ in each group.

injury with increased permeability in a model of sickle cell disease (25). We extend these findings to demonstrate that hemin can also disrupt epithelial permeability when administered IT in vivo and to lung epithelial cells in vitro. Surprisingly, IT hemin did not increase BAL RAGE as was seen with IT CFH. It may be that CFH and hemin have different molecular mechanisms of permeability, an idea supported by our in vitro results showing that hemin disrupts permeability more quickly and robustly than CFH. Alternately, elevated RAGE may occur in response to elevated CFH as a protective measure to sequester CFH to limit its toxicity. Our in vivo data also suggest that the heme moiety of CFH is not responsible for the inflammatory response to CFH. One potential explanation of this finding could be that free heme fails to reach the distal air spaces because of the avidity of binding to its scavenger protein hemopexin; however, this is less likely because of the significant alveolar permeability defect seen after IT hemin. The lack of inflammation in response to hemin is particularly interesting since circulating heme has been reported to be an important mediator of systemic inflammation (19, 34, 51). For example, iron is a key factor for oxidation of low-density lipoproteins and for membrane damage by CFH in endothelial cells and triggers neutrophil extracellular trap formation during sickle cell disease (14, 36). Furthermore, released iron is a stimulus for inflammation in the lung, in part through production of reactive oxygen species (22, 24, 37). Why heme has such different effects in mediating inflammation systemically compared with in the air space is unknown but has important implications for therapeutic targeting of CFH in ARDS and will require further study.

Based on the present studies, we propose that the release of CFH in the air space could be a novel therapeutic target for ARDS. Importantly, since CFH levels in the air space exceed concentrations of CFH in the circulation and because CFH has direct toxicity to the lung epithelium, therapies aimed at CFH will need to be targeted to the air space rather



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Cytokine (pg/mL)	CFH (n=8)	Hemin (n=8)	P value
TNF-α	22.4 (17.1-28.0)	1.2 (1.2-1.2)	<0.001
IFN-γ	20.3 (15.1-37.9)	13.5 (6.9-18.2)	0.131
IL-10	13.6 (1.2-31.8)	14.7 (1.2-29.6)	0.979
IL-12p70	9.8 (1.2-82.6)	45.4 (1.2-97.2)	0.586
IL-1β	13.0 (9.8-17.4)	3.9 (1.7-8.3)	0.002
IL-6	379.4 (344.1-444.3)	1.2 (1.2-43.0)	<0.001
KC	117.3 (76.6-136.8)	44.1 (32.6-47.0)	0.002

Values are median (interquartile range).

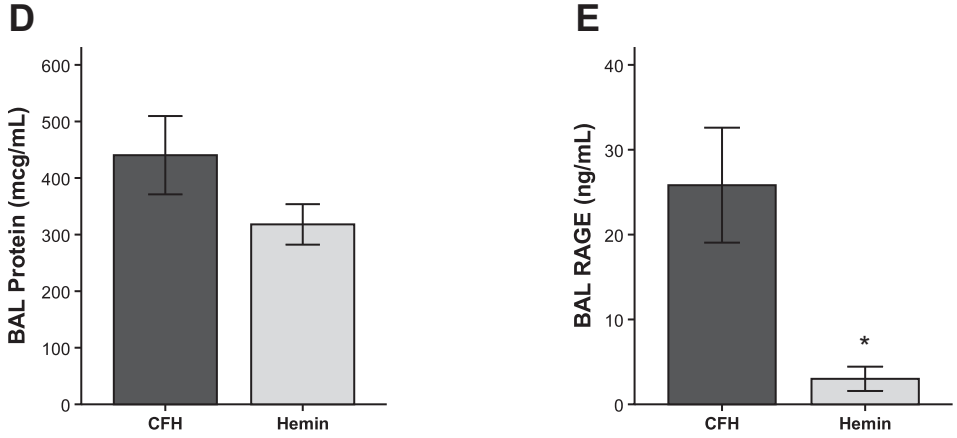

Fig. 5. The heme moiety of cell-free hemoglobin affects permeability but not inflammation during acute lung injury. C57Bl/6 mice were treated with intratracheal CFH (100 μg) or hemin (60 μM), the chloride salt of heme, for 24 h prior to sample collection. Hemin causes less alveolar inflammation as measured by bronchoalveolar cell counts (*P = 0.037) (*A*) with fewer neutrophils in the air space (*P = 0.009) (*B*). *C*: hemin induces less proinflammatory cytokines in BAL compared with CFH. BAL protein (*D*) was equivalent between CFH and hemin (P = 0.178). Hemin caused less release of BAL RAGE (*E*) compared with CFH (*P = 0.006); n = 8 in each group.

than given systemically. Augmentation of the endogenous mechanisms for CFH removal may limit the adverse effects of CFH in the air space. In the healthy state, CFH in the circulation is bound by haptoglobin and free heme is bound by hemopexin prior to uptake by monocytes via CD163 and CD91, respectively (10, 11, 60, 61). Based on our results, we would expect that haptoglobin would bind CFH, thereby improving barrier integrity and reducing inflammation, whereas hemopexin may only attenuate permeability with limited impact on inflammation. In support of this treatment approach, mice that overexpress haptoglobin in alveolar macrophages have reduced air space hemoglobin during acute lung injury, suggesting that rapid binding of CFH in the lung may limit tissue injury (67, 68). Systemic haptoglobin also reduces blood pressure, vascular injury, and renal dysfunction in animal models of hypertension (4, 11, 31) and reduces iron accumulation and vascular remodeling in a model of CFH-induced pulmonary hypertension (31). Similarly, intraperitoneal hemopexin administration improved lung permeability after bromine inhalation (1) and intravenous hemopexin improved survival and reduced liver and kidney toxicity in an animal model of sepsis (39). Because CFH and free heme have different effects in the air space compared with the circulation, air space targeting of CFH and heme in ARDS will need to be explored.

Although the current findings advance our understanding of the role of intra-alveolar CFH in the pathogenesis of ARDS, unanswered questions remain. For example, the mechanisms leading to CFH accumulation in the lung are unknown. Based on the human data showing that CFH in EF is disproportionately elevated compared with simultaneous levels in PL, we propose that CFH is actively released from red blood cells within the air space rather than passively leaking into the lung from the circulation. Increased red blood cell fragility in the injured air space may also play a role in CFH production, similar to the mechanisms of

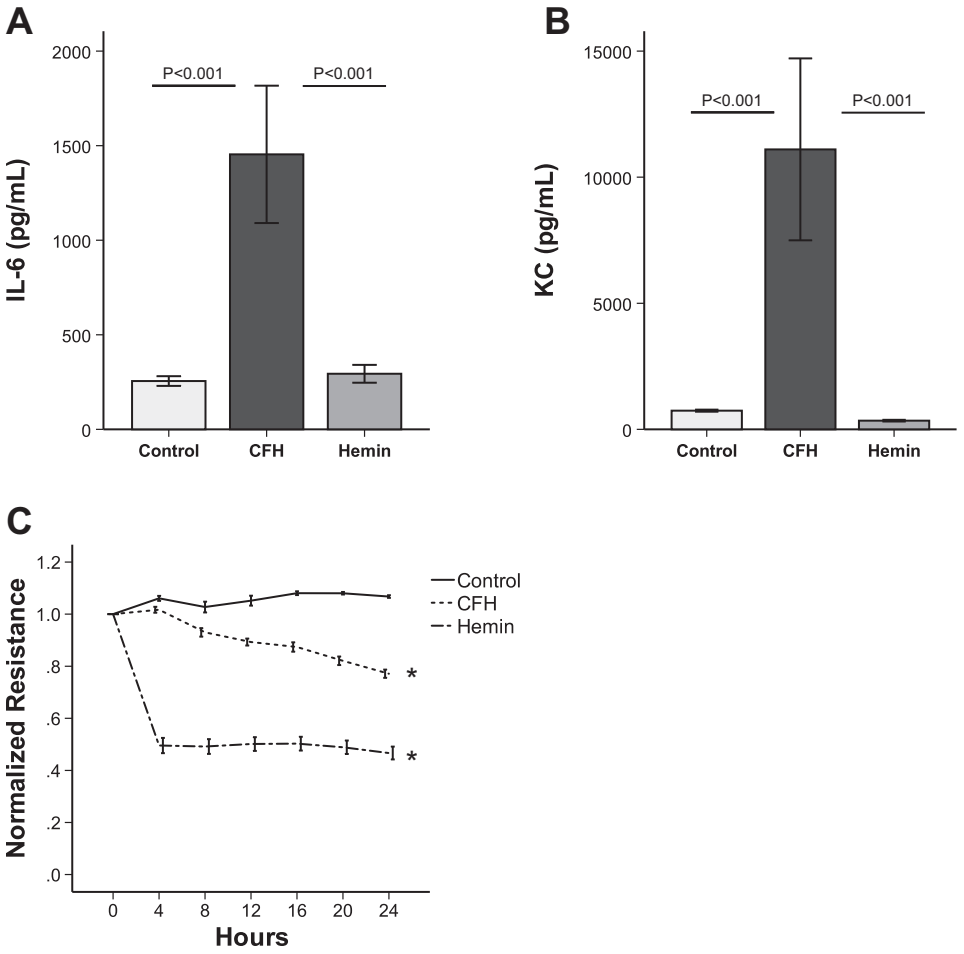

Fig. 6. The hemin moiety of cell-free hemoglobin affects permeability but not inflammation in cultured lung epithelial cells. MLE-12 cells were incubated with media control, CFH (1 mg/ml), or hemin (60 μM) for 24 h. Expression of IL-6 (*A*) and KC (*B*) protein were measured by ELISA. *C*: permeability was measured by ECIS over time (*$P < 0.001$ vs. control at 24 h); $n = 4–8$ replicates for each measurement.

hemolysis of circulating red blood cells in patients with sepsis (52, 57). Despite the possibility that red blood cell lysis could also release antioxidants (63), previous studies have demonstrated very low levels of the antioxidants glutathione, ascorbate, and urate in pulmonary EF sampled from the distal air spaces of patients with ARDS (12). As discussed above, it is also uncertain how CFH is removed from the air space. Further studies are needed to understand the balance between CFH generation in the air space and potential mechanisms of clearance. In addition, although we have specifically evaluated the effect of CFH on the lung epithelium, there are other cells in the air space that may modulate the effects of CFH. In particular, alveolar macrophages may play a key role in CFH-mediated acute lung injury because of their role in innate immunity and their hypothesized role in clearance of CFH from the air space. In fact, alveolar macrophages upregulate heme oxygenase-1, a key enzyme for heme degradation, and increase reactive oxygen species production in response to hemin exposure (29, 30).

In summary, we have shown for the first time that elevated levels of CFH are a consistent feature of human and experimental ARDS and that CFH delivered IT to mice causes robust acute lung injury independent of any other stimulus. Intact CFH results in increased inflammation, loss of alveolar capillary barrier integrity, and epithelial cell injury. In contrast, the isolated heme moiety of CFH fails to induce significant inflammation or epithelial cell injury, despite causing a similar disruption in lung epithelial permeability to CFH. These studies identify distinct effects of intact CFH and its heme moiety in the air space and suggest that CFH and heme may be specific targets for novel therapeutic agents for ARDS patients with elevated CFH in the air space.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

C.M.S., D.R.J., S.I.D., L.B.W., and J.A.B. conception and design of research; C.M.S., C.P.U., B.S.G., N.P., N.W., S.I.D., L.B.W., and J.A.B. performed experiments; C.M.S., C.P.U., B.S.G., N.P., N.W., S.I.D., L.B.W., and J.A.B. analyzed data; C.M.S., D.R.J., B.S.G., N.W., S.I.D., L.B.W., and J.A.B. interpreted results of experiments; C.M.S., L.B.W., and J.A.B. prepared figures; C.M.S., L.B.W., and J.A.B. drafted manuscript; C.M.S., D.R.J., S.I.D., L.B.W., and J.A.B. edited and revised manuscript; C.M.S., C.P.U., D.R.J., B.S.G., N.P., N.W., S.I.D., L.B.W., and J.A.B. approved final version of manuscript.

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