JCI insight

RESEARCH ARTICLE

Cell-free hemoglobin promotes primary graft dysfunction through oxidative lung endothelial injury

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Primary graft dysfunction (PGD) is acute lung injury within 72 hours of lung transplantation. We hypothesized that cell-free hemoglobin (CFH) contributes to PGD by increasing lung microvascular permeability and tested this in patients, ex vivo human lungs, and cultured human lung microvascular endothelial cells. In a nested case control study of 40 patients with severe PGD at 72 hours and 80 matched controls without PGD, elevated preoperative CFH was independently associated with increased PGD risk (odds ratio [OR] 2.75, 95%CI, 1.23–6.16, $P = 0.014$). The effect of CFH on PGD was magnified by reperfusion fraction of inspired oxygen (FiO_2) ≥ 0.40 (OR 3.41, $P = 0.031$). Isolated perfused human lungs exposed to intravascular CFH (100 mg/dl) developed increased vascular permeability as measured by lung weight (CFH 14.4% vs. control 0.65%, $P = 0.047$) and extravasation of Evans blue–labeled albumin dye (EBD) into the airspace ($P = 0.027$). CFH (1 mg/dl) also increased paracellular permeability of human pulmonary microvascular endothelial cell monolayers (hPMVECs). Hyperoxia ($FiO_2 = 0.95$) increased human lung and hPMVEC permeability compared with normoxia ($FiO_2 = 0.21$). Treatment with acetaminophen (15 μg/ml), a specific hemoprotein reductant, prevented CFH-dependent permeability in human lungs ($P = 0.046$) and hPMVECs ($P = 0.037$). In summary, CFH may mediate PGD through oxidative effects on microvascular permeability, which are augmented by hyperoxia and abrogated by acetaminophen.

Conflict of interest: JAB and LBW received research grant funding from Global Blood Therapeutics. LBW receives research grant support from Boehringer Ingelheim. DJL is a consultant for ImmuneWorks.

Submitted: November 9, 2017
Accepted: December 19, 2017
Published: January 25, 2018

Reference information:
JCI Insight. 2018;3(2):e98546.
https://doi.org/10.1172/jci.insight.98546.

Introduction

Primary graft dysfunction (PGD) is a form of acute lung injury that is characterized by hypoxemia, pulmonary edema, and lung inflammation that develops within the first 72 hours after lung transplantation (1, 2). PGD is the most common cause of short-term mortality after lung transplantation and also con-

tributes to the development of chronic lung allograft dysfunction (2). A variety of clinical risk factors for PGD have been identified with contributions from the donor (3–7), the recipient (3–5, 7–10), and operative variables (4, 5, 8, 11). In addition, a number of biomarkers have been associated with increased risk of PGD, including markers of innate and adaptive immune activation, epithelial and endothelial injury, coagulation, vascular permeability, and lipid peroxidation (2, 12–27). Despite identification of these clinical and biomarker predictors of PGD, the mechanisms leading to PGD are not well understood, and there are no specific therapeutic interventions for PGD.

Cell-free hemoglobin (CFH) is a potent proinflammatory oxidant that accumulates in the circulation in clinical conditions associated with increased RBC fragility, such as sepsis or sickle cell disease, as a result of RBC shearing during cardiopulmonary bypass or hemodialysis, or during storage of RBCs. When CFH is released into the extracellular space, it can be oxidized and drive oxidant-mediated modification of proteins and lipids (28). Elevated levels of CFH have been associated with poor clinical outcomes in a variety of clinical conditions. For example, in sepsis, elevated levels of circulating CFH are associated with increased mortality and organ dysfunction (29). In the airspace, elevated levels of CFH are associated with lung epithelial injury and disruption of the alveolar-capillary barrier (30, 31). Whether CFH contributes to the pathogenesis of PGD is unknown. However, the known association of PGD with cardiopulmonary bypass (5), pulmonary hypertension (5), and lipid peroxidation (27) — risk factors that have also been associated with elevated CFH (29, 32–34) — suggests a potential mechanistic link. Based on these data, we hypothesized that CFH may play a mechanistic role in the development of PGD via effects on vascular permeability.

It also may be feasible to specifically target CFH-mediated oxidative injury for therapeutic benefit. Based on structural homology between the heme moiety of CFH and the peroxidase moiety of cyclooxygenase, the cyclooxygenase inhibitor acetaminophen (APAP) is a specific hemoprotein reductant that can reduce oxidized ferryl (Fe^{4+}) CFH to less reactive CFH species, reducing the potential for CFH-mediated lipid peroxidation (35). APAP prevented hemoprotein-induced renal injury through reduction of oxidized myoglobin in a rat model of rhabdomyolysis (35). In addition, in a clinical trial in critically ill patients with sepsis who had elevated CFH levels, administration of enteral APAP at standard FDA-approved doses resulted in lower levels of plasma lipid peroxidation products and improved renal function (28). Whether APAP can limit development of PGD after lung transplantation has not been studied.

Based on these data, the current study had 3 goals. First, we tested the hypothesis that elevated perioperative levels of circulating CFH are associated with increased risk for PGD after lung transplantation. Second, we investigated whether CFH induces acute lung injury by increasing microvascular permeability in the isolated perfused human lung and in cultured human microvascular endothelial cells. Finally, we tested whether oxygen exacerbates or APAP attenuates the injurious effects of elevated CFH in these model systems.

Results

Patient characteristics. Patients with International Society for Heart and Lung Transplantation (ISHLT) grade 3 PGD (partial pressure arterial oxygen [PaO_2]/fraction of inspired oxygen [FiO_2] < 200 with radiographic infiltrates in the lung allograft) (1, 2) at 72 hours after transplantation were frequency matched with control patients with no PGD in the 72 hours after lung transplant according to the underlying diagnosis and type of lung transplant (Table 1). Patients on preoperative extracorporeal support were excluded. Patients with PGD were more likely to have received cardiopulmonary bypass and more likely to have a donor with a smoking history. As expected, both 90-day and 1-year mortality were significantly higher in patients with PGD.

Perioperative trends in plasma CFH. Unlike healthy subjects who have very low levels of circulating CFH (<5 mg/dl) (36), CFH was elevated (>10 mg/dl) in plasma collected preoperatively in the majority (91%) of lung transplant recipients (Figure 1A). The median level of CFH was 30 mg/dl (range 0–390 mg/dl). Plasma CFH increased within 6 hours of surgery and was downtrending by 24 hours after surgery. Levels were similar between patients with chronic obstructive versus interstitial lung disease at each time point (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.98546DS1). Patients who received intraoperative cardiopulmonary bypass had marked increases in plasma CFH between preoperative and 6-hour postoperative measurements (Figure 1B). Patient characteristics aside from PGD frequency were similar across CFH groups (Supplemental Table 1).

Preoperative CFH is independently associated with increased risk of PGD. Preoperative CFH levels greater than or equal to the median of 30 mg/dl were associated with increased frequency of grade 3 PGD at 72 hours (Figure 2A, 45% vs. 23%, $P = 0.013$). Postoperative CFH levels were not associated with risk of PGD

Table 1. Patient characteristics

Characteristic	PGD cases (*n* = 40)	Controls (*n* = 80)	*P* value
Age[A]	55 (47, 61)	56 (52, 61)	0.84
Male[B]	27 (68%)	40 (50%)	0.06
European descent[B]	32 (80%)	72 (90%)	0.13
Diagnosis[B]			0.67
COPD	16 (40%)	30 (38%)	
CF	0 (0%)	2 (2%)	
ILD	24 (60%)	47 (59%)	
Other	0 (0%)	1 (1%)	
Bilateral lung transplant[B]	24 (60%)	50 (63%)	0.85
Ischemic time (min)[A]	243 (212, 268)	234 (196, 268)	0.54
Cardiopulmonary bypass[B]	22 (55%)	25 (31%)	0.01
mPAP[A]	31.7 (22.0, 40.7)	25.0 (20.0, 33.7)	0.08
pRBC transfusion (ml)[A]	625 (313, 1,500)	500 (250, 1,000)	0.24
BMI[A]	28.3 (23.4, 30.2)	25.6 (23.2, 28.1)	0.07
Donor smoke exposure[B]	24 (60%)	34 (43%)	0.03
Reperfusion FiO_2[A]	0.62 (0.31, 0.95)	0.50 (0.30, 0.78)	0.20
90-day mortality[B]	9 (23%)	4 (5%)	0.003
1-year mortality[B]	13 (32.5%)	9 (11.3%)	0.005

Cases were identified as having grade 3 primary graft dysfunction (PGD) within 72 hours. Controls were matched for underlying diagnosis and procedural type. Data are shown as *n* (%) or median (25th percentile, 75th percentile). Comparisons were made by χ^2 test (A) or Mann Whitney *U* test (B) as appropriate. Cardiopulmonary bypass is limited to intraoperative use of bypass. COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; FiO_2, fraction of inspired oxygen; ILD, interstitial lung disease; mPAP, mean pulmonary artery pressure; pRBC, packed RBC.

(Figure 2B). In a univariate logistic regression model, a preoperative CFH level of 30 mg/dl or greater was associated with an odds ratio (OR) for PGD of 2.75 (95% CI, 1.23–6.16, $P = 0.014$) (Table 2). This relationship persisted after individually controlling for other risk factors for PGD, including cardiopulmonary bypass, mean pulmonary artery pressure, donor smoking, BMI, sex, and reperfusion FiO_2.

High reperfusion FiO_2 increases the risk of PGD. Because a higher FiO_2 at reperfusion increased the association of lipid peroxidation with risk of PGD (27), we tested whether the association of CFH with risk of PGD was greater in those with higher reperfusion FiO_2 (≥0.40). The median FiO_2 was 0.25 (interquartile range [IQR]: 0.21–0.33) in the low FiO_2 group ($n = 46$) and 0.74 (IQR: 0.58–0.95) in the high FiO_2 group ($n = 74$). In patients with a high reperfusion FiO_2, levels of CFH at or above the median were associated with increased risk of PGD (Figure 3). There was no statistically significant relationship between levels of CFH and risk of PGD in patients with low reperfusion FiO_2. There was also no significant correlation between the concentration of CFH and reperfusion FiO_2, suggesting that the impact of FiO_2 on PGD risk is from modification of CFH oxidation rather than alteration in CFH release. We then performed multivariable logistic regression to further understand the relationship between CFH, reper-

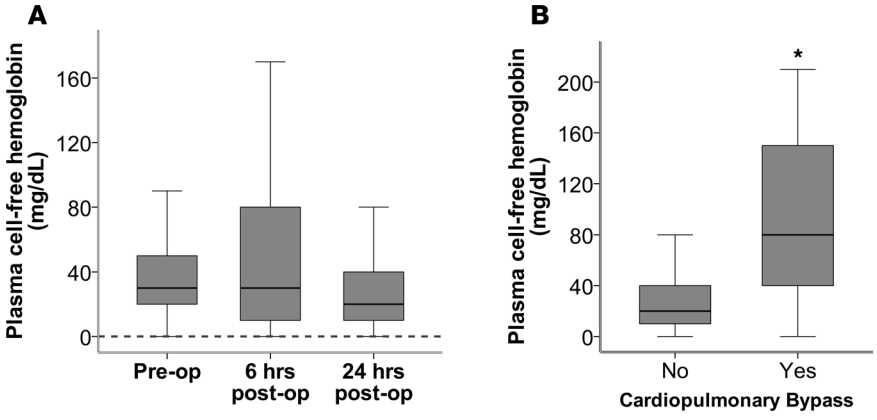

Figure 1. Plasma cell-free hemoglobin is elevated in lung transplant recipients. (**A**) Plasma cell-free hemoglobin (CFH) is detectable preoperatively as well as at 6 or 24 hours after lung transplantation. CFH was elevated in 91%, 97%, and 85% of patients at each time point, respectively, $n = 120$. The reference value of CFH in plasma in healthy patients is < 10 mg/dl. (**B**) Use of cardiopulmonary bypass significantly increases CFH at 6 hours after transplant surgery, $^*P < 0.001$ by Mann Whitney *U* test, $n = 70$ without bypass, $n = 44$ with bypass.

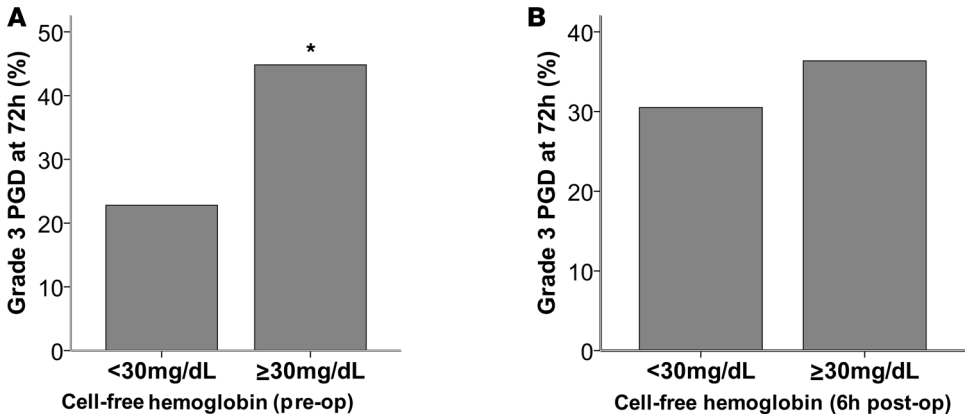

Figure 2. Preoperative plasma cell-free hemoglobin is associated with increased risk of primary graft dysfunction. (**A**) Patients with a plasma cell-free hemoglobin (CFH) level at or above the median (30 mg/dl) had a 1.9-fold increased risk of severe primary graft dysfunction (PGD) at 72 hours, **P* = 0.013 by χ^2 analysis, *n* = 105. (**B**) There was no association of CFH measured 6 hours postoperatively with risk of PGD, *P* = 0.51 by χ^2 analysis, *n* = 105.

fusion FiO_2, and PGD. Preoperative levels of CFH were independently associated with an increased risk of PGD only in patients with a reperfusion $FiO_2 \geq 0.40$ (high FiO_2, OR 3.41, 95% CI, 1.12–10.42, *P* = 0.031; low FiO_2, OR 2.78, 95% CI, 0.54–14.38, *P* = 0.22) (Table 3).

Circulating CFH increases lung vascular permeability in the isolated perfused human lung and cultured lung microvascular endothelial cells. The effect of CFH on lung vascular permeability was tested in isolated perfused human lungs obtained from deceased organ donors (Figure 4). Addition of a clinically relevant concentration of CFH (100 mg/dl) to the lung perfusate increased vascular permeability in the presence of an FiO_2 of 0.95. Compared with control, lungs exposed to CFH in the perfusate had increasing lung weight over 2 hours (14.4% weight gain with CFH vs. 0.65% with control, *P* = 0.047) (Figure 5A), an index of pulmonary edema formation. This was accompanied by increased extravasation of Evans blue–labeled albumin dye (EBD) from the perfusate into the alveolar space, a direct measure of vascular permeability (Figure 5B). Exposure of a tight monolayer of cultured human pulmonary microvascular endothelial cells (hPMVECs) to increasing doses of CFH for 24 hours increased paracellular permeability as assessed by a decrease in monolayer electrical resistance at 4,000 Hz, showing a direct time-dependent effect of CFH on microvascular endothelial monolayer permeability (Figure 6).

Hyperoxia augments the effects of CFH on vascular permeability. Because of the association of reperfusion FiO_2 with risk of PGD in our patient population, we next tested whether FiO_2 affected CFH-mediated changes in vascular permeability. For these studies, we compared the effect of CFH in the presence of an FiO_2 of 0.95 to an FiO_2 of 0.21 in paired lungs from the same donor to minimize the impact of donor-to-donor variability. Lungs exposed to CFH in the presence of an FiO_2 of 0.95 developed significantly

Table 2. Multivariable analysis of association between preoperative cell-free hemoglobin and primary graft dysfunction at 72 hours

Variable	Odds ratio	95% CI	*P* value
Unadjusted			
Preoperative cell-free hemoglobin (≥30 mg/dl)	2.75	1.23–6.16	0.014
Adjusted for			
Cardiopulmonary bypass	2.40	1.04–5.50	0.039
Pulmonary artery pressure (mean)	2.60	1.15–5.88	0.021
Donor smoke exposure	3.56	1.46–8.68	0.005
BMI	2.76	1.19–6.37	0.018
Male sex	2.89	1.26–6.66	0.012
Reperfusion FiO_2	2.88	1.27–6.51	0.011

The relationship between cell-free hemoglobin (CFH) and severe PGD, adjusted individually for each covariate is shown. CFH is measured in mg/dl in plasma. The referent group is patients with preoperative CFH below the median of 30 mg/dl. PGD, primary graft dysfunction.

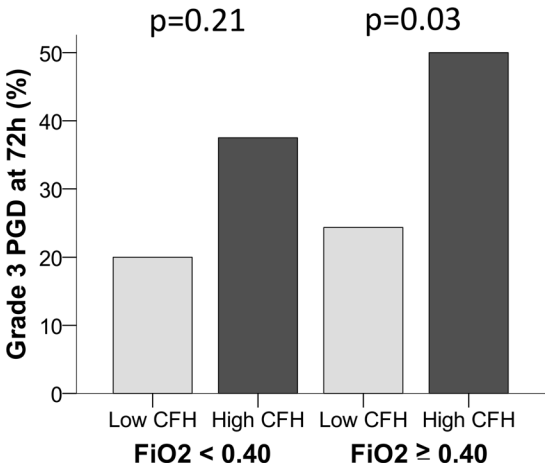

Figure 3. The association of cell-free hemoglobin with risk of primary graft dysfunction is modified by reperfusion FiO_2 greater than 0.40. Primary graft dysfunction (PGD) is most common in patients with high cell-free hemoglobin (CFH) and high reperfusion FiO_2. Comparisons were made between low (<30 mg/dl) and high (≥30 mg/dl) CFH groups by χ^2 testing. n = 105.

more pulmonary edema as measured by weight gain (Figure 7A) and extravasation of EBD (Figure 7B). Prior to addition of CFH to perfusate, there was no significant difference in weight change between normoxic and hyperoxic conditions (normoxia, median [IQR] –0.27% [–4.43%, –0.16%]; hyperoxia, –0.12% [–2.67%, 0.06%], P = 0.47 by Mann Whitney U test). Hyperoxia magnified CFH-dependent permeability changes in hPMVECs (Figure 7C). Cell viability of hPMVECs as measured by trypan blue exclusion was not affected by exposure to CFH or hyperoxia (data not shown).

APAP, a specific hemoprotein reductant, attenuates the effects of CFH on vascular permeability. Because of a structural similarity between the heme moiety of hemoglobin and the peroxidase moiety of cyclooxygenase, APAP specifically reduces oxidized Fe^{4+} hemoglobin to the less injurious reduced state (35, 37). Therefore, to determine whether the effects of hemoglobin on vascular permeability are attenuated by reduction of CFH into a less oxidized state, we next tested whether APAP could ameliorate CFH-induced lung injury in paired human lungs. Addition of APAP at levels in the clinically therapeutic range (15 μg/ml) to the perfusate of the isolated human lung attenuated both CFH-dependent lung weight gain (Figure 8A) and extravasation of EBD (Figure 8B). Prior to addition of CFH to perfusate, there was no significant difference in weight change between control and APAP (CFH alone, median [IQR] –0.92% [–2.55%, 1.05%]; CFH+APAP, 0.35% [–1.12%, 0.82%], P = 0.34 by Mann Whitney U test), indicating that, in the absence of CFH, APAP had no effect on lung weight. APAP prevented CFH-induced increases in paracellular permeability of hPMVECs as measured by monolayer resistance (Figure 8C).

Discussion

PGD is a major cause of morbidity and mortality after lung transplantation. Taken together, the clinical and experimental data in this study support a mechanistic contribution of CFH to the development of PGD. The primary finding is that elevated preoperative levels of plasma CFH are independently associated with increased risk of PGD in lung transplant recipients. Mechanistically, addition of CFH to the perfusate in the ex vivo perfused human lung increased vascular permeability and pulmonary edema formation. Exposure to CFH also increased

Table 3. Multivariable analysis of association between preoperative cell-free hemoglobin, reperfusion FiO2, and primary graft dysfunction at 72 hours

Variable	Odds ratio	95% CI	*P* value
Low reperfusion FiO_2 (<0.40)			
Preoperative cell-free hemoglobin (>30 mg/dl)	2.78	0.54–14.38	0.22
High reperfusion FiO_2 (≥0.40)			
Preoperative cell-free hemoglobin (≥30 mg/dl)	3.41	1.12–10.42	0.031

The relationship between cell-free hemoglobin (CFH) and severe PGD, adjusted for recipient diagnosis, procedure type, mean pulmonary arterial pressure, and use of cardiopulmonary bypass. CFH is measured in mg/dl in plasma. The referent group is patients with preoperative CFH below the median of 30 mg/dl.

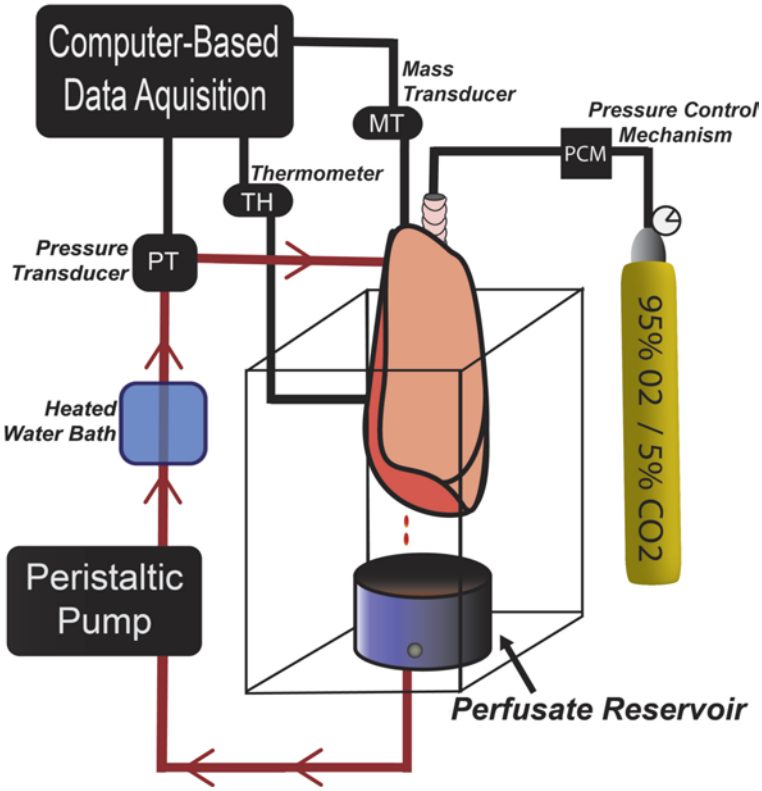

Figure 4. Schematic of ex vivo isolated perfused lung preparation. Lungs were suspended from a mass transducer and were continuously perfused at 37°C with DMEM containing 5% albumin and 16% (v/v) fresh whole human blood by roller pump to maintain a pulmonary artery pressure of 10–12 mmHg with passive drainage of perfusate through an open left atrium (53, 54). Lungs were inflated to continuous positive end-expiratory pressure of 10 mmHg with either an FiO_2 of 0.95 or of 0.21. The lung was suspended from a mass transducer for continuous measurement of lung weight as an index of pulmonary edema formation.

permeability in cultured pulmonary microvascular endothelial cell monolayers, demonstrating that CFH has direct effects on microvascular endothelial cell barrier function. Patients who received reperfusion FiO_2 at or above 0.40 had increased risk of PGD compared with those with lower reperfusion FiO_2. Similarly, the injurious effects of CFH were magnified in the presence of hyperoxia in isolated human lungs and in hPMVECs. Finally, the detrimental effects of CFH were attenuated by targeting the oxidation state of CFH with clinically relevant doses of APAP, a specific hemoprotein reductant. These findings identify CFH as a mediator of increased vascular permeability that contributes to the pathophysiology of PGD, and suggest that targeting oxidized CFH with the hemoprotein reductant APAP perioperatively could be a novel therapeutic strategy for prevention of PGD.

The current translational studies suggest that CFH contributes to increased microvascular permeability

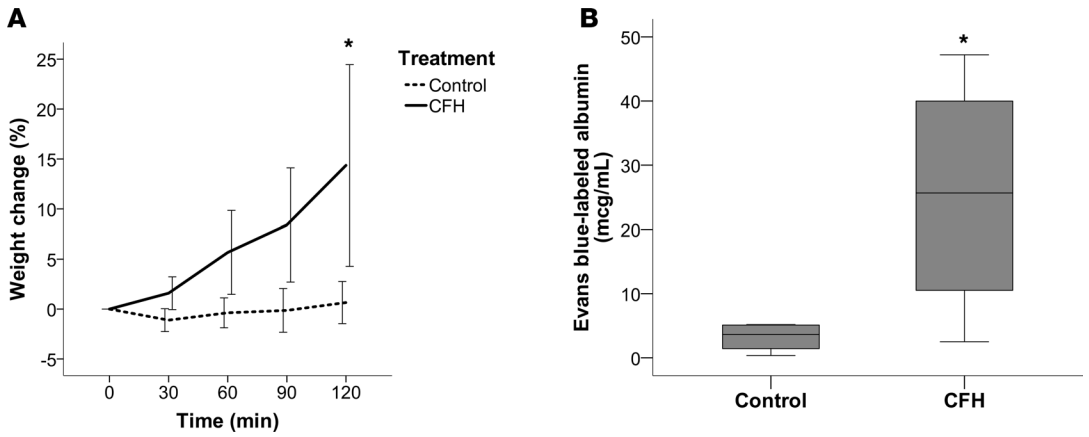

Figure 5. Cell-free hemoglobin increased pulmonary edema formation and vascular permeability in ex vivo isolated perfused lungs. (**A**) Cell-free hemoglobin (CFH) added to the perfusate (100 mg/dl) in the presence of hyperoxia (FiO_2 = 0.95) results in persistent weight gain over time, indicative of formation of pulmonary edema, n = 5 per group, *P = 0.047 vs. control. (**B**) CFH increased vascular permeability as evidenced by extravasation of Evans blue–labeled albumin into bronchoalveolar lavage fluid, n = 5 per group, *P = 0.027 vs. control at 2 hours. Comparisons were made between control and CFH groups by Mann Whitney U tests.

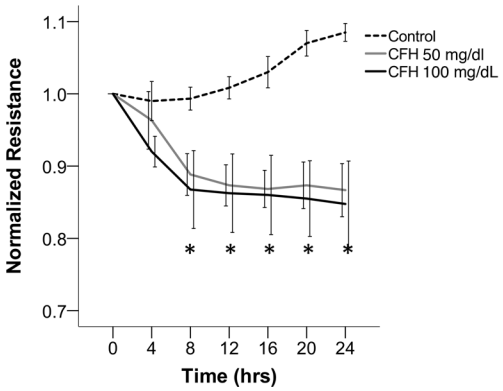

Figure 6. Cell-free hemoglobin increases pulmonary microvascular endothelial cell monolayer permeability. Cell-free hemoglobin (CFH) causes increased permeability as measured by a reduction in electrical resistance across the monolayer. Data are presented after normalization to the baseline resistance in each treatment chamber. *$P < 0.05$ vs. control by Mann Whitney *U* tests at each time point, $n = 6$ per group.

in PGD. While other studies have reported associations between circulating CFH and alterations in endothelial function in models of atherosclerosis, malaria, and sickle cell disease (38–40), there have been few prior studies of the direct effects of CFH on microvascular endothelial permeability (41). Oxidized CFH, which is present in atherosclerotic plaques, results in intercellular gap formation and increased permeability in cultured endothelial cell monolayers (42, 43). Hypoxia-responsive genes including HIF have also been implicated in hemoprotein-induced endothelial permeability in dermal microvascular endothelial cells (41). Another study showed that cross-linked CFH disrupted mesenteric permeability through actin cytoskeletal rearrangements and gap formation between intestinal endothelial cells (44). The data reported here support a potential mechanistic role for CFH in the pathogenesis of pulmonary microvascular permeability in PGD. Whether CFH impacts other known mediators of increased vascular permeability in this model, such as VEGF (24, 45, 46), angiopoeitin-2 (23), or integrin αvβ5 (47–49), will require further study.

In addition to being both a preoperative marker and a mediator of PGD, CFH can be targeted therapeutically. The rationale for a potential therapeutic effect of APAP is based on structural homology between the heme moiety of CFH and the peroxidase domain of cyclooxygenase, one of the molecular targets of APAP (35), a feature that is not present in other iron-containing proteins. APAP reduces the tyrosine radical that results from oxidation of CFH into its Fe^{4+} oxidation state (35). By this mechanism, APAP reduces the iron in the heme moiety of CFH from Fe^{4+} to Fe^{3+}, thereby diminishing the capacity for CFH to drive lipid peroxidation. The benefit of this therapeutic approach has been demonstrated in a rat model of rhabdomyolysis where APAP administration mitigated renal injury induced by release of myoglobin, another hemoprotein that can be reduced by APAP (35). In a randomized placebo-controlled clinical trial in patients with severe sepsis, APAP decreased circulating lipid peroxidation products and reduced acute kidney injury in patients with elevated plasma CFH (28). In the current study, we demonstrated that clinically relevant doses of APAP, an inexpensive, safe, and widely available drug, limited CFH-induced pulmonary edema formation and vascular permeability in both the isolated perfused human lung and cultured pulmonary microvascular endothelial cells. The association of preoperative CFH levels with PGD suggests that a treatment targeting CFH may be most effective if started prior to reperfusion. One approach would be to begin APAP administration in the lung recipient at the time of organ allocation or at the time of waitlisting. Together, these results provide compelling support for a future clinical trial of APAP for prevention or treatment of PGD.

The finding that preoperative CFH levels had a stronger association with PGD than postoperative CFH levels was unexpected and suggests that early exposure of the reperfused pulmonary vascular bed to high levels of CFH may be critical for the development of PGD. The striking relationship between high CFH, high reperfusion FiO_2, and increased risk of PGD suggests that hyperoxia at the time of reperfusion after implantation may potentiate the injurious effects of CFH. This hypothesis is strongly supported by the finding that hyperoxia augments the detrimental effects of CFH on isolated human lungs and on endothelial cells. Another therapeutic approach that may attenuate the toxicity of preexisting circulating CFH in the transplant recipient would be to limit the reperfusion FiO_2 to the lowest possible level required to achieve adequate oxygenation. In the current study, 62% of patients received a reperfusion $FiO_2 \geq 0.40$, suggesting that the majority of transplant recipients might benefit from limiting early hyperoxia.

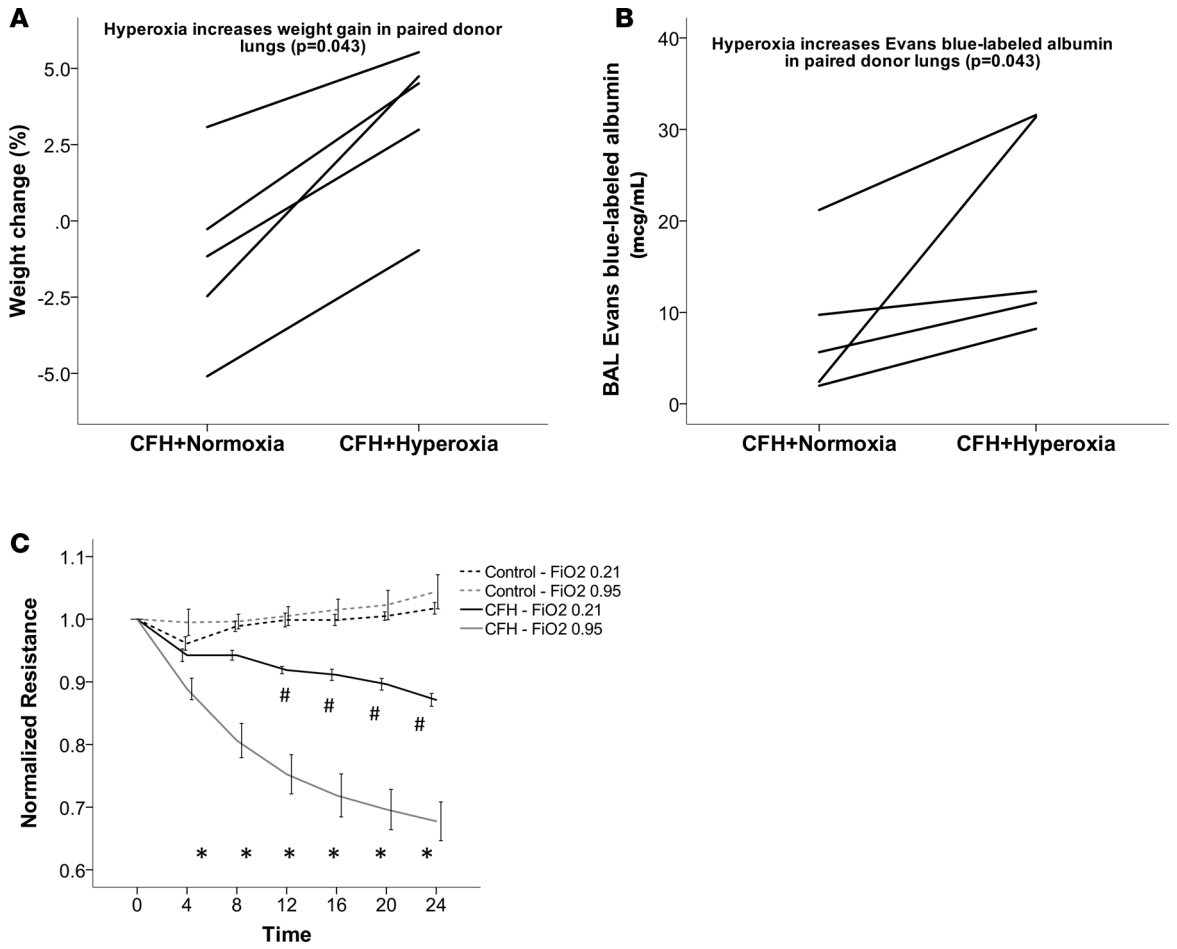

Figure 7. Hyperoxia exacerbates the toxicity of cell-free hemoglobin in the human lung and in human lung microvascular endothelial cells. (**A**) Ex vivo human isolated perfused lungs have greater weight gain over time after cell-free hemoglobin (CFH) exposure in the presence of hyperoxia. Each line connects lung weight change for paired donor lungs subjected to CFH in normoxia (FiO_2 0.21) and CFH in hyperoxia (FiO_2 0.95). n = 5, P = 0.043 by Wilcoxon rank sum testing. (**B**) Hyperoxia exacerbates extravasation of Evans blue–labeled albumin into the airspace. n = 5, P = 0.043 by Wilcoxon rank sum testing. (**C**) Hyperoxia exacerbates CFH-induced permeability of cultured human pulmonary microvascular endothelial cells. n = 8 per group, $^*P <$ 0.05 vs. control FiO_2 0.95, $^\#P < 0.05$ vs. control FiO_2 0.21 by Mann Whitney U test.

The explanation for the high preoperative levels of circulating CFH in lung transplant recipients is uncertain. One possibility is that patients with advanced obstructive or fibrotic lung disease develop elevated CFH due to chronic RBC shearing in diseased pulmonary microvasculature. Pulmonary arterial hypertension is associated with elevated CFH (33), and pulmonary hypertension due to advanced lung disease may share this association. Furthermore, chronic lung and systemic inflammation in the setting of end-stage lung disease might lead to a persistent oxidant-rich environment to trigger CFH-mediated injury, in part due to depletion of protective antioxidants. Consistent with this concept, reduced levels of the antioxidants glutathione and protein-cys-SH were reported in blood of smokers with chronic obstructive pulmonary disease (COPD) compared with smokers without COPD (50). Further studies in large patient cohorts are needed to determine the mechanisms and consequences of elevated circulating CFH in patients with advanced lung disease.

This study has some limitations. Since the experimental studies only included lungs that were declined for transplantation, it is possible that preprocurement lung injury in the donor modified the impact of CFH. However, the majority of procured donor lungs declined for transplantation had intact alveolar fluid clearance (51, 52), an indication that these donor lungs were physiologically intact. We attempted to minimize the effects of any preprocurement lung injury in 2 ways. First, to be included in the experimental protocol, each lung had to maintain a stable baseline weight for 30–60 minutes prior to the beginning of the experiment. This ensures that lungs have an intact microvascular barrier prior to the start of the experiment. Second, because of donor-

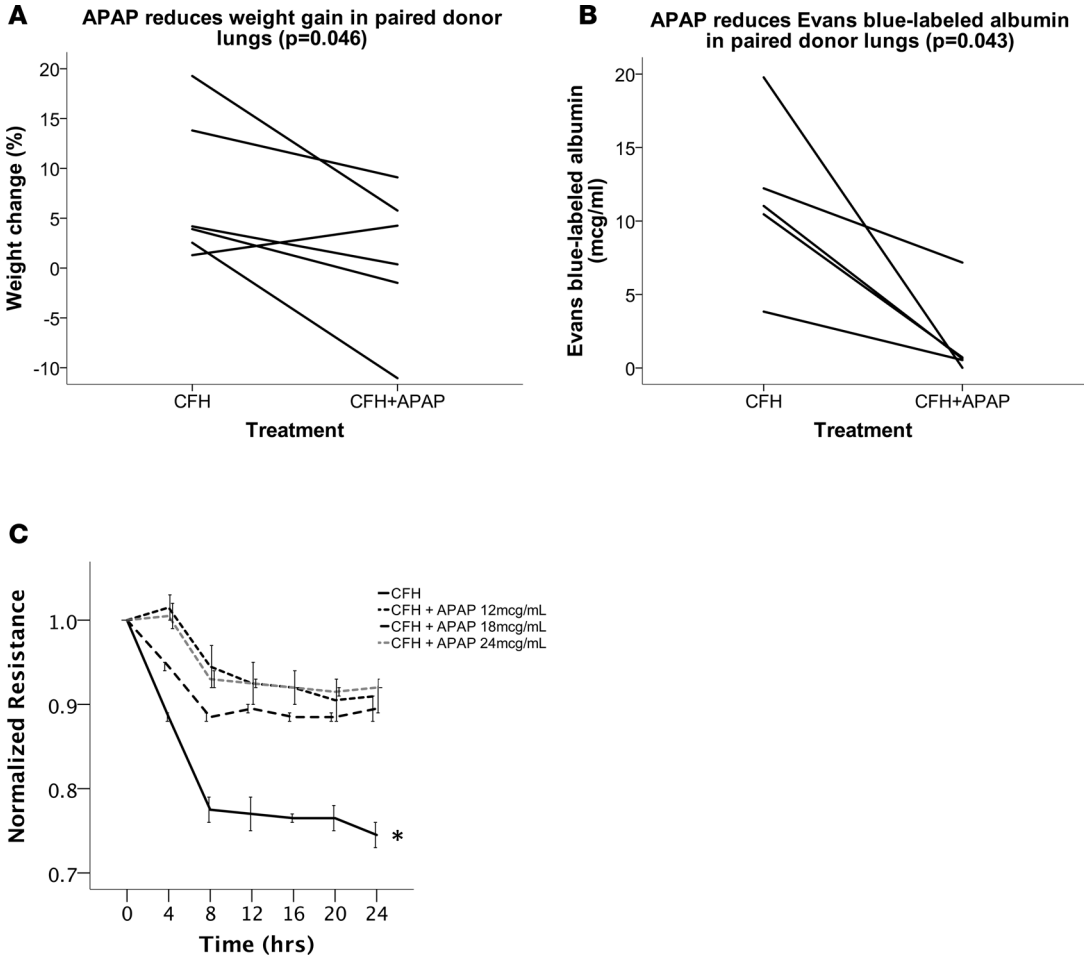

Figure 8. Acetaminophen, a specific hemoprotein reductant, attenuates microvascular permeability caused by cell-free hemoglobin. (**A**) Ex vivo human isolated perfused lungs inflated with FiO_2 0.95 have less weight gain over time after acetaminophen (APAP) therapy compared with cell-free hemoglobin (CFH) alone. Each line connects lung weight change for paired donor lungs subjected to CFH and CFH+APAP. n = 6 per group, P = 0.046 by Wilcoxon rank sum testing. (**B**) Treatment with APAP prevented extravasation of Evans blue–labeled albumin into the airspace. P = 0.043 by Wilcoxon rank sum testing. (**C**) APAP prevents CFH-induced permeability as measured by monolayer resistance in human pulmonary microvascular endothelial cells in culture. n = 2–4 per group, *P < 0.05 vs. CFH by Mann Whitney U test.

to-donor variability in responses to CFH in our initial experiments, we tested the effects of hyperoxia or APAP using paired lungs from the same donor. Another potential limitation of our study is that we restricted our observational study to patients with chronic obstructive or fibrotic lung disease as the indication for transplantation. While these diagnoses represent the majority of lung transplant recipients in the US, it is uncertain whether our findings apply to patients with other indications for lung transplantation. It is also possible that other hemoglobin-binding proteins such as haptoglobin, other cell types such as alveolar macrophages, or other antioxidant molecules may alter CFH-mediated vascular injury, and these concepts will require further study.

In summary, this translational study provides both clinical and mechanistic evidence that oxidized CFH is an important mediator of PGD and offers significant preclinical support for targeting oxidized CFH for prevention of PGD. The protective effects of APAP both in the isolated perfused human lung and in cultured hPMVECs suggest that clinical trials of perioperative APAP for prevention of PGD after lung transplantation may be warranted.

Methods

Patient population. Forty cases with grade 3 PGD within 72 hours and 80 control patients with no grade 3 PGD within 72 hours were selected from lung transplant recipients who were prospectively enrolled in the LTOG multicenter observational cohort study (9). Patients were frequency matched for underlying diagnosis and number of lungs transplanted (9). Patients in this study were primarily those recipients with

COPD or idiopathic pulmonary fibrosis (IPF), the 2 most frequent indications for lung transplantation. No patients received extracorporeal membrane oxygenation (ECMO) prior to lung transplantation. Demographic and clinical information was extracted from the LTOG database.

CFH measurement. Blood was collected into sodium citrate–containing tubes preoperatively and at 6 and 24 hours postoperatively. Samples were centrifuged and plasma collected within 2 hours of sample collection. Plasma CFH was measured by HemoCue (29). The lower limit of detection for this method is 10 mg/dl.

Isolated perfused human lung experiments. Donor lungs that were declined for transplantation were procured from donors managed by Tennessee Donor Services after consent for research was obtained from each donor's next of kin. Lungs were transported to Vanderbilt at 4°C. For the experimental preparation (Figure 4), lungs were continuously perfused at 37°C with DMEM containing 5% albumin and 16% (v/v) fresh whole human blood by roller pump to maintain a pulmonary artery pressure of 10–12 mmHg with passive drainage of perfusate through an open left atrium into a reservoir for recirculation (53, 54). Lungs were inflated to continuous positive airway pressure (CPAP) of 10 mmHg with either 95% O_2 (FiO_2 = 0.95) or 21% O_2 (FiO_2 = 0.21), and a recruitment maneuver with sustained inflation to 25 mmHg was performed prior to the experiment (53, 54). The lung was suspended from a mass transducer for continuous measurement of lung weight as an index of pulmonary edema formation. Lungs that failed to reach a stable weight over 30–60 minutes after rewarming were discarded. A baseline bronchoalveolar lavage (BAL) was performed with 40 ml normal saline. Then, purified CFH (100 mg/dl, Cell Sciences) or CFH+APAP (15 μg/ml APAP, MilliporeSigma) was added to the perfusate. After 2 hours, Evans blue–labeled albumin (0.5% in H_2O, MilliporeSigma) was added to the perfusate (15 ml) and repeat BAL was performed. Weights are expressed as percent change from baseline. The concentration of Evans blue–labeled albumin in BAL fluid (BALF) was measured spectrophotometrically at 620 nm.

Endothelial permeability assessment. hPMVECs (55) were grown to confluence on 8W10E+ PC electrode arrays (Applied BioPhysics) previously coated with 10 μM cysteine and 1% gelatin. Cells were treated with vehicle control, purified endotoxin-free CFH (0.25–1.0 mg/dl), or CFH+APAP (12-24 μg/ml) for 24 hours in tissue culture medium (MCDB131 lacking L-glutamine [Thermo Fisher Scientific], supplemented with 10 ng/ml epidermal growth factor [MilliporeSigma], 1 μg/ml hydrocortisone [MilliporeSigma], 10 mM glutamine [MilliporeSigma], and 10% FBS [Thermo Fisher Scientific]). Room air (FiO_2 = 0.21) or oxygen (FiO_2 = 0.95) was bubbled through the media prior to incubation at 37°C. Paracellular permeability was measured by monitoring monolayer resistance at 4,000 Hz using electrical cell-substrate impedance sensing (ECIS) (Applied BioPhysics). Data were normalized to the baseline resistance of each chamber prior to addition of treatments. Experiments were performed with n = 2–3 per group on at least 2 separate days, and representative data are shown.

Statistics. Continuous variables and categorical variables were compared between patient groups using Mann Whitney U or χ^2 testing, respectively. Line graphs depict the mean value at each time point with errors bars indicating ± SEM. Box plots depict the median as a dark horizontal line, the upper and lower edges of the box show the 75% and 25% quartiles, respectively, and the whiskers are ×1.5 the IQR. Multivariable logistic regression models were used to determine the effects of confounding variables over the impact of preoperative CFH on risk of PGD grade 3 at 72 hours. The multivariable analysis individually included each variable with $P < 0.2$ for an association with PGD in univariate analysis. For this analysis, preoperative CFH was entered as a categorical variable based on the median value of < 30 mg/dl or ≥ 30 mg/dl. To assess the contribution of reperfusion FiO_2 to the association between CFH and PGD, the cohort was divided into those with low (<0.40) or high (≥0.40) reperfusion FiO_2, and a multivariable regression was performed in each group. Lung weight gain and change in EBD concentration over 2 hours was performed by Mann Whitney U testing comparing CFH with control. Because of the presence of lung donor–specific effects, CFH in normoxia vs. hyperoxia and CFH vs. CFH+APAP were compared using paired lungs, and analysis was controlled for donor identification by using Wilcoxon signed rank testing. In vitro treatments of cultured endothelial cells were compared by Mann Whitney U testing. $P < 0.05$ was considered significant. Statistical analysis was performed using SPSS version 24 (IBM).

Study approval. The study was approved by the Vanderbilt University Medical Center IRB (protocol number 141499). Patients provided informed consent at the time of enrollment into the LTOG study, a multicenter prospective cohort study of patients undergoing lung transplantation designed to evaluate predictors of PGD (5).

Author contributions

CMS, JAB, and LBW conceived and designed the study, performed data analysis, and wrote the manuscript. NW, JBM, HN, and AM performed the ex vivo isolated perfused human lung experiments. SRL and JLK performed cultured endothelial cell experiments. JMD and JDC provided clinical information from the LTOG cohort and assisted with manuscript preparation. DJL, SMK, SMP, KMW, AW, VNL, MMC, JBO, PDS, CAH, EC, MKP, GD, and JM are primary investigators in the LTOG cohort study. All authors read and approved the final manuscript.

Acknowledgments

These studies were funded by NIH HL135849 (LBW, JAB), HL103836 (LBW), HL087115 (JDC), HL126176 (LBW), HL126671 (JAB), HL117676 (JAB), HL121406 (JMD), HL136888 (CMS), Vanderbilt Faculty Research Scholars (CMS), American Thoracic Society (CMS), and the Parker B. Francis Family Foundation (CMS). We would like to thank Tennessee Donor Services for their invaluable assistance in obtaining donor lungs for research. We thank the donor families for their gift to research.

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