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RESEARCH ARTICLE

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Transpulmonary generation of cell-free hemoglobin contributes to vascular dysfunction in pulmonary arterial hypertension via dysregulated clearance mechanisms

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Abstract

Circulating cell-free hemoglobin (CFH) is elevated in pulmonary arterial hypertension (PAH) and associated with poor outcomes but the mechanisms are unknown. We hypothesized that CFH is generated from the pulmonary circulation and inadequately cleared in PAH. Transpulmonary CFH (difference between wedge and pulmonary artery positions) and lung hemoglobin α were analyzed in patients with PAH and healthy controls. Haptoglobin genotype and plasma hemoglobin processing proteins were analyzed in patients with PAH, unaffected bone morphogenetic protein receptor type II mutation carriers (UMCs), and control subjects. Transpulmonary CFH was increased in patients with PAH ($p = 0.04$) and correlated with pulmonary vascular resistanc (PVR) ($r_s = 0.75$, $p = 0.02$) and mean pulmonary arterial pressure (mPAP) ($r_s = 0.78$, $p = 0.02$). Pulmonary vascular hemoglobin α protein was increased in patients with PAH ($p = 0.006$), especially in occluded vessels ($p = 0.04$). Haptoglobin genotype did not differ between groups. Plasma haptoglobin was higher in UMCs compared with both control subjects ($p = 0.03$) and patients with HPAH ($p < 0.0001$); patients with IPAH had higher circulating haptoglobin levels than patients with HPAH ($p = 0.006$). Notably, circulating CFH to haptoglobin ratio was elevated in patients with

HPAH compared to control subjects ($p = 0.02$) and UMCs ($p = 0.006$). Moreover, in patients with PAH, CFH: haptoglobin correlated with PVR ($r_s = 0.37$, $p = 0.0004$) and mPAP ($r_s = 0.25$, $p = 0.02$). Broad alterations in other plasma hemoglobin processing proteins (hemopexin, heme oxygenase-1, and sCD163) were observed. In conclusion, pulmonary vascular CFH is associated with increased PVR and mPAP in PAH and dysregulated CFH clearance may contribute to PAH pathology. Further study is needed to determine whether targeting CFH is a viable therapeutic for pulmonary vascular dysfunction in PAH.

KEYWORDS
cell-free hemoglobin, pulmonary arterial hypertension, vascular dysfunction

INTRODUCTION

Disrupted nitric oxide (NO) signaling is a key feature of pulmonary arterial hypertension (PAH). Reduced NO synthase and decreased NO bioavailability during PAH contribute to vasoconstriction, smooth muscle cell proliferation, inflammation, and thrombosis.[1] Insight into the mechanisms of impaired NO signaling may lead to new therapeutic targets that are not redundant with current strategies. Cell-free hemoglobin (CFH), which is released from red blood cells during hemolytic or inflammatory pathologies, is a potent NO scavenger known to impair vasodilation and contribute to endothelial injury.[2–7] Elevated levels of CFH are observed in several conditions with vascular dysfunction including sickle cell disease,[4,8] sepsis,[9,10] primary graft dysfunction,[11] and acute respiratory distress syndrome,[12] and are associated with poor outcomes. Patients with these conditions and animal models of CFH infusion share pathological features with PAH.[13]

We previously demonstrated that patients with PAH and unaffected carriers of a mutation in the bone morphogenetic protein receptor type II (BMPR2) gene (UMCs) have elevated levels of circulating CFH compared with healthy control subjects and patients with pulmonary venous hypertension (PVH). Moreover, elevated CFH levels were associated with higher pulmonary vascular resistance and risk for hospitalization.[14] Based on these observations, we sought to interrogate potential mechanisms by which CFH levels become elevated in PAH and contribute to PAH pathology.

There are several potential mechanisms for systemically elevated CFH levels, including lysis of red blood cells or reduced levels of CFH-processing proteins such as haptoglobin (endogenous scavenger of hemoglobin), hemopexin (endogenous scavenger of the heme moiety released from hemoglobin), or CD163 (receptor for hemoglobin-haptoglobin complexes). Given the associations between elevated CFH and the progression of PAH pathology in animal models and patients,[13,15,16] we hypothesized that remodeling in the pulmonary microcirculation in PAH may be a source of red blood cell lysis. Therefore, in this study, we specifically measured CFH generated from the pulmonary circulation by calculating the transpulmonary CFH gradient (difference between blood sampled in the pulmonary wedge and pulmonary artery positions). Additionally, previous studies have demonstrated that patients at risk for PAH may have abnormalities in hemoglobin-processing proteins.[17,18] Taken together, we hypothesized that both red blood cell lysis and dysregulated CFH processing contribute to elevated levels of CFH generated from the pulmonary circulation and contribute to PAH pathology.

METHODS

Human subjects and clinical data

This study was approved by the Vanderbilt University Institutional Review Board (IRB #9401 and #140983) and all subjects gave written informed consent. PAH patients for this study were consecutively enrolled in the Vanderbilt Pulmonary Hypertension Research Cohort (VPHRC), a prospective institutional registry containing detailed clinical information and biologic specimens collected over 30 years.[19] Patients with PAH were diagnosed by experienced clinicians according to consensus guidelines.[20] PAH was defined according to contemporary guidelines at the time the study commenced as an invasively measured mean pulmonary arterial pressure (mPAP) ≥25 mmHg, pulmonary wedge pressure (PWP), or left ventricular end diastolic pressure ≤15 mmHg and pulmonary vascular resistance (PVR) >3 Wood units.[20] PAH etiologies were idiopathic (IPAH) or

heritable (HPAH). Concomitantly, we studied several additional populations to maximize insight into CFH biology in PAH. We collected specimens from BMPR2 UMCs (either obligate based on family pedigree or confirmed by genotyping). We enrolled selected consecutive patients with PVH to determine whether differences in CFH processing proteins are specific to PAH (i.e., pulmonary vascular disease) or a nonspecific feature of general pulmonary hypertension (PH). Inclusion in this group required mPAP >25 mmHg, PWP >15 mmHg, and PVR <3 Wood units measured on right heart catheterization (RHC) on the date of transpulmonary blood draws. Finally, we also studied healthy control subjects with no known cardiovascular disease and no cardiopulmonary signs or symptoms enrolled in the VPHRC. Additionally, for haptoglobin genotyping, we used existing HP genotyping data from Vanderbilt's deidentified DNA biobank (BioVU) to determine whether the distribution of HP genotypes varies between PAH and a large clinical population.[21]

In subjects with PH, RHC was performed with a balloon tipped catheter using hemodynamic and fluoroscopic guidance. Heart rate, right atrial pressure, PAP, PWP, and cardiac output were recorded from the RHC closest to the date of blood draw. Cardiac index, pulmonary vascular resistance, and stroke volume were calculated using standard formulas. A subset of subjects underwent transpulmonary blood sampling to determine whether CFH is generated or consumed in the pulmonary vasculature. Influent blood was sampled from the main pulmonary artery with location determined by stereotypical waveform. Effluent blood was sampled from the wedge position with the balloon inflated. Wedge location was determined by stereotypical waveform and oxygen saturation >95%. The transpulmonary gradient of CFH was defined as the wedge value minus the pulmonary artery value. A positive value indicates CFH increase across the pulmonary circulation and a negative value indicates consumption.

Immunostaining

Localization and staining intensity of hemoglobin α were assessed in human lung tissue sections using goat polyclonal antibody SC-31109 (Santa Cruz). Human lung tissue paraffin blocks were acquired through the Pulmonary Hypertension Breakthrough Initiative (IRB #060203). Specimens were explanted PAH lungs from patients undergoing lung transplant, or failed donor controls ($n = 4$ control patients & $n = 7$ PAH patients). Blocks were cut onto slides at 10 μm and slides were deparaffinized. Epitope retrieval was carried out in Tris EDTA buffer with heating in a pressure cooker at 99°C for 20 min. Slides were blocked with normal serum and primary antibody was added at a 1:50 dilution in 1% BSA overnight at 4°C. After washing in 1X TBST the following day, a fluorescently conjugated secondary antibody (Alexa Fluor A11058) was added to the sections at 1:800 in 1X TBS and incubated for 1 h. Slides were then washed and mounted with DAPI mounting medium (Vector Labs; H1200) to visualize nuclei. Imaging was carried out using a Nikon Eclipse Ti Series confocal microscope at X10 for quantification and X40 for assessment of localization. Hemoglobin α was visualized in red at 595 nm. All images were acquired in the same day, under the same conditions, and were subjected to the same exposure time. Staining intensity was analyzed within ROIs (pulmonary arterioles) using NIS Elements AR 4.11.00 64-bit software. A varying number of vessels were counted and averaged for each patient. Data are reported as sum intensity (a measure of all positive pixels) divided by total area of ROI. Vessel lumen is excluded from ROI area.

Blood collection and analysis

Blood (ethylenediaminetetracetic acid [EDTA]) was collected either from central venous access catheters, pulmonary arterial catheters, or via venipuncture. EDTA tubes were centrifuged within 45 min of collection at 4000 rpm and the plasma fraction immediately aliquoted as 200 μl aliquots and stored at −80°C.

Enzyme-linked immunosorbent assays (ELISA)

Plasma concentrations of CFH (Quantichrom; DIHB-250), haptoglobin (Abcam; ab108856), hemopexin (Abcam; ab108859), heme oxygenase-1 (Enzo Life Sciences; ADI-EKS-800), and sCD163 (R&D Systems; DC1630) were assessed by ELISA. All samples were run in duplicate, and replicate readings were averaged for each sample with background subtraction. Absorbance values were obtained using Bio Rad iMark microplate reader with Bio Rad Microplate Manager 6 software.

Haptoglobin genotyping

Direct haptoglobin genotyping (HP1-1, HP1-2, or HP2-2) was performed using an established TaqMan-based real-time PCR method.[22,23] To overcome the limited sample size of healthy control subjects in our PAH cohort, we used existing HP genotyping data from Vanderbilt's deidentified DNA biobank (BioVU) to determine whether the distribution of HP genotypes varies between PAH and a large clinical

population.[21] All patients were genotyped on the Illumina Infinium Expanded Multi-Ethnic Genotyping Array (MEGA^{EX}) platform as part of various institutional and investigator-initiated projects. Genotyping quality control measures have been previously described[24]: we excluded single-nucleotide polymorphisms (SNPs) with missingness rate >0.05, minor allele frequency <0.005, and deviation from Hardy–Weinberg equilibrium with a $p \leq 1 \times 10.^{-6}$ Only directly genotyped variants (not imputed variants) were included. We extracted SNPs within a two megabase region surrounding the *HP* gene (Hg19 chr16:71,036,975–73,063,764), then imputed *HP* genotype using a phased reference panel provided by Boettger et al.[25] The reference panel consisted of 274 unrelated individuals of European ancestry from the 1000 Genomes Project[26] and HapMap3 project[27] who underwent genotyping on several array-based platforms as well as droplet-digital PCR *HP* genotyping.[25] We used Genotype Harmonizer version 1.4.20 for strand alignment,[28] SHAPEIT for haplotype estimation (prephasing) using a window size of 0.1 megabases,[29] and IMPUTE2 for genotype imputation.[30] We used the calculated genotype posterior-probabilities for number of *HP2* alleles as the surrogate for *HP* genotype, with a *HP2* genotype probability of 0.0–0.5 corresponding to a predicted *HP* genotype of *HP1-1*, a genotype probability of 0.5–1.5 corresponding to *HP2-1*, and a genotype probability of 1.5–2.0 corresponding to *HP2-2*. We have previously demonstrated the accuracy of this method compared to *HP* genotyping by real-time PCR with a balanced accuracy of 0.91 (95% confidence interval: 0.84–0.95) and Cohen's κ of 0.85.[10]

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.0.0. Differences between two groups were calculated via Mann–Whitney test and among more than two groups via Kruskal–Wallis with Dunn's multiple comparisons and are represented as median with interquartile range (IQR). Correlation coefficients were calculated via Spearman's method and represented as XY scatter plot. Frequency distribution was calculated with χ^2 test.

RESULTS

Study population

We studied 82 patients with IPAH, 39 patients with HPAH, 24 UMCs, and 41 healthy control subjects (Table 1). Patients with IPAH or HPAH were predominantly female, with a median age of 48 years (IQR: 36–60), and median New York Heart Association class of 2.3 (IQR: 2.0–3.0). The study population had a median PVR of 9.0 WU (IQR: 6.0–11.9), mean PA pressure of 52 mmHg (IQR: 42.0–59.0), and PWP of 9.0 mmHg (IQR: 6.0–12.0). None of the IPAH or HPAH patients met criteria for vasodilator responsiveness with NO inhalation.

TABLE 1 Demographics and clinical data

Variable	IPAH ($n = 82$)	HPAH ($n = 39$)	Control ($n = 41$)	UMC ($n = 24$)
Female sex, no. (%)	63 (77)	27 (73)	32 (78)	12 (71)
Age (year)	50 (15)	41 (13)	36 (11)	47 (16)
NYHA	2.4 (0.8)	2.3 (1.0)	-	-
6MWD (m)	324 (124)	397 (67)	-	-
BNP (pg/ml)	179 (183)	390 (532)	-	-
Creatinine (mg/dl)	1.0 (0.3)	0.9 (0.2)	-	-
Hemoglobin (mg/dl)	14 (2)	14 (2)	-	-
Hemodynamics				
Heart rate (bpm)	79 (17)	76 (17)	-	-
RAP (mmHg)	10 (12)	7.5 (5.3)	-	-
Systolic PAP (mmHg)	77 (21)	89 (17)	-	-
mPAP (mmHg)	49 (12)	57 (11)	-	-
PWP (mmHg)	10 (5)	9 (4)	-	-
Cardiac index (L/min/m)^2	2.7 (0.9)	2.6 (1.1)	-	-
PVR (Wood units)	8.9 (4.6)	11.5 (5.7)	-	-
M_{VO2} (%)	65 (10)	64 (9)	-	-
Δ mPAP	5.5 (5.9)	2.5 (4.8)	-	-
Δ PVR	1.0 (2.8)	0.1 (2.1)	-	-

Note: Data shown as mean (SD) except where indicated. Δ values indicate baseline minus postnitric oxide inhalation.

Abbreviations: 6MWD, 6 min walk distance; BNP, brain natriuretic peptide; mPAP, mean pulmonary arterial pressure; mPVR, mean pulmonary vascular resistance; M_{vO2}, mixed venous oxygen saturation; N/A, not available; NYHA, New York Heart Association; PAH, pulmonary arterial hypertension; PAP, pulmonary artery pressure; PVH, pulmonary venous hypertension; PVR, pulmonary vascular resistance; PWP, pulmonary wedge pressure; RAP, right atrial pressure; UMCs, unaffected carrier of bone morphogenetic protein receptor type II mutation.

Elevated levels of CFH arise from the pulmonary circulation and are associated with pulmonary vascular resistance and mPAP

We previously found that patients with PAH have increased circulating CFH compared to patients with PVH and healthy control subjects[14]; here we

hypothesized that CFH is generated specifically from the pulmonary circulation during PAH. Therefore, we measured transpulmonary CFH (the difference between wedge and PA positions) in patients with PAH (Table 2) and found a median increase of 5.5 mg/dl ($n = 12$, [IQR: −0.7 to 15.5]) in CFH levels, compared to a decrease of 3.4 mg/dl ($n = 9$, [IQR: −8.1 to 4.7]) in patients with PVH ($p = 0.04$; Figure 1a). There was no difference in baseline pulmonary artery CFH levels in patients with PAH (31 mg/dl, [IQR: 23−39]) compared to patients with PVH (29 mg/dl, [IQR: 25−46]; $p = 0.8$). In PAH patients that specifically displayed *increased* transpulmonary generation of CFH ($n = 9$), the transpulmonary CFH gradient correlated with pulmonary vascular resistance (PVR; $r_s = 0.75$, $p = 0.02$; Figure 1b) and mPAP ($r_s = 0.78$, $p = 0.02$; Figure 1c).

We confirmed increased CFH in the pulmonary microcirculation in patients with PAH by immunofluorescence staining of hemoglobin α in explanted lung tissue. Hemoglobin α in pulmonary arterioles was prominent in patients with PAH ($n = 7$, $p = 0.006$; Figure 2a,b), especially in occluded vessels (areas which are more conducive to hemolysis) compared to patent vessels ($n = 6$, $p = 0.04$; Figure 2c,d).

Patients with PAH display defects in CFH-processing proteins

Haptoglobin, the endogenous scavenger of hemoglobin, exists in three different genotypes in humans (HP1-1, HP1-2, and HP2-2). HP2-2 genotype is associated with less efficient clearance of hemoglobin from the circulation and is associated with increased hemoglobin-mediated oxidation and risk of cardiovascular disease.[31] Thus, we hypothesized that patients with PAH might have a higher prevalence of the HP2-2 genotype than control subjects. To ensure that the controls used in this analysis reflected the typical distribution of haptoglobin genotype across a robust population of healthy controls, we used genotype data from a large cohort of healthy control subjects from the Vanderbilt BioVU deidentified DNA biobank database (Table 3). However, we did not find a significant difference in the distribution of haptoglobin genotypes across control subjects ($n = 64{,}843$), patients with IPAH ($n = 171$), HPAH ($n = 129$), or UMCs ($n = 59$; Figure 3a).

Due to the lack of difference in haptoglobin genotypes across groups, we hypothesized that CFH clearance by haptoglobin may be dysregulated during PAH. To test this hypothesis, we investigated levels of circulating haptoglobin in patients with PAH. Similar to other disorders of chronic hemolysis such as sickle cell anemia, levels of haptoglobin are expected to decrease with increasing levels of CFH. Although patients with IPAH ($n = 69$, 1230 μg/ml [IQR: 476−1997]) had haptoglobin levels similar to control subjects ($n = 39$, 976 μg/ml [IQR: 542−1430]), patients with HPAH ($n = 31$, 552 μg/ml [IQR: 160−1090]) had significantly lower haptoglobin levels ($p = 0.006$). Interestingly, UMCs ($n = 24$, 1580 μg/ml [IQR: 1216−2532]) demonstrated significantly elevated haptoglobin levels compared to control subjects ($p = 0.03$) and haptoglobin levels in UMCs were more than double the levels observed in patients with HPAH ($p < 0.0001$; Figure 3b). Notably, the ratio of CFH to haptoglobin was significantly elevated in patients with HPAH ($n = 33$, 0.15 [IQR: 0.05−0.51]) compared to UMCs ($n = 24$, 0.05 [IQR: 0.03−0.08], $p = 0.006$) or control subjects ($n = 40$, 0.05 [IQR: 0.03−0.18], $p = 0.02$; Figure 3c). Moreover, in patients with PAH, CFH to haptoglobin ratio is correlated with PVR ($n = 100$, $r_s = 0.37$, $p = 0.0004$; Figure 3d) and mPAP ($n = 105$, $r_s = 0.25$, $p = 0.02$; Figure 3e).

Similar to haptoglobin, we found that levels of hemopexin, the endogenous scavenger of the heme moiety that can be released from hemoglobin and is known to contribute to cellular and tissue injury, were elevated in patients with IPAH ($n = 58$, 793 μg/ml [IQR: 562−1034], $p = 0.0001$) and UMCs ($n = 23$, 814 μg/ml [IQR: 498−991], $p = 0.046$) compared to control subjects ($n = 38$, 500 μg/ml [IQR: 406−625]), but levels in patients with HPAH were significantly lower ($n = 34$, 576 μg/ml [IQR: 400−798], $p = 0.048$; Figure 4a). We did not observe significant differences in circulating levels of heme oxygenase-1, the

TABLE 2 Characteristic information for patients that underwent RHC with transpulmonary CFH measurements

Variable	PAH ($N = 12$)	PVH ($N = 9$)
Age (years)	57 (14)	63 (11)
Sex, *n* (%) women	8 (89%)	9 (75%)
BMI (kg/m)^2	31.5 (8.2)	36.3 (9.9)
Mean PAP (mmHg)	47.2 (16.0)	34.1 (12.7)
PCWP (mmHg)	12.6 (5.0)	17.4 (9.6)
PVR (Wood units)	8.3 (4.7)	2.9 (2.2)
Etiology	Idiopathic: 9	
	Heritable: 1	
	Connective tissue: 2	

Note: Data shown as mean (SD) except where indicated.

Abbreviations: BMI, body mass index; CFH, cell-free hemoglobin; PAH, pulmonary arterial hypertension; PAP, pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; PVH, pulmonary venous hypertension; PVR, pulmonary vascular resistance; RHC, right heart catheterization.

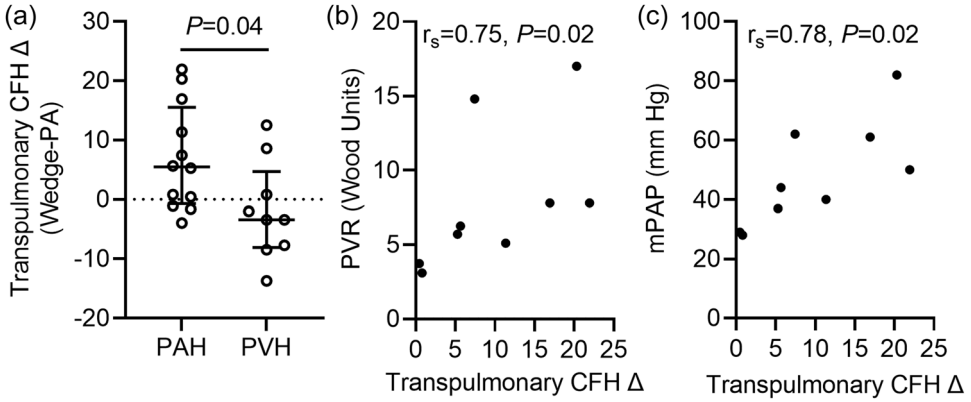

FIGURE 1 Effects of increased transpulmonary CFH on pulmonary vascular resistance and mean pulmonary arterial pressure in patients with PAH. (a) Transpulmonary CFH gradient (Wedge-PA) was higher in patients with PAH ($n = 12$) than those with PVH ($n = 9$) by Mann−Whitney analysis ($p = 0.04$). Graph represents median ± IQR. Transpulmonary CFH gradient correlated with (b), PVR ($n = 9$, $r_s = 0.75$, $p = 0.02$) and (c), mPAP ($n = 9$, $r_s = 0.78$, $p = 0.02$) by Spearman's correlation. CFH, cell-free hemoglobin; IQR, interquartile range; mPAP, mean pulmonary arterial pressure; PAH, pulmonary arterial hypertension; PVH, pulmonary venous hypertension.

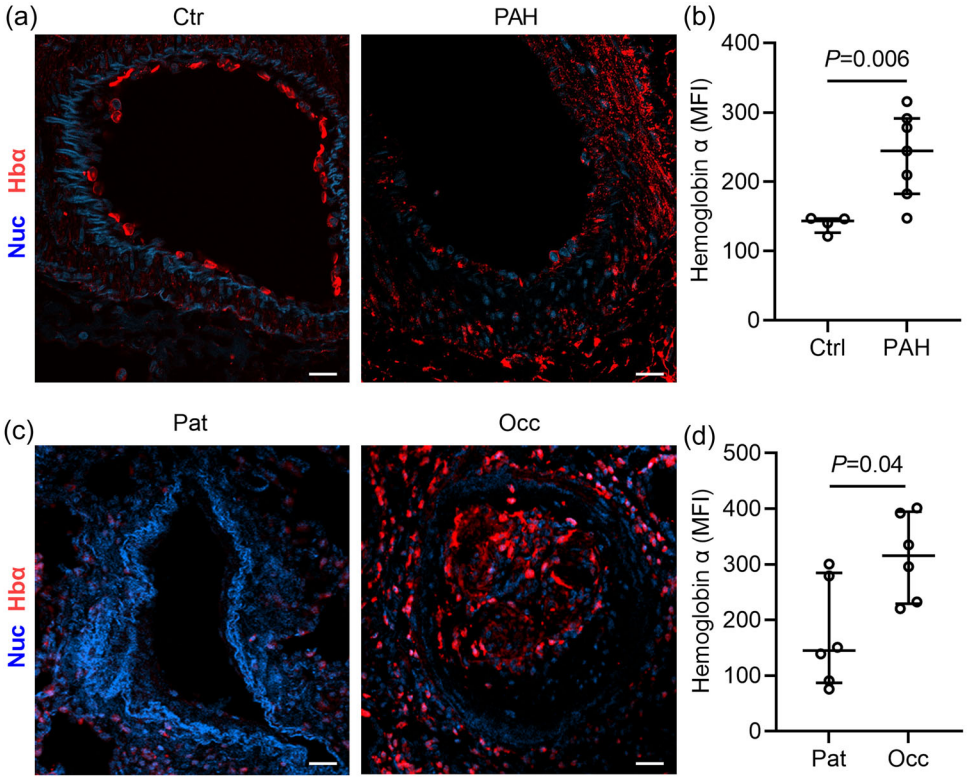

FIGURE 2 Hemoglobin α staining in explanted lung tissue from patients with PAH. (a) Representative images (X40, scale bar = 25 μm; blue = nuclei, red = hemoglobin α) and (b), quantification shows increased hemoglobin α staining in explanted lung tissue of patients with PAH ($n = 7$) compared to control subjects ($n = 4$) by Mann−Whitney analysis ($p = 0.006$). Graph represents median ± IQR. (c) Representative images (X40, scale bar = 25 μm; blue = nuclei, red = hemoglobin α) and (d), quantification shows increased hemoglobin α staining in occluded vessels ($n = 6$) versus patent vessels ($n = 6$) by Mann−Whitney analysis ($p = 0.04$). Graph represents median ± IQR. IQR, interquartile range; PAH, pulmonary arterial hypertension.

rate-limiting enzyme in the catabolism of heme, across groups (Figure 4b). However, we did find that circulating levels of sCD163, the soluble form of the hemoglobin-haptoglobin receptor on macrophages that is shed from the membrane during inflammatory macrophage activation,[32] were significantly elevated in patients with IPAH ($n = 72$, 549 ng/ml [IQR: 420−769]) compared to control subjects ($n = 40$, 417 ng/ml [IQR: 291−838], $p = 0.003$) or UMCs

($n = 24$, 361 ng/ml [IQR: 274–514], $p = 0.001$; Figure 4c). However, none of these parameters were significantly associated with higher PVR or mPAP (Supporting Information: Figure 1).

TABLE 3 Demographic data for BioVU control population

Variable	BioVU control subjects ($n = 64,843$)
Female sex, no (%)	36,073 (55.6)
Age, median year (IQR)	62 (42–75)
HP2 allele frequency	
0	9405 (14.5)
1	30,436 (46.9)
2	25,002 (38.6)

Abbreviation: IQR, interquartile range.

DISCUSSION

The purpose of this study was to investigate possible mechanisms that may explain our previous finding of elevated CFH in patients with PAH.[14] We examined the potential for in situ generation of CFH within the remodeled pulmonary vasculature, genetic susceptibility, and abnormal protein processing. In summary, our data show that patients with PAH have increased transpulmonary CFH that correlates with higher pulmonary vascular resistance and mPAP. Hemoglobin α immunostaining in explanted lung tissue from patients with PAH is localized to areas of occlusion in pulmonary arterioles. We found no differences in haptoglobin genotype frequency across study groups, but haptoglobin levels were increased in UMCs and in patients with IPAH compared to those with HPAH. Notably, the ratio of

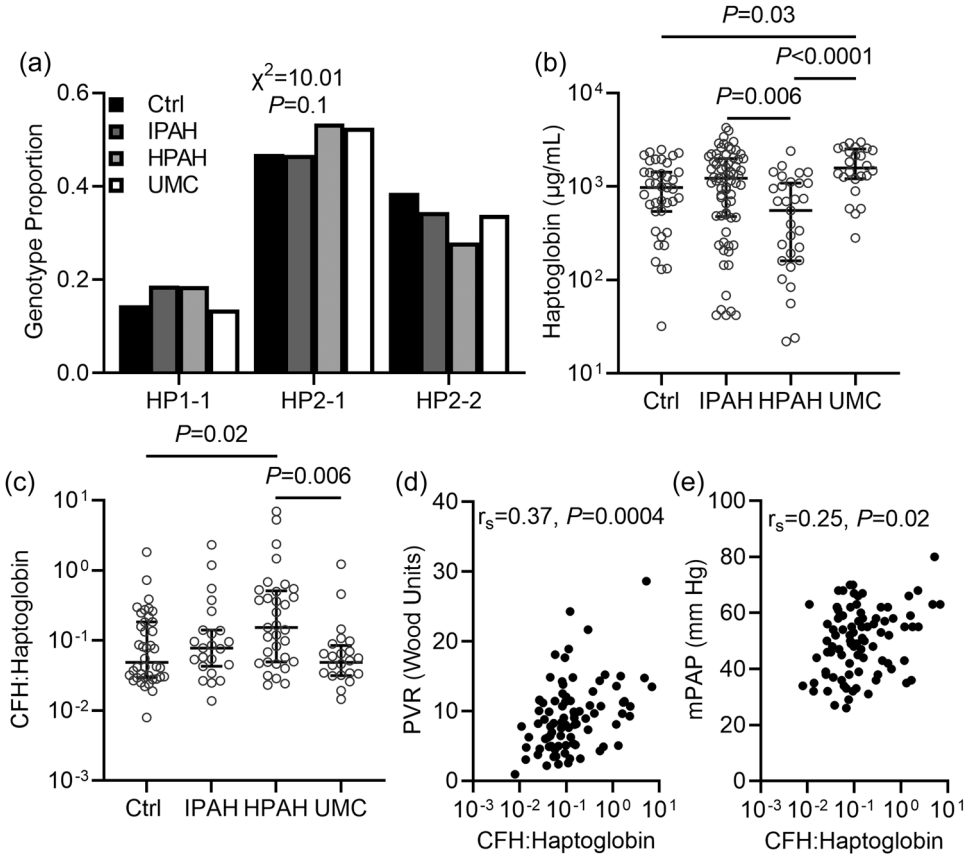

FIGURE 3 Haptoglobin genotype and circulating haptoglobin levels in patients with PAH. (a) Frequency distribution of haptoglobin genotype did not differ between control populations ($n = 64,843$), patients with IPAH ($n = 171$) or HPAH ($n = 129$), or UMCs ($n = 59$) by χ^2 analysis ($p = 0.1$). (b) Circulating levels of haptoglobin are increased in UMCs ($n = 24$) compared to control subjects ($n = 39$, $p = 0.03$) or those with HPAH ($n = 31$, $p < 0.0001$) and increased in patients with IPAH ($n = 69$) compared to those with HPAH ($p = 0.006$) by Kruskal–Wallis analysis with Dunn's post hoc multiple comparisons. Graph represents median ± IQR. (c) Ratio of circulating CFH to haptoglobin is significantly elevated in patients with HPAH ($n = 33$) compared to control subjects ($n = 40$, $p = 0.02$) or UMCs ($n = 24$, $p = 0.006$) by Kruskal–Wallis analysis with Dunn's post hoc multiple comparisons. Graph represents median ± IQR. In patients with PAH, CFH: haptoglobin is positively correlated with higher (d), PVR ($n = 100$, $r_s = 0.37$, $p = 0.0004$) and (e), mPAP ($n = 105$, $r_s = 0.25$, $p = 0.02$) by Spearman's correlation. CFH, cell-free hemoglobin; IQR, interquartile range; mPAP, mean pulmonary arterial pressure; PAH, pulmonary arterial hypertension; UMCs, unaffected carrier of bone morphogenetic protein receptor type II mutation.

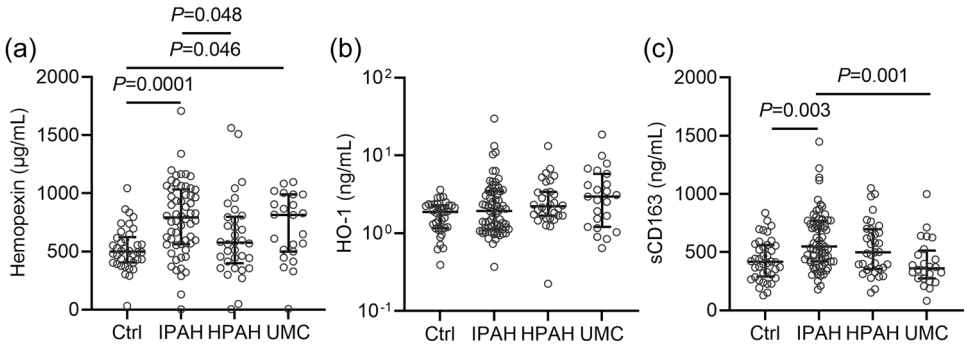

FIGURE 4 Circulating levels of hemopexin, HO-1, and sCD163 in patients with PAH. (a) Circulating levels of hemopexin were elevated in patients with IPAH ($n = 66$, $p = 0.0001$) and UMCs ($n = 24$, $p = 0.046$) compared to control subjects ($n = 38$), but levels in patients with HPAH were significantly lower ($n = 34$, $p = 0.048$). (b) There were no significant differences in circulating HO-1 across groups. (c) Circulating levels of sCD163 were significantly elevated in patients with IPAH ($n = 72$) compared to control subjects ($n = 40$, $p = 0.003$) or UMCs ($n = 24$, $p = 0.001$). PAH, pulmonary arterial hypertension; UMCs, unaffected carrier of bone morphogenetic protein receptor type II mutation.

circulating CFH to haptoglobin was significantly higher in patients with HPAH and was associated with pulmonary vascular resistance and mPAP. Circulating levels of hemopexin were elevated in patients with IPAH and UMCs, but not in patients with HPAH, circulating levels of HO-1 did not differ between groups, and circulating levels of sCD163 were elevated in patients with IPAH. However, no association was found between circulating levels of hemopexin, HO-1, or sCD163 with PVR or mPAP. These data indicate that CFH generated from the pulmonary circulation may contribute to PAH pathology via dysregulated hemoglobin clearance mechanisms, particularly in patients with HPAH.

We and others have shown increased plasma levels of CFH in patients with PAH that correlate with worse cardiopulmonary hemodynamics and associate with functional class and hospitalization risk.[14,33] Increased levels of circulating CFH have also been shown in the SU/hypoxia rat model of PAH and correlate with disease severity.[33,34] Moreover, populations at risk for developing pulmonary hypertension have abnormalities in hemoglobin processing proteins.[17,18] Hemoglobin infusion in animal models leads to pulmonary vascular injury which is mitigated by concomitant infusion of the CFH scavenger haptoglobin.[13,15,16,35,36] Collectively, these studies suggest CFH generation and incomplete metabolism are common features of pulmonary vascular dysfunction that may be modifiable. However, the mechanisms underlying elevated CFH in human PAH have not been elucidated.

In this study, we examined CFH metabolism using multisite plasma samples, human lung tissue, and genotyping data from patients with PAH as well as healthy controls and a population that is at genetic risk of PAH. We show that the transpulmonary gradient of CFH is increased in patients with PAH compared to patients with other forms of PH in the absence of pulmonary vascular disease (e.g., PVH), suggesting that CFH is generated specifically in the pulmonary circulation in PAH. Fluorescent staining of explanted lung tissue revealed increased levels of hemoglobin α in patients with PAH that were prominent in occluded vessels. These findings lend support to the idea that vascular remodeling in the pulmonary circulation and the development of vascular occlusions creates an environment conducive to shear-induced hemolysis leading to hemoglobin liberation.[37] Together, these data suggest that red blood cell damage leading to CFH generation in the pulmonary circulation could potentiate a feed-forward mechanism whereby vascular lesions generating increased CFH induce vasoconstriction (due to NO depletion), which then contributes to further red blood cell lysis. Furthermore, the extent of transpulmonary elevation of CFH correlates with pulmonary vascular resistance and mPAP, indicating that increased CFH could play a causal role in the increased vascular tone observed in patients with PAH, and conversely, that elevated CFH could be a viable target to improve vascular tone and remodeling in PAH.

The elevated transpulmonary CFH gradient and accumulation of hemoglobin α in pulmonary vasculature led us to hypothesize that mechanisms of CFH clearance may also be dysregulated in patients with PAH. We found that levels of haptoglobin, the endogenous scavenger of hemoglobin, were similar between IPAH and control subjects. The highest levels were observed in UMCs, and lowest levels were in patients with HPAH. A probable explanation for the low levels of haptoglobin observed in patients with HPAH is that haptoglobin is being consumed by the high levels of CFH being

generated (similar to other chronic hemolytic diseases such as sickle cell anemia) in the pulmonary vasculature. On the other hand, the high levels of haptoglobin in UMCs could potentially represent a heightened inflammatory state or a low-level hemolysis. Indeed, the ratio of circulating CFH to haptoglobin levels is significantly higher in patients with PAH and is associated with higher PVR and mPAP. This indicates that there may be an imbalance between increased production and decreased clearance of CFH that contributes to vascular dysfunction in patients with PAH. Differences in haptoglobin between UMCs and HPAH did not appear to be driven by haptoglobin genotype, but this specific comparison was possibly underpowered. We also observed reduced hemopexin among HPAH patients, further supporting the notion of particularly impaired clearance mechanisms in this population. Speculatively, the higher haptoglobin and hemopexin levels observed in UMCs could point to unrecognized resilience mechanisms which may confer protection from the deleterious effects of CFH and warrants further study.

Considering the decreased haptoglobin levels only in patients with HPAH despite the similarly high levels on CFH in both groups,[14] we investigated the circulating levels of sCD163, the macrophage receptor responsible for processing hemoglobin–haptoglobin complex uptake. We observed higher levels of sCD163 in patients with IPAH compared to control subjects and UMCs. Higher levels of circulating sCD163 have been shown to contribute to pulmonary hypertension and indicate macrophage activation and receptor shedding,[38] which could inhibit the ability for macrophages to clear hemoglobin-haptoglobin complexes. Enhanced receptor shedding in response to an inflammatory environment could be contributing to these observations. Likewise, during oxidative stress and inflammation, HO-1 is released into the circulation by inflammatory and vascular cells, offering protection against tissue injury. HO-1 is upregulated during CD163 mediated CFH uptake and is responsible for the breakdown of heme, which can be released from CFH during oxidative stress. We found that HO-1 was not increased in PAH patients suggesting that PAH patients have deficient CFH uptake. This is consistent with the higher levels in sCD163 in these patients. Alternatively, increased circulating levels of HO-1 could indicate that patients with PAH have a defect in upregulating HO-1, or that UMCs have a more efficient antioxidant response over patients with PAH. Together, these data provide new evidence that dysfunction in CFH clearance mechanisms contribute to CFH-mediated vascular dysfunction in humans with PAH.

Our study does have some limitations. Our initial observations of increased transpulmonary generation of CFH in patients with PAH compared these individuals to patients with PVH. While providing an exciting basis to pursue further mechanisms, these initial measurements were largely exploratory due to the lack of baseline controls and comparison to a validation cohort. Additionally, we cannot exclude the possibility that RHC blood sampling could induce hemolysis. Furthermore, this study was primarily an observational and correlative study, and no interventions were tested. Future work should utilize CFH-targeted therapies in experimental models to investigate its roles in the pathophysiology of PAH. For example, exogenous administration of haptoglobin in animal models of PAH in which CFH is elevated may protect against vascular injury and organ damage; this approach has already shown promise in several models to prevent hemolysis-induced hypertension, vascular remodeling and injury, inflammation, and organ dysfunction.[13,15,39,40] Moreover, future physiologic studies could explore using alternative agents that mitigate CFH pathology. Acetaminophen (TYLENOL®), a hemoprotein reductant, has been shown to prevent oxidative injury and reduce renal injury markers in critically ill patients with severe sepsis with elevated levels of CFH,[41] reduce kidney injury in patients with severe falciparum malaria,[42] prevent CFH-mediated vascular permeability in an ex vivo perfused human lung model,[11] and inhibit hemoprotein-induced oxidative damage and renal failure in a rat model of rhabdomyolysis.[43] Along the same lines, the precise signaling mechanisms involved in CFH-mediated contributions to PAH pathology were not investigated in this study. The haptoglobin genotyping analyses, though novel, may be underpowered. We attempted to mitigate this limitation by comparison of PAH genotype distributions with a well-powered clinical population ($N = 64{,}843$) with existing haptoglobin genotyping though this line of investigation warrants further work in larger PAH populations.

CONCLUSIONS

We found that levels of CFH generated from the pulmonary circulation are associated with pulmonary vascular resistance and mPAP in humans with PAH. Concordantly, immunofluorescence staining of hemoglobin α is elevated in patients with PAH, especially in areas of occlusion, indicating an environment conducive to hemolysis. Circulating markers of CFH clearance mechanisms are differentially regulated in patients with PAH and appear especially impaired in patients with HPAH. Further study into the mechanisms of CFH elevation and clearance is required to determine whether CFH and its processing are viable therapeutic targets to improve vascular function in PAH.

AUTHOR CONTRIBUTIONS

Concept and design: Jamie E. Meegan, Eric D. Austin, Anna R. Hemnes, Evan L. Brittain. *Data acquisition*: Vern Eric Kerchberger, Niki L. Fortune, Joel Brennan McNeil, Eric D. Austin, Anna R. Hemnes, Evan L. Brittain. *Data analysis*: Jamie E. Meegan, Vern Eric Kerchberger, Niki L. Fortune, Joel Brennan McNeil, Julie A. Bastarache, Eric D. Austin, Lorraine B. Ware, Anna R. Hemnes, Evan L. Brittain. *Data interpretation*: Jamie E. Meegan, Vern Eric Kerchberger, Niki L. Fortune, Joel Brennan McNeil, Julie A. Bastarache, Eric D. Austin, Lorraine B. Ware, Anna R. Hemnes, Evan L. Brittain. *Figure preparation and manuscript drafting*: Jamie E. Meegan, Evan L. Brittain. All authors critically revised and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

This study was approved by the Vanderbilt University Institutional Review Board (IRB #9401, #140983, #060203) and all subjects gave written informed consent.

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