Hypoxic pulmonary hypertension and the role of extra-cellular hemoglobin in perivascular macrophage accumulation

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Hemolytic anemia associated pulmonary hypertension (PH) typically occurs in chronic diseases such as sickle cell anemia and thalassemia. The contributions of chronic and intermittent hypoxia caused by the anemic state and the role of free hemoglobin (Hb) and iron have been debated in the literature. The lifetime cycle of hypoxia in hemolytic anemia is an important contributor to the worsening of pulmonary vascular remodeling and increased pulmonary vascular disease. However, the role of low, but continuous plasma Hb in combination with hypoxia has not been well studied. As a result we initially designed a rat model to evaluate the specific effects of hypoxia and continuous levels of Hb on the progression of PH in a chronic setting. In this model we observed that Hb delivered by an implanted refillable infusion pump had an additive effect on hypoxia driven PH, even at low Hb plasma concentrations (10-20 µM heme). Our observations were consistent with increased adventitia macrophage accumulation, oxidation and inflammation that consistently lead to more pronounced metrics of enhanced pulmonary vascular remodeling. As a result we suggested this model would be useful to evaluate potential treatments for PH, specifically PH associated with hemolysis. Effective treatments targeted directly at Hb induced PH associated with hemolytic anemia do not presently exist. Sickle cell anemia patients have undetectable levels of the Hb binding protein, haptoglobin (Hp). Therefore we focused our efforts on the study of repeated dose Hp therapy to increase the circulatory Hp pool and maintain plasma Hb in a non-reactive intravascular compartmentalized Hb-Hp complex. Here we review results from several studies utilizing this model, discuss the role of macrophages in PH disease progression, dissect the contributions of hypoxia and Hb in our model and suggest treatment strategies tested to reduce Hbs contribution of progressive complications of PH.

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arterial catheter measurement of $\geq 25$ mmHg at rest $^{[5,6]}$. PH caused by intermittent and chronic hemolytic anemia disease states are categorized as having multifactorial mechanisms that ultimately lead to remodeling of the pulmonary vasculature $^{[7]}$. The most well studied and accepted mechanism of intermittent and chronic extra-cellular Hb induced PH is the abnormal regulation of nitric oxide (NO) signaling $^{[1,8]}$. NO depletion is reported to be associated with the generation of reactive oxygen species such as superoxide ($O_2^-$) resulting from increased circulating or vascular wall associated xanthine oxidase $^{[9]}$. Evidence of higher Hb oxidation states (ferric-Fe$^{3+}$ and ferryl-Fe$^{4+}$) and tissue globin chain crosslinking has also been reported following exposure to Hb $^{[10,13]}$.

Hb can consume NO via two reactions: (I) NO dioxygenation of oxygenated Hb (HbFe$^{2+}$O$_2$) which generates nitrate anion (NO$^3$) and ferric Hb (HbFe$^{3+}$), and (II) iron nitrosylation of deoxyHb (HbFe$^{2+}$) a reaction that occurs by direct iron binding of NO to deoxygenated ferrous Hb in the 2$^+$ redox state.

I) Hb-Fe$^{2+}$O$_2$ + NO$\bullet$ $\rightarrow$ [Hb(Fe$^{3+}$OONO$\bullet$)] $\rightarrow$ Hb-Fe$^{3+}$ + NO$^3$$^-$$

II) Hb-Fe$^{2+}$ + NO$\bullet$ $\rightarrow$ Hb(NO)

Both of these reactions result in acute and chronic vasoactivity depending on the extent and duration of exposure to extracellular Hb. Hemolysis induced systemic and pulmonary hypertension can contribute to vascular remodeling in response to a state of NO depletion $^{[12,13]}$.

Increased tissue concentrations of peroxides [i.e., hydrogen peroxide (H$_2$O$_2$) or lipid peroxides] occur in several diseases, particularly within the context of inflammation during cycles of hypoxia/ischemia and reperfusion that occur in PH associated with hemolytic anemia. Hb biochemical reactions with physiological proxidants (peroxides) have been studied in vitro and to a some extent in vivo $^{[14]}$. Hb reactions with peroxide (H$_2$O$_2$) can be summarized as follows: deoxy-Hb (HbFe$^{2+}$) reacts with H$_2$O$_2$ generating (III) oxo-ferryl Hb [Hb(Fe$^{4+}$=O)], $^{[15]}$ ferric Hb [Hb(Fe$^{3+}$)], and (V) protein radical formation [•Hb(Fe$^{4+}$=O)] $^{[14]}$.

III) Hb(Fe$^{2+}$) + H$_2$O$_2$ $\rightarrow$ Hb(Fe$^{4+}$=O) + H$_2$O

IV) Hb(Fe$^{4+}$=O) + H$^+$ $\rightarrow$ Hb(Fe$^{3+}$) + OH$^-$

V) Hb(Fe$^{3+}$) + H$_2$O$_2$ $\rightarrow$ •Hb(Fe$^{4+}$=O) + H$_2$O

Globin chain free radicals that are produced during reaction (III) can oxidize amino acid and lipids within the Hb protein or free radicals can transfer to molecules such as lipoproteins created lipid-protein adducts (4-hydroxy-nonenal, 4-HNE). During free radical reactions within Hb, a defined peptide region that is located at the α-globin/β-globin interface is the primary site of amino acid oxidation. In particular the β-chain Cys93 as well as the α-chain Tyr42 are the primary amino acids affected following ferryl Hb radical (•Hb(Fe$^{4+}$=O)) formation and this leads to protein unfolding, intermolecular crosslinking and progressive degradation of the Hb molecule into precipitated protein, heme and iron $^{[16,17]}$.

Based on the biochemical properties of Hb we hypothesized that a concomitant insult such hypoxia, which facilitates an oxidative environment, would be necessary to assess the effects of Hb on pulmonary vascular pathology within the context of hemolytic anemia associated PH. To study this position we have evaluated the effects of extracellular Hb on pulmonary hemodynamics and remodeling in rats after chronic Hb exposures of 12 mg/day (0.5 mg/hr) and 35 mg/day (1.45 mg/hr) at 3, 5 and 7 weeks ± hypoxia (10% O$_2$, 5,500 m; 18,000 feet; barometric pressure 380 mmHg). Under normoxic conditions significant Hb induced elevations in pulmonary arterial pressures (PAP) were not observed until later than 3 weeks of exposure. PAP was significantly increased at 5 and 7 weeks of Hb exposure, the mean ± SEM did not increase to or above 25 mmHg. However, when rats were subjected to hypoxia they demonstrated clinically relevant PAP (≥ 25 mmHg) at 3 weeks. Concomitant Hb exposure lead to an additive effect on PAP at 3, 5 and 7 weeks and this was associated with increased pulmonary vascular remodeling (muscularization of distal and proximal vessels), increased Fulton index, increased inflammation (ICAM-1 and IL-6), tissue oxidative stress biomarkers (MDA and 4-HNE) and increased pulmonary blood vessel adventitia layer macrophage accumulation $^{[15]}$. In this study CD163$^+$/HO-1$^+$ and CD163$^+$/ICAM$^+$ cells were observed visually to accumulate within the adventitia of muscularized pulmonary blood vessels. Taken together these data suggested that Hb exposure was more damaging in the cardiopulmonary system if a second “pathological hit” (in this case hypoxia) was present. In general this also approximated the anemic conditions of severe genetic hemolytic disorders and the chronic microvascular occlusions that occur in sickle cell disease.

Based on data from our model that demonstrate increased CD163$^+$ cells that co-express either HO-1 or ICAM, we further hypothesized that the observation of monocyte/macrophage accumulation in the pulmonary vasculature could lead to increased tissue injury in hypoxic
and Hb rich conditions. This hypoxic and Hb rich microenvironment may also lead to a pro-oxidative and inflammatory macrophage phenotype. In our model this likely contributes to an exacerbation of pulmonary vascular remodeling and worsening PH associated with hypoxia plus extracellular Hb. Several groups have evaluated the role of inflammation on the initiation and progression of PH in hypoxia, scleroderma, parasitic infections and anemia \[18-21\]. Stenmark et al. have demonstrated compelling results to suggest that fibroblasts and immunomodulatory cells including macrophages and other cell types contribute to the expansion of the pulmonary vascular vaso vasorum during hypoxia. This occurs as a result of activation of the cells residing within the adventitia of vessels and leads to chronic generation of reactive oxygen species \[22\] and inflammation causing an “outside-in” induction/progression of pulmonary vascular remodeling and PH \[23-25\].

A critical role for tissue resident monocytes/macrophages is the clearance of Hb-haptoglobin (Hp) complexes. Hp is the Hb binding protein in plasma and tissue of mammals. In humans, Hp can exist as one of three primary phenotypes (Hp 1-1, Hp 2-1 and Hp 2-2) in a range of concentrations from 0.3 – 1.9 mg/ml \[26\]. The three Hp polymorphisms have the same Hb binding β globin (Hpβ), but differ in α globin chains (Hpα1 or Hpα2) \[26\]. This results in dimeric (Hp 1-1) or multimeric (Hp 2-1, Hp 2-2) forms, as determined by the number of α globin cysteine residues involved in disulfide bond formation. One primary function of Hp is the prevention of Hb filtration by the glomerulus and therefore Hp limits renal injury following hemolysis \[27\]. In humans, Hb-Hp complexes are cleared from circulation by the scavenger receptor CD163 complex tethered to the surface of tissue resident macrophages that express high CD163 and HO-1 (CD163+ / HO-1*).

Figure 1. (A) The sequential progression from Hb access to into the perivascular space (extravasation) to factors that lead to the progression of pulmonary vascular disease are described. (B) Describes the pathological role of Hb following extravasation into the perivascular space. In this environment Hb can oxidize to met-Hb and readily releases heme and iron. Both can contribute to tissue lipid peroxidation that leads to reactive lipid adduction to enzymatic and non-enzymatic proteins. This tissue injury can lead to the recruitment of macrophages to the adventitia and differentiation of fibroblasts within this compartment. All of these factors lead to inflammation and the progression of PH. However, Increasing the Hp pool prevents the induction of the Hb driven cascade of events by driving the process toward Hp binding, vascular compartmentalization and clearance by liver and spleen tissue resident macrophages that express high CD163 and HO-1 (CD163+ / HO-1*).
compartment where heme is degraded by HO-1 ultimately to biliverdin with the release of carbon monoxide (CO). Iron is released and bound to its cellular storage protein, ferritin and CD163 is recycled back to cell surface.

Heme can trigger changes in macrophage phenotype polarization [22]. A heme-driven macrophage phenotype with high HO-1 expression, high anti-oxidant capacity and suppressed HLA class 2 has been consistently found in atherosclerotic plaques with intra-plaque hemorrhage [30]. Results from Schaar et al. demonstrate that Hb complex formation with Hp leads to macrophage polarization favoring a CD163 – HO-1 - HLA class 2low - antioxidative phenotype. This macrophage phenotype is alternatively activated by Hb-Hp to promote healing at the site of injured tissue [22]. This scenario likely differs in our model in that Hb extravasation, oxidation, heme and iron release leads to tissue injury followed by inflammatory cell recruitment and accelerated remodeling. However, expansion of the Hp pool by repeated administrations could prevent the initial event of extravasation in this cascade and limit Hb contribution to tissue injury.

Based on cumulative data that demonstrate the role of adventitia expansion, oxidation, inflammation and the potential protective role for Hp to promote clearance and limit extra-vascular exposure we designed experiments using our rodent infusion pump model to evaluate the effects of twice weekly Hp doses (90 mg/kg, i.v.) in rats subjected to continuous Hb exposure of 35 mg/day (1.45 mg/hr) with concomitant hypoxia (10% O2, 5,500 m; 18,000 feet; barometric pressure 380 mmHg). The results of this study are presented as a visual summary in Figure 1. Experimental observations from this study confirm that extracellular Hb gains accesses to perivascular spaces in the pulmonary vasculature and this lead to a cascade of Hb oxidation and accumulation of reactive non-heme iron within the vascular tissue of rat lungs. This created a tissue environment favorable for oxidation/inflammation as visualized by increased 4-HNE immune reactivity, accumulation of inflammatory cells (including CD163+ macrophage populations) within the blood vessel adventitia and a significant increase in inflammatory molecules ICAM-1 and IL-6. Moreover, iron accumulation was observed in the cardiomyocytes and around the cardiac blood vessels of the right heart. We suggest that this progression of Hb and hypoxia induced pathophysiology lead to enhanced metrics of cardiopulmonary remodeling. Consistent with our hypothesis, the repeated administration of Hp to increase the circulatory Hp pool attenuated the molecular pathological response caused by Hb in tissue (oxidation and inflammation) and returned functional parameters cardiopulmonary vascular remodeling to that of hypoxia. Based on clear evidence of non-heme iron accumulation in pulmonary and cardiac tissue during Hb exposure absent Hp administration we suggest that Hb is the vehicle for iron deposition. Further we did not observe increased non-transferrin bound iron or transferrin saturations, which would indicate a role for free-iron.

Finally, in the future it will be important to determine how the combination of chronic hypoxia and increased Hb exposure may drive a unique pro-inflammatory macrophage phenotype in our model system. Interestingly, emerging data from Stenmarks group suggests hypoxia-induced metabolic reprogramming causes CtBP1 inhibition of HO-1 expression in fibroblasts and macrophages causing up-regulation of inflammatory and growth mediators. Thus, it’s plausible, that in our Hb plus hypoxia model, hypoxia depletes HO-1 expression in the Hb-clearance macrophage via a CtBP1 mechanism. Subsequently, this may create a macrophage phenotype with high iron and low HO-1, which functions more as a pro-inflammatory compared to anti-inflammatory macrophage. Accordingly, it has been shown that increased intra-macrophage iron retention caused by hepcidin-mediated ferroportin down-regulation could be considered as a pro-inflammatory signal [31]. However, further research is needed to determine how hypoxia may alter or interfere with the Hb-clearance macrophage

In conclusion our data highlights the potential for Hp as a therapeutic in hemolysis related PH to limit the contribution of Hb toward worsening pulmonary vascular disease. The mechanisms by which Hp binding contributes to this effect likely involves (1) a decrease in Hb’s ability to access sites of NO production, thus preserving NO bioavailability, (2) prevention of Hb induced tissue oxidation, (3) reduced accumulation of perivascular macrophages/adventitial expansion and (4) normalization of inflammation.

References


