Published in final edited form as:

Br J Haematol. 2016 November ; 175(4): 714–723. doi:10.1111/bjh.14280.

Sustained treatment of sickle cell mice with haptoglobin increases HO-1 and H-ferritin expression and decreases iron deposition in the kidney without improvement in kidney function

Patricia A. Shi¹, Erika Choi¹, Narendranath R. Chintagari³, Julia Nguyen², Xinhua Guo¹, Karina Yazdanbakhsh¹, Narla Mohandas¹, Abdu I. Alayash³, Elizabeth A. Mancini⁴, John D. Belcher², and Gregory M. Vercellotti²

¹Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY

²Department of Hematology, Oncology, and Transplantation, University of Minnesota Medical School, Minneapolis, MN

³Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, MD

⁴Department of Pathology, University of South Alabama School of Medicine, Birmingham, AL

Abstract

There is growing evidence that extracellular haemoglobin and haem mediate inflammatory and oxidative damage in sickle cell disease. Haptoglobin (Hp), the scavenger for free haemoglobin, is depleted in most patients with sickle cell disease due to chronic haemolysis. Although single infusions of Hp can ameliorate vaso-occlusion in mouse models of sickle cell disease, prior studies have not examined the therapeutic benefits of more chronic Hp dosing on sickle cell disease manifestations. In the present study, we explored the effect of Hp treatment over a three-month period in sickle mice at two dosing regimens: the first at a moderate dose of 200 mg/kg thrice weekly and the second at a higher dose of 400 mg/kg thrice weekly. We found that only the higher dosing regimen resulted in increased haem-oxygenase-1 and heavy chain ferritin (H-ferritin) expression and decreased iron deposition in the kidney. Despite the decreased kidney iron deposition following Hp treatment, there was no significant improvement in kidney function. However, there was a nearly significant trend towards decreased liver infarction.

Corresponding Author: Patricia Ann Shi, MD, New York Blood Center, 310 East 67th Street, New York, NY 10065, pshi@nybloodcenter.org, Tel 212-570-3046, Fax 212-570-3092.

Authorship Contributions and Disclosure of Conflicts of Interest

PAS: Designed and directed the study, performed research, analysed data and wrote the manuscript.

EC: Performed research and analysed data.

NRC: Performed research and analysed data.

JN: Performed research and analysed data.

XG: Performed research.

KY: Analysed data and edited the manuscript.

NM: Designed the study, analysed data and edited the manuscript.

AA: Designed the study, analysed data and edited the manuscript.

EAM: Designed the study, performed research, analysed data and edited the manuscript.

JDB: Designed the study, analysed data and edited the manuscript.

GMV: Designed the study, analysed data and edited the manuscript.

The authors report no relevant conflict of interests.

Keywords

free haemoglobin; sickle cell disease; haptoglobin; haem-oxygenase-1; H-ferritin

Introduction

There is growing evidence that extracellular haemoglobin (Hb) and haem mediate inflammatory, vascular and oxidative damage in sickle cell disease (SCD) (Rifkind, *et al* 2014, Schaer, *et al* 2013, Zhou, *et al* 2011). Haptoglobin (Hp) is a Hb-scavenging plasma glycoprotein which binds rapidly and irreversibly (K_d , 1 pM) to Hb dimers derived from the dissociation of Hb tetramers after intravascular haemolysis (Hwang and Greer 1980, Mollan, *et al* 2014). Hp thus sequesters Hb in the plasma, preventing its filtration into the kidney (Lim, *et al* 1998) and its translocation across endothelium (Lipiski, *et al* 2013), and preserving nitric oxide signalling in the vasculature (Schaer, *et al* 2015). Hp also stabilizes bound Hb from Hb oxidation, thereby preventing the release of free haem, which can cause tissue damage during oxidative stress (Buehler, *et al* 2009, Deuel, *et al* 2015). The high affinity of Hp for Hb dimers has two important implications. First, addition of exogenous Hp markedly inhibits haem loss and, second, as a result of this inhibition, globin precipitation is prevented (Mollan, *et al* 2014). Hp-Hb complexes (but not Hp alone) then bind with high affinity (K_d , 12 nM) to tissue macrophages via CD163 and are internalized by endocytosis (Kristiansen, *et al* 2001, Nielsen, *et al* 2010); haem liberated within the lysosome is then broken down into less toxic compounds by haem-oxygenase-1 (HO-1, also termed HMOX1) (Belcher, *et al* 2006), with incorporation of released Fe^{2+} into ferritin (Vercellotti, *et al* 2014).

Hp is depleted in most patients with SCD due to chronic haemolysis (Muller-Eberhard, *et al* 1968), as Hp is not recycled but rather degraded by macrophages after CD163-mediated endocytosis (Nielsen, *et al* 2010). In mouse models of SCD, single infusions of Hp ameliorate vaso-occlusion (Belcher, *et al* 2014) and Hb-mediated oxidative damage (Chintagari, *et al* 2015). Twice weekly Hp (90 mg/kg) over 5 weeks was recently tested in a mouse model of chronic extracellular Hb-mediated progression of pulmonary hypertension and decreased iron accumulation in lung and heart tissue was documented in conjunction with decreases in pulmonary vascular inflammation and resistance and right-ventricular hypertrophy (Irwin, *et al* 2015). There is increased interest in clinical trials in SCD patients of the use of haptoglobin and haemopexin, the scavenger for free haem (Quimby, *et al* 2015, Smith and McCulloh 2015). To assess the possible utility of Hp replacement in SCD, we administered Hp thrice weekly at doses of 200 and 400 mg/kg to sickle cell mice for a period of 3 months and examined the effect of Hp administration on the extent of decreases in organ damage.

Materials and Methods

Sickle mice

Male and female Townes mice (stock number 013071, The Jackson Laboratory, Bar Harbor, ME), which exclusively express human Hb, were used for all experiments, which were

approved by the New York Blood Center Institutional Animal Care and Use Committee. The studies were performed between January 2014 and January 2016 in SS and SA mice (SA used as additional controls). Genotypes were confirmed by polymerase chain reaction per the Jackson Laboratory protocol and high performance liquid chromatography (Fabry, *et al* 1995). Baseline organ damage in the spleen, liver, and kidneys that was previously described (Wu, *et al* 2006) was confirmed in SS mice, with baseline mean urine osmolalities in 1-month and 3-month animals being 1033 ± 303 mOsm/kg ($n=13$) and 1069 ± 208 mOsm/kg ($n=8$), respectively (p-value NS), compared to historical controls of 2129 ± 281 mOsm/kg (Wu, *et al* 2006)

Haptoglobin treatment

Human haptoglobin (Hp) solution was a kind gift from Bio Product Laboratory (BPL, Hertfordshire, UK), and consists of Hp that is approximately 60% Hp 1-1, 21% Hp 1-2 and 19% Hp 2-2 (Mollan, *et al* 2014). Single-dose Hp injections were performed in 3-month old mice to determine the optimal dose, based on evaluation of peak and trough concentrations. Littermate mice 1 to 3 months old were then randomized to intraperitoneal (IP, initial cohort) or subcutaneous (SC, later cohorts) treatment with Hp or an equivalent volume of phosphate-buffered saline (PBS) control on a dosing schedule of Monday (M), Wednesday (W) and Friday (F) (two 48-h and one 72-h dosing intervals) for a period of 3 months. One month of age was the earliest that we were viably able to chronically inject the Townes mice.

Peripheral blood studies

Peripheral blood was collected into EDTA while mice were alive (by retro-orbital sinus bleed) or at the time of sacrifice (by aortic puncture after euthanizing with CO₂). Complete blood counts and white blood cell (WBC) differentials (validated by manual count) were measured using the Advia 120 Hematology System (Siemens, Malvern, PA). Plasma levels of human Hp by enzyme-linked immunosorbent assay (ELISA) (GenWay Biotech, San Diego, CA), Hb/haem by the tetramethylbenzidine (TMB) haem assay (Huy, *et al* 2005), soluble VCAM1 (R&D Systems, Minneapolis, MN) and creatinine (BioAssay Systems, Hayward, CA) were measured in duplicate or triplicate after frozen storage at -85°C . The TMB haem assay does not distinguish between free Hb and free haem. Ektacytometry to measure red cell deformability was performed as previously described (Mohandas, *et al* 1980) using red cells suspended in 3% (wt/vol) polyvinylpyrrolidone exposed to increasing shear stress (0–150 dynes/cm²).

Urine studies

Unlike Berkeley mice (Manci, *et al* 2006), Townes mice do not exhibit kidney infarcts, but do have hyposthenuria (Wu, *et al* 2006). Urine osmolality was measured with an Advanced® Model 3320 Micro-Osmometer (Advanced Instruments, Norwood, MA). Urine albumin concentration was also measured by ELISA (Abcam #ab108792). Twenty-four hour urine studies were obtained by placing individual mice in metabolic cages for 24 h (#3600M021; Tecniplast, West Chester, PA) after 10 (out of 12) weeks of treatment. Spot urine for analysis was performed by pooling the urine collected at 3 time points (9am, 12pm and 3pm) of a day. Urine was stored at -20°C for no longer than 6 months before analysis. Urine values are

expressed as concentrations (the trends were the same for total absolute amounts of albumin excreted unless otherwise indicated).

Solid organ studies

Freshly harvested organs were immediately weighed using an Explorer Pro precision standard scale (Ohaus, Parsippany, NJ), and per cent body weight was calculated using body weight on the day of sacrifice. The lungs, a liver lobe and one kidney were immediately frozen for HO-1 and heavy chain ferritin (H-ferritin) analysis by Western blot as previously described (Chintagari, *et al* 2010). Equal amounts of total proteins (30 µg) were electrophoretically separated and immobilized onto a nitrocellulose membrane and probed with mouse anti-HO-1 and rabbit anti-H-ferritin antibodies (Abcam, Cambridge, MA) at 1:2500 dilutions. The proteins were visualized using chemiluminescence (Amersham ECL Western Blotting Detection Kit, GE Healthcare, Port Washington, NY).

The remaining organs (brain, heart, liver, spleen and the other kidney) were immediately fixed in neutral buffered formalin for 24 h and then transferred to ethanol for storage and routine processing for paraffin embedding. Tissue blocks were labelled with alphanumeric codes and submitted to the University of South Alabama Centralized Pathology Unit for the Study of Sickle Cell Disease. The tissue blocks were sectioned at 4 µm and stained by haematoxylin-and-eosin, Gomori trichrome and standard iron methods, with appropriate controls for each stain. Each tissue was studied in a blinded manner for infarcts (neutrophilic infiltrates and/or coagulative necrosis), iron (blue granules) and thrombosis and/or congestion. Each parameter was graded on a subjective scale (0 for absent/none; 10 for abundant/severe) by a single experienced pathologist to avoid inter-observer variation and each grading session was conducted as a unit during the same time interval to minimize intra-observer day-to-day variation. In addition to SS mice that received PBS injection, controls included SA mice that received 200 or 400 mg/kg Hp in parallel with the Hp-treated SS mice. Validation of the subjective grading was studied by comparison of the natural disease process (iron deposition and infarcts) with age in the SA and SS mice.

Statistical analysis

Groups were tested for normality and then compared using the appropriate t-test (Mann-Whitney U for non-parametric data) or repeated measures one-way ANOVA using Excel (Microsoft version 10). Significance was assessed at the $p < 0.05$ level, with figures showing mean $\pm$ standard deviation and significant p-values compared to PBS.

Results

Validation of pharmacokinetics and absence of toxicity of dosing regimen

Doses of 200 mg/kg resulted in supra-physiological levels (mean serum Hp level in untreated Townes AA mice being 39.3 ± 8 µg/ml, unpublished data) in 3/3 mice at 24 h but absent Hp levels in 2/4 mice at 48 h (Figure 1A). A 400 mg/kg SC dosing regimen maintained Hp levels in 5/5 mice at 48 h but Hp was absent in 2/3 mice at 72 h (Figure 1A). Both doses, however, were associated with decreased haem levels at 24 and 72 h (Figure 1B), and at 400 mg/kg were significantly different from baseline. Due to concern about

potential toxicity of supra-physiological peak Hp levels, heterozygous haemoglobin SA (sickle trait) mice were treated with repeated dose (M, W, F schedule) Hp 400 mg/kg intraperitoneally for 3 months after confirming that a 400 mg/kg dose in SA mice maintained Hp levels until 72 h (72-h mean 217 ± 26 ug/ml, n=2). There was no significant difference in 24-h post-dose Hp levels between SA mice naïve to human Hp treatment and mice after 3 months of Hp treatment (Figure 1C); in other words, there was no evidence of an immune response to human Hp. Nor was there evidence of toxicity to supra-physiological Hp in SA mice as measured by any notable differences from PBS controls in complete blood counts (Figure 1D), white cell differentials or organ histology (where iron deposition but no infarcts are observed, data not shown).

Repeated-dose regimens in 3-month-old SS mice

Repeated doses (M, W, F schedule) of 200 mg/kg IP and 400 mg/kg SC were performed to determine, respectively, if transient high levels as well as sustained higher levels of plasma Hp would have therapeutic benefit. No significant differences in lung and kidney expression of H-ferritin were observed in the 200 mg/kg Hp group compared to the PBS group (data not shown). Increased expression of HO-1 and H-ferritin in organs, however, was observed in all mice in the 400 mg/kg Hp compared to the PBS group (Figure 2). Organ histology showed no significant differences between the 200 mg/kg Hp and PBS groups (data not shown). However, the 400 mg/kg Hp group had a significant decrease in iron deposition in the kidney compared to the PBS group (Figure 3A), and a trend towards decreased liver infarction was also noted (Figure 3B). No significant improvements in complete blood count (CBC) (or WBC differential), splenomegaly, hyposthenuria or albuminuria, or red cell deformability were observed with either of the two doses (Figure 4). Plasma creatinine levels were within normal range and showed no significant differences between Hp and PBS groups (Figure 5A). Soluble VCAM1 (sVCAM1) levels as a biomarker of inflammation were elevated and showed no significant differences between Hp and PBS groups (Figure 5B). There were no significant differences between Hp-naïve mice and Hp-treated mice in their 24-h post-dose Hp and haem levels with either dosing regimen (Figure S1A-B). Hp-naïve mice were 3 months old, in contrast to Hp-treated mice, which were 6 months old by the end of treatment. Given that disease severity in SCD mice worsens with age (Manci, *et al* 2006), the trend towards decreased Hp after 3 months treatment is consistent with more rapid Hp utilization due to worsening haemolysis with age. The trend towards decreased haem after 3 months of treatment is consistent with lowering of circulating Hb/haem levels over time by the sustained Hp dosing.

400 mg/kg repeated-dose regimen in 1-month-old SS mice

Because of the minimal improvement in disease severity in 3-month-old mice with Hp treatment, the 400 mg/kg dosing regimen was carried out in 1-month-old mice to determine if treatment earlier in the disease course – juveniles versus adults – would result in more significant improvement in disease parameters. There was maintenance of Hp levels at 24 h- and 48 h- post-dose after 3 months of treatment, accompanied by low haem levels (Figure S1C). HO-1 in all cases, and H-ferritin in 3/5 cases, was increased compared to the PBS group (Figure 2). A significant decrease in iron deposition in the kidney was again observed, but there was no trend towards decreased liver infarction (Figure 3A-B). As with the 3-

month-old mice, no significant improvements in CBC (or WBC differential), splenomegaly, hyposthenuria or albuminuria, or red cell deformability were observed (Figure S2). There were no significant differences between Hp and PBS groups in plasma creatinine and sVCAM1 levels (Figure 5).

Discussion

This is the first report of sustained (repeated dose) treatment with Hp in a sickle mouse model. Functional binding of the administered human Hp to the human Hb of the Townes mice occurred, as evident by the significant decrease in iron deposition in the kidneys in the Hp-treated group. The macromolecular Hb-Hp complexes prevent filtration of Hb into the kidneys, due to the large size (> 100 kD) and negative charge of Hp (Allison and Rees 1957, Bhensdadia, *et al* 2013, Chintagari, *et al* 2015, Thomsen, *et al* 2013). In this regard, mouse models of human Hb with human Hp treatment are clearly different from mouse models with mouse Hb, where precipitation of Hb in the kidney is not increased in Hp knockout mice (Lim, *et al* 2000). There was no evidence of an immune response to human Hp, as evidenced by maintenance, after 3 months Hp treatment in both age cohorts, of Hp levels at 24-h and 48-h post-dosing at comparable levels to Hp-naïve mice.

The increases in HO-1 and H-ferritin observed in liver, lung and kidney in the Hp-treated group also support the validity of the human Hb model as a pre-clinical model despite the lack of Hp and CD163-dependence of Hb clearance in mouse models with mouse Hb (Etzerodt, *et al* 2012). Although there may be a low affinity interaction between Hb alone and CD163, allowing for endocytosis and degradation of Hb by macrophages in the absence of Hp (Schaer, *et al* 2006), this is less relevant in the sickle cell mouse model, where the level of haemolysis would overwhelm this mechanism.

Hp-knockout mice still have increased renal damage (Lim, *et al* 1998, Lim, *et al* 2000) and haemoglobinuria and cell-free Hb are associated with renal damage, chronic kidney disease progression, and increasing albuminuria in SCD (Chintagari, *et al* 2015, Saraf, *et al* 2014, Saraf, *et al* 2015). It was therefore disappointing that no improvement in kidney function, as measured by urine osmolality and albumin excretion and plasma creatinine, was observed in either 400 mg/kg Hp age group, despite a clear decrease in kidney iron deposition. This is despite clearly abnormal urine osmolality and microalbuminuria measurements in the sickle cell mice compared to historical controls (Gurley, *et al* 2006, Wu, *et al* 2006). Thus earlier treatment, as assessed by treatment of 1-month- versus 3-month-old animals, had no benefit. This lack of benefit might be explained by existent kidney damage possibly being irreversible (Miller, *et al* 2010, Sasongko, *et al* 2015), with our 1-month-old mice at baseline having similar defective urine concentrating ability as our 3-month-old mice (see **Materials and Methods**, *Sickle mice*). We regard this as unlikely considering that the normal mouse age span is about 2 years and so 1-month-old mice are about 3 years old in human years, assuming a normal human age span of about 80 years. Therefore 1-month-old mice probably have minimal albuminuria, although we did not formally confirm this. It is also possible that 3 months of treatment with Hp was too short a treatment period to show benefit, a hypothesis supported by the trends towards decreased albuminuria in the Hp-treated groups and possibly the lack of improvement in sVCAM1.

Although the minimal HO-1 and H-ferritin expression in PBS-treated Townes mice contrasts with significant HO-1 expression at baseline in Berkeley mice (Belcher, *et al* 2006), that HO-1 and H-ferritin was increased in the liver and lung with Hp treatment makes theoretical sense because Hp-Hb complexes are taken up by macrophages in these organs with subsequent production of HO-1 (Etzerodt and Moestrup 2013, Yang, *et al* 2003). Lung histology was unfortunately not examined, but liver histology in 3-month-old Hp-treated mice showed a trend towards decreased liver infarction, a finding consistent with data that upregulation of HO-1 or H-ferritin expression inhibits vascular stasis in sickle cell mice (Belcher, *et al* 2006, Vercellotti, *et al* 2014), with the protective effect of H-ferritin being independent of HO-1. Although improvement in liver infarction did not predictably correlate with expression of H-ferritin in our Hp-treated cohort, other data also support that neutralization of cell-free Hb reduces liver injury (Hanson, *et al* 2013), probably via HO-1 and CO expression (Beckman, *et al* 2009, Belcher, *et al* 2006, Siciliano, *et al* 2011).

The similar pattern of increased expression of HO-1 and H-ferritin in the kidney is less clear in that the Hb-Hp complex is at least 160 kDa (Thomsen, *et al* 2013) and is thus not filtered into the kidneys. The reason for the increase in HO-1 and H-ferritin expression is therefore unlikely to be CD163-mediated uptake of the complex within the kidney with subsequent increased expression of HO-1. The increased expression also appears to conflict with previous data (Chintagari, *et al* 2015), where mice given a single injection of Hp with Hb showed decreased expression of HO-1 compared to mice injected with Hb alone. This discrepancy might be explained in that CD163-mediated uptake of Hb-Hp and subsequent HO-1 expression in the liver (and lung) may have distal effects in sickle mice via diffusion of anti-inflammatory carbon monoxide, biliverdin, and ferritin into the circulation (Belcher, *et al* 2010). It is possible that carbon monoxide and ferritin may then positively feedback to increase HO-1 and Nrf2 (also termed NFE2L2) expression systemically (Belcher, *et al* 2013, Vercellotti, *et al* 2014); Nrf2 itself directly activates HO-1 expression (Keleku-Lukwete, *et al* 2015).

Given the increase in HO-1 and H-ferritin, the lack of decrease in sVCAM1 was surprising. Haem induces coagulation activation (Sparkenbaugh, *et al* 2015), and plasma sVCAM1 is a marker of both endothelial and coagulation activation (Chantrathammachart, *et al* 2012). Also, sVCAM1 is positively associated with renal dysfunction and hepatic impairment (Kato, *et al* 2005), and negatively associated with increased HO-1 expression (Belcher, *et al* 2010). One explanation is that, although local organ-specific reduction of VCAM1 occurs, such as in the kidney, liver and lung (Irwin, *et al* 2015), systemic VCAM1 expression is not reduced. This is possible because very little is known about the regulation of VCAM1 or the regulation of catabolism of soluble cell adhesion molecules *in vivo* (Ballantyne and Entman 2002), and sVCAM1 levels are positively associated with the degree of urinary albumin excretion (Polat, *et al* 2016). The lack of decrease in sVCAM1 is also consistent with continued activity of free haem despite Hp, as discussed further below.

Only dosing at 400 mg/kg and not 200 mg/kg increased HO-1 and H-ferritin levels and decreased kidney iron deposition, suggesting that Hp dosing for clinical benefit in SCD may need to achieve continuous maintenance of physiological Hp concentrations, due to the ongoing haemolysis releasing Hb continuously. This may be difficult to achieve other than

through a gene therapy approach, because it has recently been shown that large doses of Hp at a 1:1 stoichiometry with Hb are required for effective limitation of haem-driven lipid peroxidation and endothelial damage (Deuel, *et al* 2015).

Other reasons that organ damage did not improve, however, may be related to the properties of Hp itself. Binding of Hb to Hp may not change its nitric oxide reactivity (Azarov, *et al* 2008, Schaer, *et al* 2014), so that nitric-oxide induced vaso-constriction may continue unabated. The Hp 2-2 phenotype, which makes up about 19% of the Hp product used in this study, may not completely stabilize haem within the central cavity of the Hb subunits (Bamm, *et al* 2004), thus allowing haem dissociation and subsequent free haem-mediated oxidative reactions and inflammatory responses, although the weight of evidence (Lipiski, *et al* 2013, Mollan, *et al* 2014) supports no differences among Hp phenotypes in their redox activity and that Hb-Hp complexes retain haem for at least 6-8 h.

Finally, the minimal organ damage improvement may be related to the fact that haem, not Hb, is what causes actual oxidative damage (Deuel, *et al* 2015) and that not all free haem may be derived from cell-free Hb. There may be free haem within sickle red cells that is directly released as free haem (Uzunova, *et al* 2010). The red cell membrane may act as a sponge for free haem (Atamna and Ginsburg 1995), and the presence of haem-laden red cell microparticles can induce kidney vaso-occlusion (Camus, *et al* 2012).

In conclusion, our work supports the possible therapeutic efficacy of repeated dose Hp treatment in SCD (Irwin, *et al* 2015), but with the caveat that, given the amount of Hp needed to ameliorate oxidative damage (Deuel, *et al* 2015) and the degradation of Hp after CD163-mediated endocytosis (Nielsen, *et al* 2010), possibly only a gene therapy approach, in contrast to a purified or recombinant Hp product, may be effective in a chronic haemolytic state like SCD. Haemopexin, due to its efficacy at sub-stoichiometric concentrations and recycling after CD91-mediated endocytosis (Smith and Morgan 1979), may possibly be more effective as a purified or recombinant product in sickle cell disease as it was effective in ameliorating cardiovascular dysfunction and the inflammatory phenotype of macrophages in mouse models of SCD (Vinch, *et al* 2016, Vinch, *et al* 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The first author is grateful to Drs. Dominik Schaer and Paul Buehler for initial encouragement regarding this study. This work was supported by New York Blood Center (PAS), NIH P01-HL110900 (AIA), NIH N01-HB-07086 (EAM) and NIH R01 HL114567-03 (JDB, GMV).

References

Allison AC, Rees WA. The binding of haemoglobin by plasma proteins (haptoglobins); its bearing on the renal threshold for haemoglobin and the aetiology of haemoglobinuria. *Br Med J*. 1957; 2:1137–1143. [PubMed: 13472068]

- Atamna H, Ginsburg H. Heme degradation in the presence of glutathione. A proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobinopathic red blood cells. *J Biol Chem*. 1995; 270:24876–24883. [PubMed: 7559611]
- Azarov I, He X, Jeffers A, Basu S, Ucer B, Hantgan RR, Levy A, Kim-Shapiro DB. Rate of nitric oxide scavenging by hemoglobin bound to haptoglobin. *Nitric Oxide*. 2008; 18:296–302. [PubMed: 18364244]
- Ballantyne CM, Entman ML. Soluble adhesion molecules and the search for biomarkers for atherosclerosis. *Circulation*. 2002; 106:766–767. [PubMed: 12176941]
- Bamm VV, Tsemakhovich VA, Shaklai M, Shaklai N. Haptoglobin phenotypes differ in their ability to inhibit heme transfer from hemoglobin to LDL. *Biochemistry*. 2004; 43:3899–3906. [PubMed: 15049697]
- Beckman JD, Belcher JD, Vineyard JV, Chen C, Nguyen J, Nwaneri MO, O'Sullivan MG, Gulbahce E, Hebbel RP, Vercellotti GM. Inhaled carbon monoxide reduces leukocytosis in a murine model of sickle cell disease. *Am J Physiol Heart Circ Physiol*. 2009; 297:H1243–1253. [PubMed: 19617415]
- Belcher JD, Mahaseth H, Welch TE, Otterbein LE, Hebbel RP, Vercellotti GM. Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. *J Clin Invest*. 2006; 116:808–816. [PubMed: 16485041]
- Belcher JD, Vineyard JV, Bruzzone CM, Chen C, Beckman JD, Nguyen J, Steer CJ, Vercellotti GM. Heme oxygenase-1 gene delivery by Sleeping Beauty inhibits vascular stasis in a murine model of sickle cell disease. *J Mol Med (Berl)*. 2010; 88:665–675. [PubMed: 20306336]
- Belcher JD, Young M, Chen C, Nguyen J, Burhop K, Tran P, Vercellotti GM. MP4CO, a pegylated hemoglobin saturated with carbon monoxide is a modulator of HO-1, inflammation and vaso-occlusion in transgenic sickle mice. *Blood*. 2013; 122:2757–64. [PubMed: 23908468]
- Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, Smith A, Nath KA, Hebbel RP, Vercellotti GM. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*. 2014; 123:377–390. [PubMed: 24277079]
- Bhensdadia NM, Hunt KJ, Lopes-Virella MF, Michael Tucker J, Mataria MR, Alge JL, Neely BA, Janech MG, Arthur JM, Veterans Affairs Diabetes Trial study, g. Urine haptoglobin levels predict early renal functional decline in patients with type 2 diabetes. *Kidney Int*. 2013; 83:1136–1143. [PubMed: 23536133]
- Buehler PW, Abraham B, Vallelia F, Linnemayr C, Pereira CP, Cipollo JF, Jia Y, Mikolajczyk M, Boretti FS, Schoedon G, Alayash AI, Schaer DJ. Haptoglobin preserves the CD163 hemoglobin scavenger pathway by shielding hemoglobin from peroxidative modification. *Blood*. 2009; 113:2578–2586. [PubMed: 19131549]
- Camus SM, Gausseres B, Bonnin P, Loufrani L, Grimaud L, Charue D, De Moraes JA, Renard JM, Tedgui A, Boulanger CM, Tharaux PL, Blanc-Brude OP. Erythrocyte microparticles can induce kidney vaso-occlusions in a murine model of sickle cell disease. *Blood*. 2012; 120:5050–5058. [PubMed: 22976952]
- Chantrathammachart P, Mackman N, Sparkenbaugh E, Wang JG, Parise LV, Kirchhofer D, Key NS, Pawlinski R. Tissue factor promotes activation of coagulation and inflammation in a mouse model of sickle cell disease. *Blood*. 2012; 120:636–646. [PubMed: 22661702]
- Chintagari NR, Mishra A, Su L, Wang Y, Ayalew S, Hartson SD, Liu L. Vacuolar ATPase regulates surfactant secretion in rat alveolar type II cells by modulating lamellar body calcium. *PLoS One*. 2010; 5:e9228. [PubMed: 20169059]
- Chintagari NR, Nguyen J, Belcher JD, Vercellotti GM, Alayash AI. Haptoglobin attenuates hemoglobin-induced heme oxygenase-1 in renal proximal tubule cells and kidneys of a mouse model of sickle cell disease. *Blood Cells Mol Dis*. 2015; 54:302–306. [PubMed: 25582460]
- Deuel JW, Vallelia F, Schaer CA, Puglia M, Buehler PW, Schaer DJ. Different target specificities of haptoglobin and hemopexin define a sequential protection system against vascular hemoglobin toxicity. *Free Radic Biol Med*. 2015; 89:931–943. [PubMed: 26475040]
- Etzerodt A, Moestrup SK. CD163 and Inflammation: Biological, Diagnostic, and Therapeutic Aspects. *Antioxid Redox Signal*. 2013; 18:2352–2363. [PubMed: 22900885]

- Etzerodt A, Kjolby M, Nielsen MJ, Maniecki MB, Svendsen P, Moestrup SK. Plasma clearance of hemoglobin and haptoglobin in mice and effect of CD163 gene targeting disruption. *Antioxid Redox Signal*. 2012
- Fabry ME, Sengupta A, Suzuka SM, Costantini F, Rubin EM, Hofrichter J, Christoph G, Mancini E, Culbertson D, Factor SM, Nagel RL. A second generation transgenic mouse model expressing both hemoglobin S (HbS) and HbS-Antilles results in increased phenotypic severity. *Blood*. 1995; 86:2419–2428. [PubMed: 7662990]
- Gurley SB, Clare SE, Snow KP, Hu A, Meyer TW, Coffman TM. Impact of genetic background on nephropathy in diabetic mice. *Am J Physiol Renal Physiol*. 2006; 290:F214–222. [PubMed: 16118394]
- Hanson MS, Xu H, Flewelen TC, Holzhauer SL, Retherford D, Jones DW, Frei AC, Pritchard KA Jr, Hillery CA, Hogg N, Wandersee NJ. A novel hemoglobin-binding peptide reduces cell-free hemoglobin in murine hemolytic anemia. *Am J Physiol Heart Circ Physiol*. 2013; 304:H328–336. [PubMed: 23125208]
- Huy NT, Xuan Trang DT, Uyen DT, Sasai M, Harada S, Kamei K. An improved colorimetric method for quantitation of heme using tetramethylbenzidine as substrate. *Anal Biochem*. 2005; 344:289–291. [PubMed: 16091279]
- Hwang PK, Greer J. Interaction between hemoglobin subunits in the hemoglobin . haptoglobin complex. *J Biol Chem*. 1980; 255:3038–3041. [PubMed: 7358726]
- Irwin DC, Baek JH, Hassell K, Nuss R, Eigenberger P, Lisk C, Loomis Z, Maltzahn J, Stenmark KR, Nozik-Grayck E, Buehler PW. Hemoglobin-induced lung vascular oxidation, inflammation, and remodeling contribute to the progression of hypoxic pulmonary hypertension and is attenuated in rats with repeated-dose haptoglobin administration. *Free Radic Biol Med*. 2015; 82:50–62. [PubMed: 25656991]
- Kato GJ, Martyr S, Blackwelder WC, Nichols JS, Coles WA, Hunter LA, Brennan ML, Hazen SL, Gladwin MT. Levels of soluble endothelium-derived adhesion molecules in patients with sickle cell disease are associated with pulmonary hypertension, organ dysfunction, and mortality. *Br J Haematol*. 2005; 130:943–953. [PubMed: 16156864]
- Keleku-Lukwete N, Suzuki M, Otsuki A, Tsuchida K, Katayama S, Hayashi M, Naganuma E, Moriguchi T, Tanabe O, Engel JD, Imaizumi M, Yamamoto M. Amelioration of inflammation and tissue damage in sickle cell model mice by Nrf2 activation. *Proc Natl Acad Sci U S A*. 2015; 112:12169–12174. [PubMed: 26371321]
- Kristiansen M, Graversen JH, Jacobsen C, Sonne O, Hoffman HJ, Law SK, Moestrup SK. Identification of the haemoglobin scavenger receptor. *Nature*. 2001; 409:198–201. [PubMed: 11196644]
- Lim SK, Kim H, Lim SK, bin Ali A, Lim YK, Wang Y, Chong SM, Costantini F, Baumann H. Increased susceptibility in Hp knockout mice during acute hemolysis. *Blood*. 1998; 92:1870–1877. [PubMed: 9731043]
- Lim YK, Jenner A, Ali AB, Wang Y, Hsu SI, Chong SM, Baumann H, Halliwell B, Lim SK. Haptoglobin reduces renal oxidative DNA and tissue damage during phenylhydrazine-induced hemolysis. *Kidney Int*. 2000; 58:1033–1044. [PubMed: 10972668]
- Lipiski M, Deuel JW, Baek JH, Engelsberger WR, Buehler PW, Schaer DJ. Human hp1-1 and hp2-2 phenotype-specific haptoglobin therapeutics are both effective in vitro and in Guinea pigs to attenuate hemoglobin toxicity. *Antioxid Redox Signal*. 2013; 19:1619–1633. [PubMed: 23418677]
- Mancini EA, Hillery CA, Bodian CA, Zhang ZG, Luty GA, Collier BS. Pathology of Berkeley sickle cell mice: similarities and differences with human sickle cell disease. *Blood*. 2006; 107:1651–1658. [PubMed: 16166585]
- Miller ST, Wang WC, Iyer R, Rana S, Lane P, Ware RE, Li D, Rees RC, Investigators B-H. Urine concentrating ability in infants with sickle cell disease: baseline data from the phase III trial of hydroxyurea (BABY HUG). *Pediatr Blood Cancer*. 2010; 54:265–268. [PubMed: 19621454]
- Mohandas N, Clark MR, Jacobs MS, Shohet SB. Analysis of factors regulating erythrocyte deformability. *J Clin Invest*. 1980; 66:563–573. [PubMed: 6156955]

- Mollan TL, Jia Y, Banerjee S, Wu G, Kreulen RT, Tsai AL, Olson JS, Crumbliss AL, Alayash AI. Redox Properties of Human Hemoglobin in Complex with Fractionated Dimeric and Polymeric Human Haptoglobin. *Free Radic Biol Med*. 2014; 69:265–77. [PubMed: 24486321]
- Muller-Eberhard U, Javid J, Liem HH, Hanstein A, Hanna M. Plasma concentrations of hemopexin, haptoglobin and heme in patients with various hemolytic diseases. *Blood*. 1968; 32:811–815. [PubMed: 5687939]
- Nielsen MJ, Moller HJ, Moestrup SK. Hemoglobin and heme scavenger receptors. *Antioxid Redox Signal*. 2010; 12:261–273. [PubMed: 19659436]
- Polat SB, Ugurlu N, Aslan N, Cuhaci N, Ersoy R, Cakir B. Evaluation of biochemical and clinical markers of endothelial dysfunction and their correlation with urinary albumin excretion in patients with type 1 diabetes mellitus. *Arch Endocrinol Metab*. 2016; 60:117–124. [PubMed: 26886090]
- Quimby KR, Hambleton IR, Landis RC. Intravenous infusion of haptoglobin for the prevention of adverse clinical outcome in Sickle Cell Disease. *Med Hypotheses*. 2015
- Rifkind JM, Mohanty JG, Nagababu E. The pathophysiology of extracellular hemoglobin associated with enhanced oxidative reactions. *Front Physiol*. 2014; 5:500. [PubMed: 25642190]
- Saraf SL, Zhang X, Kanas T, Lash JP, Molokie RE, Oza B, Lai C, Rowe JH, Gowhari M, Hassan J, Desimone J, Machado RF, Gladwin MT, Little JA, Gordeuk VR. Haemoglobinuria is associated with chronic kidney disease and its progression in patients with sickle cell anaemia. *Br J Haematol*. 2014; 164:729–739. [PubMed: 24329963]
- Saraf SL, Zhang X, Shah B, Kanas T, Gudehithlu KP, Kittles R, Machado RF, Arruda JA, Gladwin MT, Singh AK, Gordeuk VR. Genetic variants and cell-free hemoglobin processing in sickle cell nephropathy. *Haematologica*. 2015; 100:1275–1284. [PubMed: 26206798]
- Sasongko TH, Nagalla S, Ballas SK. Angiotensin-converting enzyme (ACE) inhibitors for proteinuria and microalbuminuria in people with sickle cell disease. *Cochrane Database Syst Rev*. 2015; 6:CD009191.
- Schaer CA, Deuel JW, Schildknecht D, Mahmoudi L, Garcia-Rubio I, Owczarek C, Schauer S, Kissner R, Banerjee U, Palmer AF, Spahn DR, Irwin DC, Vallelia F, Buehler PW, Schaer DJ. Haptoglobin Preserves Vascular Nitric Oxide Signaling During Hemolysis. *Am J Respir Crit Care Med*. 2015; 193:1111–22.
- Schaer DJ, Schaer CA, Buehler PW, Boykins RA, Schoedon G, Alayash AI, Schaffner A. CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin. *Blood*. 2006; 107:373–380. [PubMed: 16189277]
- Schaer DJ, Buehler PW, Alayash AI, Belcher JD, Vercellotti GM. Hemolysis and free hemoglobin revisited: exploring hemoglobin and heme scavengers as a novel class of therapeutic proteins. *Blood*. 2013; 121:1276–1284. [PubMed: 23264591]
- Schaer DJ, Vinchi F, Ingoglia G, Buehler PW. Haptoglobin, hemopexin, and related defense pathways—basic science, clinical perspectives, and drug development. *Front Physiol*. 2014; 5:415. [PubMed: 25389409]
- Siciliano A, Malpeli G, Platt OS, Lebouef C, Janin A, Scarpa A, Olivieri O, Amato E, Corrocher R, Beuzard Y, De Franceschi L. Abnormal modulation of cell protective systems in response to ischemic/reperfusion injury is important in the development of mouse sickle cell hepatopathy. *Haematologica*. 2011; 96:24–32. [PubMed: 20851863]
- Smith A, McCulloh RJ. Hemopexin and haptoglobin: allies against heme toxicity from hemoglobin not contenders. *Front Physiol*. 2015; 6:187. [PubMed: 26175690]
- Smith A, Morgan WT. Haem transport to the liver by haemopexin. Receptor-mediated uptake with recycling of the protein. *Biochem J*. 1979; 182:47–54. [PubMed: 496916]
- Sparkenbaugh EM, Chanrathammachart P, Wang S, Jonas W, Kirchhofer D, Gailani D, Gruber A, Kasthuri R, Key NS, Mackman N, Pawlinski R. Excess of heme induces tissue factor-dependent activation of coagulation in mice. *Haematologica*. 2015; 100:308–314. [PubMed: 25596265]
- Thomsen JH, Etzerodt A, Svendsen P, Moestrup SK. The haptoglobin-CD163-heme oxygenase-1 pathway for hemoglobin scavenging. *Oxid Med Cell Longev*. 2013; 2013:523652. [PubMed: 23781295]
- Uzunova VV, Pan W, Vekilov PG. Free heme and the polymerization of sickle cell hemoglobin. *Biophys J*. 2010; 99:1976–1985. [PubMed: 20858444]

- Vercellotti GM, Khan FB, Nguyen J, Chen C, Bruzzone CM, Bechtel H, Brown G, Nath KA, Steer CJ, Hebbel RP, Belcher JD. H-ferritin ferroxidase induces cytoprotective pathways and inhibits microvascular stasis in transgenic sickle mice. *Front Pharmacol*. 2014; 5:79. [PubMed: 24860503]
- Vinchi F, De Franceschi L, Ghigo A, Townes T, Cimino J, Silengo L, Hirsch E, Altruda F, Tolosano E. Hemopexin Therapy Improves Cardiovascular Function by Preventing Heme-Induced Endothelial Toxicity in Mouse Models of Hemolytic Diseases. *Circulation*. 2013; 127:1317–29. [PubMed: 23446829]
- Vinchi F, Costa da Silva M, Ingoglia G, Petrillo S, Brinkman N, Zuercher A, Cerwenka A, Tolosano E, Muckenthaler MU. Hemopexin therapy reverts heme-induced proinflammatory phenotypic switching of macrophages in a mouse model of sickle cell disease. *Blood*. 2016; 127:473–486. [PubMed: 26675351]
- Wu LC, Sun CW, Ryan TM, Pawlik KM, Ren J, Townes TM. Correction of sickle cell disease by homologous recombination in embryonic stem cells. *Blood*. 2006; 108:1183–1188. [PubMed: 16638928]
- Yang F, Haile DJ, Berger FG, Herbert DC, Van Beveren E, Ghio AJ. Haptoglobin reduces lung injury associated with exposure to blood. *Am J Physiol Lung Cell Mol Physiol*. 2003; 284:L402–409. [PubMed: 12388365]
- Zhou Z, Behymer M, Guchhait P. Role of extracellular hemoglobin in thrombosis and vascular occlusion in patients with sickle cell anemia. *Anemia*. 2011; 2011:918916. [PubMed: 21490767]

Figure 1.

Pharmacokinetics (A-C) and toxicity (D) of Haptoglobin. (A) Plasma Hp concentrations after a single dose of human Hp administered to Hp-naïve SS mice were supra-physiological at 24 h but variably present at 48 and 72 h. (B) Haem concentrations decreased or remained stable over time after a single dose of human Hp administered to Hp-naïve SS mice. (C) There was no significant change in plasma Hp levels suggestive of an immune response to 3 months of human Hp treatment (400 mg/kg). (D) Complete blood counts showed no toxicity in heterozygous haemoglobin SA mice with 3 months of human Hp treatment (400 mg/kg). Hp, haptoglobin; Hb, haemoglobin; Hct, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RBC, red blood cell count; WBC, white blood cell count; PLT, platelet count.

Figure 2. Haem-oxygenase 1 (HO-1) and Heavy chain ferritin (H-ferritin) Western blots of lung, kidney, and liver. The first 4 lanes in the Hp group are from the 3-monthold mice. The subsequent 5 lanes in the Hp group are from the 1-monthold mice. Individual mouse samples are in the same order on all Western blots. Hp, haptoglobin; PBS, phosphate-buffered saline

Figure 3.

Histopathological findings in kidney and liver. (A) This bar graph shows significantly less iron staining in the Hp-treated kidneys at 1 and 3 months of age compared with PBS control littermates. The adjacent photomicrographs are representative iron-stained sections of kidney (arrows indicate blue iron granules) from each of these groups (400x, standard iron stain). (B) This bar graph shows no difference in the severity of liver infarcts in the 1-month-old Hp-treated versus PBS groups, but the 3-monthold Hp-treated group showed a trend toward less severe infarcts compared to PBS control. Photomicrographs of representative sections of liver at 3 months illustrate the less severe infarcts (arrows) in the Hp-treated compared to PBS littermates (200x, Haematoxylin-and-eosin). Hp, haptoglobin; PBS, phosphate-buffered saline

Figure 4.

Effect on various parameters after 3 months of 200 mg/kg and 400 mg/kg human Hp treatment in 3-month-old SS littermates. There was no significant effect on (A) complete blood count parameters, (B) organ mass, (C) urine parameters or (D) ektacytometry. Hp, haptoglobin; PBS, phosphate-buffered saline; Hb, haemoglobin; Hct, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RBC, red blood cell count; WBC, white blood cell count; PLT, platelet count; A.U. arbitrary units.

Figure 5.

Plasma creatinine and soluble VCAM1 after 3 months of Hp treatment. (A) Creatinine levels were within normal range and showed no significant differences between Hp-treated and PBS control littermates. (B) sVCAM1 levels were elevated and showed no significant differences between Hp-treated and PBS control littermates. Hp, haptoglobin; PBS, phosphate-buffered saline.