Haptoglobin Preserves Vascular Nitric Oxide Signaling during Hemolysis

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Abstract

Rationale: Hemolysis occurs not only in conditions such as sickle cell disease and malaria but also during transfusion of stored blood, extracorporeal circulation, and sepsis. Cell-free Hb depletes nitric oxide (NO) in the vasculature, causing vasoconstriction and eventually cardiovascular complications. We hypothesize that Hb-binding proteins may preserve vascular NO signaling during hemolysis.

Objectives: Characterization of an archetypical function by which Hb scavenger proteins could preserve NO signaling during hemolysis.

Methods: We investigated NO reaction kinetics, effects on arterial NO signaling, and tissue distribution of cell-free Hb and its scavenger protein complexes.

Measurements and Main Results: Extravascular translocation of cell-free Hb into interstitial spaces, including the vascular smooth muscle cell layer of rat and pig coronary arteries, promotes vascular NO resistance. This critical disease process is blocked

by haptoglobin. Haptoglobin does not change NO dioxygenation rates of Hb; rather, the large size of the Hb:haptoglobin complex prevents Hb extravasation, which uncouples NO/Hb interaction and vasoconstriction. Size-selective compartmentalization of Hb functions as a substitute for red blood cells after hemolysis and preserves NO signaling in the vasculature. We found that evolutionarily and structurally unrelated Hb-binding proteins, such as PIT54 found in avian species, functionally converged with haptoglobin to protect NO signaling by sequestering cell-free Hb in large protein complexes.

Conclusions: Sequential compartmentalization of Hb by erythrocytes and scavenger protein complexes is an archetypical mechanism, which may have supported coevolution of hemolysis and normal vascular function. Therapeutic supplementation of Hb scavengers may restore vascular NO signaling and attenuate disease complications in patients with hemolysis.

Keywords: hemolysis; hemoglobin; extravasation; PIT54; haptoglobin

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At a Glance Commentary

Scientific Knowledge on the

Subject: Diseases and medical interventions leading to acute and chronic hemolysis can cause vasoconstriction, hypertension, and eventually cardiovascular complications resulting from nitric oxide (NO) depletion by cell-free Hb.

What This Study Adds to the

Field: This study demonstrates that extravasation of cell-free Hb into perivascular sites is a critical disease process resulting in Hb-induced vascular NO resistance and vasoconstriction. Hb compartmentalization within the vascular lumen by the plasma protein haptoglobin or by a structurally unrelated evolutionary decedent Hb-binding protein, PIT54, prevents extravasation and vasoconstriction. This NO-preserving function of haptoglobin suggests a therapeutic potential for vascular protection during active hemolysis.

The red blood cell (RBC) pool of an adult human contains more than 500 g Hb. The broad reactivity of Hb necessitates stringent control of its biochemistry to avoid adverse physiological effects (1, 2). In healthy individuals, this control is provided by the compartmentalization of Hb within RBCs. However, in patients with hemolysis such as in sickle cell disease (SCD), during extracorporeal circulation, on transfusion of stored blood, or during malaria and sepsis, substantial quantities of cell-free Hb are occasionally released into plasma (3). In these conditions, cell-free Hb can promote disease complications (4–11). Some of the most severe adverse effects associated with cell-free Hb exposure, including pulmonary and systemic hypertension, are caused by depletion of the vasodilator nitric oxide (NO) (12–16).

The toxicity of cell-free Hb and the diversity of hemolytic conditions exerted an evolutionary pressure that led to the development of protective systems. In most species haptoglobin (Hp) is the principal Hb scavenger protein in plasma (17, 18). Hp binds Hb $\alpha\beta$ -dimers within Hb:Hp complexes, which are cleared from the

blood by liver and spleen macrophages (19, 20). Hp inhibits Hb-triggered protein and lipid oxidation, whereas the large size of the Hb:Hp complex prevents renal Hb excretion and kidney injury (6, 21, 22). However, the most intriguing and presently unexplained protective function of Hp is its ability to attenuate hypertension that is triggered by cell-free Hb (6, 23–25). Because the NO reaction rates are comparable for Hb and the Hb:Hp complex (23, 26), we hypothesize that the large Hb:Hp complex may shield vascular NO by limiting cell-free Hb decompartmentalization from the vascular lumen to the sites of vascular NO activity.

An independent line of Hb-binding proteins evolved over time to exploit the ancient scavenger receptor cysteine-rich (SRCR) domain as a scaffold (17). More than 1,000 different SRCR domains provide molecular pattern recognition and ligand-binding functions in the innate immune systems of primitive species, such as the sea urchin (27, 28). In vertebrates, a more restricted set of SRCR domain proteins has acquired more specialized functions. Among these proteins, the acute-phase inducible plasma protein PIT54, which is composed of four repetitive SRCR domains, has been identified as an Hb-binding protein in birds (17, 29). Thus far, the protective functions of PIT54 have not been characterized. Nonetheless, its existence suggests that the toxicity of cell-free Hb may have driven independent evolution of redundant and structurally diverse Hb scavenger systems with overlapping functions.

In the current studies, we demonstrate that Hb binding by Hp restores vascular NO signaling during hemolysis, and we define a shared function of the structurally and evolutionarily unrelated Hb-binding protein PIT54. Collectively, our data illuminate an archetypical mechanism that may have supported coevolution of hemolysis and the vascular system and that may provide therapeutic options to restore NO signaling in patients with hemolysis.

Methods

Haptoglobin from human plasma (phenotype 2-2 predominant) was obtained from CSL Behring (Bern, Switzerland). The identity, purity, and function of the protein were confirmed

by liquid chromatography–tandem mass spectrometry (LC-MS/MS), polyacrylamide gel electrophoresis, and surface plasmon resonance as described (24). Hemoglobin was purified from outdated blood, as previously described (24). Hb concentrations were determined by spectrophotometry and are given as molar concentration of total heme (1 M Hb tetramer is therefore equivalent to 4 M heme). For all Hb used in these studies, the fraction of ferrous HbO₂ was always greater than 98%, as determined by spectrophotometry, unless stated otherwise. Recombinant PIT54 was expressed in HEK293 cells.

A detailed description of the materials and methods is included in the online supplement.

Results

Hp Enhances the NO Vasodilator Reserve during Intravascular Hb Exposure in Rats

Rats were infused with 35 mg of HbO₂ (n = 4) or HbO₂ bound to Hp (n = 4). In the first 10 minutes after infusion, the mean arterial pressure (MAP) increased more than 10% with Hb treatment but only 2.5% after infusion with Hb bound to Hp (Figure 1A). After 10 minutes, a single 5-mg bolus dose of the endothelial NO synthase (eNOS) inhibitor L-N^G-nitroarginine methyl ester (L-NAME) was administered. L-NAME increased MAP by 57 and 92% compared with the mean values after infusion with Hb and Hb:Hp, respectively (Figure 1B). The stronger MAP increase after L-NAME treatment of Hb:Hp-infused rats suggested that Hp preserved NO function in Hb-infused animals.

In a second model, acetylcholine (ACh) was infused into rats to maximize eNOS activity. Over 5 minutes of ACh infusion the MAP was reduced by approximately 40% (see Figure E1 in the online supplement). Under these conditions, a dose of 35 mg of cell-free Hb or Hb bound to Hp slightly increased MAP without a significant difference between the two groups. In both groups, MAP clearly persisted below baseline levels. However, on cessation of the ACh infusion, free Hb-exposed animals demonstrated a MAP increase to 30% greater than baseline, whereas Hb:Hp-exposed animals demonstrated only a 5% overshoot of basal mean values ($P < 0.01$).

Figure 1. Haptoglobin (Hp) enhances nitric oxide (NO)-mediated arterial dilation during Hb exposure. (A, left panel) Change in mean arterial blood pressure (MAP) of rats after injection of 35 mg cell-free Hb with or without Hp. Data show the mean $\pm$ SEM, $n = 4$. (A, right panel) Comparison of maximum MAP changes ($*P < 0.01$). (B, left panel) Ten minutes after the initial Hb $\pm$ Hp injection, the animals were treated with L- N^G -nitroarginine methyl ester (L-NAME), and MAP was monitored for a further 20 minutes to estimate the “NO reserve.” (B, right panel) Comparison of maximum L-NAME-induced changes in MAP in Hb $\pm$ Hp-pretreated rats ($*P < 0.01$). (C) Relative contractile response of porcine coronary arteries pretreated with prostaglandin F $_{2\alpha}$ (PGF $_{2\alpha}$) ($\pm N^G$ -[1-iminoethyl]-L-ornithine [L-NIO]) in response to a range of concentrations of Hb or Hb:Hp complexes (mean $\pm$ SEM of $n = 11$ –15 vascular rings) ($*P < 0.001$). (D, E) PGF $_{2\alpha}$ precontracted porcine coronary arteries were dilated with either the intracellular NO donor nitroglycerin (NTG) (D) or the extracellular NO donor DETA-NONOate (DETA) (E). Each NO donor was applied at two different concentrations, and the contractile response to various concentrations of Hb and Hb:Hp was measured (mean $\pm$ SEM of $n = 15$ vascular rings) ($*P < 0.001$). (F) Relative contraction of PGF $_{2\alpha}$ precontracted and nitroglycerin (10 μ M)-dilated porcine coronary arteries in response to a range of concentrations of ferrous HbO $_2$ (Fe $^{2+}$) or ferric Hb (Fe $^{3+}$) (mean $\pm$ SEM of $n = 24$ and 9 vascular rings for ferrous and ferric Hb, respectively) ($*P < 0.001$). (G) Original tension traces of three arterial rings (prepared from different porcine hearts) from an experiment as shown in D–F. At the end of the Hb dose response, albumin or Hp were added at a concentration of 32 μ M. Black arrows mark the serial addition of the indicated compounds in half-log $_{10}$ steps. For all arterial ring tension experiments, the baseline tension before PGF $_{2\alpha}$ treatment is considered 0%, and the tension at maximum PGF $_{2\alpha}$ response is considered 100%. Responses are therefore indicated as % PGF $_{2\alpha}$. n.s. = not significant.

Figure 2. Haptoglobin (Hp) uncouples nitric oxide (NO) depletion in solution and NO-cyclic guanosine 3',5'-monophosphate (cGMP) signaling function in the vascular wall during Hb exposure. (A) An example of a stopped-flow experiment of the reaction of HbO₂ ± Hb with NO. The traces show the normalized absorption changes at 406 nm (red: free Hb; blue: Hb:Hp complex), with HbO₂ in excess at pH 7.4. (B) NO depletion after injection with either Hb or Hb:Hp into an air-tight, oxygen-free reaction chamber in which NO was continuously produced by DETA-NONOate decay in phosphate-buffered saline (pH 7.4). NO was measured online in the carrier gas phase with an ANTEK chemiluminescence NO detector. Depletion by Hb

These data suggest that maximal endogenous NO production could reverse the hypertensive effect of cell-free Hb but that Hp is critical to preserve vasodilator NO signaling under physiologic conditions.

Hp Blocks the Vasoconstrictor Activity of Hb in NO-dilated Porcine Coronary Arteries

Next, we studied the effects of HbO₂ and HbO₂ plus Hp on vascular tone in porcine coronary arteries *ex vivo*. In a first set of experiments, coronary artery rings were precontracted with prostaglandin F₂α (PGF₂α). The precontracted arteries were subsequently exposed to increasing concentrations of Hb (Figure 1C, red symbols) or Hb:Hp (Figure 1C, white symbols) in the absence or presence of the eNOS inhibitor N⁵-(1-iminoethyl)-L-ornithine (L-NIO). Free Hb, but not Hb:Hp, caused dose-dependent contractions of the arteries. When endogenous NO was depleted before Hb exposure by pretreatment with L-NIO, the additional contraction induced by cell-free Hb was significantly attenuated. These findings

Figure 2. (Continued). was set at 100% and was not statistically different from depletion by Hb:Hp. (C) The NO-mediated dilatory response of prostaglandin F₂α (PGF₂α) precontracted porcine coronary artery segments was measured after injection of MAHMA-NONOate boluses (30 nM) into the immersion buffer. Experiments were performed in buffer only, with Hb (32 μM) or with Hb:Hp (32 μM). The traces show mean ± SEM of at least 15 averaged responses recorded in three independent experiments. In parallel to the vascular response, the NO concentration was measured in vessel immersion buffer using an NO-sensitive microelectrode. The red arrows indicate addition of NO donor. (D) MAHMA-NONOate induced vascular dilation in the presence of intact red blood cells (RBCs) (32 μM Hb). No dilation was observed after lysis of RBCs (32 μM Hb) or with intact RBCs in the presence of the guanylate-cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM). The experiment was identical to that in C. Red arrow indicates the time point of MAHMA-NONOate injection. (E) MAHMA-NONOate induced vascular dilation in the presence of lysed RBCs (32 μM Hb) + Hp. The cGMP inhibitors ODQ (10 μM) and NS2028 (10 μM) blocked the NO dilatory response. The experiment was identical to that in C. Red arrow indicates the time point of MAHMA-NONOate injection. Traces in D and E represent mean ± SEM of at least 15 averaged responses recorded in three independent experiments. n.s. = not significant.

suggest that Hp may preserve dilatory signaling of endogenous NO during cell-free Hb exposure.

To directly explore the potential NO-preserving function of Hp, we examined the effects of Hb and Hb:Hp on arteries that were precontracted with PGF2 α and subsequently dilated to baseline tension with exogenous NO derived from chemical NO donors. We used either the intracellular NO donor nitroglycerin or the extracellular NO donor DETA-NONOate. The cell-free Hb-mediated contraction of the NO-dilated arteries was dose dependent and inversely correlated with the level of NO generated by different NO donor concentrations (Figures 1D and 1E, *red symbols*). This result indicates that the Hb-induced contraction in our system is a direct result of the stoichiometric consumption of NO by HbO₂, which only occurs with NO-reactive ferrous HbO₂ (Fe²⁺) but not ferric Hb (Fe³⁺) (Figure 1F). The addition of Hp almost completely blocked the Hb-mediated contractile response (*white symbols*). Furthermore, addition of Hp, but not addition of albumin, rapidly and fully reverted the Hb-induced contraction (Figure 1G). In an Hb-free system, Hp had no dilatory activity on PGF2 α precontracted arteries (data not shown).

Hp Uncouples NO/Hb Interaction and Vasoconstriction

Earlier studies have suggested that Hp does not affect the NO dioxygenation reaction of bound Hb (23, 26). To assess the validity of these observations in our system, we measured the dioxygenation kinetics of free HbO₂ and HbO₂:Hp complexes with NO by stopped-flow spectroscopy under pseudo-first-order conditions (Figure 2A). Averaged kinetic traces measured at 406 nm were fitted to an exponential curve with a second-order rate constant of $79.2 \pm 4 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ and $66.7 \pm 6 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ for free Hb and Hb:Hp, respectively (means of eight experiments). Our rate constant for free Hb is comparable to those previously published (15, 30). When we directly measured the NO consumption by cell-free HbO₂ and the Hb:Hp complex with a chemiluminescence NO analyzer, we found no statistically significant differences (Figure 2B). This finding suggests that, despite the slightly lower rate constant of the Hb:Hp complex, the two compounds may equally deplete NO in a physiologic

system. The assumption that the small (15%) difference in the reaction rates of Hb and the Hb:Hp complex could not explain the drastically reduced vasoconstrictor activity of the complex is also in agreement with findings reported in previous studies of heme-pocket mutated Hbs, which demonstrated that a greater reduction of the NO dioxygenation rate is necessary to significantly reduce vasopressor activity *in vivo* (31).

In the next set of experiments, we measured (in parallel) the vasodilatory activity of an NO donor and the accumulation of NO in the buffer solution. For this experiment an NO-sensitive electrode was placed in proximity (≈ 1 mm) to the monitored artery segments. NO was generated *in situ* after injection of the short-lived NO donor MAHMA-NONOate into the buffer solution. In the absence of Hb, repeated addition of MAHMA-NONOate caused bursts of NO accumulation in the buffer, which were accompanied by transient vascular dilations (Figure 2C, *left panel*). When Hb (32 μM) was added, no accumulation of NO and no vascular dilation were observed after addition of MAHMA-NONOate (Figure 2C, *middle panel*). In the presence of the Hb:Hp complex (32 μM), NO accumulation was still not detectable after addition of the NO donor. However, despite complete NO consumption in the buffer, a strong vasodilator response could be recorded in arteries exposed to Hb:Hp. This response was similar to the MAHMA-NONOate-induced vasodilator response observed in the control experiments. Hp also reverted Hb-induced NO resistance of dog coronary arteries, which were used in some experiments to confirm that the observed effects were not restricted to certain animal species (Figure E2).

These findings suggest that Hp uncouples the reaction of cell-free Hb-mediated NO consumption in solution from additional processes that are required to cause vascular NO resistance.

Hp Substitutes an RBC Function to Preserve Vasodilator NO-Cyclic Guanosine 3',5'-Monophosphate Signaling during Hemolysis

MAHMA-NONOate-inducible vascular dilations could also be induced in the presence of intact RBCs (Figure 2D). These dilations were blocked by the cyclic guanosine 3',5'-monophosphate (cGMP) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μM). Although lysis of the RBCs

induced complete NO resistance (Figure 2D), addition of Hp to the lysed RBCs restored the vasodilator response (Figure 2E). This experiment illustrates that Hp can substitute an essential NO-sparing function of RBCs during hemolysis. The vasodilator response in the presence of the Hb:Hp complex could also be blocked by the guanylate cyclase inhibitors ODQ (10 μM) and NS2028 (10 μM), suggesting that Hp preserved the classic NO-cGMP signaling pathway in the presence of cell-free Hb.

Formation of a Large Protein Complex Determines Vascular NO Preservation by Hp

We hypothesized that the ability of Hp to restore vascular NO signaling during cell-free Hb exposure could be related to the large molecular size of the complex, which may restrict access of free Hb to vascular smooth muscle cells. Moreover, we postulated that the large size of the Hb:Hp complex would be the principal determinant of vascular NO protection. To further explore this hypothesis, we attempted to mimic an Hp-like activity by conjugating Hb and albumin with chemical groups that rapidly and selectively react ("click") with each other in the tetrazine-trans-cyclooctene (TCO) ligation reaction to form covalent crosslinks between the two proteins (32, 33). In this chemical reaction system, the functionalized albumin should mimic the role of Hp as an Hb scavenger without exhibiting any of the Hp-typical structural features. The reaction of TCO-functionalized Hb with tetrazine-functionalized albumin resulted in large crosslinking products, which retained NO-depleting activity in solution (Figure E3). PGF2 α -precontracted and NO-dilated porcine coronary arteries contracted equally after exposure to either unmodified Hb or Hb-TCO. However, when tetrazine-functionalized albumin was added to the Hb-TCO-exposed (contracted) arteries, the vessels dilated, indicating that NO signaling was restored. This dilation was comparable to that observed when Hp was added to bind cell-free Hb (Figure 3B). No dilation occurred if either Hb or albumin was not click-functionalized (Figure 3A).

To further support the role of a size-selective mechanism, we compared the effects of free Hb and the Hb:Hp complex with the vasoactivity of glutaraldehyde-polymerized Hbs ranging in molecular size from ≈ 8 to

Figure 3. Large molecular-sized protein complex formation restores dilatory nitric oxide (NO) signaling during Hb exposure. (A) Dilatory response after *in situ* polymerization of Hb. The plot shows the dilatory response of porcine coronary arteries that were sequentially pretreated with prostaglandin F2 α (PGF2 α), nitroglycerin (NTG), and Hb, resulting in an Hb-mediated constricted state. At this point (time = 0 s) the albumin component was added. Hb polymerization and dilation were only observed when both protein components were click-functionalized to participate in the tetrazine-trans-cyclooctene (TCO) ligation reaction. The plot represents averaged tension traces of six experiments $\pm$ SEM (gray lines). (B) Identical experiment as in A, with the exception that haptoglobin (Hp) was added instead of (functionalized) albumin at time 0 s, resulting in an identical dilatory response. (C) Dose-dependent contractile response of PGF2 α - and NTG-pretreated porcine coronary artery segments during exposure to increasing concentrations of unmodified and glutaraldehyde-polymerized bovine Hbs. The number indicates the degree of polymerization (glutaraldehyde to protein ratio), whereas T/R indicates the Hb conformational state. Data represent mean $\pm$ SEM of at least 13 independent dose-response experiments per compound ($P < 0.001$). (D) Correlation plot of the molecular size of glutaraldehyde-polymerized Hb versus [1/(maximum contraction at Hb 10 $^{-5}$ M) $\times$ 100]. An analysis of variance for this data set can be found in the online supplement. n.s. = not significant.

≈ 25 nm (Figure 3C). These experiments revealed that the molecular sizes of the different Hb molecules negatively correlated with the constrictive response (Figure 3D and Table E1). In an analysis of variance model, which considered molecular size, NO reaction rates, and T- versus R-state conformation of free Hb, the Hb:Hp complex, and all tested polymerized Hbs as variables, only the molecular size was found to significantly contribute to the variation in vascular contraction that was induced by the different compounds (Table E2).

Hp Blocks Hb Translocation into Interstitial Tissue Spaces

Our functional studies support the hypothesis that sequestration of cell-free Hb in large protein complexes could prevent cell-free Hb distribution to tissue compartments of NO production and/or activity. Therefore, to test this hypothesis, we infused anesthetized rats with Hb-TCO to achieve an intravascular Hb exposure of 50 μ M Hb over 60 minutes. Hb-positive tissue regions were visualized by “click” reaction with Cy5-tetrazine. The covalent and site-specific conjugation reaction between Hb-TCO and Cy5-tetrazine supported specific and sensitive detection of Hb in tissues. We tested this labeling strategy in kidney sections (Figure 4A). No Cy5 signal was detected in control tissues or in tissues obtained from animals perfused with Hb that was functionalized with another tag (Hb-azide) unable to react with tetrazine. In contrast, the Cy5-tetrazine reaction in renal tissue from Hb-TCO-perfused animals exhibited a strong fluorescence signal (red) in the renal cortical region. Hb accumulation was markedly reduced when Hp was added to the Hb-TCO perfusion.

In the myocardium of Hb-TCO-infused and Hp-treated animals, we found strong Cy5 fluorescence (red-yellow) confined to the lumen of small capillaries (Figure 4B, lower image). However, in the absence of Hp, a very strong Cy5 signal could be observed in the interstitial spaces around cardiomyocytes (Figure 4B, upper image). This finding reflected extravasation of cell-free Hb, which occurred only in the absence of Hp. Similar Hb distribution was also detected in muscular coronary arteries. In the absence of Hp, a strong signal was detected in the interstitial regions throughout the

Figure 4. Extravascular cell-free Hb translocation in rats. Rats were infused with Hb-trans-cyclooctene (TCO), and Hb was visualized on tissue slides by the Cy5-tetrazine-TCO ligation reaction. (A) Renal sections. Red indicates the intensity of Cy5 fluorescence in Hb-TCO or Hb-TCO-haptoglobin (Hp)-exposed kidneys. Gray shows a superimposed bright-field image. (B) Myocardial sections from Hb-TCO $\pm$ Hp-infused animals. Red-yellow illustrates the intensity of the Cy5 channel (Hb); the gray image shows a superimposed bright-field image of the hematoxylin and eosin (H&E)-stained tissue. The white arrowhead in the zoom images indicate a capillary with Hb-TCO in plasma and nonfluorescent red blood cells (original magnification, $\times 250$).

vascular smooth muscle layer. Extremely weak Cy5-Hb-TCO signal was detected in Hb-TCO-perfused animals treated with Hp (Figure 5).

We also examined whether Hb may distribute into the tissues associated with porcine coronary arteries. Hearts were perfused *ex vivo* through the left main coronary artery with cell-free Hb in the absence or presence of Hp for 1 hour. The photograph in Figure 6A shows left main coronary artery segments ($n = 4$ per group) placed in glass tubes with the endothelial layer facing the glass surface (“inside-out”).

The discriminating brownish color, which was most intense in the arteries from Hb-perfused hearts, suggested extravasation of Hb into the vascular wall. Accumulation of Hb was not evident in Hb:Hp-perfused or control hearts.

Next, we perfused hearts with Hb-TCO with or without Hp. Figure 6B shows a gel analysis of coronary artery lysates that were reacted with Cy5-tetrazine before gel separation. The Cy5-signal (yellow) that associated specifically with the Hb monomer at 15 kD was significantly more intense in samples obtained from

Hb-TCO-perfused hearts than in control or Hb-TCO + Hp-perfused hearts. A comparably decreased Hb accumulation was observed when porcine hearts were perfused with large molecular weight polymerized Hbs (Figure E4). Hb-TCO was also visualized by reaction of tissue sections with Cy5-tetrazine (Figure 6C). Hb-TCO was found in the muscular layer of the large pericardial coronary arteries. The area-normalized Cy5 signal was quantified in the region between the internal and external elastic membranes by a blinded investigator. A significantly greater quantity of Hb accumulated in the smooth muscle layer of coronary arteries from Hb-TCO-perfused hearts compared with that in Hb-TCO + Hp-perfused hearts (Figure 6D).

Finally, Figure 6E shows representative electron paramagnetic resonance (EPR) spectra that were recorded at 6 K from samples shown in Figure 6A. A clear ferric heme-iron signal around $g = 6$, which was more intense in Hb-perfused arteries relative to that pertaining to control or Hb:Hp-perfused arteries, was detected. The concentration estimate of the ferric high-spin heme in the vascular wall was approximately $6 \mu\text{M}$, which corresponds to 3% of the perfusate Hb concentration. Although this suggests that a fraction of the ferrous HbO_2 in the perfusate is ultimately oxidized to ferric Hb in the vascular wall, we cannot calculate the total amount of Hb translocated into the arterial wall by this method because ferrous Hb is EPR silent.

Hb Sequestration and Protection of Vascular NO Signaling by PIT54

To support the assertion that Hb sequestration in large protein complexes could be a very fundamental and evolutionarily redundant mechanism to prevent cell-free Hb-induced vascular NO resistance, we examined whether the SRCR Hb-binding protein PIT54 shared the NO-shielding mechanism of Hp (Figure 7A). Surface plasmon resonance experiments demonstrated that chicken Hb (cHb), but not human Hb, binds to recombinant PIT54 with a high affinity ($K_d = 0.37 \mu\text{M}$), although it did not show the irreversible complex formation that can be observed with Hp (Figure 7B). Size exclusion chromatography demonstrated that a stable, large cHb-PIT54 complex was

Figure 5. Cell-free Hb translocation in the smooth muscle layer of rat coronary arteries. Coronary artery sections from Hb-trans-cyclooctene (TCO) $\pm$ haptoglobin (Hp)-treated animals. The different images pertain to two different animals per treatment. Images on the *left* show hematoxylin and eosin (H&E) staining of tissues, whereas those on the *right* show the superimposed Cy5 channel intensity (Hb-TCO) acquired on the identical sections (original magnification, $\times 100$).

formed under physiological conditions (Figure 7C). As with the Hb:Hp complex, PIT54 complex formation only minimally reduced the NO reaction rate of cHb (Figure 7D). The NO dioxygenation rate constant for cHbO₂ of $81.6 \pm 0.1 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ is almost identical to the rate of human HbO₂. The NO dioxygenation rate constant of the cHbO₂:PIT54 complex was $66.9 \pm 5 \mu\text{M}^{-1} \cdot \text{s}^{-1}$.

cHbO₂ at 10 μM completely suppressed the NO-dependent dilation of porcine coronary artery segments, which was induced by injection of MAHMA-NONOate into the buffer solution. Analogous to the effect of Hp, addition of 15 μM PIT54 restored NO sensitivity almost to the extent observed in control experiments (Figures 7E and 7F).

The cGMP inhibitor ODQ could block the dilatory response that occurred in the presence of the cHb-PIT54 complexes.

Discussion

Our findings support the hypothesis that extravasation of cell-free Hb is a critical disease process in hemolysis, which ultimately leads to interference of Hb with vascular NO signaling. Moreover, we demonstrated that sequestration of Hb within large protein complexes that block the extravasation is sufficient to restore vascular NO function. The archetypical Hb scavenger proteins Hp and PIT54 thus represent evolutionarily independent developments to extrapolate the protective

paradigm of Hb compartmentalization beyond RBCs.

Our *in vivo* experiments in rats suggested that Hp limits arterial blood pressure increases during Hb infusion by preserving NO signaling in the systemic circulation. Although Hp only marginally slowed the NO reaction with HbO₂, it blocked constriction of NO-dilated coronary artery segments exposed to cell-free HbO₂ *ex vivo*. Moreover, we found that addition of Hp fully reversed Hb-induced vasoconstriction, indicating that functional NO signaling can be rapidly restored if the translocation process of HbO₂ into the vascular wall is interrupted. In more specific *in vitro* experiments, we demonstrated that Hp uncoupled the two processes of vasodilatory NO signaling in the vascular wall and NO consumption by cell-free Hb in solution. We could demonstrate that this Hp activity is solely determined by the large molecular size of the complex. The established antioxidant functions of Hp may further enhance its vasoprotective activity in certain pathophysiologic conditions (34–36).

NO consumption by cell-free Hb has been studied as a disease-related pathway in patients with SCD (12, 13, 37, 38). Prior research has indicated that chronic NO depletion may cause pulmonary hypertension in some patients with SCD having more severe hemolysis, and recent clinical data have reinforced this concept (39, 40). Most extant studies have focused on NO-consuming reactions of cell-free Hb in plasma, particularly within the RBC-free zone adjacent to the luminal surface of the endothelium. Our data further extend these concepts by demonstrating that cell-free Hb can also redistribute directly into the smooth muscle cell compartment of the arterial wall as well as into other extravascular spaces, such as the myocardium. Leakage of cell-free Hb into tissue interstitial spaces may also be a fundamental mechanism underlying other hemolysis-driven processes, such as Hb-triggered tissue oxidation and inflammatory signaling (37–40), which may be much more pronounced in tissues having lower heme- and radical-buffering capacity than does blood.

NO consumption is also reported to be a harmful consequence of Hb-based oxygen carrier blood substitutes (31, 41–44). Polymerized or conjugated Hb molecules, with larger molecular sizes but comparable NO and O₂ reaction kinetics, were shown

Figure 6. Extravascular cell-free Hb translocation in porcine coronary arteries. (A) Porcine hearts ($n = 4$) were perfused with buffer with or without Hb and haptoglobin (Hp). Coronary artery segments were placed inside-out into glass tubes. Images show a view of the endothelial surface with slight red discoloration of Hb-perfused, but not of control or Hb:Hp-perfused, arteries. The lower panel shows a color-deconvoluted image, whereby the most discriminating color is extracted and quantified. The right panel shows quantitative data from the color-deconvoluted image. (B) Detection of fluorescent Hb-trans-cyclooctene (TCO) in lysates from coronary artery segments after reaction with Cy5-tetrazine and polyacrylamide gel electrophoresis separation. Blue fluorescence channel indicates total protein; yellow-orange indicates the Cy5-tetrazine (Hb) signal. Right: Quantitative analysis of Hb-TCO detection in lysates from coronary arteries. The box plots represent data from 12 perfused porcine hearts per condition. Cy5 channel intensities were corrected for total protein intensities. (C) Visualization of Hb-TCO in porcine coronary arteries after conjugation with Cy5-tetrazine (yellow-orange). The white color indicates the orcein positive staining of the internal (*) and external (**) elastic membranes. The scale bar is 200 μm ; original magnification $\times 100$. (D) Quantitative image analysis of the intensity of Cy5 fluorescence. Data represent four different coronary arteries with 10 slides per artery. (E) Exemplary electron paramagnetic resonance traces of coronary arteries from control (ctrl) and Hb $\pm$ Hp-perfused porcine hearts showing a typical ferric Hb(Fe^{3+}) signal. AU = arbitrary units.

to have less pronounced vasopressor effects (16, 45). Although extravasation of smaller Hb compounds or Hb-based oxygen carrier fragments has been postulated as an explanation for these observations, translocation of cell-free Hb into the vascular wall has not been extensively

documented (16). This lack of evidence may be related to methodological difficulties associated with Hb tracking *in vivo*. In our study, we exploited an indirect labeling technique with small, biochemically inert click-chemistry tags that allowed us to track free Hb and

Hb:Hp complexes *in vivo*. Our findings demonstrate that, in the absence of Hp, free Hb extravasates into the kidneys and the interstitial spaces of the myocardium as well as into the muscular layer of coronary arteries where local NO consumption would be expected to cause vasoconstriction. In

Figure 7. PIT54 sequesters chicken Hb in a large protein complex and restores the nitric oxide (NO) dilatory vascular response during Hb exposure. (A) Gene organization of PIT54 on chicken chromosome 4. The soluble protein consists of four scavenger receptor cysteine-rich domains. (B) Binding of human Hb (hHb) and chicken Hb (cHb) to PIT54 or human Hp immobilized on a Proteon SPR chip. (C) Size exclusion high-performance liquid chromatography shows that cHb and PIT54 form a large protein complex eluting at 17.8 minutes when mixed under physiologic conditions. (D) An example of a stopped-flow experiment of the reaction of chicken HbO₂ ± PIT54 with NO. The curves show the normalized absorption changes at 406 nm (red: free cHb; blue: cHb:PIT54 complex). (E) The NO-mediated dilatory response of prostaglandin F_{2α} (PGF_{2α}) precontracted porcine coronary artery segments was measured after injection of MAHMA-NONOate boluses into the immersion buffer. Experiments were performed in buffer only, or with 10 μ M cHb, with or without 15 μ M PIT54. The PIT54-dependent NO dilatory response was blocked by the cGMP inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). The traces show mean $\pm$ SEM of averaged responses obtained with nine arterial rings from three porcine hearts. The red arrows indicate addition of NO donor. (F) Scatterdot plot of dilatory responses (maximum dilation per response) across all experimental conditions. DMBT1 = deleted in malignant brain tumors 1; Hp = haptoglobin.

contrast, when Hp was available, tissue extravasation was blocked, and Hb remained sequestered in the vasculature. These data were supported by the demonstration of

extravasation of untagged Hb in *ex vivo* perfused porcine hearts and by the detection of Hb by EPR in the vascular wall. Within the concentration range of

free Hb tested in our studies, which may represent most hemolytic conditions, the vascular NO sparing effect of the Hb:Hp complex was comparable to that provided

by the compartmentalization function of normal RBCs. Of course, it is likely that at higher Hb concentrations approaching the physiologic Hb concentration in blood of ≈ 15 g/dL, other characteristics that are unique to RBCs, such as the cell-free zone in the periphery of the blood flow, become increasingly important (46).

Intriguingly, we found that the evolutionarily and structurally unrelated plasma Hb-binding protein PIT54 provided the same mechanism for restoring vascular NO signaling via sequestration of cell-free Hb in a large protein complex. Given these observations, it is likely that the harmful effects of cell-free Hb promoted independent evolutionary processes that ultimately merged toward a single mechanism, recapitulating the concept of Hb compartmentalization within the intravascular space. In humans, another evolutionary event resulted in three primary Hp phenotypes with dimeric (Hp 1-1) and multimeric (Hp 2-1 and Hp 2-2) structure

and differing molecular weights. Studies presented here are restricted to the multimeric Hp phenotype. However, based on earlier studies, in which we confirmed that monomeric and multimeric have identical activity against Hb-triggered hypertension, we do not assume that this polymorphism would significantly affect the compartmentalization function of Hp (24).

Compartmentalization of Hb in the Hb:Hp complex extends the plasma half-life of the complex in animal models compared with free Hb because renal elimination is blocked (34). It is therefore possible that Hp-facilitated clearance of the Hb:Hp complex by the scavenger receptor CD163 occurred as a secondary evolutionary development to provide a nonrenal Hb clearance pathway and heme-iron recovery by macrophages (20).

In conclusion, we found that the balance of compartmentalization and extravascular translocation of cell-free

Hb determines deregulation of vascular NO signaling during hemolysis. Control of these fundamental disease-defining processes is provided by evolutionarily redundant protection systems, which support compartmentalization of cell-free Hb in the intravascular space. Supplementation of plasma-purified or recombinant Hp, or analogous Hb scavengers, may support vascular NO homeostasis and prevent vascular disease complications in patients with hemolysis. ■

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