Hemopexin Therapy Improves Cardiovascular Function by Preventing Heme-Induced Endothelial Toxicity in Mouse Models of Hemolytic Diseases

Running title: Vinchi et al.; Hemopexin therapy in hemolytic diseases

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Abstract:

Background—Hemolytic diseases are characterized by enhanced intravascular hemolysis resulting in heme-catalyzed reactive oxygen species (ROS) generation, that leads to endothelial dysfunction and oxidative damage. Hemopexin (Hx) is a plasma heme scavenger able to prevent endothelial damage and tissue congestion in a model of heme overload. Here, we tested whether Hx could be used as a therapeutical tool to counteract heme toxic effects on the cardiovascular system in hemolytic diseases.

Methods and Results—By using a model of heme overload in Hx-null mice, we demonstrated that heme excess in plasma, if not bound to Hx, promoted the production of ROS and the induction of adhesion molecules and caused the reduction of nitric oxide (NO) availability.

Then, we used β-thalassemia and Sickle Cell Disease (SCD) mice as models of hemolytic diseases to evaluate the efficacy of a Hx-based therapy in the treatment of vascular dysfunction related to heme overload. Our data demonstrated that Hx (i) prevented heme-iron loading in the cardiovascular system, thus limiting the production of ROS as well as the induction of adhesion molecules and the oxidative inactivation of NOS/NO, and (ii) promoted heme recovery and detoxification by the liver mainly through the induction of Heme Oxygenase (HO) activity.

Moreover, we showed that in SCD mice, endothelial activation and oxidation were associated with increased blood pressure and altered cardiac function and the administration of exogenous Hx was found to almost completely normalize these parameters.

Conclusions—Hemopexin treatment is a promising novel therapy to protect against heme-induced cardiovascular dysfunction in hemolytic disorders.

Key words: heme, hemolytic disorders, oxidant stress, endothelial dysfunction, cardiac dysfunction
Introduction

Hemolytic diseases characterized by intravascular hemolysis are associated with a state of endothelial dysfunction, leading to vasomotor instability and ultimately producing a proliferative vasculopathy\(^1\). Enhanced expression of adhesion molecules on the endothelial wall, high levels of circulating pro-inflammatory cytokines and activated leukocytes, pro-oxidant stress and coagulopathy have been reported in patients suffering from several hemolytic diseases, including paroxysmal nocturnal hemoglobinuria, sickle-cell disease (SCD), thalassemias, and hereditary spherocytosis\(^2\)\(^-\)\(^4\). Oxidative stress plays a central role in promoting vascular inflammation, primarily through the induction of adhesion molecules on the vascular endothelium and the promotion of monocyte and neutrophil activation\(^5\)\(^,\)\(^6\).

Heme represents a major source of reactive oxygen species (ROS) in hemolytic patients. Because of enhanced rates of red blood cells (RBC) hemolysis, the endothelium of these patients is exposed to high levels of ROS catalyzed by plasma hemoglobin, heme and free iron\(^7\)\(^,\)\(^8\). High levels of ROS lead to lipid, protein and DNA damage and eventually to cell death and favour endothelial activation\(^6\)\(^-\)\(^9\) and leukocyte recruitment, thus promoting a chronic inflammatory state\(^10\). In these hemolytic anemias, the presence of circulating free hemoglobin that avidly buffers the nitric oxide (NO) and the generation of ROS that oxidatively inactivate NO, results in reduced bioavailability of the main bio-vasodilator NO and abnormal vascular homeostasis, leading to endothelial dysfunction\(^1\)\(^,\)\(^11\).

Mammals have evolved a tissue and vasculo-protective program of heme metabolism that includes the plasma hemoglobin/heme scavengers haptoglobin (Hp) and hemopexin (Hx) and the cellular enzyme heme oxygenase (HO)-1\(^12\)\(^-\)\(^14\). Hp and Hx, by binding with high affinity hemoglobin and heme respectively, block their pro-oxidant effect. HO-1 degrades the heme ring...
into iron, carbon monoxide (CO) and biliverdin, thus exerting primary anti-inflammatory, anti-oxidant and anti-apoptotic effects. In hemolytic diseases, the high rate of hemolysis results in the saturation and depletion of the plasma hemoglobin/heme scavenging systems and leads to a buildup of hemoglobin and heme in the circulation that mediates pro-oxidant and pro-inflammatory effects on vessel endothelial cells.

Although many mechanisms contribute to the complex pathophysiology of hemolytic diseases as SCD and β-thalassemia, a unifying theme is represented by the dysfunction of the vascular endothelium and the high pro-oxidant plasma environment.

SCD is characterized by recurring episodes of painful vaso-occlusion, leading to ischemia/reperfusion injury and organ damage. Endothelial dysfunction, inflammation as well as activated monocytes, neutrophils, platelets and dense red cells all contribute to sickle cell crisis. β-Thalassemia is frequently complicated by thromboembolic events due to coagulation abnormalities and damaged red cells exposing phosphatydyl-serine to which endothelial activation and oxidative stress strongly contribute. Moreover, severe forms of SCD and β-thalassemia require a blood transfusion regimen that further increases the amount of circulating hemoglobin/heme, thus exacerbating oxidative stress. Although an iron chelation therapy is routinely associated to a transfusion regimen, no heme chelation therapy has been developed to date, to specifically prevent heme-induced endothelial damage and oxidative stress.

We previously showed that heme-overloaded Hx-null mice suffered from endothelial damage and vascular congestion thus highlighting the critical role of Hx in preventing vascular damage. Therefore we hypothesized that Hx could be administered as a drug in hemolytic diseases to prevent heme-driven endothelial dysfunction and oxidative injury. To test this hypothesis, we chose two mouse models of SCD and β-thalassemia, and we evaluated the...
effect of an Hx-based therapy in these animals.

Our findings support the hypothesis that the replenishment of the plasma Hx pool by exogenous Hx administration is beneficial in preventing endothelial dysfunction and ameliorating the vasculopathy in hemolytic disorders.

Materials and Methods

Mice treatment

Hx-null mice, knock-in HbS SCD mice and Hbb\textsuperscript{th1/th1} \(\beta\)-Thalassemia mice were previously described\textsuperscript{30-33}. SV129 wild-type and Hx-null mice were injected into the tail vein with 30\(\mu\)mol/kg freshly prepared hemin. Hx treatment in SCD and \(\beta\)-thalassemic mice was performed by injecting ip 700\(\mu\)g of purified human Hx (Athens Research, Georgia, USA) twice a week for one month, from 1 month of age. All experiments were approved by the animal studies committee of the University of Torino (Italy).

Cells treatment

Hemin Chloride was dissolved in DMSO to obtain a 4 mM stock solution, diluted and mixed 1:1 with human serum albumin (Sigma-Aldrich) or Hx. HUVECs and primary hepatocytes were treated with heme-albumin or heme-Hx 5 to 10 \(\mu\)M in culture medium.

Heme and Iron content

Heme content in tissues and bile was quantified fluorometrically by the method of Sassa. Tissue non-heme iron content was determined by a colorimetric method using 4,7-diphenyl-1, 10-phenanthroline disulphonic acid (BPTS; Sigma) as chromogen.

Lipid Peroxidation Assay

Lipid peroxidation from tissue extracts was measured using the colorimetric assay kit Bioxytech.
LPO-586 from Oxis International (Portland, OR)\textsuperscript{29}, according to the manufacturer’s instructions.

**Heme Oxygenase activity assay**

HO activity was measured by spectrophotometric determination of bilirubin produced from hemin added as substrate.

**ROS Production Assay**

Accumulation of ROS in HUVECs and aortic rings was assessed by using the fluorescent oxidant-sensitive dye 2,7'-dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCFDA; Molecular Probes, Eugene, OR).

**Annexin V/propidium iodide staining**

After treatment, HUVECs were double stained with fluoresceinisothiocyanate-conjugated annexin V and propidium iodide (PI) for 15 min at room temperature and then analyzed by flow cytometer.

**NOS Activity Assay**

Activity of NOS in liver and aorta extracts was determined by monitoring the conversion of L-[\textsuperscript{3}H]arginine to L-[\textsuperscript{3}H]citrulline, by using NOS Activity Assay Kit, N.781001, Cayman Chemical Company. The reaction was performed following the manufacturer’s instructions. Results were expressed as pmol citrulline/min/g tissue.

**Blood Pressure Measurement**

Heart rate, systolic, diastolic and mean blood pressure were measured in conscious mice by using a non-invasive computerized tail cuff system (CODA, Kent Scientific Corporation).

**Echocardiography**

Mice were anesthetized with 1% isoflurane and analyzed with a Vevo770 High Resolution Imaging System (Visual Sonics Inc, Toronto, Ontario, Canada). Echocardiographic parameters
were measured in the long-axis M mode. Cardiac function was assessed when heart rate was 500 to 600 bpm.

**Statistical Analysis**

Results were expressed as mean ± SEM. Comparisons between two groups were performed with two-sided Welch t-tests and among more than two groups with one- or two-way Anova (with repeated measures when the same mice are measured in different conditions) followed by Bonferroni post-test. Specific comparisons were performed by reverting to t-tests and adjusting the P-values with Bonferroni correction. In Table S1 the statistic test and the number of comparisons used per each panel of each figure are reported. A P value of less than 0.05 was considered significant.

Further details on Methods are reported in Supplemental Materials and Methods

**Results**

**β-thalassemic and SCD mice show vascular dysfunction associated to Hemopexin depletion and serum heme overload**

Both β-thalassemia and SCD patients experience a condition of vascular dysfunction\(^1\). We confirmed that this occurred also in mouse models of these diseases. Both β-thalassemic and SCD mice showed evident signs of increased endothelial activation, enhanced oxidative stress, reduced NO bioavailability and inflammation (Figure 1a-d), hallmarks of vascular dysfunction. The phenotype of thalassemic and SCD mice resembles that of heme-overloaded Hx-null mice, showing increased endothelial activation and oxidative stress, altered vascular permeability, inflammation\(^29\) and vascular anti-oxidant response hyperactivation (not shown). The comparison suggests that in β-thalassemia and SCD, heme overload consequent to hemolysis and plasma
hemoglobin and heme scavengers consumption may contribute to the observed vasculopathy and indicates that, although these pathologies have specific clinical outcomes, they share hemoglobinemia-related sequelae\textsuperscript{2}. Consistently, in the serum of both thalassemic and SCD mice, Hp and Hx were almost completely depleted (Figure 1e), as occurs in human patients\textsuperscript{17}. This was associated with a significant increase in serum heme levels (Figure 1f, thalassemic: 75 vs 20\textmu M; SCD: 110 vs 55\textmu M).

**The lack of Hemopexin favours heme loading in the vascular endothelium**

To understand how heme overload consequent to Hx consumption may concur to vascular dysfunction, we took advantage of the model of heme-overloaded Hx-null mice that, as reported in the previous section, mimics Hx depletion and serum heme overload occurring in \(\beta\)-thalassemia and SCD.

The quantification of heme content in the aortas from wild-type and Hx-null mice intravenously injected with 35 \textmu mol/kg hemin revealed that in the absence of Hx, a greater amount of heme accumulated in vessels (Figure 2a). Consistently, the heme degrading enzyme HO-1 as well as L-Ferritin and Ferroportin (FPN), involved in heme-derived iron storage and export respectively, were induced to an higher extent in the aorta of heme-treated Hx-null mice than in that of wild-type counterpart (Figure 2b-d). Heme overload in vessels of Hx-null mice was associated to an enhanced induction of VCAM-1 and a strong increase in intracellular ROS (Figure 2e-g).

NOS activity was significantly reduced in aortas from heme-treated Hx-null mice but not in wild-type counterpart, suggesting a reduced NO production in Hx-null endothelium (Figure 2h). Moreover, nitrotyrosine formation was significantly higher in both the aorta and serum of Hx-null mice compared to wild-type ones, after heme injection, suggesting an oxidative
consumption of NO and indicating an increased production of reactive nitrogen species (RNS) in the absence of Hx (Figure 2i,l).

**Hemopexin limits heme uptake by endothelial cells**

Data in the previous section strongly support the conclusion that Hx limits heme uptake by the vascular endothelium. To demonstrate this, we measured heme uptake in HUVECs incubated with 7.5 μM heme bound to albumin or Hx in a 1:1 ratio. After 30 min incubation, heme levels were significantly higher in HUVECs treated with albumin-heme than in those treated with Hx-heme (Figure 3a) proving that Hx strongly limits heme uptake by endothelial cells. Consistently with in vivo findings, incubation of HUVECs with Hx-heme blunted the up-regulation of HO-1, L-Ferritin and FPN (Figure 3b-d), as well as the induction of adhesion molecules, such as ICAM-1 and E-selectin, compared to incubation with albumin-heme (Figure 3e,f). Furthermore, treatment of HUVECs with the Hx-heme complex slightly modulated iNOS mRNA levels and eNOS phosphorylation whereas they were strongly increased after treatment with albumin-heme (Figure 3g,h). The upregulation of iNOS as well as the increased eNOS phosphorylation in albumin-heme treated cells plausibly occur as an attempt to compensate for the reduced NOS activity and increased NO oxidative consumption, as above described in aorta from heme-loaded Hx-null mice. In contrast with albumin, Hx strongly suppressed heme-induced ROS generation and oxidative stress in HUVECs, (Figure 3i-m). As a result, Hx limited heme-induced endothelial cell death (Figure 3n).

Taken together these data confirmed the in vivo results and further proved that Hx prevents endothelial heme overload. Similarly, Hx limited heme uptake by vascular smooth muscle cells in vitro (not shown).

**Hemopexin prevents endothelial heme loading and toxicity by promoting hepatic heme**
detoxification

Data reported in the previous paragraph suggest that Hx may very efficiently counteract heme loading and toxicity on the vascular endothelium. To address the specific mechanism through which heme is inactivated after Hx binding, we analyzed heme-overloaded Hx-null mice and we found that these animals had a defect in hepatic heme accumulation and catabolism. As shown in Figure 4a,b, heme loading in the liver as well as in isolated hepatocytes was strongly reduced in heme-overloaded Hx-null mice compared to wild-type controls. Measurement of heme uptake on primary hepatocytes confirmed that heme entered very efficiently into these cells if bound to Hx but not if bound to albumin (Figure 4c).

Consistently, HO-1 protein was induced to a significantly lower extent in the liver of heme-overloaded Hx-null mice than in that of wild-type animals and this correlated with a lower HO activity as well as with a reduced induction of H- and L-Ferritin (Figure 4d-f) indicating that following heme overload, Hx-null liver produced less CO and biliverdin and accumulated less iron. This was further demonstrated by the reduced bilirubin excretion into the bile of heme-overloaded Hx-null mice compared to wild-type animals (Figure S2a). Consistently with data on bilirubin excretion, heme-overloaded Hx-null mice excreted a lower amount of intact heme into the bile compared to wild-type animals (Figure S2b,c; additional data on hepatic heme metabolism in Supplemental Results and Figures S1-S3).

Together these data indicate that the lack of Hx in serum significantly affects the liver heme detoxifying potential and explain why in these conditions heme excess remains into the bloodstream, thus causing endothelial cell damage.

Hemopexin therapy suppresses heme-driven endothelial activation in β-thalassemic and SCD mice
Data shown in the previous sections clearly linked vascular dysfunction to heme overload not buffered by plasma Hx. Since we observed that thalassemic and SCD mice experienced a condition of vascular damage associated to heme overload and Hx consumption (Figure 1), we hypothesized that a Hx-based therapy might be beneficial in hemolytic pathologies to limit endothelial dysfunction, by increasing hepatic heme detoxifying potential.

To test this hypothesis, we treated 1-month-old thalassemic and SCD mice with 700 μg of purified human Hx twice a week for one month and, at the end of the treatment, we analyzed the aortic endothelium. In Hx-treated thalassemic and SCD mice, we observed that iron accumulation was strongly reduced in the aortic endothelium as well as in the heart (Figure 5a,b) thus demonstrating that the exogenous Hx reduced endothelial heme loading in these mouse models of hemolysis. Accordingly, HO-1 was not induced in aortas of Hx-treated anemic mice and this correlated with an attenuated oxidative stress and with the reduced induction of adhesion molecules both in large vessels and in tissue vasculature (Figure 5c-e).

eNOS mRNA level and NOS activity were increased, whereas nitrotyrosine formation was strongly suppressed in aorta of Hx-treated animals compared to untreated ones (Figure 5f,g), thus suggesting an increased availability of bioactive NO following Hx treatment.

Similarly to what occurs in large vessels, NOS activity was strongly suppressed in the liver of non-treated animals and restored to normal levels after Hx administration indicating that the whole vasculature was positively affected by Hx therapy (Supplemental Results and Figure S4). The same parameters were analyzed in Hx-treated wild-type and HbA mice and no significant effect of exogenous Hx was detected in these animals (not shown).

Thus, Hx administration successfully alleviates heme-induced endothelial alterations in thalassemic and SCD mice.
Hemopexin-mediated endothelial protection in β-thalassemic and SCD mice is due to an enhanced hepatic heme detoxification

We asked whether the Hx protective effect on the endothelium of thalassemic and SCD mice was specifically due to the enhancement of their hepatic heme detoxifying potential mediated by exogenous Hx.

Analysis of heme-overloaded Hx-null mice demonstrated that exogenous human Hx was able to fully rescue their hepatic heme recovery capacity (Supplemental Results and Figure S5). In Hx-treated thalassemic and SCD mice, we observed that iron accumulated in a significantly higher amount in the liver compared to untreated animals (Figure 6a). Consistently, in these animals Hx administration reduced the amount of circulating heme (not shown) and total bilirubin (Figure S6a), while enhancing HO-1 mRNA levels in the liver (Figure 6b), as well as bilirubin and heme excretion in the bile (Figure S6b,c).

All together these data demonstrated that Hx therapy enhanced liver detoxifying potential and restored iron homeostasis in thalassemic and SCD mice. This was associated to an attenuated oxidative stress and an improved control of the inflammatory response and to an amelioration of the liver status (Figure 6c-f). On the other hand, we did not observed changes in red cell indices in Hx treated mice from both mouse strains compared to baseline values (Table S2).

Hemopexin therapy normalizes blood pressure and improves cardiac function in SCD mice

Our results on anemic mice showing an enhanced endothelial activation and a reduced NO bioavailability, support the idea that these animals could have altered cardiovascular function. Consistently, it has recently been reported that 10-14 month-old thalassemic mice show left ventricle hyperthrophy and decreased fractional shortening and ejection fraction34. Since our data on endothelium (Figure 5) indicated that the damage was worst in SCD mice, we supposed that
cardiac function might become altered earlier in these animals. And indeed, measurement of blood pressure and echocardiography analysis on 2 month-old SCD mice demonstrated that HbS mice were hypertensive compared to HbA controls, showing a 1.6 fold increase in mean arterial blood pressure (MAP) (Figure S7a). An enhancement in both systolic and diastolic pressure contributed to MAP increase, as reported in Figure S7b. Moreover, SCD mice showed increased cardiac output and aortic valve peak pressure, that were respectively 2.8 and 4.5 fold higher compared to HbA mice (Figure S7c,d; Table S3). Increased blood pressure and cardiac output was found associated to an increased hypertrophic response and a significant reduction in myocardial performance (Table S3).

Since we demonstrated that Hx therapy decreased ROS production and NO oxidative consumption in the vascular endothelium of SCD mice, we hypothesized that Hx administration might positively affect cardiovascular function in these animals. To test this hypothesis, we monitored blood pressure in Hx-treated HbS mice during the therapy. Blood pressure was significantly reduced by Hx treatment starting from the first administration and almost completely normalized by the 4th injection (Figure 7a). A reduction in both systolic and diastolic pressure accounted for blood pressure normalization (Figure 7b). This potent anti-hypertensive effect of Hx was further confirmed on 5 month-old HbS mice, that showed a full rescue in MAP after a single 3 mg Hx injection (Figure 7c). Accordingly, cardiac output as well as aortic valve peak pressure were restored to normal values by Hx treatment (Figure 7d-f, Figure S8, Table S3). As a result, the performance index of the left ventricle was improved in Hx-treated HbS mice (Figure 7g).

These data highlight the critical importance of Hx in preventing heme-mediated vascular oxidative stress and in rescuing cardiovascular function in a mouse model of SCD.
Discussion

Here we showed that Hx infusion alleviates heme-induced endothelial activation and maintains vascular homeostasis in two mouse models of β-thalassemia and SCD, thus suggesting important implication for a therapeutic use of Hx in the treatment of vasculopathy in hemolytic disorders. Moreover, we reported that in SCD mice vascular damage was associated to altered cardiac function and this was restored by Hx therapy. These data demonstrate that heme has a strong impact on cardiovascular function and highlight the efficacy of a therapy specifically aimed at chelating free heme.

Hx exerts its protective effect mainly by promoting heme recovery and detoxification through HO activity in the liver and by limiting heme-iron loading and HO-1 induction in the vascular endothelium. Patients and mice suffering from SCD showed an adaptive upregulation of HO-1 in response to hemolysis, nonetheless being often insufficient to completely handle the excessive heme burden, particularly during acute bouts of hemolysis. Hx therapy demonstrates that a major benefit is obtained by HO activity induction in hepatocytes, likely because the liver is well equipped to manage high amounts of heme. The enhanced heme-iron accumulation in the liver of Hx-treated anemic mice resulted in a strong increase in hepatic heme detoxifying potential and in the protection of non-hepatic tissues from heme accumulation and its toxic effects. Indeed, an important outcome of Hx therapy is the prevention of heme-iron loading in the vascular endothelium and in the heart. This is clinically relevant as hemolysis-driven iron overload, further exacerbated by transfusion regimen, strongly contributes to heart failure in β-thalassemia patients, the latter representing the most common cause of death in these subjects.

The critical role of heme-driven endothelial activation in the pathophysiology of β-thalassemia and SCD has been recently recognized and its contribution to vascular instability and
vaso-occlusive events has been described\(^5,9,40,41\). Because of chronic hemolysis, vessels of thalassemic and SCD patients are exposed to great amount of ROS catalyzed by heme-derived redox-active iron\(^8,1,5,6,8\) that lead to endothelial activation and adhesion molecule expression on the vessel wall, which in turn favours the adhesion of RBCs and leukocytes, resulting in vascular instability and vaso-occlusion\(^9,40-42\). Serum heme overload properly correlates with the increased tissue oxidation and anti-oxidant response as well as with endothelial activation, inflammation and plasma Hb/heme scavenger depletion.

Here we demonstrated that Hx administration, by scavenging free heme, alleviates heme-induced tissue oxidative injury, limits the induction of adhesion molecules and the formation of ROS in the vascular endothelium as well as the production of pro-inflammatory cytokines in both β-thalassemia and SCD mouse models. This indicates that Hx may confer protection against heme-driven endothelial activation, oxidative stress and inflammation\(^43\).

The vascular dysfunction common to both β-thalassemia and SCD is further amplified by the reduced bioavailability of NO as major vasodilator, resulting in imbalance of vascular tone towards vasoconstriction\(^4,11,22,44,45\). In β-thalassemia and SCD, consumption/inactivation of NO is accelerated due to synergistic effects of chronic oxidative stress and persistent hemolysis\(^1,4\). In these patients, increased ROS production is implicated in NO consumption and formation of peroxynitrite (ONOO\(^-\)) that resulted in nitrotyrosine formation\(^46,47\). NOS itself can be uncoupled by oxidation of the essential cofactor BH\(^4\) and uncoupled NOS produces superoxide in place of NO\(^1\). Excessive peroxynitrite formation further contribute to NOS activity reduction and NOS dimer disruption. Consistently, elevated nitrotyrosine levels correlate with impaired NOS activity and loss of NOS dimerization in SCD mice\(^35\). Furthermore, NO is rapidly destroyed by its reaction with the iron contained in free heme/hemoglobin present in the plasma\(^1\). Hemolysis
also releases red cell arginase-1 into plasma, thus reducing the levels of NOS substrate, L-arginine, and further limiting NO bioavailability\(^1,4,11\). As a result, in these pathologic conditions, vascular endothelium is likely to be in a perpetually activated state because of chronic oxidative stress and reduced NO consequent to hemolysis. Agents directed at restoring NO homeostasis could be promising to alleviate vascular instability in patients suffering from \(\beta\)-thalassemia and SCD. Here, we observed enhanced NOS activity in the vascular endothelium of Hx-treated thalassemic and SCD mice, suggesting that Hx could promote NO production by reducing the oxidative consumption of NOS cofactors and NOS uncoupling. Moreover, Hx treatment reduced nitrotyrosine formation, a footprint of NO-ROS interaction and peroxynitrite production in both mouse models of \(\beta\)-thalassemia and SCD. These results together demonstrate that, after Hx administration, more NO is produced and less NO is oxidatively-inactivated in the endothelium, thus resulting in an increased availability of bioactive NO, that could be beneficial to counteract endothelial dysfunction associated with these hemolytic pathologies.

We found that in SCD mice, oxidative stress and reduced NO availability are associated to systemic hypertension and Hx therapy, by restoring normal NO level as well as reducing ROS production, also normalizes blood pressure. This strong antihypertensive effect of Hx opens the possibility of the use of Hx-based drugs to counteract systemic vasoconstriction promoted by free heme.

Although traditionally associated with systemic vasoconstriction, endothelial dysfunction was recently proposed to play a central role even in heart failure pathogenesis. The failing heart is characterized by an altered redox state with ROS overproduction, and increasing evidence suggests that the abnormal cardiac and vascular phenotypes characterizing the failing heart are caused in large part by imbalances between NO bioavailability and oxidative stress\(^48\). Our results
are consistent with these findings as we showed that low NO and high ROS in SCD mice were associated to an impairment of cardiac performance. Our data strenthen the central role of heme in triggering these processes. Taking into account that heme is released not only during hemolysis associated to hemoglobinopathies, but also after ischemia-reperfusion injury and cardiac remodeling, these observations could be of a broader importance.

The administration of antioxidants or endothelial activation inhibitors or NO-donors has been shown to positively affect vascular function in hemolytic diseases. In this scenario, the Hx therapy could contribute to restoration of cardiovascular homeostasis targeting multiple steps involved in the pathogenesis of vasculopathy and consequent cardiac decay. Indeed, we showed that in SCD mice, Hx treatment not only beneficially affected the unbalance between vasodilator/vasoconstrictor factors but also significantly improved cardiac performance.

Consistently with our results on SCD mice, others have recently reported that even thalassemic mice developed cardiovascular dysfunction with aging, thus suggesting that the deterioration of cardiovascular function may occur more slowly in these animals. This is consistent with our data showing a milder endothelial damage in thalassemic compared to SCD mice and further strenthen the relationship between endothelial dysfunction and heart damage.

Based on the observation that heme overload had similar toxic effects on the endothelium of both mouse models, it is likely that a long-term Hx therapy might beneficially affect cardiovascular function also in β-thalassemia.

In conclusion, we propose a pivotal role for Hx as a potent free heme scavenger to treat vasculopathy related to hemolytic disorders. In fact, Hx avoids heme intercalation in cell membranes, thus limiting lipid peroxidation, cell oxidative stress and hemolysis amplification. Moreover, Hx prevents free heme-mediated generation of ROS that directly act on the
endothelial wall and inactivate NO, thus impairing vascular function (Figure 8). The final outcome of the Hx therapy is the preservation of the cardiovascular function.

Thus, purified or recombinant Hx might be used pharmacologically for the treatment of patients with hemolytic diseases. Since Hx acts as a heme chelator, it could be used, together with iron chelators28 whose administration is usually associated with blood transfusion25, 26 in anemic patients, as a specific therapy to counteract heme toxicity, thus enhancing the effectiveness of the chelation therapy and preserving cardiovascular function.

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Figure Legends:

Figure 1. β-thalassemic and SCD mice show vasculopathy associated to Hx depletion and serum heme overload. Data on β-thalassemic (β-Thal) and SCD (HbS) mice are shown on the left and right respectively. (a) Endothelial activation in β-Thal and HbS mice. Western blotting showing VCAM-1 and E-Selectin protein expression in aorta and qRT-PCR analysis showing ICAM-1/P-Selectin and E-Selectin mRNA levels in the liver. (AU: Arbitrary Units; RQ: Relative Quantity) (b) Oxidative stress in vessels of β-Thal and HbS mice as evinced by SOD1 protein expression and ROS production. (c) Reduced availability of active NO in vessels of β-Thal and HbS mice as demonstrated by increased amount of nitrated proteins and by reduced NOS activity. (d) Increased inflammation in β-Thal and HbS mice demonstrated by increased leukocyte number. (e, f) Representative Western blots showing Hp and Hx and heme content in the serum of β-Thal and HbS mice. Wt, HbA n=4; β-Thal, HbS n=8. Values represent mean ± SEM. *P<0.05; **P<0.01; ***P<0.001.

Figure 2. The lack of Hx favors heme loading in the vascular endothelium. (a) Heme content in aorta of wild-type and Hx-null mice 5h after heme injection (not-treated (NT):n=4; heme:n=5). (b-e) qRT-PCR analysis of HO-1, L-Ferritin, FPN and VCAM-1 mRNA level in the aorta of wild-type and Hx-null mice 5h after heme injection (NT:n=4; heme:n=5). (f) Representative Western blot showing VCAM-1 expression, (g) ROS production assay (NT:n=6;heme:n=9) and (h) NOS activity (NT:n=3; heme:n=5) in aorta from wild-type and Hx-null mice 5h after heme injection. (i,l) Representative Western blots showing Nitrotyrosine formation in aorta and serum from wild-type and Hx-null mice 5h after heme injection. (NT:n=3; heme:n=5). Results shown are representative of three independent experiments. Values represent mean±SEM.
*P<0.05; **P<0.01.

**Figure 3.** Hx limits heme uptake by HUVECs. (a) Heme uptake in HUVECs incubated with 7.5 μM Hx-heme or HSA-heme for 15, 30 or 60 min (n=5). (b,d,e,g,i) qRT-PCR analysis of HO-1, FPN, ICAM-1, E-selectin, iNOS and Xanthine-Oxidase mRNA levels in HUVECs incubated with 7.5 μM Hx-heme or HSA-heme for 4h (n=6). (c,h,l) Representative Western blots of HO-1 and L-Ferritin expression, P-eNOS and eNOS and SOD1 expression in HUVECs incubated with 7.5 μM Hx-heme or HSA-heme for 4h (n=3). (m,n) Representative FACS analysis of H2DCFDA fluorescence and Annexin V-PI staining in HUVECs treated with 10 μM Hx-heme or HSA-heme for 20 hours (n=5). Values represent mean±SEM. *P<0.05; **P<0.01; ***P<0.001. Results shown are representative of three independent experiments.

**Figure 4.** Hx promotes heme uptake and detoxification by the liver. (a) Heme content in liver of wild-type and Hx-null mice at different time points after heme injection (NT:n=4; 1h:n=3; 3h:n=6; 5h:n=5) (liver: Hx-/ NT vs +heme *P<0.05). (b) Heme content in hepatocytes isolated from heme-overloaded wild-type and Hx-null mice 1 and 3h after heme injection. (NT:n=3; 1h:n=6; 3h:n=7). (c) Heme content in primary hepatocytes treated with 5 μM Hx-heme or human albumin (HSA)-heme in 1:1 ratio for 30 min (n=11). (d) Representative Western blot showing HO-1 expression in total liver extracts from wild-type and Hx-null mice 6h after heme injection (NT:n=3; heme:n=6). (e) HO activity in hepatocytes isolated from wild-type and Hx-null mice 1 and 3h after heme injection (n=4). (f) Representative Western blots showing H- and L-Ferritin expression in isolated hepatocyte extracts from wild-type and Hx-null mice 6h after heme injection (NT:n=3; heme:n=6). Values represent mean±SEM. *P<0.05; **P<0.01; ***P<0.001.
Figure 5. Hx therapy suppresses heme-driven endothelial activation in β-thalassemic and SCD mice. Data on wild-type, β-Thal and Hx-treated β-Thal mice and HbA, HbS and Hx-treated HbS mice are shown on the left and right respectively. (a) Representative Western blot analysis of L-Ferritin expression in aorta. (b) Heart iron content. (c) Representative Western blot showing HO-1 expression in aorta. (d) Representative Western blot showing SOD-1 expression (left) and ROS production (right) in aorta. (e) Representative Western blot showing VCAM-1 expression in aorta and qRT-PCR analysis of E-Selectin and ICAM-1/P-Selectin mRNA level in the liver. (f) qRT-PCR analysis of eNOS mRNA level (left) or calcium-dependent NOS activity (right) in extracts of aorta (n=4). Calcium-dependent NOS activity assay measures the activity of both iNOS (calcium-independent) and eNOS (calcium-dependent), the latest being the most abundant NOS expressed in aorta. (g) Representative Western blot showing Nitrotyrosine formation in extracts of aorta. n=4. Values represent mean±SEM. *P<0.05; **P<0.01; ***P<0.001. Results shown are representative of three independent experiments.

Figure 6. Hx therapy promotes hepatic heme detoxification in β-thalassemic and SCD mice. Data on wild-type, β-Thal and Hx-treated β-Thal mice and HbA, HbS and Hx-treated HbS mice are shown on the left and right respectively. (a) Iron content and (b) qRT-PCR analysis of HO-1 mRNA level in liver. (c) Liver MDA content. (d) qRT-PCR analysis of NQO1 and γGCS mRNA level and (e) of TNFα and IL-6 mRNA level in the liver. n=4. Values represent mean±SEM. *P<0.05; **P<0.01; ***P<0.001. Results shown are representative of three independent experiments. (f) Representative liver sections stained with hematoxylin and eosin. Arrows indicate sites of cell necrosis (left), evident in β-Thal mice but not in Hx-treated animals, and leukocyte aggregates (right), whose number was strongly reduced by Hx administration in SCD mice.
**Figure 7.** Hx administration normalizes blood pressure and improves cardiac function in SCD mice. (a) Mean arterial pressure in 1 month-old HbA, HbS and Hx-treated HbS mice, measured before and during the treatment (0.7mg Hx each injection; n=5). HbA vs. HbS: ***P<0.001 at each point. (b) Systolic and diastolic pressure measured after the 4th Hx injection (n=5). (c) Mean arterial pressure in 5 month-old HbA, HbS and Hx-treated HbS mice (a single 3 mg Hx dose; n=4). (d) Representative images of Color Doppler on the left ventricular outflow (LVOT) in HbA, HbS and Hx-treated HbS mice. (e-g) Cardiac output, aortic valve peak pressure and left ventricular myocardial performance index measured in HbA, HbS and Hx-treated HbS mice by echocardiography (n=6). Values represent mean±SEM. *P<0.05; **P<0.01; ***P<0.001. Results shown are representative of three independent experiments.

**Figure 8.** Proposed mechanism for Hx action. Hx acts as a heme chelator that promotes heme uptake and detoxification by the liver (left). In hemolytic diseases, the exhaustion of the Hx plasma pool leads to a significant reduction of the hepatic heme detoxifying potential and finally to heme recovery by vascular endothelial cells and heart. Upon this condition, heme directly acts on the endothelial wall, causing oxidative damage; it intercalates into RBC membrane, amplifying hemolysis and Hb release, and strongly enhances ROS production. All these mechanisms contribute to oxidative damage, NO depletion and endothelial activation, thus altering vascular homeostasis. Hx administration in hemolytic pathologies, by scavenging free heme, blocks heme toxic effects and improves cardiovascular function (right).
Figure 4.

a) Liver Heme Content

b) Isolated Hepatocytes Heme Content

c) Primary Hepatocytes Heme Uptake

d) Liver HO-1 Protein

e) Isolated Hepatocytes HO Activity Assay

f) Isolated Hepatocytes H-Ferritin Protein

L-Ferritin Protein

AU
Figure 8.

Sickle Cell Disease / Thalassemia

Endogenous Hx Pool

Heme-driven ROS Formation and Tissue Injury

Liver

Hx-heme

Hx-heme

Hb

Hp-Hb

Hemolytic RBC

Spleen

Heart

Endothelium

Cardiovascular Dysfunction Vasoconstriction

Hx-heme

Hx

Hx-heme

ROS

Hb

Heme

Hemopexin Administration

Therapeutical Hx administration

RBC: Red Blood Cells

Hb: Hemoglobin

Hp: Haptoglobin

Hx: Hemopexin

RBC: Red Blood Cells

Heme Uptake

Reticuloendothelial macrophages

ROS: Reactive Oxygen Species
Hemopexin Therapy Improves Cardiovascular Function by Preventing Heme-Induced Endothelial Toxicity in Mouse Models of Hemolytic Diseases
Francesca Vinchi, Lucia De Franceschi, Alessandra Ghigo, Tim Townes, James Cimino, Lorenzo Silengo, Emilio Hirsch, Fiorella Altruda and Emanuela Tolosano

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Hemopexin therapy improves cardiovascular function by preventing heme-induced endothelial toxicity in mouse models of hemolytic diseases

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SUPPLEMENTAL MATERIAL

Supplemental Material and Methods

Mice and treatments
A mouse strain that underexpresses β-globin chains, the Hbb$^{th1/th1}$ mouse, was used as a model of β-thalassemia intermedia$^1$. This mouse model has arisen as a spontaneous DNA deletion of the β major gene$^2, 3$. Wild-type animals of the same genetic background were used as controls. As a model of SCD, a humanized knock-in mouse in which the murine α-globin and β-globin genes were replaced with human α-globin and with human Aγ and β$^5$ (sickle) globin genes respectively, was employed$^2$. SCD mice (HbS) were compared to their counterpart carrying the human Aγ and β alleles in homozygosity (HbA).

Mice used in these studies were 2/3-month-old littermates, maintained on a standard chow diet and kept with free access to food and water. All experiments were approved by the animal ethical committee of the University of Torino (Italy).

Hemin and Tin-protoporphyrin IX (Frontier Scientific, Logan, Utah) were freshly prepared as previously reported$^4$, and injected into the tail vein of wild-type and Hx-null mice at a dose of 30μmol/kg. Control mice were injected with PBS. Mice were sacrificed at different times after hemin injection. Mice were anesthetized, tissue samples collected and kept frozen until analysis. Blood was collected by retro-orbital bleeding.

For rescue experiment, 1500μg of human Hx (Athens Research, GA, USA) were injected iv in Hx-null mice. After 1 hour, the same mice were subjected to heme injection and sacrificed 60 min later.

Primary Hepatocyte culture preparation
Hepatocytes were isolated from single hepatic lobules$^5$. After liver dissection, the single liver lobe was perfused using Hepatocyte Liver Perfusion Medium and then Hepatocyte Liver Digest Medium (Gibco). Perfusion with Digest Medium was kept until the liver lobe felt very soft. This is a critical step, as this medium contains collagenase, and excessive digestion should be avoid to prevent cell death. Subsequently, the perfused liver lobe was disrupted and the cell suspension was forced through a 100 μm Cell Strainer (BD). Cells were centrifuged and then cell suspension was applied
over a Percoll gradient. After Percoll gradient centrifugation the two upper layers that contain cell debris and non-parenchymal cells were carefully pipetted out and discarded. Then, the lowest layer that contains the live hepatocytes was collected. Cells were washed, centrifuged and then plated onto collagen-coated well plates.

**Heme Content Quantification**

Heme content in tissues and in bile samples was quantified fluorometrically by the method of Sassa\(^6\). Briefly, tissues were homogenized in phosphate buffer saline (PBS) and protein content was determined by using the Bio-Rad protein assay system (Bio-Rad, Munchen, Germany). 10 µg of protein samples were incubated with 0.5 ml of 2 M Oxalic Acid (Sigma-Aldrich) at 95°C for 30 min. Samples were subsequently centrifuged at 14000 rpm for 5 min. Fluorescence emission in the supernatant was determined spectrofluorimetrically (Glomax, Promega). Excitation and emission wavelengths were set at 405 and 662 nm, respectively. The background was evaluated by measuring fluorescence in non-boiled samples. A standard curve of hemin was run in parallel.

**HO Activity Assay**

Heme-Oxygenase activity was measured by spectrophotometric determination of bilirubin produced from hemin added as the substrate\(^7\). Isolated hepatocytes were lysed with a hypotonic buffer (0.1 M potassium phosphate, 2 mM MgCl\(_2\), Complete Protease Inhibitor Cocktail, Roche Diagnostics Corp., Milano, Italy, pH 7.4) for 15’ on ice. After brief sonication, 0.6 M sucrose was added to cell lysates in order to obtain an hypotonic solution (final 0.25 M sucrose). Lysates were centrifuged at 1000 x g for 10 min at 4°C to pellet nuclei, and supernatants centrifuged at 12000 x g for 15 min at 4 °C to pellet mitochondria. Finally, supernatants were ultracentrifuged at 105000 x g for 1 hour at 4°C. Microsomal fractions were resuspended in 100 mM potassium phosphate buffer pH 7.4, containing 2 mM MgCl\(_2\) and Complete protease inhibitor. Protein concentration was determined using a small aliquot of these suspensions (Bio-Rad, Munchen, Germany). The microsomal supernatant fraction (cytosol) from the liver of a normal rat served as source of biliverdin reductase. Liver supernatant was prepared fresh from rat liver by homogenization in 0.1 M sodium citrate buffer, pH 5, containing 10% glycerol. HO-1 activity assay was carried out by incubating 600 µg microsome proteins with a reaction mixture containing 1 mM NADPH, 2 mM glucose-6-phosphate, 1U glucose-6-phosphate dehydrogenase (Sigma-Aldrich), 25 µM hemin, 2 mg of rat-liver cytosol and 100 mM potassium phosphate buffer, pH 7.4 (400 µl final volume). The reaction was conducted in the dark for 1h at 37 °C and terminated by placing tubes on ice for 2 min. The amount of bilirubin was determined by the difference in absorption between 464 and 530 nm (extinction coefficient, 40
mM\(^{-1}\) cm\(^{-1}\) for bilirubin). HO activity was expressed in picomoles of bilirubin formed per milligram microsomal protein per hour.

**Biliary excretion study**

In gallbladder cannulation experiments mice were anesthetized by intramuscular injection. Body temperature was maintained at 37°C by heating pads. After opening the abdominal cavity, the cystic duct was ligated and an i.v. cathether 24GA (BD Insyte, Spain) attached to a PE-10 tubing (Portex limited, Hythe, UK) inserted in the common bile duct and fixed with an additional ligation. After bile flow equilibration for 10 min, bile was collected into preweighed tubes for 15 min. A bolus of heme (30 µmol/kg body weight) was then infused into the tail vein of mice. Bile was collected through the cannula after heme infusion for 1,15 hr at 15 min intervals and then up to 4.15 at 1hr intervals. Bile flow was determined by weighing the collected bile samples, assuming a density of 1.0 g/ml for bile. Bile samples were frozen immediately and stored at -20°C.

In gallbladder removal experiments, mice were intravenously infused with heme (30 µmol/kg body weight) or with SnPP (30 µmol/kg body weight) and 1h later, with heme. After 1, 3 or 5 hours an upper midline laparotomy was realized, the cystic duct was ligated and transected and a cholecystectomy performed. Gallbladder was removed and its volume was determined by water displacement. Bile was collected and stored at -20°C until analysis.

**Bilirubin and Heme Concentration in serum and bile**

Direct and Total bilirubin concentrations in serum and bile were determined colorimetrically using the QuantiChrom bilirubin assay kit DIBR-180 from BioAssay Systems (Hayward, CA). Heme concentrations in serum were determined colorimetrically using the QuantiChrom Heme assay kit DIHM-250 from BioAssay Systems.

**Measurement of Total Bile Acid Concentrations in bile**

Total Bile Acid (TBA) concentrations in bile was measured using a total bile acids assay kit according to the procedure supplied by the manufacturer (Diazyme, San Diego, California). The concentration of bile acids is expressed as pmol TBA excreted per min per gram of liver.

**Cell culture**

HUVECs were isolated from human umbilical vein and cultured in Medium199 (Invitrogen) added with bFGF 10ng/ml, heparin, 20%FBS and 1% penicillin/streptomycin on 0.1% gelatin (Sigma).
Measurement of intracellular ROS accumulation
Accumulation of ROS in HUVECs and aortic rings was assessed by using the oxidant-sensitive fluorescent dye 29,79-dichlorodihydrofluoroscein diacetate (H$_2$DCFDA; Molecular Probes, Inc., Eugene, OR). H$_2$DCFDA penetrates easily into the cells. Upon crossing the cellular membrane, H$_2$DCFDA undergoes deacetylation by intracellular esterases producing a non-fluorescent compound that becomes highly green fluorescent following oxidation by intracellular ROS. Within the cell the probe reacts with ROS to form fluorescent 28,78 dichlorofluoroscein (DCF), which is detected with spectrofluorometry. HUVECs untreated or treated for 20 hrs with 15 µM Hx-heme, HSA-heme or heme alone were incubated with 5µM H$_2$DCFDA in Hanks’ balanced salt solution (HBSS) for 30 min at 37 °C under 5% CO$_2$ atmosphere. Then cells were washed twice with HBSS, trypsinyzed and analyzed by flow cytometry using a FACS flow cytometer$^9$.

Similarly, aortic rings from HbA, HbS and Hx-treated HbS mice were incubated with 20µM H$_2$DCFDA in Krebs-Henseleit buffer for 60 min at 37 °C under 5% CO$_2$ atmosphere. Fluorescence was recorded at excitation and emission wavelengths of 485 and 530 respectively by a fluorimeter plate reader (Promega). The background fluorescence caused by buffer and DCF was subtracted from the total fluorescence in each well generated by aortic rings in presence of DCF. Fluorescence intensity units were normalized by mg of weight tissue for each aortic rings and expressed as arbitrary fluorescence units/mg tissue$^{10}$.

Tissue Iron Measurement
Tissue non-heme iron content determined with a colorimetric method using 4,7-diphenyl-1,10-phenanthroline disulphonic acid (BPS) as chromogen$^{11}$. Briefly, 0.1 g of dry tissue was incubated overnight in a mixture of trichloroacetic (10%) and hydrochloric (4N) acids, and 100 µl of supernatant reduced with thioglycolic acid (Sigma-Aldrich) and acetic acid-acetate buffer (pH4.5). Ferrous iron content was determined spectrophotometrically at 535 nm following addition of BPS and incubation for 1 hr at 37°C. Results were expressed as µg iron/g dry tissue weight.

LDH Activity Assay
LDH activity in serum was determined colorimetrically using the QuantiChrom Lactate Dehydrogenase kit DLDH-100 from BioAssay Systems. Serum LDH activity was expressed as Unit of LDH(1U/L) catalyzing the conversion of 1 µmole of lactate to pyruvate per minute at pH 8.2.

Echocardiography
Transthoracic echocardiography was performed with a small animal high-resolution imaging system (VeVo2100, VisualSonics, Inc, Toronto, Canad) equipped with a 22-55 MHz trasducer (MicroScan Transducers, MS500D). The mice, anesthetized by isoflurane (2%) inhalation and maintained by mask ventilation (isoflurane 1%), were placed in a shallow left lateral decubitus position, with strict thermoregulation (37+1°C) to optimize physiological conditions and reduce hemodynamic variability. Fur was removed from the chest by application of a cosmetic cream to gain a clear image. Echocardiographic parameters were measured at the level of the papillary muscles in the parasternal short-axis view (M mode). LV fractional shortening was calculated as follows: $FS = ((LVEDD - LVESD)/ LVEDD) \times 100$, where LVFS indicates LV fractional shortening; LVEDD, LV end-diastolic diameter; and LVESD, LV end-systolic diameter. LV ejection fraction was calculated automatically by the echocardiography system. Cardiac output was calculated as the product of stroke volume and heart rate. All measurements were averaged on 5 consecutive cardiac cycles per experiment and cardiac function was assessed when heart rate was 450-500 bpm.

**Quantitative Real-Time Polymerase Chain Reaction Analysis**

Total RNA was extracted using Pure Link RNA Mini Kit (Ambion, Invitrogen, US). 1µg of total RNA was reverse transcribed by using M-MLV reverse transcriptase (Invitrogen) and random primers (New England Biolabs, Ipswich, MA). qRT-PCR was performed on a 7300 Real Time PCR System (Applied Biosystems, California). Primers and probes were designed using the ProbeFinder software (www.roche-applied-science.com).

**Protein Extraction and Western Blotting**

Tissue and cell proteins were extracted as previously reported and concentration was determined using the Bio-Rad protein assay system (Bio-Rad, Germany). One µl of plasma or 50µg of total protein extracts were separated on 8/12% SDS-PAGE and analyzed by Western blotting using antibodies against Hx, Hp (Sigma H5015), HO-1 (Stressgen, Victoria, Canada), VCAM-1 (R&D, Minneapolis, US), Nitrotyrosine (Millipore,#06-284), L- and H-Ferritin, FPN, eNOS (BD Transduction Lab.), P-eNOS (Cell Signaling), SOD-1 and actin (Santa Cruz).

**Histology**

Animals were anesthetized and transcardially perfused with PBS. Tissues were collected, fixed in 10% formalin overnight at room temperature and embedded in paraffin. Microtome sections, 5 µm thick, were stained with hematoxylin and eosin.
Supplemental Results

Additional data on Hepatic Heme Metabolism

Regulation of HO-1 expression

We observed that heme accumulated to a significantly lower extent in the liver of heme-overloaded Hx-null mice than in that of wild-type animals. In agreement with liver heme uptake results, ALAS-1 mRNA, which is transcriptionally down-regulated by intracellular heme\(^34\), was decreased in the liver of heme-treated wild-type mice but not in that of Hx-null mice (Figure S1a).

It is likely that heme accumulation in the liver also contributed to HO-1 induction as previous works demonstrated that HO-1 expression is mainly regulated by heme-mediated removal of the repressor Bach1 and binding of the transcriptional factor Nrf2 to HO-1 promoter\(^32\), \(^33\). Accordingly, we observed an higher amount of Nrf2 in the nuclei of hepatocytes isolated from heme-treated wild-type mice than in those from heme-treated Hx-null animals (Figure S1b) that could account for the enhanced HO protein level found in these mice.

Bilirubin and Heme excretion in the bile

We demonstrated that biliary bilirubin was strongly increased after heme injection in both wild-type and Hx-null mice, with a peak 30-45 minutes after heme injection and this increase was significantly higher in wild-type mice compared to Hx-null animals (Figure S2a). This resulted in a significantly higher amount of bilirubin excreted in the bile of wild-type mice (150 nmoles) compared to Hx-null mice (80 nmoles) in the first four hours after heme injection. A similar difference was observed in heme excreted into the bile. We observed that heme excretion in the bile was significantly higher in heme-overloaded wild-type mice than in Hx-null counterpart (Figure S2b,c) and this resulted in higher amount of heme excreted in the bile in the first four hours after heme injection (40 nmoles in wild-type vs. 15 nmoles in Hx-null).

Bile flow and total bile acid (TBA) excretion were monitored before and after heme infusion. Under basal condition bile flow (Figure S2d) and TBA excretion (not shown) appeared comparable in wild-type and Hx-null mice, thus indicating that biliary excretion was not altered in Hx-deficient animals. In both wild-type mice and Hx-null mice bile flow was not significantly affected by heme treatment. Only Hx-null mice showed a trend toward a slower flow (Figure S2d). Total bile acid excretion was slightly but not significantly decreased in both wild-type mice and Hx-null animals after heme injection (not shown). The slight reduction in bile flow and TBA excretion after heme infusion may occur as a consequence of increased oxidative stress that has been demonstrated to
impair bile excretion. Due to more pronounced alteration in the oxidative status, this effect is more evident in heme-treated Hx-null mice. The observed decrease in TBA excretion could be alternatively due to an increase in the bile acid-independent component of the bile flow versus the bile acid-dependent one.

Thus, our results indicate that the liver heme detoxifying potential is mainly accounted for by HO activity and, to a lesser extent, by biliary heme excretion and both these mechanisms are strongly induced following Hx-mediated heme uptake. Putting together the data on catabolism and excretion, we conclude that the presence of Hx in serum increases by two-fold the amount of heme detoxified in the liver.

**Contribution of heme catabolism and heme excretion to Hx-mediated heme detoxification**

Other than through catabolism, Hx-mediated heme delivery to the liver promotes heme detoxification through direct excretion of the molecule as an intact porphyrin in the bile. Interestingly, we found an inverse correlation between biliary excretion of bilirubin and heme: wild-type mice that excreted less bilirubin showed an increased excretion of intact heme and viceversa (Figure S3a). To evaluate whether heme excretion may be affected by the rate of catabolism, we analyzed biliary excretion of heme in wild-type mice treated with the HO inhibitor Tin-protoporphyrin IX before heme injection. As expected Tin-protoporphyrin IX treatment completely blocked biliary excretion of bilirubin in heme-overloaded wild-type mice (Figure 3b). Moreover, treatment with Tin-protoporphyrin IX resulted in a higher accumulation of heme in the liver of these animals and enhanced excretion of intact heme in the bile (Figure S3c,d), indicating that these two mechanisms of detoxification work together to efficiently remove heme excess.

**NOS expression and activity in the liver of SCD mice**

Previous works demonstrated that hemin, thanks to its ability to induce HO-1, is able to attenuate iNOS expression and activity\[^{13, 14}\]. HO-1 activation can negatively modulate iNOS by releasing CO, which is able to interact with iNOS heme moiety, thus causing its inactivation, and iron, which downregulates iNOS transcription\[^{13, 14}\]. We demonstrated that Hx treatment of SCD mice resulted in a significantly higher iron accumulation in the liver as well as in an enhanced induction of HO-1 (Figure 6a,b Main Text; Figure S4a). Moreover, we observed that iNOS expression was reduced in the liver of SCD mice after Hx treatment (Figure S4b). Both the enhanced Hx-mediated heme-iron uptake by the liver and the increased HO-1-mediated CO production could account for iNOS downregulation. Even though iNOS expression is increased in the liver of sickle mice and
downregulated after Hx administration, its activity has an opposite modulation, being strongly suppressed in non treated animals and restored after Hx treatment (FureS4c). This observation suggests that iNOS upregulation occurs as an attempt to compensate for the reduced activity of the enzyme, most likely related to oxidative stress and cofactor/substrate consumption. NOS oxidative inactivation is completely prevented by administering the anti-oxidant Hx. The restoration of NOS activity in Hx-treated animals could further contribute to HO-1 upregulation, that in turn may downregulates iNOS expression.

**Rescue of the phenotype of Hx-null mice through the administration of purified human Hx**

To demonstrate that injected human Hx is able to restore hepatic heme detoxifying potential, we evaluated the ability of human Hx to rescue heme recovery capacity in heme-overloaded Hx-null mice. Hx-null mice were injected with human Hx at physiological concentration 1 h before heme injection and liver heme content and biliary bilirubin and heme were analyzed. As shown in Figure S5, Hx-treated Hx-null mice fully recovered the capacity to take up heme by the liver and to detoxify it (Figure S5a,c,d). Heme uptake by the liver in Hx-treated Hx-null mice was further proved by the modulation of ALAS1 mRNA (Figure S5b).
Figure S1. Heme does not accumulate in the liver when Hx is lacking. (a) qRT-PCR analysis of ALAS1 mRNA in the liver of wild-type and Hx-null mice at different time points after heme injection (n=6). (b) Representative Western blots showing nuclear Nrf2 expression in isolated hepatocyte extracts from wild-type and Hx-null mice 6h after heme injection (NT:n=3; heme:n=6). AU: Arbitrary Units. Values represent mean±SEM. *P<0.05.
Figure S2. Hx promotes heme detoxification by the liver. (a,b) Excretion rate and cumulative excretion of bilirubin and heme in the bile of heme-overloaded wild-type and Hx-null mice (NT:n=4; heme:n=12). (c) Heme content in the bile fraction 75'-135' of heme-overloaded mice (n=7). Representative fractions of 2 Hx-null and 2 wild-type mice are shown. (d) Bile flow in wild-type and Hx-null mice before (time 0) and at different time points after heme injection (n=12). Values represent mean±SEM. *P<0.05; **P<0.01; ***P<0.001.
Figure S3. Heme degradation and heme excretion are inversely correlated. (a) Biliary excretion rate of heme plotted on biliary excretion rate of bilirubin. Mice were arbitrarily divided into two groups according to bilirubin excretion (more or less than 900 pmol/min/g liver) (n=4). (b-d) Biliary bilirubin (b) and liver (c) and biliary (d) heme content in Tin-protoporphyrin IX (SnPP)-treated heme-overloaded wild-type mice at different time points after heme injection. Gallbladder was ligated and removed at the indicated time points and bile collected. (Wt -Tin-protoporphyrin IX: n=9; Wt +Tin-protoporphyrin: n=6). Values represent mean ± SEM. *P<0.05; **P<0.01.
Figure S4. Hx modulates hepatic iNOS expression and activity in SCD mice. (a,b) qRT-PCR analysis of HO-1 and iNOS mRNA level in the liver of HbA, HbS and Hx-treated HbS mice (n=4). (c) Calcium-independent NOS activity in extracts of liver from HbA, HbS and Hx-treated HbS mice. (n=4). Calcium-independent NOS activity assay measures the activity of iNOS, that is the most abundant NOS expressed in the liver. Results shown are representative of three independent experiments. Values represent mean±SEM. *P<0.05; **P<0.01.
Figure S5. Hx injection in Hx-null mice rescues the hepatic capacity of detoxifying heme. (a) Heme content in the liver of heme-overloaded wild-type mice, Hx-null mice and Hx-treated Hx-null mice at 1 and 3 hours after heme injection (n=8). (b) qRT-PCR analysis of ALAS1 mRNA in the liver of heme-overloaded wild-type mice, Hx-null mice and Hx-treated Hx-null mice at 1 hour after heme injection (NT: n=4; heme: n=7). (c-d) Bilirubin (c) and heme (d) content in the bile of heme-overloaded wild-type mice, Hx-null mice and Hx-treated Hx-null mice at 1 and 3 hours after heme injection (n=8). Bile was collected after cholecystectomy. Values represent mean ± SEM. *P<0.05; **P<0.01; ***P<0.001. Results shown are representative of three independent experiments.
Figure S6. Hx administration increases biliary bilirubin and heme excretion in thalassemic and SCD mice. Data on wild-type, β-Thal and Hx-treated β-Thal mice and HbA, HbS and Hx-treated HbS mice are shown on the left and right respectively. (a) Serum bilirubin and bile bilirubin (c) and heme content (d) (n=4). Bile was collected after gallbladder ligation. Values represent mean±SEM. *P<0.05; **P<0.01; ***P<0.001. Results shown are representative of three independent experiments.
Figure S7. Altered blood pressure and cardiac output in SCD mice. (a) Mean arterial pressure in Hba and Hbs mice. (b) Systolic and diastolic pressure in Hba and Hbs mice. (c,d) Cardiac output and aortic valve peak pressure measured in HbA and HbS mice by echocardiography. HbA n=6; HbS n=10. Results shown are representative of three independent experiments. Values represent mean±SEM. *P<0.05; **P<0.01; ***P<0.001.
Figure S8. Pulsed wave Doppler on the left ventricular outflow (LVOT) in HbA, HbS and Hx-treated HbS mice. The velocity time interval (VTI) was measured for inclusion in the CO calculation. VTI was higher in HbS mice compared to HbA mice and restored after Hx treatment.
## Supplemental Tables

**Table S1. Statistic Analysis.** Table showing the statistic tests used for each panel of each figure. On the right it is reported the total number of comparisons that was applied for each Bonferroni adjustment

<table>
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<th>Figure number (panel)</th>
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<td>Figure 1 (a,b,c,d,e,f,g,h)</td>
<td>Welch T-test</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2 (a,b,c,d,e,f,g,h,i,l)</td>
<td>Two-way ANOVA</td>
<td>2+2 = 4</td>
</tr>
<tr>
<td>Figure 3 (a)</td>
<td>Two-way ANOVA (between Hx-heme and HSA-heme)</td>
<td>3+3+3 = 9</td>
</tr>
<tr>
<td>Figure 3 (b,d,e,f,g,i,m)</td>
<td>One-way ANOVA</td>
<td>3</td>
</tr>
<tr>
<td>Figure 3 (c,h,l)</td>
<td>Two-way ANOVA</td>
<td>2+2+2 = 6</td>
</tr>
<tr>
<td>Figure 3 (n)</td>
<td>Welch T-test</td>
<td>1</td>
</tr>
<tr>
<td>Figure 4 (a)</td>
<td>Two-way ANOVA</td>
<td>4+3+3 = 10</td>
</tr>
<tr>
<td>Figure 4 (b,e)</td>
<td>Two-way ANOVA</td>
<td>3+2+2 = 7</td>
</tr>
<tr>
<td>Figure 4 (c)</td>
<td>One-way ANOVA</td>
<td>3</td>
</tr>
<tr>
<td>Figure 4 (d,f)</td>
<td>Two-way ANOVA</td>
<td>2+2 = 4</td>
</tr>
<tr>
<td>Figure 5 (a,b,c,d,e,f,g,h)</td>
<td>One-way ANOVA</td>
<td>3</td>
</tr>
<tr>
<td>Figure 6 (a,b,c,d,e)</td>
<td>One-way ANOVA</td>
<td>3</td>
</tr>
<tr>
<td>Figure 7 (a)</td>
<td>Two-way ANOVA RM (between HbS and Hx-treated HbS)</td>
<td>4+4+4 = 12</td>
</tr>
<tr>
<td>Figure 7 (b,c,e,f,g)</td>
<td>One-way ANOVA</td>
<td>3</td>
</tr>
<tr>
<td>Figure S1(a)</td>
<td>Two-way ANOVA</td>
<td>4+3+3 = 10</td>
</tr>
<tr>
<td>Figure S1(b)</td>
<td>Two-way ANOVA</td>
<td>2+2 = 4</td>
</tr>
<tr>
<td>Figure S2 (a,b)</td>
<td>Two-way ANOVA RM (between Wt-heme and Hx-/- +heme)</td>
<td>9</td>
</tr>
<tr>
<td>Figure S2 (c)</td>
<td>Welch T-test</td>
<td>2</td>
</tr>
<tr>
<td>Figure S2 (d)</td>
<td>Two-way ANOVA RM (between Wt-heme and Hx-/- +heme)</td>
<td>8</td>
</tr>
<tr>
<td>Figure S3(a)</td>
<td>Welch T-test</td>
<td>1</td>
</tr>
<tr>
<td>Figure S3 (b,c,d)</td>
<td>Two-way ANOVA</td>
<td>4+3+3 = 10</td>
</tr>
<tr>
<td>Figure S4 (a,b,c)</td>
<td>One-way ANOVA</td>
<td>3</td>
</tr>
<tr>
<td>Figure S5(a,b,c,d)</td>
<td>One-way ANOVA (between Wt+heme, Hx-/-+heme and Hx-/-+heme +Hx, per each time point)</td>
<td>3</td>
</tr>
<tr>
<td>Figure S6 (a,b,c)</td>
<td>One-way ANOVA</td>
<td>3</td>
</tr>
<tr>
<td>Figure S7 (a,b,c,d)</td>
<td>Welch T-test</td>
<td>2</td>
</tr>
<tr>
<td>Table S2, S3</td>
<td>One-way ANOVA</td>
<td>3</td>
</tr>
</tbody>
</table>
Table S2. Hx treatment does not recover anemia. Analysis of blood samples from wild-type, β-Thal and Hx-treated β-Thal mice and from HbA, HbS and Hx-treated HbS mice (n=6). Values represent mean ± SEM. WBC: white blood cells; RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; Retic: reticocytes. *P<0.05.

### Blood Analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>Thal</th>
<th>Thal + Hx</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (*10^3 cells/μl)</td>
<td>1.37 ± 0.15</td>
<td>4.53 ± 0.57</td>
<td>3.34 ± 0.18</td>
</tr>
<tr>
<td>RBC (*10^6 cells/ml)</td>
<td>9.53 ± 0.22</td>
<td>7.25 ± 0.18</td>
<td>7.12 ± 0.08</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>13.9 ± 0.26</td>
<td>7.42 ± 0.23</td>
<td>7.4 ± 0.1</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>45.4 ± 1.15</td>
<td>29.92 ± 0.73</td>
<td>28.9 ± 0.7</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>47.53 ± 0.42</td>
<td>41.35 ± 0.77</td>
<td>40.63 ± 0.81</td>
</tr>
<tr>
<td>Retic (%)</td>
<td>3.28 ± 0.47</td>
<td>22.75 ± 0.93</td>
<td>21.27 ± 0.34</td>
</tr>
</tbody>
</table>

N=5 for each genotype

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HbA</th>
<th>HbS</th>
<th>HbS + Hx</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (*10^3 cells/μl)</td>
<td>7.48 ± 1.36</td>
<td>17.11 ± 0.88</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>RBC (*10^6 cells/ml)</td>
<td>12.37 ± 0.01</td>
<td>4.68 ± 0.12</td>
<td>4.65 ± 0.25</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>12.3 ± 0.3</td>
<td>5.56 ± 0.13</td>
<td>5.45 ± 0.25</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>51.15 ± 3.15</td>
<td>24.9 ± 0.6</td>
<td>24.75 ± 0.25</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>41.3 ± 2.6</td>
<td>54.25 ± 1.95</td>
<td>51.15 ± 1.15</td>
</tr>
<tr>
<td>Retic (%)</td>
<td>10.26 ± 5.47</td>
<td>71.7 ± 3.7</td>
<td>70.35 ± 1.35</td>
</tr>
</tbody>
</table>

N=5 for each genotype
Table S3. Echocardiographic and Hemodynamic Parameters in HbA, HbS and Hx-treated HbS mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HbA</th>
<th>HbS</th>
<th>HbS + Hx</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>710 ± 36</td>
<td>671 ± 7.63</td>
<td>714 ± 32</td>
</tr>
<tr>
<td>FS (%)</td>
<td>43.42 ± 2.4</td>
<td>40.76 ± 3.0</td>
<td>39.71 ± 3.25</td>
</tr>
<tr>
<td>EF (%)</td>
<td>75.28 ± 2.43</td>
<td>71.49 ± 3.29</td>
<td>71.04 ± 4.2</td>
</tr>
<tr>
<td>IVSTD (mm)</td>
<td>0.93 ± 0.06</td>
<td>1.218 ± 0.048**</td>
<td>1.099 ± 0.11</td>
</tr>
<tr>
<td>IVSTS (mm)</td>
<td>1.40 ± 0.07</td>
<td>1.839 ± 0.097**</td>
<td>1.682 ± 0.11</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.32 ± 0.11</td>
<td>3.591 ± 0.129</td>
<td>3.50 ± 0.16</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.89 ± 0.12</td>
<td>2.142 ± 0.155</td>
<td>2.12 ± 0.22</td>
</tr>
<tr>
<td>LPWTD (mm)</td>
<td>0.94 ± 0.05</td>
<td>1.164 ± 0.056*</td>
<td>1.13 ± 0.06</td>
</tr>
<tr>
<td>LPWTS (mm)</td>
<td>1.32 ± 0.05</td>
<td>1.645 ± 0.086*</td>
<td>1.49 ± 0.08</td>
</tr>
<tr>
<td>CO (ml/min)</td>
<td>18.04 ± 1.49</td>
<td>55.32 ± 12.3**</td>
<td>23.29 ± 9.5 #</td>
</tr>
<tr>
<td>AV Peak Press (mmHg)</td>
<td>4.22 ± 0.37</td>
<td>16.97 ± 6.02*</td>
<td>6.4 ± 2.61 #</td>
</tr>
<tr>
<td>LV MPI</td>
<td>1.02 ± 0.11</td>
<td>0.72 ± 0.08*</td>
<td>0.90 ± 0.11</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>3.94 ± 0.14</td>
<td>5.64 ± 0.47*</td>
<td>5.26 ± 0.25</td>
</tr>
</tbody>
</table>

HR, heart rate; bpm, beats per minute; FS, percent fractional shortening; EF, precent ejection fraction; IVSTD, interventricular septum thickness in end diastole; IVSTS, interventricular septum thickness in end systole; LVEDD, left ventricle end diastolic diameter; LVESD, left ventricle end systolic diameter; LPWTD, left posterior wall thickness in end diastole; LPWTS, left posterior wall thickness in end systole; CO, cardiac output; AV Peak Press, aortic valve peak pressure; LV MPI, left ventricular myocardial performance index; LVW, left ventricular weight; BW, body weight.
* P<0.05; **P<0.01 HbS versus HbA mice
# P<0.05 HbS+Hx versus HbS mice

Older HbS mice (8-12 month-old) showed right ventricle dilation and developed pulmonary hypertension, accordingly to what occur in the human sickle cell disease\textsuperscript{15,16}. 
Supplemental References


