Human Sickle Cell Blood Modulates Endothelial Heme Oxygenase Activity
Effects on Vascular Adhesion and Reactivity

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Objective—Sickle cell disease (SCD) is characterized by extensive hemolysis, increased cellular adhesion, and vaso-occlusion. Tissues from sickle patients express heme oxygenase-1 (HO-1), the enzyme that degrades free heme/hemoglobin to the signaling molecule carbon monoxide, and the antioxidants biliverdin/bilirubin. Here, we examined the HO response in endothelial cells exposed to human sickle blood and determined whether this response is beneficial for SCD.

Methods and Results—We measured HO activity in human and bovine aortic endothelial cells incubated with human sickle or normal blood. Sickle blood increased HO activity, which was enhanced by hypoxia and was caused mainly by the red cell components of sickle blood. Oxidized hemoglobin was higher in sickle blood and increased markedly over time. Interestingly, HO activity correlated inversely with patients’ hemoglobin levels and positively with bilirubin and lactate dehydrogenase. HO-1 induction, exogenous biliverdin, or carbon monoxide markedly decreased adhesion of sickle blood to the endothelium, and sickle red cells partially inhibited relaxation mediated by carbon monoxide in isolated aortas.

Conclusions—Our results highlight important associations between SCD and HO byproducts, which may counteract vascular complications of SCD. 

Key Words: adhesion ■ carbon monoxide ■ endothelium ■ heme oxygenase ■ sickle cell disease

Sickle cell disease (SCD) is caused by a mutation in the β-globin gene, resulting in abnormal hemoglobin (HbS), either in a homozygous or in a compound heterozygous form with another atypical hemoglobin. The mutated HbS polymerizes in the deoxygenated state, leading to unusually shaped red cells. Sickle erythrocytes hemolyze easily, causing chronic anemia and small vessel occlusion, triggering acute painful episodes and other complications of SCD, such as acute chest syndrome, avascular necrosis, leg ulcers, and renal failure.1,2 Presently, hydroxyurea is the only drug widely used for treating SCD. Hydroxyurea stimulates production of fetal hemoglobin and lowers the concentration of HbS;3 however, most patients receiving it still exhibit hemolysis and an underlying inflammatory state,4 indicating the need for new therapeutic strategies to help patients with SCD.

Despite a well-characterized genetic origin, SCD may be viewed as a chronic inflammatory condition,4–6 with studies revealing abnormally high levels of leukocytes in subjects with SCD.7 Circulating endothelial cells from SCD patients also display an activated state with expression of adhesion molecules,8 and a genetic profile of blood mononuclear cells from 27 patients in steady-state SCD showed upregulation of genes related to oxidative stress and inflammation and global upregulation of proinflammatory markers.4 Interestingly, genes of heme metabolism, including heme oxygenase-1 (HO-1) and biliverdin reductase, were also increased, as already reported for circulating endothelial cells and kidneys of SCD subjects.9

HO-1 is the inducible isoform of HO, the enzyme that converts heme to iron, carbon monoxide (CO), and biliverdin. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase.10 Given that sickle erythrocytes release large amounts of hemoglobin after hemolysis, endothelial HO-1 induction may serve to control excessive heme load. However, the antioxidant and antiinflammatory action attributed to the HO system11 suggest that HO-1 upregulation in SCD may attenuate ischemia-reperfusion injury induced by vaso-occlusive crises. Few published reports addressing this possibility indicate contrasting effects. For instance, a recent investigation in transgenic sickle mice showed that HO-1 induction (via administration of hemin or HO-1 adenovirus)
reduced inflammation and vaso-occlusion, partly by decreasing expression of the adhesion molecules vascular cell adhesion molecule-1 and intercellular adhesion molecule-1. Furthermore, exogenous CO or biliverdin afforded similar protection. Conversely, inhibiting HO activity protected kidneys of sickle mice against ischemic-mediated injury,13 suggesting that a better characterization of the HO system in SCD is required.

In the present study, we investigated the response of human and bovine aortic endothelial cells and rat aortic tissue on exposure to human sickle blood. We measured HO activity at 21% oxygen. BAEC and HAEC were incubated for 6 or 24 hours for the cell adhesion assay, and 18 were used for vasorelaxation experiments.

Materials and Methods

Blood Collection and Patients Characteristics

Blood was collected from 196 SCD patients and 20 healthy controls; detailed information about patients’ characteristics is reported in the Table. Subjects gave written informed consent and the protocol was approved by the National Research Ethics Service (formerly COREC) in England. Patient eligibility criteria included all SCD forms (homozygous HbSS, HbSC, HbSβ thalassemia), age older than 12 months, and receiving hydroxyurea, blood transfusion, or no treatment. Current disease status (steady-state or acute crisis), date of last transfusion or last crisis, and number and date of crises in the previous 2 years were recorded. An acute crisis was defined as acute sickle pain requiring analgesics and hospital admission of the patient, whereas patients with steady-state were asymptomatic. Blood samples were analyzed for hemoglobin, bilirubin, and lactate dehydrogenase (LDH) by the hematology laboratory. Of the 196 blood samples collected, 141 were used for the HO assay (102 samples used with bovine aortic endothelial cells [BAEC] and 39 with human aortic endothelial cells [HAEC]), 37 were used for the cell adhesion assay, and 18 were used for vasorelaxation experiments.

Cell Culture and Experimental Protocol

BAEC were purchased from Coriell Cell Repositories (Camden, NJ) and HAEC were purchased from Promocell (Heidelberg, Germany). Blood was collected in EDTA-coated tubes, stored at 4°C, and used within 24 hours for all experiments. Blood components were separated by centrifugation of whole blood at 1000 g for 15 minutes; RBC were washed with phosphate-buffered saline 3 times before use, and plasma not immediately used for experiments was stored at −80°C.

We first investigated whether sickle blood affected endothelial HO activity at 21% oxygen. BAEC and HAEC were incubated for 6 or
18 hours with medium containing 20% whole sickle or control blood. BAEC were also exposed to 20% plasma or washed RBC for 6 and 18 hours; 20% sickle blood was chosen because this was the minimal dilution affecting HO in preliminary experiments (not shown). We also examined endothelial HO activation by whole sickle blood at 1% O₂, because lack of oxygen stimulates HbS polymerization with deformation of erythrocytes and hemolysis.

**Assay for Endothelial HO Activity**

Cellular HO activity was determined as previously described by our group.¹⁵

**Measurement of Oxidized Hemoglobin**

Oxidized hemoglobin (methemoglobin, oxidized hemoglobin) was determined in the culture medium after incubation of cells with 20% sickle or normal blood for different times as described previously.¹⁶

**RBC Adhesion to the Endothelium**

Adhesion of RBC was measured using an in vitro static assay (gravity sedimentation method) adapted from a previously published method.¹⁷ BAEC and HAEC were exposed to either 5% sickle or normal blood for 45 minutes before measurement of RBC adhesion. Furthermore, BAEC were treated with hemin (1, 10, 30 μmol/L) for 6 hours to induce HO-1 before addition of 5% whole sickle blood. To assess the contribution of HO byproducts, cells were also treated with biliverdin or CO using 2 water-soluble CO-releasing molecules (CO-RMs), CORM-3 and CORM-A1.¹⁸,¹⁹ For this purpose, cells were incubated with biliverdin (0.1, 0.5, 1 μmol/L), CORM-3 (1, 10, 30 μmol/L), or CORM-A1 (1, 5, 10 μmol/L) 30 minutes before exposure to sickle blood. Alternatively, sickle blood treated with biliverdin, CORM-A1, or CORM-3 for 30 minutes was added to endothelial cells for the adhesion assay.

**Isolated Rat Aortic Ring Model**

Transverse ring sections of rat aortas were prepared as previously described.²⁰,²¹ Aortic rings were incubated with 1% sickle or normal blood components and challenged with CORM-A1 (please see http://atvb.ahajournals.org).

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**Statistical Analysis**

Data were analyzed for normal distribution using the Kolmogorov-Smirnov test, and data sets fulfilling the normality criteria were further analyzed by ANOVA or unpaired t test to determine significance. For data not normally distributed (data sets in Figure 2), nonparametric tests (Mann-Whitney) were applied. Pearson correlation coefficients examined the relationship between patients’ hemoglobin values (n=47), plasma bilirubin (n=48), LDH (n=26), and age (n=43) with HO activity. Results were expressed as means±SEM and considered significant at P<0.05.

**Results**

**Sickle Blood Induces HO in Endothelial Cells**

As shown in Figure 1A (BAEC) and B (HAEC), 20% whole normal blood did not affect cellular HO activity after 6 or 18 hours of incubation. In contrast, 20% whole sickle blood significantly increased the activity, with the increase being more pronounced at 18 hours than at 6 hours. Interestingly, 20% normal or sickle plasma incubated for 6 hours or 18 hours did not change activity levels (Figure 1C), whereas sickle RBC caused a significant increase in HO compared with normal red cells (Figure 1D). We note that HO induction was more pronounced with whole sickle blood than sickle RBC. Compared to normoxic conditions, hypoxia further enhanced (≈2-fold) the increase in HO activity elicited by sickle blood after 18 hours of incubation (Figure 1E), whereas normal whole blood caused no changes.

Basal levels of methemoglobin were higher in sickle than normal whole blood (please see http://atvb.ahajournals.org).
HO Activity Levels Analyzed According to Patient Treatment Regimen and Time Elapsed Since Last Sickle Crisis

Considering that patients were experiencing different stages of SCD and were undergoing different treatment regimens when the blood was collected, we hypothesized that these differences could be reflected in the levels of HO activity stimulated by sickle blood. The sickle sample population included HbSS, HbSC, and HbSβ thalassemia traits and, as reported in Figure 2, blood from patients receiving hydroxyurea (n=7; Figure 2A), transfusion (n=5; Figure 2B), or in acute crisis (n=10; Figure 2C) did not elicit any changes in endothelial HO upregulation when compared with samples from patients not receiving treatment and stable. Conversely, HO levels grouped according to time elapsed from the last crisis indicated that blood from SCD subjects who experienced vaso-occlusive crises in the previous year (n=23) elicited a significantly higher activity (*P<0.05) compared to blood from subjects experiencing crises >2 years before (n=6; Figure 2D). It is acknowledged that the small number of subjects in certain subgroups is a limitation that reduces the power of the statistical analysis of interpatient variability.

Correlations of HO Activity With Patients’ Hemoglobin, Bilirubin, LDH Levels, and Age

Correlation analysis performed comparing HO activity with patient variables revealed that: (1) HO activity correlated inversely with hemoglobin levels (n=47; r=-0.39;
P=0.007; Figure 3A), indicating that higher activity was stimulated by sickle blood containing progressively lower hemoglobin; (2) there was a positive relationship between HO and plasma bilirubin levels (n=48; r=0.58; P<0.001; Figure 3B), implying that the in vivo production of bilirubin in SCD subjects significantly matched the activity levels induced by blood in vitro; (3) HO correlated positively with LDH (n=26; r=0.45; P=0.02; Figure 3C), a marker of cell injury, suggesting that higher vascular damage in patients was accompanied by greater HO activation; and (4) HO activity tended to decline with increasing age of patients (n=43; r=-0.34; P=0.03; Figure 3D).

**HO-1 Induction, Biliverdin, and CO Attenuate the Adhesion of Sickle RBC to the Endothelium**

As expected, normal blood did not adhere to the BAEC in culture, whereas sickle blood adhered extensively (Figure 4A; P<0.05; for data on HAEC, see http://atvb.ahajournals.org). Pretreatment with hemin (1, 10, and 30 μmol/L), which leads to upregulation of HO-1, reduced sickle cell adhesion in a concentration-dependent manner, and inhibition of HO activity with tin protoporphyrin IX reversed the protection elicited by hemin (Figure 4B). Pretreating cells with biliverdin (0.1, 0.5, 1 μmol/L) also decreased sickle cell adhesion (Figure 4C). To examine whether CO influenced RBC adhesion, we used 2 CO-RMs recently developed in our laboratory. CORM-3 and CORM-A1 have distinguished chemical characteristics affecting their kinetic of CO release in physiological buffers (CORM-3 half life=3.6 minutes; CORM-A1 half-life=21 minutes) and their interactions with cells and tissues.22,23 Pretreating endothelial cells with CORM-3 had little effect on sickle cell adhesion (Figure 4D), whereas CORM-A1 reduced adhesiveness in a concentration-dependent manner (Figure 4E). Neither iCORM-3 nor iCORM-A1 influenced the adhesion response. Furthermore, longer or shorter preincubation times did not change the outcome of the experiment. Importantly, direct incubation of sickle blood with biliverdin, CORM-3, or CORM-A1 before addition to cells markedly decreased adhesion (Figure 5A–C). Adhesion was also lower when sickle blood was pretreated with i-CORM-A1. Thus, induction of endothelial HO-1 prevents sickle red cell adhesion, as does treatment of either endothelial cells or sickle blood with HO-1-derived products. The kinetics of CO release by CORM-3 and CORM-A1, and possibly their diverse chemical nature, explain their different effects.

**Sickle RBC Modulate CORM-A1 Vasodilation in Isolated Aortas**

In experiments studying aortic relaxation by CORM-A1, we found that sickle blood components decreased CORM-A1 relaxation, whereas normal blood did not (please see http://atvb.ahajournals.org).
Discussion

Chronic hemolysis in SCD stimulates vascular tissue to counteract the pro-oxidative and proinflammatory environment created by free heme/hemoglobin. HO-1, the inducible protein responsible for heme degradation, is an essential enzyme recruited by the vasculature in the active response to high heme exposure. Here, we examined whether human sickle blood affects HO activation in HAEC and BAEC, and whether HO-1–derived products exert antiadhesive and vasodilatory properties in the presence of sickle blood. We found that sickle blood increased endothelial HO activity in a time-dependent manner, and hypoxia further amplified this effect. The RBC fraction of sickle blood seemed the major contributor to HO induction. Sickle blood exhibited higher basal levels of oxidized hemoglobin when compared to normal blood, and these changes correlated closely with the ability of sickle blood to induce HO. Analysis of patient profiles revealed that the time elapsed from the last vaso-occlusive crisis influenced the extent of HO activity. In addition, HO values correlated with patients’ hemoglobin, bilirubin, and LDH levels, indices of hemolysis, and vascular damage. Most specifically, sickle blood taken from patients with the most severe SCD complications elicited the highest endothelial HO response, perhaps suggesting a homeostatic mechanism. Upregulation of HO-1, exogenous biliverdin, or CO markedly decreased adhesion of sickle erythrocytes to the endothelium. Furthermore, sickle red cells partially inhibited the relaxation elicited by CORM-A1 in precontracted aortic rings. Our results indicate an important association between SCD and HO, suggesting that heme degradation products may reduce vascular complications elicited by sickle blood.

We expected to find that both sickle red cells (a source of heme) and plasma (containing extraerythrocytic factors such as free heme and inflammatory molecules) would contribute to inducing HO in our experiments; however, increased HO activity was only stimulated by RBC. We do not exclude that plasmatic components may synergize with erythrocytes to upregulate HO because the activity was higher with whole blood than RBC. Accordingly, reports have shown that sickle plasma modifies endothelial gene expression toward a proangiogenic and anti-inflammatory phenotype. We also found that hypoxia enhanced HO activity, potentially mimicking the pathological scenario occurring during vaso-occlusive crises and ischemia-reperfusion episodes. It is known that oxidized hemoglobin is susceptible to heme loss, and heme is a strong inducer of HO-1. Therefore, our data detecting high basal levels of oxidized hemoglobin in sickle blood and its continuous increase during the incubation with cells strongly implicate that heme derived from oxidized hemoglobin contributes HO activation. Altogether, these results point to sickle red cell hemolysis, which is stimulated under hypoxia, as one of the causes of endothelial HO induction. Nevertheless, the participation of factors such as heme-induced oxidative stress is possible and we are currently exploring this hypothesis.

Differences within the SCD population in the present study were analyzed retrospectively, allowing us to explore variables that could influence the extent of HO induction. The analysis and discussion of these results are made with acknowledgement of some limitations concerning our approach. Because data were evaluated subsequently to testing blood samples, we could explore only a restricted number of variables. For example, fetal hemoglobin levels, an important determinant of SCD severity, were not routinely measured in our patients, and therefore we could not examine their influence on HO induction. Furthermore, despite our total study group consisting of 196 patients and 20 controls, the statistical analysis of interpatient variability was underpowered because only few patients represented some subgroups investigated. Nevertheless, the analysis uncovered interesting
trends that future exploration in a larger number of patients may corroborate. For instance, although not statistically significant, blood from patients receiving hydroxyurea or transfusion elicited lower HO compared to blood from patients not receiving treatment. Hydroxyurea, usually prescribed to patients with severe and recurrent chest crises, stimulates fetal hemoglobin production and ameliorates clinical symptoms. Regular blood transfusion is performed to improve anemia and prevent complications in SCD patients who already had strokes. Thus, induction of HO may reflect disease severity and a better clinical management of SCD may diminish the factor(s) in blood responsible for enzyme activation. The results showing that blood from patients experiencing a crisis within the previous year stimulated higher HO than blood from subjects whose last crisis was ≥2 years before support this idea. Therefore, the clinical and biochemical stress imposed by vaso-occlusive crises may be linked to HO expression in SCD.

In our analysis correlating patients’ blood parameters with HO activity values, the decline in patients’ hemoglobin levels correlated with increasing endothelial HO in vitro. This again suggests a relationship between the severity of the disease and HO response, because higher free hemoglobin/heme liberated during hemolysis and vaso-occlusive crises could increase the activity. Accordingly, patients’ bilirubin levels also correlated with HO, suggesting that the stimuli inducing production of heme metabolites in SCD subjects also promoted HO upregulation in vitro. Moreover, LDH correlated positively with HO, linking the levels of activity to the extent of vascular damage. In this study, we also found that HO-1 overexpression attenuated adhesiveness of sickle blood to the endothelium. This effect was reversed by an inhibitor of the enzyme activity, potentially implicating all heme degradation products. In fact, exogenous application of biliverdin or CO significantly decreased adhesion, presumably via antioxidant and antiinflammatory mechanisms that remain to be investigated. Preincubation of blood with biliverdin, CORM-3, or CORM-A1 inhibited blood adhesion more than preincubation of cells, suggesting that sickle blood plays a predominant role in stimulating adhesion and may be a target for therapeutic intervention. However, vascular endothelial cells of SCD subjects exhibit a proinflammatory/activated phenotype and might affect the adhesion process more than our cells in culture. Collectively, our results support recent findings showing that biliverdin and CO gas inhibited vascular stasis and expression of adhesion molecules in a transgenic mice model of SCD. It was also interesting that sickle erythrocytes inhibited CORM-A1–mediated relaxation in precontracted aortas, suggesting that defective sickle erythrocytes bind CO with different affinity, thus scavenging CO and preventing its vascular activities more effectively than normal RBC.

**Conclusion**

In conclusion, human sickle blood enhances endothelial HO and the positive effect of HO-1 induction, biliverdin, and CO-RMs in reducing sickle blood adherence and in promoting vasodilation, indicating the need to further explore the therapeutic potentials of the HO pathway in the treatment of SCD.

**Acknowledgments**

The authors thank Professor Brian Mann and Professor Roger Alberto for synthesis of CO-RM.

**Sources of Funding**

This research was supported by a grant from the Dunhil Medical trust (R.M., R.F).

**Disclosure**

R.M. is a shareholder, co-founder, and member of the Board of Directors of hemoCORM. C.J.G. is co-founder and member of the Board of Directors of hemoCORM. Northwick Park Institute for Medical Research and R. Motterlini have financial interests with Alfama.

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Arterioscler Thromb Vasc Biol. 2010;30:305-312; originally published online December 3, 2009;
doi: 10.1161/ATVBAHA.109.196360

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplement Material

Figure I

Figure I. Hemoglobin from sickle blood is more susceptible to oxidation than hemoglobin from normal blood. Human aortic endothelial cells were incubated with medium containing 20% whole sickle (n=5) or normal blood (n=5) for 6 or 18 h. Aliquots of medium were collected at 0, 6 and 18 h and the levels of methemoglobin (MetHb) determined. As shown in the graph, sickle blood exhibited at basal conditions (0 h) higher levels of MetHb than normal blood. Furthermore, hemoglobin oxidation in sickle blood rose progressively and dramatically over the time of incubation while MetHb levels remained steady and unchanged in normal blood. Data represent the mean±SEM of independent experiments. * P<0.05 vs normal blood.
Figure II

A

No. of adherent RBC/mm²

Normal  SCD

B

No. of adherent RBC/mm²

SCD  BV (1 µmol/L)  CORM-A1 (10 µmol/L)  CORM-3 (10 µmol/L)

C

No. of adherent RBC/mm²

SCD  BV (1 µmol/L)  CORM-A1 (10 µmol/L)  CORM-3 (10 µmol/L)
**Figure II.** Biliverdin and carbon monoxide inhibit sickle red cell adhesion in human aortic endothelial cells. A, adhesion of red blood cells determined after exposure of HAEC to normal (n=5) or sickle blood (n=10). B, the adhesion assay was also performed in HAEC incubated with biliverdin (BV, 1 µmol/L, n=5), CORM-A1 (10 µmol/L, n=5) or CORM-3 (30 µmol/L, n=5) for 30 min before exposure to sickle blood (SCD). C, alternatively, sickle blood pre-treated with biliverdin, CORM-A1 or CORM-3 (n=5 for each treatment) for 30 min was added to endothelial cells for the adhesion assay. As observed with BAEC, pre-incubation of endothelial cells with biliverdin, CORM-A1 or CORM-3 resulted in a reduction in the adhesion of sickle red blood cells to the endothelium; sickle red cell adhesion was further and significantly diminished when sickle blood was pre-incubated with the compounds (p<0.001). Data represent the mean±SEM of independent experiments. * P<0.05 vs normal blood in graph A; * P<0.05 vs SCD in graphs B and C.
Figure III

A. Whole blood

B. RBC

C. Plasma
Figure III. Sickle red blood cells modulate CORM-A1 vasodilation in isolated aortas. Transverse ring sections of aortas from male Sprague Dawley rats were suspended under 2 g tension in oxygenated Krebs Henseleit buffer. A 1% solution of blood was warranted in this model as higher concentrations of blood would lead to scavenging of CO by hemoglobin. The protocol used was based on previous studies performed in a similar model but assessing the effect of nitric oxide\textsuperscript{19}. (A) Aortic rings in Krebs buffer (CORM-A1, n=10), 1% whole sickle (Sickle blood+CORM-A1, n=5) or normal blood (Normal blood+CORM-A1, n=5) were challenged with 1 \( \mu \text{mol/L} \) phenylephrine and 100 \( \mu \text{mol/L} \) CORM-A1 was added at the peak of phenylephrine contraction. Contraction was followed for 60 min from addition of CORM-A1. (B) Results of similar experiments conducted in the presence of 0.5% sickle (Sickle RBC+CORM-A1, n=7) or normal (Normal RBC+CORM-A1, n=4) RBCs or (C) 0.5% sickle (Sickle plasma+CORM-A1, n=7) or normal (Normal plasma+CORM-A1, n=4) plasma. We found that whole sickle blood only marginally reduced CORM-A1-mediated dilation with an overall response similar to that of normal blood or buffer. However, sickle RBCs significantly decreased CORM-A1 relaxation, while normal red cells did not. Moreover, normal and sickle plasma inhibited the dilation elicited by CORM-A1 to a similar extent. Data represent the mean±SEM. * P<0.05 vs. CORM-A1 and normal blood.