MP4CO, a pegylated hemoglobin saturated with carbon monoxide, is a modulator of HO-1, inflammation, and vaso-occlusion in transgenic sickle mice

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Key Points
• Carbon monoxide treatment of murine sickle mice can ameliorate inflammation and vaso-occlusion.
• MP4CO induces heme oxygenase-1 and Nrf2 to mediate these salutatory effects.

Transgenic sickle mice expressing βthal hemoglobin have activated vascular endothelium in multiple organs that exhibits enhanced expression of NF-κB and adhesion molecules and promotes microvascular stasis in sickle, but not normal, mice in response to hypoxia/reoxygenation (H/R), or heme. Induction of heme oxygenase-1 (HO-1) or administration of its products, carbon monoxide (CO) or biliverdin, inhibits microvascular stasis in sickle mice. Infusion of human hemoglobin conjugated with polyethylene glycol and saturated with CO (MP4CO) markedly induced hepatic HO-1 activity and inhibited NF-κB activation and H/R-induced microvascular stasis in sickle mice. These effects were mediated by CO; saline or MP4 saturated with O2 (MP4OX) had little to no effect on H/R-induced stasis, though unmodified oxyhemoglobin exacerbated stasis. The HO-1 inhibitor, tin protoporphyrin, blocked MP4CO protection, consistent with HO-1 involvement in the protection afforded by MP4CO. MP4CO also induced nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), an important transcriptional regulator of HO-1 and other antioxidant genes. In a heterozygous (hemoglobin-AS) sickle mouse model, intravenous hemin induced cardiovascular collapse and mortality within 120 minutes, which was significantly reduced by MP4CO, but not MP4OX. These data demonstrate that MP4CO induces cytoprotective Nrf2 and HO-1 and decreases NF-κB activation, microvascular stasis, and mortality in transgenic sickle mouse models. (Blood. 2013; 122(15):2757-2764)

Introduction

Heme oxygenase-1 (HO-1) is a cytoprotective enzyme that degrades free heme to carbon monoxide (CO), biliverdin, and iron. Induction of HO-1 activity elicits protective effects in a variety of models of inflammatory, ischemic, and oxidant injury.1,2 and conversely, humans or mice lacking HO-1 are sensitized to oxidant injury.3,4 In transgenic sickle mice, HO-1 plays a vital role in the inhibition of endothelial activation, rolling and adhesion of leukocytes to the vessel wall and vaso-occlusion.5 The cytoprotective properties of HO-1 are mimicked by the products of the heme/HO-1 reaction, CO and biliverdin.6

CO is a potent mediator of cell protection and has a number of properties that make it an attractive therapeutic option for treating sickle cell disease (SCD) including vasodilator, anti-inflammatory, left-shift of the hemoglobin (Hb)–oxygen dissociation curve, up-regulation of HO-1, and activation of cytoprotective cell signaling pathways.6,7 Beneficial effects of exogenous CO administration have been observed in SCD5,6 because CO may prevent HbS polymerization,8 Hb oxidation, and heme release9 and provide anti-inflammatory/antiadhesive actions.5 Additionally, CO induces HO-1 expression indirectly by its action on the stress-induced, antioxidant response element transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2).10

The development of inhaled CO as a therapeutic agent is limited by questions of safety and the need for controlled delivery systems. MP4CO is a solution of polyethylene glycol (PEG)–conjugated human Hb saturated with CO gas. We have previously demonstrated that CO from MP4CO administered intravenously (IV) rapidly equilibrates with red cell Hb and exerts cytoprotective effects, reducing myocardial infarct size in rats.11 The objectives of the current research were to examine the potential benefits of CO by intravenous administration of MP4CO in transgenic sickle mouse models before and after a vaso-occlusive stimulus, hypoxia/reoxygenation (H/R). Our hypothesis was that the potential anti-inflammatory actions of MP4CO would diminish H/R-induced stasis in transgenic sickle mice. Additionally, we examined the mechanism of MP4CO action by comparing MP4CO with MP4 saturated with O2 (MP4OX) and the effects on NF-κB, Nrf2, and HO-1 expression.
Methods

Reagents
All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified differently. MP4CO, MP4OX, and stroma-free Hb were formulated in lactated Ringer’s solution (LRS) and prepared by Sangart, Inc. (San Diego, CA), as described previously.12

Mice
All animal breeding and experiments were approved by the Institutional Animal Care and Use committees at the University of Minnesota Medical School and Sangart, Inc., and conducted in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals. We used male and female NY1DD13 and heterozygous HbAS-Townes14 transgenic sickle mice at 8-13 weeks of age as models for human SCD and SCD trait, respectively. The NY1DD mice and HbAS mice are on C57BL/6 and mixed genetic backgrounds, respectively. The NY1DD mice are homozygous for deletion of the mouse β\textsuperscript{mow} globin locus and express a human α and β\textsuperscript{b} globin transgene. Heterozygous HbAS mice were derived by breeding male HbSS-Townes mice (α\textsuperscript{b-};β\textsuperscript{b+};hbα\textsuperscript{a+};β\textsuperscript{b+}) with female HbAS-Townes mice (α\textsuperscript{b-};β\textsuperscript{b+};hbα\textsuperscript{a+};β\textsuperscript{b+}). Globin phenotypes in both strains were determined by isoelectric focusing.

Mouse treatments
Mice were treated with LRS, MP4CO, MP4OX, or unmodified Hb. MP4CO and MP4OX are 4.3-g/dL solutions of human Hb conjugated with PEG and saturated with CO or O\textsubscript{2}, respectively, and identical in all aspects (colloid osmotic pressure, molecular weight, concentration, production process) except the heme ligand, as previously published.11,12 Stroma-free Hb (without pegylation) is a 4.3-g/dL solution of human Hb saturated with O\textsubscript{2}. Mice with implanted dorsal skin-fold chambers (DSFCs) (see below) were given a bolus injection via the tail vein of 2, 4, 8, or 12 mL/kg of LRS, MP4CO, MP4OX, or Hb 24 hours pre-H/R, 30 minutes post-H/R (1 hour hypoxia at 7% O\textsubscript{2}/93% N\textsubscript{2}, followed by 30 minutes in room air), or both. In some control mice, hemin chloride was injected intraperitoneally (IP) (40 μmoles/kg/day × 3 days) to induce HO-1, a known inhibitor of vascular stasis.5 Additionally, in some mice, tin protoporphyrin (SnPP) was injected IP (40 μmoles/kg/day × 3 days) to inhibit HO-1 activity.

Collection and measurement of exhaled CO
Volume of expired CO was measured in mice using a rebreathing collection technique and gas chromatography, as previously described.15

Vascular stasis in dorsal skinfold of NY1DD mice
H/R-induced stasis of venular blood flow in the subcutaneous skin was measured in NY1DD mice with implanted DSFCs using intravital microscopy as previously described 3 days after DSFC implantation.10 At baseline, with the mice in ambient air, flowing venules were selected at random, and their relative locations were mapped and recorded. The mice were then subjected to 1 hour hypoxia (7% O\textsubscript{2}/93% N\textsubscript{2}), followed by reoxygenation in room air. After 1 hour of reoxygenation, the same venules were reexamined for blood flow. Venules with no flow were counted as static, and the percentage of static venules was calculated. A minimum of 33 subcutaneous venules were examined in each mouse.

Mouse tissue collection
After 4 hours of reoxygenation, the mice were sacrificed and tissues harvested as described.10 Mice were asphyxiated in a CO\textsubscript{2} chamber for approximately 2 minutes. Blood was collected by cardiac puncture for isolation of EDTA plasma. Livers were removed, frozen in liquid nitrogen, and stored at −85°C. Nuclear extracts and microsomes were prepared from livers as described.17

Measurement of HO enzyme activity
HO activity was measured in liver microsomes as described.17

Western blots of liver NF-κB phospho-p65, Nrf2, and HO-1
Liver nuclear extracts (NF-κB phospho-p65 and Nrf2) or microsomes (HO-1) were analyzed by western blot. The membranes were probed with rabbit anti-NF-κB phospho-p65 (Ser536) (Cell Signaling Technology, Beverly, MA), rabbit monoclonal anti-Nrf2 (Cell Signaling Technology) or mouse monoclonal anti-HO-1 (Enzo Life Sciences, Farmingdale, NY). Primary antibodies were visualized with alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX). Immunoreactive bands were visualized with ECF substrate (GE Healthcare, Piscataway, NJ) and a Storm Reader (GE Healthcare). Microsomal HO-1 blots were stripped (Restore Stripping Buffer; Thermo Scientific, Waltham, MA) and reprobed with rabbit anti-GAPDH (Sigma-Aldrich).

Heme-induced cardiorespiratory collapse and mortality
Heterozygous HbAS-Townes mice were anesthetized with 2% isoflurane in 30% oxygen followed by maintenance with IP injection of a fentanyl (225 μg/kg)/ketamine (120 μg/kg) cocktail. Animals were tracheotomized and mechanically ventilated with 30% oxygen to maintain eucapnia (Kent Scientific, Torrington, CT). The right femoral artery and vein were cannulated for measurement of arterial pressure and introduction of drugs/fluids, respectively, after which supplemental oxygen was discontinued, and animals were ventilated with room air. Core temperature was maintained at 38°C. Arterial pressures were recorded at 100 Hz using a BIOPAC data acquisition system (BIOPAC Systems, Goleta, CA).

Following 10 minutes stabilization, hemin chloride (50 μmol/kg; Frontier Scientific, Logan, UT) was injected (0.3 mL bolus) IV. Ten minutes after hemin injection, mice were infused with 2, 4, or 8 mL/kg of saline, MP4OX, or MP4CO over 30 minutes. Immediately upon death or at 150 minutes posthemin, blood samples were taken by cardiac puncture.

Statistical analyses
All statistical analyses were performed with SigmaStat 2.0 for Windows (SPSS Inc., Chicago, IL) or JMP statistical software (SAS Institute, Cary, NC). Comparisons of vascular stasis in multiple treatment groups were made using 1-way analysis of variance with pairwise multiple comparisons made using the Holm-Sidak method. For the analysis of arterial pressure following hemin injection, differences between groups of animals were analyzed using a 2-way repeated-measures analysis of variance with treatment group and time as the 2 factors. Survival data were plotted as Kaplan-Meier graphs and analyzed by log-rank test.

Results

MP4CO rapidly releases CO in vivo
Exhaled CO was collected and measured 24 hours after infusion (baseline), 0-3 hours after infusion, and 24-27 hours after infusion of MP4CO or MP4OX (12 mL/kg) (Figure 1). At baseline, sickle mice exhaled 0.7-1.2 nmols/h/g CO, which increased to 4.2 nmols/h/g at 0-3 hours after MP4CO infusion (P < .025, baseline vs 0-3 hours). Exhaled CO had returned to baseline (1.1 nmols/h/g) by the time the 24- to 27-hour sample was collected. Exhaled CO was not significantly different from baseline at any time after MP4OX infusion. These data indicate that CO is rapidly bioavailable after MP4CO infusion.

MP4CO inhibits H/R-induced stasis in NY1DD sickle mice
To determine the effect of MP4CO on H/R-induced stasis, we infused LRS, MP4CO, MP4OX, or Hb (8 mL/kg) into NY1DD sickle mice
24 hours before hypoxia (Figure 2). Mice infused with MP4CO had significantly less stasis (9.1%) after 1 hour of hypoxia and 1 hour reoxygenation than did mice infused with LRS (25.1%, \( P < .009 \)). Stasis in mice infused with MP4OX (21.8%) was not significantly different from that of LRS-treated mice. Mice infused with Hb had significantly more stasis (36.6%) than did mice treated with LRS (\( P < .017 \)). As previously shown,3 heme pretreatment IP for 3 days increases HO-1 expression several-fold in tissues and prevents H/R-induced stasis. Mice pretreated for 3 days with hemin IP had significantly lower stasis (2.8%) than did LRS-treated mice (\( P < .006 \)). These data show that pretreatment with MP4CO, but not MP4OX, inhibits stasis, whereas pretreatment with Hb exacerbates stasis.

Sickle mice infused with MP4CO 30 minutes after H/R had significantly less stasis (11.0%) after 1 hour of hypoxia and 1 hour reoxygenation than did mice infused with LRS (25.6%, \( P < .017 \)) (Figure 3). Stasis in sickle mice infused with MP4OX also had stasis (17.6%) lower than mice treated with LRS (\( P < .025 \)), but higher than MP4CO-treated mice (\( P = .052 \)). Unmodified Hb induced significantly greater stasis (44.3%) than did LRS treatment (\( P < .013 \)). Sickle mice infused with MP4CO twice (\( > 2 \) IV) had less stasis (5.8%) than did mice infused with MP4OX twice (\( > 2 \) IV, 11.9%, \( P < .027 \)) or MP4CO (\( P = .054 \)) or LRS (\( P = .029 \)) once.

**MP4CO inhibits NF-κB activation**

Activated nuclear NF-κB is a key driver of the inflammatory response. NF-κB expression was examined in liver nuclear extracts by western blot (Figure 4A). The livers of NY1DD mice were removed and frozen 4 hours posthypoxia, 29 hours after LRS, MP4CO, MP4OX, or Hb infusion (8 mL/kg). Activated nuclear NF-κB phospho-p65 was markedly reduced in the livers of sickle mice treated with MP4CO, indicating that MP4CO has a long-acting (>29 hours) inhibitory effect on NF-κB activation.

Thin sections of lungs and livers taken from NY1DD sickle mice treated with LRS, MP4CO, or MP4OX (8 mL/kg) 4 hours posthypoxia/29 hours posttreatment were immunostained for NF-κB phospho-p65 to examine NF-κB activation around blood vessels. In mice treated with LRS or MP4OX, NF-κB phospho-p65 could be seen in endothelial cells around blood vessels in the lungs (supplemental Figure 1A-B, found on the Blood website) and liver (supplemental Figure 2), as well as lung epithelial cells and hepatocytes. At higher magnification in mice treated with LRS or MP4OX, staining was primarily localized to nuclei, which indicates enhanced NF-κB activation in these tissues. MP4CO treatment markedly reduced NF-κB phospho-p65 nuclear staining in cells, indicative of decreased NF-κB activation in mice treated with MP4CO.

**MP4CO activates Nrf2**

Activated nuclear Nrf2 is an important transcriptional regulator of antioxidant response genes including HO-1. Nrf2 expression in liver nuclear extracts was examined by western blot (Figure 4B). Four hours posthypoxia—ie, 29 hours after LRS, MP4CO, MP4OX, or Hb infusion (8 mL/kg)—Nrf2 was markedly increased in MP4CO-treated sickle mice in comparison with mice treated with LRS, MP4OX, or Hb, indicating that MP4CO also has long-acting (>29 hours) effects on Nrf2 activation.

**MP4CO induces HO-1**

HO-1 protein expression was examined in liver microsomes by western blot (Figure 4C). Four hours posthypoxia—ie, 3.5 hours and 29 hours after LRS, MP4CO, MP4OX, or Hb infusion (8 mL/kg)—HO-1 was significantly elevated in liver microsomes after MP4CO infusion in comparison with LRS or MP4OX. Hb did not induce liver HO-1 at 24 hours (data not shown). The same liver microsomes were used to measure HO activity (Figure 4D). MP4CO treatment induced HO activity at both 3.5 hours and 29 hours postinfusion in comparison with LRS (\( P < .017 \)) and MP4OX (0.025). HO activity following MP4OX infusion was not significantly different from LRS at either time.

Pretreatment of sickle mice with SnPP, a potent inhibitor of HO activity (40 μmole/kg/d IP \( \times 3 \) days), inhibited HO activity 29 hours after MP4CO infusion (8 mL/kg) in comparison with mice treated with MP4CO alone (Figure 4D; \( P < .025 \)). SnPP also abrogated the protective effect of MP4CO against H/R-induced stasis (Figure 4E; \( P < .025 \)).

Thin sections of lungs and livers taken from NY1DD sickle mice treated with LRS, MP4CO, and MP4OX (8 mL/kg) 4 hours posthypoxia/29 hours posttreatment were immunostained for HO-1. In mice treated with MP4CO, HO-1 expression was upregulated in all
cells around blood vessels in the lungs (supplemental Figure 3A-B) and liver (supplemental Figure 4) in comparison with mice treated with LRS and MP4OX, which is consistent with reduced H/R-induced vaso-occlusion and a lower inflammatory tone in NY1DD sickle mice treated with MP4CO.

**MP4CO limits cardiorespiratory collapse and mortality in HbAS mice infused with hemin**

Intravenous hemin chloride has been reported to induce acute lung injury and mortality in transgenic sickle mice. We examined the ability of MP4CO to moderate the acute effects of intravenous hemin in heterozygous HbAS mice. These mice have a red blood cell turnover rate that is intermediate between Hb-AA and HbSS mice. Hemin infusion induced vaso-occlusion and a lower inflammatory tone in NY1DD sickle mice treated with MP4CO.

**Discussion**

The effects of CO in alleviating the consequences of SCD have been studied for a number of years. CO inhibits Hb-S polymerization, red cell shape change, and vaso-occlusion by direct actions on Hbs and also via indirect actions preventing red cell lysis, inflammation, adhesion, and apoptosis, and by activating guanylate cyclase leading to vasodilation. These actions of CO are also elicited by increased expression and activity of HO-1, leading to the conclusion that production of CO and biliverdin and induced-ferritin iron sequestration are beneficial in a number of hemolytic scenarios. The current results corroborate previous data from inhaled CO and demonstrate (1) the unique observation that intravenous administration of exogenous CO protects sickle mice from the vascular effects of the pro-oxidant stimuli hypoxia or hemin; (2) that the increased expression and activity of HO-1 are integral in the mechanism of protective effects against hypoxia; and (3) that the mechanism of this effect may be through CO-mediated increases in nuclear Nrf2, enhanced expression and activity of HO-1, and decreases in NF-kB activation and expression of adhesion molecules such as P-selectin and VWF on the vessel wall.

Delivery of CO in animals is accomplished typically using the inhaled gas, although pharmacologic effects have been demonstrated
with CO-releasing molecules, cell-free CO-Hb, and CO-saturated red blood cells. MP4CO is a solution of human Hb tetramer conjugated with 5 kD PEG on the 4 globin chains and saturated with 100% CO. MP4OX is the identical molecule saturated with oxygen. The CO from MP4CO rapidly distributes in blood and has a half-life in circulation of approximately 90 minutes (Cabrales et al and unpublished data). After the release and distribution of CO from MP4CO, the fate of the PEG-Hb is identical to that of MP4OX, ie, uptake by tissue macrophages with predominantly renal excretion over the ensuing days. The half-life of the PEG-Hb moiety is approximately 20 hours in rats and humans. Comparison of MP4CO and MP4OX in both the NY1DD and the HbAS mice allows clear distinction of CO gas from the solution properties of PEG-Hb, which includes effects on plasma volume and peripheral perfusion demonstrated previously with MP4OX. The bioavailability of CO from MP4CO is demonstrated in the current study by the elevated concentration of CO in expired air from sickle mice treated with MP4CO, but not with MP4OX. Although the tissue distribution of CO is unknown, our data suggest that CO freely dissociates from MP4CO and is available to bind native red cell Hb or distribute into endothelial cells and other tissues. We have demonstrated that MP4CO reduced myocardial infarct size after ischemia/reperfusion in rats, confirming the cytoprotective effects of CO demonstrated by others. In the present study, MP4CO inhibited venular stasis in NY1DD mice in response to H/R and reduced cardiovascular collapse and mortality in HbAS mice in response to hemin, neither of which was observed following administration of MP4OX. Similarly, MP4CO substantially reduced NF-κB activation, increased HO-1 expression and activity, and increased nuclear Nrf2, none of which was observed with MP4OX.
and CO.

Although basal HO-1 expression is elevated in transgenic induction of ferritin and release of protective products biliverdin to toxic heme by a number of mechanisms, including the counteracting these deleterious actions, an important protective cellular adhesion/aggregation, vaso-occlusion, and increased mortality.

Consequences in transgenic sickle cell mice characterized by cellular properties, or the high nitrite reductase activity imparted by the size, extravasation, heme retention, solution peroxidation, and iron-catalyzed oxidation, promoting vascular stasis when administered pre- or post-H/R. MP4CO and MP4OX tend to reduce venular stasis, and unmodiﬁed HbS exacerbated stasis when administered pre- or does unmodiﬁed Hb, perhaps because of differences in size, extravasation, heme retention, solution properties, or the high nitrite reductase activity imparted by the stabilized R-state conformation and low PS0 of the PEG-Hb molecule.

A number of mechanisms may contribute to the observed effects of MP4CO. CO potentially prevents HbS polymerization and increases the delay time for red cell shape change. The CO administered in the current study is approximately 1/20 of the molar heme load (or ancillary hemodynamic and volume effects) introduced by MP4OX, MP4CO, or Hb. MP4CO infusion elicited a peak CO-Hb concentration of 6.5% following a 12 mL/kg dose, consistent with the demonstration of CO-mediated cytoprotection with CO-Hb saturation near 5%. It is also important to note that the oxidogenated molecule, MP4OX, showed a slight tendency to reduce venular stasis, and unmodiﬁed HbS polymerized in the current studies. Moreover, the current observations of reduced vascular stasis 24 hours after dosing MP4CO are unlikely because of direct CO binding to HbS, because the short residence time of CO leaves little CO-Hb in blood beyond 90 minutes after dosing.

Our work has focused on an alternative mechanism for the actions of CO, based on the chronic hemolysis and enhanced oxidative and inﬂammatory state in SCD. Elevated plasma Hb generates reactive oxygen and nitrogen species from nitric oxide binding, oxidation of the globin moiety, generation of free heme, lipid peroxidation, and iron-catalyzed oxidation, promoting vascular consequences in transgenic sickle cell mice characterized by cellular adhesion/aggregation, vaso-occlusion, and increased mortality. Counteracting these deleterious actions, an important protective consequence of hemolysis, is the induction of HO-1 to limit the toxicity of free heme by a number of mechanisms, including the induction of ferritin and release of protective products biliverdin and CO. Although basal HO-1 expression is elevated in transgenic sickle mice, additional protective effects are observed by HO-1 gene therapy or IP administration of low-dose exogenous heme, such that therapeutic effects might be expected from increased activity of HO-1. The current results with IV MP4CO corroborate observations that inhaled CO protects transgenic sickle mice from adverse effects of oxidant stimuli and agree with numerous studies documenting the beneﬁcial effects of HO-1 overexpression or administration of the enzymatic products, CO and biliverdin.

Candidate mechanisms for the beneﬁcial effects of CO include binding to cell-free deoxyHb to stabilize and prevent heme loss, upregulation of antioxidant and anti-inﬂammatory pathways, and inhibition of NF-κB-mediated inﬂammation. We have previously demonstrated that inhaled CO and overexpression of HO-1 prevent vascular stasis and are associated with activated p38 MAP kinase and Akt pathways, reduction in activated NF-κB, and decreased circulating adhesion molecules. The current results are consistent with a lower overall inﬂammatory state in mice receiving MP4CO as evidenced by increased HO-1 expression, decreased nuclear NF-κB phospho-p65, and the inhibition of cell-surface P-selectin and VWF on endothelium throughout the lungs and liver. These observed anti-inﬂammatory effects by MP4CO are relevant to the decrease in vaso-occlusion and protection from hemin lethality and the fall in MAP. This is supported by the concurrent increase of nuclear Nrf2 with administration of MP4CO. CO plays a central role in nuclear Nrf2 localization and induction of HO-1 and other antioxidant enzymes. MP4CO-mediated Nrf2 translocation confers cytoprotection and anti-inﬂammatory actions. The importance of Nrf2 in HO-1 expression is highlighted by the low expression of HO-1 and poor survival of Nrf2 knockout mice.

**Table 1.** Descriptive parameters for heterozygous HbAS mice in 3 treatment groups (saline, MP4OX, MP4CO) and reference data from C57BL/6 mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Age (weeks)</th>
<th>Body weight (g)</th>
<th>Total Hb (g/dL)</th>
<th>Spleen weight (g)</th>
<th>Hb-β% (% SD)</th>
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<tr>
<td>Saline</td>
<td>11 ± 2</td>
<td>23.8 ± 0.6</td>
<td>10.1 ± 0.31</td>
<td>296 ± 27</td>
<td>39.4 ± 0.7</td>
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<tr>
<td>MP4OX</td>
<td>13 ± 1</td>
<td>25 ± 1.0</td>
<td>9.6 ± 0.42</td>
<td>312 ± 43</td>
<td>39.0 ± 1.0</td>
</tr>
<tr>
<td>MP4CO</td>
<td>11 ± 2</td>
<td>24.4 ± 0.6</td>
<td>9.8 ± 0.27</td>
<td>290 ± 30</td>
<td>39.0 ± 0.7</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>10 ± 1</td>
<td>26.3 ± 0.2</td>
<td>14.0 ± 0.26</td>
<td>87 ± 30</td>
<td>n/m</td>
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</tbody>
</table>

**Figure 5.** Effect of treatment on MAP and survival after hemin infusion into HbAS mice. (A) MAP in heterozygous HbAS mice infused with hemin followed by saline (n = 12, solid squares), MP4CO (n = 6, gray circles), or MP4OX (n = 6, open triangles). Values are means ± SD. *P < .016 vs saline. (B) Survival plot of HbAS mice infused with hemin followed by an 8 mL/kg infusion of saline (solid squares), MP4CO (gray circles), or MP4OX (open triangles). Arrows indicate the point of hemin administration and the duration of infusion of saline, MP4CO, or MP4OX. Values are fraction alive at indicated time points. *P < .016 vs saline.
following infection with *Plasmodium berghei* or *Escherichia coli*. Our observation that the actions of MP4CO were prevented by SnPP suggests that HO activity is critical for prevention of stasis by CO and that CO acts intracellularly, primarily by a feed-forward mechanism to amplify the expression and protective actions of HO-1, including further generation of CO. This concept is supported by earlier observations that HO-1 expression is an intermediate step in the anti-inflammatory activity of CO.\(^4\)\(^3\) and that endogenous, physiologic concentrations of CO are sufficient to elicit cellular signaling events.\(^2\)\(^4\)\(^4\)

The varied responses of sickle mice to the different Hb molecules in this study are noteworthy. MP4CO decreased NF-κB activation, increased HO-1 expression, inhibited P-selectin and VWF expression on endothelial cells, and reduced vascular stasis, whereas MP4OX had little effect on NF-κB, HO-1, and P-selectin/VWF and a smaller effect on stasis. Unmodified Hb had no effect on HO-1 in liver and dramatically enhanced stasis following H/R. These differences existed despite equal doses of Hb administered with each test solution. The enhanced stasis with unmodified Hb is likely due to decreased stability in vivo, with more rapid dimerization, greater heme loss,\(^4\)\(^5\)\(^6\) and a greater resultant activation of the vascular endothelium.\(^2\)\(^0\)\(^7\) The differential effect of these solutions on HO-1 induction could be explained by differences in the time course and magnitude of CO or heme concentration available to induce expression. For example, the effects of Hb, but not CO, might be expected to be modulated by haptoglobin or hemopexin.\(^4\)\(^8\) Because both unmodified Hb and pegylated Hb complex with haptoglobin and interact with the CD163 receptor (D.J. Schaer, Division of Internal Medicine, University Hospital, Zurich, Switzerland, personal communication, April 13, 2013), the general disposition of Hb is unlikely to be different for the 3 solutions. Alternatively, differences may exist in the potency and mechanism of HO-1 induction for CO, Hb, and heme via Nrf2 and Bach-1 regulation.\(^4\)\(^9\)\(^5\)\(^0\) The differential effects of CO versus Hb or heme on HO-1 induction will require further study.

The events precipitating a vaso-occlusive crisis in patients are not well delineated. Our results demonstrate that MP4CO inhibits the vascular consequences of 2 different stimuli, ie, hypoxia and hemin, generated in 2 different transgenic sickle models. Intravenous hemin is known to induce acute lung injury, hypotension, severe vaso-occlusion, and death in sickle mice.\(^1\)\(^8\)\(^2\)\(^0\) Heme toxicity may be ameliorated by rapid (<1 hour) HO-1 induction by CO, which would enhance heme degradation, generate more CO, inhibit vaso-occlusion, and blunt NF-κB-driven inflammatory responses.\(^5\)\(^2\) An integral role of reduced NF-κB activation in the effects of CO/Nrf2/HO-1 is supported by the current results and those of others.\(^1\)\(^8\)\(^5\)\(^1\)\(^3\) CO\(^4\) and Nrf2\(^5\)\(^2\) may play important roles in modulating toll-like receptor 4 (TLR4) signaling. Recently, Belcher and colleagues\(^2\) proposed a pathway by which hemolysis, and oxidative/inflammatory heme, triggers vaso-occlusion via endothelial TLR4 signaling, NF-κB activation, PKC signaling, and exocytosis of Weibel Palade body P-selectin and VWF. Thus, while still speculative, a potential pathway is emerging that links our current observations to inhibition of the TLR4 pathway. TLR4 signaling can be initiated by lipopolysaccharide, heme, or H/R.\(^2\)\(^0\)\(^5\)\(^3\) CO broadly suppresses the inflammatory response of human monocytes to lipopolysaccharide by reshaping events in TLR4 signal transduction such as stress kinase responses and NF-κB activation.\(^4\) CO suppression of TLR4-mediated signaling events, especially in the pulmonary vasculature, may explain the reduced mortality and blunted fall in MAP in the HbAS mice in response to heme. The current data demonstrate that CO is effective in episodes of inflammation initiated by either H/R or hemin administration and suggest that the durable expression of Nrf2 and HO-1 observed in response to CO might play a tonic suppression role in limiting the evolution of acute vaso-occlusive crisis.

In summary, the current results present evidence that MP4CO inhibits the effects of stimuli that induce vaso-occlusive crises in transgenic sickle mice. The actions of MP4CO are attributed to CO, because the identical molecule delivering oxygen did not elicit the same results. Our results document the upregulation of Nrf2 and HO-1 with administration of CO and support the extensive body of work implicating CO, Nrf2, and HO-1 as potential therapeutic targets. Recent clinical trials of agents intended to alleviate or shorten the duration of sickle cell crisis have failed to demonstrate robust and clinically meaningful effects.\(^5\)\(^4\)\(^5\)\(^5\) The current results extend work begun with CO in sickle disease more than a half-century ago\(^2\) and offer MP4CO as a potential therapy to limit the evolution and duration of painful vaso-occlusive events in patients with SCD.

### Acknowledgments

The authors thank Dr Robert Hebbel and Fuad Abdulla for breeding and phenotyping the NY1DD mice, Stephanie Spann and Rachell Sigan for phenotyping the HbAS mice, and Dr Tim Townes and the University of Alabama, Birmingham Research Foundation, for license to the HbAS mice. We also thank Dr Michael Levitt and Julie Furr for measurement of expired CO and Phong Nguyen and Minh Nguyen for preparation of the immunohistochemistry panels in the online supplemental Data.

This research was funded by a grant from Sangart, Inc., to G.M.V. and J.D.B.

### Authorship

**Contribution:** J.D.B., M.Y., K.B., and G.M.V. designed experiments, analyzed data, and wrote the paper; C.C., J.N., and P.T. collected the data; and M.Y. and K.B. also provided valuable reagents (MP4CO, MP4OX, and Hb).

**Conflict-of-interest-disclosure:** M.Y., K.B., and P.T. are employees of Sangart, Inc. The remaining authors declare no competing financial interests.

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### References


MP4CO, a pegylated hemoglobin saturated with carbon monoxide, is a modulator of HO-1, inflammation, and vaso-occlusion in transgenic sickle mice

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