Supplemental Data

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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Supplemental Methods

Mouse Treatments and Tissue Collection. NY1DD mice were infused (8ml/kg) via the tail vein with LRS, MP4CO or MP4OX. After 24h, mice were exposed to 1h hypoxia (7% O₂) followed by 4h of reoxygenation in room air. At 29h post-infusion, the mice were sacrificed and the lungs and livers were removed, placed into optimal cutting temperature (O.C.T.) compound (Sakura Finetek), snap-frozen, and stored at -85°C.

HbAS-Townes mice were infused (8ml/kg) via the tail vein with saline, hemin+saline, hemin+MP4CO or hemin+MP4OX. After 60 minutes, mice were sacrificed; lungs and livers were removed and processed as described above for NY1DD mice.

Immunohistochemistry. Six-micrometer tissue sections were mounted on glass slides and fixed in 4% paraformaldehyde. Slides were blocked with 3% donkey serum + 0.2% TritonX-100, and stained with IgG to CD31 (Santa Cruz), HO-1 (Enzo) and NF-κB phospho-p65(Ser 276) (Bioss). Primary antibody binding was visualized with the appropriate secondary antibodies conjugated to Cy2 or Cy3 (Jackson ImmunoResearch). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Images were acquired using a FluoView FV1000 BX2 upright confocal microscope and software (Olympus). Nonspecific staining was assessed using nonimmune IgG. Final images were prepared using Adobe Photoshop software.

For immunohistochemistry of P-selectin and von Willebrand factor (VWF) in HbAS-Townes mice, tissue sections of lungs and livers were processed as described above without Triton X-100 to examine extracellular expression of P-selectin and VWF.

Supplemental Results

NY1DD Mice
Thin sections of lungs and livers taken from NY1DD sickle mice treated with LRS, MP4CO or MP4OX (8ml/kg) 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 expression could be seen in endothelial cells around blood vessels in the lungs (Supplemental Figures S1A-B) and liver (Supplemental Figure S2), as well as epithelial cells in lungs (Supplemental Figures S1A-B) and hepatocytes in liver (Supplemental Figure S2). In the higher magnification insets, NF-κB phospho-p65 is primarily localized in nuclei around blood vessels in the lungs and livers of mice treated with LRS or MP4OX, which indicates enhanced NF-κB activation in the lungs and livers of these mice. MP4CO treatment markedly reduced NF-κB phospho-p65 nuclear staining in nuclei, indicative of decreased NF-κB activation in the lungs and livers of mice treated with MP4CO.

Thin sections of lungs and livers taken from NY1DD sickle mice treated with LRS, MP4CO and MP4OX (8ml/kg) 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 Figures S3A-B) and liver (Supplemental Figure S4) compared to 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.

HbAS-Townes Mice

To examine the mechanism of MP4CO protection in HbAS mice following injection of hemin, lungs and livers from treated HbAS mice were removed at 1h post-infusion and immunostained for CD31, HO-1, NF-κB phospho-p65, P-selectin and VWF. CD31 and VWF staining are specific for endothelium. HO-1 expression was highest in mice infused with hemin+MP4CO in both lungs (Supplemental Figures S5A-B) and livers (Supplemental Figure S6) compared to all other treatments including hemin+saline. HO-1 staining was present in all cells surrounding the blood vessels, including endothelial cells. HO-1 staining was lowest in mice treated with saline or hemin+MP4OX, with little to no staining around the blood vessels. Lungs and livers from mice treated with hemin+saline had intermediate levels of HO-1 expression in all cells.

NF-κB phospho-p65 staining was highest in the lungs (Supplemental Figures S7A-B) and livers (Supplemental Figure S8) of mice treated with hemin+saline. In the higher magnification insets, NF-κB phospho-p65 staining is apparent in nuclei and cytoplasm of pulmonary arteries and veins. In blood vessels of the liver, staining is primarily nuclear with less intense cytoplasmic staining, which indicates enhanced NF-κB activation in these tissues. MP4CO treatment markedly reduced NF-κB phospho-p65 staining in lungs and liver, indicative of decreased NF-κB activation with MP4CO.

Hemin infusion induces profound vaso-occlusion in HbSS and HbAS mice that requires cell surface expression of endothelial cell Weibel-Palade body constituents P-selectin and VWF. P-selectin and VWF expression on the cell surface of vessels in the lungs (Supplemental Figures S9A-B) and liver (Supplemental Figures S10) was stimulated in HbAS mice infused with hemin+saline and hemin+MP4OX and was absent in mice infused with saline or hemin+MP4CO. Thus, protection from hemin lethality by MP4CO correlates with a rapid (<1h) increase in HO-1, a reduction in NF-κB activation and decreased P-selectin and VWF on blood vessels in the lungs and livers.
Supplemental Figure S1A. NF-κB phospho-p65

NY1DD Lung Arteries 29h Post-infusion

CD31

NF-κB Phospho p65

Merged

Magnified inset
Supplemental Figure S1B. NF-κB phospho-p65

NY1DD Lung Veins 29h Post-infusion

- CD31
- NF-κB Phospho p65
- Merged
- Magnified Inset
**Supplemental Figure S1.** Lungs were collected 29h after infusion of LRS, MP4CO or MP4OX (8ml/kg) into NY1DD sickle mice. Five hours before organ harvest, mice were exposed to H/R. MP4CO inhibits NF-κB phospho-p65 expression (red) in lung arteries (A) and veins (B) relative to mice treated with LRS or MP4OX. CD31 (green) is specific for endothelial cells. Nuclei are stained with DAPI (blue). White bar = 40µm. In mice treated with LRS and MP4OX, but not MP4CO, NF-κB phospho-p65 expression is primarily localized in nuclei as seen in magnified insets of shaded boxes (60µm X 60µm) in the merged images.
Supplemental Figure S2.  NF-κB phospho-p65

Supplemental Figure S2. Livers were collected 29h after infusion of LRS, MP4CO or MP4OX (8ml/kg) into NY1DD sickle mice. Five hours before organ harvest, mice were exposed to H/R.
MP4CO inhibits NF-κB phospho-p65 expression (red) in liver vessels relative to mice treated with LRS or MP4OX. CD31 (green) is specific for endothelial cells. Nuclei are stained with DAPI (blue). White bar = 40 μm. In mice treated with LRS and MP4OX, but not MP4CO, NF-κB phospho-p65 expression is primarily localized in nuclei as seen in magnified insets of shaded boxes (60μm X 60μm) in the merged images.
Supplemental Figure S3A. HO-1

NY1DD Lung Arteries 29h Post-infusion

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Supplemental Figure S3B. HO-1

Supplemental Figure S3. Lungs were collected 29h after infusion of LRS, MP4CO or MP4OX (8ml/kg) into NY1DD sickle mice. Five hours before organ harvest, mice were exposed to H/R. MP4CO increases HO-1 expression (red) in lung arteries (A) and veins (B) relative to mice treated with LRS or MP4OX. CD31 is specific for endothelial cells (green). Nuclei are stained with DAPI (blue). White bar = 40 µm.
Supplemental Figure S4. HO-1

Supplemental Figure S4. Livers were collected 29h after infusion of LRS, MP4CO or MP4OX (8ml/kg) into NY1DD sickle mice. Five hours before organ harvest, mice were exposed to H/R. MP4CO increases HO-1 expression (red) around liver blood vessels relative to treatment with LRS or MP4OX. CD31 (green) is specific for endothelial cells. Nuclei are stained with DAPI (blue). White bar = 40 µm.
Supplemental Figure S5A. HO-1

HbAS-Townes Lung Arteries 1h Post-infusion

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Supplemental Figure S5B. HO-1

Supplemental Figure S5. Lungs were collected 1h after infusion of saline, hemin+saline, hemin+MP4CO or hemin+MP4OX (8ml/kg). HO-1 expression (red) in lung arteries (A) and veins (B) is rapidly increased in HbAS-Townes mice infused with hemin+MP4CO relative to mice infused with hemin+saline, hemin+MP4OX or saline alone. CD31 (green) is specific for endothelial cells. Nuclei are stained with DAPI (blue). White bar = 40 µm.
Supplemental Figure S6. Livers were collected 1h after infusion of saline, hemin+saline, hemin+MP4CO or hemin+MP4OX (8ml/kg) into HbAS-Townes mice. Treatment with hemin+MP4CO markedly increases HO-1 expression (red) around blood vessels and in hepatocytes relative to mice treated with hemin+saline, hemin+MP4OX or saline alone. CD31 (green) is specific for endothelial cells. Nuclei are stained with DAPI (blue). White bar = 40 µm.
Supplemental Figure S7A. NF-κB phospho-p65
Lungs were collected 1h after infusion of saline, hemin+saline, hemin+MP4CO or hemin+MP4OX (8ml/kg) into HbAS-Townes mice. Hemin+MP4CO decreases NF-κB phospho-p65 expression (red) in lung arteries (A) and veins (B) relative to mice treated with hemin+saline or hemin+MP4OX. CD31 (green) is specific for endothelial cells. Nuclei are stained with DAPI (blue). White bar = 40 µm. NF-κB phospho-p65 is present in nuclei and cytoplasm as seen in magnified insets of shaded boxes (60µm X 60µm) in the merged images.
Supplemental Figure S8. NF-κB phospho-p65

Livers were collected 1h after infusion of saline, hemin+saline, hemin+MP4CO or hemin+MP4OX (8ml/kg) into HbAS-Townes mice. Treatment with hemin+MP4CO decreases NF-κB phospho-p65 expression (red) around liver blood vessels relative to mice treated with hemin+saline, or hemin+MP4OX. CD31 (green) is specific for endothelial cells. Nuclei are stained with DAPI (blue). White bar = 40 µm. NF-κB phospho-p65 is present primarily in nuclei with lower amounts of cytoplasmic staining as seen in magnified insets of shaded boxes (60µm X 60µm) in the merged images.
Supplemental Figure S9A. P-selectin and VWF
Supplemental Figure S9B. P-selectin and VWF

Supplemental Figure S9. Lungs were collected 1h after infusion of saline, hemin+saline, hemin+MP4CO or hemin+MP4OX (8ml/kg). Extracellular P-selectin (green) and von Willebrand factor (VWF, red) expression on lung arteries (A) and veins (B) is blocked in HbAS-Townes mice treated with hemin+MP4CO relative to mice stimulated with hemin+saline or hemin+MP4OX. Nuclei are stained with DAPI (blue). White bar = 40 µm.
Supplemental Figure S10. Livers were collected 1h after infusion of saline, hemin+saline, hemin+MP4CO or hemin+MP4OX (8ml/kg) into HbAS-Townes mice. Hemin+MP4CO blocks extracellular P-selectin (green) and VWF (red) expression around liver blood vessels relative to mice treated with hemin+saline or hemin+MP4OX. Nuclei are stained with DAPI (blue). White bar = 40 µm.