SUPPLEMENTARY APPENDIX

Excess of heme induces tissue factor-dependent activation of coagulation in mice

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SUPPLEMENTARY METHODS

Western blot analysis of plasma levels of hemopexin

Plasma from mice treated with 0 – 35 μmol/kg heme was diluted in PBS, and 20 μg protein was mixed with 4X LDS buffer (Life Technologies) and boiled for 5 minutes, then applied to 4-20% Tris-Glycine gels (Bio-Rad Laboratories) to separate proteins using electrophoresis. Proteins were transferred to PVDF membranes (Millipore), and membranes were blocked for 1 hour with Odyssey blocking buffer (LI-COR Biosciences). Membranes were incubated overnight at 4˚C with primary antibody against mouse hemopexin (1:1000 rabbit anti-mouse hemopexin, Abcam). Washed membranes were then incubated with IRDye 680RD goat anti-rabbit fluorescently-labelled secondary antibody (1:10,000 dilution, LI-COR Biosciences) for 1 hour. Membranes were washed three times and analyzed using an Odyssey Infared Imaging System (LI-COR Biosciences). Membranes were stained with Coomassie Brilliant Blue to ensure equal protein loading.

Measurement of vascular permeability

Vascular permeability in organs was determined by the Evans Blue method (1). Two and a half hours after heme administration, mice received Evans Blue dye (30 mg/kg) via RO injection. Thirty minutes later, mice were anesthetized and the IVC was slit below the renal artery. Mice were first perfused through the right ventricle with 5 mL of cold PBS, then through the left ventricle with 30 mL of PBS. The heart, lung, liver and kidney were harvested, weighed, and incubated with formamide (4 mL/g organ weight) at 37°C for 48 hours to extract Evans Blue. The concentration of Evans Blue was determined by reading absorbance at 620 nm, and corrected for heme contamination by reading the absorbance at 740 nm wavelength. The following formula was used to determine the corrected value of Evans blue: A_{620} (corrected) = A_{620} – (1.426 x A_{740} + 0.03) (2).

Isolation of human PBMCs

Blood was obtained from healthy volunteers (n=4) for isolation of peripheral blood mononuclear cells (PBMCs) after informed consent with the Declaration of Helsinki and with the approval of the University of North Carolina Institutional Review Board. PBMCs were isolated as previously described (3). Briefly, blood was collected by venipuncture into citrate (3.2%) and centrifuged at 250 x g for 10 minutes at room temperature. The buffy coat was collected and diluted 1:1 with HBSS (with 5mM EDTA) layered over Ficoll-sodium diatrizoate (Ficoll-Paque Premium; GE Healthcare) and centrifuged at 400 x g for 30 minute at room temperature. The PBMC layer was collected and washed twice with HBSS with EDTA. PBMCs were plated at a density of 1 x 10^6 cells per mL of culture medium (RPMI 1640 with L-glutamine with 10 mM HEPES, 1%

Penicillin/Streptomycin, 100 μM sodium pyruvate and 2% fetal bovine serum; all reagents from Life Technologies). Cells were treated with 0-25 μM heme for 24 hours at 37˚C and 5% CO_2, after which cells were collected via centrifugation at 500 x g for 5 minutes at 4˚C. The cell pellet was resuspended in 1 mL of HBSA and frozen at -80˚C until procoagulant activity was assessed by one stage clotting assay.

Culturing of mouse macrophages

RAW 264.7 mouse macrophage-like cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (Life Technologies) at 37C in 5% CO_2. Cells were harvested by detachment with a sterile spatula and plated at a density of 4 x 10^4 cells/well in a 24-well plate. Cells were allowed to attach for twenty four hours, then the cells were synchronized by replacing growth medium with DMEM plus 0.5% FBS for 18 hours. Cells were exposed to 0 – 50 μM heme in DMEM in the presence of 0.5% FBS for 6 hours. The cell pellet was collected by detachment and centrifugation at 500 x g for 5 minutes at 4C. The cell pellet was resuspended in 1 mL of HBSA and procoagulant activity was assessed by one stage clotting assay.

One stage clotting assay

Procoagulant activity in cells was determined using a 1-stage clotting assay with a Start4 analyzer (Diagnostica Stago) as previously described (4). The procoagulant activity of the sample was calculated by reference to a standard curve generated using recombinant human relipidated TF (Innovin™, Dade Behring), and normalized to total protein concentration with the BioRad DC protein assay (Bio-Rad, Hercules, CA).

Immunochemical staining for TF

C57Bl/6 mice were treated with vehicle or heme for 6 hours, and blood was collected from the IVC as described above. White blood cells were isolated from 500 μL of blood after removing red cells with lysis buffer (4.14 g NH_4Cl, 0.5 g $NaHCO_3$, 0.0185 g EDTA in 500 mL sterile ddH_2O). White blood cells were re-suspended in 500 μL of PBS, spun onto slides with a Cytospin 4 centrifuge (Thermo Scientific) and air-dried. Cells were fixed with ice cold methanol for 30 seconds and washed in PBS/0.05% Tween 20. Slides containing cells and lung tissue were incubated with rat anti-mouse TF monoclonal antibody (20 μg/mL, 1H1) overnight at 4˚C. Then they were incubated with a biotinylated anti-rat secondary antibody (Vector Laboratories) followed by Vectastain ABC kit reagents. Slides were developed using ImmPACT DAB peroxidase substrate (Vector Laboratories) and counterstained with hematoxylin (Dako), then mounted with aqueous mounting medium (Vector Laboratories). Slides were visualized with an Olympus DX51W1 microscope using the 40X Olympus UPlanFln aperture for a final

magnification of 400x. Images were acquired with the Olympus DP70 digital camera and DP controller software V02.03 (Olympus America). In a similar study, human PMBCs were prepared as described above, and human TF was detected with goat anti-human monoclonal TF antibody (25 µg/mL, generously provided by Dr. Tim Nichols) and biotinylated anti-goat secondary antibody (Vector Laboratories).

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