Supplementary Information for

Thiol catalyzed heme-nitrosyl complexes regulate canonical intravascular nitric oxide signaling

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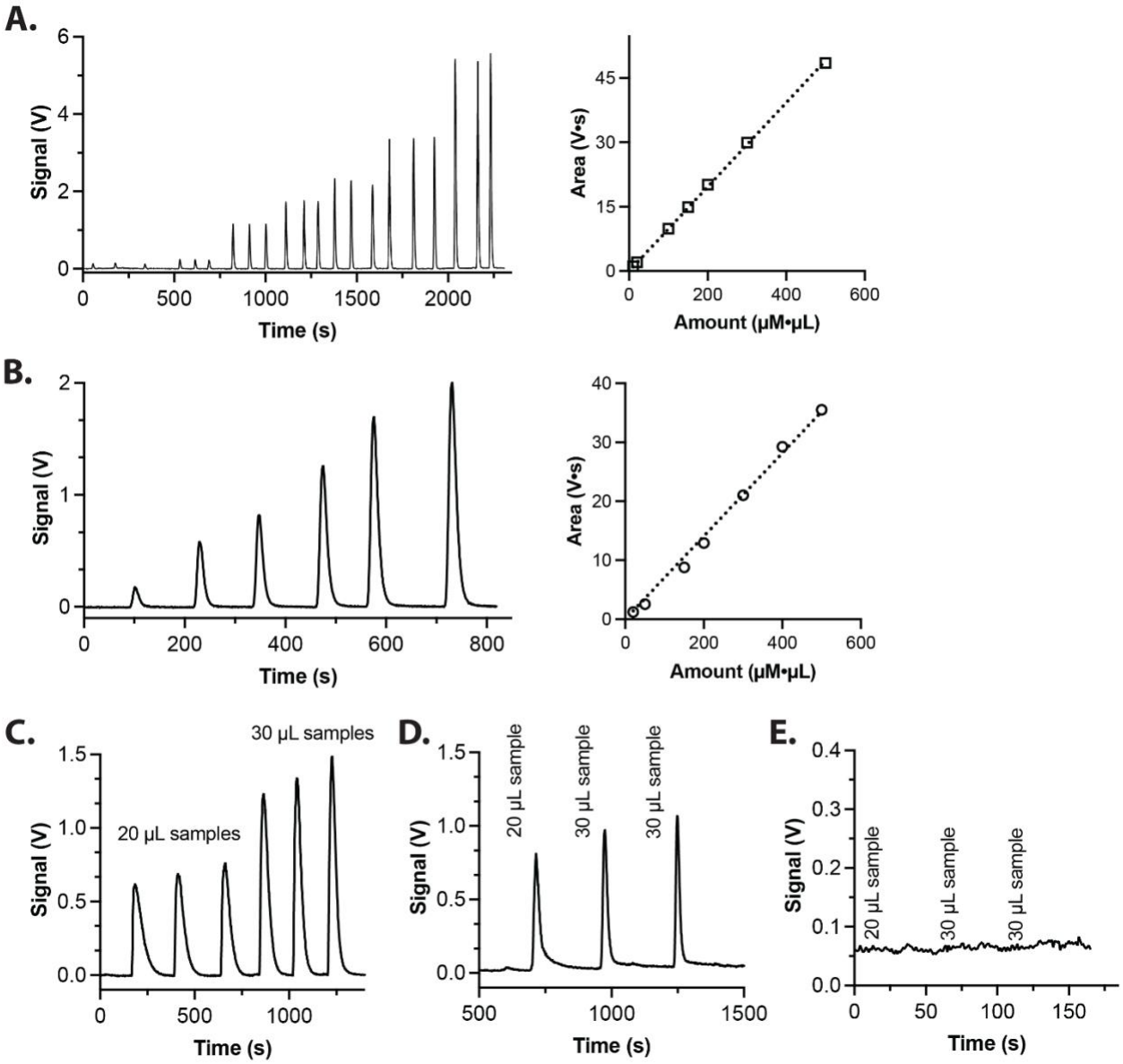
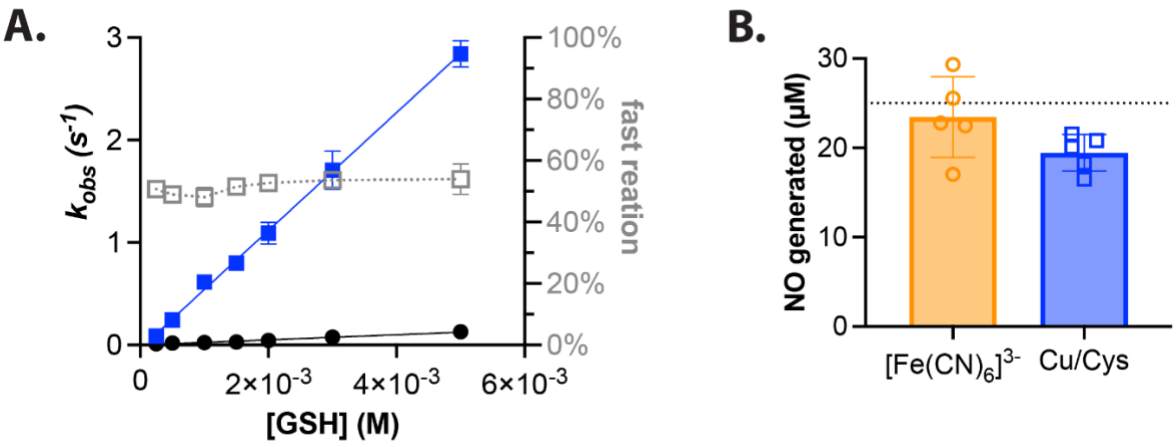


Supplementary Figure 1 Example NO traces from NO chemiluminescence analyzer. **A)** Example NO response from ferricyanide calibration traces (*left*) and example calibration curve (*right*) using known quantities of sodium nitrite at 25°C. **B)** Example NO response from 2C (copper/cysteine) calibration traces (*left*) and example calibration curve (*right*) using known quantities of fresh S-nitrosoglutathione at 54°C. **C)** Example injections of NO-ferroheme prepared in MeOH:PBS buffer using glutathione into a chemiluminescence analyzer purge vessel containing ferricyanide solution at 25°C. **D)** Example injections of the same NO-ferroheme solution into a chemiluminescence analyzer purge vessel containing the 2C solution at 54°C. **E)** Same as in **D**, except the solution was first pretreated with excess DMPO to block GSNO formation before synthesis of NO-ferroheme: no NO originating from S-nitrosothiols is detectable. In all cases, solutions are purged briefly (~1-2 min) with nitrogen to remove dissolved free NO before addition to the chemiluminescence analyzer.

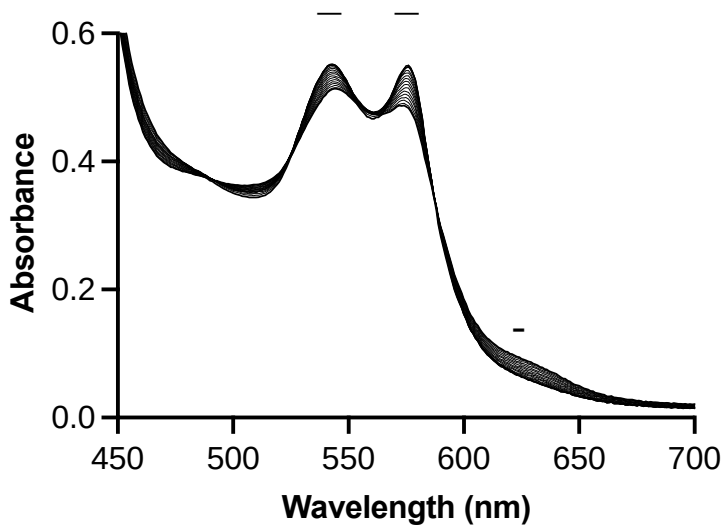
Unlike in the MeOH:PBS system, the reaction of ferric heme with NO and GSH in serum albumin exhibits biphasic kinetics under pseudo first order conditions. Both observed rate constants increase with glutathione concentration, and each phase remains ~50% of the total exponential. One possibility is that two different heme binding sites exist in albumin where one is more glutathione accessible than the other. Another is possibly a difference in electron transfer mechanism. Finally, mixed disulfide formation resulting in reduction of an otherwise oxidized albumin cysteine made available to react in the system and/or an associated change in protein dynamics are possible as well.



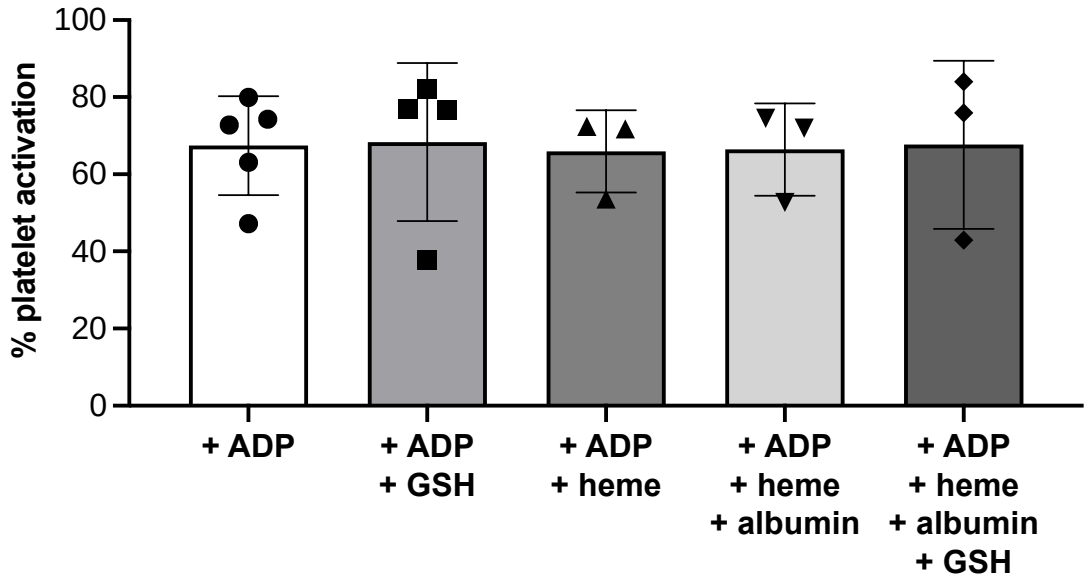
Supplementary Figure 2 Formation of NO-ferroheme via glutathione catalyzed reductive nitrosylation of ferric heme in serum albumin. **A)** Observed pseudo first order rate constants vs concentration of added reduced glutathione of reaction of ferric heme, NO, and GSH in solution containing serum albumin. The reaction in this system exhibits two phases, comprising roughly 50% of the reaction at each GSH concentration (right y-axis, gray open squares). The fast phase (left y-axis, blue closed squares) exhibits an observed 2nd order rate constant of 700 $M^{-1}s^{-1}$, while the second (left y-axis, black closed circles) is 20 $M^{-1}s^{-1}$. The two phases are linear under pseudo first conditions with varying GSH, suggesting they are both GSH dependent. Reactions were conducted at 22 °C. **B)** Determined stoichiometry of this reaction in albumin using the chemiluminescence NO detection with the ferricyanide test (orange open circles) and the copper/cysteine (2C) test (blue open squares), as described in **Figure 1** and main text. Here, 25 μM ferric heme was mixed with 50 μM GSH and 50 μM NO in 75 μM serum albumin under anaerobic conditions; thus, the expected amount for both NO-ferroheme and GSNO should be 25 μM (hashed line). 23.4 ± 4.5 μM NO-ferroheme was detected in the albumin system, along with 19.4 ± 2.1 μM GSNO.

The stability of NO-ferroheme in albumin in the presence of oxygenated hemoglobin was assessed by absorption spectroscopy. NO-ferroheme albumin (25 μM) was prepared as

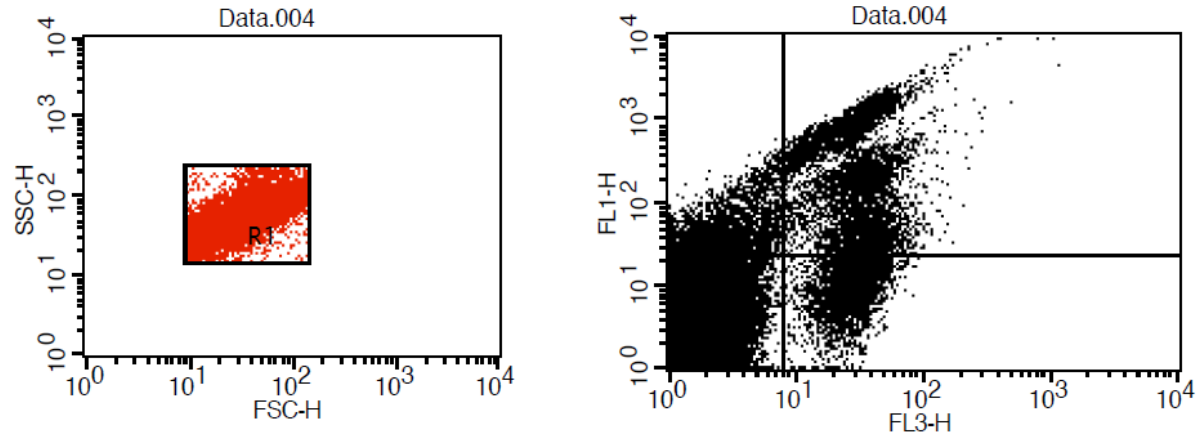
described in the main body of text via initial reduction by dithionite and adding an equivalent of NO. GSH (50 μM) was added just prior to addition of equimolar (25 μM) oxygenated hemoglobin was added to the solution and spectroscopically monitored for five hours. Dissociation of NO from NO-ferroheme albumin, should result in NO dioxygenation with oxyhemoglobin to form methemoglobin, which has a characteristic absorption peak around 630 nm (3.9 $mM^{-1}cm^{-1}$). The absorption spectra are shown below. Over five hours, only about 7 μM methemoglobin is made, corresponding to a rate constant for NO loss from NO-ferroheme of 2.1 x 10^{-5} s^{-1} at 23 °C.



Supplementary Figure 3 NO-ferroheme albumin stability in the presence of an equivalent of oxyhemoglobin. 25 μM NO-ferroheme in 75 μM albumin was added to 25 μM oxyhemoglobin in an aerobic atmosphere and monitored via UV-Visible spectroscopy over the course of 5 hours. Roughly 7 μM of methemoglobin is generated. Spectra were collected at 23 °C.



Supplementary Figure 4 Other relevant platelet control experiments. Experimental conditions for platelet-rich plasma activation are described in the main body of the text. 2 µM adenosine diphosphate (ADP) was added to activate platelets. Addition of 2.5 µM heme or 25 µM GSH, with or without 7.5 µM serum albumin exhibits little effect on activated platelets. Other combinations and controls are found with the experimental data in **Figure 4** in the main body of the text.



Supplementary Figure 5 Typified flow cytometry gating for platelet sorting and platelet activation determination. The left panel shows events plotted by side scatter (SSC-H) vs forward scattering (FSC-H) which is used to separate red blood cells from platelets by size. These events are then plotted by CP-CD61 fluorescence on the x-axis (right panel) vs PAC1-FITC fluorescence on the y-axis indicating activated platelets (upper right corner).