

Supporting Information

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SI Materials and Methods

Reagents. All of the chemicals were purchased from Sigma unless stated otherwise. GAPDH siRNA (439084) as well as the negative control (catalog number AM4611) were purchased from Ambion. Antibodies were purchased from following companies: mouse anti-GAPDH was from RDI, rabbit polyclonal anti-HA was from Santa Cruz, and mouse anti-inducible nitric oxide synthase (iNOS) was from BD Transduction laboratory (BD Biosciences). Glutathione-NO (GSNO) and Noc18 were from Alexis. IFN- γ was purchased from PeproTech. Hemin agarose was prepared as described elsewhere (1). HA-tagged wild-type, C152S, K227A, and K162A GAPDH in pRK5 expression vector were a gift from Solomon H. Snyder (The Johns Hopkins University School of Medicine, Baltimore, MD). GAPDH^{Y41F} mutant was generated using site directed mutagenesis kit (Invitrogen Inc.) and the following primers: Y41F_F: CATTGACCTCAACTTCATGGTTACATGTTCC Y41F_R: GGAACATGTAAACCATGAGTTGAGGTCAATG. Mammalian expression vector for human iNOS was as described (2).

Determination of Protein-Protein/Ligand Interaction by Surface Plasmon Resonance. All surface plasmon resonance (SPR) experiments were carried out using CM5 sensor chip and BIACORE 3000 instrument (Biacore). The ligand, GAPDH or mutant proteins (11 $\mu\text{g}/\mu\text{L}$) in PBS buffer, were diluted in the 10 mM acetate (pH 5.5) coupling solution and pumped over the activated surface at 5 $\mu\text{L}/\text{min}$ in flow cell 2, 3, or 4. About 2,000 resonance units of GAPDH were immobilized on the CM5 sensor chip. The first flow cell was activated and deactivated in the same way to yield a blank surface. HES-N buffer (0.01 M hepes, 150 mM NaCl, pH 7.4) was used through out the experiment. Heme solution was made in 0.01 N NaOH and diluted in degassed HES-N buffer. The binding was carried out at 25 $^{\circ}\text{C}$ with a flow rate of 25 $\mu\text{L}/\text{min}$, and data were collected for 120 s of association followed by 240 s of dissociation. To eliminate nonspecific interaction, response from the control flow cell was subtracted from the response of protein. Sensograms were fitted using BIA evaluation 4.1 software with nonlinear fitting, primarily 1:1 (Langmuir) binding with drift model. For S-nitrosylation, 400 μM GSNO diluted in the HBS-N buffer was

pumped over the GAPDH-activated surface at 10 $\mu\text{L}/\text{min}$ for 30 min in the dark followed by two priming cycles to washout any residual GSNO. To test the reversibility of GSNO effect, three injections of 3 mM DTT solution were made over the S-nitrosylated chip and the binding experiment was repeated. For the SPR experiment, the GAPDH-heme complex was generated as mentioned in the UV-visible spectra section.

Immunofluorescence. Cells were cultured on glass coverslips and treated as per the experiment. During the 2-h heme insertion, 1 μM of PIF was added to the media. After treatment, cells were fixed in 3.7% formaldehyde (in PBS) for 7 min at room temperature followed by permeabilization in 0.5% triton X-100 for 7 min. Cells were washed with PBS and incubated with primary antibody in humidified chambers for 90 min at room temperature. After three washes in PBS, cells were stained with fluorescence-conjugated secondary antibodies. Cells were rinsed three times with PBS and once with distilled water followed by mounting on the glass microscope slides using Vectashield mounting medium containing DAPI (Vector Laboratories). Coverslips were mounted on the glass microscope slides using Vectashield mounting medium containing DAPI (Vector Laboratories). Confocal XY images were taken using a 63 \times objective lens (zoom 2) of Leica TCS-SP/SP-AOSB laser confocal microscope using Leica confocal software version 2.5.

Methods for SI Figures. ATP measurement. An ATPlite (Perkin-Elmer) kit was used to measure the ATP content of cell lysates. **GAPDH activity measurement.** Activities of purified proteins were measured at room temperature using a KDalert GAPDH assay kit (Ambion, Applied Biosystems).

2D gel electrophoresis and protein identification. The detailed protocol is described elsewhere (3).

Overexpression studies. Human GAPDH construct was used to transfect RAW264.7 cells using lipofectamine 2000 (Invitrogen) for 30 h followed by cytokine induction and iNOS activity measurement. In case of HEK293 cells, GAPDH was overexpressed with iNOS plasmid using lipofectamine 2000.

1. Tsutsui K, Mueller GC (1982) Affinity chromatography of heme-binding proteins: An improved method for the synthesis of hemin-agarose. *Anal Biochem* 121:244–250.
2. Panda K, et al. (2005) Visualizing inducible nitric-oxide synthase in living cells with a heme-binding fluorescent inhibitor. *Proc Natl Acad Sci USA* 102:10117–10122.

3. Aulak KS, Koeck T, Crabb JW, Stuehr DJ (2004) Proteomic method for identification of tyrosine-nitrated proteins. *Methods Mol Biol* 279:151–165.

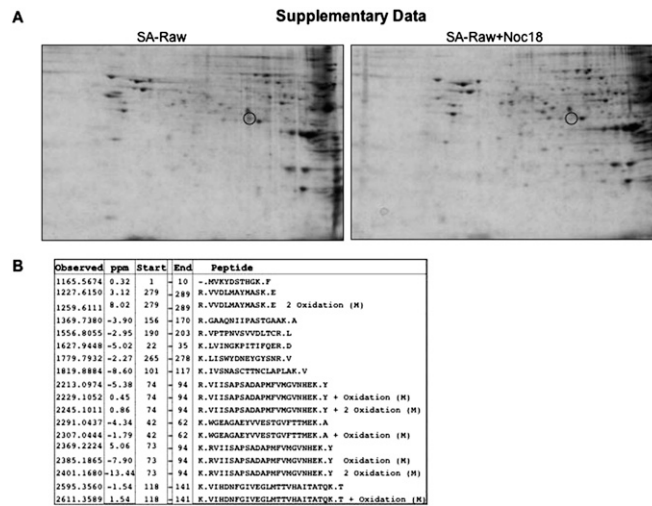


Fig. S1. Proteomic identification of GAPDH as an NO-sensitive heme binding protein. (A) Samples of cell cytosol from succinyl acetone (SA)-treated RAW264.7 cells were treated or not treated with NO donor (125 μ M NOC18) and then the heme binding proteins were pulled down with hemin agarose and separated by 2D electrophoresis. Coomassie-stained gels showed a 37-KD band is missing in the NOC18 treated lysates. (B) Circled protein from A was excised and identified to be GAPDH by MS (100% coverage by peptides in the range of 1,000–3,000 Da) by Mascot peptide fingerprint analysis following in-gel tryptic protein digestion. Matching peptides are shown. Searches were performed with a mass tolerance of 0.005% error (50 ppm).

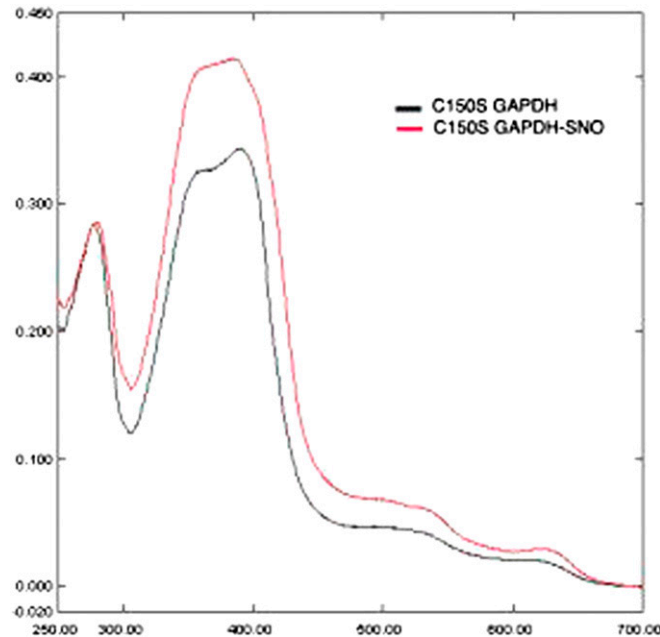


Fig. S2. Heme binding to C152S GAPDH is resistant to the effect of GSNO. UV-visible spectra of the purified heme complexes that were formed using native or GSNO-treated C152S GAPDH. The GSNO-treated protein was run through a PD-10 column to remove excess GSNO before heme complex formation.

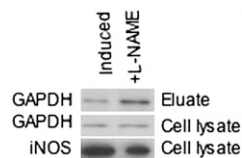


Fig. S3. GAPDH binds to iNOS in the presence of L-NAME. ADP pull-downs of equal amount of proteins generated from activated macrophages (\pm L-NAME) shows more GAPDH binds iNOS when L-NAME is present.

GAPDH interaction with different isoforms of NOS

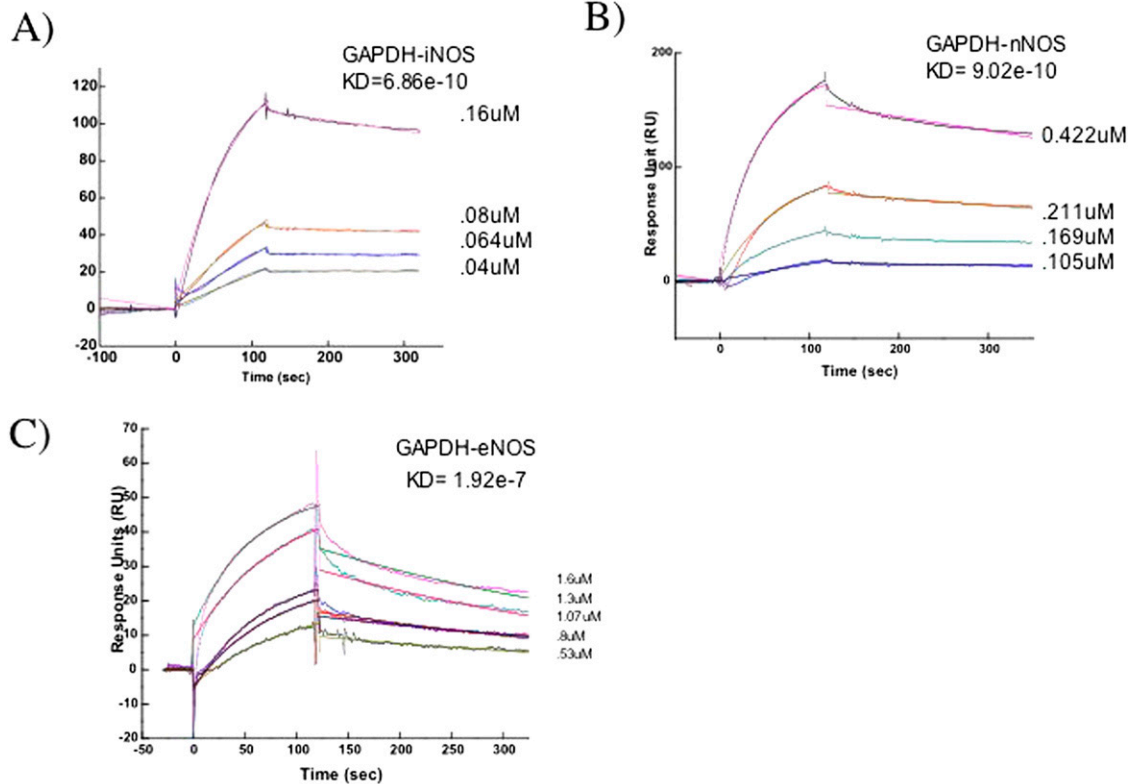


Fig. 54. GAPDH binds all three isoforms of NOS. GAPDH was immobilized on CM5 chip and was titrated with different concentrations of bacterially purified three NOS [(A) iNOS, (B) nNOS, and (C) eNOS] proteins individually. Details of methods and fittings are mentioned in the main text.

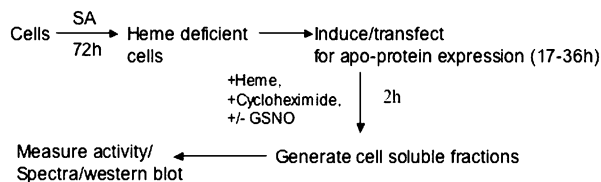


Fig. 55. General experimental design used to study heme insertion into apo-protein targets in cell culture. Adapted from Waheed et al. (1).

1. Waheed SM, et al. (2010) Nitric oxide blocks cellular heme insertion into a broad range of heme proteins. *Free Radic Biol Med* 48:1548–1558.

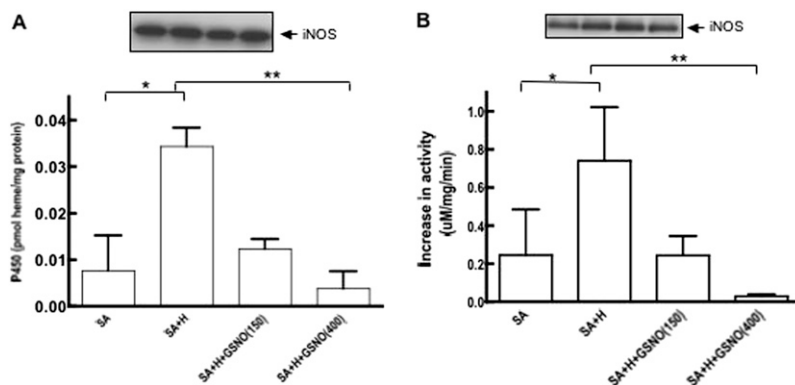


Fig. 56. GSNO inhibits cellular heme insertion into apo-iNOS. GSNO caused a dose-dependent inhibition of heme insertion into apo-iNOS as measured (A) spectroscopically (P450) ($n = 2$, mean \pm SEM; $*P < 0.05$; $**P < 0.005$), or (B) by the gain in iNOS NO synthesis activity for the respective cell supernatants ($n = 3$, mean \pm SEM; $*P < 0.05$; $**P < 0.005$). Western blot analyses showed that total iNOS protein was equivalent in the various cell supernatants. H, heme; GSNO concentrations are in micromolar.

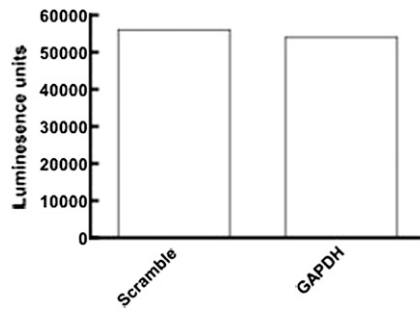


Fig. 57. Knockdown of GAPDH expression does not affect cellular ATP level. ATP content of RAW 264.7 cells was measured after siRNA treatment followed by iNOS induction ($n = 2$, mean \pm SEM).

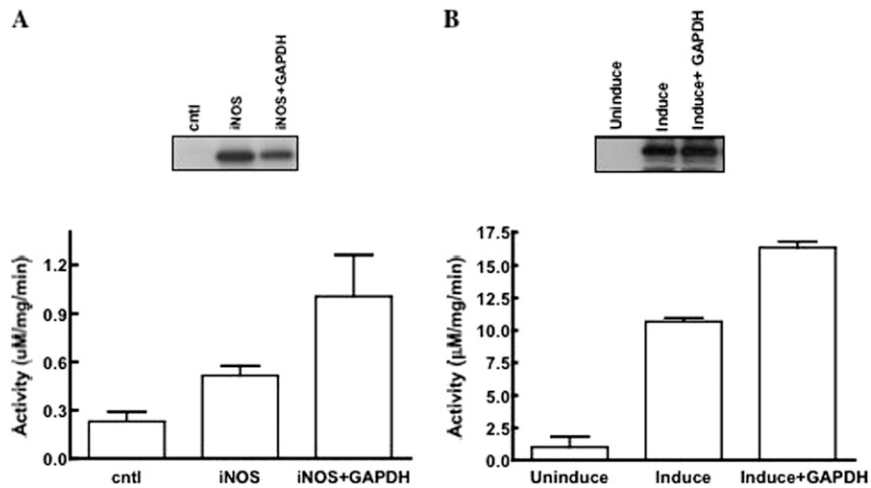


Fig. 58. Overexpression of GAPDH enhances heme insertion into apo-iNOS. Overexpression of GAPDH in (A) HEK cells cotransfected with iNOS or (B) RAW264.7 cells followed by cytokine induction enhanced heme insertion into iNOS as measured by the increased NO synthesis activity. Western blots for iNOS expression in the respective cell lysates are shown.

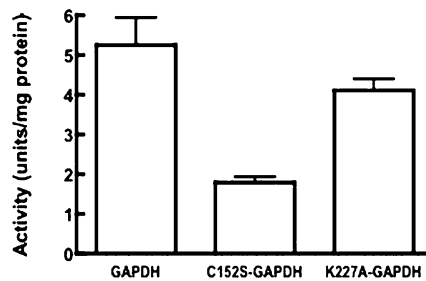


Fig. 59. Enzyme activities of purified GAPDH proteins. Enzymatic activities of purified proteins at room temperature are shown.

