Supplementary Materials

Hemodynamic effects of glutathione-liganded binuclear dinitrosyl iron complex: evidence for nitroxyl generation and modulation by plasma albumin

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Supplementary Methods

Surgical procedures in sheep

Neutered male sheep nine to ten months old and weighing 37±2 kg were surgically instrumented as previously described [1; 2]. While mechanically ventilated with 1.5 to 2.5% isoflurane (Baxter U. S., Deerfield, Illinois) in oxygen, a catheter was inserted into a side-branch of a femoral artery and its tip then advanced to within 2mm of the main

femoral artery. Another catheter was inserted into a brachial artery to measure arterial blood pressure, heart rate, and blood gases. Other catheters were placed in a brachial vein to administer L-N^{G}-nitro arginine methyl ester (L-NAME; Sigma Aldrich, St Louis, MO) and hexamethonium (Sigma Aldrich), and in a jugular vein to administer anesthetics. After surgical instrumentation, isoflurane was discontinued and anesthesia was maintained with ketamine (1mg/kg) and vecuronium (0.1 mg/kg; Sun Pharmaceutical, Mumbai, India) given intravenously and supplemented hourly or as required.

Surgical procedures in rats

Female rats weighing 301±3 g were surgically instrumented while anesthetized with 2.5% isoflurane in oxygen. A polyvinyl catheter was inserted into a femoral artery and its tip was advanced to within 2 mm of its origin from the lower abdominal aorta. A catheter was inserted into a carotid artery to measure arterial blood pressure, heart rate, and blood gases. A catheter was placed in a jugular vein to administer hexamethonium. The other jugular vein was exposed for blood sample collection by puncture through the subclavius

muscle.

After surgical instrumentation, isoflurane was discontinued and anesthesia was maintained with an intraperitoneal injection of urethane (1000 mg/kg) and thereafter supplemented as required. A bolus of hexamethonium (1 mg/kg, iv) followed by a continuous infusion (2 $mg \cdot h^{-1} \cdot kg^{-1}$, iv) was given to limit neural influences on vascular tone. Rectal temperature was monitored and body temperature was maintained with a warming pad and heat lamp. The rats were mechanically ventilated with room air, with tidal volume and ventilatory rate adjusted to maintain arterial carbon dioxide tension in the range of 35 to 45 mmHg and pH from 7.35 to 7.45.

Preparation of GSNO, DNICs, and NO gas

5 mM GSNO: 5 mM GSH in 0.5 M HCl was incubated with 5 mM sodium nitrite for 30 min on ice. The stock solution was neutralized with NaOH and diluted with 0.1 M of physiological buffered saline (PBS) at pH=7.4 containing 0.1 mM diethylene triamine pentaacetic acid (DTPA) immediately before use.

2.5 mM glut-BDNIC [(Glut)$_2$Fe2(NO)$_4$]: 20 mM GSH, 10 mM $FeSO_4$, and 10 mM $NaNO_2$ were mixed and incubated for two hours at room temperature. The solution was neutralized with NaOH to pH=7.2 and incubated for another 15 h. Then the stock solution was collected after passing through a filter paper.

5 mM glut-MDNIC [(Glut)$_2$Fe(NO)$_2$]: To the 2.5 mM glut-BDNIC 50 mM GSH was added. The solution was alkalized with NaOH to pH=11.0 and incubated for three hours at room temperature.

Because DTPA eliminates EPR signals of glut-MDNIC, PBS or saline that does not contain DTPA was used for dilution of DNICs.

NO gas was generated by reaction of 1 M HCl and 1 M nitrite in syringes. The gas was then introduced into 1 M NaOH and shaken vigorously to eliminate the trace amount of nitrogen oxides. All solutions were deoxygenated before use. Excess amount of purified NO gas was introduced into deoxygenated saline to make a saturated solution of NO (~2 mM). NO solution was prepared freshly each time before use.

Effects of ODQ on stability of DNICs

Experiments were performed to test the effects of ODQ on the stability of glut-MDNIC and glut-BDNIC (n=3). Incubation of 100 μM ODQ with 50 μM glut-MDNIC at room temperature for 5 min did not decrease the EPR signal of glut-MDNIC (98±5% recovered), indicating that ODQ did not degrade MDNIC. Because glut-BDNIC is EPR-silent, it was quantified as MDNIC after conversion into MDNIC-like metabolite by incubation with plasma. Two experiments were performed: First, ODQ was incubated with glut-BDNIC for 5 min and then with plasma for another 5 min; Second, glut-BDNIC was incubated with plasma for 5min and then with ODQ for another 5 min. Both experiments showed that the yield of MDNIC-like metabolite derived from glut-BDNIC was not affected by ODQ (97±5% recovery).

Hydroxylamine assay

This colorimetric assay is based on the formation of indooxine in the reaction between hydroxylamine and 8-hydroxyquinoline[3]. The lower limit of quantification is 5 μM.

Three reagent solutions were used: A: 10% (w/v) trichloroacetic acid in water; B: 1% (w/v) 8-hydroxyquinoline in ethanol; C: 1 M sodium carbonate in water. Solution A (48 μl) and the sample (240 μl) in potassium phosphate buffer (432 μl, 100 mM), pH 6.8, were placed in a 1.5 ml Eppendorf tube. Solution B (240 μl) was added with rapid mixing and the solution was allowed to stand for 3 min at room temperature. Solution C (240 μl) was added with thorough mixing for 10 to 20 s. The mixture was heated at 95°C for 5 min, followed by cooling at room temperature for 15 min. The absorbance at 705 nm was measured using a spectrophotometer. Calibration curves were generated using a dilution series of a standard hydroxylamine hydrochloride solution in water.

Supplementary Figures

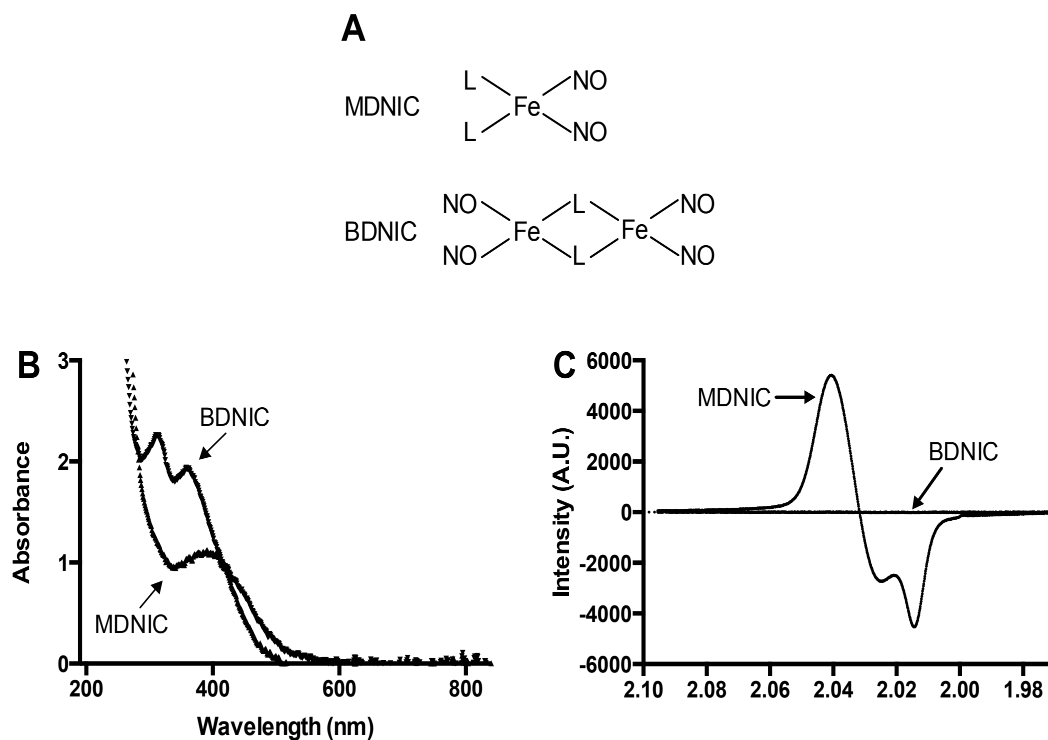

Figure S1. Verification of laboratory-prepared glut-DNICs by optical and EPR methods. **A**) Structures of MDNIC and BDNIC, where "L" represents ligand, glutathione in the present study. **B**) UV-Vis spectra of 5 mM glut-MDNIC and 2.5 mM glut-BDNIC measured by NanoDrop 2000/2000c spectrophotometer. **C**) EPR spectra of 50 μM glut-MDNIC and the lack of signal from 25 μM glut-BDNIC (EPR-silent) measured at 150 K.

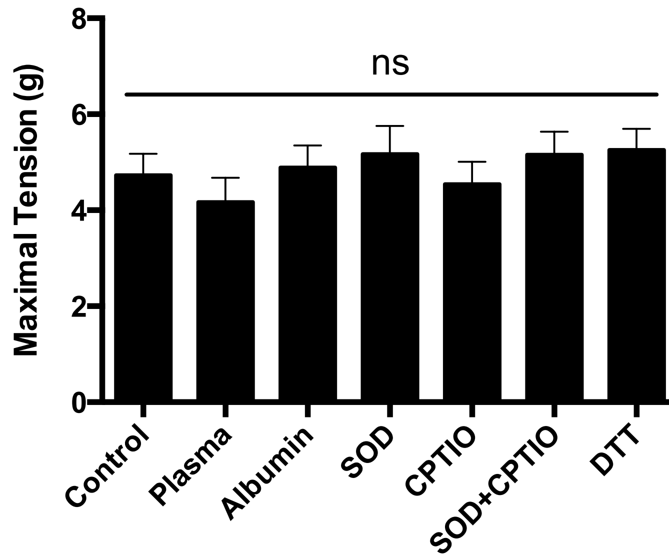

Figure S2. Maximal tension of sheep mesenteric arterial rings in the absence and presence of different test compounds, including 2% (v/v) plasma, 15 μM albumin, 1000U/ml SOD, 200 μM CPTIO, 1000U/ml SOD+200 μM CPTIO, or 1mM DTT (n≥4; p=0.7930; one-way ANOVA).

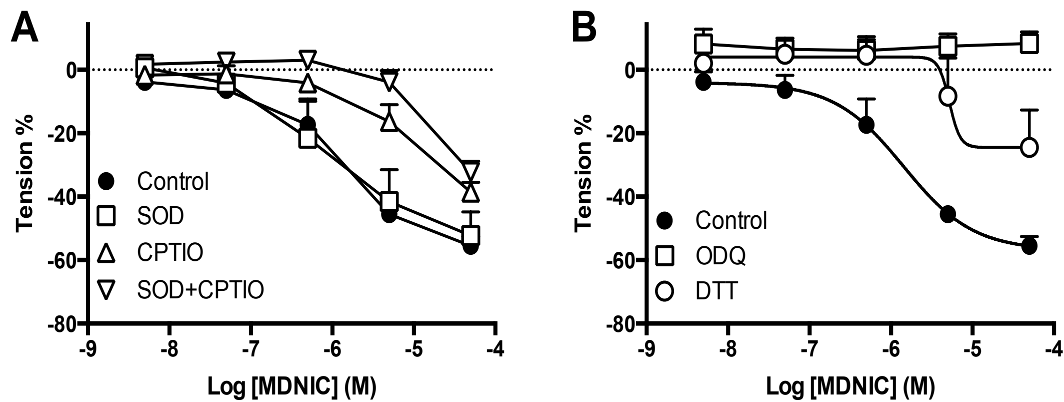

Figure S3. *Role of HNO in glut-MDNIC-mediated relaxation of isolated sheep mesenteric arteries* (*n*≥4). glut-MDNIC-mediated relaxation in the absence and presence of 1000U/ml SOD (converts HNO into NO·), 200µM CPTIO (NO· and HNO scavenger), or 1000U/ml SOD + 200µM CPTIO (**A**), the sGC inhibitor 10µM ODQ or the HNO scavenger 1mM DTT (**B**). Relaxation was normalized to the tension (100%) prior to first addition of the drug.

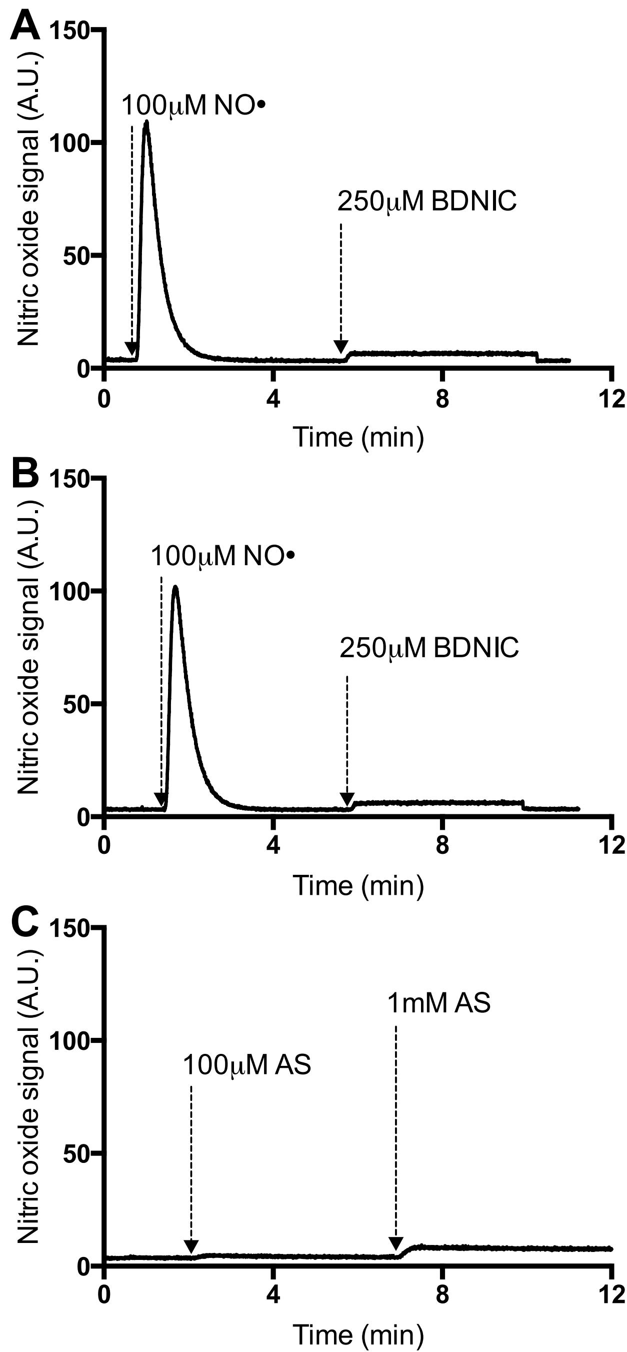

Figure S4. In vitro measurements seeking to detect NO· release from glut-BDNIC. 50 μl sample was injected into 20 ml hepes buffer (37 Celsius; pH=7.4) in a purge vessel in line with a chemiluminescence NO· analyzer. Arterial segments were added into the

purge vessel right before sample injection in **B**, but not in **A** and **C**. Each recording lasted for around 4min. In contrast to the robust NO· signal from 100μM NO· injection, 250 μM glut-BDNIC (1000 μM NO moieties; **A** and **B**) and 1mM Angeli's salt (AS; HNO donor) (**C**) resulted in little NO· signal. A representative trace of three similar experiments is shown.

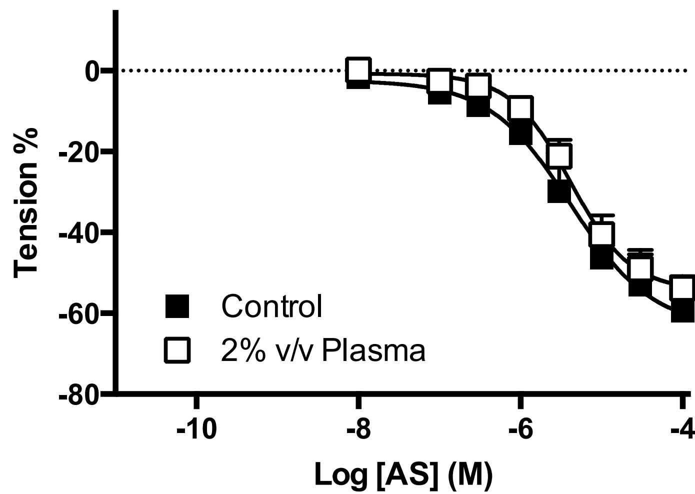

Figure S5. Effects of plasma on relaxation by the HNO donor AS (*n*=4). Plasma does not significantly alter AS-mediated relaxation ($p=0.67$ for pEC_{50}) indicating the attenuating effects of plasma on glut-BDNIC-mediated relaxation may not result from the scavenging of HNO by plasma. Relaxation was normalized to the tension (100%) prior to the first addition of the drug.

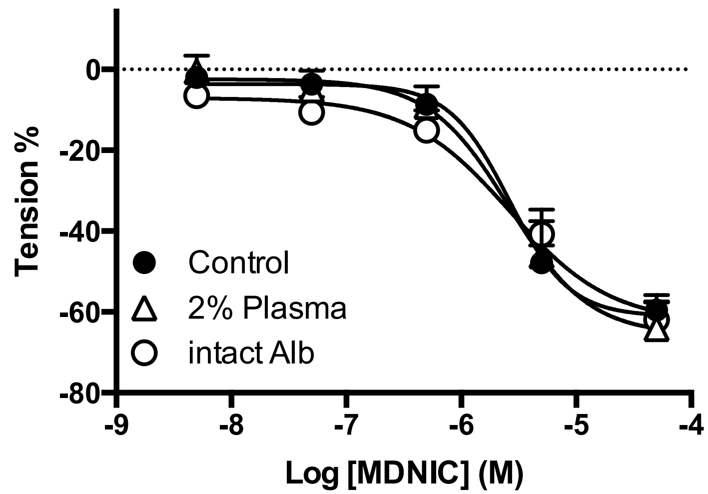

Figure S6. Effects of plasma (2% v/v) and albumin (15μM) on glut-MDNIC -mediated relaxation (n=6). In contrast to the attenuation of glut-BDNIC-mediated vasodilation (Figure 5), plasma and albumin did not alter vasodilation by glut-MDNIC. Relaxation was normalized to the tension (100%) prior to the first addition of the drug.

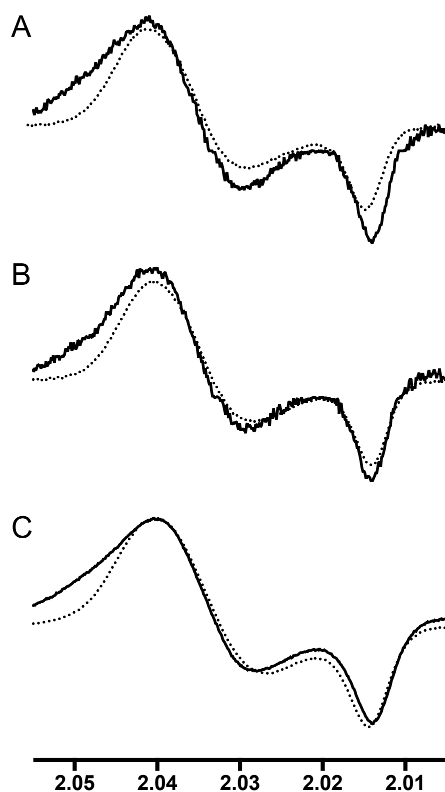

Figure S7. EPR spectrum (150 K) of metabolites of 25μM glut-BDNIC and 50μM glut-MDNIC in (**A**) plasma, (**B**) blood, and (**C**) albumin solutions in vitro. Solid lines represent the metabolite of glut-BDNIC, while interrupted lines represent the metabolite of glut-MDNIC. Addition of EPR-silent glut-BDNIC to isolated plasma, blood, and albumin solutions creates an EPR signal that is similar in all three matrices. These spectra differ slightly from those created following addition of glut-MDNIC to these matrices in that the glut-BDNIC samples all have a shallow shoulder in the left peak (at g factor=

~2.05). These findings suggest that glut-BDNICs are rapidly converted into MDNIC-like species whether infused in vivo (Fig 6C) or added into blood, plasma, or albumin solutions in vitro. The difference in the spectra between the metabolites of glut-BDNIC and glut-MDNIC suggests that the HMW metabolites of these compounds differ in the circulation. Information for intensity is omitted for the sake of shape comparison between the spectra.

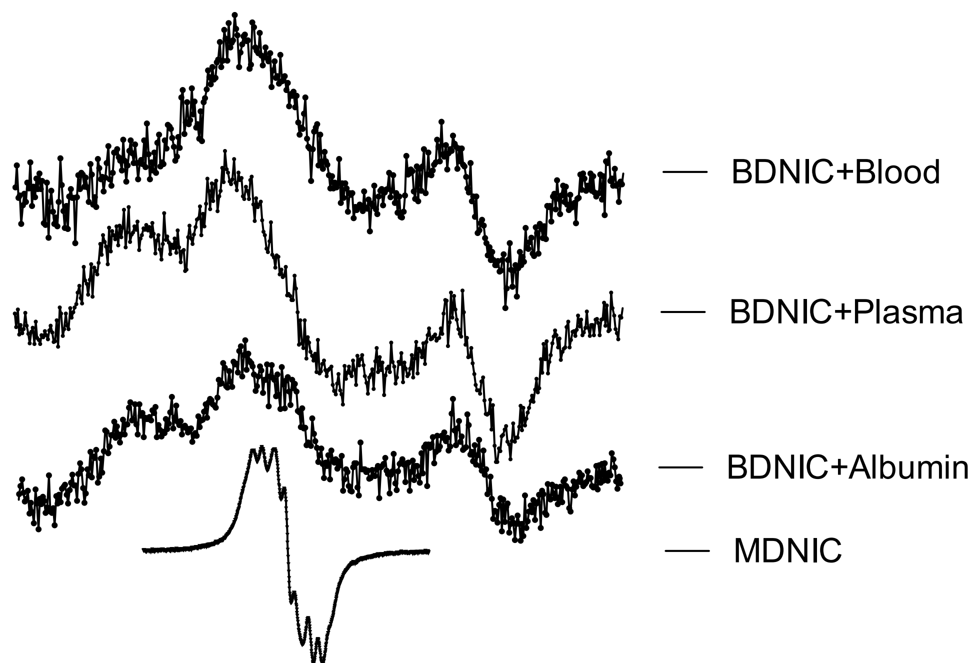

Figure S8. Representative, room temperature EPR spectra of glut-BDNIC following addition to whole blood, plasma, and albumin solution, as well as that of low molecular weight glut-MDNIC standard (N^{15} labeled). The room temperature EPR spectra of glut-BDNIC metabolites in blood, plasma, and dissolved albumin are similar to each other, and none show hyperfine structure (the mirroring dents up at left vs down at right) such as that observed for LMW MDNIC (bottom tracing), indicating that the glut-BDNIC metabolites in blood and plasma are HMW complexes containing albumin.

References:

[1] T. Liu, H.J. Schroeder, L. Barcelo, S.L. Bragg, M.H. Terry, S.M. Wilson, G.G. Power, A.B. Blood, Role of blood and vascular smooth muscle in the vasoactivity of nitrite, Am J Physiol Heart Circ Physiol 307 (2014) H976-86.

[2] T. Liu, H.J. Schroeder, S.M. Wilson, M.H. Terry, L.D. Longo, G.G. Power, A.B. Blood, Local and systemic vasodilatory effects of low molecular weight S-nitrosothiols, Free Radic Biol Med 91 (2015) 215-23.

[3] L.S. Choi, H. Bayley, S-Nitrosothiol Chemistry at the Single-Molecule Level, Angew Chem Int Ed Engl 51 (2012) 7972-7976.