Supplementary Material

Material and Methods

All Materials were obtained from Sigma Chemical Company unless otherwise stated. Chemicals were >99% pure. Reagent methanol was HPLC grade.

Exclusion criteria

In an effort to minimize potential differences in iNO metabolism due to underlying differences in lung function and ensure focus on the organ of interest, the liver, patients with hepatopulmonary syndrome (HPS) were excluded. The criteria employed for HPS were 1) evidence of intrapulmonary vascular shunting assessed by transthoracic bubble contrast echocardiography and 2) abnormal arterial oxygenation defined as an alveolar-arterial oxygen gradient of > 20mmHg or an arterial PaO2 < 70 mmHg on arterial blood gas testing in the absence of significant intrinsic cardiopulmonary disease. Additionally, minors and patients > 70 years of age (greater risk than usual) were also exclude for reasons of patient homogeneity and anesthetic and surgical risk. All liver transplantation candidates at the University of Alabama at Birmingham underwent preoperative transthoracic echocardiography and were not deemed suitable candidates if their estimated mean pulmonary artery pressures were > 35 mm Hg . Pulmonary artery catheters are rarely utilized in our practice, thus pulmonary artery pressures are not typically measured after the induction of anesthesia, unless there is a clinical trigger.

In an effort to minimize differences in the patients studied and to ensure focus on the organ of interest with inhaled NO, the liver, we decided to exclude patients with hepatopulmonary syndrome (HPS). The criteria employed for HPS were platypnea, defined as dyspnea induced by the upright position and relieved by recumbency and orthodeoxia, defined as arterial deoxygenation induced by the upright position and relieved by recumbency. Additionally, minors and patients > 70 years of age (greater risk than usual) were also exclude for reasons of patient homogeneity and anesthetic and surgical risk. All liver transplantation candidates at the University of Alabama at Birmingham underwent preoperative transthoracic echocardiography and were not deemed suitable candidates if their estimated mean pulmonary artery pressures were > 35 mm Hg . Pulmonary artery catheters are rarely utilized in our practice, thus pulmonary artery pressures are not typically measured post-induction unless there is a clinical trigger.

Blood Collection and Processing

Arterial or venous blood was collected into syringes and treated as described below for measurement of different NOx. The protocol employed was based on methodology developed by a number of laboratories (1-8). For each blood draw

duplicate samples were collected. Note recent studies question the validity of the employed protocols for measuring specifically RBC S-nitrosothiols, although the underlying reasons for discrepant results remain unclear (9). Using SNOHb as a standard, 98.7% recovery was observed (supplementary Figure 6) using the protocols described below and consistent with previous reports (1, 2, 4, 6, 10).

Whole Blood Nitrite and Nitrate: Whole blood (500µl) was immediately (~15-30sec) mixed with a whole blood stabilization solution (100µl) comprising potassium ferricyanide ($K_3(FeCN)_6$), N-ethylmaleimide (NEM) and the non-ionic detergent IGEPAL-CA630. All reagent stocks where applicable were made in PBS + 100µM DTPA. Final concentrations were $K_3(FeCN)_6$ = 133mM, NEM = 1.67mM, NP-40 = 1% v/v. Samples were mixed vigorously and incubated at room temperature for 5 min to allow reaction with $K_3(FeCN)_6$ to reach completion and then snap frozen in liquid N_2 and stored at -80°C until analysis. For analysis, samples were thawed on ice, nitrite/nitrate extracted by adding ice-cold methanol (0.6mL). Samples were vortex mixed (1min) and centrifuged at 15,000g, 2min. Supernatants were taken and nitrite and nitrate measured as described below.

Plasma Nitrate, Nitrite, S-nitrosothiols, XNO and RBC S-nitrosothiol and XNO: Whole blood (4.5mL) was immediately (~15-30sec) mixed with stabilization solution 1 (SS1, 500uL) comprising sodium citrate (anticoagulant), NEM and DTPA in PBS. Final concentrations were sodium citrate (1.5% w/v), NEM = 5mM and DTPA = 100µM. Blood was centrifuged (2000g, 90sec, 15-20°C) to separate plasma and RBC. Plasma (800µl) was then mixed with stabilization solution 2 (SS2, 800µl) comprising NEM and DTPA. Final concentrations were NEM = 1mM and DTPA = 100µM. Samples were mixed, snap frozen in liquid N_2 and stored at -80°C until analysis. For RBC (250µl) were mixed with stabilization solution 3 (SS3, 1.4 ml) comprising $K_3(FeCN)_6$, NEM, DTPA, NP-40 in PBS. Final concentrations were $K_3(FeCN)_6$ = 10mM, NEM = 20mM, DTPA = 100µM, NP-40 = 1% v/v. Recovery of added SNOHb in a RBC lysate using this procedure was >98%) After mixing samples were flash frozen in liquid N_2 and stored at -80°C until analysis. For analysis, plasma or RBC were thawed on ice and in the dark, and nitrate, nitrite, S-nitrosothiols and XNO measured by NO-chemiluminesence (see below). Note plasma and RBC stabilization was performed simultaneously resulting in average times between sample collection and freezing of different blood components of <7min. Plasma and RBC levels of NOx were normalized to protein and heme concentrations respectively.

RBC HbNO measurement. Whole blood (1ml) was centrifuged (2000 g, 90sec) and RBC (500µl) snap frozen in liquid N_2 for subsequent analysis.

Plasma cGMP and XOR activities. Aliquots of plasma collected from whole blood mixed with SS1, were snap frozen in liquid N2 for subsequent analysis of cGMP concentrations and xanthine oxidoreductase (XOR) activities. cGMP was measured using commercially available ELISA assays (Cayman) and normalized to mg protein. XOR activity was determined via HPLC using electrochemical

detection of substrate (xanthine) to product (uric acid) formation. Endogenous substrate and product are removed by gel exclusion chomatography prior to kinetic analysis. XOR containing fractions were incubated with 75μM xanthine, in the absence and presence of 150μM allopurinol followed by uric acid extraction with cold acetonitrile. Samples were then dried, resuspended in mobile phase and analyzed by HPLC. Samples were run using a thirty minute isocratic separation with a mobile phase of 50mM sodium phosphate, 4mM dodecyltrimethyl ammonium chloride, and 2.5% methanol, pH = 7.0. The flow rate was 0.6 ml/min. The column used was a Rainin Microsorb – MV (100mm x 4.6mm I.D.). The analytical system consisted of an ESA model 582 pump, ESA model 542 refrigerated autosampler and an ESA model 5600A electrochemical CoulArray detector with applied potentials of -100, 90, 180, and 300mV. A complete set of five uric acid standards was run at the beginning and at the end of each analysis; standards were also routinely run after every fifth sample. Samples were run in duplicate, any activity in the presence of allopurinol was subtracted and the average used for activity calculations. XOR activity was normalized per mg protein.

NOx detection by NO-chemiluminesence.

All measurements were made using a Sievers NOA (nitric oxide analyzer). To avoid excessive foaming in the reaction chamber, purge vessel containing Antifoam (GE).

Nitrate: Total NOx was measured by reduction of all NO-containing species to NO using vanadium (III) chloride (51mM) in 1N HCl at 90°C. Nitrate values were calculated by subtraction of nitrite, S-nitrosothiol and XNO. Signals were integrated and compared to sodium nitrate standard curves to calculate concentrations.

Nitrite, S-nitrosothiol and XNO: Reductive acidic triodiode (KI/I_3) based chemiluminesence was used as previously described. Samples were either untreated, treated with acid sulfanilamide (1% w/v final in 1N HCl final concentration) to remove nitrite, or treated with acid sulfanilamide + $HgCl_2$ (5mM) to remove nitrite and S-nitrosothiols for 15 min in the dark at room temperature and then injected into the purge vessel containing a solution containing potassium iodide (66.8mM) and iodine (28.5mM) + acetic acid (78%v/v). Signals were integrated and compared to sodium nitrite standard curves to calculate concentrations. Signals from samples with no pre-treatment reflect nitrite + S-nitrosothiol + XNO concentration, those from samples treated with acid-sulfanilamide reflect S-nitrosothiol + XNO concentrations those from samples treated with acid sulfanalimide + $HgCl_2$ reflect XNO. For RBC measurements purge vessel was washed and fresh $KI/I_3/KI$ solution used per sample injection. RBC nitrite and nitrate were calculated by subtracting plasma levels from WB levels after normalizing to RBC volume (i.e. hematocrit).

RBC HbNO: HbNO was measured as previously described (11). RBC samples were injected into purge vessel containing 8ml $K_3(FeCN)_6$ (50mM) + 80µl SE-15 Antifoam (Sigma) at 70°C. Purge vessel was washed and fresh $K_3(FeCN)_6$ solution used per sample injection. RBC containing HbNO were synthesized using open-flow respirometry as previously described and concentrations measured using UV-Vis spectroscopy after hypotonic RBC lysis. RBC standards with known HbNO concentrations were prepared and used to construct standard curves in the NO-chemiluminesence based assay.

In all cases, background levels of NOx were measured in all buffers, reagents and stabilization solutions and subtracted from sample NOx values. Due to potential changes in blood volume during surgery, all plasma levels are normalized to protein and RBC levels normalized to heme.

Heme determination: Heme concentrations were measured using Drabkins assay.

Protein concentration: Protein concentrations were determined using the Bradford assay (BioRad).

Liver Biopsy collection *:* Biopsies of donor livers obtained during transplantation were either formalin fixed or frozen in liquid nitrogen. Frozen samples were then stored at -80^{o} C. After formalin fixation (24h), samples were transferred to 70% ethanol and then embedded into paraffin and 5µm sections prepared. Frozen samples were used for western blotting and ELISA based procedures. Formalin fixed samples were used for histochemical evaluation of hepatic injury, immunofluorsence detection of MPO, 3-NT and for TUNEL staining.

Western Blot. Tissues were homogenized on ice in RIPA buffer for 20s using a PowerGen 125 homogenizer (Fisher Scientific). Homogenates were then centrifuged at 4^{o}C for 10min at 10,000 rpm to remove particulate matter. Supernatants were collected and protein content determined using BCA reagent. Samples were prepared for western analysis by diluting with Laemmli sample buffer (Biorad) plus BME at a final concentration of 2mg/ml, boiled for 5min then stored at -80^{o}C. Equal amounts of protein (40µg /lane) were subjected to SDS-PAGE (10% gels). Following electrophoresis the proteins were transferred to nitrocellulose membrane. After transfer, the membranes were blocked in TBST buffer containing 0.1% tween and 5% milk. Membranes were exposed to appropriate primary antibody in blocking buffer, washed, then exposed to the appropriate horseradish peroxidase-conjugated secondary antibody (specific dilutions described below). Blots were incubated with SuperSignal substrate solution (Pierce #34095), exposed to film and then densitometry was performed using Scion image software. Bands were normalized to β-actin which was detected after blots were stripped and reprobed with primary antibody to β-actin. Antibody source and dilutions were: eNOS (BD biosciences #6102960) at a

1:2500 dilution; Xanthine oxidoreductase (Neomarkers #MS-474-P) at a 1:1000 dilution, β-actin (Cell Signaling #4967) at a 1:3000 dilution.

ELISA for ICAM-1 and VCAM-1. Capture antibody (R & D Systems; #MAB720) was diluted to 4µg/ml in PBS and 100µl placed in 96 well ELISA plate overnight at 4°C. Plates were then washed 3X with PBS + 0.05% Tween. Wells were blocked by adding 300µl of PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 and incubated for 1.5 hours at room temperature. Plates were then washed 3X again with same wash buffer. Sample tissue homogenates were diluted in PBS to 10µg protein / ml and 100µl placed in sample wells. ICAM-1 standard (R & D Systems; # ADP4) was diluted in PBS to concentrations ranging from 0.016 to 2.0 ng/ml and 100µl placed in standard curve wells. Plates containing samples and standards were incubated for 2 hours at room temperature. Plates were then washed 3X with wash buffer. 100µl Biotinylated detection antibody (R & D Systems; # BAF720) at a concentration of 100ng/ml in PBS containing 0.1% BSA was added and incubated at room temperature for 2 hours. Plates were then washed 3X with wash buffer. 100µl streptavidin HP (R % D Systems; #DY998) diluted 1:200 in PBS containing 0.1% BSA was added and incubated for 20 minutes. Plates were then washed again 3X with wash buffer. 100µl substrate solution (3,3,5,5- Tetramethyl-benzidine; Sigma # T4444) was added and incubated at room temperature in the dark. Once appropriate development had occurred the reaction was stopped by adding 50µl of 1M H_2SO_4 and optical density measured at 450nm. Appropriate control wells containing capture antibody and detection antibody alone were measured and their average optical density subtracted from all standard and sample measurements. All standards and samples were measured in triplicate. The final ICAM-1 and VCAM-1 concentrations were calculated based on the standard curve. Similar protocol was used for VCAM-1 with following antibody details: capture antibody (R & D Systems; #MAB809) was diluted to 2µg/ml. VCAM-1 standard (R % D Systems; #809-VR) was diluted in PBS to concentrations ranging from 0.008 to 1.0 ng/ml. Biotinylated detection antibody (R & D Systems; #BAF809) was diluted to 100ng/ml.

TUNEL staining: Hepatocellular apoptosis was determined using the TUNEL staining kit from Promega, Inc according to manufacturers recommendations. 5µm formalin fixed sections were prepared and mounted with Vectashield plus 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, Ca). Immunofluorescence (green TUNEL staining at 488nm) was measured from five random fields per slide for quantitation of the extent of apoptosis. All images for quantitation were obtained using oil immersion at 25X magnification. Data represents the number of fluorescent positive objects per field.

3-nitrotyrosine detection: 2 approaches were used for 3-nitrotyrosine detection. Immunofluoresence staining on 5µm formalin fixed sections and by immunoblotting of dot blots using polyclonal anti-3-nitrotyrosine antibody from Upstate. For the latter also, nitrated bovine serum albumin standards were

synthesized and quantitated as previously described (12-14). Parallel incubations of samples with anti-3-NT antibody pre-adsorbed with 3-NT-BSA were used to demonstrate specificity of antibody binding. Detection limit using this approach was 250pg using 3-NT-BSA as a standard.

MPO detection: Liver biopsies were immersion-fixed in 10% neutral-buffered formalin and stored in 70% ethanol until paraffin-embedding and sectioning. 5µm paraffin embedded sections were mounted on slides, deparaffinized in xylene, and rehydrated in 100%, 95%, 85%, 70% solutions of ethanol. High temperature antigen retrieval was performed on the tissue by boiling in antigen unmasking solution (Vector Labs #H3300) for 2-3 minute periods for a total of 10-12 minutes. Sections were blocked with 5% goat serum in 1% bovine serum for 1 hour at RT. The primary antibody for MPO 1:100 (Abcam #9535) in an appropriate volume of 5% goat serum was applied to sections for overnight incubation at 4°C. The following day the sections were washed, then blocked with 5% goat serum in 1% bovine serum for 1 hour at RT. Tissue was then incubated with Alexa Fluor 594 goat anti-rabbit 1:400 (Molecular Probes) diluted in 5% goat serum to visualize MPO. Sections were mounted with Vectashield plus 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, Ca). Image acquisition was performed on a Leica DMRXA2 epifluorescence microscope (Leica Microsystems, Bannockburn, IL) with a Hamamatsu ORCA ER cooled CCD camera and SimplePCI software (Compix, Inc., Cranberry Township, PA).

Protein Carbonyl detection: Protein carbonyl concentrations were determined on liver biopsy homogenants. We used a protein carbonyl ELISA kit from Alexis (#ALX-850-312-KI01). The assay was performed according to manufacturers specifications for low protein containing samples.

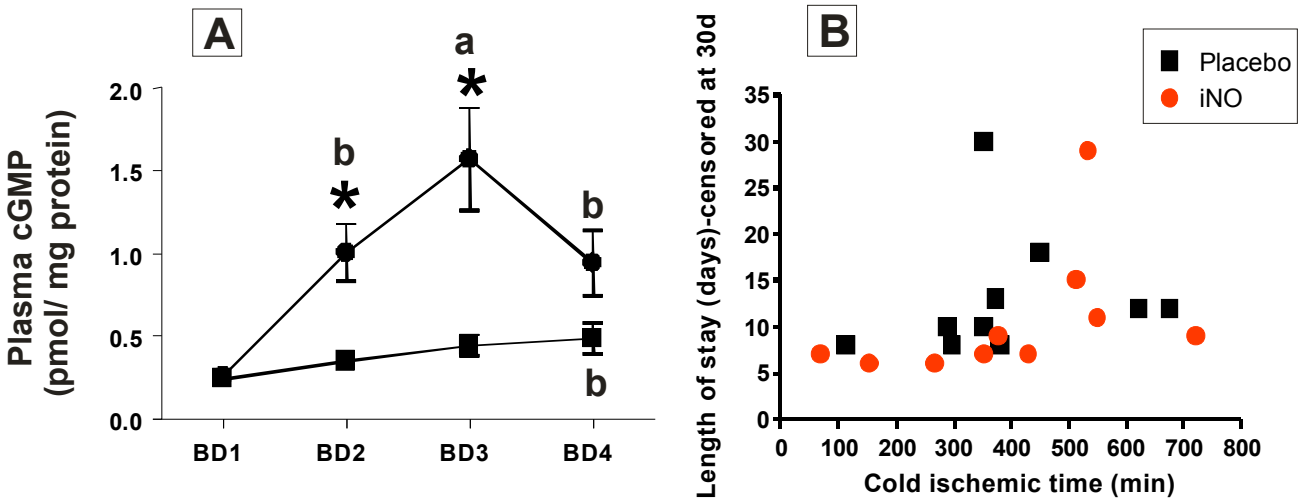
Supplementary Table 1: Profile of immunosuppressant used in perioperative period for patients enrolled in this study.

		Immunsuppresant						
		Prednisone	Cellcept	Prograf	Rapamine	Imuran	Neoral	Dicyclomine
Placebo	1	X	X	X	X			X
	2	X	X		X			
	3	X	X	X				
	4	X	X	X				
	5	X	X	X				
	6	X	X	X				
	7	X	X			X		
	8	X	X	X				
	9	X	X	X	X	X		
	10	X	X		X			X
iNO	1	X	X	X				
	2	X	X	X				
	3	X	X	X				
	4	X	X	X	X			
	5	X	X	X				
	6	X	X		X			
	7	X	X	X	X			
	8	X	X	X	X			
	9	X	X		X			
	10	X	X	X				

5 different immunosuppressants' (Prednisone, Cellcept, Prograf, Rapamine, Imuran, Neoral, Dicyclomine) were used. For the placebo group, 7/10 received 3 immunosuppressants, 1/10 received 4 immunosuppressants and 2/10 received 5 immunosuppressants. For the iNO group 7/10 received 3 immunosuppressants and 3/10 received 4 immunosuppressants. Average number of immunosuppressant's were not significantly different ($P = 0.53$) and were 3.5 ± 0.27 and 3.3 ± 0.15 (mean ± SEM, n=10) for placebo and iNO groups respectively.

Supplementary Figures:

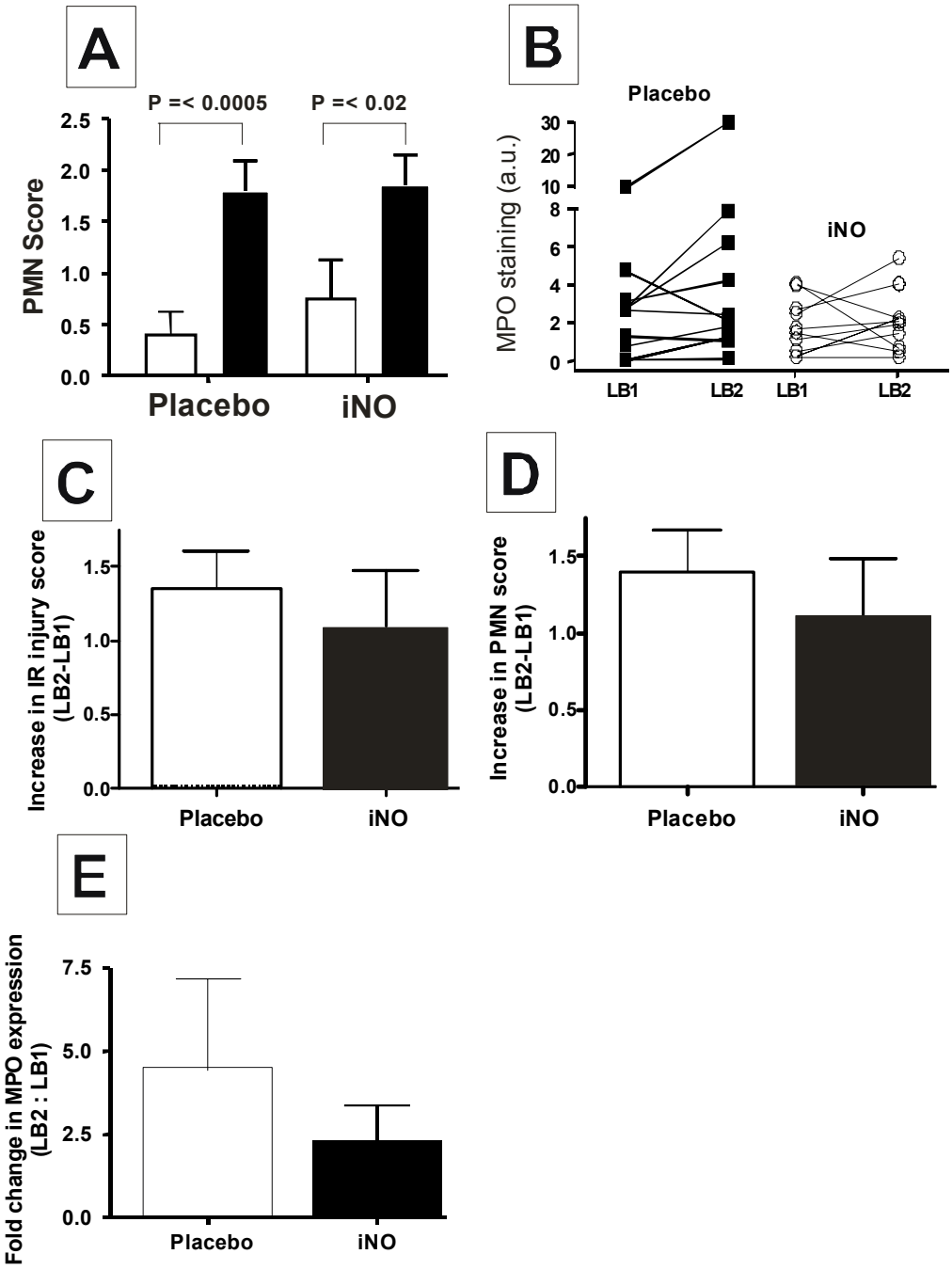
Supplementary Figure 1



Panel A: Time dependent changes in venous plasma cGMP. Key: iNO = -●-; placebo = -■-. * P = < 0.003 for placebo vs iNO at corresponding blood draw by Mann-Whitney test. [a] P = < 0.001, [b] P = < 0.05 for cGMP at indicated BD vs corresponding BD1 value by repeated measures ANOVA with Tukey post hoc analysis. All values are mean ± SEM.

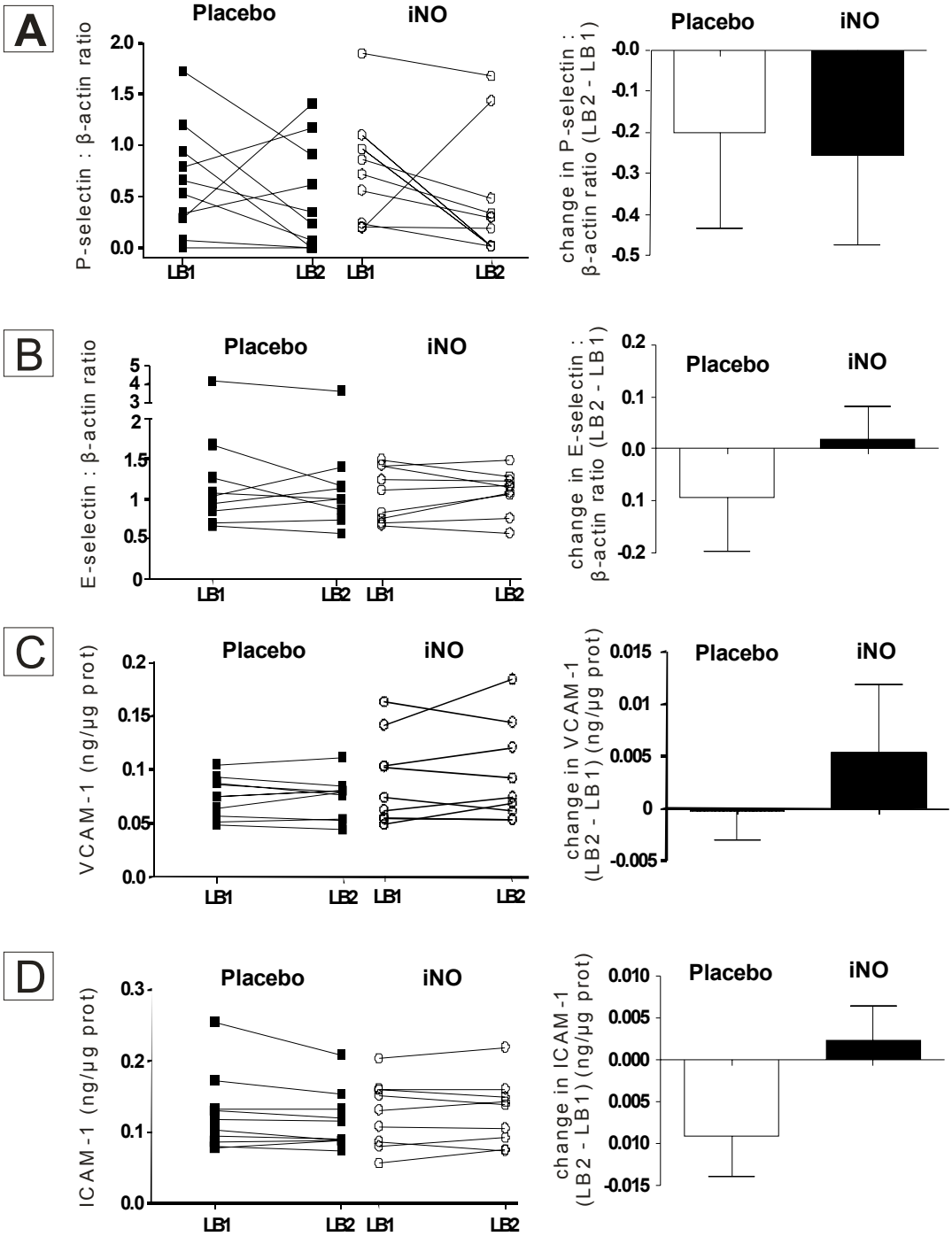
Panel B: Plot of length of stay vs cold ischemic time. Length of stay is censored at 30 days (censored time point was for 1 placebo patient whose length of stay was 95 days. For a given cold ischemic time, patients receiving iNO had a trend towards lower length of stays.

Supplementary Figure 2



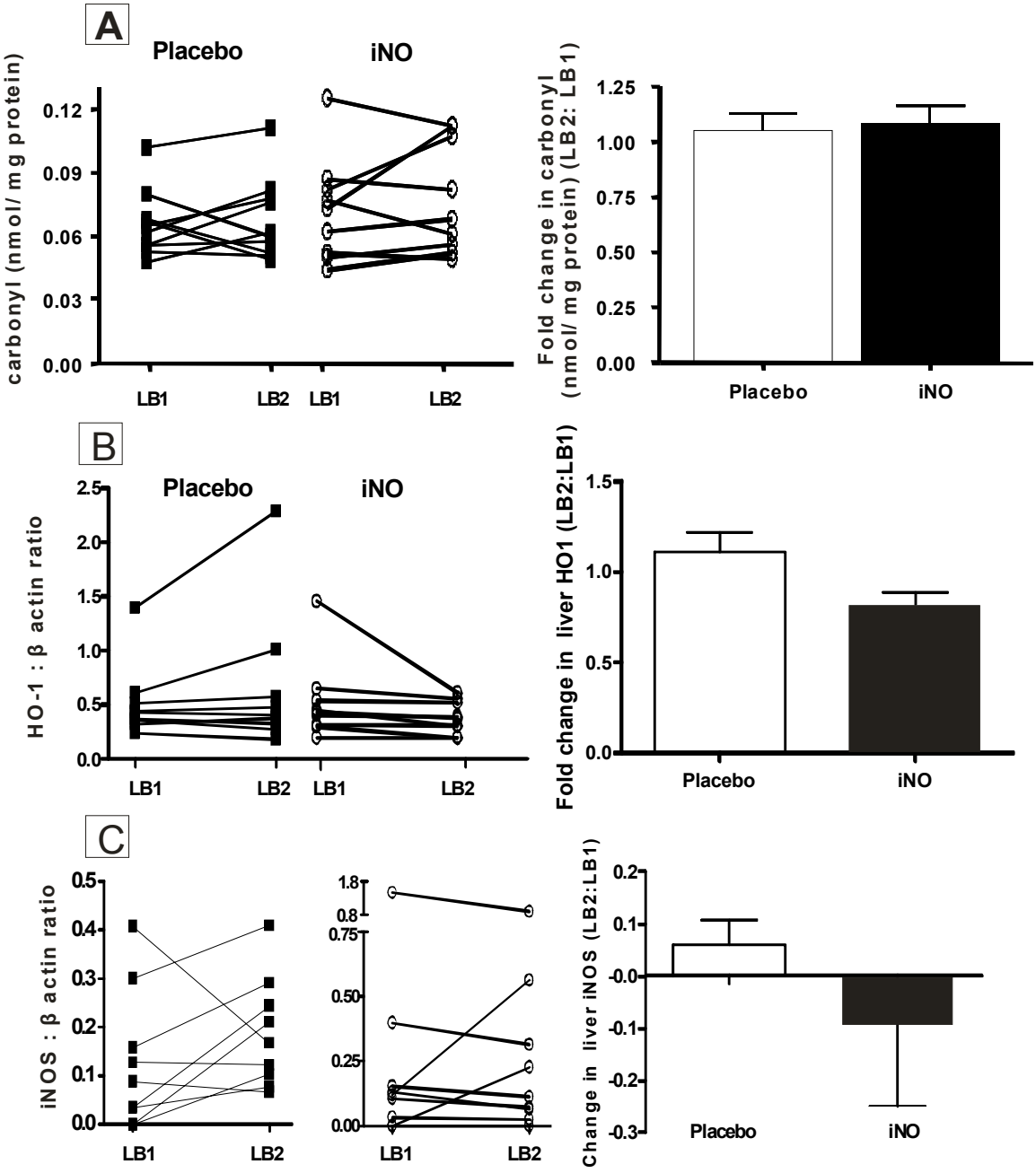
Panel A: Histopathologic scoring for changes in PMN infiltration in hepatic tissue samples pre- (open bars) and 1h post reperfusion (black bars). P-values represent significance calculated by paired t-test. **Panel B** Changes in MPO expression assessed by immunofluoresence of 5µm formalin fixed sections. **Panels C-E** show average increase in scoring for hepatic IR injury (panel C) or PMN infiltration (panel D) or fold change in MPO expression (panel E). In all cases differences were not significant by unpaired t-test.

Supplementary Figure 3



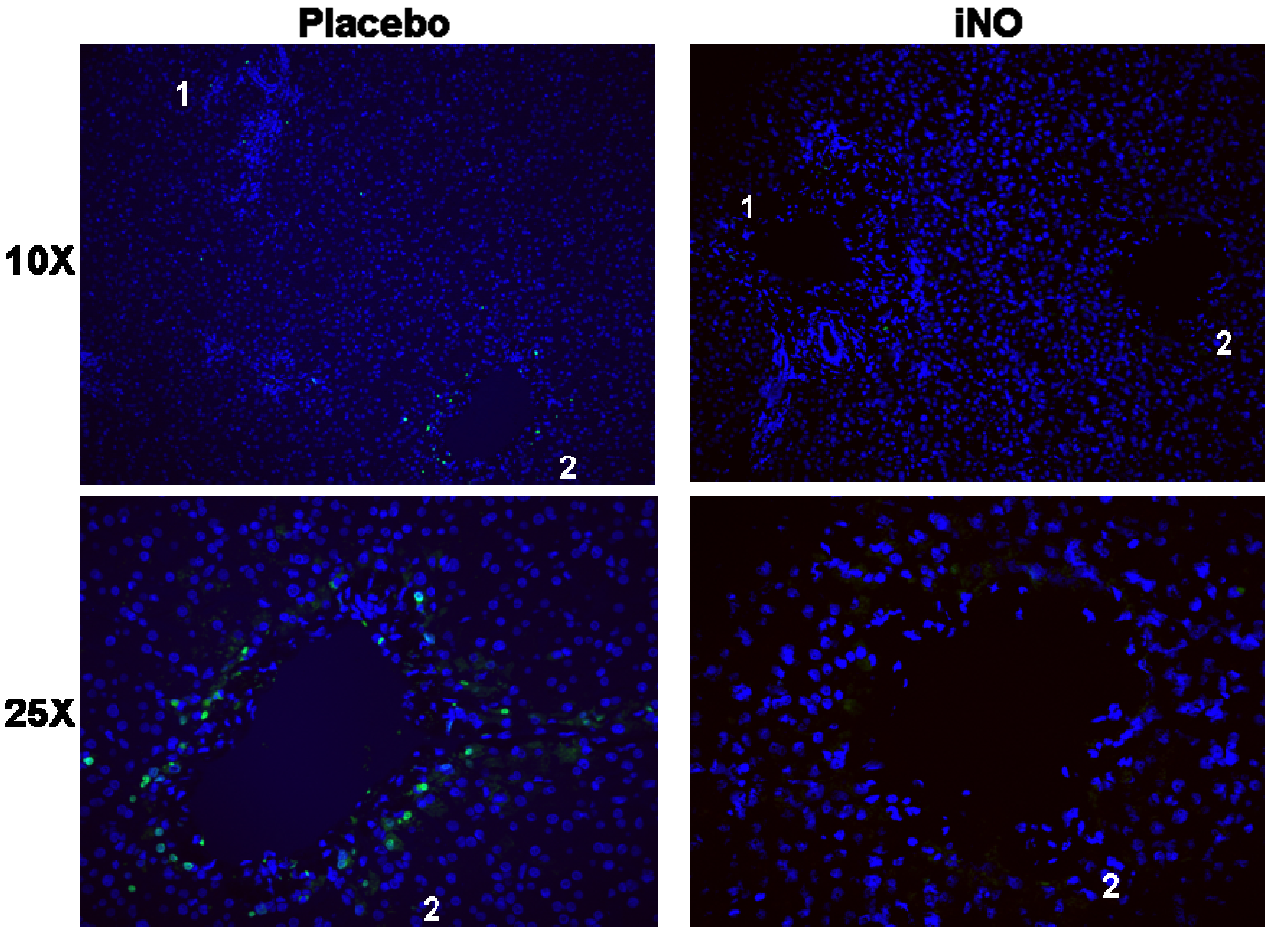
Panels A-D: Paired changes and average change in expression of P-selectin, E-selectin, VCAM-1 and ICAM-1 respectively. No significant change in these adhesion molecules was observed either in response to reperfusion or between placebo and iNO exposure.

Supplementary Figure 4



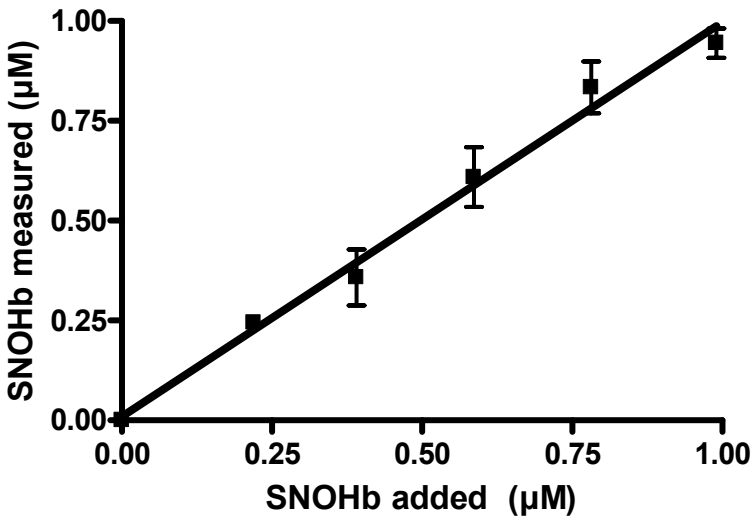
Paired changes and average fold-change in protein carbonyl expression (Panel A), HO-1 (Panel B) and iNOS (Panel C). No significant changes in either carbonyl expression, HO-1 expression nor iNOS expression was observed either in response to reperfusion or between placebo and iNO exposure.

Supplementary Figure 5



Representative fluorescence micropgraphs depicting hepatic distribution of TUNEL positive cells in LB2 (liver biopsy 1h post-reperfusion). Shown are images from placebo and iNO groups and at 10X and 25X magnification. 10X magnification shows general hepatic architecture and illustrates a portal triad (1) and a central vein (2). The central vein region is magnified in the 25X image. TUNEL positive cells are distributed around the central vein area and consistent with hepatocyte localization of apoptosis. iNO administration inhibited IR dependent apoptosis. TUNEL staining = green, Nuclei = blue (DAPI).

Supplementary Figure 6



SNOHb was synthesized as previously described using SNAP (15) followed by purification using gel-filtration chromatography. S-nitrosothiol concentrations were measured by the Saville assay and then increasing concentrations of SNOHb added to RBC lysates, mixed 15sec followed by addition of stabilization solutions. Samples were handled as described above in methods describing how samples for RBC S-nitrosothiols were collected, stored and measured by I_3^- chemiluminesence. Data show a 98.7% recovery SNOHb using these protocols (line shows best fit by linear regression, $y = 0.987x + 0.0099$, $r^2=0.99$). Data shown mean ± SEM, n = 3.

References (Supplementary Material)

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