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SNO–Hemoglobin Not Essential for Red Blood Cell Dependent Hypoxic Vasodilation

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Introductory Paragraph

The coupling of hemoglobin sensing of physiological oxygen gradients to stimulation of nitric oxide (NO) bioactivity is an established principle of hypoxic blood flow. One mechanism proposed to explain this O₂ sensing/NO bioactivity linkage postulates an essential role for the conserved hemoglobin β93Cys residue and, specifically, for S–nitrosation of β93Cys to form S–nitrosohemoglobin (SNO–Hb)¹. The SNO–Hb hypothesis, which conceptually linked hemoglobin and NO biology, has been debated intensely in recent years^{2,3}. This debate has precluded a consensus on physiological mechanisms and on assessment of the potential role of SNO–Hb in pathology. Here we describe novel mouse models that express exclusively either human wild type hemoglobin or human hemoglobin in which the β93cys residue is replaced with alanine to assess the role of SNO–Hb in red cell mediated hypoxic vasodilation. Substitution of this residue, precluding hemoglobin S–nitrosation, did not change total red cell S–nitrosothiol levels but shifted S–nitrosothiol distribution to lower MWt species, consistent with the loss of SNO–Hb. Loss of β93cys resulted in no deficits in systemic nor pulmonary hemodynamics under basal conditions and, importantly, did not affect isolated red cell dependent hypoxic vasodilation. These results demonstrate that SNO–Hb is not essential for the physiologic coupling of erythrocyte deoxygenation with increased NO–bioactivity in vivo.

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Author Contributions

TSI, CWS, LCW, XT, DAV and KMP were responsible for performing experiments. TSI, CWS, DAV, RPP and TMT were responsible for planning all experiments, analyzing data and writing manuscript. MBR contributed to mass spectrometry assays, LS was responsible for exercise related studies, CGK and BGB for capillary density measurements, NP and JW contributed to blood pressure measurements and NA for assessment of pulmonary hemodynamics. JR did the ES cell injections to generate the chimeras.

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In addition to hemoglobin oxygen affinity, blood flow is a key component of the processes that match oxygen delivery to demand. Increased blood flow in response to hypoxia is a critical physiological response which does not correlate with dissolved oxygen tensions but does correlate with hemoglobin oxygen fractional saturation⁴. These observations have led to the concept that the red blood cell (RBC) itself is a regulator of flow and to the general paradigm that RBC/hemoglobin deoxygenation is coupled to the stimulation of vasodilation^{1,5,6}. Three mechanisms for this coupling have been proposed (illustrated in supplementary Figure 1) and involve either i) ATP–release and subsequent activation of endothelial nitric oxide synthase⁷, ii) nitrite reduction to NO by deoxyhemoglobin⁵ and iii) S–nitrosohemoglobin (SNO–Hb) dependent pathways¹. In the latter proposal, hemoglobin becomes S–nitros(yl)ated on a specific and conserved cysteine residue on the β –chain (β 93Cys) as RBCs become oxygenated in the lungs (forming SNO–Hb). In the R–state, SNO–Hb remains relatively unreactive but upon deoxygenation, T–state SNO–Hb can rapidly react with thiols [GSH or anion exchanger–1 (AE–1) thiols] and transmit a vasodilatory signal out of the RBC (suppl Fig 1)^{8–12}. This hypothesis has been extended recently to suggest that dysfunction in this pathway leads to a variety of systemic vascular and pulmonary diseases and contributes to pathologic effects associated with diabetes, congestive heart failure and with transfusion of banked blood^{13–17}. These pioneering studies also began to resolve a long standing question in hemoglobin biology, namely the role of the highly conserved β 93Cys residue. However, the function of SNO–Hb *in vivo* and the potential role in disease has been debated intensely over the past few years. Many aspects of the SNO–Hb hypothesis have been disputed including the measurements of SNO–Hb concentrations and the mechanisms of SNO formation and bioactivity (suppl Figure 1).

To directly address this issue and explore the role of the conserved β 93cys residue, we created knockin mouse models in which RBCs contained exclusively wild–type (WT) human hemoglobin (HbC93) or exclusively human hemoglobin in which the β 93cys residue was replaced with Ala (HbC93A) (Fig. 1a) (see suppl Fig. 2a, b for knockin scheme). Sequencing of mouse genomic DNA (Fig. 1b), isoelectric focusing (IEF) of hemolysates (Fig. 1c), β –globin protein sequencing by mass spectrometry (Fig. 1d, e) and a decrease of 2 reduced thiols per hemoglobin tetramer (Fig. 1f and inset) demonstrate unequivocally that β 93cys was changed to alanine. No change in total glutathione levels (Fig. 1g) was observed suggesting no compensatory up–regulation of the other major thiol pool in RBC. Finally, replacement of β 93cys with Ala resulted in a modest, but significant increase in RBC oxygen affinity (Fig. 1h and inset) consistent with cell–free recombinant hemoglobin studies¹⁸.

Supplementary Table 1 shows hematological indices and urine concentrations in mice. Small increases in hemoglobin levels, red cell distribution widths, mean corpuscular volumes and mean corpuscular hemoglobin were observed in HbC93A compared to HbC93 mice. However, reticulocyte levels, which are the most sensitive indicator of anemia in mice, were equivalent in HbC93A and HbC93 mice. No differences in phenotypic evaluations (weight and visual inspection of whole organs) or systematic evaluation [hematoxylin and eosin stained tissues sections, (suppl table 1 and suppl Fig. 2–3)] of HbC93 and HbC93A mice were observed. Finally, embryonic development (suppl Fig. 2) and the size of litters derived from each group were not significantly different. These results suggest that the HbC93A mice develop normally.

To evaluate the role of β 93cys S–nitrosation in RBC–dependent modulation of NO–bioactivity, we first characterized basal nitric oxide metabolism in the circulation by measuring NO–metabolites nitrate, nitrite, S–nitrosothiols and C– or N–nitroso compounds (referred to as XNO) in the plasma and RBC. Total NO production (Fig. 2) in HbC93A and HbC93 mice is similar (nitrate levels were not different; $33.6 \pm 10.6 \mu\text{M}$ in HbC93 mice and $29.5 \pm 1.2 \mu\text{M}$ in HbC93A mice, $n=3$ per group). Interestingly, no differences in circulating NO–metabolites were observed except for plasma nitrite which was lower in HbC93A mice. A trend towards

lower whole blood nitrite levels was also observed. Suppl Fig. 4 shows lower nitrite levels are not associated with different levels of eNOS expression nor activity. Recent studies show that loss of the $\beta 93\text{cys}$ residue increases the nitrite reductase activity of deoxyhemoglobin^{19,20}, a result also observed with HbC93A RBC (not shown) and suggesting that this activity accounts for observed lower steady-state nitrite levels. Collectively, Fig. 2 suggests that loss of $\beta 93\text{cys}$ does not result in adaptive increases in endogenous NO formation. RBC levels of S-nitrosothiols (Fig. 2e) were not significantly different in HbC93A and HbC93 mice. Interestingly however, the distribution of S-nitrosothiols was altered. In HbC93 RBC, $78.2 \pm 14.3\%$ of the SNO's were on the high Mwt fraction consistent with a predominantly hemoglobin localization. In the HbC93A RBCs, however, high Mwt SNO's were undetectable. More than 99% of SNO's were in the low Mwt fraction, most likely S-nitrosoglutathione (GSNO) underscoring a dynamic transnitrosation equilibrium between SNO-Hb and low Mwt thiols (as previously indicated)^{8,21} which in the absence of the $\beta 93\text{cys}$ is shifted to GSNO. This conclusion is further supported by the absence of any effect of $\beta 93\text{cys}$ substitution on XNO distribution in RBC (Fig 1f). In summary, the data in Fig. 1–2 demonstrate no functionally significant phenotypic or biochemical differences between HbC93 and HbC93A mice, except for a relatively small (~20%) decrease in circulating nitrite levels and re-distribution of RBC SNO's from $\beta 93\text{cys}$ to a non-hemoglobin fraction.

To evaluate potential hemodynamic effects of the altered RBC SNO profile, mean arterial pressure (Fig. 3a and suppl Fig. 5a), systolic pressure (suppl Fig. 5b), diastolic pressure (suppl Fig. 5c), and heart rate (suppl Fig. 5d) were measured. No differences between HbC93 and HbC93A mice were observed suggesting that SNO-Hb is not essential for physiologic modulation of blood pressure.

Recent studies suggest that depletion of SNO-Hb leads to pulmonary hypertension¹⁶. However, no differences in right ventricular pressures between HbC93 and HbC93A mice were observed (Fig. 3b). Moreover, assessment of right ventricular hypertrophy (Fig. 3c), or pulmonary arterial wall thickness (Fig. 3d), which are diagnostic features for pulmonary hypertension, showed no differences. Collectively these data indicate that loss of $\beta 93\text{Cys}$ and associated loss of SNO-Hb does not adversely impact systemic nor pulmonary hemodynamics. These data do not rule out a role for other RBC SNO's²², but indicate that allosteric regulation of SNO-Hb bioactivity is not an essential component of physiologic mechanisms that control pulmonary blood flow.

Given the multiple studies documenting a critical role for SNO-Hb, we were surprised by a lack of phenotype in $\beta 93\text{cys}$ deficient mice under physiological conditions. Since hypoxia is a critical element in the SNO-Hb hypothesis, we first considered that a role for this residue may only be evident under conditions of hypoxic stress. To test this, HbC93 and HbC93A mice were exposed to two types of exercise stress that model acute (analogous to sprinting) and chronic (analogous to long-distance running) exercise resulting in varying degrees of hypoxic stress. Figs. 4a, b demonstrate that HbC93 and HbC93A mice perform equally well at both exercise regimes suggesting that SNO-Hb is not essential for global exercise capacity. Adaptive responses of the vasculature, such as differences in capillary densities, could compensate in the absence of SNO-Hb; however no changes in capillary density in the heart were observed (suppl Fig. 6).

Finally, since multiple mechanisms contribute to regulation of hypoxic blood flow *in vivo*, which could be up-regulated to compensate for loss of SNO-Hb bioactivity, we tested the ability of isolated RBCs to stimulate hypoxic vasodilation responses using rabbit pulmonary arteries in vessel bioassay experiments. Previous studies have shown that addition of RBCs under oxygenated (21% oxygen) conditions constricts vessels (consistent with scavenging of endogenous NO) whereas RBC addition to vessels pre-equilibrated at $\leq 1\%$ O₂ (tensions which

result in RBC deoxygenation) elicits a transient vasodilation response which is followed by vasoconstriction¹¹. These data were pivotal in the generation of the SNO-Hb hypothesis. Fig. 4c demonstrates that addition of RBCs from HbC93 mice reproduce these data, with vasoconstriction observed at 21% oxygen but a transient vasodilation followed by vasoconstriction observed at 0% oxygen. Fig. 4d illustrates similar effects with HbC93A RBCs; the hypoxic vasodilation response with HbC93A and HbC93 RBCs are essentially identical (Fig. 4e) demonstrating that the β 93Cys and hence SNO-Hb are not required for RBC-dependent hypoxic vasodilation. We have reported previously that under nitrite-free conditions, this dilation response, which has been ascribed to RBC SNO-Hb, is most likely due to ATP release from hypoxic RBCs¹⁹. In this model, ATP is released from hypoxic RBC, binds to endothelial purinergic receptors (P_{2Y}) and thereby stimulates NO production by eNOS resulting in vasodilation⁷. Consistent with ATP release as the mediator of vasodilation with HbC93 and HbC93A RBCs, hypoxic vasodilation was inhibited by >90% in the presence of the eNOS inhibitor, LNMMA (1 mM) (Fig. 4f). It should be pointed out that hypoxic vasodilation responses ascribed to SNO-Hb have also been stated to occur with denuded vessels^{15,23}. Denudation removes the endothelium, and hence eNOS, conceivably precluding ATP-dependent pathways. However, both HbC93 and HbC93A RBCs dilated denuded vessels to similar extents at low oxygen tensions (Suppl Fig. 7). Moreover, ATP also dilated denuded vessels (suppl. Fig 7). This response is most likely due to metabolism of ATP to adenosine, an endothelium independent vasodilator. A role for adenosine is indicated by significant inhibition of hypoxic RBC dependent dilation of denuded vessels by the adenosine receptor antagonist theophylline; this inhibition is equivalent with RBCs from HbC93 and HbC93A animals (suppl Fig. 7). We present this data i) to demonstrate that dilation of denuded vessels by hypoxic RBCs does not directly exclude ATP-dependent effects and that SNO-Hb is not required for vasodilation in this assay; and ii) to underscore the potential for SNO-Hb independent mechanisms for RBC-induced vasodilation. Further studies are required to determine how ATP-released from hypoxic RBC can be metabolized to adenosine and mediate dilation of denuded vessels.

In summary, our data demonstrate that i) β 93cys is not essential for normal development or physiology under basal or exercise-stress conditions, ii) lack of SNO-Hb does not result in pulmonary hypertension under normal conditions and iii) SNO-Hb is not required for RBCs to stimulate hypoxic vasodilation ex-vivo. Although it is possible that the use of a humanized mouse model may mask a physiological effect of β 93cys-deletion our studies strongly suggest that SNO-Hb does not play an essential role as a physiological regulator. We hypothesize that the conserved β 93Cys residue plays a functional role in diseases characterized by vascular oxidative or nitrosative stress²⁴⁻²⁶. The use of HbC93 and HbC93A mouse models should provide important insights into the role of β 93cys and SNO-Hb in these disorders.

Methods Summary

Detailed methods are provided in on-line supplementary material.

Production of knockin mice

The replacement of mouse alpha- and beta-globin genes with human alpha- and beta-globin genes was performed essentially as described²⁷. See supplementary data for knockin scheme. All studies using animals were performed according to institutionally (IACUC) approved protocols.

Red Blood Cell Thiol Measurements

RBC glutathione, hemoglobin total and surface accessible reduced thiol were measured as described in supplementary information.

Hematology

Hematology profiles were determined as previously described²⁷.

RBC–Hemoglobin Oxygen Affinity

Hemoglobin oxygen affinity in intact RBC was determined as previously described¹⁹.

Hemodynamics

Hemodynamic parameters were measured non-invasively using a specialized differential pressure transducer tail cuff (Kent Scientific). Right ventricular pressures and measurement of Fulton index to assess pulmonary hypertension were determined as previously described²⁸.

Vessel Bioassays

Vessel studies were performed using female rabbit pulmonary arteries precontracted with phenylephrine in Krebs Heineleit buffer as previously described¹⁹. Whole blood from either HbC93 or HbC93A mice was collected and the RBC pellet washed once with PBS. A 0.3% final Hct of either HbC93 or HbC93A RBC was then immediately added to the vessel baths perfused with either 21% or 0% oxygen. Vasodilatory effects were determined by measuring the change in tension and expressing this as a percent relaxation with respect to the maximal PE constriction. Vessel bioassay experiments were performed in decreased lighting to protect photo-labile RSNO.

Measurement of Nitric Oxide Metabolites

NO-metabolites were measured as previously described²⁹. For RBC measurements, samples were also passed through Sephadex G-25 gel-filtration column (pre-equilibrated with PBS + 10 μ M DTPA) to assess S-nitrosothiol distribution between high Mwt (>10 kDa, hemoglobin containing) and low Mwt (<10kDa) fractions.

Exercise Stress Tests

Male mice were exposed to either graded or endurance exercise tests on a treadmill (Exer6M, Columbus Instruments) and in each case time to fatigue recorded when mice could no longer maintain a running pace despite external stimuli.

LC-MS/MS of globin polypeptides

See supplementary information for details.

Capillary Density Determination

Capillary density in the heart was determined as previously described³⁰.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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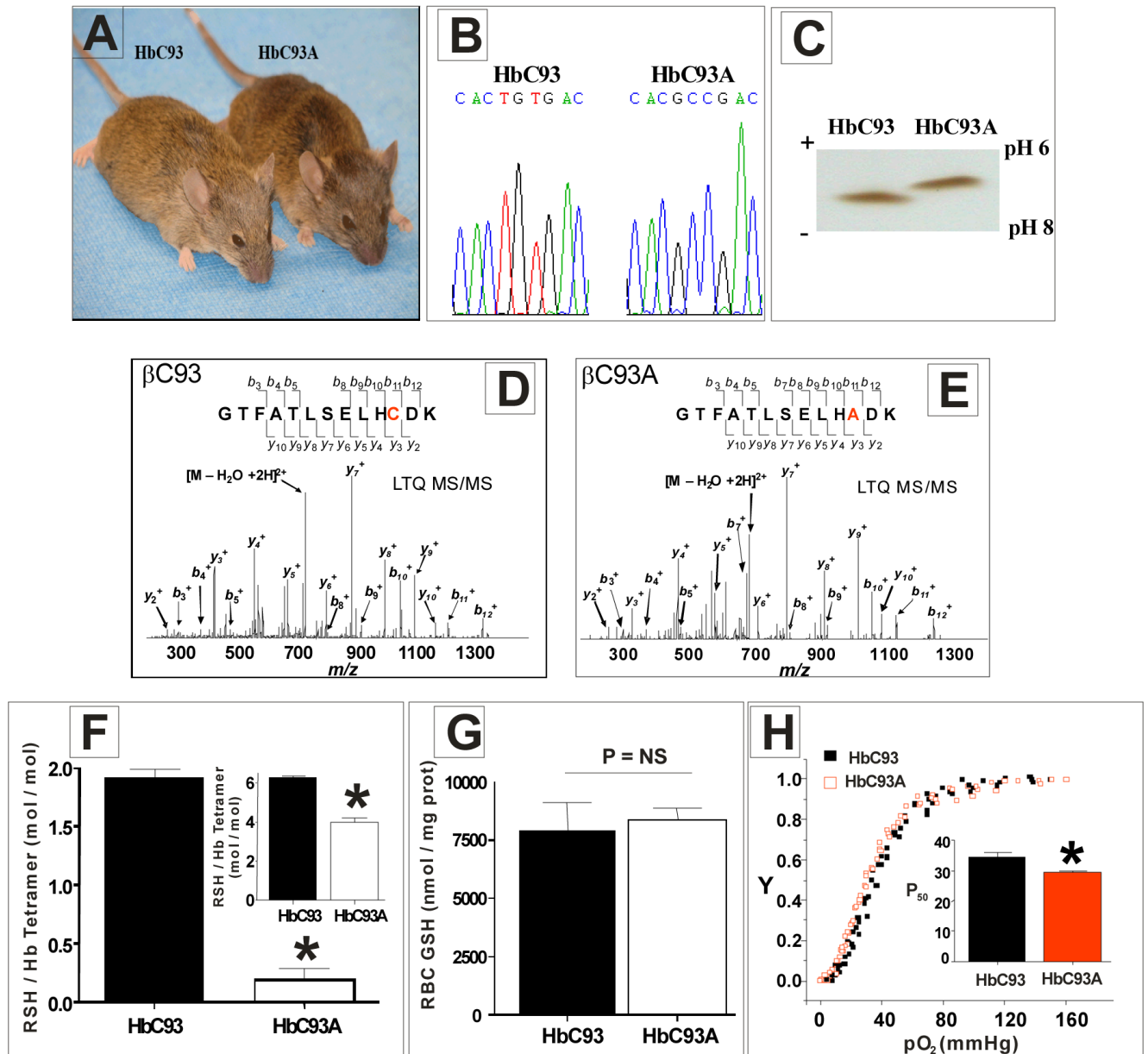


Figure 1. Genotypic, phenotypic and biochemical characterization of HbC93 and HbC93A mice
a) HbC93 and HbC93A knockin mice. The animals are phenotypically indistinguishable. **b)** Genomic DNA sequence analysis of the β -globin gene in HbC93 and HbC93A knockin mice. The animals are homozygous for human β C93 or β C93A genes. **c)** Isoelectric focusing gel analysis of hemolysates from HbC93 and HbC93A mice. The animals synthesize a single, human adult hemoglobin consistent with HbC93 and HbC93A, respectively. **d)** and **e)** Mass spectrometry analysis (LTQ MS/MS) of β C93 and β C93A polypeptides respectively. Peptide sequence analysis unequivocally demonstrates the C93A substitution. **f)** Hemoglobin was isolated from HbC93 and HbC93A RBC, and surface accessible and total reduced thiols (inset) measured as described in methods using the DTNB assay. Consistent with 2 β 93cys residues per hemoglobin tetramer, surface and total hemoglobin thiol decreased by 2 in HbC93A mice. Data show mean \pm SEM (n = 3–4), * P = < 0.0002 relative to HbC93 hemoglobin by unpaired

t-test. **g**) RBC concentrations of total GSH (reduced + oxidized) are not different between HbC93 and HbC93A RBC, Data shown mean \pm SEM, $P = 0.72$, by unpaired t-test. NS= not significant. **h**) Hemoglobin oxygen affinity was measured in intact HbC93 or HbC93A RBC suspensions (0.3% Hct) in PBS, 37°C. Oxygen binding curves represent cumulative data points obtained from RBC isolated from 4 mice per group. Inset shows P_{50} values determined by fitting the oxygen binding curves to the Hill Equation. HbC93A RBC have lower P_{50} (increased oxygen affinity) compared to HbC93 RBC. Data shown mean \pm SEM ($n = 4$), * $P = < 0.006$ by unpaired t-test.

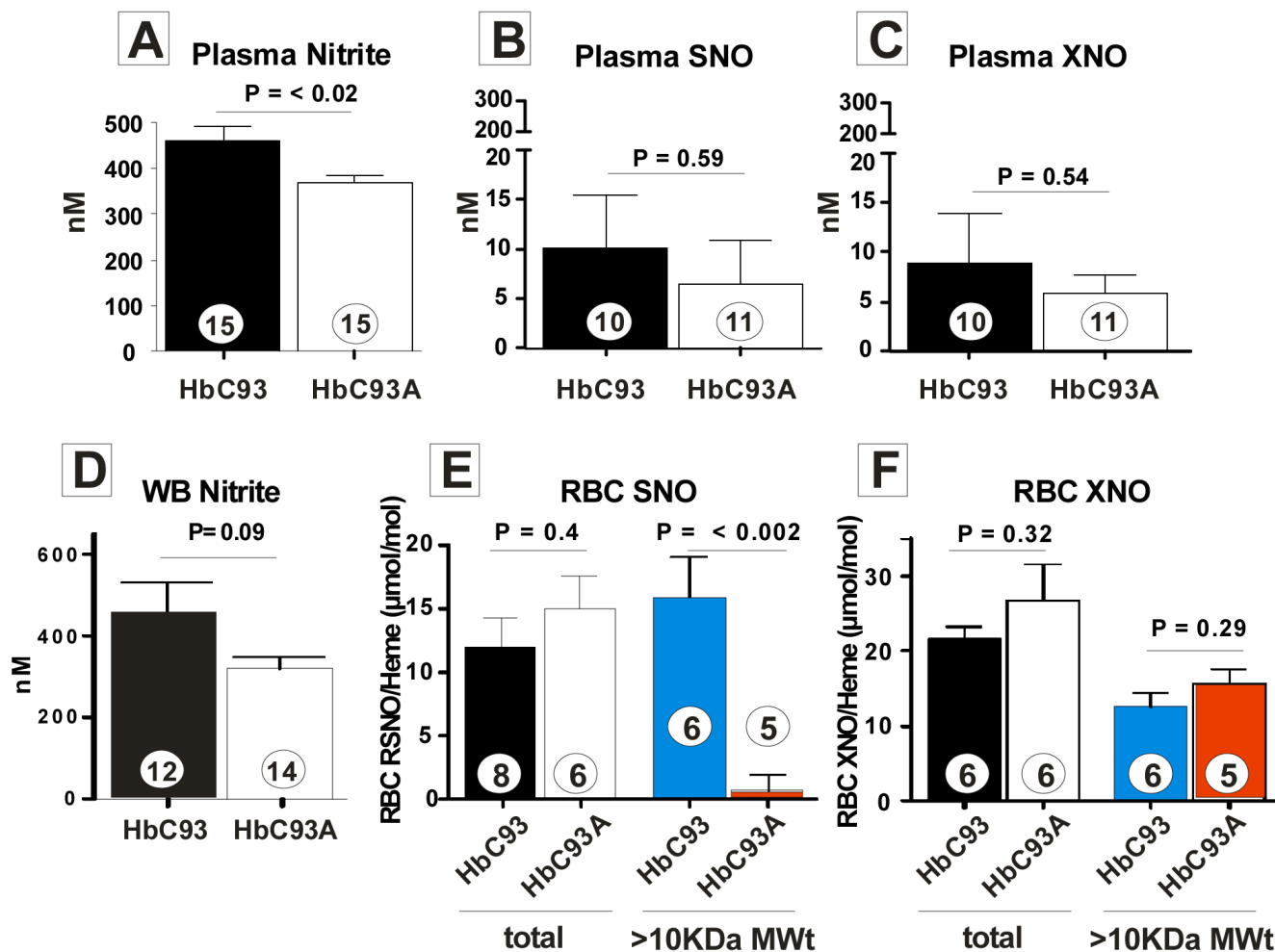


Figure 2. Circulating nitric oxide metabolite profiles in HbC93 and HbC93A mice

Panel A–F show different NO–metabolites in plasma, whole blood (WB) or RBC as indicated. Shown P–values were calculated by unpaired t–test with ‘n’ values indicated in each data bar and referring to number of mice. All values represent mean \pm SEM. For SNO and XNO in RBC, samples lyzed and collected in stabilization solutions were divided into two aliquots, one of which was analyzed for total RBC SNO/XNO and the other fractionated on a gel–filtration column (Sephadex, G–25, using PBS + DTPA (10 μ M) as the eluant). The high MWt fraction (> 10KDa, predominantly hemoglobin) was collected and SNO/XNO measured and normalized to heme. RBC SNO concentrations (in packed RBC) were 214.6 ± 37 nM and 147.9 ± 28.6 nM for HbC93A and HbC93 mice respectively, n=6). NO–metabolites were measured in plasma and RBC compartments as described in methods.

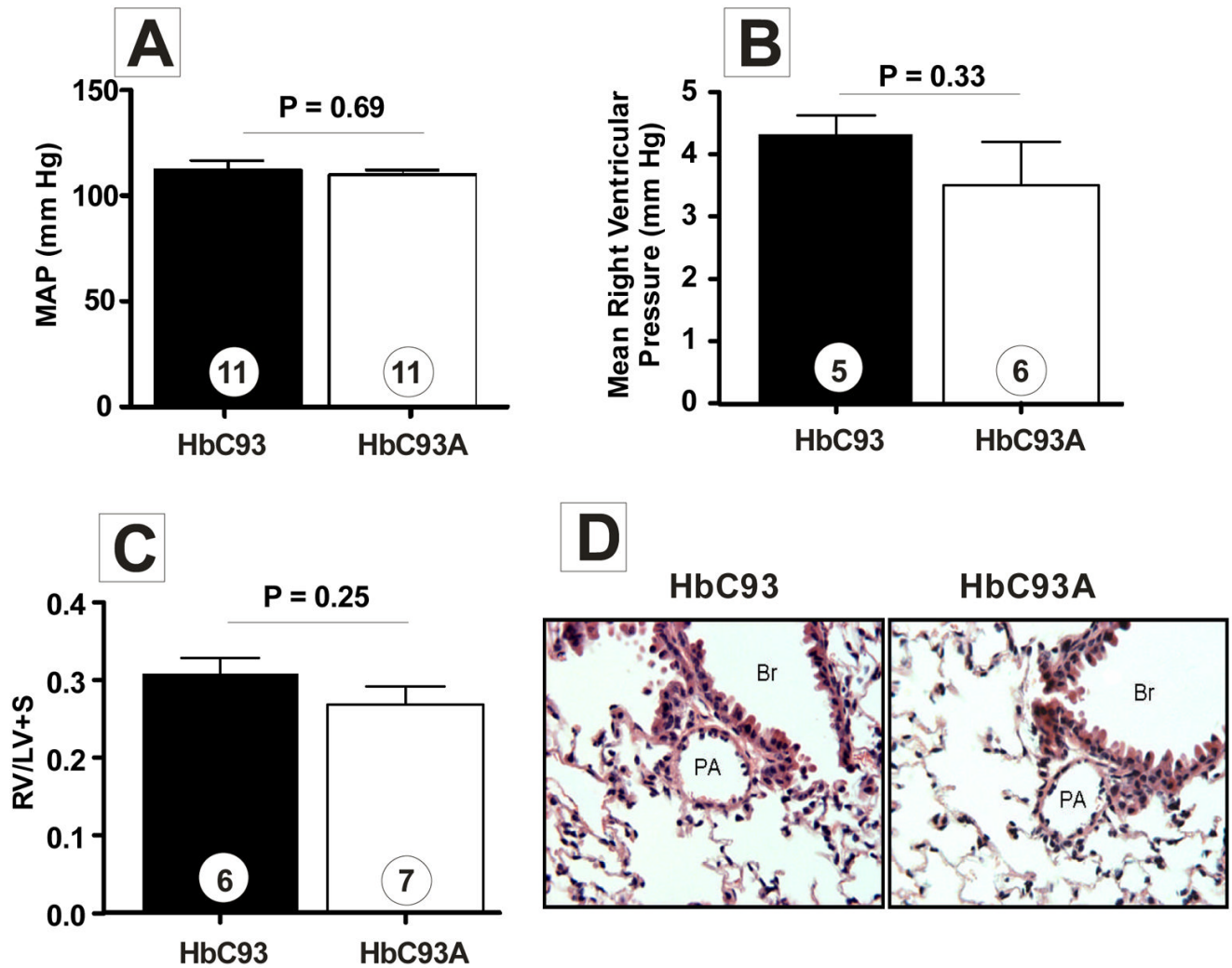


Figure 3. Loss of β 93Cys affects neither systemic nor pulmonary hemodynamics

A) Mean arterial blood pressure (MAP) was measured by tail-cuff on conscious mice. Note, similar MAP values were obtained in non-restrained mice via carotid artery catheters (suppl Fig B) shows mean right ventricular pressures in anesthetized mice. C) shows Fulton index (right ventricle (RV): left ventricle (LV) + septum (S) weight ratio) for assessing right ventricular hypertrophy secondary to pulmonary hypertension.. D) shows representative micrographs of H&E stained pulmonary arteries from HbC93 and HbC93A mice. No changes in vascular structure were observed (magnification = 40X). PA = pulmonary artery, Br = bronchus. Unpaired t-tests were used to evaluate significance with P-values and 'n' values shown on figure. In all cases data represent mean \pm SEM

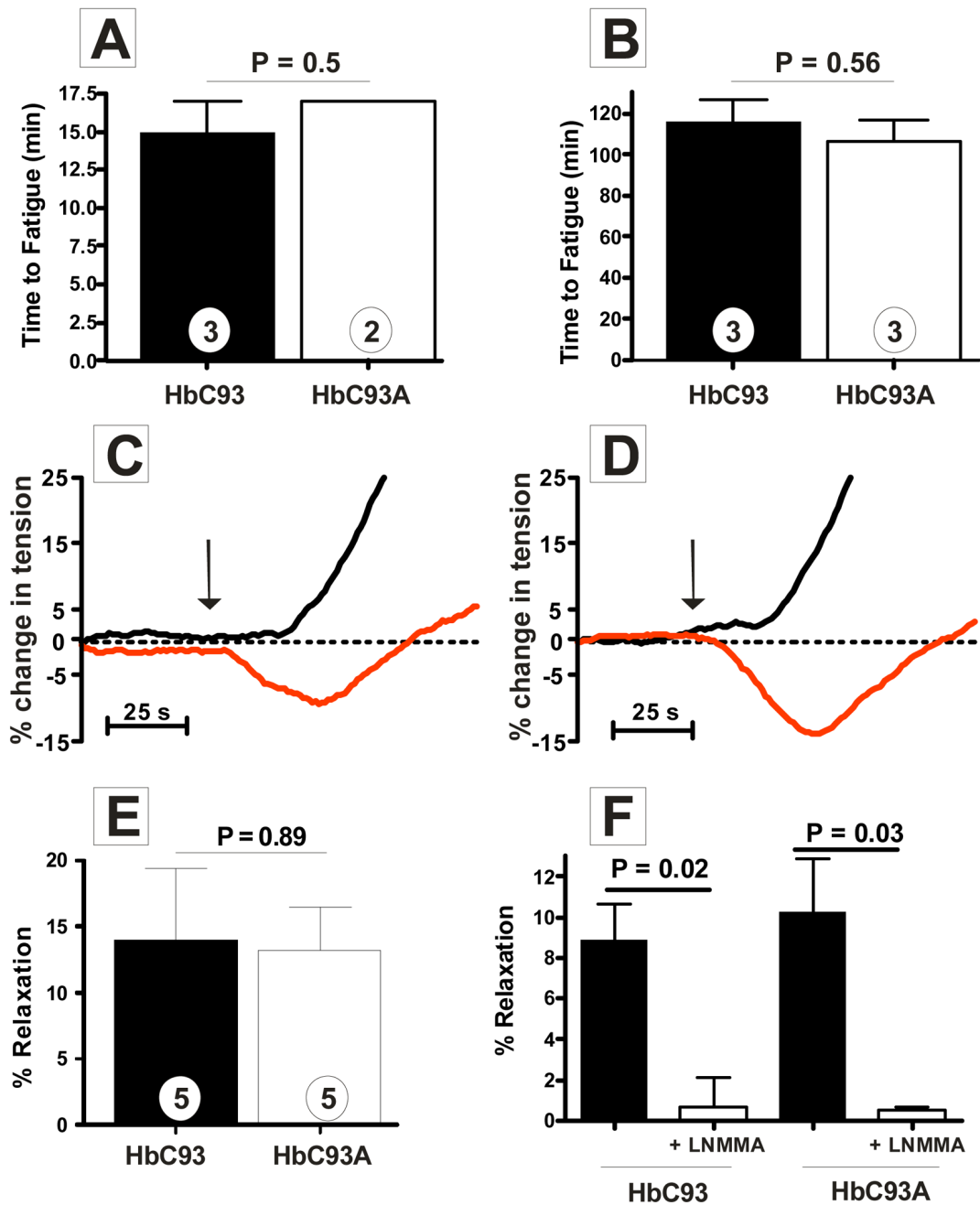


Figure 4. Effects of $\beta 93\text{Cys}$ on exercise tolerance and RBC dependent modulation of hypoxic vasodilation

A) Mice were exposed to a graded exercise test and time to fatigue measured. Mice were allowed an initial warm up for 5 min at 10m/min and then speed increased by 3 meters every 3 minutes to a maximum speed of 30m/min. B) Mice were exposed to an endurance exercise test and time to fatigue determined. Mice were allowed to warm up for 10 minutes at 10m/min and speed increased to 15m/min for 30 minutes. Thereafter speed was increased by 3 meters every 30 minutes to a final of 27m/min. For A) and B) time to fatigue was recorded when mice could no longer maintain a running pace despite external stimuli (see methods). P-values shown were calculated by unpaired t-test and data represent mean \pm SEM where shown with

indicated 'n' values. Panels C) and D) are representative traces showing changes in tension of isolated rabbit pulmonary arteries exposed to HbC93 or HbC93A RBC respectively at 21% oxygen (—) or 0% oxygen (—). Pulmonary arteries were equilibrated at each oxygen tension for 5 min then pre treated with indomethacin (5 μ M) and pre-contracted with PE (200nM). Upon reaching a stable contractile tone RBC were added for a final 0.3 Hct in the baths (indicated by arrow). RBC from either HbC93 or HbC93A mice were collected by cardiac puncture, washed once with air equilibrated PBS (2000 \times g, 90s, 22 $^{\circ}$ C) and then added to vessel bioassay chambers resulting in average times between blood collection and RBC addition of <5 mins. E) Average hypoxic vasodilation stimulated by either HbC93 or HbC93A RBC. F) Hypoxic vasodilation stimulated by HbC93 or HbC93A RBC (0.3% Hct) was measured in the absence or presence of LNMMA (1mM). Data show mean \pm SEM (n=3). P-values calculated by unpaired t-test.