

# Low Concentrations of Nitric Oxide Increase Oxygen Affinity of Sickle Erythrocytes In Vitro and In Vivo

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## Abstract

The hallmark of sickle cell disease (SCD) is the polymerization of deoxygenated sickle hemoglobin (HbS). In SCD patients, one strategy to reduce red blood cell (RBC) sickling is to increase HbS oxygen affinity. Our objective was to determine if low concentrations of nitric oxide (NO) gas would augment the oxygen affinity of RBCs containing homozygous HbS (SS). Blood containing normal adult hemoglobin (AA) or SS RBCs was incubated in vitro in the presence of varying concentrations of NO up to 80 ppm, and oxygen dissociation curves (ODCs) were measured. In addition, blood was obtained from three AA and nine SS volunteers, before and after breathing 80 ppm NO in air for 45 min, and the ODCs were measured. Exposure of SS RBCs to 80 ppm NO in vitro for 5 min or longer decreased the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen ( $P_{50}$ ), an average of 15% ( $4.8 \pm 1.7$  mmHg mean  $\pm$  SE;  $P < 0.001$ ). The increase in SS RBC oxygen affinity correlated with the NO concentration. The  $P_{50}$  of AA RBCs was unchanged ( $P > 0.1$ ) by 80 ppm NO. In SS volunteers breathing 80 ppm NO for 45 min, the  $P_{50}$  decreased ( $P < 0.001$ ) by  $4.6 \pm 2.0$  mmHg. 60 min after NO breathing was discontinued, the RBC  $P_{50}$  remained decreased in five of seven volunteers in whom the ODC was measured. There was no RBC  $P_{50}$  change ( $P > 0.1$ ) in AA volunteers breathing NO. Methemoglobin (Mhb) remained low in all subjects breathing NO (SS Mhb  $1.4 \pm 0.5\%$ ), and there was no correlation ( $r = 0.02$ ) between the reduction in  $P_{50}$  and the change in Mhb. Thus, low concentrations of NO augment the oxygen affinity of sickle erythrocytes in vitro and in vivo without significant Mhb production. These results suggest that low concentrations of NO gas may offer an attractive new therapeutic model for the treatment of SCD. (*J. Clin. Invest.* 1997; 100: 1193–1198.) Key words: antisickling agents •  $P_{50}$  • therapy • anemia

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## Introduction

The primary features of sickle cell disease (SCD)<sup>1</sup> include severe hemolytic anemia, frequent vasoocclusive episodes, and shortened longevity. SCD is caused by a single point mutation in the DNA encoding the sixth amino acid of the hemoglobin  $\beta$  chain. The mutation results in the replacement of a negatively charged amino acid, glutamine, with a neutral hydrophobic residue, valine. Upon deoxygenation, sickle hemoglobin (HbS) aggregates and produces a viscous gel composed of multistranded helical polymers, resulting in rigid and deformed red blood cells (RBCs). These RBCs have impaired ability to traverse the microcirculation, transiently or permanently blocking the microvasculature and decreasing oxygen supply to surrounding tissues. The resulting acute and chronic organ damage is a major cause of pain, morbidity, and mortality associated with SCD (1, 1b).

Therapeutic strategies for SCD are based on reducing HbS polymerization by increasing the cellular concentration of hemoglobin F (HbF), reducing the cellular concentration of HbS, or chemically modifying HbS. As shown in the Multicenter Sickle Hydroxyurea study, the increase in HbF induced by hydroxyurea (HU) therapy was associated with a very significant reduction in pain rates, acute chest crises, and transfusion requirements (2). Oral clotrimazole has been shown to reduce sickle cell dehydration in a short-term study in patients with SCD (3). Combination therapy using agents with different mechanisms of action is being considered for the treatment of SCD.

Another therapeutic approach is based on reducing HbS polymerization by increasing the affinity of HbS for oxygen. As measured by the hemoglobin oxygen dissociation curve (ODC), homozygous HbS (SS) erythrocytes have markedly reduced affinity for oxygen as compared to normal adult hemoglobin (AA) erythrocytes containing hemoglobin A (HbA). The reduction in oxygen affinity of sickle erythrocytes is due to an increase in intraerythrocytic 2,3-diphosphoglycerate (DPG) concentrations as compared to normal HbA erythrocytes, and to the presence of HbS polymers (4–6). This decreased RBC oxygen affinity is reflected in an increase in the partial pressure of oxygen at which hemoglobin is half-saturated with oxygen ( $P_{50}$ ). Compared to AA red cells, the tight (T) deoxyhemoglobin conformational state is favored over the relaxed (R)

1. Abbreviations used in this paper: AA, normal adult hemoglobin; DPG, diphosphoglycerate; HbS, sickle hemoglobin; HU, hydroxyurea; Mhb, methemoglobin; NO, nitric oxide; ODC, oxygen dissociation curve; oxyHb, oxyhemoglobin;  $P_{50}$ , partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen; R, relaxed; RBC, red blood cell; SCD, sickle cell disease; SpO<sub>2</sub>, pulse oximetry; SS, homozygous sickle hemoglobin; T, tight.

oxyhemoglobin (oxyHb) conformation at any given oxygen tension in SS red cells. Polymerization only occurs when HbS is in the deoxyhemoglobin conformation. Sunshine et al. suggested that therapeutically significant inhibition of intracellular HbS polymerization could be accomplished by increasing the oxygen affinity (e.g., reducing  $P_{50}$  by 4 mmHg) (7).

Modification of hemoglobin affinity for oxygen has been shown to increase survival under hypoxic conditions in a transgenic mouse model of SCD (8). Other investigators have demonstrated that increasing oxygen affinity of HbS by exposure to sodium cyanate or carbon monoxide (CO) reduces HbS RBC sickling in vitro (9, 10). However, these agents are too toxic for clinical use (11). High concentrations of NO have also been demonstrated to increase oxygen affinity in AA RBCs; however, significant methemoglobin (Mhb) was produced (12). The ability of low, nontoxic concentrations of NO gas to alter the oxygen affinity of SS erythrocytes has not been reported. In this study, the effect of low concentrations of NO on the oxygen affinity of erythrocytes with SS or AA was evaluated by measuring the ODC and  $P_{50}$  in vitro and in human subjects breathing low levels of NO. We report here that exposure of SS RBCs to low concentrations of NO gas in vitro and in vivo increases oxygen affinity without producing significant Mhb levels.

## Methods

**Subjects.** All protocols were approved by the Massachusetts General Hospital Subcommittee on Human Studies, and all subjects gave signed informed consent. Three male AA volunteers (ages 25–40 yr) and nine (six males and three females) clinically stable SS volunteers (ages 18–36 yr) were studied. Three SS volunteers were receiving HU therapy and had been taking HU for > 6 mo.

**ODC determinations.** 50  $\mu$ l whole blood was obtained by venipuncture from AA or SS volunteers and diluted with 4 ml phosphate buffer, 10  $\mu$ l antifoam solution, and 20  $\mu$ l 20% albumin. The blood samples were desaturated by exposure to 100% nitrogen ( $N_2$ ) gas and then reoxygenated with air using a Hemox analyzer (TCS Medical Products Co., Huntingdon Valley, PA) to measure the ODC, as reported previously (13).  $P_{50}$  was determined as the partial pressure of oxygen at 50% oxyHb saturation. To ensure the accuracy of repeated measures over time using SS RBCs, blood samples obtained from three SS volunteers had repeated ODC measurements made at 0, 15, 30, and 60 min without NO gas exposure, and changes in  $P_{50}$  were not detected.

**In vitro NO exposure of SS and AA RBCs.** NO gas was added using a rotameter during RBC reoxygenation and displaced equal volumes of  $N_2$ . Concentrations of NO were continuously monitored using an electrochemical analyzer (model SAAN TM-100; Taiyo Sanso, Tokyo, Japan), which was frequently calibrated by an NO chemiluminescence analyzer (model CLD-700 AL; ECO-Physics, Inc., Ann Arbor, MI). For all samples, ODCs were measured first using air to determine a baseline. RBCs were then exposed to air with 10, 40, or 80 ppm NO for 1–60 min, and ODCs were repeated. Mhb levels were measured before and after exposure to NO using a CO-Oximeter (model 270; Ciba Corning, Medfield, MA).

**NO inhalation in SS and AA volunteers.** Three normal and nine SCD volunteers were studied. One SS volunteer was studied twice, with 1 mo between studies. Blood pressure, electrocardiogram, respiratory and heart rates, and pulse oximetry ( $SpO_2$ ) were monitored continuously. Subjects breathed air and then 80 ppm NO in air via a nonbreathing circuit for 45 min. Venous blood was sampled before and immediately after NO breathing. The normal subjects and seven SCD patients had an additional blood sample drawn 1 h after NO breathing. ODCs were measured ex vivo, as described above.

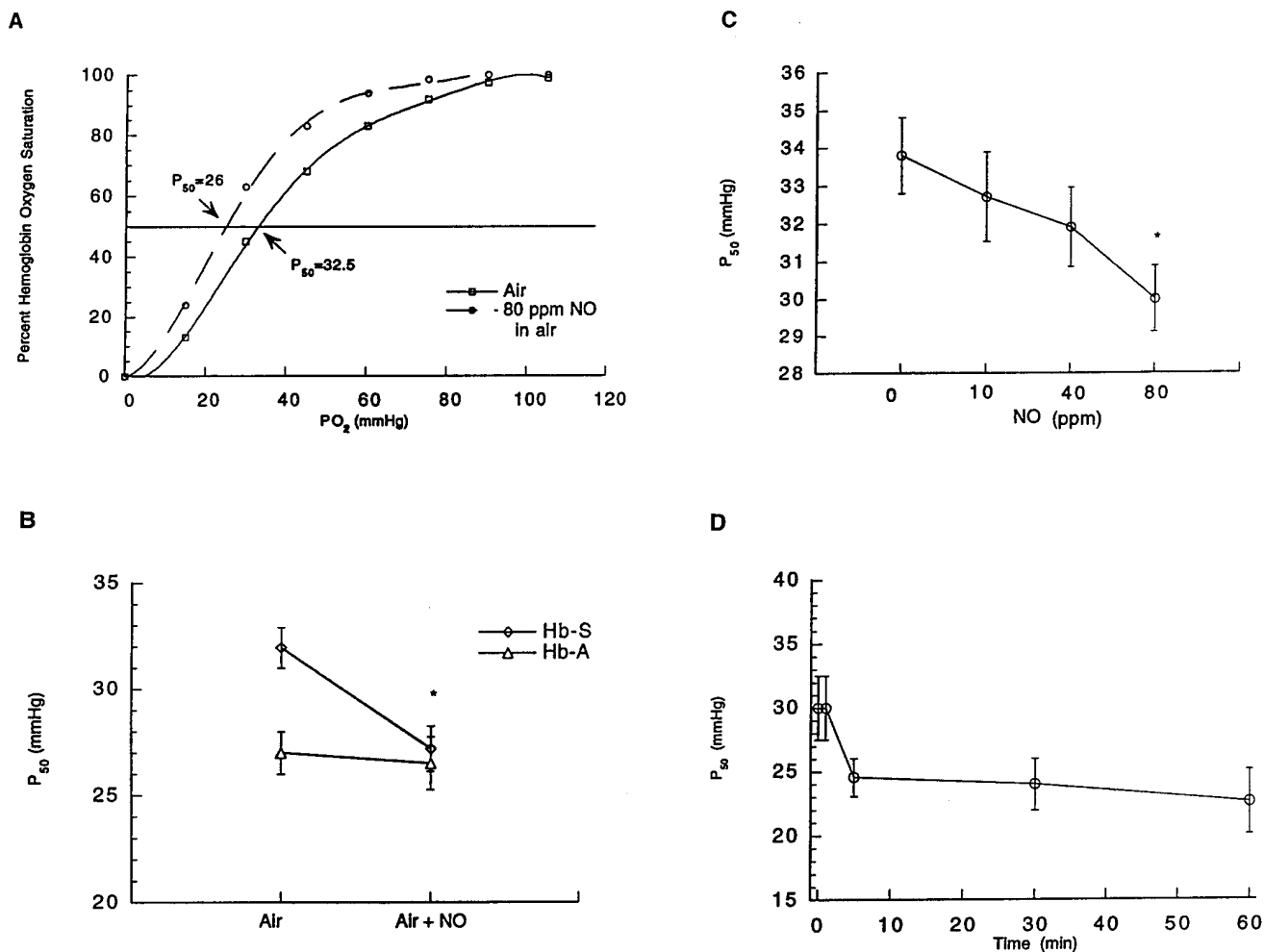
In addition, red cell ATP and 2,3-DPG concentrations were determined as reported previously (14). Mhb was measured using a CO-Oximeter, as described above. Venous pH and blood gases were measured using a pH/blood gas analyzer (model 170; Ciba Corning). In two sickle cell volunteers (SS volunteers 9 and 10) and two HbA volunteers, we performed Mhb analysis by both the CO-Oximeter and spectrophotometrically, using a spectrophotometer (Cary 2000; Varian Corp., Sugarland, TX). This instrument did not show any production of Mhb at the absorption peak of 630 nm in blood of SS volunteers after NO therapy, even though the CO-Oximeter showed an increase in Mhb. Therefore, this commercial analyzer appears to overestimate the value of Mhb in NO-modified sickle erythrocytes.

**Spectrophotometry.** Whole blood samples taken before and after NO breathing were immediately frozen at  $-80^\circ\text{C}$  and transported on dry ice to Northeastern University (Dr. James Manning's laboratory), where they were stored at  $-80^\circ\text{C}$  until spectral analysis. Storage in this manner does not affect their integrity, since control studies showed that storage at this temperature for months does not cause formation of Mhb from oxyHb. Before analysis, the samples were thawed and centrifuged at 2,000 g. The clear supernatant was then analyzed in two modes in a spectrophotometer (Cary 2000; Varian Corp.). This instrument is well-suited for this purpose because of its high quality optics and ability to measure absorbance values up to 4.0 U with no deviation from linearity. In the first mode, the spectral bands at 577, 560, and 415 nm were measured, and their ratios to one another were calculated. For all samples (obtained from HbA and HbS volunteers before and after NO breathing), the spectra indicated only oxyHb. Nevertheless, this is an indirect manner of measuring Mhb, which has a separate absorbance band at 630 nm, but an extinction coefficient only one-fifth that of oxyHb at 577 nm, making its determination impossible from the first spectra. Therefore, it was necessary to use relatively concentrated hemoglobin samples (easily done in the Cary 2200) in which all other absorbance bands were off-scale, but any Mhb, if present, would be observed at 630 nm. No Mhb was observed in any of the blood samples. An upper limit of 0.1% was estimated by determining what could have been observed at 630 nm (if present) with the off-scale values of the other wavelengths. Hence, using two criteria on the Cary 2200 (direct and indirect), no Mhb was measured in any of the blood samples.

**Statistical analysis.** Data are expressed as mean  $\pm$  SEM, except where indicated. Both paired and unpaired Student's *t* test were used with a *P* value < 0.05 indicating statistical significance. All tests were two-sided. Correlations were evaluated by computing the Pearson correlation coefficient.

## Results

**In vitro NO exposure to SS and AA RBCs.** To determine the effect of low concentrations of NO gas on SS RBC oxygen affinity, blood from SS volunteers was incubated with varying concentrations of NO, and ODCs were measured. Exposure of SS RBCs to 80 ppm NO gas in air for 15 min increased oxygen affinity, producing a significant shift (towards normal) of the ODC (Fig. 1 A). The shape of the ODC curve during NO exposure was maintained, suggesting that the cooperativity of oxygen binding was preserved. In RBCs containing SS, exposure to NO for 15 min decreased ( $P < 0.001$ ) the  $P_{50}$  an average of 15% ( $4.8 \pm 1.7$  mmHg; Fig. 1 B). The dose of NO administered was directly proportional to the increase in SS RBC oxygen affinity (Fig. 1 C). The effect of NO exposure on  $P_{50}$  was dependent upon the duration of exposure, with 5 min producing a significant reduction (Fig. 1 D). When SS RBCs were exposed to 80 ppm NO for 15 min and then exposed to air without NO, the reduction in  $P_{50}$  persisted for at least 2 h (data not shown). Exposure to 80 ppm NO for 15 min did not alter the oxygen affinity of normal RBCs containing AA (Fig. 1 B). In



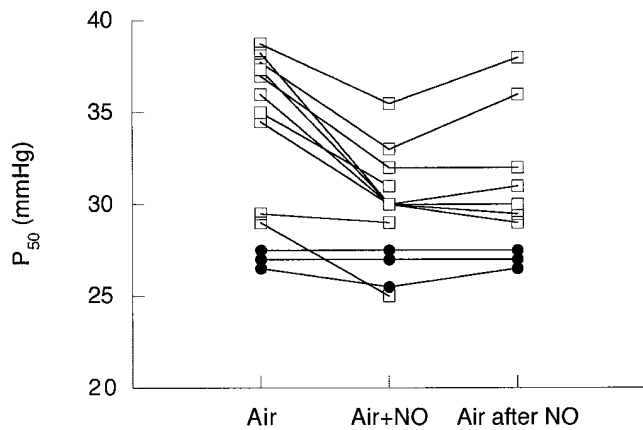
**Figure 1.** In vitro exposure to low concentrations of NO gas increased SS RBC oxygen affinity. (A) Representative SS RBC ODC without (□) and with (○) 15 min of exposure to 80 ppm NO in air. Exposure to NO shifted the SS RBC ODC to the left, and the sigmoid shape was maintained. (B) The mean value ( $\pm$ SE) RBC P<sub>50</sub> of blood samples SS (◇,  $n = 10$ ) and AA (△,  $n = 3$ ) were measured after exposure to air or air with 80 ppm NO for 15 min. NO exposure decreased the SS RBC P<sub>50</sub> by 15% or 5 mmHg ( $*P < 0.001$ ), as compared to the ODC derived without NO exposure. There was no difference ( $P > 0.05$ ) in the AA RBC P<sub>50</sub> with or without NO. (C) The SS RBC P<sub>50</sub> ( $\pm$ SE) of SS volunteers ( $n = 3$ ) exposed to air or air containing concentrations of 10, 40, or 80 ppm NO for 15 min. There was a reduction of SS RBC P<sub>50</sub> at each dose; however, only the 80 ppm NO dose was significant ( $*P < 0.05$ ). (D) The mean RBC P<sub>50</sub> using blood from SS volunteers ( $n = 3$ ) exposed to air or air with 80 ppm NO added for periods of 1, 5, 30, and 60 min. No change in P<sub>50</sub> was noted after 1 min. However, a reduction in the SS RBC P<sub>50</sub> was noted after 5 min, and this was unchanged after longer exposures to NO. RBC Mhb levels measured with the Corning CO-Oximeter were low ( $< 3\%$ ) after 60 min of 80 ppm NO exposure.

these experiments, exposure of RBCs containing SS or AA to 80 ppm NO for up to 60 min produced low Mhb levels ( $< 3\%$  by CO-Oximeter). These results suggest that in vitro, the oxygen affinity of SS erythrocytes is uniquely sensitive to low concentrations of NO.

**NO inhalation in SS and AA volunteers.** To determine whether low concentrations of NO could alter HbS in vivo, blood P<sub>50</sub> from AA and SS volunteers was measured before and after breathing 80 ppm NO in air for 45 min. In SS volunteers breathing 80 ppm NO, the RBC P<sub>50</sub> was decreased ( $P < 0.001$ ), with an average reduction in RBC P<sub>50</sub> of  $4.6 \pm 2$  mmHg (Fig. 2 and Table I). In contrast, the RBC P<sub>50</sub> did not change ( $\leq 1$  mmHg;  $P = \text{NS}$ ) in the AA volunteers breathing NO. One SS volunteer was studied twice with 1 mo between studies and demonstrated a significant reduction in P<sub>50</sub> on both occasions. In seven SS volunteers, the ODC was measured 1 h after

NO inhalation was discontinued. In five of the seven, the RBC P<sub>50</sub> remained decreased, suggesting that the effect of NO on the oxygen affinity of SS RBCs may persist after NO is discontinued (Fig. 2 and Table I).

In all subjects breathing 80 ppm NO in air for 45 min, the RBC ATP and 2,3-DPG concentrations did not change. The blood pressure, respiratory and heart rates, SpO<sub>2</sub>, venous blood pH, and electrocardiogram were unchanged during NO breathing. Volunteers with SS red cells had a higher baseline Mhb ( $0.5 \pm 0.2\%$ ) compared to those with AA red cells ( $0.1 \pm 0.1\%$ ). Exposure to NO led to a small but significant increase in Mhb levels in both SS ( $1.4 \pm 0.7\%$ ) and AA ( $0.7 \pm 0.1\%$ ) volunteers, with a return toward baseline values at 60 min after NO exposure ( $0.6 \pm 0.3$  and  $0.2 \pm 0.1\%$  for SS and AA volunteers, respectively). There was no correlation between the increase in Mhb levels and the decrease in P<sub>50</sub> values



**Figure 2.** Inhaled NO increased RBC oxygen affinity in SS volunteers. The average reduction of the RBC  $P_{50}$  of 10 studies with nine stable SS volunteers ( $\square$ ) was  $\sim 5$  mmHg (range 3–8 mmHg;  $P < 0.001$ ) after 80 ppm NO breathing for 45 min. In one SS volunteer, the RBC  $P_{50}$  did not change. Normal volunteers ( $\bullet$ ) had no change ( $\leq 1$  mmHg) in the AA RBC  $P_{50}$  during NO breathing. Blood samples were taken from three AA and seven SS volunteers while breathing air 1 h after NO breathing. Five of seven SS volunteers maintained the RBC  $P_{50}$  reduction for at least 1 h. In all subjects, erythrocytic 2,3-DPG and ATP levels, venous pH, and blood gas tensions did not change after 45 min of NO breathing. In all subjects, there were no clinical side effects noted. The mean Mhb levels after 45 min of NO breathing were low (see Table I) for SS RBCs and returned to baseline after 60 min, though NO effects persisted.

(Fig. 3,  $n = 10$ ,  $r = 0.02$ ). In fact, the shift in  $P_{50}$  persisted at 60 min after NO exposure in five of seven SS volunteers tested, while the Mhb levels had returned to baseline values. In addition, in SS volunteers 9 and 10, Mhb levels were measured with spectrophotometric analysis. These two patients had among the highest changes in Mhb levels after NO treatment. After 45 min of NO treatment, there was no Mhb detectable at 630 nm.

## Discussion

The most important finding of this study is that hemoglobin oxygen affinity increases when SS erythrocytes are exposed to low concentrations of NO. This effect was observed when RBCs were exposed to NO in vitro or during NO inhalation in vivo. Exposure to NO did not produce clinically significant Mhb levels. Increased SS RBC oxygen affinity was observed within 5 min of NO exposure in vitro, and the increase persisted for 2 h. In five of seven SS volunteers in whom it was measured, the RBC  $P_{50}$  remained decreased at least 60 min after NO breathing was discontinued. In contrast, AA RBC oxygen affinity was unaffected by exposure to low concentrations of NO, either in vitro or in vivo.

The mechanisms by which low concentrations of NO augment the oxygen affinity of SS erythrocytes but not AA erythrocytes are unknown. The reaction between NO and the heme moiety of hemoglobin has been studied in great detail using extremely high NO concentrations (up to 100%) (12, 14, 15). However, there is no information available on the effects of

**Table I.** Effects of Inhaled NO in SS and AA Volunteers

Trial	Age	Sex	Transfusion	HU	Crisis per yr	Hb (g/dl)	SpO <sub>2</sub>	P <sub>50</sub> (mmHg)			Mhb%		
								Baseline	Inhaled NO	Post NO	Baseline	Inhaled NO	Post NO
<b>SS Volunteers</b>													
1	27	M	Yes	No	1	6.5	97%	38.5	35.5	38	0.5	1.1	0.7
2	32	M	No	No	2	6.9	96%	37	32	32	0.3	2.7	0.4
3	33	M	No	No	1	10.1	97%	35	30	30	0.8	1.3	1.3
4	30	M	Yes	No	8	8.2	99%	36.5	30	29	0.3	1.1	0.5
5	27	F	No	Yes	12	6	94%	38	30	31	0.3	1.2	0.1
6	36	M	No	No	3	6.1	93%	29	29	x	0.6	1.2	0.8
7	30	M	Yes	No	14	6.1	90%	29	25	x	0.5	1.1	0.6
8	18	F	No	Yes	10	6.9	97%	36.5	33	36	0.3	1.5	0.7
9	28	F	No	Yes	9	6	98%	37	30	30	0.9	1.5	0.5
*10	27	F	Yes	Yes	12	7.3	95%	35	31	x	0.9	3.2	0.5
<b>AA Volunteers</b>													
1	25	M	NA	NA	NA	13.2	100	27	27	27	0.1	0.8	0.2
2	40	M	NA	NA	NA	12.5	100	26.5	25.5	26.5	0.1	0.8	0.3
3	37	M	NA	NA	NA	13	100	27	27	27	0.2	0.5	0.2

Nine SS volunteers were studied (\*SS volunteer data 5 and 10 are from the same patient with 1 mo between studies and after a blood transfusion). Transfusion is yes if blood was received within 1 mo of study date. HU was not an exclusion to our study. SpO<sub>2</sub> was monitored continuously during the study. Crisis over the past 12 mo is shown as well as total hemoglobin (Hb) level at time of study. The average reduction of the RBC  $P_{50}$  of 10 studies with nine stable SS volunteers was  $\sim 5$  mmHg (range 3–8 mmHg;  $P < 0.001$ ) after 80 ppm NO breathing for 45 min. In one SS volunteer, the RBC  $P_{50}$  did not change. Normal volunteers had no change ( $\leq 1$  mmHg) in the AA RBC  $P_{50}$  during NO breathing. Blood samples were taken from three AA and seven SS volunteers while breathing air 1 h after NO breathing had been discontinued. Five of seven SS volunteers maintained the RBC  $P_{50}$  reduction for at least 1 h. In all subjects, erythrocytic 2,3-DPG and ATP levels, venous pH, and blood gas tensions did not change after 45 min of NO breathing. In all subjects, there were no clinical side effects noted. The mean Mhb levels (CO-Oximeter) after 45 min of NO breathing were low ( $1.4 \pm 0.5\%$ ) for SS RBCs and returned to baseline after 60 min, though NO effects persisted in five of seven patients evaluated.

**Changes from baseline:  
MHb vs. P50**

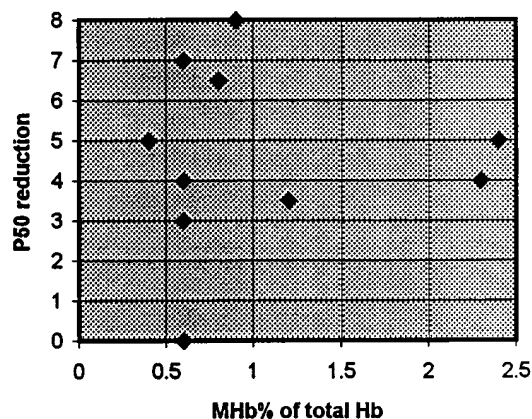


Figure 3. Change in  $P_{50}$  versus change in Mhb levels (CO-Oximeter) induced by inhaled NO in SS volunteers. Change in  $P_{50}$  was calculated from the difference between the value obtained after 45 min of breathing 80 ppm of NO in air and the baseline value for each of the SS volunteers. Mhb levels were measured with the CO-Oximeter after 45 min of breathing 80 ppm of NO in air, and expressed as percentage of the total Hb. No correlation could be established between these two parameters, MHb and  $P_{50}$  ( $r = 0.024$ ).

low doses of NO (80 ppm = 0.008%) on sickle erythrocytes. Breathing low concentrations of NO has been used safely to dilate the pulmonary vasculature in various pulmonary disorders, such as adult respiratory distress syndrome and pulmonary hypertension (16, 17). NO binds avidly to the heme moiety in hemoglobin and releases more slowly than oxygen (18, 19). Therefore, NO may increase the tendency for the HbS conformation to be maintained in the R (high affinity) state. Since only deoxyhemoglobin S (i.e., T state HbS) polymerizes, any experimental or therapeutic intervention that favors the R over the T state may result in a reduction in HbS polymerization. NO modification of HbS may reduce polymerization and thereby explain the increase of oxygen affinity in SS red cells while having no effect on oxygen affinity of AA red cells. By comparison, potassium cyanate modifies the oxygen affinity of both SS and AA red cells to the same extent *in vitro* (9). Therefore, the selective effect of NO on SS RBCs indicates a different mechanism (e.g., as a polymerization inhibitor for HbS, see Fig. 1 B), than an oxygen-based HbS modifier like potassium cyanate.

Another possibility is that NO increases erythrocyte Mhb levels, thereby altering oxygen affinity (20–22). However, since our *in vitro* and *in vivo* studies recorded only very low levels of Mhb in both AA and SS RBCs, it is unlikely that Mhb contributed to the selective increase in HbS RBC oxygen affinity. Moreover, we found no correlation between Mhb and  $P_{50}$  changes (Fig. 3), and the decrease in  $P_{50}$  persisted 60 min after exposure to NO, while the Mhb levels had returned to baseline values. Another theoretical concern would be the generation of additional hemoglobin oxidation products during NO breathing. Detailed spectrophotometric analysis from two SS volunteers after NO breathing failed to show any increase in Mhb or the production of abnormal hemoglobin or any additional hemoglobin oxidation products. However, additional studies are needed to evaluate this issue carefully.

NO can also affect other portions of the hemoglobin molecule by forming an adduct. Moriguchi et al. showed that the amino-terminal valine and possibly other amino groups of HbA within the 2,3-DPG cleft are modified by NO exposure, becoming more electronegative (23). HbS carries a Glu → Val substitution at  $\beta 6$ , making HbS less negative than HbA. Since this site determines HbS polymerization, NO-mediated modification might reduce intracellular polymerization, thereby increasing HbS oxygen affinity.

Thiol groups (such as  $\beta 93$  Cys) also interact with NO, producing S-nitrosothiols which may play a role in the control of vascular tone (24). Interestingly, Hb Okazaki (a  $\beta 93$  Cys-Arg variant) demonstrates increased oxygen affinity (25). It is possible that the oxygen affinity of S-nitrosothiol HbS behaves like that of Hb Okazaki. Moreover,  $\beta 93$  Cys plays a crucial role in both hemoglobin oxygen affinity and HbS polymerization: when this residue is reacted with a thiol reagent, a significant reduction in both  $P_{50}$  and HbS polymer formation is observed (26, 27).

We found no increase in oxygen affinity of AA RBCs after exposure to 80 ppm NO gas. However, Kon et al. have shown that high concentrations of NO can increase oxygen affinity in HbA RBCs (12). Of note, Briehl and Salhany (18) observed that high concentrations (100%) of NO in the presence of inositol hexaphosphate promoted the gelation of HbS *in vitro*, and suggested that NO induces the switch of HbS from the R to the T conformational state. Our data show that low concentrations of NO (80 ppm = 0.008% NO) do not promote an R to T switch. This apparent discrepancy may be related to the very low concentrations of NO that we studied.

To assess the ability of NO to increase HbS oxygen affinity *in vivo*, we measured the ODCs in blood from SS volunteers before and after NO breathing. We chose this approach for the administration of NO because RBCs are efficiently exposed to NO gas as they transit the lungs, and because inhaled NO does not produce systemic vasodilation (16, 28, 29). Our *in vitro* analysis suggested NO gas could be safely administered to SCD patients without detrimental effects on SS RBCs. In contrast, NO donor compounds would be expected to decrease systemic perfusion pressure and could aggravate the occlusive phenomena associated with SCD. In this study, nine SS volunteers breathed 80 ppm NO for 45 min without significant methemoglobinemia, systemic hypotension, or any other adverse effects. Moreover, measurements of ODCs *ex vivo* suggested that NO inhalation rapidly and markedly increases SS RBC oxygen affinity, an effect which persisted for at least 1 h after NO breathing in five of seven SS volunteers studied. However, a theoretical concern of NO therapy involves nonuniform modification of HbS within the RBC, where unmodified HbS may unload more oxygen, suggesting that reaching a critical concentration of intracellular NO-modified HbS could be important. No study yet has shown that nonuniform HbS modification has enhanced RBC sickling either *in vitro* or *in vivo*. Another concern for NO therapy is the possibility of a reduction in oxygen carrying capacity. However, this is unlikely to be clinically significant, since we did not observe a reduction in oxygen saturation during NO breathing, and neither did two large clinical trials using NO therapy in hypoxic patients (30, 31).

Another theoretical concern for the increase in oxygen affinity with NO would be an increase in hematocrit or blood viscosity. This could partially negate the benefit of the increased oxygen affinity. However, there are no prior studies

for comparison. We did not evaluate the oxygen affinity beyond 1 h after NO breathing had stopped. Therefore, we do not know the duration of NO's effect on HbS RBCs beyond 1 h in vivo. Additional studies are needed in the mouse model and in patients to determine effectively the duration of NO's effect and its long-term outcome.

In summary, our results demonstrate that inhaling low concentrations of NO gas increases the oxygen affinity of SS RBCs in vitro and in vivo. Low concentrations of NO did not alter oxygen affinity of AA RBCs, suggesting the effect of NO was selective for SS RBCs. Similar effects of NO on SS RBC oxygen affinity were observed in SS volunteers breathing NO gas, and were not associated with significant Mhb levels, changes in RBC 2,3-DPG, ATP, or systemic hypotension. Increases in oxygen affinity were also found in some of our SS volunteers receiving HU therapy, suggesting combination therapy is possible. Our studies were conducted over short time periods, and additional studies are needed to determine the long-term effects of NO therapy in patients with SCD. However, because interventions designed to increase SS erythrocyte oxygen affinity decrease RBC sickling, our results suggest that breathing low concentrations of NO gas may represent a novel therapeutic approach to the treatment of SCD.

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