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Nitric oxide reduces sickle hemoglobin polymerization: Potential role of nitric oxide-induced charge alteration in depolymerization

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Abstract

We previously demonstrated that inhaling nitric oxide (NO) increases the oxygen affinity of sickle red blood cells (RBCs) in patients with sickle cell disease (SCD). Our recent studies found that NO lowered the P_{50} values of sickle hemoglobin (HbS) hemolysates but did not increase methemoglobin (metHb) levels, supporting the role of NO, but not metHb, in the oxygen affinity of HbS. Here we examine the mechanism by which NO increases HbS oxygen affinity. Because anti-sickling agents increase sickle RBC oxygen affinity, we first determined whether NO exhibits anti-sickling properties. The viscosity of HbS hemolysates, measured by falling ball assays, increased upon deoxygenation; NO treatment reduced the increment. Multiphoton microscopic analyses showed smaller HbS polymers in deoxygenated sickle RBCs and HbS hemolysates exposed to NO. These results suggest that NO inhibits HbS polymer formation and has anti-sickling properties. Furthermore, we found that HbS treated with NO exhibits an isoelectric point similar to that of HbA, suggesting that NO alters the electric charge of HbS. NO–HbS adducts had the same elution time as HbA upon high performance liquid chromatography analysis. This study demonstrates that NO may disrupt HbS polymers by abolishing the excess positive charge of HbS, resulting in increased oxygen affinity.

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Disclosure

The Massachusetts General Hospital has received a patent (#5885,621) for the treatment of hemoglobinopathy with NO and has licensed INO-Therapeutics, Inc. Drs. C. Alvin Head and Warren M. Zapol are the inventors of the patent.

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Keywords

Sickle cell disease; Nitric oxide; Oxygen affinity; Anti-sickling; Polymer formation

Introduction

Sickle cell disease (SCD) arises from a point mutation of the β-globin chain that substitutes a negatively charged glutamic acid for a neutral hydrophobic valine [1]. When sickle hemoglobin (HbS) releases its ligand, the conformational state changes from the relaxed (R) oxyhemoglobin (oxyHb) structure to the tight (T) deoxyhemoglobin (deoxyHb) state at any given oxygen tension in red blood cells (RBCs). Polymerization of HbS occurs only when HbS is in the deoxyHb T conformation. Under low-oxygen tension, HbS chains form a hydrophobic contact between valine on one chain and other amino acids such as alanine, phenylalanine, and leucine on the other chain, which subsequently polymerize and form rigid, rod-like fibers with a diameter of 20 nm, and precipitate inside RBCs, resulting in deformed sickle RBCs [2,3]. The kinetics of HbS polymerization is critical to determine the level of clinical severity in this disorder [4]. HbS polymerization is dependent on oxygen tension in capillaries as well as on hemoglobin concentrations in RBCs. Very few polymers are generated in capillaries for a short period, which is the delay time [5]. If the delay time is longer than the time required for RBCs to pass through capillaries, most RBCs would be allowed to escape such small vessels [6,7]. In contrast, a shorter delay time would cause microvascular obstruction [8]. If sickle RBCs occlude the microvasculature, including capillaries and small vessels, oxygen supply to peripheral tissues is impaired. HbS polymerization under hypoxic conditions thus plays a central role in the pathophysiology of SCD.

Despite widespread use of hydroxyurea in the treatment of SCD, several clinical problems with this chemical, including non-responsive patients, require us to develop novel treatment modalities [9]. One potential therapeutic procedure is to inhibit intracellular HbS polymerization by increasing the oxygen affinity of HbS; for instance, a reduction in P_{50} of HbS by 4 mm Hg efficiently inhibits HbS polymerization [7]. Several molecules have been tested as to their ability to modify the oxygen affinity of HbS. Carbon monoxide, which binds to hemoglobin at a high affinity, significantly prolongs RBC survival in SCD patients [10] and depolymerizes HbS fibers by two mechanisms, endo- and side-depolymerization [8], suggesting that carbon monoxide may increase the oxygen affinity of HbS. Potassium cyanide was also found to increase the oxygen affinity of HbA and HbS [11]. Both agents, however, are too toxic for clinical applications.

Nitric oxide (NO) binds hemoglobin with a much higher affinity than carbon monoxide [12]. NO is generated when nitrite is reduced by deoxyhemoglobin [13]. Low concentrations of NO have been used for patients with respiratory disorders [14] or SCD [15–17]. We previously examined the physiologic effects of low concentrations of NO in SCD patients and found that NO increases the oxygen affinity of sickle RBCs in vivo [18], however, our results have not been corroborated by other investigators [19,20]. This study was performed to gain further insight into the biophysical mechanisms by which the oxygen affinity of sickle RBCs increases in response to treatment with low concentrations of NO. NO shares many biophysical and physiological properties with carbon monoxide, which has been shown to inhibit the polymerization of HbS fibers [21]. Oxygen affinity of HbS is also altered by chemical anti-sickling agents [22,23]. These lines of evidence led us to speculate that NO may increase the oxygen affinity of sickle RBCs by inhibiting HbS polymerization and improving the access of oxygen to HbS chains. In this study we demonstrate that low

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concentrations of NO inhibit HbS polymer formation and alter the electric charge of HbS, which may have relevance to the NO-induced increase in the oxygen affinity of HbS.

Materials and methods

Blood samples from normal and SCD subjects

Peripheral blood was collected in tubes containing anti-coagulant (EDTA) from 10 normal (A/A)[3] and 10 SCD (S/S) subjects who were not receiving hydroxyurea. Informed consent was obtained from all subjects and the study was conducted according to the protocol approved by the Human Assurance Committee of Georgia Health Sciences University.

Preparation of hemolysates

Freshly drawn blood was washed three times at 4 °C with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4, 300 mOsm/L) and lysed using a sonicator (Model S60, Fisher Scientific, Pittsburgh, PA). Hemolysates (HbA or HbS) were centrifuged at 4 °C at 16,000 *g* for 90 min to remove cell debris and diluted with PBS by ~5-fold to the final concentration of 4 mM or 6 g/dl, the concentration of which was comparable to those used in other polymerization studies [8]. Polymerization of HbS hemolysates was examined as described below.

Tonometry of HbS hemolysates and sickle RBCs

Tonometry of hemolysates was carried out by two procedures. *Protocol 1:* To examine the effects of NO on HbS polymer formation, freshly prepared hemolysates were equilibrated at 37 °C in a continuous bubble tonometer (Equilibrator™ 300, RNA Medical, Acton, MA) at 80 bubbles/min with 21% oxygen and balanced nitrogen for 15 min. Hemolysates were then quickly switched to 3.5% oxygen for 15 min with or without 40 ppm NO gas (INO Therapeutics, Clinton, NJ). The same procedure was used to examine the effects of NO on HbS polymerization within RBCs. *Protocol 2:* To examine the effects of NO on "pre-formed" HbS polymers, hemolysates were initially exposed to 21% O_2 for 15 min and then deoxygenated by exposing them to 3.5% O_2 for 30 min. Hemolysates were then treated with 3.5% O_2 and 40 ppm NO for an additional 30 min. The net treatment was 60 min of exposure to 3.5% oxygen with NO gas included during the last 30 min. Levels of oxygen and nitrogen dioxide were continuously monitored by Servomex Model 570A (Crowborough, Sussex, England) and Sievers Model 280 (Boulder, CO) analyzers, respectively. Nitrogen dioxide levels were consistently less than 1 ppm by volume.

Determination of oxygen dissociation curves (ODCs)

Four ml of 30 mM deoxy-HbA or deoxy-HbS were exposed to 0% O_2 with or without 40 ppm NO for 30 min. Each sample was then exposed to 12 different O_2 concentrations (0–150 mm Hg) for at least 5 min. The oxygen saturation value was obtained using the UV–Vis spectrophotometry method based on the observation that ligand binding to hemoglobin at the heme site results in a change in the visible spectrum [24]. We monitored the differences in visible bands for deoxy-Hb (λ_{max} = 555 nm), Hb–NO (λ_{max} = 540 and 574 nm) and HbO_2 (λ_{max} = 540 and 577 nm) [24]. Isosbestic points were measured at 547 and 569 nm, and the formation of metHb was monitored by measuring the isosbestic point at 634 nm. ODCs for HbS or HbA hemolysates were plotted after calculating the HbO_2 saturation percentage at 12 different oxygen tensions with or without 40 ppm NO exposure using the following formula:

[3]*Abbreviations used*: A/A, normal subjects; Hb, hemoglobin; HbA, normal adult hemoglobin; HbS, sickle hemoglobin; metHb, methemoglobin; NO, nitric oxide; ppm, parts per million; SCD, sickle cell disease; S/S, subjects homozygous for β^S mutation.

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$$[\mathrm{HbO_2}]\% = \frac{[\mathrm{HbO_2}]_{\mathrm{obs}} - [\mathrm{Hb}] - [\mathrm{HbNO}]}{[\mathrm{HbO_2}]_o - [\mathrm{HbNO}]} \times 100$$

where $[HbO_2]_{obs}$ is observed oxyhemoglobin, $[HbO_2]_o$ is fully saturated oxyhemoglobin, [Hb] is deoxyhemoglobin and [HbNO] is nitrosyl hemoglobin. The best fit curves were plotted using the Hill equation.

Measurement of deoxy Hb, oxyHb, NO–Hb, and mtHb by UV–Vis spectrophotometry

Hemolysate samples were prepared and tonometered using Protocol 1 as described above. For control absorbance spectra, hemolysates were diluted either 1:100 or 1:200 in a deoxygenated 0.9% NaCl solution. Diluted samples were transferred anaerobically to 0.1 cm path glass cuvettes that had been purged with N_2 gas for at least 30 min. Spectra were obtained at a scan rate of 600 nm/min using a Beckman DU-650 spectrophotometer. Basis spectra of oxy-Hb, deoxyHb, NO–Hb, and metHb were obtained from 350 to 700 nm in a 0.1 cm path glass cuvette. NO–Hb was prepared by adding a 5-fold molar excess of sodium nitrite per heme to deoxygenated Hb in the presence of a 20-fold molar excess of sodium dithionite per heme, as described by Xu et al. [25]. The basis spectra were normalized to their extinction coefficients in the 500–700 nm regions and then used to quantitate the relative concentration of each species in the sample. Quantitation was performed using an additive spectral method, where $A_{tot}(\lambda) = (C_{deoxy} * B_{deoxy}(\lambda) + (C_{oxy} * B_{oxy}(\lambda)) + (C_{NO} * B_{NO}(\lambda)) + (C_{met} * B_{met}(\lambda))$, and C represents the concentration of each species, and $B(\lambda)$ represents the basis spectrum of each species, as described previously [26]. All spectral manipulations were performed using Grams AI (Thermo Scientific, West Palm Beach, FL), and the spectral addition and least squares minimization were performed using Microsoft Excel.

Analysis of NO–Hb adducts by electron spin resonance (ESR)

NO–Hb adducts were monitored by ESR spectrometer (Bruker Instruments Inc., Billerica, MA), as described [27]. Tonometry with or without 40 ppm NO was performed by Protocol 1 and hemolysate samples were then frozen in liquid nitrogen. ESR spectroscopy was performed at the following settings: field sweep, 300 G; microwave power, 10 mW; microwave frequency, 9.40 GHz; modulation amplitude, 3 G; conversion time, 2624 ms; time constant, 5248 ms.

Examination of apparent viscosity of hemolysates using falling ball assays

Stokes' law predicts that for the steady state motion in a Newtonian fluid, the velocity of a spherical particle driven by a constant force is inversely proportional to the viscosity of the fluid. This principle has been extended in an application called the falling ball assay to measure apparent viscosity of even non-Newtonian fluids such as a solution of actin filaments [28]. The apparent viscosities of hemolysates were compared using a falling ball apparatus described by Pollard [28]. Hemolysates were prepared from 10 normal (A/A) and 10 SCD (S/S) subjects. Tonometry of hemolysates was carried out using Protocol 1 or Protocol 2 as described above. A small stainless steel metal ball of uniform size (Grade 10, gauge + 0.00001; Winsted Precision Ball Company, Winsted, CT) was introduced into a 100 μl glass capillary tube (Fisher Scientific, Pittsburgh, PA) filled with 50–60 μl of hemolysates, and the distance that the ball traveled over a fixed time (1 s) was measured automatically using photocells to detect the ball interrupting a beam of light. The velocities (cm/s) of the metal ball falling in hemolysates with and without NO added were directly compared, bearing in mind that the measured velocity is inversely proportional to the apparent viscosity of the lysates.

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Multiphoton image analysis of HbS polymers

After tonometry with or without NO, as described above, 4 μl of hemolysates or RBCs that were prepared from S/S or A/A RBCs were placed on glass slides. Cover slips were rapidly sealed onto the slides with a clear sealant to protect them from atmospheric oxygen. A BioRad MRC 1024ES multiphoton imaging system (BioRad, Inc., Hercules, CA) coupled to a Zeiss Axiovert S100 inverted microscope was used for imaging the samples in transmitted light mode in a blinded fashion [29]. RBC images were recorded at intervals of 0.1 μm depths along the Z-axis over the entire thickness of RBCs. The images of HbS hemolysates or sickle RBCs were then reconstructed and processed using BioRad LaserSharp and MetaMorph software (Universal Imaging, West Chester, PA).

Isoelectric focusing gel electrophoresis

Four ml of 65 μM deoxy-Hb in 0.1 M potassium phosphate buffer, pH 7.4, was sealed with a rubber stopper, fully deoxygenated and then treated with or without 80 ppm NO under different O_2 states (0%, 3.5%, and 21%) at 37 °C for 30 min as mentioned above. A higher concentration of NO (80 ppm) was used to maintain heme:NO ratios comparable to those of previous experiments [18]. Isoelectric focusing gel electrophoresis was performed at 15 °C for 30 min using Resolve-Hb IEF system gels (pH 6–8) (Perkin Elmer, Waltham, MA) at constant power (20 W in 26-well gel) in Amersham Biosciences Multiphor II electrophoresis apparatus. Gels were soaked in 10% trifluoroacetic acid for 5 min and washed with distilled water for 40 min. Gels were stained using JB-2 staining system (Code FR9367 or FR-9460), washed with distilled water, and dried overnight at room temperature.

High performance liquid chromatography (HPLC) for HbS-NO adducts

Five ml of 90 μM hemolysates from A/A and S/S were deoxygenated by N_2 gas for 30 min and then exposed to 80 ppm NO under different oxygen concentrations at 37 °C using Equilibrator tonometer (RNA Medical, Devens, MA). NO (80 ppm) was used to maintain heme:NO ratios comparable to those of previous experiments [18]. The levels of O_2 and NO in the air mixture were measured by respective analyzers (O_2, Servomex, Oxygen Analyzer; NO, Sievers, Nitric Oxide Analyzer, NOA 280). Hb solution (500 μl) was sealed in HPLC tubes and then subjected to cation-exchange HPLC using SynChropak CM 300 columns (Eprogen, Darien, IL) and Bis–Tris developers, as described [30].

Statistical analysis

All experiments were repeated at least three times to ensure reproducibility of results. Data were expressed as mean ± standard error of mean. Paired Student t-test was used to compare mean velocities, and values with $p < 0.05$ were considered significant.

Results

Effects of NO on oxygen affinity of HbS and metHb formation

Our previous in vitro and in vivo studies found decreases in P_{50} values of sickle RBCs exposed in vitro to 80 ppm NO as well as of RBCs of SCD patients who inhaled 80 ppm NO [18]. To verify these observations, we examined whether NO reduces the P_{50} of HbS hemolysates. Hemolysates prepared from S/S and A/A were used to generate ODCs and P_{50} values in the absence and presence of 40 ppm NO (Fig. 1). P_{50} values are shown in Table 1. Upon exposure to 40 ppm NO, the P_{50} values of HbS hemolysates decreased from 16.4 ± 1.0 to 13.5 ± 0.9 mm Hg ($p < 0.001$), while no changes were seen with those of HbA hemolysates (Fig. 1A and B, Table 1). The O_2 saturation levels of untreated HbS hemolysates did not reach 100%, but those treated with 40 ppm NO did (Fig. 1A). To verify the results with P_{50} values of RBCs in our previous study [18], we examined P_{50} values of

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RBC preparations from both normal (HbA) and SCD (HbS) subjects. In these assays we used 80 ppm NO to confirm reproducibility (Table 1). The results in the current study were consistent with those of our previous study. To exclude possible effects of metHb on P_{50} of HbS exposed to NO [31,32], we examined the metHb levels in those HbS hemolysates. The metHb levels of HbS hemolysates did not change significantly after NO exposure (Table 2). Also, a separate absorbance band at 630 nm, which is useful for estimating metHb levels [33], did not increase in HbS hemolysates (Fig. 2A). Formation of NO–Hb adducts in HbS hemolysates exposed to 40 ppm NO was confirmed by UV–Vis spectrophotometry (Table 1) as well as by ESR (Fig. 2B).

NO reduces viscosity of HbS hemolysates

Although microviscometers have been used to measure the viscosity of sickle RBCs [22,34,35], we employed falling ball assays described previously [28]. The falling ball assay was successfully used to study “rapid” changes in the viscosity of the gelation process of actin and accessory proteins [36]. HbS promptly polymerizes under hypoxic conditions and exhibits gel-like rheological behavior [37–39]. Such rapid rheological changes in HbS hemolysates can be detected accurately by falling ball assays.

Under 21% O_2, the falling particle velocity in HbS hemolysates was the same as that of HbA hemolysates, indicating no HbS polymerization under normoxia (Fig. 3A). Soon after the oxygen tension was reduced to 3.5%, the velocity of falling balls decreased from 2.53 to 1.18 cm/s, indicating an increase in HbS hemolysate viscosity. However, when the oxygen level was decreased to 3.5% in combination with 40 ppm NO, a significantly smaller decline in the falling ball velocities, from 2.53 to 1.72 cm/s, was observed, demonstrating that HbS polymer formation under low oxygen tension is partially inhibited by 40 ppm NO. To examine whether NO is capable of dissociating HbS polymers, we performed falling ball assays using “preformed” HbS polymers. HbS hemolysates were tonometered using Protocol 2. The falling ball velocity of HbS hemolysates under 3.5% O_2 decreased from 1.73 to 0.30 cm/s (Fig. 3B, left and middle columns), indicating HbS polymer formation. Further treatment with 3.5% O_2 and 40 ppm NO restored the velocity to 0.80 cm/s (Fig. 3B, right column). These results show that NO inhibits HbS polymerization under low oxygen tension and even disrupts preformed HbS polymers.

Multiphoton image analyses show that NO reduces HbS polymerization

To confirm that NO inhibits HbS polymerization under hypoxic conditions, we next examined HbS polymer formation in hemolysates and RBCs using multiphoton microscopy in the transmission mode. Pseudo-color images of HbS hemolysates demonstrated no polymer formation under 21% O_2, as confirmed by the presence of a uniform imaging field in both two (2D) and three (3D) dimensions, (Fig. 4A, left panel). In contrast, under hypoxic conditions (3.5% O_2), a large number of polymers of various sizes and shapes were observed with HbS hemolysates, as indicated by dots in 2D and peaks in 3D, respectively (Fig. 4A, middle panel). However, when deoxygenated HbS hemolysates were treated with 40 ppm NO, the polymers were dissolved into much smaller entities (Fig. 4A, right panel). Fig. 4B demonstrates HbS polymers that were observed in the transmission mode under hypoxic conditions in the absence (middle row) and presence (bottom row) of 40 ppm NO. It is obvious that the size of HbS polymers observed with 40 ppm NO are much smaller than those formed without NO. These observations were further verified by measuring the width and length of the polymers shown in Fig. 4B. Adding 40 ppm NO to deoxygenated HbS hemolysates reduced the length of HbS polymers from 25.28 ± 1.04 to 5.86 ± 0.37 μm and the width from 5.86 ± 0.17 to 2.93 ± 0.08 μm. Next, using multiphoton microscopy in the transmission mode, we examined the effects of NO on HbS polymers inside sickle RBCs (Fig. 5). Under hypoxic conditions (3.5% O_2), large HbS polymers, which were seen as

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thick black fibers (Fig. 5, left panel), were observed in the cytoplasm of sickle RBCs. In contrast, such dense and solid HbS polymers disappeared in deoxygenated sickle RBCs exposed to 40 ppm NO gas (Fig. 5, right panel). It is of note that the shape of sickle RBCs exposed to NO was less distorted compared with those under 3.5% O_2 (Fig. 5, compare right and left panels). These results, together with the falling ball assay findings (Fig. 3), demonstrate that low concentrations of NO inhibit HbS polymerization under hypoxic conditions.

NO alters electric charge of HbS

NO interacts with heme or amino acid residues of hemoglobin such as Cysβ93 in a complex manner [40,41]. To gain further insight into the mechanism by which NO disrupts HbS polymers, we examined whether NO modulates the electric charge of HbS molecules. Under various oxygen tensions, HbS hemolysates (65 μM) were treated with 80 ppm NO and then subjected to isoelectric focusing gel electrophoresis; 80 ppm NO rather than 40 ppm NO was used to facilitate the interactions between Hb and NO. A representative gel is shown in Fig. 6. Without NO exposure and irrespective of oxygen tension, HbS and HbA demonstrated isoelectric points (pIs) of 7.8 and 7.4, respectively (Fig. 6, lanes 1–9). This is consistent with evidence that HbS has an excess of positive charge compared to HbA [1]. In contrast, HbS, when exposed to 80 ppm NO, exhibited an additional band with a pI of 7.4, which migrated to a position similar to that of HbA. The intensities of HbS bands with a pI of 7.4 positively correlated with oxygen levels (Table 3). These results suggest that HbS bands with pI of 7.4 are likely due to the formation of NO–HbS adducts, and more importantly, an excess of positive charge of HbS is abolished by NO binding. To confirm that NO–HbS adducts have an electric charge similar to that of HbA, HbS hemolysates treated with NO were subjected to cation-exchange HPLC (Fig. 7). HbS and HbA were eluted at different retention times; HbA and HbS were eluted at 17.2 min and 25.7 min, respectively (Fig. 7, left panel), indicating a difference of the electric charges of these molecules. However, HbS treated with 80 ppm NO generated an additional peak due to the formation of NO–HbS adducts, which was eluted at a retention time of 17.6 min. The retention time of the new peak was similar to, but distinct from, the retention time of HbA (17.24 ± 0.05 vs. 17.61 ± 0.12; $p < 0.00017$, Fig. 7, right panel). This again indicates that NO–HbS adducts have an electric charge similar to that of HbA. Taken together, NO is likely to abrogate an excess of positive charge from HbS, making the charge status of HbS molecules similar to that of HbA.

Discussion

We had previously shown that the oxygen affinity of sickle RBCs increases in SCD patients who were administered low concentrations of NO [18], but the underlying mechanisms remain unclear. Moreover, our results were not reproduced by other investigators [19,20], prompting us to further investigate the biophysical effects of NO on HbS.

To gain insight into the mechanism by which NO modulates the oxygen affinity of HbS, in this study we investigated the biophysical properties of NO and obtained multiple new lines of evidence. First, we confirmed the effects of low concentrations of NO on the oxygen affinity of HbS using hemolysates, as sickle RBCs were used in our previous studies [18]. After exposure to NO, HbS hemolysates, but not HbA hemolysates, demonstrated substantially lower P_{50} values, indicating an increase in the oxygen affinity of HbS (Fig. 1 and Table 1 for the summary of P_{50} values). In addition, although the levels of O_2 saturation of untreated HbS hemolysates did not reach 100%, those treated with 40 ppm NO did reach 100% O_2 saturation. This may suggest that HbS molecules without NO treatment cannot be fully saturated O_2 or fully liganded, presumably due to the tertiary structure resulting from T

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conformation or partial ligation, while NO may induce conformational changes in HbS, which may allow HbS to be fully liganded with O_2. We also saw no significant increases in the metHb levels of HbS hemolysates (Fig. 2A and Table 2), excluding the possible effects of metHb on the P_{50} values [33,42]. These results may support the conclusion that low concentrations of NO increase the oxygen affinity of HbS [18]. Second, as stated above, NO is known to share a number of common biological and biophysical activities with carbon monoxide [43], which has been shown to depolymerize HbS polymers [8]. It is reasonable to assume that NO could exhibit anti-sickling activities by depolymerizing HbS polymers. Prior studies demonstrating increases in the oxygen affinity of HbS after treatment with anti-sickling agents such as nitrogen mustard [22] and 5-hydroxy-methyl-2-furfural [23] have also led us to speculate that NO-induced increases in the oxygen affinity of HbS may be associated with the inhibition of HbS polymerization. Our in vitro studies, including falling ball assays (Fig. 3) and multiphoton microscopic analyses (Figs. 4 and 5), have shown that NO as low as 80 ppm has anti-sickling properties. Because HbS polymerizes only when it is in the tight (T) deoxyhemoglobin conformation, this result may suggest that NO may be converting HbS molecules from the T deoxyhemoglobin conformation to the relaxed (R) oxyhemoglobin conformation, leading to the depolymerization of HbS polymers. This view is supported by recent reports by Ferrone and colleagues that NO significantly decreases the polymerization rate by inhibiting the number of T state HbS molecules [44]. Collectively, it seems likely that NO increases the oxygen affinity of HbS in part by disrupting HbS polymers or converting their conformation, thereby improving HbS molecule access to oxygen. To our knowledge, this is the first demonstration of HbS depolymerization induced by NO.

Inhalation of low concentrations of NO has been shown to benefit patients with pulmonary hypertension [45] or adult respiratory distress syndrome by dilating pulmonary arteries and improving oxygenation of the lungs [14]. Regarding the dilatation of pulmonary arteries by inhaled NO, increased levels of nitrite after inhalation likely act as a pool for NO generation [13]. NO inhalation may contribute to the improvement of SCD pathophysiology through multiple mechanisms. Anti-platelet effects of NO may play a role in alleviating pulmonary complications in SCD [46]. Furthermore, the present study suggests that SCD patients may benefit from NO-mediated HbS depolymerization and a subsequent increase in the oxygen affinity of HbS, which would contribute to improved peripheral blood circulation in the lungs. These multiple biophysical and hematological properties of NO may contribute to the successful treatment of SCD patients with acute chest syndrome [15–17].

As to mechanisms that mediate HbS depolymerization, low concentrations of carbon monoxide, which, like NO, binds hemoglobin with a high affinity, were shown to depolymerize HbS polymers from the fiber ends [8]. Our multiphoton microscopic analysis of HbS polymers exposed to NO demonstrated much smaller HbS polymers with intact nuclei (Fig. 4B), suggesting that NO may depolymerize HbS fibers by mechanisms similar to those employed by carbon monoxide. Both NO and carbon monoxide are low-spin ligands for hemoglobin [47] and are capable of dissociating HbS polymers, however, the biophysical and biochemical mechanisms underlying HbS depolymerization induced by these active gases remain unclear. An interesting finding in this study – that NO modifies the electric charge status of HbS – may provide a clue. NO–HbS adducts exhibited an electric charge similar to but distinct from that of HbA; NO–HbS adducts had a pI similar to that of HbA and exhibited a retention time comparable to that of HbA upon cation-exchange HPLC analysis (Figs. 6 and 7). It is still unclear at this point, however, whether NO–HbS adducts are nitrosylated HbS [46] or some other products [40] due to NO-induced reactions. NO readily reacts with a number of molecules with unpaired electrons in their outer orbital, including other free radicals or proteins containing heme [48]. One possible mechanism for the NO-induced HbS depolymerization may be that NO, a free radical with an unpaired

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electron molecule [48], may confer a negative charge to HbS molecules after binding to heme. It is thus possible that NO binding abolishes the excess positive charge of HbS and allows HbS to have an electric charge similar to that of HbA. HbS polymerization is strongly inhibited and HbS solubility is greatly increased in an alkaline solution; even a fraction of a pH unit suffices to inhibit polymerization [49]. Also, the formation of hydrophobic contacts between valine and other amino acid residues appears to be very sensitive to alterations of the electric charge of HbS [39,50]. A similar change in the electric charge of HbS was reported with aldehyde–HbS adducts formed after treatment with the anti-sickling agent 5-hydroxymethyl-2-furfural [23]. Although it is reasonable to speculate that NO-mediated charge alterations in HbS molecules may destabilize HbS polymers and lead to depolymerization, further studies are necessary to define the effects of NO-mediated charge alterations on HbS depolymerization.

A controversial issue is that inhalation of low concentrations of NO up to 80 ppm failed to produce a significant effect on the oxygen affinity of HbS in SCD patients in other laboratories [19,20]. It is difficult at this point to reconcile the difference between the results of our laboratory and those of others. However, there have been reports that lend support to our conclusion that NO, which we show here has anti-sickling properties, increases the oxygen affinity of HbS. For instance, as stated above, anti-sickling agents such as 5-hydroxymethyl-2-furfural and nitrogen mustard significantly decreased P_{50} values of sickle RBCs and HbS hemolysates [22,23]. Administration of the anti-sickling chemical tucaresol to SCD patients resulted in an increase in the oxygen affinity of HbS [51]. Furthermore, a recent study showed a substantial increase in the oxygenated HbS levels in the peripheral skin of SCD patients after inhalation of 80 ppm NO [52]. Although several mechanisms presumably contribute to the increase in oxygenated HbS levels, one possibility is that an elevated oxygen affinity of HbS by NO increases oxygenated HbS levels. The present study has shown that NO may increase the oxygen affinity of HbS by dissociating HbS polymers and improving the access of oxygen to HbS. Although one might argue that the NO-modified HbS levels achieved by the inhalation of 80 ppm NO are too low to modify overall oxygen affinity, inhalation of 80 ppm NO for 90 min has been shown to generate a substantial increase in the oxygenated HbS levels in peripheral blood [52]. Furthermore, we recently found that sickle RBC adhesion in peripheral bone marrow venules is reduced in mice lacking endothelial nitric oxide synthase after inhalation of 20 ppm NO for 15 min (Gutsaeva et al., manuscript submitted). NO inhalation may generate systemic effects that could modulate the pathophysiology in SCD patients.

It was suggested that increases in metHb levels are responsible for the decrease in the P_{50} values of HbS in SCD patients [20], however, several lines of experimental evidence do not support this possibility. First, in our previous study [18], we had not seen a positive correlation between the reduction of P_{50} values and the incremental levels of metHb levels. Second, although we measured metHb levels in HbS hemolysates treated with 40 ppm NO, no significant increase in metHb levels was observed in the hemolysates (Table 2). SCD patients have higher metHb levels than normal subjects [53]. Although our patients had metHb levels of up to 4% after 80 ppm NO inhalation, these levels were comparable to those observed in other NO inhalation studies [15–17]. Further studies are necessary to determine the effects of metHb on the oxygen affinity of HbS exposed to NO.

Conclusions

The present study has found a novel biophysical activity of NO: its anti-sickling properties. It is logical to assume that NO dissociates HbS polymers, presumably by mechanisms similar to those by which low-dose carbon monoxide depolymerizes [8], and allows oxygen to bind to the heme moiety in HbS. We showed an increase in the oxygen affinity of HbS in

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SCD patients who inhaled low concentrations of NO [18]. Our SCD mouse study showed that NO exerts a rapid and protective effect from severe hypoxic stress [54]. Previous studies have already suggested the beneficial effects of NO inhalation on pulmonary disorders [14,45]. Furthermore, our recent double-blind, randomized, placebo-controlled clinical trial demonstrated that NO inhalation reduces pain scores in adult SCD patients [55]. Thus, NO inhalation may provide a rational and effective therapeutic intervention for pulmonary complications in SCD. However, in vitro experiments in this study were performed using diluted hemolysates and NO concentrations that are higher than the normal levels. Under such conditions, the stoichiometry of NO to HbS molecules deviates from that which is observed under physiologic conditions. It is thus necessary to re-evaluate the roles of inhaled NO in HbS polymerization and the interactions between NO and HbS in an in vivo setting. Such studies are currently under way to further investigate biophysical and biochemical effects of inhaled NO using SCD model mice.

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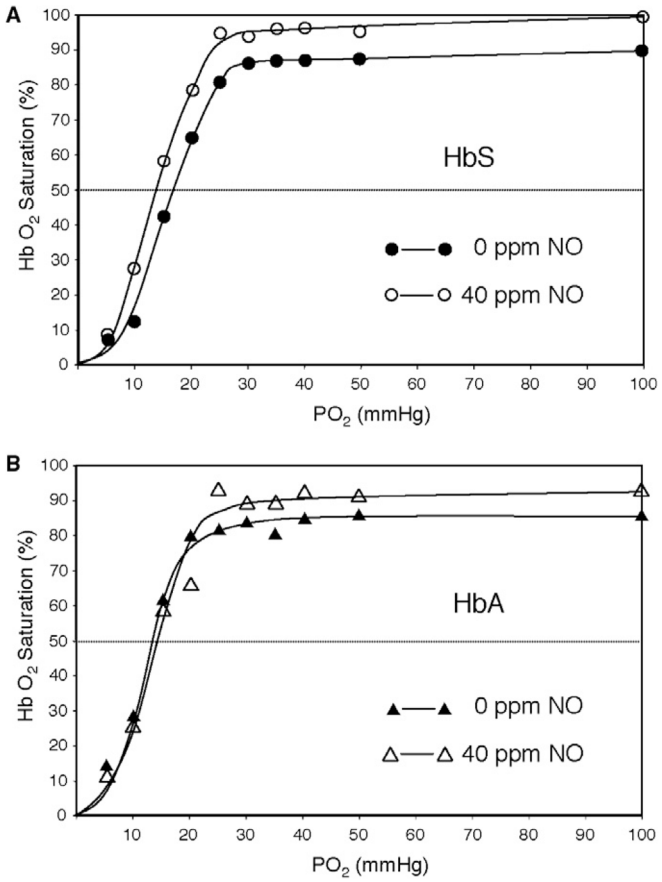

Fig. 1.

Low concentrations of NO increase the oxygen affinity of HbS hemolysates. Oxygen dissociation curves (ODCs) of HbS (A) and HbA (B) were generated using hemolysates that were treated with or without 40 ppm NO, as described in "Materials and Methods." Assays were repeated at least three times and representative ODCs are shown. The Hill equation was used for best-fit curves.

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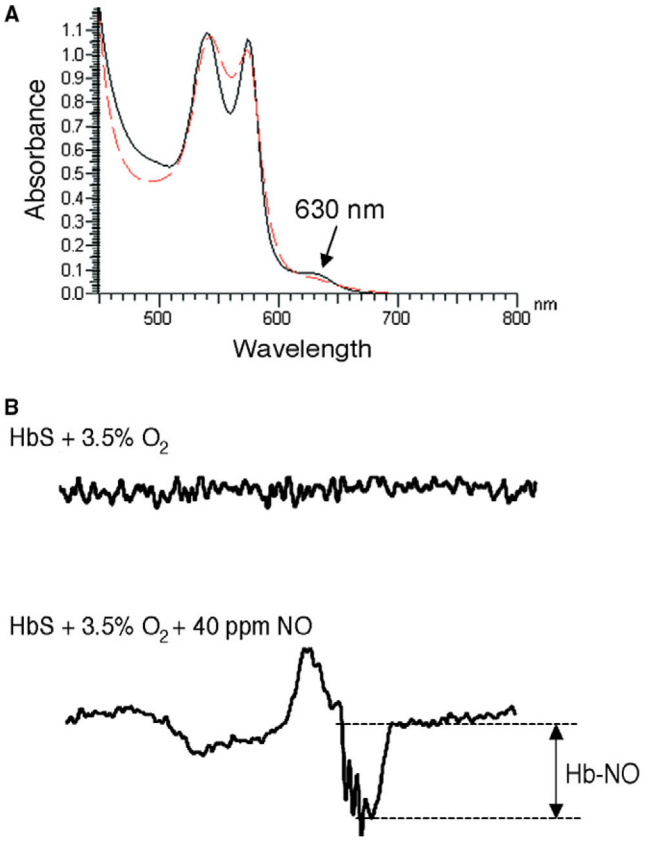

Fig. 2.

Levels of metHb and NO–HbS in HbS hemolysates exposed to NO. (A) MetHb levels in HbS hemolysates were quantitated by UV–Vis spectrophotometry as described in "Materials and Methods." Black solid line, HbS with 3.5% O_2; red dotted line, HbS with 3.5% O_2 and 40 ppm NO. (B) NO–HbS adducts were assayed by electron spin resonance. Representative assay results are shown from three experiments that gave similar results.

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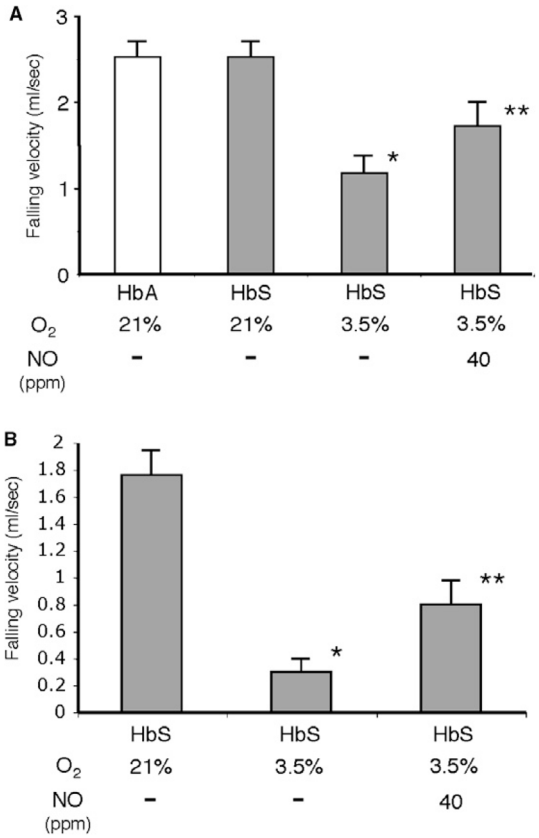

Fig. 3.

Examination of viscosity of HbS hemolysates by falling ball assays. (A) Effects of 40 ppm NO on HbS polymer formation. Falling ball assays were performed using HbS hemolysates that were tonometered with Protocol 1 as described in "Materials and Methods." *, $p < 0.001$ compared to HbS with 21% O_2. **, $p < 0.05$ compared to HbS with 3.5% O_2. (B) Effects of 40 ppm NO on pre-formed HbS polymers. Falling ball assays were performed using HbS hemolysates that were tonometered with Protocol 2. *, $p < 0.0001$ compared to HbS with 21% O_2. **, $p < 0.05$ compared to HbS with 3.5% O_2.

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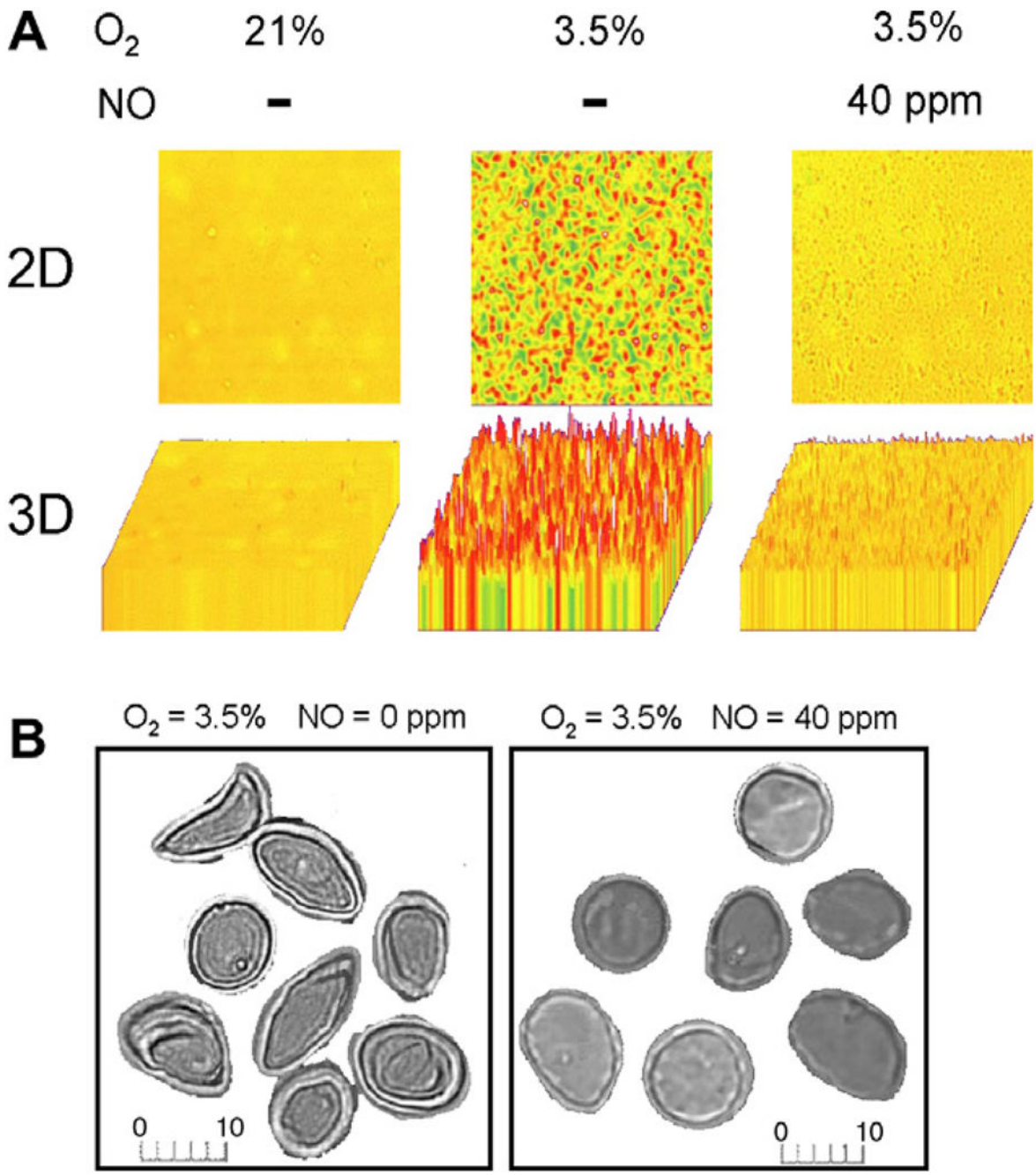

Fig. 4.

NO inhibits HbS polymerization at low oxygen tensions. (A) Multiphoton image analysis was carried out for HbS hemolysates that were tonometered with Protocol 1. Pseudo-color images of HbS polymers were created in two (2D) or three dimensions (3D); the Metamorph image processing software utilized the quantum yield from the samples to render 2D and 3D images. No HbS polymers were observed at an oxygen tension of 21% (left panel), but prominent HbS polymers were seen at 3.5% O_2 (middle panel). NO markedly inhibited HbS polymerization at 3.5% O_2 (right panel). The pixel intensity profile in the lower panels (3D) more graphically demonstrates polymer formation. The height, width and intensity of the pseudo-colored peaks represent the size and shape of HbS polymers. (B) NO dissociates pre-

formed HbS polymers. HbS hemolysates were tonometered with Protocol 2 and examined by multiphoton microscopy in the transmission mode. Top row, HbS hemolysates with normal oxygen tension; middle row, HbS hemolysates with 3.5% O_2; bottom row, HbS with 3.5% O_2 for 30 min and with 3.5% O_2 and 40 ppm NO for an additional 30 min.

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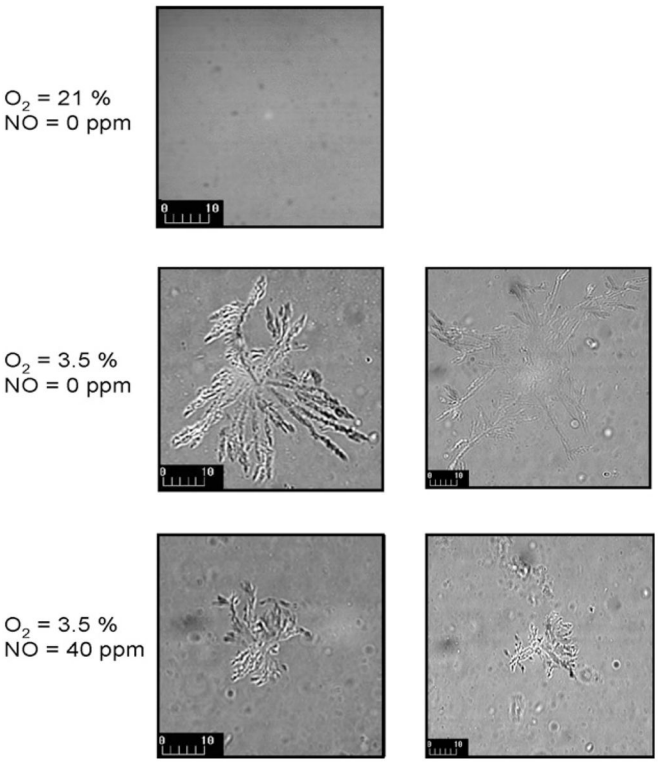

Fig. 5.

Analysis of sickle RBCs under hypoxia with 40 ppm NO. Sickle RBCs were tonometered with Protocol 1 and analyzed by multiphoton microscopy in the transmission mode. Intracellular HbS polymers in the form of folded structures (black fibers) were observed throughout sickle RBCs exposed to 3.5% O_2 (left panel). In contrast, when 40 ppm NO was added to the 3.5% O_2, virtually no polymers developed in sickle RBCs (right panel).

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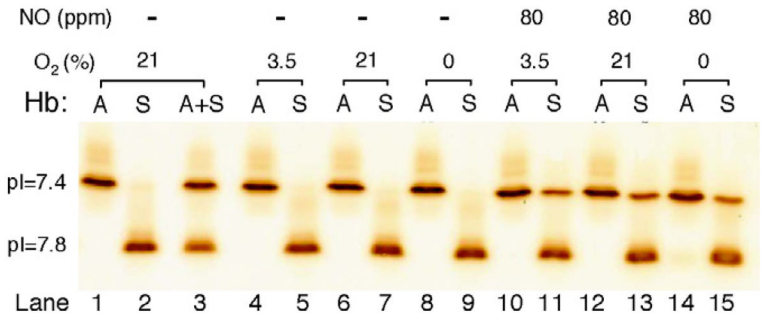

Fig. 6.

NO alters electric charge of HbS. Hemolysates of HbS (S) and HbA (A), both of which were 65 μM, were tonometered with Protocol 1 and analyzed by isoelectric focusing gel electrophoresis. Levels of O_2 and NO are shown on top of the figure; 80 ppm NO was used to enhance the interactions between Hb molecules and NO. Electrophoresis was repeated at least three times. A representative gel is shown.

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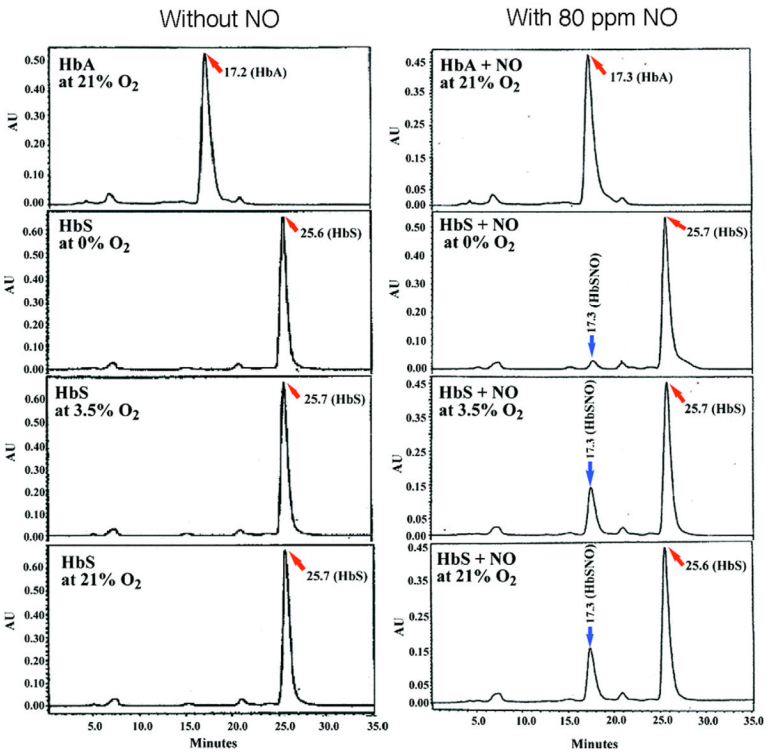

Fig. 7.

Analysis of HbS hemolysates (90 μM) by cation exchange HPLC. Hemolysates of HbA and HbS were deoxygenated by N_2 gas from 30 min and then exposed to 80 ppm NO under different oxygen states at 37 °C using Equilibrator tonometer. As stated above, 80 ppm NO was used to enhance the interactions between Hb molecules and NO. Representative elution profiles without NO (left panel) and those with 80 ppm NO (right panel) are shown from three independent experiments that gave similar results.

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Table 1

P_{50} values of hemoglobin hemolysates and RBCs with or without NO.

	P_{50} (mm Hg) of HbA		**P_{50} (mm Hg) of HbS**	
Hemolysates	Without 40 ppm NO	With 40 ppm NO	Without 40 ppm NO	With 40 ppm NO
	13.2 ± 1.1	13.5 ± 1.0	16.4 ± 1.0	13.5 ± 0.9[b]
RBCs[a]	Without 80 ppm NO	With 80 ppm NO	Without 80 ppm NO	With 80 ppm NO
	27.0 ± 1.5	27.3 ± 1.8	35.2 ± 1.0	30.5 ± 0.9

[a] P_{50} values for RBCs were determined using 80 ppm NO to compare with those measured in our previous study [18].

[b] $P < 0.001$ compared with P_{50} without 40 ppm NO.

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Table 2

Concentrations of deoxy Hb, oxyHb, NO–Hb, and metHb.

HbS samples	Hb concentration (mM)[a]	Deoxy Hb (%)[b]	OxyHb (%)[b]	NO–Hb (%)[b]	MetHb (%)[b]
3.5% O_2	24.6 ± 3.3	74 ± 2	23 ± 4	NA	3 ± 2
3.5% O_2 + 40 ppm NO	25.1 ± 2.2	71 ± 5	19 ± 9	6 ± 1	3 ± 2

[a]Concentrations are an average of 3 independent experiments.

[b]Percentages are an average of 3 independent experiments and are measured relative to total Hb concentrations. The concentrations of individual Hb species are determined from the spectral fits as described in “Materials and Methods.”

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Table 3

Analysis of HbS treated with NO by isoelectric focusing gel electrophoresis.[a]

Hemolysate	Treatment condition	HbS concentration (μM)	HbS concentration (μM) at pI 7.4
HbS	0% O_2, 80 ppm NO	65	10.05 (15.5%)[b]
HbS	3.5% O_2, 80 ppm NO	65	16.80 (25.9%)[b]
HbS	21% O_2, 80 ppm NO	65	20.87 (32.1%)[b]

[a] HbS hemolysates (65 μM) were treated with 80 ppm NO in 0.1 M potassium phosphate buffer, pH 7.4, for 30 min to facilitate the interactions between Hb and NO.

[b] Numbers in parentheses indicate percentages of modified HbS with NO relative to total HbS.