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Nitric Oxide Transport on Sickle Cell Hemoglobin: Where Does it Bind?

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We have recently reported that nitric oxide inhalation in individuals with sickle cell anemia increases the level of NO bound to hemoglobin, with the development of an arterial-venous gradient, suggesting delivery to the tissues. A recent model suggests that nitric oxide, in addition to its well-known reaction with heme groups, reacts with the β-globin chain cysteine 93 to form S-nitrosohemoglobin (SNO-Hb) and that SNO-Hb would preferentially release nitric oxide in the tissues and thus modulate blood flow. However, we have also recently determined that the primary NO hemoglobin adduct formed during NO breathing in normal (hemoglobin A) individuals is nitrosyl (heme)hemoglobin ($HbFe^{II}NO$), with only a small amount of SNO-Hb formation. To determine whether the NO is transported as $HbFe^{II}NO$ or SNO-Hb in sickle cell individuals, which would have very different effects on sickle hemoglobin polymerization, we measured these two hemoglobin species in three sickle cell volunteers before and during a dose escalation of inhaled NO (40, 60, and 80 ppm). Similar to our previous observations in normal individuals, the predominant species formed was $HbFe^{II}NO$, with a significant arterial-venous gradient. Minimal SNO-Hb was formed during NO breathing, a finding inconsistent with significant transport of NO using this pathway, but suggesting that this pathway exists. These results suggest that NO binding to heme groups is physiologically a rapidly reversible process, supporting a revised model of hemoglobin delivery of NO in the peripheral circulation and consistent with the possibility that NO delivery by hemoglobin may be therapeutically useful in sickle cell disease.

Abbreviations: NO, nitric oxide; SNO-Hb, S-nitrosohemoglobin; $HbFe^{II}NO$, nitrosyl(heme)hemoglobin; F_IO_2, fraction of inspired oxygen

INTRODUCTION

It has been known for decades that nitric oxide (NO) reacts with oxyheme groups on hemoglobin to form methemoglobin and nitrate and reacts with deoxyhemoglobin to form nitrosyl(heme)-

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hemoglobin ($HbFe^{II}NO$). It has recently been proposed that nitric oxide also reacts with the β-globin chain cysteine 93 of R-state hemoglobin to form a covalent S-nitrosothiol bond (SNO-Hb).[1–3] SNO-Hb appears to be energetically linked to oxygenation, such that deoxygenation favors NO release from the cysteine, particularly in the presence of glutathione.[4] This model suggests that SNO-Hb would preferentially release nitric oxide in hypoxic vascular regions and thus modulate blood flow. Physiologic studies of nitric oxide inhalation, demonstrating systemic effects, have supported a model of NO transport on hemoglobin.[5–7]

We have recently demonstrated in individuals with sickle cell anemia that NO breathing augments NO binding to hemoglobin without increasing hemoglobin oxygen affinity.[8] Furthermore, an arterial-venous gradient in nitrosylated hemoglobin develops, suggesting delivery of NO from hemoglobin. However, we were unable to determine whether the NO is bound on the heme or on cysteine 93. Using a new methodology that is highly sensitive and specific for both $HbFe^{II}NO$ and SNO-Hb we have recently determined that the primary NO hemoglobin adduct formed during NO breathing in normal (hemoglobin A) individuals is $HbFe^{II}NO$, with very little SNO-Hb formation.[9] The relative reaction products of NO with hemoglobin S have not yet been reported and it is possible that nitric oxide will react differently with hemoglobin S.[10] We therefore applied these new methodologies to the study of NO hemoglobin adducts in three previously studied individuals breathing NO at 40, 60, and 80 ppm.[8]

METHODS

Protocol

The study protocol was approved by the National Heart, Lung and Blood Institute's Institutional Review Board, and all subjects signed an informed consent. All volunteers had hemoglobin S documented by electrophoresis. Peripheral intravenous and radial artery catheters were placed, and blood samples were collected hourly. Following an hour of baseline measurements, subjects breathed NO gas at a concentration of 40 ppm (Ohmeda INOvent Delivery System, Madison, WI) for one hour, 60 ppm for one hour and 80 ppm for a final hour. A room air gas condenser with an oxygen blender was used to deliver an F_IO_2 of 0.21 at 40 L/minute with an in-line reservoir bag.[8]

Processing of Blood and Ozone-based Chemiluminescent Detection of Nitrosyl(heme)hemoglobin and S-nitrosohemoglobin

Blood samples were drawn and centrifuged at $750 \times G$ for five minutes. The red blood cell pellet was washed two times in five volumes of phosphate buffered saline (PBS). Red cell samples in duplicate were then lysed in both 1:4 dilution of 0.5 mM EDTA in nitrite free molecular biology grade water (Biofluids, Bethesda, MD) or 0.2 M KCN and 0.2 M $K_3Fe(CN)_6$ in 0.5 mM EDTA distilled water. Following 30 minute incubation, 500 µl was passed through a 9.5 ml bed volume Sephadex G25 column to remove nitrite, small thiols, and $KCN/K_3Fe(CN)_6$. The hemoglobin concentration of the Sephadex G25 effluent was measured by conversion to cyanomethemoglobin ($\varepsilon_{540} = 11$ for heme). Note that all hemoglobin concentrations are reported in terms of heme. Hemoglobin samples (200 µl), with and without $KCN/K_3Fe(CN)_6$ pretreatment, were immediately drawn into 250 µl Hamilton syringes and reacted with I_3^- to release NO for chemiluminescent detection.

This method for the measurement of nitrite and S-nitrosothiols by reaction with I_3^- to release NO gas[8,11] was applied to hemoglobin that had been pre-treated with and without KCN and $K_3Fe(CN)_6$. Pretreatment with and without KCN and $K_3Fe(CN)_6$ was used to selectively

remove the NO from heme while preserving the S-nitrosothiol bond (Figure 1). A molar excess of $KCN/K_3Fe(CN)_6$ (0.2 M) oxidizes the Fe-NO to an intermediate species not detectable in the chemiluminescent I_3^- reaction, while preserving the S-NO bond of SNO-Hb.[9] Injection of samples into this I_3^- reactant stoichiometrically releases NO with linear sensitivity for SNO-Hb, and $HbFe^{II}NO$ from 0.001% to 100% (SNO-Hb $r^2 = 0.996$, $p < .001$, $n = 5$; Hb(FeII)NO $r^2 = 0.999$, $p < .001$, $n = 5$).[9] Results are corrected for hemoglobin concentration and expressed as nM concentration in whole blood.

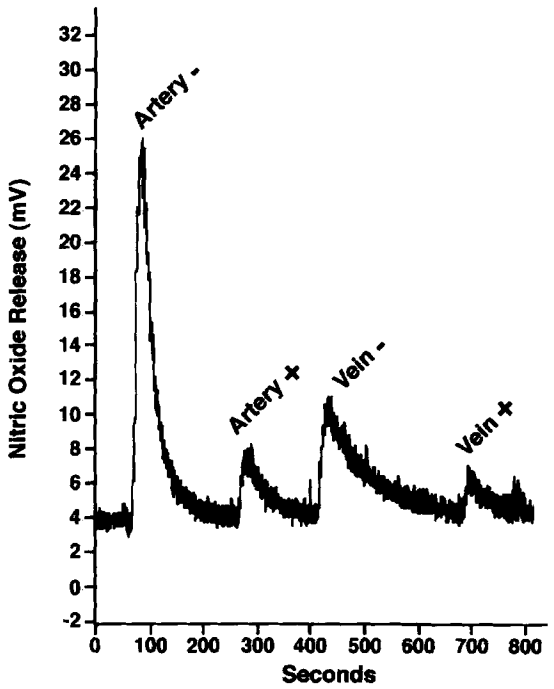

FIGURE 1 Levels of $HbFe^{II}NO$ and SNO-Hb in a volunteer with sickle cell anemia, breathing 80 ppm inhaled NO, measured by the I_3^-/ozone-based chemiluminescent assay. This demonstrates the effects of KCN and $K_3Fe(CN)_6$ pretreatment, which selectively removes the NO from heme without affecting NO covalently bound to cysteine 93, on the signal of NO released from hemoglobin in I_3^- from an individual with sickle cell anemia breathing 80 ppm NO for two hours. KCN and $K_3Fe(CN)_6$ pretreatment is indicated by a (+) sign and no pretreatment by a (−) sign. A significant reduction in the NO signal after KCN and $K_3Fe(CN)_6$ pretreatment provides evidence that the majority of the NO associated with hemoglobin is bound to the heme group $HbFe^{II}NO$. The residual signal is still greater than baseline suggesting that SNO-Hb does form at lower levels. Arterial (A) – venous (V) gradients are observed.

RESULTS AND DISCUSSION

To determine whether the NO is transported as $HbFe^{II}NO$ or SNO-Hb, we measured these two hemoglobin species in three sickle cell volunteers, and similar to previous observations in normal individuals, the predominant species formed was $HbFe^{II}NO$ (Figures 1 and 2), in addition to methemoglobin formation which increased from 0.26 to 1.2%.[12] Mean baseline levels of $HbFe^{II}NO$ were undetectable in arterial and venous blood; during NO breathing there was a significant dose dependent increase in arterial and venous levels, with a significant arterial-venous gradient developing at all three doses of inhaled NO. The mean (±SEM) levels in the arterial circulation were 1,270 ± 119 nM, 2,514 ± 73 nM and 3,856 ± 68 nM at 40, 60 and 80 ppm NO respectively, while the mean levels in the venous circulation were 570 ± 93 nM, 1,199 ± 39 nM and 1,479 ± 256 nM at 40, 60 and 80 ppm NO respectively. The significant arterial-venous difference at all NO doses suggests a very rapid dissociation rate of NO from heme with possible delivery of NO to the tissues.

These results demonstrate that NO-heme reaction pathways predominate in patients with sickle cell anemia and suggest that NO binding to heme groups is a rapidly reversible process, supporting a revised model of hemoglobin delivery of NO in the peripheral circulation. Direct NO, NO+, or nitrite release from $HbFe^{II}NO$, with SNO-Hb possibly serving as an intermediary, may explain the peripheral vascular effects reported during NO breathing and represent a salvage pathway for maintenance of vascular flow in regions of tissue stress.[9] Studies to determine if the delivered NO from heme results in physiologic increases in vascular flow are in progress.

The physiological implications of $HbFe^{II}NO$ formation in sickle cell individuals are unclear.

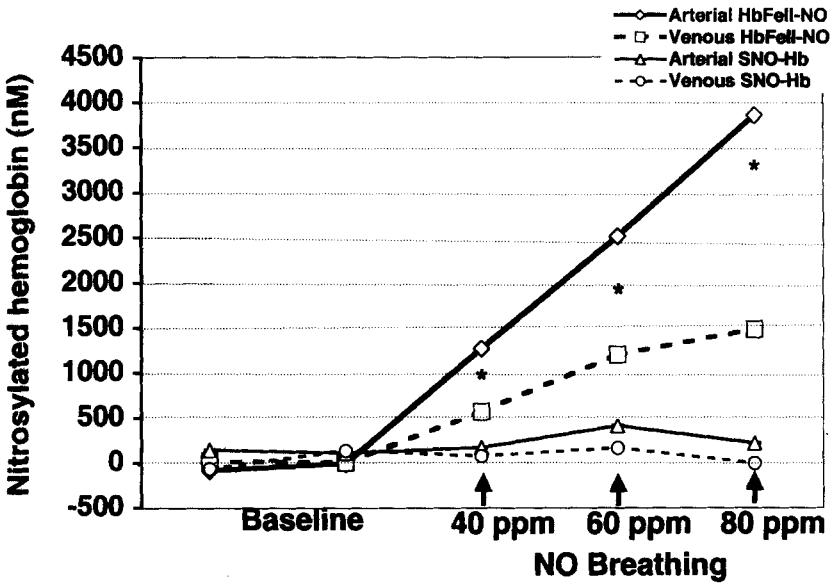

FIGURE 2 Nitric oxide inhalation augments $HbFe^{II}NO$ formation with minimal SNO-Hb formation. Mean baseline levels of $HbFe^{II}NO$ and SNO-Hb were nearly undetectable in the arterial and venous circulation. During dose titration of 40, 60, and 80 ppm inhaled nitric oxide there was a significant dose-dependent increase in arterial and venous $HbFe^{II}NO$, with a significant arterial-venous gradient developing at all three doses of inhaled NO ($^*p < .05$). Mean ± the standard error of the mean $HbFe^{II}NO$ levels in the arterial circulation (solid line, diamonds) were 1,270 ± 119 nM, 2,514 ± 73 nM and 3,856 ± 68 nM at 40, 60 and 80 ppm NO respectively, while the mean levels in the venous circulation (dashed line, squares) were 570 ± 93 nM, 1,199 ± 39 nM and 1,479 ± 256 nM at 40, 60 and 80 ppm NO, respectively. NO inhalation resulted in minimal SNO-Hb formation with arterial levels (solid line, triangles) of 172 ± 61 nM, 412 ± 146 nM, and 217 ± 62 nM, at 40, 60 and 80 ppm NO respectively. Venous levels (dashed line, circles) were slightly lower but this difference was not significant in the three individuals studied.

Yonetani and his research group and Kosaka and colleagues have shown that upon initial NO binding, $HbFe^{II}NO$ is a 6-coordinate species (iron binds four nitrogens, a proximal histidine, and NO) with an increased affinity for oxygen. However, upon deoxygenation of the other heme groups, the proximal histidine bond stretches or breaks, forming a 5-coordinate species with a significantly lowered oxygen affinity.[13–17] $HbFe^{II}NO$ also demonstrates an enhanced Bohr effect, promoting oxygen release and increasing sensitivity to tissue acidosis.[13] On one hand, despite the loss of an oxygen binding site (replaced by NO), overall $HbFe^{II}NO$ may more effectively deliver oxygen than pure oxyhemoglobin to regions with very low oxygen tensions[16] but on the other hand, these events could promote hemoglobin S polymerization.[18]

NO inhalation resulted in minimal SNO-Hb formation (Figures 1 and 2). Mean baseline levels of SNO-Hb were at the limits of detection (149 ± 90 nM in the arterial and 169 ± 30 nM in the venous circulation), and during NO breathing these values increased slightly, but not significantly, in the arterial circulation to 172 ± 61 nM, 412 ± 146 nM, and 217 ± 62 nM at 40, 60 and 80 ppm NO respectively. An insignificant trend toward an arterial-venous gradient was observed (Figure 2). The levels of SNO-Hb measured are not consistent with significant transport of NO using this pathway, but they do provide evidence that this pathway exists.

While we have been unable to document an increase in hemoglobin S oxygen affinity with 80 ppm NO gas treatment *in vivo*[8] or *in vitro*, other investigators have reported an increase in hemoglobin S oxygen affinity,[19] a finding potentially attributable to the formation of SNO-Hb.[20, 21] Indeed, pure synthesized SNO-Hb has an increased oxygen affinity consistent with

the energetic linkage of β-cysteine 93 ligands and β-heme oxygen binding.[4,22] It is clear from these studies that high level modification of hemoglobin with NO is necessary to increase oxygen affinity, an event that does not occur during 80 ppm NO inhalation. Modification of β-globin cysteine 93 to form a mixed disulfide, such as glutathionyl-hemoglobin, interferes with polymerization, increases oxygen affinity, and inhibits sickling. Again, this effect requires a high proportion of reacted hemoglobin (ranging from 8 to 25% labile glutathionyl-hemoglobin and greater than 95% irreversible covalent modification with thiols)[23,24] and we observed only an absolute 0.0012% increase in arterial SNO-Hb formation. Other NO donors, such as S-nitroso-penicillamine, appear to nitrosate the cysteine residue more effectively than NO gas.[22,25] The demonstration that SNO-Hb becomes measurable during NO breathing does suggest that this pathway exists *in vivo* and efforts to increase the level of cysteine 93 modification with other NO donors seems a promising strategy.

In conclusion, nitric oxide binds primarily to the heme groups of hemoglobin S during NO inhalation. Arterial-venous gradients in $HbFe^{II}NO$ suggest that delivery occurs and studies to evaluate peripheral effects of NO inhalation in normal and sickle cell individuals are indicated. Whether NO and oxygen delivery effects of $HbFe^{II}NO$ can enhance perfusion to ischemic regions and outweigh a potential reduction in nitrosylated hemoglobin S oxygen affinity (during deoxygenation) remains to be determined.

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