Inhaled Nitric Oxide Improves Survival Rates during Hypoxia in a Sickle Cell (SAD) Mouse Model

Ricardo Martinez-Ruiz, M.D.,* Pedro Montero-Huerta, M.D.,† Jonathan Hromi, M.S.,† C. Alvin Head, M.D.‡

Background: The hallmark of sickle cell disease (SCD) is erythrocyte sickling during deoxygenation of the abnormal hemoglobin S (HbS). When HbS is deoxygenated, it aggregates into polymers, resulting in distortion of the erythrocyte structure, producing microvascular thrombosis and ischemia. The transgenic SAD mouse produces three types of human hemoglobin: S, Antilles, and D-Punjab (HbSAD) and provides an animal model for SCD. We studied the effects of nitric oxide (NO) breathing at various doses and time regimens in the presence of severe hypoxia (6% oxygen) using the SAD mouse model.

Methods: Age- and sex-matched control and SAD mice were exposed to 6% oxygen breathing in an environmental chamber and assessed for survival up to 1 h. Animals received different inhaled NO concentrations before and/or during hypoxia. Blood was obtained to evaluate the oxyhemoglobin dissociation curve and measure methemoglobinemia.

Results: Pretreatment by breathing NO at 20 ppm by volume in air for 30 min, and continuing to breathe 20 ppm NO during hypoxia resulted in improvement in survival rates in the SAD mouse (75%, n = 8) as compared with control SAD mice (11%, n = 9; P < 0.001). Pretreatment alone or breathing lower doses of NO were not protective. Changes in HbSAD oxygen affinity were not detected with NO breathing, and methemoglobin levels were low in all surviving mice.

Conclusions: Breathing NO produced a rapid, protective effect to severe hypoxic stress in SAD mice. There appears to be a required loading period between NO breathing and its beneficial effect during hypoxic stress, possibly because of the total amount of NO delivered to SAD hemoglobin, blood cell components, and endothelium. NO breathing may be beneficial as a therapeutic intervention in SCD.

SICKLE cell disease (SCD) is a molecular disease produced by the replacement of the amino acid glutamic acid by the neutrally charged valine in the sixth position of the hemoglobin chain. When sickle hemoglobin (HbS) is deoxygenated, it aggregates into large polymers, resulting in distortion of the shape of the erythrocyte and a marked decrease in its deformability. These rigid cells are responsible for the vaso-occlusive phenomena that are the hallmark of this disease. In addition, adhesive interactions between sickle erythrocytes and the microvasculature are likely to play an important ancillary role in the pathogenesis of SCD. Even though the disease is produced by a single DNA point mutation, the unpredictable nature of vaso-occlusion is probably a manifestation of the multiple and complex interactions of the sickle cell erythrocyte and the microvasculature, modulated by integrins, adhesion molecules, and plasma proteins.

Multiple therapeutic strategies are aimed at decreasing HbS polymerization, including pharmacologic increases of erythrocyte fetal hemoglobin, a reduction of the intracellular concentration of HbS, and chemical inhibition of HbS polymerization. Sickle hemoglobin has a low affinity for oxygen, partly because of increased levels of intra-erythrocytic 2,3-biphosphoglycerate and the presence of HbS polymers. Polymerization occurs with HbS in the deoxygenated state. Interventions that increase HbS affinity for oxygen will have the beneficial effect of reducing the tendency of erythrocytes to sickle. Carbon monoxide and cyanate have been used to increase the affinity of HbS for oxygen and thereby reduce sickling in vivo but are too toxic to be used in vivo. Head et al. reported that low-dose inhaled nitric oxide (NO) increases the oxygen affinity of human HbS erythrocytes in vitro and in vivo as assessed by oxygen hemoglobin dissociation curves (ODCs). Production of methemoglobin was minimal, and no complications were noted in human volunteers breathing 80 ppm NO gas for 45 min. The increase in erythrocyte HbS oxygen affinity or decrease of oxygen tension at 50% saturation (P50) was of sufficient magnitude to favorably improve the pathophysiology of SCD.

Transgenic murine models have been used to investigate the pathogenesis and assess therapeutic interventions in SCD. Some of these models have produced an erythrocyte that will sickle during hypoxia in vitro, but irreversible sickle cells in vivo are rarely observed. SAD mice express three human hemoglobin mutations: S, Antilles, and D-Punjab with the human-globin gene, resulting in 15–26% HbSAD expression in the erythrocyte of these transgenic mice. These groups of mutations have created a transgenic mouse that, when stressed by hypoxia, develops the phenotypic end organ damage of SCD.

We studied the tolerance of SAD mice to acute, severe hypoxia stress with and without breathing NO gas using
inspired oxygen [FIO2] protocol I, SAD mice breathed NO in air (fraction of oxygen after three different NO exposure protocols. In were exposed to NO gas and evaluated for survival in 6% oxygen, and survival was assessed for 1 h. In the SAD 5 and a SAD control group (n = 59) were divided into groups (table 1) and exposed either to hypoxia (6% oxygen) or hypoxia plus NO gas, as described below. Survival was recorded up to 1 h or until death. A 500-ml environmental chamber was used with a continuous flow of oxygen, nitrogen, and NO gases (average total flow, 8 l/min).

Materials and Methods

Animals
A colony of 70 SAD mice was propagated from a breeding pair provided by Dr. J. L. Degen. All mice (body weight < 25 g) were housed in a viral-free environment in standard approved chambers (5 mice/cage). Mice ate and drank ad libitum. Genotyping was performed with polymerase chain reaction. The Massachusetts General Hospital Subcommittee on Research Animal Care approved all protocols. All mice were sex- and age-matched for comparison. SAD mice (n = 59) were divided into groups (table 1) and exposed either to hypoxia (6% oxygen) or hypoxia plus NO gas, as described below. Survival was recorded up to 1 h or until death. A 500-ml environmental chamber was used with a continuous flow of oxygen, nitrogen, and NO gases (average total flow, 8 l/min).

Protocols
There were two control groups: A wild-type control group (n = 8) of the same SAD mouse strain (black C57) and a SAD control group (n = 9) were exposed to 6% oxygen, and survival was assessed for 1 h. In the SAD mice breathing 6% oxygen, six SAD mice groups (A–F) were exposed to NO gas and evaluated for survival in 6% oxygen after three different NO exposure protocols. In protocol I, SAD mice breathed NO in air (fraction of inspired oxygen [FiO2] = 0.21) for 30 min before 6% oxygen exposure and continued breathing NO during the entire 1-h hypoxic period (group A: n = 8, 20 ppm NO; group B: n = 8, 10 ppm NO). In protocol II, SAD mice breathed NO in air for 30 min, and then NO was discontinued when the 1-h hypoxic breathing started (group C: n = 5, 20 ppm NO). In protocol III, inhaled NO began with the 1-h 6% oxygen breathing without preexposure to NO (group D: n = 9, 20 ppm NO; group E: n = 10, 40 ppm NO; group F: n = 10, 60 ppm NO).

Table 1. Protocol Design and Results

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>NO Prehypoxia</th>
<th>NO During Hypoxia</th>
<th>NO Dose (ppm)</th>
<th>Survival (%)</th>
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<td>0</td>
<td>100</td>
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<tr>
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<td>0</td>
<td>11</td>
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<td>X</td>
<td>20</td>
<td>75*</td>
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<tr>
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<td>X</td>
<td>X</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
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<td>X</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>SAD D</td>
<td>9</td>
<td>X</td>
<td>20</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>SAD E</td>
<td>10</td>
<td>X</td>
<td>40</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>SAD F</td>
<td>10</td>
<td>X</td>
<td>60</td>
<td>30</td>
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</table>

Prehypoxia period consisted of 30 min of breathing 21% oxygen with or without nitric oxide (NO) gas. Hypoxic period consisted of 60 min of breathing 6% oxygen with or without NO gas. X indicates the mice received NO gas during that time period. The wild-type (WT) control group survived the entire hypoxic study period.

* Group A survival rate was significantly different (P < 0.05) from all other groups, except the WT control, group E, and group F. ppm = parts per million by volume; SAD = SAD transgenic mice.

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Gas Administration
Oxygen was diluted with 100% nitrogen using rotameters to deliver either air (FiO2 = 0.21) or a hypoxic gas mixture (FiO2 = 0.06) into the breathing chamber. The oxygen concentration was continuously monitored inside the chamber (ServoMex 570A, Crowborough, Sussex, United Kingdom). NO at 800 ppm by volume in nitrogen was obtained from INO Therapeutics, Inc. (Clinton, NJ). NO was administered via a rotameter into the breathing chamber. The nitrogen gas was adjusted to maintain the same FiO2 and deliver the proper NO concentration. The concentrations of NO and nitrogen dioxide within the breathing chamber were continuously monitored with electrochemical NO and nitrogen dioxide analyzers (model SAAN TM-100; Taiyo Sanso, Tokyo, Japan).

SAD Mice Time-matched Death Group
Another group of SAD mice (n = 4) of the same sex and age were studied to compare erythrocyte morphology and tissue histology with and without NO exposure during 6% oxygen breathing. The methods and NO dose were the same as in group A and the SAD control group. The SAD mice were placed in adjacent chambers, group 1 without NO (n = 2) and group 2 with 20 ppm NO (n = 2). Mice in the NO-exposed group were killed in a paired fashion with the SAD mice without NO exposure (such that at each time a non–NO-exposed mouse died, a NO-exposed mouse was euthanized). Euthanasia was performed by cervical dislocation. This technique reduces the effect of hypoventilation secondary to sedation. After they were euthanized, the mice were immediately weighed and dissected. The heart, lungs, liver, spleen, and kidneys were fixed in 10% phosphate-buffered formaldehyde. Tissue samples were embedded in paraffin according to standard methods. Sections were cut and stained for light microscopy. This permitted a time-matched comparison between SAD mice with and without NO exposure.

Erythrocyte Structure
Tail blood samples were obtained for erythrocyte structure study before and after hypoxia. Blood was fixed with a buffered formaldehyde solution. Cells were placed in a grid-rule slide, and 500 erythrocytes were counted using light microscopy. The percent erythrocytes that were sickled, deformed, or normal were compared between prehypoxic and posthypoxic samples with and without NO exposure.
In Vivo Oxygen Hemoglobin Dissociation Curves and Methemoglobinemia Determination

Oxygen hemoglobin dissociation curves were determined in an additional five SAD mice before and after 60-min exposure to 20 ppm NO breathing at 21% O2 (as described above). Tail blood (50 μl) was collected and diluted with 4 ml of phosphate buffer, 10 μl antifoam solution, and 20 μl 20% albumin. Blood was desaturated by exposure to 100% nitrogen gas and then reoxygenated with air using a Hemox analyzer (TCS Medical Products Co., Huntingdon Valley, PA) to measure the ODC, as previously reported.16 The P50 was determined as the partial pressure of oxygen at 50% oxyhemoglobin saturation. Another sample collected in a microcapillary tube was used to determine methemoglobin concentration (before and after NO exposure) using a CO-Oximeter (model 270; Ciba Corning, Medfield, MA). An additional two SAD mice received 60 ppm NO for 60 min, and the methemoglobin level was determined.

Statistical Analysis

Data are expressed as mean ± SEM, except where indicated; the Kaplan-Meier method with log-rank tests was used to compare the 1-h survival rate between groups. The Student t test was used for P50 comparisons. The level of significance was P < 0.05.17

Results

The results are summarized in table 1 and figure 1. In the control groups, all wild-type mice (n = 8) survived for the entire 60-min study period breathing 6% oxygen, whereas the SAD control mice (n = 9) died with an average survival time of 8.2 ± 1.2 min, except for one SAD mouse that survived the entire 60-min period.

In protocol I, in group A (n = 8, 20 ppm NO), six mice survived for the entire hour of hypoxic breathing. Two mice died, with an average survival time of 23.1 ± 2.8 min. As shown in figure 1, the survival time in this group was significantly longer than in SAD controls (P < 0.001), group B (P < 0.001), group C (P = 0.011), group D (P = 0.001), group E (P = 0.026), or group F (P = 0.026). Similarly, the survival rate at 60 min (75%) was significantly higher than in the SAD control group (11.1%) or groups B (0%) or D (11.1%) (P < 0.001, P = 0.007, and P = 0.015, respectively; table 1). In group B (n = 8, 10 ppm NO), all of the mice died, with an average survival time of 5.9 ± 0.8 min. The survival time was shorter when compared with the SAD control group (P = 0.12, fig. 1), even though the survival rate at 60 min was not significantly different (0% vs. 11.1%; P = NS; table 1).

In protocol II, in group C (n = 5, 20 ppm NO), one mouse survived and four mice died, with an average survival time of 6.2 ± 1.9 min. Survival time was not significantly different from the SAD control group (fig. 1), nor was the survival rate at 60 min (20% vs. 11.1%; P = NS; table 1).

In protocol III, in group D (n = 9, 20 ppm NO), one mouse survived and eight mice died, with an average survival time of 7.8 ± 0.5 min. Survival time was not significantly different than the SAD control group (fig. 1), nor was the survival rate (11.1% vs. 11.1%; P = NS; table 1). In group E (n = 10, 40 ppm NO), three mice survived. The average survival time among those who died was 9.0 ± 4.6 min. The survival rate was not significantly different from the SAD control group (fig. 1), nor was the survival rate (11.1% vs. 11.1%; P = NS; table 1).
significantly different from the SAD control group (table I). In group F (n = 10, 60 ppm NO), three mice survived the hypoxic period. Average survival time was 6.9 ± 1.1 min. Survival rate was not significantly different from the SAD control group (table I).

In the SAD mice time-matched death group, in both control and NO groups, there were no gross histologic differences between the tissue sections. With regard to erythrocyte structure, there were no significant differences between the percentage of sickled or deformed cells in the posthypoxic period as compared with the prehypoxic samples with or without NO breathing.

The P50 values averaged 48 ± 4.5 mmHg before NO breathing and were not significantly changed after NO breathing (P > 0.05). Methemoglobin levels were less than 1.5% in five mice breathing 20 ppm NO in air for 60 min. Two mice breathing 60 ppm of NO in air for 60 min had methemoglobin levels of 7.1 ± 1.2%.

Discussion

We have shown improved survival rates of transgenic (SAD) sickle cell mice when these mice breathed 20 ppm NO gas in air for 30 min before and continued 20 ppm NO during acute, severe hypoxic gas exposure. Unlike other therapies for SCD, the speed at which NO can confer protection and survival in this animal model is within minutes. Minimal amounts of circulating methemoglobin (< 1.5%) were measured at the optimal NO dose of 20 ppm, suggesting that the beneficial effect was not directly related to the methemoglobin concentration.

Our study evaluates the ability of inhaled NO to improve outcome and allow survival during hypoxic exposure of SAD mice. Death as the primary end point was used because of the difficulty of assessing the sickling phenomena in acute studies. This is demonstrated in our study, showing no significant differences in circulating erythrocytes or time-matched tissue changes after short severe hypoxic stress, with or without NO exposure. Long-term exposure to milder hypoxia for longer time intervals may produce different results. However, another study successfully used death as an end point using this SAD animal model, demonstrating therapeutic effects.

The salutary effects of inhaled NO appeared to be rapid and dose-dependent. Group A was pretreated with 20 ppm in air and continued breathing NO during hypoxia. This group tolerated hypoxia as well as the control wild-type mouse. However, mice in group B, which breathed 10 ppm NO before and during hypoxia, did not have a survival benefit as compared with those in group A, which breathed a higher NO dose (20 ppm).

The strategy of breathing NO before and during hypoxia, as in protocol I, was based on our hypothesis that nitrosylated HbSAD prevents and/or melts HbSAD polymers within the erythrocyte and thereby prevents sickling and improves survival. Based on our previous work using human HbS erythrocytes in vitro, we noted that a critical time of NO gas exposure was necessary to increase erythrocyte oxygen affinity. Therefore, mice were “loaded” with inhaled NO for 30 min before hypoxic exposure. In addition, we found that it was vital to continue NO breathing during hypoxia. As postulated by Stamler et al., oxygen delivery to tissue may be improved by microvascular dilation by NO or S-nitrosothiols carried by hemoglobin, especially in hypoxic regions. As oxygen is unloaded in the microvasculature, NO is also unloaded. Therefore, NO may be required to be replenished during hypoxic stress. This would maintain adequate microvascular blood flow during crisis with impaired flow. Interestingly, increased vascular tone has been implicated as a factor in erythrocyte sickling. In addition, hypoxia has been associated with increased adherence of sickle erythrocytes to the endothelium. Therefore, hypoxia and increased vascular tone may accentuate occlusion of the vascular lumen by adhering sickled erythrocytes. Preexposure to NO breathing may ameliorate some of the effects produced by acute hypoxia in the microcirculation. In a recent article by Space et al., using a parallel plate flow chamber, NO was shown to attenuate sickle erythrocyte adherence to pulmonary endothelium. A combination of decreased erythrocyte sickling by a reduction in HbSAD polymerization coupled with improved microvascular flow conditions may reduce the erythrocyte transit time and reduce deoxy-HbSAD erythrocyte levels within microvessels.

In protocol II, we explored whether preexposure to NO alone would be protective. We attempted to load the HbSAD erythrocyte by breathing 20 ppm NO in air before hypoxia, and then we ceased NO breathing during the hypoxic exposure. The turnover rate of NO and S-nitrosothiol on HbsAD is unknown, but is probably as high as in HbA. Therefore, NO and S-nitrosothiol would be quickly exhausted and would not protect during the prolonged hypoxic study period. This was demonstrated in group C, where the same dose of 20 ppm NO, which was protective in group A, did not improve survival when NO was not replenished during the hypoxic stress. Therefore, there appears to be a critical level of NO availability in the lung and blood that is necessary to demonstrate beneficial effects during acute, severe hypoxia using this murine model. An insufficient NO exposure time or a low NO dose, in our current work 10 ppm, does not appear to convey a protective effect and augment survival. Studies of the kinetics and binding of NO at therapeutic doses are needed in the intact erythrocyte.

In protocol III, we examined whether survival would be improved if NO gas was given only during hypoxia without prior NO exposure. We administered 20, 40,
and 60 ppm NO during hypoxia and found that there was a trend toward better survival at 40 and 60 ppm, but this did not reach statistical significance. Higher inhaled NO doses in mice produce higher methemoglobin levels, with 60 ppm NO breathing for 60 min producing 7.1 ± 1.2%.

Nitric oxide gas has been reported to increase human HbS erythrocyte oxygen affinity both in vitro and in stable volunteers, independent of methemoglobin production or apparent changes in 2,3-bisphosphoglycerate concentration. NO may stabilize the HbS heme structure, possibly in the R-state, and therefore may reduce HbS polymerization. McDade et al. (personal communication, October 1997) have shown that NO increases the solubility of deoxy-HbS solutions and promotes unsickling of erythrocytes in vitro. The precise site of NO and HbS interaction remains uncertain. It has been postulated that NO binds either at the -Cys93 site or the amino terminal valine and/or possibly other amino groups within the 2,3-bisphosphoglycerate cleft. NO binding at these sites could alter HbS polymerization.

Increased HbSAD erythrocyte oxygen affinity was not detected in this animal model after NO breathing. This lack of oxygen affinity augmentation (reduction of P50) differs from our previous study of human homozygous HbS erythrocytes. Gladwin et al., studying SCD patients, did not detect a P50 change after 2 h of NO breathing. They attributed the lack of a P50 change to the generation of low levels of nitrosylated HbS. However, Bonaventura et al. have shown, using purified HbS in vitro, that nitrosylated Hbs leads to an increase in oxygen affinity. Additional studies are needed to better understand these differences. Small animals such as mice have high specific metabolic rates as compared with humans, and their hemoglobin has a low affinity for oxygen to facilitate its unloading to tissue. SAD mice have ODCs further shifted to the right with even less affinity for oxygen than a wild-type mouse. The nature of the parent hemoglobin and its environment makes it possible for nitrosylated hemoglobin forms to express varied oxygen affinities. The lack of an oxygen affinity change may also reflect the limited amount of HbSAD, approximately 19%, in the SAD mouse erythrocyte. Finally, NO may have “washed out” because of the severity of hypoxic stress. Although NO may be modifying the HbSAD oxygen dissociation, these changes may be below the sensitivity of our analyzer.

We did not observe any significant difference in tissue sections with or without NO exposure. This is not surprising, as tissue samples may not reflect acute changes at the microvascular level. In the brief time required to kill an animal and obtain and fix tissues, significant hypoxia can occur. This would preclude the possibility of visualizing any beneficial or deleterious effects of an intervention as compared with controls. In addition, we did not find evidence of decreased sickling in the peripheral-blood samples of animals exposed to NO as compared with those not receiving NO during hypoxia. Again, the amount of sickling in peripheral venous samples may not closely reflect acute changes at the tissue level, as deformed erythrocytes can be selectively trapped in the microvasculature.

In normal humans and sheep, inhaled NO completely reverses acute hypoxic pulmonary vasoconstriction during hypoxic breathing. Pulmonary vasodilation reduces right heart strain and improves right ventricular performance during hypoxic stress. We did not measure pulmonary artery pressure; however, in other mice studies with low environmental oxygen, 20 ppm NO has been shown to reduce pulmonary artery pressure by attenuating hypoxic pulmonary vasoconstriction. In our group D, SAD mice breathing 20 ppm NO in 6% oxygen without preloading of NO did not have an improved survival. Because this NO concentration should have reduced pulmonary artery pressure, it is unlikely that the attenuation of pulmonary artery pressure elevations alone during NO breathing was sufficient to increase the murine survival rate.

Nitric oxide breathing improves ventilation-perfusion matching in some clinical settings. This has been shown in SCD patients with acute chest syndrome. We believe these effects would not play a major role augmenting survival in our study, because in the presence of environmental hypoxia, there would not be improved systemic oxygenation associated with improvement of ventilation-perfusion matching during NO breathing.

Inhaled NO may produce other beneficial effects such as inhibiting platelet aggregation and adhesion. A nocuous interaction between the endothelium, platelets, and HbS erythrocytes also contributes to vaso-occlusive pathophysiology in SCD. Platelets display an activated state in this disease, with increased circulating levels of platelet-activating factor and platelet-derived adhesion molecules. Animal models have demonstrated platelet adhesion is reversibly inhibited by NO breathing via the NO–cyclic guanosine monophosphate pathway. It is interesting to note that SCD patients in crisis, who have higher levels of plasma NO metabolites, have lower pain scores. Studies assessing sickle erythrocyte adhesiveness to cerebral blood vessels in rats have also shown increased adhesion to the microvasculature. Inhibition of NO synthase in this model produced total obstruction of blood flow within minutes. Although we could not determine the exact cause of death in our study, it is likely that in survivors, a reduction of platelet and erythrocyte adhesion to the endothelium plays an important role.

In summary, NO breathing produced an improvement in survival rates in the sickle cell (SAD) mouse when given both before and during acute hypoxia compared with those without NO breathing. The onset of this protective effect is rapid (minutes) and requires loading...
with a sufficient NO dose before hypoxia exposure. Multiple mechanisms and sites (e.g., hemoglobin interactions, vascular and blood cell components) may be involved in the beneficial effects of inhaled NO. Therefore, inhaled NO may be beneficial as a therapeutic intervention for SCD. Further studies are needed and ongoing to define mechanisms responsible for this apparent improvement in survival.

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References