

Detection of Nitrosyl Hemoglobin in Venous Blood in the Treatment of Sick Cell Anemia with Hydroxyurea

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ABSTRACT

The clinical efficacy of hydroxyurea (HU) in the treatment of sickle cell anemia has mainly been attributed to increased levels of fetal hemoglobin (HbF), which reduces the tendency for sickle hemoglobin to polymerize, thereby reducing the frequency of the vaso-occlusive phenomena associated with the disease. However, benefits from HU treatment in patients have been reported in advance of increased HbF levels. Thus, it has been suggested that other hydroxyurea-dependent mechanisms may, in part, account for its clinical efficacy. We have previously demonstrated that HU is metabolized in rats to release nitric oxide and, therefore, postulated the same to

occur in humans. However, to our knowledge, evidence of nitric oxide production from HU metabolism in humans has yet to be demonstrated. Here we report that oral administration of HU for the treatment of sickle cell anemia produced detectable nitrosyl hemoglobin. The nitrosyl hemoglobin complex could be detected as early as 30 min after administration and persisted up to 4 h. Our observations support the hypothesis that the ability of HU to ease the vaso-occlusive phenomena may, in part, be attributed to vasodilation and/or decreased platelet activation induced by HU-derived nitric oxide well in advance of increased HbF levels.

Sickle cell anemia was the first disease to be characterized at the molecular level (Pauling et al., 1949). The fault has since been found to be on the gene encoding the human β -globin subunit, with the resulting replacement of $\beta 6$ glutamic acid by valine (Bunn, 1997). In a low-oxygen-tension environment, the valine replacement can bind to a complementary hydrophobic site on a β subunit of another hemoglobin tetramer in a polymerization process that leads to the sickling of the red blood cells. Polymerization of deoxygenated sickle hemoglobin (HbS) tetramers is central to the process of vaso-occlusion (Kaul et al., 1996; Bunn, 1997). However, it is likely that other factors are involved in the pathology of the sickle cell disease. These include increased adherence of sickle red blood cells to vascular endothelium modulated by plasma proteins (Brittain et al., 1993; Duits et al., 1996), cytokines (Croizat, 1994; Natarajan et al. 1996), and platelet activation (Westwick et al., 1983; Wun et al., 1997), along with endothelial (Solovey et al., 1997) and vaso-motor tone (Rodgers et al., 1993) abnormalities.

Sickle cell-induced infarction as a result of inadequate tissue perfusion (i.e., vaso-occlusion) can contribute to the pain crisis, organ dysfunction, and, in severe cases, even death. Hydroxyurea (HU), a ribonucleotide reductase inhibitor (Nyholm et al., 1993) used to treat a variety of myeloproliferative disorders (Lofvenberg and Wahlin, 1988), has

gained increased importance in the management of sickle cell anemia (Charache, 1991; Orringer and Parker, 1992). The efficacy of HU has been mainly attributed to its ability to stimulate the production of fetal hemoglobin (HbF) in patients with sickle cell anemia (Goldberg et al., 1990; Rodgers et al., 1990), thereby decreasing the concentration of HbS and reducing the number of painful episodes. However, clinical benefits from HU treatment in some patients have been reported in advance of increased HbF levels (Orringer and Parker, 1992; Charache et al., 1996). Thus, it has been suggested that other HU-dependent mechanisms may, in part, account for its clinical efficacy. The exact mechanism(s) by which HU induces increased HbF levels and other HbF-independent changes that are observed and contribute to its efficacy in the treatment of sickle cell anemia remains to be fully elucidated.

Increased nitric oxide (NO) formation detected as increased serum nitrite levels in steady-state sickle cell disease and during the acute painful crisis (Rees et al., 1995) has been suggested as a compensatory mechanism required to improve delivery of oxygen to the tissues in the chronic anemic state. Selective vasodilation by diminishing the entrapment of sickle erythrocytes might be expected to decrease some of the hemolytic and vaso-occlusive manifestations of the sickle cell disease (Rodgers et al., 1988). Recently,

ABBREVIATIONS: HU, hydroxyurea; HbNO, nitrosyl hemoglobin; NO, nitric oxide; EPR, electron paramagnetic resonance; HbS, sickle hemoglobin; HbF, fetal hemoglobin; G, Gauss; g, g-factor.

we have shown that HU is metabolized *in vivo* by the rat to release NO (Jiang et al., 1997). It is therefore probable that HU-derived NO may, in part, account for the clinical efficacy observed by mediating events such as inhibition of platelet activation and/or vasodilation. However, to date, no evidence for NO production from HU administration has been demonstrated in humans.

In this report, electron paramagnetic resonance (EPR) spectroscopy, also known as electron spin resonance, was used to present evidence for HU metabolism to NO in the treatment of sickle cell anemia. Specifically, we took advantage of the binding of NO to deoxyhemoglobin to yield characteristic nitrosyl hemoglobin (HbNO) EPR spectra at 77 K.

Materials and Methods

HU Administration. All protocols were approved by the National Institute of Environmental Health Sciences Institute Review Board. A 26-year-old male with homozygous sickle cell anemia (141 lb or 64 kg) who had been on HU (Bristol-Myers Squibb, Princeton, NJ) treatment for 2 years gave informed consent to participate in this clinical trial. At the beginning of the study, blood was collected into vacuum tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ). Whole blood was transferred into a 1-ml Monoject syringe (Sherwood Medical, St. Louis, MO) and immediately frozen. Red blood cells (1 ml volume) were obtained by centrifugation of whole blood at 4°C without further washing and were immediately frozen in a 1-ml Monoject syringe. His normal oral dose of 1 g/day HU (16 mg/kg) was administered, and samples of whole blood and red blood cells were then obtained every 30 min for a period of up to 4 h. Red blood cells from three female sickle cell patients on a similar dose of HU were also collected, before and after (60–90 min) treatment. The samples were immediately frozen and stored at –80°C before EPR analysis.

Exposure of Whole Blood to Authentic NO. EDTA-treated whole blood (1 ml) obtained before HU administration was deoxygenated using N₂ gas for 30 min with continual stirring. The sample was then exposed to NO gas (National Specialty Gases, Durham, NC) for approximately 1 s (approximately 5 bubbles of NO gas), and the sample was degassed further with N₂ for an additional 2 h at 37°C. Treatment of blood with NO as just described was not expected to alter its pH significantly (Eriksson, 1994). The NO-treated blood (200 µl) was then transferred into a quartz EPR tube (3 mm i.d.) (Wilma, Buena, NJ), frozen immediately, and stored at –80°C before EPR analysis.

EPR Analysis. All EPR measurements were carried out at liquid nitrogen temperature with samples held in a quartz finger dewar (Wilma) using a Bruker ESP300 spectrometer (Bruker Instruments, Billerica, MA) operated at 9.5 GHz with 100-kHz modulation frequency. Typical spectrometer settings were 31.86 mW power, 5 Gauss (G) modulation amplitude, 5.242 s time constant, 2684 s scan time, and 300 G scan range. The EPR signal from Cr³⁺ was used as a g-factor (g) value marker (g = 1.9800 ± 0.0006) (Low, 1957).

Results

Typical EPR spectra obtained from venous whole blood before and 60 min after administration of 16 mg/kg HU are shown in Fig. 1, A and B, respectively. The broad signal at g = 2.06 has been attributed to Cu²⁺ arising from the serum protein ceruloplasmin (Hall et al., 1994). Figure 1C is the difference spectrum resulting from the spectral subtraction of Fig. 1A from B. The resulting spectrum shows a weak but resolved triplet hyperfine structure (g = 2.011) (Fig. 1C) with coupling of 16.8 G due to the ¹⁴N assigned to the HbNO complex. The HbNO complex could be seen in whole blood at

30 and 60 min after HU administration and, on one occasion, up to 2 h (data not shown). The data presented is consistent with that obtained when sickle blood was exposed to authentic NO gas (Fig. 1D).

Preferential binding of NO to the α subunit over the β subunit has been demonstrated by Henry and Cassoly (1973). The iron coordination of the α subunits is dependent on whether the β subunits are deoxygenated or oxygenated or, more generally, whether the hemoglobin is in the R “oxy-like” state or in the T “deoxy-like” state. These states correspond to the pentacoordinate and the hexacoordinate species, which are the dominant species in venous and arterial blood, respectively (Kosaka et al., 1994). The spectral features of sickle blood treated with authentic NO showed a combination of two HbNO species (Fig. 1D): the characteristic three-line hyperfine pattern at g = 2.011 of the pentacoordinate species and the hexacoordinate species, which can be seen as the trough at g = 1.986 (Westenberger et al., 1990). A combination of both the pentacoordinate- and the hexacoordinate-nitrosyl species can also be seen after HU treatment (Fig. 1C).

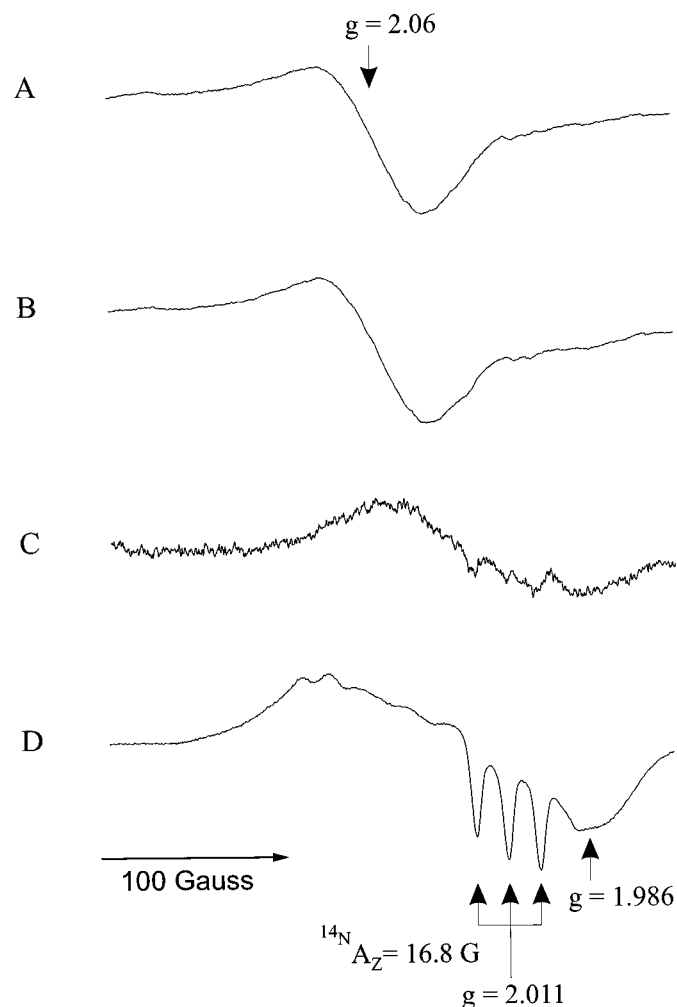


Fig. 1. Typical EPR spectra obtained from whole blood before (A) and 60 min after (B) 16 mg/kg HU administration (see *Materials and Methods* for EPR parameters). C, difference spectrum (gain $\times 5$) resulting from subtraction of A from B. D, spectrum obtained from whole blood exposed to authentic NO; spectral conditions were the same as for whole blood, except for 1 mW power, 655 ms time constant, and 671 s scan time.

To increase the concentration of the HbNO complex, red blood cells obtained by centrifugation were studied. Figure 2 shows typical spectra obtained from red blood cells after HU treatment. The broad signal at $g = 2.06$ arising from serum ceruloplasmin (Hall et al., 1994) associated with the red blood cells has been diminished relative to the whole blood spectrum (Fig. 1A). EPR spectra from red blood cells obtained before oral administration of HU also showed an unidentified free radical signal at $g = 2.005$ (Fig. 2). The unidentified radical was present in most of the samples with varying intensities (Svistunenko et al., 1997) and partially overlapped the HbNO signal of interest.

Figure 3 shows the difference spectra obtained by the subtraction of the predose spectrum from the spectra of samples collected after HU administration after given times. The triplet hyperfine structure ($g = 2.011$) with coupling of 16.8 G due to the ^{14}N assigned to the HbNO complex could be detected as early as 30 min after oral administration and persisted up to 4 h. The characteristic HbNO was also detected in red blood cells from three other sickle cell anemia

patients on a similar dose of hydroxyurea, 60 to 90 min after oral administration (data not shown). The time course of the formation of HbNO in the red blood cells (Fig. 4) was not inconsistent with the results of human pharmacokinetic studies, which showed that HU reaches peak plasma concentration at about 80 min after oral administration (Rodriguez et al., 1998). The HbNO appeared to have reached a steady-state concentration as early as 30 min after administration of HU.

Discussion

Sickle cell disease results from the inheritance of the sickle β -globin gene, in which the $\beta 6$ glutamic acid is replaced by valine. NO binding to HbS should not be affected because NO preferentially binds to the α subunit, which is normal. Previously, we demonstrated that HU metabolism in rats leads to the generation of NO (Jiang et al., 1997). In the four patients studied, we confirmed that HU is metabolized to release NO in the treatment of sickle cell anemia. The data presented show EPR spectra representative of a five-coordinate nitrosyl-heme complex derived from NO binding to deoxyhemoglobin. In particular, the three-line hyperfine coupling at $g = 2.011$, which results from HbNO complexes in which NO is bound to the heme iron of the α subunit, can be seen. The measured hyperfine coupling constant (16.8 G) and g value (2.011) are in good agreement with values reported in

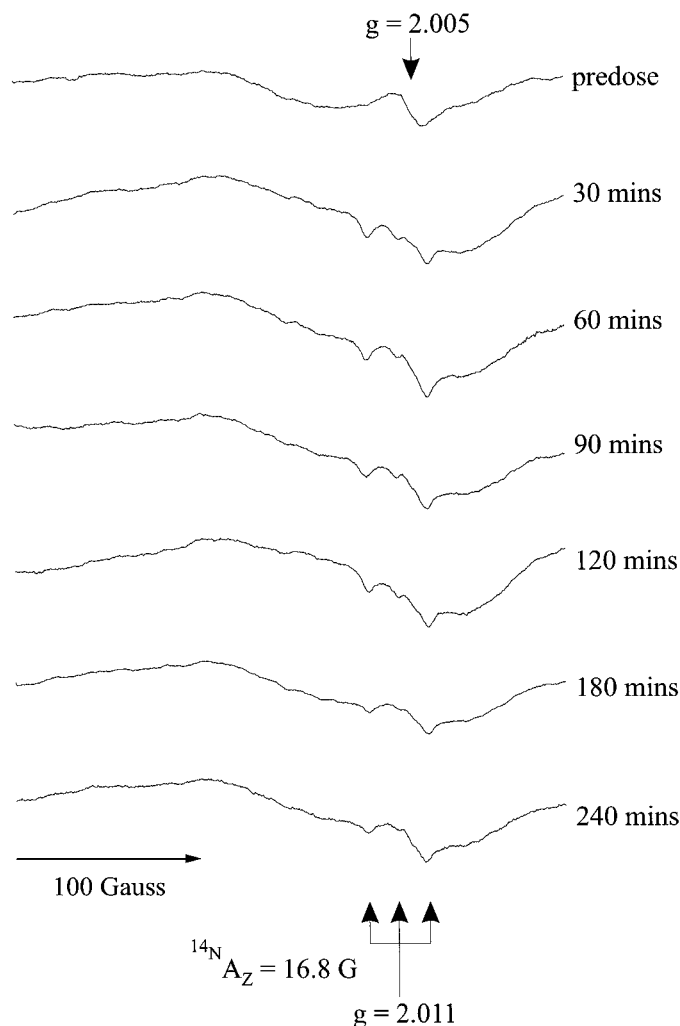


Fig. 2. Formation of HbNO EPR signal in human red blood cells after a single oral dose of 16 mg/kg HU. EPR measurements were carried out on a Bruker ESP300 spectrometer operated at 9.5 GHz with 100-kHz modulation frequency at liquid nitrogen temperatures, with samples held in a quartz finger dewar. Typical spectrometer settings were 31 mW power, 5 G modulation amplitude, 5.242 s time constant, 2684 s scan time, and 300 G scan range.

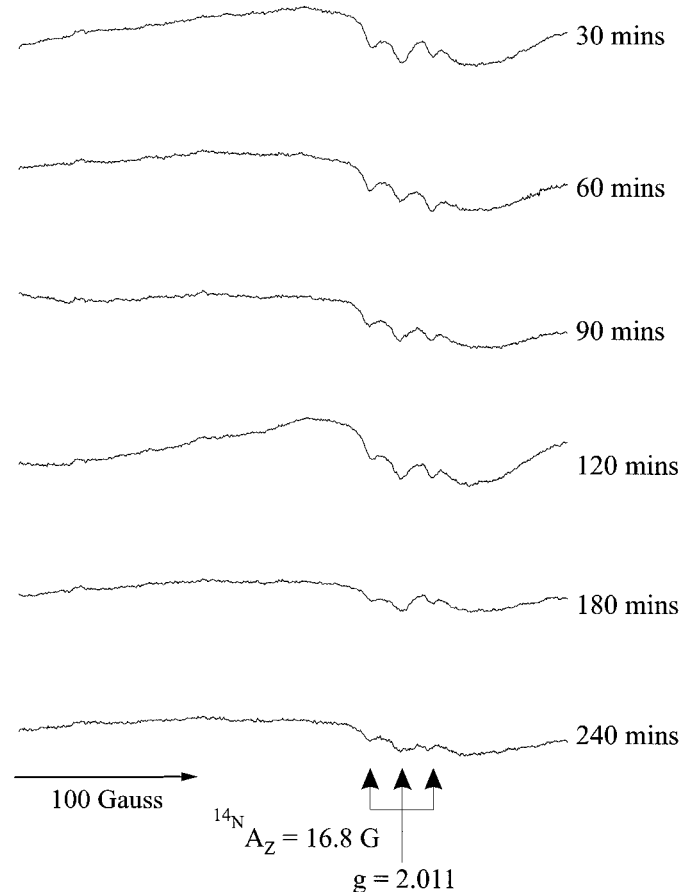


Fig. 3. Typical difference EPR spectra obtained from red blood cells resulting from the subtraction of the predose spectrum from spectra obtained at given times after HU administration (16 mg/kg). The spectra obtained are representative of three separate experiments.

the literature. We believe that the data presented represents the first definitive EPR spectra of HbNO in the systemic circulation of humans receiving HU therapy.

We previously demonstrated, using ^{15}N -labeled HU, that the NO generated originated from the NOH moiety (Jiang et al., 1997). However, the mechanism and site of HU metabolism in vivo remains to be fully elucidated. HU is known to be decomposed in vitro to NO by a number of mechanisms: 1) H_2O_2 and CuSO_4 (Kwon et al., 1991); 2) oxyhemoglobin (Stolze and Nohl, 1990; Jiang et al., 1997; Kim-Shapiro et al., 1998); 3) H_2O_2 and Cu, Zn-SOD, or ceruloplasmin (Sato et al., 1997); and 4) more generally, hydrogen peroxide and heme proteins (Pacelli et al., 1996). We also previously demonstrated that in vitro incubations of whole blood with HU does result in the formation of HbNO complex, although at much higher concentrations of HU than would occur pharmacologically after HU administration (Jiang et al., 1997).

In healthy humans the level of NO in the serum has been estimated to be a few nanomolar (Stamler et al., 1992), below the limits of detection by EPR. Therefore, the detection of the HbNO complex in red blood cells after HU treatment represents significant NO production well above that required for vasomotor regulation. Increased hemolysis of red blood cells normally accompanies the vaso-occlusive episodes. It is well known that deoxyhemoglobin can bind to NO with a high affinity and is the basis for the data presented (Westenberger et al., 1990; Hall et al., 1994; Kosaka et al., 1994). In addition, NO reacts rapidly with oxyhemoglobin (Liu et al., 1998). This raises the possibility that relative hypertension may be associated with the vaso-occlusive manifestations of the sickle cell disease (Rodgers et al., 1993). Therefore, peripheral vasodilation due to daily or regular treatment with HU

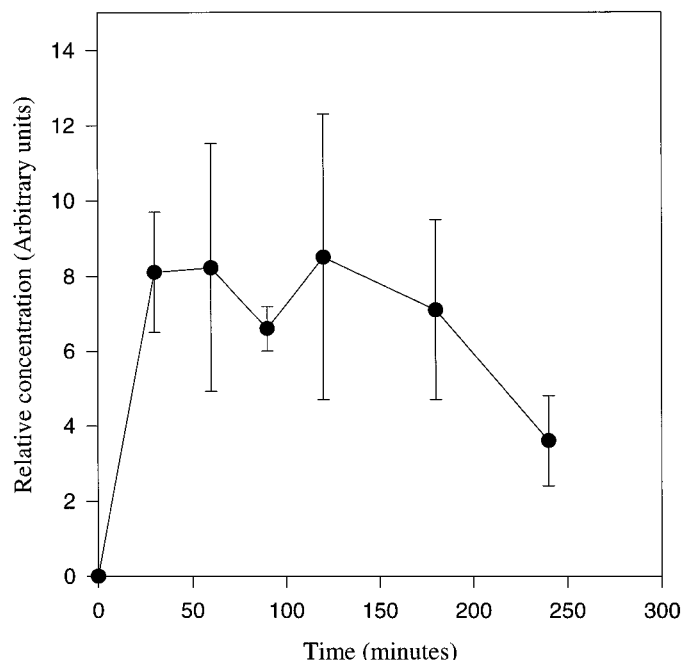


Fig. 4. Time course of formation of HbNO in human red blood cells after a single oral dose of 16 mg/kg HU. After subtraction of background signals, EPR spectra were double-integrated and plotted with time. EPR spectra are classically displayed as a first derivative of absorption spectra; therefore, double integral of the EPR spectrum is directly proportional to concentration of HbNO present. Data is presented as mean $\pm$ S.E. of three separate experiments.

may aid in maintaining vasomotor control by releasing NO and reducing the frequency of occlusion (Rodgers et al., 1988). However, to date there is no evidence that HU-derived NO can reduce blood pressure at the concentrations used in the treatment of sickle cell anemia.

The role of activated platelets in the pathogenesis of microvascular vaso-occlusion in sickle cell disease is controversial. Previous reports have given conflicting results, with some providing evidence for enhanced platelet activity (Westwick et al., 1983; Wun et al., 1997) and others showing minimal platelet activation (Buchanan and Holtkamp, 1983). It is possible that HU-derived NO contributes to the inhibition of platelet activation, thereby reducing the possibility of platelet-erythrocyte-induced vaso-occlusion. Pawloski et al. (1998) have recently shown that both cell-bound and cell-free nitrosylated hemoglobin (S-nitrosohemoglobin) inhibit human platelet aggregation. Alternatively, the small decrease in platelet numbers induced by HU in patients with sickle cell anemia, although not to a significant extent as reported in a recent multicenter study (Charache et al., 1996), may contribute to fewer occlusive episodes. However, the exact events contributing to the sickling of red cells and ultimately resulting in the sickle cell crisis remain to be elucidated. The success of HU in the management of sickle cell anemia may, in part, be attributed to its effects on NO-mediated events such as inhibition of platelet activation and/or vasodilation.

Leukocytes, neutrophils in particular, have been implicated in the sickling process and in the genesis and propagation of tissue damage that patients recognize as pain episodes (Charache et al., 1996). Adherent neutrophils could prevent the passage of stiffened sickle cells in the small blood vessels as well as enhance cytokine production, thereby contributing to vascular occlusion. Thus, the observed reduction of circulating neutrophils associated with HU treatment may contribute to the beneficial effects of the drug (Charache et al., 1996). It has been shown that a white blood cell count greater than 15×10^9 cells/liter is associated with an increased risk of early death in sickle cell anemia (Platt et al., 1994). HU-mediated cyto-reduction may be attributed in part to the ability of NO to inhibit ribonucleotide reductase, thereby decreasing the rate of leukocyte proliferation (Kwon et al., 1991).

Data from our studies and others would suggest that HU has multiple mechanisms of action that contribute to its clinical efficacy that cannot be attributed solely to increased HbF levels (Orringer and Parker, 1992; Charache et al., 1996). HU therapy does not eliminate the clinical manifestations of sickle cell anemia. It has been suggested that HU exerts its effect only while the patient continues to ingest it or for a short time thereafter (Charache et al., 1996). NO production from HU occurs within a few minutes as compared with other physiological changes already discussed, which require continual daily dosing and a time lapse from a few days to weeks to be of any clinical benefit. NO-mediated events such as decreased platelet activation and/or vasodilation may, in part, account for the efficacy of HU. Selective vasodilation along with decreased platelet activation may contribute to the decrease of some of the hemolytic and vaso-occlusive phenomena by diminishing the entrapment of sickle erythrocytes and may, in part, account for the effectiveness of HU on the time scale of hours. Interestingly, Head et al. (1997) provided data showing that low levels of NO

augmented the oxygen affinity of sickle erythrocytes both in vivo and in vitro without significant methemoglobin production. The same authors also noted that hemoglobin from some of their sickle cell volunteers on HU had an increased oxygen affinity (Head et al., 1997). This observation provides yet another mechanism by which HU may exact its beneficial effects in vivo. These results would suggest that low levels of NO inhalation and/or the use of NO-donors may offer an alternative therapeutic target for the treatment of sickle cell anemia.

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