Published in final edited form as:

Biochem Biophys Res Commun. 2016 May 13; 473(4): 1084–1089. doi:10.1016/j.bbrc.2016.04.019.

S-nitrosylated fetal hemoglobin in neonatal human blood

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Abstract

Background—Nitric oxide (NO) and its derivatives play important roles in the cardiopulmonary transition upon birth and in other oxygen-sensitive developmental milestones. One mechanism for the coupling of oxygen sensing and signaling by NO species is via the formation of an S-nitrosothiol (SNO) moiety on hemoglobin (Hb, forming SNO-Hb) and its release from the red blood cell in hypoxia. Although SNO-Hb formed on adult-type Hb (HbA, forming SNO-HbA) has been documented in physiological and pathophysiological human states, the fetal variant, SNO-HbF, has thus far not been isolated or characterized in human blood.

Methods and Results—We developed a technique capable of separating Hbs A and F under conditions that preserve SNO. We then measured SNO-HbF in the blood of healthy and premature or otherwise ill neonates using the gold standard for SNO measurement, mercury-coupled photolysis-chemiluminescence. SNO-HbF levels were in the range of those previously reported for HbA in adults. We found that SNO-HbF was more abundant at earlier gestational age (<30 weeks), even when accounting for the absolute HbF level.

Conclusions—The ability to monitor SNO-HbF could provide new insights into fetal development and the perinatal transition, and has potential as a biomarker relevant to the management of neonatal diseases.

Keywords

perinatology; nitric oxide; S-nitrosothiols; prematurity; erythrocyte; hydroxyurea

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Background

The O₂ sensor hemoglobin (Hb) and red blood cell (RBC)-derived vasoactive mediators,¹ such as the vasodilator nitric oxide (NO) and its derivatives, influence blood flow distribution. NO binds reversibly to the thiol group at the cysteine (Cys) 93 residue on the beta (β) chain of adult Hb (HbA), forming the stable SNO carrier *S*-nitrosohemoglobin (SNO-Hb).² Hb may also scavenge and bind NO at heme iron sites (Hb[Fe]NO), where NO is not bioactive. Hb[Fe]NO can then be converted to SNO-Hb during the oxygenation-induced allosteric transition in Hb.^{3–5} Release of the (S)NO derivative from SNO-Hb occurs in areas of tissue hypoxia along with O₂ release, allowing a nitrosonium (NO⁺) equivalent to exit the RBC and interact with the vessel wall, resulting in vascular smooth muscle relaxation and therefore increased blood flow and O₂ delivery.⁶

Evidence for NO/SNO coupling with Hb has largely been generated using adult studies in which HbA predominates.^{7–10} However, newborn infants primarily carry fetal Hb (HbF), which contains a gamma-chain Cys residue analogous to Cysβ93 of HbA and possesses a greater O₂ affinity than HbA.^{11,12} During the perinatal transition period and for several months after birth, HbA increases and HbF declines.¹² SNO-HbF physiology may be particularly important in newborns requiring stored RBC transfusions or with pulmonary hypertension, as altered SNO-Hb levels and function may play a role in related adverse events.^{4,13,14} While recent studies have attempted to investigate the levels and physiology of SNO-Hb in newborns as well as establish a correlation with HbF,^{11,15–18} none have isolated HbF for SNO analysis, and none has employed the gold standard for measuring SNO, mercury-coupled photolysis-chemiluminescence (MPC).^{19,20}

We conducted an exploratory study of the presence and levels of SNO-HbF, SNO-HbA, and the Hb[Fe]NO of each Hb variant in infants during their perinatal transition period after developing a novel preparative Hb separation method to assay bound NO and SNO via MPC in each Hb species. We hypothesized that SNO-HbF levels would be higher than SNO-HbA in newborn infants due to the high O₂ affinity of HbF. Our secondary hypothesis is that SNO-HbF levels would be greater in infants born at earlier gestational ages (GA), reflecting a role for SNO-HbF in regulating the fetal circulation.

Methods

Study cohort

This study was approved by the Duke University Institutional Review Board. Blood from two cohorts of patients was studied. In the first series, umbilical cord venous and arterial blood samples, upon delivery of healthy infants by Caesarean section, were collected in Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) and processed within one hour. In the second series, we prospectively identified all infants admitted to the Duke Intensive Care Nursery within the first 48 hours after birth. Inclusion criteria were the presence of umbilical venous or arterial catheters to allow for ease of blood sampling. Exclusion criteria were prior transfusions, use of inhaled NO (iNO), anemia, or bleeding abnormalities. Additionally, the clinical care team was notified to give final approval for blood sampling from study participants. For infants < 1500 grams, 0.3 mL was obtained

from each available umbilical catheter site. For infants > 1500 grams, 0.5 mL was taken from each umbilical site. Blood samples were placed in EDTA tubes, kept on ice, and protected from light. We collected data on demographics, medication exposure, respiratory support, and outcomes including mortality.

Sample preparation

Samples were prepared for analysis within three hours of blood draw. RBCs were separated from plasma and white blood cells by centrifugation, washed with excess phosphate buffered saline (PBS) with 0.1 mM diethylenetriaminepentaacetic acid (DTPA; a chelator that prevents SNO degradation), and lysed with hypotonic water. Hb was partially purified from the RBC lysate by exchange over a G-25 Sephadex column equilibrated with PBS/0.1 mM DTPA. Hbs were separated using anion exchange chromatography. Specifically, 200 μ L of the partially purified total Hb was loaded onto a HiTrap Q HP exchange column and subjected to a 100 mL linear gradient of increasing ionic strength from 0.0 to 0.15 M NaCl in pH 8.4 Tris buffer. Eluted HbA and HbF fractions were collected from the column and reconcentrated via a 10 kDa MW cutoff centrifugal filter. Samples of each Hb variant and unseparated total Hb were injected into a photolysis-coupled chemiluminescence instrument. Measurement of SNO-Hb was determined from the difference between signals generated from Hb pretreated with six-fold molar excess HgCl_2 vs. those that were not. The amount of Hb[Fe]NO was calculated from the HgCl_2 -treated samples, as HgCl_2 decomposes SNO, but not FeNO . The SNO values were normalized to the actual Hb concentrations.

Statistical analyses

We reported continuous variables for demographics as medians and range (minimum – maximum) and categorical variables as counts and proportions. We reported individual values of Hb[Fe]NO , SNO-Hb, and total Hb-bound NO and plotted the median and interquartile range (IQR). We compared values from infants < 30 weeks to those $\geq$ 30 weeks using Wilcoxon rank sum tests. We conducted all analyses using GraphPad Prism software and considered a $p < 0.05$ to be statistically significant except where otherwise noted.

Results

We identified and consented 21 newborn patients for the postnatal SNO-HbF study. From these 21 patients, a total of 32 samples (14 venous, 18 arterial) were collected. One patient was excluded because the sample was collected after 48 hours. The samples from seven patients were discarded due to technical issues (technician non-availability or equipment malfunction). Our final specimen cohort included 18 freshly tested blood samples from 14 patients (Table 1). The median GA of the entire cohort was 29.5 weeks (range 24–36 weeks) and the median birthweight was 1166 grams (500–3842 g). The indications for delivery were similarly distributed between infants < 30 weeks GA and those $\geq$ 30 weeks GA, and included preterm labor, fetal distress, and preeclampsia or other maternal indications (Table 1).

A typical chromatogram from our anion-exchange protocol is depicted in Fig. 1. Elution peaks for HbF and HbA were well resolved. Isoelectric focusing with inclusion of standards

for Hbs A, F, S, and C confirmed the successful isolation of Hbs A and F (Fig. 1B), and revealed the additional presence of acetylated HbF, as previously described.^{21,22} Hb-bound SNO levels in an unfractionated purified Hb sample was similar to that in the recombined HbF and HbA eluates, indicating that the bound SNO was stable during the separation technique (not shown).

Umbilical cord blood RBCs from term infants delivered by Caesarean section contained substantial SNO-HbF (Fig. 2), and the SNO:Hb ratios were comparable to those for HbA in the samples and to published values for unfractionated Hb in adult and neonatal human blood from healthy individuals.^{17,23–26} The levels of SNO-HbF and SNO-HbA did not differ significantly in arterial and venous cord blood samples.

Blood concentrations of SNO-Hb and Hb[Fe]NO in postnatal blood were also in a range similar to what has been documented previously in normal adult and neonatal human blood.^{16,24} HbF-bound SNO and the total NO (SNO + FeNO) were significantly greater in babies <30 weeks than those older than 30 weeks GA (Fig. 3, left), relevant to our secondary hypothesis. Similar but nonsignificant trends were seen for unfractionated Hb. Hb[Fe]NO values showed a nonsignificant trend toward higher values in the HbF fraction and for unfractionated Hb for babies <30 weeks (Fig. 3, left).

When the arterial and venous cord blood specimen subgroups were examined (Fig. 3 middle, right), similar trends were seen, but in this smaller number of data points, only the GA-dependent differences in arterial (low-O₂) Hb[Fe]NO values reached statistical significance. Trends toward GA-dependent increase in SNO and total NO bound to both HbF and unfractionated Hb were seen only in arterial blood, but did not reach statistical significance.

Discussion

The salient findings of this study are that HbF bears substantial and relevant quantities of the adduct SNO, a reversibly bound NO derivative that participates in the O₂-sensitive regulation of vascular tone. We designed and incorporated a Hb fractionation scheme to isolate HbF and HbA from human blood in a manner that did not disturb NO or SNO binding. In the umbilical cord blood of term infants delivered by uncomplicated Caesarean section, SNO-HbF and SNO-HbA ratios were similar. In addition, we report that infants <30 weeks GA have higher SNO-HbF values compared to infants ≥30 weeks GA. In deoxygenated arterial blood, Hb[Fe]NO was also significantly more preponderant at <30 weeks GA.

The “SNO-Hb hypothesis” has been criticized, but many elements have now been demonstrated experimentally.^{27–29} A critical role for Hb S-nitrosylation was suggested by recent work demonstrating baseline cardiac ischemia and impaired survival in hypoxic mice bearing exclusively humanized Hb and in which the Cysβ93 residue of HbA was mutated to alanine.²⁹ These mice exhibit not only a clear phenotype with deficient hypoxic vasodilation and an impaired ability to optimize O₂ delivery through blood flow autoregulation, but also the compensatory appearance of SNO-HbF. Therefore, SNO-HbF could play a role not only in the normal cardiopulmonary adaptation to air-breathing at birth, but could in theory

provide a mechanism whereby the stress of tissue hypoxia can be mitigated. Such adaptation also characterizes disorders that may be mitigated by the persistence or reappearance of HbF itself, as exemplified by sickle cell disease. In sickle cell disease, augmentation of HbF through treatment with hydroxyurea (HU)³⁰ is beneficial in preventing deoxygenation-induced polymerization of HbS. Interestingly, HU may also directly release NO,³¹ although the degree to which this action contributes to the therapeutic benefit of HU is unclear.

SNO-Hb has previously been measured in term infants.^{16,17} One study showed that in premature infants ranging from 25–27 weeks GA there was an increase in total SNO-Hb 12–24 hours after birth, and that total SNO-Hb levels correlated with HbA levels; however, HbA and HbF were not fractionated in order to determine which Hb bound the SNO.¹⁸ In the present study we did not report SNO-HbA levels in relation to GA, as these levels were too low to be reliably obtained. Differences between our results and prior studies could be attributed to their use of a chemiluminescent assay that relied on I₃⁻, which may generate artifacts that render results unreliable.³² Additionally, we developed a novel method of separating fetal and adult SNO-Hb types, and our study is accordingly the first to separate HbF and HbA in order to quantify NO and SNO bound to each. Importantly, our assay does not introduce any reagent that would effect HbA or HbF conformation.³³

The ability to isolate and measure SNO-HbF could enable new insights into development and the perinatal transition. Endogenous NO appears to be critical in the development of normal fetal anatomy and physiology. Animal and *in vivo* human studies have linked NO signaling to cell proliferation and differentiation, angiogenesis, myogenesis, cardiogenesis, neurogenesis, lymphangiogenesis, and bone formation, among other critical developmental events.^{34–40} NO is also central to fetal and neonatal hemodynamics during the perinatal transition period. NO appears to be involved in maintenance of a relatively high cardiac output state of the fetus. Levels of the NO derivatives nitrite and nitrate climb throughout gestation, reaching a peak at term.⁴¹ As a pulmonary vasodilator coupled to the O₂ sensor Hb, the SNO-HbF remaining could contribute to the fall in pulmonary vascular resistance that normally occurs upon birth.

Additionally, NO/SNO regulation is altered in a variety of fetal pathologic processes. Pisaneschi *et al* found that endothelial cells from umbilical veins of late preterm infants who were growth restricted *in utero* produced higher levels of SNO-Hb and nitrite. Also, they expressed lower levels of RNA transcripts that tend to produce vasoconstrictive proteins and higher levels of those linked to vasodilation.⁴² Similarly, umbilical vein endothelial cells collected from cords with umbilical vascular disease had higher levels of NO synthase mRNA compared to healthy controls.⁴³ Cord samples from infants who experienced meconium aspiration, a condition that may lead to pulmonary hypertension, had higher levels of NO and lower of arginase. This result suggests that arginase downregulation interferes with its competitive inhibitory effect on NO production via catabolism of L-arginine, the NO synthase substrate.⁴⁴ NO and SNO are apparently also produced at higher levels during sepsis in term and preterm infants, as in adults.^{41,45} SNO-HbA, in particular, increases during sepsis, and could act as a sink to protect the host from promiscuous SNO activity, or could propagate tissue injury and hypotension, for example via excessive vasodilation. With its higher affinity for both O₂ and NO/SNO, HbF could be expected to act

more as a detoxifying sink for superfluous SNO than is HbA, with SNO delivery disfavored. NO production has also shown to be downregulated in other disease states, such as in infants of diabetic mothers who have been found to produce lower levels of NO and downregulate production of NO synthase.⁴⁶

Our findings of our exploratory study indicate for the first time that GA-sensitive Hb-bound SNO is in fact bound to HbF. Larger sample size studies are needed to confirm our findings. We cannot determine from the available data whether these findings reflect primarily a developmental event, or whether the greater prematurity-induced stress in the <30-week cohort might account for the differences. Although the sample size is limited, the differences in HbF-SNO do not appear to be substantially driven by the indications for delivery, which were similarly distributed between the two GA groups. There were, however, other differences in clinical characteristics between the two groups, such as respiratory support device use, that suggest that the younger cohort was a sicker group. Sampling for both GA groups occurred in the first 48 postnatal hours but demonstrated a wide range, a possible source of variability since SNO-Hb levels may change as infants progress through transition in the first postnatal hours and days.¹⁸ We were unable to compare SNO-HbF levels in our cord blood cohort with age-matched samples from our GA cohort, as our sample size was too low. Also, our study did not find differences in arterial and venous sampling, possibly due to the low sample size in our postnatal cohort. Furthermore, we did not rigorously preserve the O₂ tension during sample handling in our cord blood cohort, so the lack of arterial vs venous difference may be partly artefactual.

Hb-derived NO/SNO signaling is responsive to the tissue O₂ content, and HbF-bound NO/SNO could participate in O₂-sensitive fetal development and the perinatal transition. Thus, the ability to isolate and measure the SNO/NO content of each Hb variant independently provides visibility to the promise of these analytes as potential biomarkers for syndromes and disease states presenting during the perinatal transition, including persistent pulmonary hypertension of the newborn and complications of prematurity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Hongmei Zhu, M.S., Kim Fisher, Ph.D., Brendan Huang, and Brian Antczak for technical and logistical assistance, and Dr. Joanne Kurtzberg for scientific advice and logistical support. The work was supported by grants from the NIH (GM-113838, HD-040492, HL-105702 and HL-107608) and VA (BX-000281).

Abbreviations

RBC	Red blood cell
NO	Nitric Oxide
Hb	Hemoglobin
Cys	Cysteine

β	Beta
HbA	Adult type hemoglobin
SNO-Hb	S-nitrosohemoglobin
Hb[Fe]NO	Heme iron-bound nitric oxide
NO⁺	Nitrosonium
HbF	Fetal type hemoglobin
MPC	Mercury-coupled photolysis chemiluminescence
GA	Gestational age
iNO	Inhaled nitric oxide
DTPA	Diethylenetriaminepentaacetic acid
PBS	Phosphate buffered saline
IQR	Interquartile range
HU	Hydroxyurea

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Highlights

- Anion exchange chromatography can be used to separate fetal hemoglobin (HbF) from HbA while preserving bound S-nitrosothiol.
- S-nitrosylated fetal hemoglobin is identified and quantified in human blood for the first time.
- Gestational age appears to be a key determinant of HbF-SNO concentrations.

Figure 1.

(A), Typical chromatogram resulting from the ion-exchange procedure and indicating distinct elution profiles for HbA relative to HbF. Peak identities were confirmed using isoelectric (gel) focusing (**B**) alongside known (“AFSC”) human Hb standard mixtures. IEF gel results from either unfractionated (“Unfr”) or fractionated (HbF, HbA) arterial and venous cord blood samples from two donors are shown.

Figure 2.

Hb-bound SNO (SNO-Hb, upper panels), Hb[Fe]NO (middle panels), and the total Hb-bound NO (lower panels) in the umbilical arterial (left) or venous (right) umbilical cord blood of term infants delivered by uncomplicated Caesarean section. Values are moles of (S)NO per tetramer of the respective Hb. Each color represents an individual patient.

Levels of Hb-bound NO/SNO by gestational age

Arterial and venous blood

Arterial blood

Venous blood

Figure 3.

NO bound to Hb as SNO-Hb (upper row), heme-NO ("FeNO", middle row), and the sum of NO bound to Hb (lowest row) measured either in unfractionated Hb samples ("Hb total") or on isolated HbF from arterial or venous umbilical cord catheters in neonates. Median and interquartile ranges are shown. * indicates $p < 0.05$.

Table 1**Demographics**

	< 30 weeks GA N = 7	30 weeks GA N = 7
GA (weeks)	28 (24 – 29)	31 (30 – 36)
Birthweight (g)	1050 (500 – 1460)	1480 (1040 – 3842)
Delivery indication		
Preterm labor	4 (57)	3 (43)
Fetal distress	1 (11)	2 (29)
Preeclampsia/maternal indication	2 (29)	2 (29)
Age at sample collection (hours)	26 (3.3 – 46.5)	21 (13 – 45)
Respiratory support		
Room air	1 (11)	5 (71)
Nasal Cannula or CPAP	1 (11)	2 (29)
SIMV	4 (57)	0 (0)
Jet ventilator	1 (11)	0 (0)
Hematocrit	35 (29 – 43)	44 (41 – 52)
Umbilical artery catheter	6 (86)	3 (43)
Umbilical vein catheter	3 (43)	6 (86)
Both umbilical catheters	2 (29)	3 (43)

Continuous variables – median (min – max); Discrete variables – median (%)

GA = gestational age

FiO₂ = fractional inspired oxygen

CPAP = continuous positive airway pressure

SIMV = synchronized intermittent minute ventilation