Nitric Oxide Metabolism in Adults With Cyanotic Congenital Heart Disease

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Endothelial-derived nitric oxide (NO) diffuses abluminally to regulate blood flow by activating soluble guanylate cyclase in medial smooth muscle. However, a significant fraction of NO diffuses luminally, where the extremely high reaction rate with red blood cell hemoglobin (Hb) effectively reduces luminal concentration to zero. The erythrocytosis of cyanotic congenital heart disease has potentially opposing effects, namely, a reduction in medial smooth muscle NO bioavailability because of the increase in luminal consumption of the molecule and, conversely, an increase in the elaboration of NO in response to the high endothelial shear stress of the erythrocytotic perfusate. NO metabolism in cyanotic congenital heart disease is unknown. Accordingly, this study aimed to establish the metabolic fate of NO and to determine the degree to which its levels are altered. Blood samples from 25 nonfasting patients with cyanotic congenital heart disease and 25 nonfasting normal controls were collected in Vacutainer tubes containing citrate dextrose and in separate Vacutainer tubes containing a solution that specifically preserves S-nitrosated Hb. Total NO species, plasma S-nitrosated proteins, iron nitrosyl Hb, and S-nitrosated Hb were quantified using chemiluminescence. In conclusion, a significant increase in plasma concentrations of NO metabolites and a modest increase in iron nitrosyl Hb levels were found, suggesting increased luminal consumption caused by erythrocytosis and further suggesting that hypoxemia might activate nonoxidative NO metabolic pathways and enhance tissue oxygen delivery. **(Am J Cardiol 2007; 99:691–695)**

The erythrocytosis of cyanotic congenital heart disease (CCHD) has potentially opposing effects on the bioavailability of nitric oxide (NO): (1) a reduction in bioavailability because increased luminal consumption decreases abluminally mediated vasodilation and (2) an increase in bioavailability because the high endothelial shear stress of the erythrocytotic perfusate and the hypoxemia of CCHD enhance the formation of iron nitrosyl hemoglobin (HbNO). Endothelial NO elaborated in response to high shear stress diffuses abluminally, activating soluble guanylate cyclase in medial smooth muscle cells and catalyzing the conversion of guanosine triphosphate to cyclic guanosine monophosphate, thus initiating a cascade of reactions that culminate in vasodilation. The abluminal effect is not linear but instead is characterized by a pronounced initial response and then a plateau, followed by no further response.[1] Because NO diffusion, like oxygen diffusion, is governed by Fickian principles, endothelial NO diffuses into medial smooth muscle cells and into the lumen according to the concentration gradient in each direction.[2] The effective concentration of NO in the center of the lumen is zero, so the maximum concentration gradient is necessarily from endothelium to lumen. The extreme rapidity with which NO is consumed by luminal red blood cell (RBC) hemoglobin (Hb) curtails its effect on the media. Elevated Hb levels implicit in the erythrocytosis of CCHD potentially influence NO-related vascular behavior by increasing luminal RBC NO consumption. The formation of HbNO from endothelial NO is facilitated by a combination of a high luminal concentration of Hb, RBC hypoxemia, and the increased elaboration of NO in response to high endothelial shear stress.

NO alters its own rate of entry into RBCs by forming HbNO,[3] which has been linked to increased oxygen delivery to tissues. Because the molecule is rapidly degraded by oxygen-bound Hb,[4,5] HbNO is formed only under hypoxemic conditions, during which NO accelerates its own consumption by increasing its entry into RBCs. Low NO concentrations increase RBC membrane deformability, further enhancing oxygen release, whereas high concentrations decrease deformability.[6] If the formation of HbNO is increased in CCHD, oxygen transfer from RBCs to tissues might also increase.[3]

The measurement of NO metabolites is a necessary step in determining the molecule's metabolic fate and in establishing the extent to which a reduction in bioavailability is the consequence of a shift from abluminal to luminal diffusion. Because the metabolism of NO in CCHD is unknown, we sought to provide this information.

Methods

Subject selection: Twenty-five adults with CCHD and 25 normal, acyanotic, age-matched controls were studied (Table 1). Patients in neither category smoked, were hyper-

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Table 1
Patient profile

Description	Gender (M/F)	Age Range (yrs)	Age (yrs) (mean ± SD)
Acyanotic	14/11	22–43	31 ± 5
Cyanotic	10/15	22–44	29 ± 4

tensive, were obese, had personal or family histories of cardiovascular disease, or were currently taking or had previously taken NO-containing pharmaceuticals (nitroglycerin or L-arginine). The protocol was approved by the Institutional Review Board, Office for Protection of Research Subjects, University of California, Los Angeles. All participants signed consent forms.

Chemicals: Water (high-performance liquid chromatography grade) was purchased from EMD Chemical (Gibbstown, New Jersey). Sodium chloride, potassium cyanide, potassium ferricyanide, sodium hydroxide, and acetic acid were purchased from Fisher Scientific (Tustin, California). Iodine, iodide, Sephadex G-25, α-cellulose, microcrystalline cellulose, sulfanilamide, mercuric chloride, diethylenetriaminepentaacetic acid, and sodium phosphate were purchased from Sigma (St. Louis, Missouri), and N-ethylmaleimide was purchased from Alfa Aesar (Ward Hill, Massachusetts).

Blood samples: Two vials of nonfasting blood were drawn by venipuncture. Nine milliliters were drawn into a Vacutainer (Becton, Dickinson and Company, Franklin Lakes, New Jersey) containing the anticoagulant citrate dextrose at a final concentration of 10%; 0.5 ml was drawn into another Vacutainer containing a solution of 4 mmol/L ferricyanide and 4 mmol/L cyanide with 10 mmol/L N-ethylmaleimide, 0.1 mmol/L diethylenetriaminepentaacetic acid, and 1% Nonidet-P40 (Fluka, Buchs, Switzerland) in a phosphate buffer saline solution (pH 7.4). We and others have shown experimentally that this solution preserves S-nitrosated Hb (SNO-Hb) for a minimum of 24 hours when the sample is kept at 4°C.[7,8] The blood samples were iced and stored in a light-protected Styrofoam box (The Dow Chemical Company, Midland, Michigan) immediately after withdrawal, until NO metabolites were measured.

Chemiluminescence: The release of NO from plasma NO species (NO_x), plasma S-nitrosated low-molecular-weight proteins, HbNO, and SNO-Hb was measured using chemiluminescence.[7] This method has been used previously, and the reproducibility of results has been established.[8] Each NO metabolite was converted to NO using a variety of chemical methods in a reaction chamber inline with a gas wash bottle containing 1 mol/L sodium hydroxide. NO that evolved from the reaction was carried in ultra-high-purity-grade helium (Airgas, Radnor, Pennsylvania) to a Sievers Nitric Oxide Analyzer 280 (Ionics Instruments, Boulder, Colorado) to measure quantities by the chemiluminescence reaction with ozone. Data were recorded with NOAnalysis 3.21 liquid software provided by Sievers and analyzed with PeakFit 4.11 (Systat Software, Richmond, California).

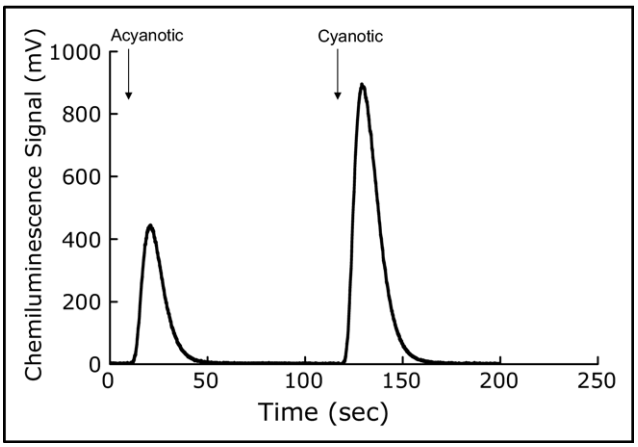

Figure 1. Representative data of total NO_x measurements from the plasma of an acyanotic control and a cyanotic patient. The area under the curve proportionally correlates with the total NO_x in each sample. Similar data were acquired to measure the other NO metabolites.

Measurement of NO metabolites: Total NO species were measured using chemiluminescence as described. A solution was prepared by dissolving 0.2 g vanadium (III) chloride in 25 ml of 1 mol/L hydrogen chloride in a light-protected bottle. After thorough mixing, the vanadium solution was filtered into a light-protected flask using Whatman paper. The solution was stored at 4°C and prepared fresh every 2 weeks. Approximately 15 ml of the vanadium solution was transferred to the reaction vessel. The water jacket surrounding the reaction vessel was set at 95°C. Once the chemiluminescence signal stabilized (typically in 1 hour), samples were injected. Plasma samples were mixed with 100% ethanol at a 1:2 ratio for 5 minutes. The samples were then centrifuged at 16,000*g* for 15 minutes at 4°C. The supernatant was collected, and 20 μl were injected into the reaction chamber.

Measurement of SNO-Hb and plasma S-nitrosated low-molecular-weight proteins: SNO Hb was measured according to Yang et al,[9] with minor modifications. A solution of triidoide anion was prepared by dissolving 1.0 g potassium iodide and 0.65 g iodine in a 70% acetic acid solution. This mixture was prepared fresh each day of sample measurements. The Hb fraction was isolated using a 3-ml bed volume Sephadex G-25 fine column and separated into 2 vials: 1 containing mercuric chloride in an isotonic 10 mmol/L phosphate buffer (100 μmol/L diethylenetriaminepentaacetic acid, pH 7.4) to displace NO from SNO-Hb and the other in the same buffer but without mercuric chloride. Acidified sulfanilamide was added after 10 minutes to remove background nitrite. Samples were iced for 15 minutes and then tested for NO-bearing Hb species by the same protocol used for nitrite detection. Because mercuric chloride selectively displaces NO from the thiol, the difference in the NO signal between the mercuric chloride and phosphate samples is the amount of SNO-Hb in the original Hb sample.

Measurement of HbNO: RBCs were isolated by centrifugation and lysed in high performance liquid chromatography–grade water containing 0.5 mmol/L ethylenedia-

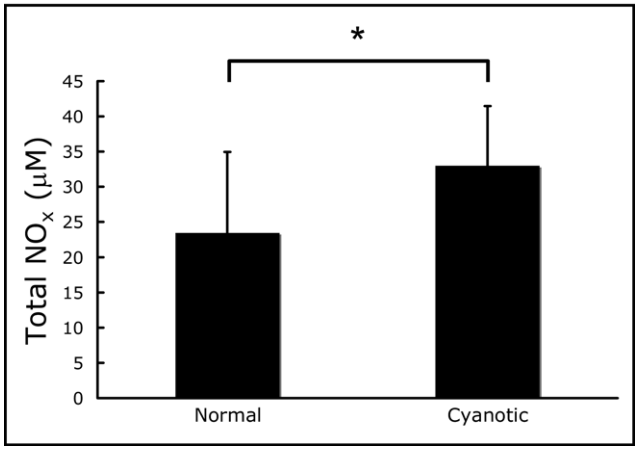

Figure 2. Total NO_x was increased in the plasma of adults with CCHD. Plasma total NO_x of subjects with CCHD (33.0 ± 8.5 μmol/L) was significantly higher than the plasma total NO_x of acyanotic controls (23.5 ± 11.5 μmol/L), indicating the increased elaboration of NO from endothelial cells ($p = 0.002$ by 2-sample Student's t test, $n = 25$ per group). Further analysis identified nitrate as the primary component of total NO_x.

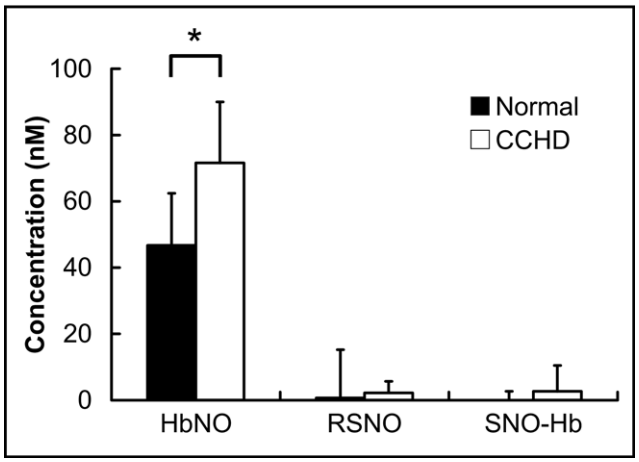

Figure 3. HbNO concentration was increased in the RBCs of patients with CCHD. The intracellular concentration of HbNO in the blood of these patients (71.6 ± 18.3 nmol/L) was statistically higher, although modestly so, compared with acyanotic, normal controls (46.7 ± 14.6 nmol/L) ($p < 0.0001$ by 2-sample Student's t test, $n = 25$ per group). The measured value of HbNO represents the lower bound, because HbNO is a labile species. Intracellular SNO-Hb and plasma low-molecular-weight nitrosothiols (RSNO) were at the detection limit of the instrument and not statistically different between patients with CCHD and acyanotic normal controls.

minetetraacetic acid in a 1:2 ratio. The Hb lysate (0.2 ml) was added to 0.6 ml of 0.2 mol/L potassium cyanide and 0.2 mol/L ferricyanide in phosphate-buffered saline with 5 mmol/L ethylenediaminetetraacetic acid, and another aliquot of lysate was added to 0.4 ml of 0.5 mol/L ethylenediaminetetraacetic acid in phosphate-buffered saline. After 30 minutes of incubation, 0.5 ml of each Hb sample was passed through an extensively washed Sephadex G-25 column. Each isolated Hb fraction (0.2 ml) was injected into the reaction chamber containing the triidoide anion reagent. The quantity of HbNO was calculated from the difference in NO evolved from the 2 samples. Although methods to reduce HbNO degradation were carefully used before its measurement, the possibility that some HbNO was degraded cannot be entirely excluded. The results presented here therefore represent the lower boundary of HbNO in CCHD.

Results

Concentrations of plasma total NO_x metabolites measured using chemiluminescence (Figure 1) were statistically higher in adults with CCHD (33.0 ± 8.5 μmol/L) than in normal, acyanotic controls (23.5 ± 11.5 μmol/L) ($p < 0.002$ by 2-sample Student's t test; Figure 2), supporting the hypothesis that increased RBC mass is accompanied by the increased production of NO. The primary NO_x metabolite was nitrate, suggesting that the primary metabolic route of increased NO production was degradation by RBC Hb. Low-molecular-weight S-nitrosthiol concentrations were at the instrument detection limit.

Because total NOx were increased in the plasma of the cyanotic subjects, conditions facilitating formation of the NO-Hb adduct were assessed. The concentration of intracellular HbNO was statistically higher in adults with CCHD (71.6 ± 18.3 μmol/L) than in normal, acyanotic controls (46.7 ± 14.6 μmol/L) ($p < 0.001$ by 2-sample Student's t test; Figure 3). However, the absolute concentration of HbNO was modest in the 2 groups, suggesting that most of the NO produced was degraded into nitrate. The concentration of SNO-Hb was near the detection limit of the instrument, and detection might have been limited by the inherent instability of this species in RBCs.[7]

Discussion

In CCHD, oxygen delivery to metabolizing tissues is increased by systemic vasodilation, which is in response to the endothelial elaboration of NO provoked by increased shear stress of the viscous erythrocytotic perfusate.[1] Oxygen delivery is also increased by erythrocytosis. There is a uniform consensus that an increase in RBC mass is a desirable adaptive response to the systemic arterial hypoxemia of CCHD, serving to improve oxygen delivery to metabolizing tissues.[10] NO might also play an important role by enhancing oxygen delivery from luminal RBC oxygen-bound Hb to tissues. NO metabolism involves a complex interplay between the rate of production, storage, and degradation. Because NO metabolism is unknown in CCHD, we sought to establish the molecule's metabolic fate and to determine whether and to what extent its levels were altered.

Abluminal and luminal diffusion of NO from vascular endothelium proceeds according to Fickian principles (see Introduction). Abluminal diffusion regulates blood flow by activating soluble guanylate cyclase in medial smooth muscle cells,[11–14] but a significant fraction of NO diffuses luminally, where its fate is determined by the extremely high reaction rate with RBC Hb, making the effective luminal concentration zero.[15,16] If luminal NO consumption were increased by erythrocytosis, abluminally mediated vasodilatation might reciprocally decrease.

A recent investigation of NO in CCHD disclosed a striking impairment of endothelium-dependent vasodilation in

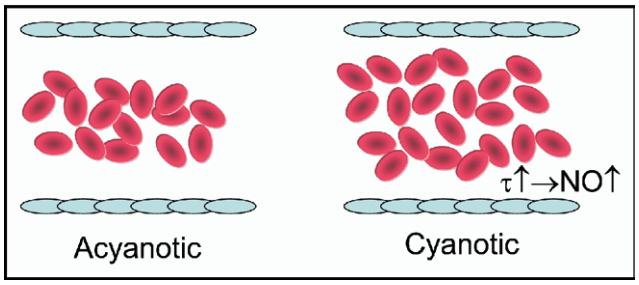

Figure 4. The erythrocytosis of CCHD increases endothelial shear stress and initiates a cascade of interactions that result in increased NO elaboration. The diffusion of NO is abluminal and luminal according to Fickian principles. Abluminal diffusion activates soluble guanylate cyclase in endothelial smooth muscle cells, and luminal diffusion reacts with RBC Hb.

response to the agonist acetylcholine, implying reduced bioavailability and downregulation of NO synthase despite enhanced NO release provoked by increased endothelial shear stress.[17] Endothelial-derived NO synthase, which is responsible for producing vasoregulatory NO, is activated by increased shear stress (Figure 4).[1] Oechslin et al[17] proposed that chronic hypoxia alters endothelial function so that the potential increase in NO elaboration is insufficient to overcome impairment of endothelial dependent vasodilation.

If NO production were significantly increased in CCHD, plasma nitrate concentrations should also increase. An increase in RBC mass reportedly increases NO consumption in porcine coronary arterioles.[16] Because partially oxygenated Hb exists in CCHD, a distribution of NO metabolites might be expected. We found that the concentration of NO metabolic products is increased in CCHD, implying that the rate of production is also increased. The primary detected NO metabolite was nitrate, suggesting that increased endothelial-derived NO is consumed by oxygen-bound Hb.

In light of enhanced NO production associated with the hypoxemia and increased RBC mass of CCHD, it was anticipated that HbNO concentration would increase in proportion to the increase in nitrate concentration. The formation of HbNO might serve as a pivotal mechanism by which each RBC compensates for its reduced oxygen content by increasing the rate of tissue oxygen delivery. In a rat model, HbNO formation reportedly increases tissue oxygen transport.[3] It has also been shown that HbNO increases the rate of NO consumption by RBCs,[18] suggesting that RBCs sense hypoxemia and alter tissue oxygen delivery by increasing NO uptake, which then increases HbNO formation. HbNO concentrations were statistically higher in the blood of patients with CCHD compared with that of normal, acyanotic controls. Although the absolute magnitude of the increase was modest, the data show that HbNO formation is enhanced in CCHD.

The storage of bioactive NO as a nitrosothiol has been proposed as a mechanism for RBC-mediated vasoregulation,[19] a mechanism that might be operative in CCHD. We found very reduced concentrations of low-molecular-weight nitrosothiols in plasma and in RBC SNO-Hb, and there was no statistical difference between samples from patients with CCHD and normal controls. The low concentration of detectable SNO-Hb may have been due to the inherent instability of SNO-Hb in the reducing environment of the RBC.[7]

If it were possible to increase NO production by administering L-arginine or by chemical compounds that release NO or that were metabolized to NO (e.g., sodium nitroprusside and nitroglycerin), the modest increase in HbNO identified herein might be increased, potentially enhancing tissue oxygen delivery. NO itself may extend the tissue diffusion capacity of oxygen, playing a further role in CCHD.[20]

Concentrations of NO metabolites in our patients with CCHD were measured to determine whether the increased RBC mass might alter luminal NO consumption. Our results indicate that the total NO products, primarily nitrate, were significantly increased, together with a modest increase in HbNO concentrations, suggesting that an increase in luminal NO consumption is caused by erythrocytosis and suggesting that nonoxidative NO metabolic pathways might be activated by hypoxemia, thus enhancing tissue oxygen delivery.

Study limitations: Oxygen delivery depends on the local oxygen concentration gradient. Blood samples in this study were aggregates that may not have represented local concentrations. HbNO, for example, is likely to be formed in higher concentrations in regions of lower oxygen tension to deliver oxygen to tissues in which demand is higher.

HbNO measurements represent the lower bound in the blood of cyanotic patients and acyanotic controls. Because HbNO is an unstable species in the presence of oxygen, some degradation might have occurred during or after sample collection, even though appropriate steps were taken to minimize degradation by icing the sample immediately and by protecting it from light.

The measurement of SNO-Hb represents the lower bound of SNO-Hb in plasma of cyanotic patients and acyanotic controls. Separate aliquots of blood were collected in a solution that has been shown experimentally to preserve SNO-Hb for 24 hours. The low concentration of SNO-Hb might have been due to the inherent instability of SNO-Hb in the reducing environment of the RBCs.[7]

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