Oxidation of Iron-nitrosyl-hemoglobin by Dehydroascorbic Acid Releases Nitric Oxide to Form Nitrite in Human Erythrocytes[†]

Nathawut Sibmooh,[‡,§] Barbora Piknova,[‡] Fabiola Rizzatti,[‡] and Alan N. Schechter*[,‡]

Molecular Medicine Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, and Department of Pharmacology, Faculty of Science, Mahidol University, Bangkok, 10400, Thailand

Received October 26, 2007; Revised Manuscript Received January 10, 2008

ABSTRACT: The reaction of deoxyhemoglobin with nitric oxide (NO) or nitrite ions (NO_2^-) produces iron-nitrosyl-hemoglobin (HbNO) in contrast to the reaction with oxyhemoglobin, which produces methemoglobin and nitrate (NO_3^-). HbNO has not been associated with the known bioactivities of NO. We hypothesized that HbNO in erythrocytes could be an important source of bioactive NO/nitrite if its oxidation was coupled to the ascorbic acid (ASC) cycle. Studied by absorption and electron paramagnetic resonance (EPR) spectroscopy, DHA oxidized HbNO to methemoglobin and liberated NO from HbNO as determined by chemiluminescence. Both DHA and ascorbate free radical (AFR), the intermediate between ASC and DHA, enhanced NO oxidation to nitrite, but not nitrate; nor did either oxidize nitrite to nitrate. DHA increased the basal levels of nitrite in erythrocytes, while the reactions of nitrite with hemoglobin are slow. In erythrocytes loaded with HbNO, HbNO disappeared after DHA addition, and the AFR signal was detected by EPR. We suggest that the ASC−AFR−DHA cycle may be coupled to that of HbNO-nitrite and provide a mechanism for the endocrine transport of NO via hemoglobin within erythrocytes, resulting in the production of intracellular nitrite. Additionally, intracellular nitrite and nitrate seem to be largely generated by independent pathways within the erythrocyte. These data provide a physiologically robust mechanism for erythrocytic transport of NO bioactivity allowing for hormone-like properties.

The reactions of hemoglobin with NO and its metabolites are considered to be the main mechanism limiting NO bioavailability *in vivo*.The reactions include conversion of NO to inactive nitrate and methemoglobin under aerobic conditions by oxyhemoglobin ($k = 5 \times 10^7$ $M^{-1}\cdot s^{-1}$) (*1*) or iron-nitrosyl-hemoglobin (HbNO[1]) under anaerobic conditions by reacting with deoxyhemoglobin ($k = 2.6 \times 10^7$ $M^{-1}\cdot s^{-1}$) (*2*). HbNO is considered to be a stable and inert end product of NO−deoxyhemoglobin interaction because of its slow dissociation rate (*2–4*); however, its half-time *in vivo* has been estimated as 40 min after NO inhalation in human subjects (*5*). The biological function and metabolic pathways of HbNO in erythrocytes have not been described. HbNO in erythrocytes could be an important source of bioactive NO and could explain aspects of the role of erythrocytes in the transport of NO to tissues (*6*) and other endocrine effects of NO. HbNO could also be generated directly from nitrite or indirectly following nitrite reduction to NO catalyzed by the reductase activity of hemoglobin (*4*). The nitrite reductase activity of deoxyhemoglobin had been described to produce HbNO (*7*) and is now believed to be a mechanism of hypoxic vasodilation driven by deoxyhemoglobin and acidosis (*8, 9*). However, the explanation of how HbNO is itself metabolized remains unknown.

We have also addressed the hypothesis that nitrite can be generated from HbNO in erythrocytes. Most nitrite in blood resides in erythrocytes, (∼300 nM) (*10*), and this has been postulated to serve the endocrine delivery of NO (*11*). Nitrite is also present in plasma, presumably generated from autoxidation of NO (*12*) or NO oxidase activity of ceruloplasmin (*13*). However, the autoxidation of NO to nitrite ($k = 2 \times 10^6$ $M^{-2}\cdot s^{-1}$) (*12*) at physiologic NO and oxygen levels (400 nM and 150 μM, respectively) is considered unlikely to compete with the very high concentrations of oxyhemoglobin (∼10–20 mM) in erythrocytes, which would rapidly convert NO to the nitrate anion (NO_3^-). Thus, the mechanism to maintain nitrite levels through either generation from HbNO or transport of NO into erythrocytes remains unknown in view of competition with high hemoglobin concentrations.

NO donors have been shown to produce ascorbic acid in erythrocytes (*14*). In this case, ascorbic acid is thought to be produced intracellularly from its oxidized forms, ascorbate free radical (AFR) or dehydroascorbic acid (DHA), and this reaction could yield nitrite. Ascorbic acid is unstable and oxidized rapidly to DHA in aqueous solution (*15*); both

[†] This research was supported by the Intramural Research Program of the NIH, Molecular Medicine Branch, NIDDK. Low temperature EPR was supported by NIH Grant P41 EB001980.

* To whom correspondence should be addressed. NIH, NIDDK, Molecular Medicine Branch, 10 Center Dr., Building 10, Room 9N314, Bethesda, MD 20892. Tel: (301) 496-5408. Fax: (301) 402-0101. E-mail: aschecht@helix.nih.gov.

[‡] National Institutes of Health.

[§] Mahidol University.

[1] Abbreviations: AFR, ascorbate free radical; ASC, ascorbic acid; DHA, dehydroascorbic acid; DTPA, diethylenetriaminepentaacetic acid; EPR, electron paramagnetic resonance; HbNO, iron-nitrosyl-hemoglobin; NO, nitric oxide; PBS, phosphate-buffered saline; PRNO, proli NONOate; SPNO, spermine NONOate.

10.1021/bi702158d CCC: $40.75
Published on Web 01/29/2008

ascorbic acid and DHA, however, are dietary forms of vitamin C in humans and possess equivalent bioavailability (*16*). Although they are metabolically converted to each other, only DHA is rapidly taken up by human erythrocytes but is thought to be immediately reduced to ascorbic acid, which could reach intracellular concentrations of up to millimolar levels (*16–18*). Since NO increases ascorbic acid in erythrocytes, it is likely that nitrite is produced from the reaction of DHA or AFR with NO. In the presence of high levels of NO, DHA consumes NO efficiently *in vitro* (*19*), and this process would be expected to yield nitrite. DHA concentration values in blood are controversial, depending on a variety of methods of measurement; plasma DHA values have been reported ranging from zero (*20*), 2–11 μM (*21–24*), and up to 53 μM (*25*), while DHA concentrations in erythrocytes were reported as zero (*20*) and ~9 μM (*24*). The basal levels of HbNO in human blood are reported varying from 0.2 to 0.4 μM (*26*) to 5 μM (*27*) as detected by tri-iodide-based and photolysis-based chemiluminescence, respectively, or 20–200 nM (*5, 28*) by electron paramagnetic resonance (EPR).

In this study, we demonstrate that DHA could oxidize HbNO and liberate NO to form nitrite in erythrocytes. Vitamin C recycling could convert inert HbNO to bioactive NO/nitrite and may contribute to the endocrine delivery of NO by blood.

MATERIALS AND METHODS

Reagents. Sodium nitrite, dehydroascorbic acid (DHA), and all other reagents were purchased from Sigma-Aldrich (Saint Louis, MO). Proli NONOate (PRNO) was purchased from Alexis (San Diego, CA). Spermine NONOate (SPNO) and PRNO were initially dissolved in 1 M NaOH as 0.1 M stock solution and kept at 4 °C. DHA, sodium nitrite, and other reagents were freshly prepared. DHA and ascorbic acid solution was adjusted to pH 7 by NaOH prior to use.

Visible Absorption Spectroscopy and Preparation of HbNO. The stock hemoglobin solution (3 mM) was prepared from human blood (*29*) and kept at −80 °C. The 10%H_2/90%N_2 gas mixture was used to deoxygenate hemoglobin. Thirty micromolar of HbNO in phosphate-buffered saline (PBS) was prepared by incubating 30 μM deoxyhemoglobin with 50 μM SPNO for 3 h at 37 °C under anaerobic conditions. A UV/visible spectrophotometer model HP 8453 (Agilent Technologies, Santa Clara, CA) equipped with 8-cell transporter and water bath temperature control was used. The purity of deoxyhemoglobin and HbNO was calculated through spectral deconvolution. All measurements were performed at 37 °C.

Measurement of NO Release. NO gas liberated from a reaction mixture was measured by chemiluminescence NO analyzer (CLD88Y et; Eco Physics Inc., Ann Arbor, MI) in real-time. Helium or 21% O_2/79% N_2 gas mixture was used as the carrier gas without bubbling in studies under anaerobic and aerobic conditions, respectively, and the flow rate was strictly regulated. The reactions were carried out at 37 °C in 25 mL-filter flasks. Calculated amounts of NO were validated by injection of sodium nitrite standards into tri-iodide solution.

Preparation of HbNO-Loaded Erythrocytes. Washed human erythrocytes (40% hematocrit in PBS) were deoxygenated by helium for 1 h at room temperature with gentle stirring. This would produce at least 70% deoxyhemoglobin as determined by the deconvolution of absorption spectra. Deoxygenated cells were treated with 100 μM SPNO at room temperature for an additional 1 h to produce intracellular HbNO and then washed to remove excess SPNO. This method produced ~1 μM HbNO in 40% erythrocyte suspension as measured by tri-iodide chemiluminescence. In some experiments, DHA (500 μM) was incubated for 30 min with the HbNO-loaded erythrocytes at 37 °C. Then, 300 μL of cell suspension was transferred to EPR tubes, which were immediately frozen in liquid nitrogen.

EPR Determination of HbNO. Erythrocyte suspensions and hemoglobin solutions taken before and after the addition of DHA were frozen in liquid nitrogen and then kept at −80 °C. The EPR spectra at 100 K were recorded on MiniScope MS200 EPR spectrometer (Magnettech, Berlin, Germany) equipped with temperature controller H02 using the following conditions: modulation amplitude 10 G and scan time 60 s. In separate experiments, samples were scanned from the $g = 6$ to $g = 2$ region to reveal the presence of methemoglobin and HbNO at 6 K using a Bruker Elexys EPR spectrometer (Bruker, MA) with a modulation frequency of 100 kHz, a modulation amplitude of 10 G, and a sweep time of 86 s.

Measurement of Nitrite and Nitrate in Aqueous Buffer. In order to measure nitrite and nitrate simultaneously, HPLC with subsequent postcolumn derivatization with the Griess reagent (ENO-20 analyzer, Eicom Corporation, San Diego, CA) was used. The reaction between DHA and NO donors in PBS containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) at 37 °C was stopped by injecting the reaction mixture into the HPLC system. The values were calculated after subtraction from the basal levels in PBS.

Measurement of Ascorbate Free Radical (AFR). AFR was generated in PBS containing 0.1 mM DTPA from 1 mM ascorbic acid and 0.3 unit/mL ascorbate oxidase and recorded at different times by EPR spectroscopy (MiniScope) at room temperature. The concentration of AFR was determined following calibration of the instrument using 10 μM 2,2,6,6-tetramethyl-4-hydroxy-piperidine-*N*-oxyl (*17*).

Measurement of Nitrite and HbNO in Erythrocytes. Nitrite levels in erythrocytes were determined by tri-iodide-based chemiluminescence with nitrite-stabilizing solution (*10*). Apart from spectral deconvolution, HbNO was also determined by this method, which showed similar results. Identification of HbNO was done by pretreating samples with 50 mM $HgCl_2$ and 5% acidified sulfanilamide to eliminate SNOHb and nitrite, respectively (*30*).

Statistical Analysis. Data processing and statistical test were done by Prism, version 4.0 (GraphPad Software Inc., San Diego, CA). Reported data in figures and text are given as the mean ± SE and the mean ± SD, respectively from at least three experiments. Analysis of variance (ANOVA) with Tukey's multiple comparison test was used to compare with acceptable p value less than 0.05.

RESULTS

DHA Oxidized HbNO to Methemoglobin and Caused NO Release. We tested the hypothesis that DHA could oxidize HbNO and release NO for nitrite formation. The purity of HbNO was checked by spectroscopy, and HbNO was stable

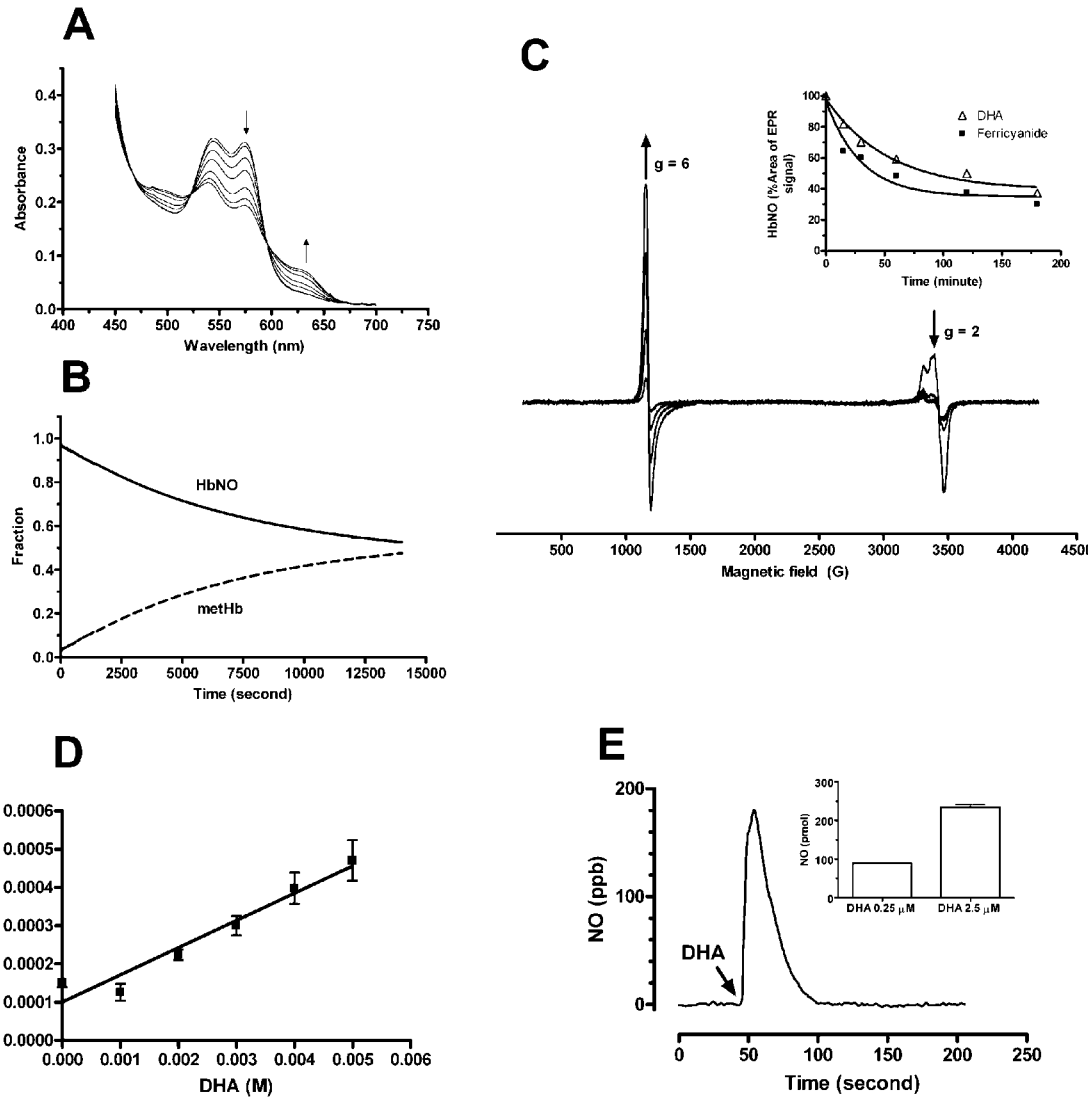

FIGURE 1: Oxidation and NO release from HbNO by DHA. Thirty micromolar HbNO (prepared as described in Materials and Methods) was incubated with 25 μM DHA in PBS at 37 °C under aerobic conditions. (A) Spectral evolution showing a decrease of HbNO (575 nm) and increase of methemoglobin (630 nm) at time 0, 5, 30, 60, 120, 180, and 240 min. (B) Time course of HbNO and metHb. (C) EPR spectra of control HbNO (30 μM) and HbNO incubated with 25 μM DHA for 1, 2, and 3 h at 37 °C. The EPR spectra were recorded at 6 K. The inset depicts the time-course profile of HbNO oxidation by 25 μM DHA or ferricyanide. HbNO was calculated from the double integration of the EPR signal. Each point represents the mean ± SE ($n = 3$). (D) Apparent rate constants as a function of DHA concentrations. Thirty micromolar HbNO was incubated with DHA under aerobic conditions at 37 °C. (E) NO release from HbNO by DHA. NO release was measured by chemiluminescence after the addition of 2.5 μM DHA to hemoglobin solution (4 mL) containing 30 μM HbNO in PBS at 37 °C, under 21% O_2. Inset: Calculated NO release (in pmol) after injection of 0.25 and 2.5 μM DHA.

at 4 °C under anaerobic conditions for 24 h, but slowly decayed with a rate constant of $1.46 \pm 0.16 \times 10^{-4}$ s^{-1} (half-life ~79 min) at 37 °C under aerobic conditions. The addition of 25 μM DHA was followed by a more rapid decrease of the HbNO peak at 575 nm and an increase of the methemoglobin (metHb) peak at 630 nm under aerobic conditions (Figure 1A). The fraction of HbNO and metHb was calculated using spectral deconvolution and plotted against time (Figure 1B). There was no change in the absorption spectra of HbNO with DHA under anaerobic conditions. The data from absorption spectrosocopy were consistent with the parallel EPR results: a decrease in the EPR signal of 30 μM HbNO ($g = 2$) and an increase in the signal of methemoglobin ($g = 6$) (Figure 1C) after 3 h of incubation with 25 μM DHA. The area of EPR signal of HbNO was calculated from double integration. The ability of DHA in oxidizing HbNO was comparable with that of ferricyanide as shown in the time course of the area of the HbNO EPR signal (inset of Figure 1C). The second-order rate constant of the reaction between HbNO and DHA under aerobic conditions is 0.071 ± 0.016 $M^{-1} \cdot s^{-1}$ (Figure 1D) as calculated from the disappearance of HbNO. Furthermore, the NO release from HbNO was detected by chemiluminescence immediately after DHA injection (Figure 1E). Under aerobic conditions, NO was released from 30 μM HbNO after adding DHA.

AFR and DHA Enhanced NO Oxidation to Nitrite in PBS. Oxidation of NO released from PRNO (10 μM) or SPNO (1 μM) in PBS containing 0.1 mM DTPA was studied with and without DHA or AFR at 37 °C. AFR was generated using 1 mM ascorbic acid and 0.3 unit/mL ascorbate oxidase, which yielded ~2.5 μM AFR as determined by EPR. The formation of nitrite and nitrate was measured by HPLC (ENO-20 analyzer). One mole of PRNO (half-life ~1.8 s) or SPNO (half-life ~39 min) gives 2 mol of NO. AFR and

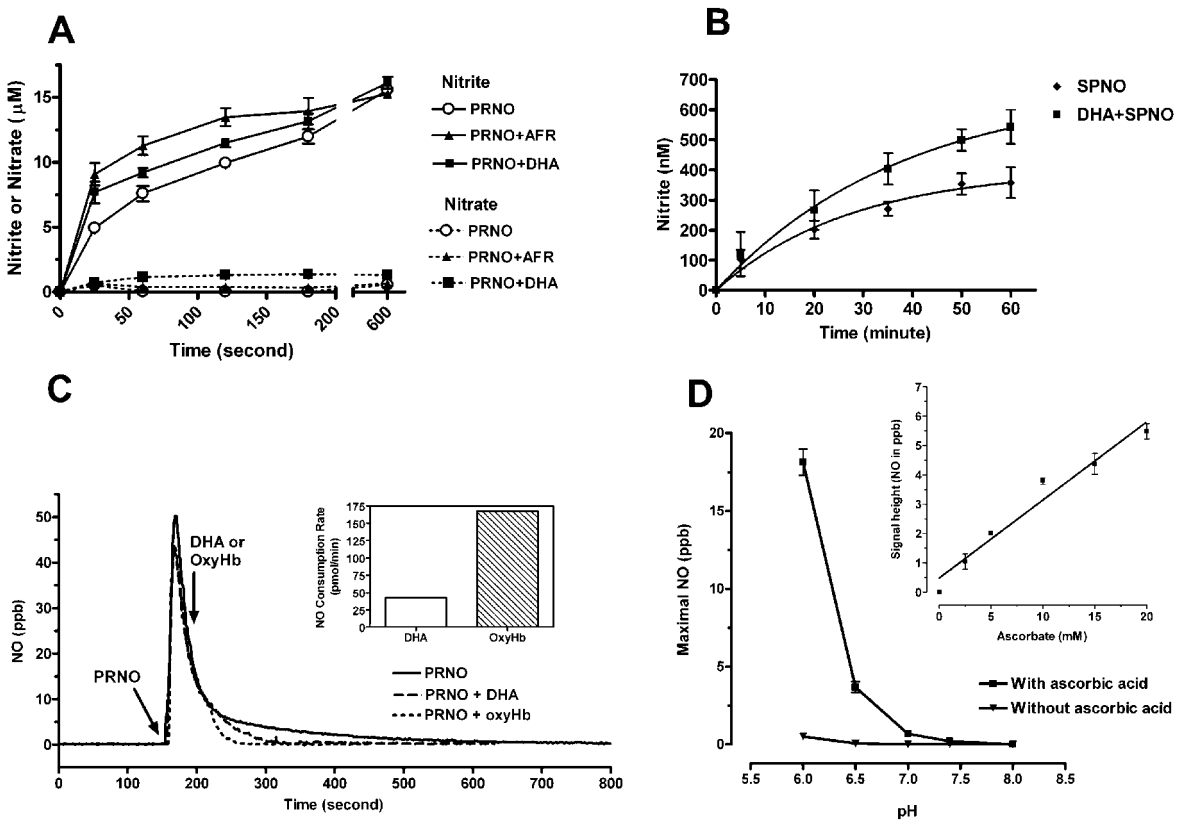

FIGURE 2: Nitrite formation from proli NONOate (PRNO) (A) and spermine NONOate (SPNO) (B) in PBS containing 0.1 mM DTPA at 37 °C. Nitrite and nitrate were measured simultaneously by HPLC. [PRNO] = 10 μM, [SPNO] = 1 μM, and [DHA] = 500 μM. AFR (~2.5 μM) was generated from 1 mM ascorbic acid and 0.3 unit/mL ascorbate oxidase. (C) NO consumption by DHA and oxyhemoglobin in PBS at 37 °C. The inset depicts comparison of the NO consumption rate by DHA and oxyhemoglobin. The amount of consumed NO was calculated from the area after the addition of DHA or oxyhemoglobin. [PRNO] = 0.1 μM, and [DHA] = [oxyhemoglobin] = 1 μM. (D) Effect of pH on nitrite reduction to NO by ascorbic acid in PBS containing 0.1 mM DTPA. [Nitrite] = [ascorbic acid] = 1 mM. Inset: Dependence of NO formation on ascorbic acid concentrations in PBS (pH 7.4) containing 1 mM nitrite and 0.1 mM DTPA.

DHA (500 μM) enhanced NO oxidation as shown in the time-course plots of nitrite formation (Figure 2A and B). Addition of DHA (500 μM) had no effect on nitrite (1 μM) in PBS. No increase in nitrate levels was observed from the reactions between NO and DHA or AFR. These suggest that nitrite is stable and that DHA does not react with NO and nitrite to form nitrate in aqueous buffer. These results are consistent with the ability of DHA to consume NO as measured by chemiluminescence (Figure 2C). The rate of NO consumption by DHA was ~4 times less than that by oxyhemoglobin (inset of Figure 2C), and the relative rate constant of the reaction of NO and DHA was $1.26 \pm 0.02 \times 10^7$ $M^{-1} \cdot s^{-1}$. In PBS, ascorbic acid, only at high concentrations (millimolar), was able to reduce nitrite (1 mM) to NO as determined by chemiluminescence, and the NO generation increased exponentially when pH was <7.4 (Figure 2D).

DHA Increased Nitrite Levels in Erythrocytes. Nitrite-stabilizing solution containing ferricyanide was used to inhibit nitrite oxidation to nitrate by oxyhemoglobin in erythrocytes. We have shown previously that nitrite is unstable in erythrocytes (half-time in whole blood ~10 min after drawing), but it is stable over 24 h at room temperature with the stabilizing solution (*10*). Because ferricyanide oxidizes HbNO (Figure 1C) and might release NO to form nitrite, we tested the nitrite formation from HbNO solution and erythrocytes after mixing them with the stabilizing solution. Hemoglobin (30 μM) was deoxygenated and subsequently incubated with 100 μM SPNO to generate HbNO with continuous monitoring by absorption spectroscopy. The ultrafiltration with 10,000-molecular-weight-cutoff filters (Amicon Ultra, Millipore, Bedford, MA) was used to remove excess SPNO and concentrate HbNO. The filters were washed extensively with PBS to remove contaminated nitrite before use. The retentate was redissolved in PBS. Addition of the stabilizing solution fully eliminated HbNO, while the nitrite levels were unchanged (Figure 3A). In erythrocytes with additional HbNO, the nitrite levels were unaffected. These suggest that HbNO is oxidized to nitrate, which is undetectable by tri-iodide chemiluminescence without producing artificial nitrite.

We used the PBS-washed erythrocytes throughout the studies, which had average nitrite levels of 66 ± 21 nM (Figure 3B). The nitrite levels in these washed erythrocytes were partially elevated, up to 114 ± 12 nM ($p < 0.01$) by the addition of DHA (50 μM) after washing. As endothelial nitric oxide synthase (eNOS) had been described in erythrocytes (*31*), we hypothesized that nitrite could be generated from NO produced by eNOS. To test this hypothesis, we preincubated erythrocytes with an eNOS inhibitor L-N^G-nitro-L-arginine methyl ester (L-NAME, 1 mM) for 30 min. The effect of DHA on erythrocytic nitrite formation was unaffected by L-NAME. Ascorbic acid at physiologic levels in erythrocytes (60–100 μM) (*17*) had no effect on the erythrocytic nitrite (Figure 3C). Furthermore, to test whether nitrite production by DHA occurred intracellularly, we used

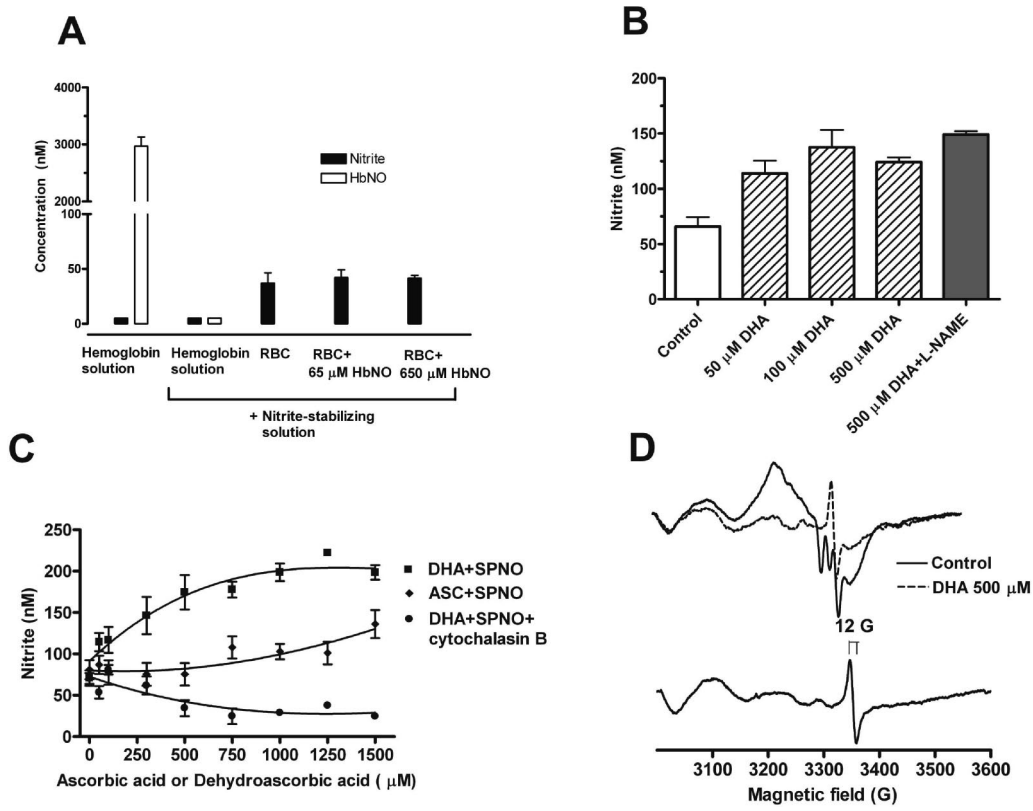

FIGURE 3: Increase of nitrite in erythrocytes in the presence of DHA. (A) Validation of method using the nitrite-stabilizing solution. (B) Nitrite generation from endogenous NO in erythrocytes after 15 min incubation with DHA. L-NAME (1 mM) was incubated with erythrocytes for 30 min before the addition of DHA. The washed erythrocytes were resuspended in PBS at 40% hematocrit and incubated with DHA at 37 °C. (C) Effect of DHA and ascorbic acid (ASC) on erythrocytic nitrite in the presence of 0.15 μM SPNO. Cytochalasin B (20 μM) was used to inhibit DHA transport. (D) Upper panel: EPR spectra of the HbNO (1 μM)-loaded erythrocyte samples in the presence of 500 μM DHA at 37 °C for 30 min. Lower panel: EPR spectrum of reference ascorbate free radical (AFR), which was prepared by incubating DHA (500 μM)-loaded erythrocytes with 5 mM ferricyanide for 1 min. All spectra were recorded at 100 K.

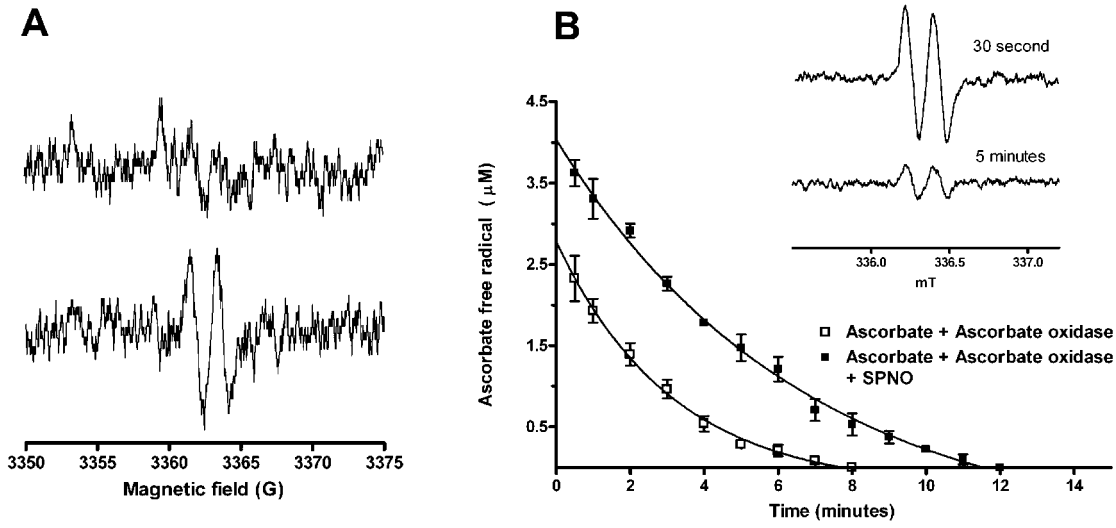

FIGURE 4: (A) Formation of AFR from the reaction of DHA with PRNO (50 mM each) in PBS (pH 7.4) containing 0.1 mM DTPA at room temperature. (B) Time course of AFR in the presence and absence of 1 mM SPNO. AFR was generated from 1 mM ascorbic acid and 0.3 unit/mL ascorbate oxidase in PBS containing 0.1 mM DTPA at room temperature. Inset: Original EPR trace of AFR recorded at room temperature.

cytochalasin B, an inhibitor of glucose transporters, to inhibit DHA uptake (*32, 33*). Cytochalasin B (20 μM) abolished the effect of DHA, suggesting that nitrite was generated inside the cells.

In HbNO (1 μM)-loaded erythrocytes, there was a small NO peak (~0.4 nM) after the addition of 500 μM DHA under aerobic conditions, but the release was undetectable under anaerobic conditions. Thirty minutes after DHA addition, erythrocytes had a marked loss of HbNO as detected by EPR (Figure 3D). Instead, a spectrum with identical features with those of the ascorbate free radical (AFR) was observed with a narrow singlet (12 G) at 100 K. A reference AFR signal (Figure 3D, lower panel) was prepared from erythrocytes preloaded with 500 μM DHA (*17*) and incubated with 5 mM

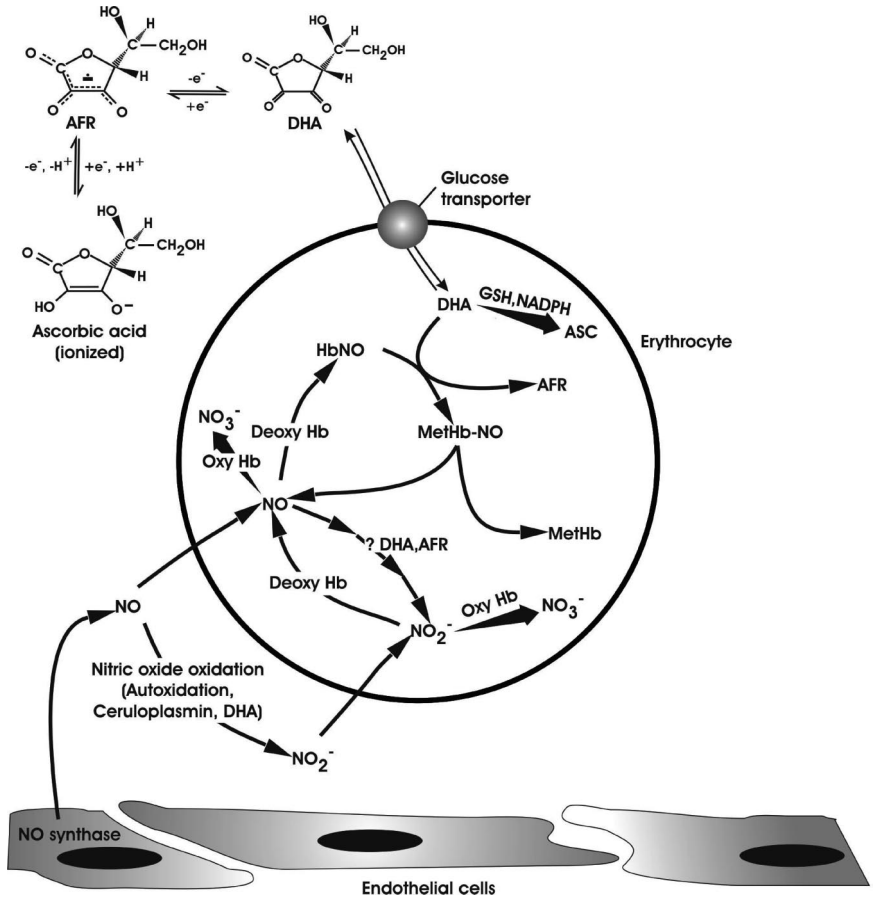

FIGURE 5: Proposed role of DHA in the NO cycle. The complete cycle of NO consists of the NO-producing reactions catalyzed by endothelial NO synthase and nitrite reductase of deoxyhemoglobin (Deoxy Hb). NO would interact with deoxyhemoglobin to form HbNO, which could be reused after oxidation by DHA.

ferricyanide for 1 min (*17, 34*) and recorded at 100 K. However, some contribution to the spectrum (arrow, upper panel) might also come from protein-based radicals (*5*).

AFR Generation from DHA and NO. Generation of AFR from the reaction of DHA with PRNO was demonstrated by the studies in PBS containing 0.1 mM DTPA (Figure 4A). In separate experiments, AFR (~2.5 μM) was generated from 1 mM ascorbic acid and 0.3 unit/mL ascorbate oxidase. In the presence of SPNO, AFR formation was enhanced (Figure 4B).

DISCUSSION

The primary finding of our study is that DHA oxidizes HbNO and releases NO to form nitrite in erythrocytes. The nitrite levels in erythrocytes are presumably maintained in equilibrium between the production and uptake, and metabolism. We suggest here the association of the ascorbic acid cycle to NO in the erythrocytes, namely, DHA could be a part of the pathway for NO release and nitrite generation in erythrocytes.

Although ascorbate is the predominant form of vitamin C in blood, it is possible that DHA is produced extracellularly during oxidative stress (*21, 23, 24, 35*). DHA is known as an oxidized product of ascorbic acid, forming at the end of protective antioxidant process of vitamin C, and is recycled. DHA has an important physiologic role because it can be used to regenerate ascorbic acid inside cells. Although ascorbate recycling utilizes intracellular reducing agents including glutathione (GSH), NADH, or NADPH (*16–18*), little is known about its association with NO. In this work, we showed that DHA accelerated NO oxidation to nitrite but not nitrate. This is in accord with the reduction potentials of DHA/AFR (−0.17 V) and AFR/ASC (0.28 V) (*36*) and the potentials where nitrite reduction is observed (−0.3 V) (*37*), predicting that NO oxidation by DHA is more favorable than nitrite reduction to NO by ascorbic acid. Decreased intracellular levels of GSH or increased DHA during oxidative stress may favor the oxidation of HbNO, NO release, and nitrite formation in erythrocytes.

The decay of HbNO under aerobic conditions corresponds to the autoxidation of its heme group, and our rate constant ($1.46 \pm 0.16 \times 10^{-4}$ s^{-1}) is in a range similar to those of NO dissociation from HbNO (4.2×10^{-4} to 10^{-3} s^{-1}) (*38*). The autoxidation of HbNO alone (half-life of 79 min) is unlikely to explain an arterial−venous gradient of HbNO in human subjects breathing NO gas (*26*). Since the circulatory time from artery to vein is very fast (less than 30 s from forearm artery to vein), the rapid decrease of HbNO from arterial to venous blood could be due to its oxidation accelerated by oxidants such as DHA, which is more likely to happen in arterial blood.

DHA could oxidize HbNO to metHb at a rate comparable with that of ferricyanide in the presence of oxygen. Oxidation of the heme group of hemoglobin would favor the release of NO from HbNO (*4, 6, 26*) to generate nitrite in erythrocytes, which could explain an increment of nitrite when the hemoglobin oxygen saturation increases (*39*). However, the liberated NO is believed to react fully with

hemoglobin in erythrocytes to form nitrate. Hemoglobin causes three electron oxidation of NO to nitrate; however, NO in aqueous solution free of biological material undergoes one electron oxidation to form nitrite but not nitrate (*40*). Inside erythrocytes, the majority of NO reacts with oxyhemoglobin to form nitrate ($k = 5 \times 10^7$ $M^{-1}\cdot s^{-1}$), but it is likely that the significant levels of nitrite could also be formed. The slow reactions of nitrite with oxyhemoglobin (0.21 $M^{-1}\cdot s^{-1}$; Piknova, B., unpublished data) and deoxyhemoglobin (0.2–0.3 $M^{-1}\cdot s^{-1}$) (*41*) may allow nitrite to accumulate in erythrocytes.

The increase in erythrocytic nitrite by DHA is consistent with the previous finding that oral ascorbate supplements increased erythrocytic nitrite in healthy humans (*42*) and deprivation of ascorbate in pregnant rats increased vasoconstrictive response probably due to a decrease in NO (*43*). We found that ascorbic acid at physiological levels had no direct effect on nitrite levels in PBS and erythrocytes; however, at pharmacological concentrations (>1 mM), it increased erythrocytic nitrite, probably by increasing DHA. This may explain in part the effect of ascorbic acid (*44*) and DHA (*45*) at pharmacological doses on vasodilation regarding the HbNO−NO−nitrite pathway. For example, at high doses of ascorbic acid, human erythrocytes take up DHA and reduce it intracellularly by the reductase system, or DHA could interact with HbNO or NO to form nitrite. Ascorbic acid, at high levels in erythrocytes, may subsequently convert nitrite to NO. In addition, DHA can be transported across the blood−brain barrier, and the intravenous injection of DHA is shown to increase postischemic cerebral blood flow in mice (*45*).

The increase of nitrite by DHA was not inhibited by the NOS inhibitor, L-NAME; thus, we have results contrary to the NOS enzyme (*31*) as a significant source of NO in erythrocytes. The raised nitrite levels in erythrocytes would presumably come from HbNO. This nitrite production would likely happen intracellularly because the increase of nitrite was inhibited by cytochalasin B, an inhibitor of the glucose transporter that blocked DHA entry (*32, 33*). SPNO does not enter erythrocytes (*46, 47*). In fact, SPNO can produce the NO signal in the chemiluminescence machine when directly injected, but we found that the signal was zero after pretreatment of erythrocyte samples with acid sulfanilamide, a specific nitrite scavenger (*48*), indicating that SPNO did not enter the cells and that only nitrite in erythrocytes was measured. Therefore, we suggest that NO was released from SPNO outside the cells, entered the erythrocytes, and was subsequently oxidized to nitrite by DHA. Taken together, DHA would be expected to be able to generate approximately 50 nM nitrite from the basal levels of HbNO found physiologically in erythrocytes.

In Figure 5, we present a proposed pathway of the HbNO−NO−nitrite cycle in relation to the ASC−AFR−DHA cycle in erythrocytes. The NO cycle had been proposed previously (*4*); NO is generated as a result of the NO synthase reaction in endothelial cells and oxidized to nitrite/nitrate, and nitrite is recycled to NO by the reductase activity of deoxyhemoglobin. In erythrocytes, NO also interacts with deoxyhemoglobin to form HbNO. HbNO could be oxidized easily by DHA, regenerating bioactive NO, and nitrite, which could contribute to the endocrine activities of NO/nitrite. Furthermore, both DHA and AFR could enhance NO oxidation to nitrite, but most NO becomes nitrate.

Administration of ascorbic acid or DHA could have therapeutic benefit in terms of regenerating NO and nitrite apart from the antioxidant properties on vascular system. DHA obtained from dietary intake would enhance NO and nitrite formation in erythrocytes, which serves as a supply for the endocrine delivery of NO.

ACKNOWLEDGMENT

Low temperature EPR was carried out with the assistance of Dr. Brian Bennett from National Biomedical EPR Center, Department of Biophysics, Medical College of Wisconsin.

REFERENCES

1. Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. J., Johnson, K. A., Smith, R. D., Phillips, G. N., Jr., and Olson, J. S. (1996) Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry 35*, 6976–6983.
2. Cassoly, R., and Gibson, Q. (1975) Conformation, co-operativity and ligand binding in human hemoglobin. *J. Mol. Biol. 91*, 301–313.
3. Sharma, V. S., and Ranney, H. M. (1978) The dissociation of NO from nitrosylhemoglobin. *J. Biol. Chem. 253*, 6467–6472.
4. Reutov, V. P., and Sorokina, E. G. (1998) NO-synthase and nitrite-reductase components of nitric oxide cycle. *Biochemistry (Moscow) 63*, 874–884.
5. Piknova, B., Gladwin, M. T., Schechter, A. N., and Hogg, N. (2005) Electron paramagnetic resonance analysis of nitrosylhemoglobin in humans during NO inhalation. *J. Biol. Chem. 280*, 40583–40588.
6. Nagababu, E., Ramasamy, S., Abernethy, D. R., and Rifkind, J. M. (2003) Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. *J. Biol. Chem. 278*, 46349–46356.
7. Huang, Z., Shiva, S., Kim-Shapiro, D. B., Patel, R. P., Ringwood, L. A., Irby, C. E., Huang, K. T., Ho, C., Hogg, N., Schechter, A. N., and Gladwin, M. T. (2005) Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. *J. Clin. Invest. 115*, 2099–2107.
8. Zweier, J. L., Wang, P., Samouilov, A., and Kuppusamy, P. (1995) Enzyme-independent formation of nitric oxide in biological tissues. *Nat. Med. 1*, 804–809.
9. Hunter, C. J., Dejam, A., Blood, A. B., Shields, H., Kim-Shapiro, D. B., Machado, R. F., Tarekegn, S., Mulla, N., Hopper, A. O., Schechter, A. N., Power, G. G., and Gladwin, M. T. (2004) Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator. *Nat. Med. 10*, 1122–1127.
10. Dejam, A., Hunter, C. J., Pelletier, M. M., Hsu, L. L., Machado, R. F., Shiva, S., Power, G. G., Kelm, M., Gladwin, M. T., and Schechter, A. N. (2005) Erythrocytes are the major intravascular storage sites of nitrite in human blood. *Blood 106*, 734–739.
11. Lang, J. D., Teng, X., Chumley, P., Crawford, J. H., Isbell, T. S., Chacko, B. K., Liu, Y., Jhala, N., Crowe, D. R., Smith, A. B., Cross, R. C., Frenette, L., Kelley, E. E., Wilhite, D. W., Hall, C. R., Page, G. P., Fallon, M. B., Bynon, J. S., Eckhoff, D. E., and Patel, R. P. (2007) Inhaled NO accelerates restoration of liver function in adults following orthotopic liver transplantation. *J. Clin. Invest.*, in press.
12. Ford, P. C., Wink, D. A., and Stanbury, D. M. (1993) Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett. 326*, 1–3.
13. Shiva, S., Wang, X., Ringwood, L. A., Xu, X., Yuditskaya, S., Annavajjhala, V., Miyajima, H., Hogg, N., Harris, Z. L., and Gladwin, M. T. (2006) Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nat. Chem. Biol. 2*, 486–493.
14. Spagnuolo, M. S., Carlucci, A., Cigliano, L., and Abrescia, P. (2006) Nitric oxide stimulates the erythrocyte for ascorbate recycling. *Nitric Oxide 14*, 272–277.
15. Jansson, P. J., Jung, H. R., Lindqvist, C., and Nordstrom, T. (2004) Oxidative decomposition of vitamin C in drinking water. *Free Radical Res. 38*, 855–860.
16. Wilson, J. X. (2005) Regulation of vitamin C transport. *Annu. Rev. Nutr. 25*, 105–125.

17. May, J. M., Qu, Z. C., and Cobb, C. E. (2004) Human erythrocyte recycling of ascorbic acid: relative contributions from the ascorbate free radical and dehydroascorbic acid. *J. Biol. Chem. 279*, 14975–14982.
18. Linster, C. L., and Van Schaftingen, E. (2007) Vitamin C. Biosynthesis, recycling and degradation in mammals. *FEBS J. 274*, 1–22.
19. Kytzia, A., Korth, H. G., Sustmann, R., de Groot, H., and Kirsch, M. (2006) On the mechanism of the ascorbic acid-induced release of nitric oxide from N-nitrosated tryptophan derivatives: scavenging of NO by ascorbyl radicals. *Chemistry 12*, 8786–8797.
20. Dhariwal, K. R., Hartzell, W. O., and Levine, M. (1991) Ascorbic acid and dehydroascorbic acid measurements in human plasma and serum. *Am. J. Clin. Nutr. 54*, 712–716.
21. Schorah, C. J., Downing, C., Piripitsi, A., Gallivan, L., Al-Hazaa, A. H., Sanderson, M. J., and Bodenham, A. (1996) Total vitamin C, ascorbic acid, and dehydroascorbic acid concentrations in plasma of critically ill patients. *Am. J. Clin. Nutr. 63*, 760–765.
22. Nakayama, H., Akiyama, S., Inagaki, M., Gotoh, Y., and Oguchi, K. (2001) Dehydroascorbic acid and oxidative stress in haemodialysis patients. *Nephrol., Dial., Transplant. 16*, 574–579.
23. Trepanier, L. A., Yoder, A. R., Bajad, S., Beckwith, M. D., Bellehumeur, J. L., and Graziano, F. M. (2004) Plasma ascorbate deficiency is associated with impaired reduction of sulfamethoxazole-nitroso in HIV infection. *J. Acquired Immune Defic. Syndr. 36*, 1041–1050.
24. Wann, J. G., Lin, C. S., Chang, L. C., Hsu, Y. H., Chien, C. T., Tai, D. W., Chen, J. S., Yang, C. C., Yeung, L. K., and Chen, C. F. (2006) Enhanced expression of glucose transporter 1 on erythrocyte membrane in hemodialysis patients: the possible role in erythrocyte ascorbate recycling. *Am. J. Kidney Dis. 47*, 1055–1063.
25. DeBari, V. A., Frank, O., Baker, H., and Needle, M. A. (1984) Water soluble vitamins in granulocytes, erythrocytes, and plasma obtained from chronic hemodialysis patients. *Am. J. Clin. Nutr. 39*, 410–415.
26. Gladwin, M. T., Ognibene, F. P., Pannell, L. K., Nichols, J. S., Pease-Fye, M. E., Shelhamer, J. H., and Schechter, A. N. (2000) Relative role of heme nitrosylation and beta-cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation. *Proc. Natl. Acad. Sci. U.S.A. 97*, 9943–9948.
27. McMahon, T. J., Moon, R. E., Luschinger, B. P., Carraway, M. S., Stone, A. E., Stolp, B. W., Gow, A. J., Pawloski, J. R., Watke, P., Singel, D. J., Piantadosi, C. A., and Stamler, J. S. (2002) Nitric oxide in the human respiratory cycle. *Nat. Med. 8*, 711–717.
28. Fink, B., Dikalov, S., and Fink, N. (2006) ESR techniques for the detection of nitric oxide in vivo as an index of endothelial function. *Pharmacol. Rep. 58*, 8–15.
29. Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1961) Studies on the relations between molecular and functional properties of hemoglobin. I. The effect of salts on the molecular weight of human hemoglobin. *J. Biol. Chem. 236*, 391–396.
30. Yang, B. K., Vivas, E. X., Reiter, C. D., and Gladwin, M. T. (2003) Methodologies for the sensitive and specific measurement of S-nitrosothiols, iron-nitrosyls, and nitrite in biological samples. *Free Radical Res. 37*, 1–10.
31. Kleinbongard, P., Schulz, R., Rassaf, T., Lauer, T., Dejam, A., Jax, T., Kumara, I., Gharini, P., Kabanova, S., Ozuyaman, B., Schnurch, H. G., Godecke, A., Weber, A. A., Robenek, M., Robenek, H., Bloch, W., Rosen, P., and Kelm, M. (2006) Red blood cells express a functional endothelial nitric oxide synthase. *Blood 107*, 2943–2951.
32. May, J. M., Qu, Z. C., and Whitesell, R. R. (1995) Ascorbic acid recycling enhances the antioxidant reserve of human erythrocytes. *Biochemistry 34*, 12721–12728.
33. Rumsey, S. C., Kwon, O., Xu, G. W., Burant, C. F., Simpson, I., and Levine, M. (1997) Glucose transporter isoforms GLUT1 and GLUT3 transport dehydroascorbic acid. *J. Biol. Chem. 272*, 18982–18989.
34. Dunne, J., Caron, A., Menu, P., Alayash, A. I., Buehler, P. W., Wilson, M. T., Silaghi-Dumitrescu, R., Faivre, B., and Cooper, C. E. (2006) Ascorbate removes key precursors to oxidative damage by cell-free haemoglobin in vitro and in vivo. *Biochem. J. 399*, 513–524.
35. Hubel, C. A., Kagan, V. E., Kisin, E. R., McLaughlin, M. K., and Roberts, J. M. (1997) Increased ascorbate radical formation and ascorbate depletion in plasma from women with preeclampsia: implications for oxidative stress. *Free Radical Biol. Med. 23*, 597–609.
36. Buettner, G. R. (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch. Biochem. Biophys. 300*, 535–543.
37. Davis, J., Moorcroft, M. J., Wilkins, S. J., Compton, R. G., and Cardosi, M. F. (2000) Electrochemical detection of nitrate and nitrite at a copper modified electrode. *Analyst 125*, 737–742.
38. Azizi, F., Kielbasa, J. E., Adeyiga, A. M., Maree, R. D., Frazier, M., Yakubu, M., Shields, H., King, S. B., and Kim-Shapiro, D. B. (2005) Rates of nitric oxide dissociation from hemoglobin. *Free Radical Biol. Med. 39*, 145–151.
39. Rogers, S. C., Khalatbari, A., Datta, B. N., Ellery, S., Paul, V., Frenneaux, M. P., and James, P. E. (2007) NO metabolite flux across the human coronary circulation. *Cardiovasc. Res. 75*, 434–441.
40. Ignarro, L. J., Fukuto, J. M., Griscavage, J. M., Rogers, N. E., and Byrns, R. E. (1993) Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc. Natl. Acad. Sci. U.S.A. 90*, 8103–8107.
41. Huang, K. T., Keszler, A., Patel, N., Patel, R. P., Gladwin, M. T., Kim-Shapiro, D. B., and Hogg, N. (2005) The reaction between nitrite and deoxyhemoglobin. Reassessment of reaction kinetics and stoichiometry. *J. Biol. Chem. 280*, 31126–31131.
42. Sureda, A., Batle, J. M., Tauler, P., Ferrer, M. D., Tur, J. A., and Pons, A. (2006) Vitamin C supplementation influences the antioxidant response and nitric oxide handling of erythrocytes and lymphocytes to diving apnea. *Eur. J. Clin. Nutr. 60*, 838–846.
43. Ramirez, R. J., Hubel, C. A., Novak, J., DiCianno, J. R., Kagan, V. E., and Gandley, R. E. (2006) Moderate ascorbate deficiency increases myogenic tone of arteries from pregnant but not virgin ascorbate-dependent rats. *Hypertension 47*, 454–460.
44. Gokce, N., Keaney, J. F., Jr., Frei, B., Holbrook, M., Olesiak, M., Zachariah, B. J., Leeuwenburgh, C., Heinecke, J. W., and Vita, J. A. (1999) Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation 99*, 3234–3240.
45. Huang, J., Agus, D. B., Winfree, C. J., Kiss, S., Mack, W. J., McTaggart, R. A., Choudhri, T. F., Kim, L. J., Mocco, J., Pinsky, D. J., Fox, W. D., Israel, R. J., Boyd, T. A., Golde, D. W., and Connolly, E. S., Jr. (2001) Dehydroascorbic acid, a blood-brain barrier transportable form of vitamin C, mediates potent cerebroprotection in experimental stroke. *Proc. Natl. Acad. Sci. U.S.A. 98*, 11720–11724.
46. Moulinoux, J. P., Le Calve, M., Quemener, V., and Quash, G. (1984) In vitro studies on the entry of polyamines into normal red blood cells. *Biochimie 66*, 385–393.
47. Vaughn, M. W., Huang, K. T., Kuo, L., and Liao, J. C. (2000) Erythrocytes possess an intrinsic barrier to nitric oxide consumption. *J. Biol. Chem. 275*, 2342–2348.
48. Feelisch, M., Rassaf, T., Mnaimneh, S., Singh, N., Bryan, N. S., Jourd'Heuil, D., and Kelm, M. (2002) Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *FASEB J. 16*, 1775–1785.

BI702158D