Nitric oxide (NO) synthesized by endothelial nitric-oxide synthase diffuses both to the smooth muscle cells as well as the lumen. The reaction of NO with smooth muscle cell guanylate cyclase plays a crucial role in the regulation of vascular tone and blood flow (3). The reaction of NO with smooth muscle cell synthase diffuses both to the smooth muscle cells as well as the lumen (1, 2). The reaction of NO with oxyhemoglobin to form methemoglobin (Hb(III)) and nitrate and with deoxyhemoglobin (deoxyHb) to form heme-nitrosylated hemoglobin (Hb(II)NO) (6, 7). Earlier studies, therefore, assumed that the only effect of red blood cells on the bioactivity of nitric oxide was to scavenge and inactivate NO limiting its availability for vasodilation.

This perspective was challenged by studies of Stamler and co-workers (8–13) who proposed an active role for red blood cells in the delivery of NO. Their model is based on the potential binding of NO released from arterial endothelial cells to the few vacant deoxyhemep to form Hb(II)NO and to the β-3 cysteine to form S-nitrosohemoglobin (SNOHb) (11). The relative affinity for these two hemoglobin sites was assumed to depend on the hemoglobin quaternary conformation with heme-bound NO favored by the T-state present in deoxygenated hemoglobin transferred to the cysteine in the R-state formed during hemoglobin oxygenation. The destabilization of the cysteine bound NO during hemoglobin deoxygenation was assumed to facilitate the transfer of NO to low molecular weight and membrane-associated thiols resulting in an increase in the availability of NO for vasodilation in the microcirculation (8–13).

The Stamler proposal has attracted much attention because it provides a mechanism for the delivery of relatively unstable NO produced in the larger arteries to the smaller blood vessels where the regulation of blood flow for the delivery of oxygen is crucial.

Many investigators have challenged the validity of this hypothesis. The areas of disagreement include almost all aspects of the hypothesis. 1) The hypothesis was based on determinations of red cell Hb(II)NO and SNOHb in arterial (A) and venous (V) blood indicating micromolar levels of red blood cell NO with A/V differences in rats and humans consistent with a conformationally linked transfer between both sites (9, 13). Although Rassaf et al. (14) have been able to confirm the data of Stamler used in the initial studies on rodents, their results indicate no detectable heme-NO with human subjects, completely contradicting the Stamler results. Gladwin et al. (15) have also studied human subjects and report levels of red cell Hb(II)NO and SNOHb in humans much lower than those of Stamler with no indication of the necessary A/V differences. 2) The chemical processes required by the Stamler hypothesis have been challenged. (a) NO should not bind specifically to the low levels of deoxyHb present in arterial blood where most of the endothelial NO is produced (16–18). (b) NO tightly bound to Hb(II) should not be transferred to the β-3 cysteine residue producing SNOHb, and (c) the R to T quaternary conformation does not seem to facilitate the release of NO from SNOHb (19–21).

In order to address the levels of red cell NO in human subjects, we have developed a new chemiluminescence assay...
that can measure heme-NO (Hb(II)NO and Hb(III)NO) without any sample processing and without interference from nitrate, nitrite, or nitrosodihydroxylation down to a level of 10 pm in red blood cells. By this method we find appreciable levels of heme-NO and significant A/V differences with more heme-NO in the venous blood. Furthermore, by combining electron paramagnetic resonance (EPR) with chemiluminescence we have shown that most of the heme-NO in arterial Hb(III)NO, which other investigators have focused on, but Hb(III)NO. The Hb(III)NO represents a labile form of heme-NO, which is formed by nitrite reduction in the red cell.

EXPERIMENTAL PROCEDURES

Reagents—All reagents and chemicals were obtained from Sigma-Aldrich unless mentioned. 2.5% NO gas and argon (Research grade) were obtained from Matheson Gas Products. All reactions were conducted in 46 mM NaCl, 4 mM sodium phosphate buffer, pH 7.4, 100 μM EDTA (NaCl-phosphate buffer) unless otherwise mentioned.

Arterial and Venous Blood—Arterial and venous blood were obtained from normal healthy human volunteers after overnight fasting. The Johns Hopkins Bayview Medical Center IRB approved the study protocol. Arterial samples (3 ml) were obtained from the cannulated brachial artery and venous samples (3 ml) from a cannula in the ipsilateral antecubital artery. Blood was immediately plated on ice and 30 min the cells were centrifuged to remove plasma and stored at 77 K.

Hb(II)NO Preparation—Human hemoglobin was purified from fresh hemolyate by gel filtration using a Sephadex G-100 column equilibrated with NaCl-phosphate buffer and stored in liquid nitrogen. 50 μM hemoglobin was completely deoxygenated by flushing with argon while stirring until the spectrum of hemoglobin corresponded to that of deoxyHb. The NO gas (2.5%) was purified by passing through concentrated NaOH with small amounts of NO added to a deoxyHb solution to produce Hb(II)NO as confirmed spectrophotometrically. The Hb(II)NO was then quickly passed down a Sephadex G-25 at 4 °C to remove unreacted NO. The final concentration of Hb(II)NO was determined by visible spectrophotometry using the molar extinction coefficient for Hb(II)NO of 11.4 at 544 nm. Multiple aliquots of the sample were stored in liquid nitrogen.

Chemiluminescence Method to Determine Hb(II)NO—The Model 280 Nitric Oxide Analyzer (NOA) from Sievers Instruments was used to determine heme-NO. The purge vessel contained 5.5 ml of glacial acetic acid, 1.5 ml of 0.8 M potassium ferricyanide in water and 1 ml of 100 mM sulfanilamide in 2 M acetic acid. Potassium ferricyanide was used to help release the NO from Hb(II)NO by oxidizing the hemoglobin. Sulfanilamide was used to react with nitrite under acidic conditions to minimize the NO signal coming from nitrite. Acetic acid enhances the release of NO from Hb(II)NO and prevents protein from formation. The temperature of the purge vessel was maintained at 30 °C. Arsenic bunted through the purge vessel carries any released NO to the detector, which quantitates the NO by measuring the gas-phase chemiluminescent NO reaction with oxygen. Volumes corresponding to 2.5, 5, 10, 15, and 25 pmol of Hb(II)NO were injected into the purge vessel to obtain concentration dependent chemiluminescence signals. Origin 6.1 software was used to integrate the peak areas. A calibration curve was prepared by plotting concentrations of Hb(II)NO versus area. The absence of interference from other nitric oxide species in this assay was verified by injecting 10 pmol of Hb(II)NO premixed with 25 pmol of nitrite, nitrate, or S-nitrosothioglutathione (GSNO).

Determination of Heme-NO (Hb(II)NO and Hb(III)NO) by Chemiluminescence—Heme-NO formed during nitrite reduction by deoxyHb: 1 mM deoxyHb was incubated with varying concentrations (0.5, 1, 2.5, and 5 μM) of freshly prepared sodium nitrite in NaCl-phosphate buffer in septum-sealed cuvettes at 22 °C. 20–50 μl of sample was withdrawn from the reaction mixture with a gas tight syringe at varying time points and was injected into the NOA purge vessel to determine the release of NO. NOA purge vessel consisted of total heme-NO. The concentration of NO released was determined by comparing the area of the observed signal with that for an Hb(II)NO standard curve (above). Hb(II)NO was determined after oxygenation of the sample during, which time the NO bound to Hb(II)NO reacted with oxygen producing nitrite. Subtraction of the Hb(II)NO values from the total heme-NO provides values for Hb(III)NO.

(B) Heme-NO formed during nitrite reduction by red blood cells: deoxygenated red blood cells (30% hct) were incubated in phosphate-buffered saline, pH 7.4 (PBS) with 1, 5, and 10 μM nitrite for 30 min at 22 °C in septum-sealed cuvettes. After the reaction, the cells were washed twice with cold PBS and then lysed in 1 ml of water. 50 μl of this lysate was injected into the purge vessel. Under the conditions of this experiment, without removing oxygen from the PBS used to wash the cells and the water used for lysis, the detected heme-NO is predominantly Hb(II)NO.

(C) In vivo formation heme-NO in arterial and venous red blood cells: Just prior to analysis, 100 μl of freshly thawed cells were transferred using a gas tight syringe to an anaerobic cuvette containing 300 μl of deoxygenated double distilled water to determine total heme-NO or 300 μl oxygenated double distilled water to determine Hb(II)NO. 100 μl of the lysed samples were injected into the purge vessel in triplicate (the reagent in the purge vessel was changed after 3–4 determinations). The concentration of hemoglobin in the injected sample was determined by visible spectrophotometry using the millimolar heme extinction coefficient for oxyHb of 15.37 at 577 nm. The concentration of NO released was calculated using the Hb(II)NO standard curve. The concentrations of NO and hemoglobin were used to calculate the percent hemoglobin nitrosylated. The Origin 6.1 program was used to integrate the area of the smoothed chemiluminescence signals.

Determination of Heme-NO (Hb(II)NO and Hb(III)NO) by EPR—Samples prepared for electron paramagnetic resonance (EPR) studies were transferred to 4 mm clear fused quartz EPR tubes (707 SQ250M-WILMAD) and frozen by submerging the EPR tubes in liquid nitrogen. Samples were stored at 77 K until recorded. EPR spectra were measured using an IBM ER-200D SRC spectrometer with 100 kHz modulation. An Oxford cryo-cryogenic flow ESR900 cryogenic unit with an ITC 502 temperature controller was used to maintain the temperature of the sample in the spectrometer at 3.6–4.0 K.

(A) Hb(II)NO formed during nitrite reduction by deoxyHb: 1 mM deoxyHb was reacted with 250 μM nitrite in NaCl-phosphate buffer and transferred anaerobically to 4-mm quartz EPR tubes and immediately frozen in liquid nitrogen. 6–9 mm of Hb(II)NO was used to correct the observed spectrum of Hb(II)NO in the region from 3150–3550 gauss for the g = 2 Hb(III) signal. The concentration of Hb(II)NO was determined by comparing the spectrum obtained during nitrite reduction with that of a standard solution of Hb(II)NO.

(B) Hb(II)NO formed during nitrite reduction of deoxyHb: Hb(II)NO due to the interaction of paramagnetic Hb(III) with the unpaired electron on NO. Hb(III)NO can, however, be quantitated by flushing the sample with argon to remove the weakly bound NO. 1 mM deoxyHb was reacted with 250 μM nitrite in NaCl-phosphate buffer for 10 min. One aliquot of the sample was anaerobically transferred to a 4-mm EPR tube and frozen. The remaining sample was flushed with argon for 3 min before a second aliquot was transferred to an EPR tube. The EPR signals were measured in the region of 900–1300 gauss to quantitate the amount of Hb(III)NO. The increase in the signal after flushing with argon was a measure of the amount of Hb(III)NO in the sample. The observed spectra were compared with that of a standard solution of Hb(III)NO.

(C) Hb(III)NO in red blood cells from arterial and venous blood: The blood was spun to remove plasma within 30 min of being drawn. Cells were then diluted with 4 volumes of deoxygenated PBS to measure the basal Hb(III) or oxygenated PBS to measure the additional Hb(III) that formed after the reaction of oxygen with Hb(III)NO. The samples were transferred to EPR tubes and frozen in liquid nitrogen to measure the Hb(III) NO signal as described above. The difference between the intensities of these two samples of Hb(III) was a measure of Hb(III)NO.

RESULTS

Chemiluminescence Assay of Hb(II)NO Standard—Chemiluminescence assays for NO are highly sensitive with a detection limit of 0.5 pmol. Despite this sensitivity there are a number of difficulties with the conventional NO assays. Since NO is highly unstable, the conventional method for the determination of NO is to reduce all nitric oxide related species (nitrite, nitrate, Hb(II)NO, SNOHb etc.) back to NO gas under strongly reducing conditions (strong reducing agents in strong acid at high temperature). This method, however, does not differentiate between the different NO species. It, therefore, becomes essential to utilize separation procedures involving ultrafiltration, column chromatography etc. to remove nitrate, nitrate, and other NO-related species from the species of interest. Such procedures have difficulties associated with contaminants picked up during the purification procedure and the possible loss of signal from unstable NO species of interest.
Our chemiluminescence method, bypasses all of these difficulties by directly determining heme-NO levels as low as 10 nM, even in hemolysate without any sample purification, and without interference of other NO species. This is accomplished by placing all of the reagents necessary to specifically release NO from heme in the chemiluminescence purge vessel. Fig. 1 shows the results obtained for the determination of Hb(II)NO with the sample being injected into the purge vessel containing potassium ferricyanide, sulfanilamide, and glacial acetic acid. The concentration-dependent increase in signal is shown in Fig. 1A. The inset shows the stoichiometric release of NO. A recovery of 95 (±5) was obtained when standards of Hb(II)NO were added to concentrated hemoglobin or a 25% hct suspension of red blood cells. With red blood cells the peaks were slightly broader, but the total area was the same (data not shown). The specificity of the assay is shown in Fig. 1B where the addition of nitrate (25 pmol), nitrite (25 pmol), or GSNO (25 pmol) to 10 pmol of Hb (II) NO did not have any effect on the signal intensity.

Nitrite Reduction to NO by DeoxyHb—The chemiluminescence method was used to follow the reaction of nitrite with deoxyHb under conditions approaching the 500 nM physiological level of nitrite (22) with a large excess of deoxyHb. The reaction leveled off within 5 min with 60–80% of the nitrite converted to NO (Fig. 2A). The assay used (see above) will measure heme-NO with the NO bound to either Fe(II) or Fe(III). The binding of NO to Fe(II) is very strong with a very slow off-rate (23) resulting in a stable complex. However, the binding of NO to Fe(III) is much weaker (24) and this complex is not expected to be stable in the presence of oxygen, which readily reacts with NO producing nitrite. To help identify the heme-NO species present during nitrite reduction, the sample was oxygenated after the anaerobic reaction of deoxyHb with nitrite proceeded for 40 min (Fig. 2B). The 42% reduction in signal intensity after oxygenation indicates that even 40 min after nitrite reduction was initiated a major fraction of the heme-NO is not stable in the presence of oxygen and is presumably Hb(III)NO. Although free nitric oxide will also disappear as a result of the reaction with oxygen, in the presence of an excess of deoxyHb no free NO is expected to be present. To confirm that Hb(II)NO does not contribute to this unstable pool of NO, it was shown that the heme-NO signal was not affected by oxygenation when Hb(II)NO was prepared by directly mixing deoxyHb with NO gas at a ratio of 1000:1 (data not shown).

EPR Spectroscopic Determination of Hb(III), Hb(II)NO and Hb(III)NO during Nitrite Reduction by DeoxyHb—EPR can unambiguously identify both Hb(III) and Hb(II)NO. EPR is, however, less sensitive than chemiluminescence, and it was necessary to perform these experiments with higher concentrations of reactants. These experiments were performed at 250 μM nitrite with a 4-fold excess of deoxyHb (1 mM). Fig. 3A shows an increase in the Hb(III) signal in the region of g = 6 over the basal level and the generation of an Hb(III)NO signal in the region of g = 2 during the reaction of nitrite with deoxyHb.

EPR, however, does not directly detect Hb(III)NO, because of the coupling of the unpaired electron on the nitric oxide with that of the paramagnetic Hb(III). The amount of NO bound to Hb(III) can, however, be quantitated by displacing this weakly bound NO with argon resulting in an increase in the Hb(III) signal (25). The increase in the intensity of the Hb(III) signal by flushing with argon (Fig. 3A) is indicative of the presence of labile Hb(III)NO. Also shown in Fig. 3B is the increase in the g = 2 signal for Hb(II)NO after flushing with argon. This effect is attributed to the binding of the NO released from Fe(III) to other Fe(II) sites present in deoxyHb. On the basis of the EPR data, after a 10-min reaction of 1 mM Hb with 250 μM nitrite, Hb(III)NO accounts for most of the NO formed with 77 μM Hb(II)NO and only 11.5 μM Hb(II)NO.

To quantitate the time-dependent formation of Hb(III)NO, we have compared the chemiluminescence assay of total heme-NO and the EPR determination of Hb(II)NO at the same 4:1 hemin: nitrite molar ratio (Fig. 3C). The finding that the reaction detected by chemiluminescence is so much faster than that of Hb(II)NO measured directly by EPR clearly indicates that the heme-NO detected by chemiluminescence in the early stages of the reaction (Figs. 2A and 3C) is primarily Hb(III)NO and not Hb(II)NO.

Nitrite Reduction in Vivo—Fig. 4 shows that Hb(II)NO was produced when deoxygenated red blood cells (30% hct) were incubated with low levels of nitrite approaching the 500 nM plasma level of nitrite (22). As shown in Fig. 4 the basal Hb(II)NO signal corresponding to 0.056 μM Hb(II)NO increased to 0.11, 0.32, and 0.84 μM when the cells were incubated with 1, 5, and 10 μM nitrite, respectively.

To determine whether nitrite reduction actually occurs in vivo, we have quantitated the level of Hb(III)NO in red blood cells utilizing the instability of Hb(III)NO relative to Hb(II)NO in the presence of oxygen (Fig. 2B). We measured the total heme-NO by diluting the hemolysate (arterial and venous) in argon flushed water and only the more stable Hb(II)NO by diluting in oxygenated water (Fig. 5A). The decrease in signal for the oxygenated sample is a lower limit of the level of Hb(III)NO present in the sample. The presence of unstable
Hb(III)NO in red blood cells was also confirmed by EPR results on two samples of fresh venous blood for which the Hb(III) intensity increased when NO bound to Hb(III) decomposes in the presence of oxygen (Fig. 5B). Fig. 5C and Table I shows the average values for heme-NO of arterial and venous blood determined by chemiluminescence. The results indicate that the major fraction of heme-NO (~75%) consists of Hb(II)NO with red blood cells obtained from both arterial and venous blood (Fig. 5C). The arterial red blood cells had \(0.00108 \pm 0.00004\%\) Hb(II)NO that corresponds to 201 \(\pm 72\) nM. The venous red blood cells had \(0.00176 \pm 0.00003\%\) Hb(III)NO, which corresponds to 353 \(\pm 61\) nM. These values would correspond to values in whole blood of 80.4 nM Hb(III)NO in arterial blood and 141.2 nM Hb(III)NO in venous blood.

The values of Hb(II)NO in arterial RBCs was \(0.0004 \pm 0.00016\%,\) which corresponds to \(-81 \pm 56\) nM, whereas venous red blood cells contained \(0.00052 \pm 0.000157\%,\) which corresponds to \(103 \pm 32\) nM. The levels Hb(II)NO would be 32.4 nM in arterial circulation and 41.2 nM in venous circulation.

**DISCUSSION**

The reaction of nitric oxide with smooth muscle guanylate cyclase plays a crucial role in the regulation of vascular tone and blood flow (1). Although most of the nitric oxide is synthe-
arterial blood. In arterial blood they also reported 2.5/100 g red cell NO. The use of the KI/I3 method picking up of impurities, which increase the determination, as reducing agents are used. Sample processing can both result in will contribute to the putative heme-NO measured when re-
include the uncertainty generated by the usual requirement to
by Stamler than other investigators. It is also necessary to
suggested (14) to explain the much higher NO values obtained
by other investigators (15, 18) eliminates nitrate contamina-
75% of the red cell
contaminants or generating NO (see above). Gladwin’s values (15) for heme-NO are actually comparable with our values for total heme-NO even though his purification procedure results in the oxygenation and loss of Hb(III)NO. Since his values are ~4 fold higher than our values for Hb(II)NO of 32.4 nM and 41.2 nM in arterial and venous blood respectively (Table I), the purification procedure presumably picks up nitrite impurities at the same time that the Hb(III)NO reacts with oxygen.

Hb(III)NO in Red Blood Cells—By developing a method that requires no manipulation we have been able to quantify the presence of a previously unquantified, labile form of heme-NO. This labile heme-NO accounts for ~75% of the total heme-NO and is not stable in the presence of oxygen (Fig. 5, A and C). The oxygen-dependent increase in the g = 6 Hb(III) EPR signal (Fig. 5B) that coincides with the decrease in the chemiluminescence signal indicates that the labile heme-NO involves NO bound to Hb(III), which is EPR silent. However, this Hb(III)NO can not originate from the direct reaction of Hb(III) with NO in the red cell. Although there are traces of Hb(III) present in the red cell, it reacts with NO nearly 4 orders of magnitude more slowly than either oxyHb (reacts to form nitrate) or deoxyHb (reacts to form Hb(III)) (23, 24).

How Do We Explain the Observation That 75% of the Red Cell Heme-NO Is Hb(III)NO?—Hb(III)NO in the red cell can best be explained by nitrite reduction in the red cell. Plasma contains ~500 nM of nitrite (22). This nitrite at least in part originates from the reaction of oxygen with the NO released into the arterial circulation as shown in Reaction 1.

4NO + O2 + 2H2O → 4HNO2 → 4H+ + 4NO3-
REACTION 1

The 500 nM nitrite represents a steady state level with nitrite continuously being taken up by the red cell (27). In the red cell nitrite reacts with both oxyHb and deoxyHb. With oxygenated hemoglobin the nitrite is converted to nitrate and the hemoglobin is oxidized to metHb (28) as shown in Reaction 2.

Hb(II)O2 + 2NO2- + 2H+ → Hb(III) + NO3- + NO2+H2O
REACTION 2

While the reaction with deoxygenated hemoglobin also produces oxidized hemoglobin, it results in the nitrite being reduced to NO resulting in the eventual formation of Hb(II)NO in Reaction 3.

2Hb(II) + ONO- + H+ → Hb(III) + Hb(II)NO + OH-
REACTION 3

This reaction involves Hb(III)NO as an intermediate (29) (see below). The relatively high fraction of red cell NO present as Hb(III)NO indicates that this intermediate is relatively long lived with its rate of formation appreciably higher than the rate for its eventual conversion to the stable relatively inert Hb(II)NO (see below). Nitrite reduction therefore provides a source for a pool of labile bioactive NO in the red cell.

Nitrite Reduction by deoxyHb—Nitrite reduction by de-
oxyHb has been previously reported (30, 31). However these early studies used visible spectroscopy to measure the final products (Reaction 3) formed during the reaction (Hb(III) and Hb(II)NO), but not the intermediates. They reported that the reaction at neutral pH was very slow requiring a large excess of nitrite with essentially no observable reaction under the conditions present in red blood cells. Recent studies using EPR have focused on the formation of the /Hb(II)NO (32) and also neglected the importance of the intermediates formed.

Fig. 2A, however, shows that when chemiluminescence is used to measure total heme-NO (Hb(II)NO + Hb(III)NO) 60–80% of the nitrite is used up within 3–5 min even when a large excess of hemoglobin is present and the nitrite level is in the range of nitrite present in blood. By combining the chemiluminescence data with EPR, which can be used to distinguish between the two forms of heme-NO (Hb(II)NO has a distinct EPR signal in the g = 2 region, while Hb(III)-NO is only detected as an increase in the Hb(III) g = 6 signal after the NO is removed by argon), it was possible to show (Fig. 3C) that the initial rapid formation of heme-NO observed by chemiluminescence is due to Hb(III)NO and that the Hb(II)NO is actually formed very slowly as reported in the earlier studies (30, 31).

These results can be explained in terms of the reported mechanism for nitrite reductase, by heme cd1 nitrite reductase, a bacterial hemeprotein (29). The initial step in the reaction involves the cleavage of the protonated complex of nitrite with the Fe(II)heme to produce a nitrosonium ion presumably still associated with Fe(II) in Reaction 4.

$$E-Fe(II)-ONOH \rightleftharpoons E-Fe(II)-NO^+ + OH^-$$

**REACTION 4**

The ferrous nitrosonium ion complex is in rapid equilibrium with the Fe(III)-NO complex (Reaction 5).
FTIR spectroscopy has shown the presence of such an intermediate with properties of Fe(II)NO” and Fe(III)NO both during nitrite reduction by heme cd, nitrite reductase and when NO binds to Hb(III) (29).

In the nitrite reductase enzyme the weakly bound Fe(III)NO releases NO. However, in deoxyHb with a large preponderance of deoxygenated Fe(II) hemes with a very high affinity for NO, the final NO product is expected to be Hb(II)NO shown in Reaction 6.

\[
\text{Hb(III)-NO-Hb(II)} \rightarrow \text{Hb(II)-NO} \]

**Reaction 6**

The accumulation of the Hb(II)NO intermediate during nitrite reduction is explained by the relatively slow conversion of Hb(III)NO to Hb(II)NO (Reaction 6), despite the very high affinity of NO for Hb(II). This paradox is explained by two factors. 1) The added stability generated by equilibrium between the resonance forms Hb(II)NO” and Hb(III)NO (Reaction 5). 2) The transfer to Hb(II) requires that the NO migrate between the hemes on different hemoglobin chains separated by 25–40 Å. The resultant accumulation of Hb(III)NO (Fig. 5) produces a pool of labile, potentially bioactive red cell NO.

**Significance of the Formation of Labile Hb(III)NO**—The reduction of nitrite in the red cell under hypoxic conditions provides a completely new perspective on the role of the red cell in regulating vascular tone. Instead of requiring that the red cell pick up the NO in the arterial circulation and then release it under hypoxic conditions in the arterioles, the nitrite formed when NO reacts with oxygen in the arterial circulation is reutilized under hypoxic conditions. The reduced partial pressure of oxygen as the blood enters the arterioles increases the concentration of deoxygenated hemoglobin chains thereby turning on the nitrite reduction process. This mechanism produces NO in the microcirculation where it is needed instead of transporting unstable NO from the arterial circulation to the microcirculation.

This mechanism, however, not only generates the needed NO, but also traps it in a labile form bound to Hb(III). This form of NO is the dominant species formed during nitrite reduction and found in both arterial and venous blood. Because of the equilibrium between Hb(II)NO and Hb(II)NO” (Reaction 5), we have also shown that NO” in this intermediate state reacts with thios to produce S-nitrosothiols. The lability and reactivity of this intermediate provides a realistic mechanism for releasing some of this NO from the red cell making it available to regulate vascular tone as the blood enters the microcirculation.

This mechanism explains how the red cell can provide a source of bioactive NO in the microcirculation, which bypasses all of the difficulties associated with the Stamler hypothesis. The bioactive red cell NO can be measured and quantitated under conditions where the relatively unstable Hb(III)NO is not lost. The NO is produced in the microcirculation by nitrite reduction and there is, thus, no need to bind NO to the few percent deoxygenated chains and we are not faced with the difficulties of removing very tightly bound NO from Fe(II)-hemes. This mechanism, thus, provides a realistic chemically sound method whereby the red cell can regulate vascular tone. Additional work is, however, necessary to establish that the Hb(III)NO formed can be released from the red cell and that it is vasoactive.

In the earlier studies a difference between arterial and venous blood was used as an indicator that this pool of NO is released as the blood passes through the microcirculation. Thus, Stamler has argued that the lower values of SNOHB that they find in venous blood indicates that NO is released from SNOHB as the blood passes through the microcirculation with some of it picked up by the heme resulting in an increase in venous Hb(II)NO and some of it available to regulate vascular tone. We have also found a small, but significant, increase in Hb(II)NO in venous blood relative to arterial blood (Fig. 5C). This increase can in part be attributed to the release of NO from SNOHB, which then bind to Hb(II). However, much of the recent data has not been able to detect reliable measure of SNOHB in either arterial or venous blood (14, 26, 33). The increase in venous Hb(II)NO is thus probably more likely attributed to the transfer of NO from Hb(III)NO to Hb(II)NO (Reaction 6) in the venous circulation where nitrite reduction is augmented. However, we recognize that once NO binds to Hb(II) the NO is essentially inert having no bioactivity and only very slowly decomposing to produce nitrate in the presence of oxygen.

Of greater potential significance is the increase in Hb(III)NO in venous blood relative to arterial blood (Fig. 5). This increase represents a major fraction of the red blood cell NO and is indicative of the activation of the nitrite reduction process when hemoglobin becomes partially deoxygenated. The decrease in the level of arterial Hb(III)NO is, however, not actually a measure of the NO used in the circulation. It instead reflects the instability of Hb(III)NO at elevated oxygen pressures. This decomposition prevents the buildup of high levels of NO, which would impair the ability of the red cell to transport oxygen. Unlike Stamler, the A/V difference is not actually a measure of the NO used to facilitate blood flow. Instead, at the same time that Hb(III)NO is increasing in the microcirculation, some of this NO is presumably available to regulate vascular tone and blood flow.

Although a direct effect of nitrite on the vasculature has been ruled out (34), it has been reported that nitrite does produce a decrease in blood pressure in rats indicative of a vasodilatory effect (35, 36). These results are thus supportive of our hypothesis where nitrite exerts an indirect vasodilatory effect by activating red cell nitrite reduction and, thereby, providing a larger pool of labile bioactive red cell NO that can react with the vasculature.

This mechanism can also explain the important physiological phenomenon whereby hypoxia causes vasodilation and hyperoxia causes vasoconstriction (37–39). Thus, nitrite reduction stimulated by hypoxic conditions and the increased levels of deoxygenated hemes produce vasactive nitric oxide and vasodilation. Under hypoxic conditions this process is turned off, and the NO in the lumen is converted to nitrate and/or nitrate reducing the levels of NO available to react with guanylate cyclase resulting in vasoconstriction.

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