Vasorelaxation by Red Blood Cells and Impairment in Diabetes
Reduced Nitric Oxide and Oxygen Delivery by Glycated Hemoglobin

Philip E. James, Derek Lang, Timothy Tufnell-Barret, Alex B. Milsom, Michael P. Frenneaux

Abstract—Vascular dysfunction in diabetes is attributed to lack of bioavailable nitric oxide (NO) and is postulated as a primary cause of small vessel complications as a result of poor glycemic control. Although it has been proposed that NO is bound by red blood cells (RBCs) and can induce relaxation of blood vessels distal to its site of production in the normal circulation, the effect of RBC glycation on NO binding and relaxation of hypoxic vessels is unknown. We confirm RBC-induced vessel relaxation is inversely related to tissue oxygenation and is proportional to RBC S-nitrosohemoglobin (HbSNO) content (but not nitrosylhemoglobin content). We show more total NO bound inside highly glycated RBCs (0.0134 versus 0.0119 NO/Hb, respectively; P<0.05) although proportionally less HbSNO (0.0053 versus 0.0088 NO/Hb, respectively; P<0.05). We also show glycosylation impairs the vasodilator function of RBCs within a physiological range of tissue oxygenation. These findings may represent an important contribution to reduced NO bioavailability in the microvascularity in diabetes. (Circ Res. 2004;94:976-983.)

Key Words: nitric oxide • hemoglobin • glycosylation • relaxation

The endothelial dysfunction associated with diabetes has been attributed to a lack of bioavailable nitric oxide (NO).1–3 The mechanisms proposed to explain this deficiency include reduced production of NO by NO synthase (NOS)4,5 and inactivation of NO by reactive oxygen species (ROS) produced either by glycated proteins or directly from vascular endothelium.6,7 However, these only incompletely explain reduced relaxant responses of microvessels to agonists such as bradykinin in the presence of glycohemoglobin (HbA1c).

NO released into the vascular lumen is metabolized (1) by oxyhemoglobin to form methemoglobin and nitrate, (2) by reaction with oxygen (O2) to produce nitrite and nitrate (NOx), (3) through binding to deoxyhemoglobin to produce iron nitrosyl hemoglobin (HbNO), and (4) by reaction with thiol-containing proteins in blood (RSNO) that include glutathione (GSNO) and hemoglobin (S-nitrosohemoglobin, HbSNO).8–11 It has been proposed that NO bioavailability in vivo may be governed in part by its reformation from these circulating metabolites as well as by the above local factors.12,13 This NO “reserve” is likely to be critical in tissue regions where blood supply is limiting and where O2 demand is increased. The conformational change that occurs during oxygenation of HbNO in the lungs is thought to induce transfer of the NO to an adjacent thiol on the cysteine9 group of the β-chain, producing HbSNO. The levels of HbSNO in human red blood cells (RBCs) are reflected in the physiological O2 gradient as first shown by Funai et al.14 and the occurrence of transpulmonary gradients in HbSNO at the expense of HbNO has recently been demonstrated in normal subjects.15 The HbSNO is thought to pass on its NO to other smaller molecular weight proteins in blood and these can donate NO to cause vasodilatation in the peripheral microvasculature.16–19,21 The occurrence and physiological relevance of this NO reserve in the normal circulation has been questioned21,22 with arguments both for and against.13,23–26

We hypothesized that because protein glycation can produce changes in configuration and ligand binding properties,27–29 increased HbA1c may directly influence NO metabolism in blood. In a previous study in patients with type 1 diabetes, we demonstrated increased concentrations of HbNO and RSNO, which correlated strongly with increased HbA1c.30 This may represent an important mechanism of NO inactivation in vivo, particularly because elevated HbA1c has been linked to the development of the microvascular complications neuropathy and retinopathy.31–33

McMahon et al15 have previously described hypoxic regulation of vasomotor tone by RBCs. We used this model system and report in this article on the effect of HbA1c on O2 and NO binding capacity after addition of normal and highly glycated RBCs to an isolated aortic ring preparation. The O2 tension in the tissue bath was carefully controlled and varied to give a range that mimicked tissue O2 tensions in vivo. We

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investigated relaxation of vessels induced by these RBCs and the potential source of the vasoactive NO.

**Materials and Methods**

**Aortic Ring Preparation**

Endothelium intact and denuded rings of thoracic aorta from male New Zealand White rabbits (2 to 2.5 Kg) were prepared for isometric tension recordings as previously described. Under conditions of varying O₂ concentrations (see later), the tissues were preconstricted with phenylephrine (PE; 10⁻⁶ mol/L). When the response had reached a plateau, RBC preparations were added (as described later). Relaxations (and additional constrictions) were calculated as a percent of the tension induced by 10⁻⁶ mol/L PE and retrospectively corrected for HB concentration. The responsiveness of the tissues was assessed at the end of each experiment by constricting with PE followed by GSNO (10⁻⁷ mol/L).

The concentration of O₂ in the Krebs buffer (KB) bathing the tissues was controlled by bubbling gas of the appropriate mix directly into the bottom of the tissue baths. This was monitored throughout the experiment using an O₂ electrode (World Precision Instruments) (for experimental details, refer to the online data supplement at http://circres.ahajournals.org).

In some experiments, in order to inhibit soluble guanylate cyclase and prevent smooth muscle relaxation, ODQ (1H-[1,2,4]oxadiazole[4,3-a] quinoxalin-1-one; 10⁻⁴ mol/L; Alexis) was added to the tissue bath for 1 hour. In order to study the influence of ROS on vessel tension, superoxide dismutase (SOD; 300 enzyme units/mL; Alexis) was added to the tissue bath for 10 minutes. Pretreatment (15 minutes) of vessels with N’N-di-(4-aminophenyl)-L-arginine (L-NMMA; 10⁻⁴ mol/L; Alexis) was used to inhibit tissue basal NO release. In separate experiments, the influence of HB on NO-RBC–induced relaxations was assessed by addition of free HB (final concentration 10⁻⁷ mol/L) directly to the tissue bath.

**Preparation of HbNO/HbSNO Within RBCs**

After euthanasia, a blood sample was drawn from the aorta. RBCs were washed free of plasma and made up to the original hematocrit using KB were either incubated for 20 minutes at 37°C with or without addition of exogenous NOC-9 ((Z)-1-(N-methyl-N-[6-(N-methylammonioxyethyl)amino]diazen-1-1M1,2-dioate; Alexis) that provided a burst of NO after a change from alkaline to neutral pH (refer to online data supplement for details). The saturation of RBCs with O₂ (HbO₂ sat) was 94±3%. The final concentration of NO-RBCs added to a final volume of 8 mL KB and 80 mL of this suspension was added to the RBC samples was varied from 0.24×10⁻⁹ to 24×10⁻⁹ mol/L to produce NO-RBCs. Samples were then washed 3 times in KB and 80 μL NO-RBCs added to a final volume of 8 mL KB surrounding the aortic ring. In order to account for discrepancy in the number of RBCs between samples, HB concentration was measured (hemoglobin cyanide method).

**RBC Ghosts**

In a separate group of experiments, RBCs were subjected to exogenous addition of NO (0.24×10⁻⁹ mol/L) and then lysed by bathing for 5 minutes 1:1 in EDTA (0.5 mmol/L)/NOx-free water on ice. Washed NO-RBC ghost were made up to the original concentration of RBCs, and 80 μL of this suspension was added to the tissue bath after preconstriction of the aortic vessels with PE.

**Normal Versus Highly Glycated Red Blood Cells**

Blood was drawn each day from an antecubital vein of a normal subject and that of a patient with type 1 diabetes mellitus, providing fresh, viable RBCs. The HbA1c throughout the duration of the study was 5.4±0.2% versus 10.7±0.4%, respectively. NO was introduced by adding 0.24×10⁻⁵ mol/L NO to RBC samples, and 80 μL NO-RBCs were added to preconstricted aortic ring preparations.

**Electrode-Based Method for Measurement of HbNO and Total HbSNO**

Duplicate RBC samples were processed either for relaxation or for analysis of Hb-bound NO. We modified the method of Gladwin et al. to (see online data supplement for details) for selective cleavage of NO from either HbNO or HbSNO. The NO released was detected by an NO electrode (AMI 700; Harvard) in a stream of O₂-free N₂. The NO concentration in each Hb sample was then expressed as percent NO per HB mol/L.

**Relaxation of Aortas by GSNO at Different O₂**

In a separate group of experiments, a relaxation concentration response curve was established for GSNO at each O₂ tension. Preconstricted aortic rings (10⁻⁶ mol/L PE) were subjected to increasing GSNO concentrations (10⁻¹² to 10⁻⁴ mol/L) while keeping the O₂ in the bath constant. This experiment was undertaken six times at each O₂.

**Measurement of p50**

An ABL System 625 clinical blood gas analyser (Radiometer) was used to analyze pH, PO₂, HbO₂ sat, metHb, and p50. An RBC sample (500 μL) in KB was drawn into preheparinized syringes and analyzed immediately.

**Data Analysis**

In a typical experiment, eight aortic ring preparations were run in parallel each day. Four were subjected to addition of normal RBCs and four received highly glycated RBCs. Within each group, RBCs (without addition of NO) were added to one ring. The average value recorded across the three NO-RBC rings was taken as a single data point at each O₂ tension. This was repeated >8 times for each study at different O₂ concentrations. Values are expressed as mean±SD unless otherwise stated. The SPSS statistical package was used, and groups were compared using a standard t test. Correlations were assessed using Pearson’s correlation.

**Results**

**Relaxation Versus Constriction of Aortic Rings Induced by NO-RBCs**

Tension recording from a typical experiment is shown in Figure 1A. Addition of rabbit NO-RBCs (prepared by incubating RBCs with 0.24×10⁻⁶ mol/L NO) to PE preconstricted aortic rings incubated in 95% O₂ resulted in a further 35% constriction (above that induced by PE). Preincubation of rings in NO-RBCs (without addition of NO) were added to one ring. The average value recorded across the three NO-RBC rings was taken as a single data point at each O₂ tension. This was repeated >8 times for each study at different O₂ concentrations. Values are expressed as mean±SD unless otherwise stated. The SPSS statistical package was used, and groups were compared using a standard t test. Correlations were assessed using Pearson’s correlation.
to a maximum of approximately 3% after addition of NO-RBCs at all NO concentrations studied (Figure 2A). We found no significant difference in relaxation/constriction effects of NO-RBCs on endothelium-intact versus -denuded vessels (30±4%/22±8% and 35±5%/24±6%, respectively; n=5). Studies performed in the presence of SOD showed similar vessel responses to PE and to addition of NO-RBCs (37±6%/22±7%; P=NS compared with SOD). Preincubation of vessel rings with LNMMA resulted in a significant elevation of basal resting tension (10±4%). Accepting
this increased basal resting tension with LNMMA, we found no effect on the NO-RBC-induced relaxation under hypoxic conditions (36 ± 7% of the PE-induced constriction). Addition of free Hb to the tissue bath simultaneously with NO-RBCs significantly reduced the relaxation observed with NO-RBCs alone (14 ± 7%; P = 0.04). The ensuing constriction was not significantly reduced under these conditions (19 ± 4%).

Analysis of HbNO and HbSNO in RBCs exposed to a range of NO concentrations showed HbNO was relatively constant (0.04 ± 0.008 NO/Hb mol/L %), whereas HbSNO content increased significantly with increasing NO added to RBCs (from 0.026 ± 0.003 to 0.063 ± 0.005 NO/Hb mol/L %; P = 0.01). Values were significantly different from the lowest NO concentration other than at 24 × 10⁻⁶ mol/L. HbSNO correlated positively with increased relaxation (r = 0.88).

Figure 2. A, NO-RBC–induced vessel relaxation in 1% O2 was dependent on the amount of NO added to the RBCs (r = 0.81). All values were significantly different (P < 0.04) from the lowest NO concentration (0.24 × 10⁻⁶ mol/L). Top 2 concentrations (12 and 24 × 10⁻⁶ mol/L) were not significantly different from each other. % Overshoot indicates constriction above that induced by PE. All values were significantly different from the lowest NO concentration (P < 0.02), and constriction showed an inverse relation to the amount of NO added (r = −0.84). Maximal O2 measured in the tissue bath after addition of NO-RBCs is also shown and did not change significantly across the range of NO concentrations used (~3%). B, HbNO and HbSNO (expressed as percent NO per Hb) in NO-RBCs exposed to a range of NO concentrations. HbNO was relatively constant (0.04 ± 0.008 NO/Hb mol/L %), whereas HbSNO content increased significantly with increasing NO added to RBCs (from 0.026 ± 0.003 to 0.063 ± 0.005 NO/Hb mol/L %; P = 0.01). Values were significantly different from the lowest NO concentration other than at 24 × 10⁻⁶ mol/L. HbSNO correlated positively with increased relaxation (r = 0.88).

Potential for Contaminating NOx to Interfere With NO-RBC–Induced Relaxations

Contamination does not appear to be directly responsible for the observed NO-RBCs induced relaxation in our system because (1) RBC washes (supernatants) were essentially NO₃⁻/NO₂⁻ free with no bioactivity (relaxation), and (2) addition of NO₃⁻ or NO₂⁻ directly to the hypoxic tissue bath did not produce a response at any O2 studied. Residual NOC-9 in the NO-RBC sample is unlikely because (1) we found no evidence of NO (NO electrode) or NOx generating potential in RBC washes/supernatants, (2) NO-RBC samples did not generate significant levels of NO/NOx over 15 minutes, and (3) addition of NOC-9 directly to the tissue bath produced only vessel relaxation (data not shown).

Normal Versus Highly Glycated NO-RBCs

Typical recordings of vessel tension on addition of normal or highly glycated NO-RBCs to preconstricted aortic rings incubated at 1% O2 are shown in Figure 3A. Relaxation was greater after addition of highly glycated NO-RBCs compared with normal NO-RBCs (P < 0.03). The ensuing constriction was also greater for highly glycated NO-RBCs (P < 0.04). Repeat experiments conducted at higher O2 (2%, 3.5%, 21%, and 95%) did not have the same outcome. Highly glycated NO-RBCs showed significantly less relaxation followed by less constriction at 2% and 3.5% O2 (P < 0.04 compared with normal NO-RBCs incubated under the same conditions). Relaxation was very low in both groups at 21% and 95% O2. These data are summarized in Figure 3B.

Addition of normal or highly glycated RBCs (ie, without added NO) to PE preconstricted tissues incubated at 1% O2 also resulted in a biphasic response; an initial relaxation followed by a period of constriction. We found no significant difference between relaxation due to normal versus highly glycated RBCs (5 ± 4% versus 6 ± 3%, respectively). These relaxations were typically <25% of the respective NO-RBC–induced relaxation and reflects basal HbSNO/HbNO within the normal and highly glycated RBC samples at this hematocrit.

Biochemical analysis of HbNO, HbSNO, metHb, and p50 in RBC/NO-RBC samples is shown in Table 1. Increased total NO binding was observed by highly glycated compared with normal samples. The bound NO was primarily in the form of HbNO and also showed less HbSNO. Highly glycated and normal NO-RBCs showed a reduced p50 compared with their RBC counterparts. Methemoglobin levels were not significantly different but typically higher in highly glycated RBCs after addition of NO.

Vessel Relaxation at Various O2

Aortic rings were hyperresponsive to a given GSNO concentration under hypoxic conditions (Figure 3B). This enhanced relaxation was compared by calculating the fold increase in relaxation at each O2 concentration (Table 2) and was 2.32 ± 0.4-fold regardless of GSNO dose. NO-RBCs showed a significant increase (3.67-fold) and glycated NO-RBCs a 5.96-fold increase in relaxation induced at 1% O2.

Discussion

This study demonstrates that glycosylation impairs the NO vasodilator function of RBCs within a physiological range of tissue oxygenation. This impairment of relaxation capacity is linked to the oxygenation/deoxygenation of Hb and may be...
The hypothesis that delivery of vasoactive NO by normally glycated RBCs to hypoxic tissue relies on binding and transport of NO is not new. This hypothesis is consistent with the observations of McMahon et al., who observed an immediate and transient relaxation following the addition of NO-RBCs to preconstricted aortic rings (Figure 3A). The relaxation induced by NO-RBCs under hypoxic conditions was greater for highly glycated NO-RBCs than for normal NO-RBCs. These findings are consistent with other studies that have demonstrated the importance of allosteric effectors (in this case, glycation) in the mechanism of RBC-induced vasodilation. Increased HbSNO in rabbit RBCs correlated positively with increased relaxation of hypoxic aortic rings, although we acknowledge this does not necessarily demonstrate cause and effect.

The following findings are consistent with an allosteric mechanism of delivery of a “relaxing” factor. The NO-RBC-induced relaxation was not endothelium dependent and was inversely proportional to O2 in both normal and highly glycated NO-RBCs. Allosteric effectors (in this case, glycation) influence this activity. That the NO/cGMP pathway mediated the relaxation response was confirmed by inhibiting sGC activity with ODQ. Preincubation of vessel rings with LNMMA resulted in increased basal resting tension, but had no effect on the NO-RBC-induced relaxation, whereas addition of free Hb partially inhibited the response.

Several recent studies implicate RBC-mediated conversion of NO− to bioactive NO to be responsible for dilatation of blood vessels over longer exposure to hypoxia. Given that HbSNO (and to a lesser extent HbNO) are in equilibrium with NO−, it is not unreasonable to speculate that these may be linked to the initial formation and equilibrium of NO metabolites within RBCs. However, addition of NO− to the tissue bath had no effect on the transient (≈1 minute) relaxations induced by RBCs in our studies, and we found no evidence that NO− within RBCs may be associated with this response.

Modulation of vessel tension by O2 and reduced vessel constriction during long-term hypoxia has previously been demonstrated. Explanations for this O2-mediated response include alleviation of energy limitation, effects on the Na+ pump, eicosanoid pathways, alteration in plasma membrane electrical properties, Ca+ permeability, and ATP-dependent K+ channels. Consistent with the observations of McMahon et al., we observed an immediate and transient relaxation followed by constriction on addition of NO-RBCs to aortic rings. Tension increased 30% above that induced by PE for a short period of time before returning to the original plateau, presumably because the O2 measured in the tissue bath also increased transiently (to around 3% O2). The constriction observed after addition of NO-RBCs to vessels incubated at 95% O2 was not transient and is probably due to inactivation by either oxy- or deoxy-Hb of basal NO produced by the endothelium.

The fact that relaxation of rings to an exogenous NO donor (GSNO) was also dependent on the O2 within the tissue bath is important. We compared the relaxation induced by NO-RBCs at each O2 with that induced by a range of GSNO concentrations at the same O2 and found that the hyperresponsiveness observed cannot account for the increased relaxation induced by NO-RBCs under hypoxic conditions (in agreement with other studies), or the observed difference between highly glycated and normally glycated NO-RBCs. Crawford et al. conclude there is no significant difference between the fold decrease in EC50 for relaxation at low oxygen tension (∼3 fold) between free SNOoxyHb,
GSNO, or Angeli’s salt (an NO\(^{-}\) donor), providing additional weight to our finding that a 2.3-fold change in vessel responsiveness due to hypoxia does not account for changes in total NO-RBC–induced relaxations at lower O\(_2\).

Highly glycated RBCs (Hb A1c=10.7%) showed increased NO binding in the form of HbNO. These findings are consistent with those of Gow et al.,\(^{10}\) who found increased binding of NO to the hemes of R structured molecules. The baseline p50 of these RBCs was similar to normal RBCs, whereas addition of NO resulted in a significant decrease in p50 of glycated NO-RBCs only. At first glance the fact that glycated RBCs bind NO primarily in the form of HbNO appears to contradict our conclusion from rabbit RBC experiments (see earlier) that HbSNO confers vasoactivity. However, when the O\(_2\) concentration in the tissue bath was 2% or 3.5%, representing a tissue P O\(_2\) between 16 and 28 mm Hg (and Hb sat O\(_2\) between 15% to 40%), highly glycated NO-RBCs induced significantly less relaxation and O\(_2\)-induced hyperconstriction of aortic rings compared with normal NO-RBCs. Consistent with this, normal NO-RBCs exhibited a higher proportion of NO bound as HbSNO than HbNO. Taken together, these results suggest that Hb glycation results in proportionally increased HbNO and less release of vasoactive NO (and O\(_2\)) in the physiologically normal O\(_2\) range. Although we cannot explain the opposing effects of normal versus glycated NO-RBCs at 2% compared with 1% O\(_2\), this suggests that increased relaxation by NO-linked Hb molecules with high O\(_2\) affinity may only be observed when O\(_2\) is reduced further (~1%, equivalent to 8 mm Hg), consistent with an allosteric mechanism of action. Enhanced formation of HbSNO and RSNO by glycosylated protein compared with nonglycosylated counterparts has been demonstrated previously in vitro (using purified proteins) and also in animals.\(^{46}\) However, HbNO was not measured in these studies.

The importance of inactivation of NO by glycated RBCs and the effect on regeneration of vasoactive NO under low O\(_2\) conditions have not been considered to date. Perhaps of greater significance is the fact that 2,3-diphosphoglycerate (DPG), the endogenous cofactor modulating Hb-O\(_2\) binding within RBCs, binds to a site on the \(\beta\)-chain of Hb that is in close proximity to the \(\beta\)-93 cysteine residue (the so-called “back pocket” HbSNO).\(^{27,28,47}\) We cannot conclude from our studies whether NO binding in the form of HbNO or HbSNO directly affects 2,3-DPG binding to Hb within RBCs, but it is tempting to speculate this might be involved in the reduced tissue oxygenation observed in diabetes.\(^{29}\)

Recent studies demonstrate increased inactivation of endothelial NO by ROS produced by glycated Hb.\(^{6,7}\) However, these observations may also be explained in terms of an altered NO binding and release by glycated RBCs. The documented influence of SOD may have been to inactivate superoxide released from endothelial cells resulting in increased available NO. That glycated proteins, including Hb, generate ROS and lipid peroxidation in diabetic patients,\(^{48}\) suggests they may play an important but as yet undefined role in the inactivation of NO in Type I diabetes. We have previously found increased HbNO and RSNO in diabetic patients (200% above normal) even in the absence of oxidative stress (measured as F\(_2\) isoprostane levels).\(^{30}\) In the present study, SOD had no effect, suggesting ROS generation is unlikely to be the cause of the observed difference in NO-RBC–induced relaxations.

The question remains whether the amount of NO inactivated by highly glycated RBCs is physiologically relevant. Although only a small proportion of total circulating Hb is nitrosylated (<1%) under basal conditions, our previous data indicated that this stored approximately 260\(\times\)10\(^{-9}\) mol/L NO in the form of HbNO in control subjects.\(^{30}\) From the data presented in this study, we calculate the levels of HbSNO in blood would be 800\(\times\)10\(^{-9}\) mol/L (normal RBCs) and 500\(\times\)10\(^{-9}\) mol/L (highly glycated RBCs) based on 10\(\times\)10\(^{-3}\) mol/L heme. That \(= 25 \times 10^{-9}\) mol/L GSNO in the forearm of healthy subjects results in significant increase in radial artery diameter and blood flow\(^{49}\) comparable to the vasodilator effect of infusion of aqueous NO solution or stimulation of endogenous NO formation with acetylcholine or bradykynin,\(^{15}\) provides evidence that the concentrations of transported

**TABLE 1.** Biochemical Characteristics of RBCs and NO-RBCs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Normal NO-RBCs</th>
<th>Glycated NO-RBCs</th>
<th>P</th>
<th>Normal RBCs</th>
<th>Glycated RBCs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbNO</td>
<td>0.013</td>
<td>0.044</td>
<td>0.05</td>
<td>0.0031</td>
<td>0.008</td>
<td>0.05</td>
</tr>
<tr>
<td>HbSNO</td>
<td>0.032</td>
<td>0.025</td>
<td>0.03</td>
<td>0.0088</td>
<td>0.0053</td>
<td>0.03</td>
</tr>
<tr>
<td>Total bound NO</td>
<td>0.045</td>
<td>0.069</td>
<td>0.02</td>
<td>0.0119</td>
<td>0.0134</td>
<td>0.05</td>
</tr>
<tr>
<td>MetHb, %</td>
<td>0.031</td>
<td>0.052</td>
<td>NS</td>
<td>0.030</td>
<td>0.039</td>
<td>NS</td>
</tr>
<tr>
<td>p50, mm Hg</td>
<td>32.29</td>
<td>27.40</td>
<td>*</td>
<td>34.72</td>
<td>32.99</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Significant difference compared to normal RBCs.

**TABLE 2.** Enhanced Relaxation due to Hypoxia, Expressed as a Fold Increase in Relaxation (mean±SD, n=6)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fold Difference in Relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO-RBCs</td>
<td>3.64±0.6*</td>
</tr>
<tr>
<td>Glycated NO-RBCs</td>
<td>5.76±0.9†</td>
</tr>
<tr>
<td>2(\times)10(^{-3}) mol/L GSNO</td>
<td>1.77±0.9</td>
</tr>
<tr>
<td>20(\times)10(^{-3}) mol/L GSNO</td>
<td>2.70±0.5</td>
</tr>
<tr>
<td>200(\times)10(^{-3}) mol/L GSNO</td>
<td>2.37±0.6</td>
</tr>
<tr>
<td>2000(\times)10(^{-3}) mol/L GSNO</td>
<td>2.44±0.5</td>
</tr>
</tbody>
</table>

*Significant difference (P<0.05) compared to GSNO. †Significant difference (P=0.03) between NO-RBCs and glycated NO-RBCs. Fold increase in relaxation was independent of GSNO dose (2.32±0.4).
NO we report in this study may represent a physiologically significant pool of NO.

In summary, we documented for the first time enhanced NO binding and reduced RBC-induced relaxation and O2 release by glycated RBCs within a physiologically pertinent range of O2 concentrations. Highly glycated RBCs bind NO more avidly at these O2 concentrations. Taken together with our previous observation of elevated levels of HbNO and RSNO in patients with Type I diabetes (200% compared with controls), these findings may represent an important contribution to reduced NO bioavailability in the microvasculature. Moreover, elevated HbNO is associated with poor glycemic control and may have implications for our understanding of microvascular disease in diabetes.

Acknowledgments

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References


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**Modulation of O₂ Concentration**

The concentration of oxygen in the Krebs buffer (KB) was controlled by bubbling gas of the appropriate mix directly into the bottom of the tissue baths. This was monitored throughout the experiment using an oxygen electrode (World Precision Instruments). The concentration of CO₂ was kept constant at 5%. When perfused with 95% N₂ and 5% CO₂, we found the concentration of oxygen within the chamber was 1.0+/−0.3 % (corresponding to an approximate tissue pO₂ of 8 mmHg). When perfused with 1% O₂, 94% N₂ and 5% CO₂, the concentration of O₂ in the chamber was 2.2+/−0.4 % (corresponding to an approximate tissue pO₂ of 17.6 mmHg). When perfused with 2.5% O₂, 92.5% N₂ and 5% CO₂, the concentration of O₂ in the chamber was 3.5+/−0.2%. We refer to these concentrations as 1%, 2% or 3.5% for the remainder of this report. We also conducted experiments in air (21 % O₂) and in 95% O₂.

**Addition of NOC-9**

The yield of NO from NOC-9 in saline was >90% based on measurements using an NO electrode system and compared with standards prepared from a NO saturated solution (assuming NOC-9 is capable of releasing two NO equivalents). The latter was prepared by perfusing deoxygenated saline with NO gas in an InVivO₂ hypoxia work chamber (Ruskin, Biotrace International). The saturation of RBC with O₂ (HbO₂ sat) was 94+/−3 %. The final concentration of NO added to the RBC samples was varied from 0.24x10⁻⁶mol/L to 24x10⁻⁶mol/L to produce NO-RBC. Samples were then washed three times in KB and 80 uL NO-RBC added to a final volume of 8mL KB surrounding the aortic ring.
Selective detection of HbNO/HbSNO by NO electrode

We modified the method of Gladwin et al.\textsuperscript{1-3} and incorporated a detection system using an NO electrode (AMI 700; Harvard). Briefly, potassium iodide (0.2M) in acetic acid was used to liberate the NO from Hb present in RBC lysates. Hb (comprising HbNO and HbSNO) was separated from the remainder of the lysate on a PD10 sephadex column. We can confirm both nitrite ($\text{NO}_2^-$) and nitrate ($\text{NO}_3^-$) were removed from the RBC lysate. Fluorometric analysis of NOx in the column fractions (as described in \textsuperscript{4}) showed peaks for both species far removed from that for Hb. We also tested the effect of spiking our lysate sample with low ($10^{-6}$ mol/L) $\text{NO}_2^-$ or pre-treating our sample with acidified sulfanilamide and found little effect on the NO liberated from HbNO or HbSNO. The NO released was detected by the electrode on-line current in a stream of O$_2$-free N$_2$. Samples were prepared with and without ferricyanide (FeCN;0.2x$10^{-3}$ mol/L) for 5 mins which oxidizes HbNO in the sample to a species that does not liberate NO\textsuperscript{3,5}. In this way, NO liberated from the sample without pre-treatment with FeCN arose from HbNO and HbSNO, whereas NO liberated with FeCN treatment arose from HbSNO only. The area-under-the-curve was used for analysis and the system calibrated using the NO donor NOC-9. The Hb fractions used in the electrode system were also analyzed for Hb (hemoglobincyanide method). The NO concentration in each sample was then expressed as % NO per Hb mol/L.
References


