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Nitrite-Induced Improved Blood Circulation Associated with an Increase in a Pool of RBC-NO with NO Bioactivity

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Abstract

The reduction of nitrite by RBCs producing NO can play a role in regulating vascular tone. This hypothesis was investigated in rats by measuring the effect of nitrite infusion on mean arterial blood pressure, cerebral blood flow and cerebrovascular resistance in conjunction with the accumulation of RBC-NO. The nitrite infusion reversed the increase in MAP and decrease in CBF produced by L-NAME inhibition of e-NOS. At the same time there was a dramatic increase in RBC-NO. Correlations of RBC-NO for individual rats support a role for the regulating vascular tone by this pool of NO. Furthermore, data obtained prior to treatment with L-NAME or nitrite is consistent with a contribution of RBC reduced nitrite in regulating vascular tone even under normal conditions. The role of the red cell in delivering NO to the vasculature was explained by the accumulation of a pool of bioactive NO in the red cell when nitrite is reduced by deoxygenated hemoglobin chains. A comparison of R and T state hemoglobin demonstrated a potential mechanism for the release of this NO in the T-state present at reduced oxygen pressures when blood enters the microcirculation. Coupled with enhanced hemoglobin binding under these conditions the NO can be released to the vasculature.

1. Introduction

Nitric oxide (NO) synthesized from L-arginine by endothelial nitric oxide synthase plays an important role in regulating vascular tone and maintaining blood flow ¹. NO has a short half-life and needs to be produced/released in close proximity to the site where it reacts. Recent data reports that the reduction of nitrite to NO may play an important role in supplying NO for the regulation of blood flow ^{2,3}.

Although mammals do not possess enzymes specifically designed for nitrite reduction, it has been shown that nitrite is reduced to NO in mammals. A physiological role for RBC nitrite reduction in regulating blood flow is supported by the observation that nitrite infusion results in an increase in RBC NO in conjunction with improved forearm blood flow, inhibition of hypoxic pulmonary vasoconstriction and in the prevention of delayed cerebral vasospasm ³⁻⁵. In this study we have again confirmed a role for the red cell in delivering NO to the vasculature. Cerebral blood flow (CBF) and mean arterial blood pressure (MAP) were measured in a group of rats prior to any treatment, after injection of L-NAME to inhibit nitric oxide synthase and during the subsequent infusion of nitrite. The potential involvement of the red cell in the observed vascular changes ⁶ was indicated by a dramatic increase in the levels

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of RBC-NO and their correlation with the observed physiological changes when results from different rats were compared.

The ability to release NO from the red cell, however, requires that the NO formed by nitrite reduction is not scavenged by the well studied rapid reactions with both oxyhemoglobin (oxyHb) and deoxyhemoglobin (deoxyHb) before it can be released to the vasculature. In earlier studies we have shown that during nitrite reduction a metastable delocalized intermediate is formed²⁷ that makes it possible to build up a pool of potentially bioactive NO in the red cell that is not scavenged by deoxyhemoglobin or oxyhemoglobin. We have now extended these studies to show how this NO can be released to the vasculature at low oxygen pressures when hemoglobin assumes the T-unliganded conformation that has an increased affinity for the red cell membrane.

2. Materials and Methods

2.1. Animal Preparation and Experimental Design

All experiments were performed in accordance with the Animal Care Committee of Bar-Ilan University Guidelines. A total of 8 male Wistar rats of 250-300 gr (2.5-3 months old) were used. Two of these rats were controls, which were not injected with L-NAME or infused with nitrite. The rats were anesthetized with Equithesin (0.3 ml/100gr body weight, i.p) (each ml contains: pentobarbital 9.72 mg, chloral hydrate 42.51 mg, magnesium sulfate 21.25 mg, propylene glycol 44.34 % w/v, alcohol 11.5 % and distilled water). During the duration of the experiment steady anesthesia was maintained by 0.1ml Equithesin injection every 30 minutes. Additionally, the rats were placed on a warming tray and body temperature was maintained at 37°C. Polyethylene catheters were introduced into the femoral vein for drug administration and into the femoral artery for systemic MAP monitoring. The jugular vein was also cannulated for obtaining blood samples.

After a period of stabilization, a control sample of 0.6 ml blood was withdrawn and L-NAME (50mg/kg body wt. I.V.) was injected. Approximately 10 min after L-NAME administration (when MAP became stabilized at an elevated value) a second sample of blood was taken followed by a 10 min period of nitrite infusion. The rate of NaNO₂ infusion was 1 µmol/kg body wt./min. At the end of the infusion period a third sample of blood was taken. CBF and MAP were continuously monitored through the entire experimental period. For the control animals the injections of saline and the withdrawal of blood had no effect on the CBF or MAP.

2.2. Cerebral Blood Flow

CBF was monitored by laser Doppler flowmetry (LDF), which measures relative changes in microcirculatory blood flow (0-100% range)⁸. To attach the Doppler probe to the brain cortex, the rat was placed on an operation table with a special mouth holder. A 2 mm diameter hole was drilled in the parietal bone, and the bone was removed (the dura matter remained intact). The Doppler probe, which uses light of 632.8 nm, was then placed on the brain cortex using a special micromanipulator and fixated by dental acrylic cement^{9,10}. The relative changes in cerebrovascular resistance (CVR) during nitrite infusion was determined from MAP/CBF¹¹.

2.3. Analysis of Red Cell NO

Blood samples were immediately centrifuged at 3000 rpm for 10 min and the plasma removed. The RBC pellet was stored in liquid nitrogen without further washing. NO was determined by a Nitric Oxide Analyzer (Sievers model 280) on thawed hemolysate as described earlier². Total red cell NO was determined by lysing the cells in 4 volumes of deoxygenated distilled water in a septum sealed cuvette. Oxygen stable NO was determined by lysing the cells in 4

volumes of distilled water. 100 μ l of the sample was injected into the purge vessel containing 5.5 ml glacial acetic acid 20 mM sulfanilamide (to react with any free nitrite present) and 100 mM potassium ferricyanide (to release NO tightly bound to Fe(II) hemoglobin). While sulfanilamide reacts with free nitrite, it may not react with nitrite bound to deoxyhemoglobin or methemoglobin. The released NO was flushed through the NO chemiluminescence analyzer and quantitated.

2.4. Preparation of Hemoglobin

Hemoglobin was prepared from fresh RBCs as described earlier². Cleavage of the terminal histidine and tyrosine in the β -chains of hemoglobin was accomplished by digesting oxygenated hemoglobin with carboxypeptidase A obtained from Sigma Chemical Co. at pH 7.4 for 30 min¹².

Hemoglobin samples in 50 mM NaCl and 4 mM phosphate buffer, pH 7.4 were deoxygenated in an anaerobic Coy glove box. The glove box uses hydrogen and a palladium catalyst to remove any residual oxygen. The oxygen level when used is < 1 ppm. The sample was placed in a septum sealed cuvette in the glove box and then removed for spectral analysis. The total hemoglobin concentrations in the samples were $\sim$ 100 μ M. Stock solutions of nitrite in the same buffer were also deoxygenated in the glove box. The reaction was initiated by using a gas tight syringe to add nitrite to the sealed cuvette containing hemoglobin.

The spectra of hemoglobin were recorded from 490 nm to 640 nm on a Perkin Elmer Lambda 35 spectrophotometer for 35 min at a 1:1 molar ratio of nitrite to heme. Parent spectra of deoxyHb, metHb, Hb(II)NO, and nitrite bound metHb were prepared. The series of spectra obtained in any experiment were analyzed using a least squares multicomponent fitting program (Perkin Elmer Spectrum QuantC v 4.51) including these 4 spectra.

2.5. Statistical Analysis

Origin 6.1 (Microcal Software, Northhampton MA) was used for analysis of the data. The paired student's t test was used for comparing samples before treatment with samples after L-NAME injection and after nitrite infusion. Linear regression analysis was used to compare parameters for the different rats studied. Two tailed values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. The contribution of RBCs to Nitrite induced Vasoactivity in Rats

The effect of nitrite infusion on vasoactivity was obtained by investigating the effect of 10 min nitrite infusion after e-NOS was inhibited by L-NAME⁶. MAP measured in the femoral artery was used as a measure of systemic vasoactivity. The inhibition of NO synthesis by L-NAME produced a very significant ($p < 0.001$) almost 100% increase in MAP that was completely reversed by nitrite infusion. The changes in relative CBF and relative CVR, which reflect the highly regulated cerebral circulation were less pronounced. L-NAME decreased CBF and increased CVR, but the changes were not significant. However, the increase in CBF and decrease in CVR obtained when nitrite was infused after L-NAME were significant ($p < 0.05$).

After nitrite infusion (Fig. 2) there is a 20 fold increase in oxygen stable RRBC-NO ($p < 0.0001$) and a 40 fold increase in total RBC-NO ($p < 0.0001$) indicating that a major fraction of the infused nitrite is taken up by the red cell and reduced to a potentially bioactive form of NO. A role for this NO in regulating vascular tone is supported by the correlation between the level of oxygen stable RBC-NO in each rat and both MAP (Fig. 3A; $r = -0.83$; $p < 0.05$), as a measure

of peripheral vasoactivity, and CVR (Fig. 3B; $r = -0.843$; $p < 0.05$), as a measure of cerebral vasoactivity.

3.2. The Release of Bioactive NO from the RBC

In the red cell, NO reacts very rapidly with oxyhemoglobin forming nitrate.

And with deoxyhemoglobin producing Hb(II)NO¹³.

A role for RBC-NO in the regulation of vascular tone, however, requires that NO bioactivity be released from the red cell without being scavenged by hemoglobin. In earlier studies we have shown that during nitrite reduction intermediates including a metastable delocalized species are formed that makes it possible to build up a pool of potentially bioactive NO in the red cell that is not scavenged by deoxyhemoglobin or oxyhemoglobin^{2,7}. We have recently developed a method to quantitate the intermediates using visible spectroscopy. This procedure involves a comparison of the consumption of deoxyhemoglobin with the formation of methemoglobin and nitrosylhemoglobin, the final products in the nitrite reduction process.

The concentration of intermediates is then given by the equation

$$[\text{intermediates}] = [\text{decrease in deoxyHb}] - 2 * [\text{Hb(II)NO}] \quad (4)$$

From the time course for the consumption of deoxyhemoglobin and Hb(II)NO and the concentration of the intermediates the rate constants for the initial formation of intermediates (k_1) and for the release of NO from the intermediate (k_2) can be calculated.

$$-\{d[\text{Hb(II)}]/dt\}_0 = k_1 [\text{Hb(II)}]_0 [\text{NO}_2^-]_0 \quad (5)$$

$$\{d[\text{Hb(II)NO}]/dt\}_{\text{final}} = k_2 [\text{intermediate}]_{\text{final}} \quad (6)$$

The release of NO from the red cell requires that the release of NO from the intermediate is coupled to the transport of the NO out of the red cell. To investigate a possible mechanism for such a process we have investigated the effect of the hemoglobin quaternary conformation that is involved in cooperative uptake and release of oxygen on the nitrite reaction. Fully deoxygenated hemoglobin is in the low oxygen affinity T-state. By removing the histidine and tyrosine on the amino terminus of the hemoglobin β -chains using carboxypeptidase A hemoglobin remains in the high oxygen affinity R-state even when fully deoxygenated. We then compared the nitrite reaction for fully deoxygenated T and R state hemoglobin. The results

shown in Figure 5 indicate that while the formation of the intermediate is faster in the R-state, the release of NO from the intermediate is faster in the T-state.

4. Discussion

The rat perfusion studies demonstrate that nitrite has a vasodilatory effect⁶. A role for red cell nitrite reduction in this process is suggested by the dramatic increase in red cell NO after nitrite infusion. The correlation of the changes in MAP and CVR for each rat studied and the level of red cell NO accumulation further support this contention. In fact correlation of basal red cell NO with levels of MAP suggest that, even under normal conditions, red cell reduction of the plasma nitrite generated from NO oxidation also plays a role in regulating vascular tone⁶.

A proposal that the red cell nitrite reduction to NO regulates vascular tone requires that the reduced nitrite is not immediately scavenged by the high concentrations of oxyHb and/or deoxyHb always present in the red cell. To explain this process we have studied the formation of the metastable delocalized species that retains reduced nitrite in a form that does not react with hemoglobin. This intermediate represents a pool of potentially bioactive NO that can accumulate in the red cell. Our studies of R and T state hemoglobin and the associated changes in the uptake and release of NO (Fig. 5) provide a model to explain the role of the red cell in delivering NO to the vasculature. As the blood enters the arterioles and begin to release oxygen, the hemoglobin retains the R-state but has unliganded subunits that can react with nitrite. This results in the accumulation of the bioactive intermediate. As more oxygen is removed and the quaternary conformation shifts to the T-state NO is released from this intermediate. Since T-state hemoglobin has a higher affinity for the red cell membrane¹⁴, some fraction of this NO is released while hemoglobin is bound to the membrane. This NO can diffuse through the membrane before being scavenged by hemoglobin. Once out of the cell it can diffuse to the vasculature and react with guanylyl cyclase causing vasodilation.

Additional studies are necessary to demonstrate to what extent this mechanism contributes to the nitrite induced vasodilatory effects. However, the contribution of the nitrite induced vasodilatory effects and their coupling with red cell NO, strongly suggest that the pathway described for the transfer of NO-bioactivity from the red cell to the vasculature needs to be considered.

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Figure 1.
Total red cell NO before any treatment, after injection of L-NAME and after infusion with nitrite. Error bars are the SE of the mean.

Figure 2. Relationship with oxygen stable red cell nitric oxide after nitrite infusion. (A), mean arterial blood pressure (MAP); (B), cerebrovascular resistance (CVR)

Figure 3.

A: The Time course for the reaction of a 1:1 molar ratio of nitrite and deoxyHb Fig. 1. (●), deoxyHb; (■), Hb(II)NO; (▲), Total metHb (Hb(III) + Hb(III)NO₂⁻); B; the formation of intermediates from eq. 4.

Figure 4.
The effect of the quaternary T and R states on the formation of the intermediates and the release of NO from the intermediates.