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Regulation of Oxygen Delivery by the Reaction of Nitrite with RBCs Under Hypoxic Conditions

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1 Introduction

Nitric oxide (NO) as a vasodilator plays a major role in regulating blood flow and vascular tone [1]. The primary source for the synthesis of NO in the circulatory system involves endothelial nitric oxide synthase [2]. Since NO has a life time in plasma of <0.1 s [3], effective delivery of NO to the vasculature requires that NO is synthesized at the site where it is needed. This requirement limits the ability of endothelial nitric oxide synthase to supply the NO in the microcirculation where the reduced partial pressure of oxygen reduces the activity of nitric oxide synthase. To resolve this dilemma it has been proposed that red blood cells (RBCs) play a role in transporting NO to the vasculature. One of the several mechanisms proposed for RBC transport of NO involves the reduction of nitrite by deoxygenated hemoglobin back to NO [4, 5]. The feasibility of this mechanism, however, requires that the nitrite reduced back to NO is not quenched by hemoglobin (Hb). Although no satisfactory mechanism that explains how NO can escape from the RBCs without being quenched by Hb has been proposed, a number of studies indicate that the reaction of red cell Hb with nitrite does have a vascular effect. Deoxygenated hemoglobin binds to the cytoplasmic end of band 3 in the same region as glycolytic enzymes. Since the release of NO from Hb bound to the membrane can possibly diffuse out of the cell without reacting with Hb, a role for the known binding of Hb to the RBC membrane has been suggested by most investigators. However, the nanomolar range of RBC-NO found in vivo [4] would not be able to compete with the non-nitrite reacted Hb for the limited number of band 3 binding sites on the RBC membrane and a negligible fraction of the nitrite reacted Hb is expected to be on the membrane. This dilemma is in part resolved by recent studies [6], which indicate that nitrite reacted Hb has a much higher affinity for the red cell membrane than deoxyhemoglobin (deoxyHb). In this manuscript we review the data indicating an unusually high affinity of nitrite reacted Hb with the red cell membrane. We then propose two different pathways for nitrite induced vasodilation that result from nitrite reacted Hb interacting with the RBC membrane.

2 Methods

Male Wistar rats 250–350 g (3–4 month old) were housed and studied in accord with the NIH Guide for the Care and Use of Laboratory Animals, manual 3040-2 (1999) and

approved by the Institutional Animal Care and Use Committee (protocol # 369-MDS-2010). RBCs were prepared by centrifuging the blood at $1,125\times g$ for 10 min at 4°C using an Allegra 21R centrifuge (Beckman Coulter, Fullerton, California). The plasma and buffy coat were removed by aspiration. Ghosts were prepared from RBCs hemolyzed by adding 5PBS8 (5 mM sodium phosphate, NaH_2PO_4 , pH 8.0) in a 1:40 (RBC:buffer) volume ratio. White ghosts were prepared from these lysed cells by previously published procedures [7]. Hb was prepared from fresh RBCs as described earlier [5]. RBCs or Hb were deoxygenated in a Coy glove box (Coy Laboratory, Michigan), which uses a gas mixture of 5% hydrogen and 95% nitrogen together with a palladium catalyst to remove any residual oxygen. Cells or Hb were rocked for 2 h inside the glove box at 37°C for complete deoxygenation.

ATP levels were determined by the luciferin–luciferase luminescence technique using Invitrogen kits (Invitrogen, Carlsbad, California) with a reported sensitivity of 0.1 pmol. The assay for ATP was performed according to the manual provided with the kit. A suspension of RBCs (1% hct) in TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) was pre-equilibrated under anoxic conditions for 2 h at 37°C . Nitrite was then added to this suspension and rocked to initiate a reaction of nitrite with RBC Hb. The resultant 50 μM nitrite concentration corresponded to a 4:1 heme:nitrite molar ratio. The sample was equilibrated with nitrite for 30 min at 37°C and centrifuged at $2,200\times g$ for 3 min. The supernatants were analyzed for ATP by the luminescence method. The difference in ATP before nitrite incubation and 30 min after nitrite incubation measures the nitrite induced increase in the release of ATP.

Blood pressure was measured using CODA-6 pressure cuffs (Kent Scientific Co.), which were placed around the tail. Animals were given 10 min to stabilize followed by 5 min monitoring of the basal level of blood pressure. The change in blood pressure was then monitored for 15 min after 0.8 ml of nitrite reacted RBCs prepared under anoxia was injected into the femoral vein of the rat. The change in blood pressure was determined with and without the addition of apyrase (40 U/ml of 50% hct RBCs) to the RBCs before reacting with nitrite.

Analysis of NO was performed using a model 280 Nitric Oxide Analyzer (Sievers Instruments). The total heme associated NO was determined when the purge vessel contained 5.5 ml of 85% glacial acetic acid containing 100 mM sulfanilamide and 1.2 ml potassium ferricyanide (0.8 M). Prior to sample injection, the reagents in the purge vessel are completely deoxygenated by flushing with argon. The concentration of heme-NO was determined by comparing the chemiluminescence signal obtained with data from a calibration curve generated by different concentrations of nitrite. For determinations of NO released from nitrite reacted Hb, 250 μl of the gas phase above the sample was injected into the purge vessel.

Deoxygenated Hb was reacted anaerobically with nitrite for 30–60 min. To determine the affinity of the nitrite reacted Hb for the membrane, 200 μl of white ghosts was added to a sample of nitrite reacted Hb. The reduction in heme-NO chemiluminescence when the sample was centrifuged indicates the amount of the nitrite reacted Hb bound to the membrane. With intact RBCs the binding of the basal RBC-NO to the membrane was

determined by lysing the cells and comparing the total heme-NO chemiluminescence before and after centrifugation.

Origin 6.1 (Microcal Software, Northampton MA) was used for analysis of the data. Data are presented as means $\pm$ se. ANOVA and the paired Student's *t* test were used for comparing groups of samples with *P* 0.05 considered statistically significant.

3 Results

3.1 Increased Membrane Affinity for NO Reacted Hemoglobin

RBCs contain 200–500 nM heme associated NO [4] and 20 mM total heme. Some of this NO is thought to be the result of the reaction of nitrite with Hb. Figure 27.1 shows that under anoxic conditions, a significant fraction of the total RBC heme-NO detected by chemiluminescence is associated with the membrane, as indicated by the reduced heme-NO detected in the supernatant when the sample was centrifuged.

The high affinity of nitrite reacted Hb in the presence of a large excess of deoxyHb was investigated with RBC ghosts (Fig. 27.2). Hb reacted with a 1:10 nitrite:heme molar ratio was added to white membrane ghosts. The Hb in the supernatant after centrifugation was determined when different amounts of excess deoxyHb were added. Even with a 100-fold excess of deoxyHb, >20% of the nitrite reacted Hb was still bound to the membrane.

3.2 Nitrite and ATP Induced Vasodilation

The reaction of nitrite with deoxygenated RBCs results in an increase in ATP synthesis [6] and an increase in the hypoxic release of ATP (Fig. 27.3). This effect can be attributed to an increase in glycolysis due to the displacement of glycolytic enzymes from the RBC membrane by nitrite reacted Hb [8].

The affect of this ATP release on blood pressure was observed when nitrite reacted RBCs are injected into the femoral vein (Fig. 27.4). In the presence of apyrase (which degrades any ATP released), the decrease in blood pressure as a result of ATP release is eliminated.

3.3 Membrane Induced Release of NO from Hemoglobin

NO released during the reaction of nitrite with deoxyHb binds to the excess deoxyHb forming Hb(II)NO. The Hb(II)NO binds NO very tightly quenching NO bioactivity. However, most of the nitrite that reacts with an excess of deoxyHb is retained in intermediates. One of these intermediates is a delocalized species [9] with properties of Hb(III)NO and Hb(II)NO⁺. This species retains potential NO bioactivity.

As shown in Fig. 27.5, a negligible amount of NO is released into the gas phase above the reaction mixture when nitrite reacts with Hb. However, in the presence of the RBC membrane a significant fraction of the nitrite/NO associated with the Hb is released into the gas phase. A similar fraction of NO is released into the gas phase when nitrite reacts with Hb that is already bound to the membrane.

4 Discussion

The dramatic increase in the binding of nitrite reacted Hb to the RBC membrane (Figs. 27.1 and 27.2) results in a pool of nitrite/NO that is removed from the large excess of cytoplasmic Hb that rapidly quenches any NO released.

Our studies indicate that the increased membrane affinity of nitrite reacted Hb can increase vasculature NO by two processes. (1) Glycolytic enzymes that bind in the same region of band 3 as Hb [10] are released from the membrane resulting in an increased rate of glycolysis. Increased glycolysis increases ATP production and the hypoxic release of ATP [8]. The released ATP interacts with purinergic receptors on the endothelium that activate eNOS [11]. This process bypasses the requirement to release NO from the RBC. (2) NO can, however, actually be released from the RBC into the vasculature (Fig. 27.5) as a result of membrane interactions that stimulate NO release from the nitrite reacted intermediates [9]. These studies provide important new insights into the contribution of RBCs to NO bioactivity.

NO released into the vasculature is known to cause dilatation of blood vessels. We have used blood pressure to monitor this dilatation, although a decrease in cardiac output can also contribute to the observed decrease in blood pressure. It should also be noted that the RBC induced NO dilatation cannot fully explain autoregulation whereby individual tissues maintain highly specific oxygen delivery to consumption ratios [12].

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Fig. 27.1.
Association of basal RBC heme-NO with the membrane. Heme-NO for lysed cells was determined by chemiluminescence before and after centrifugation

Fig. 27.2.

Binding of nitrite reacted Hb to RBC ghosts. Hb was reacted with nitrite and the binding to ghosts in the presence of different concentrations of excess deoxyHb (1–100 fold) was determined by centrifugation

Fig. 27.3.

Enhanced released of ATP from hypoxic RBCs by nitrite. ATP release after anoxic incubation was determined by luminescence before and after reacting with nitrite

Fig. 27.4. Reduction in blood pressure by nitrite reacted RBCs. The decrease in blood pressure when 0.8 ml of nitrite reacted RBCs are injected into a rat is eliminated if apyrase is added to degrade the released ATP

Fig. 27.5.

A significant level of NO was detected by chemiluminescence in the gas phase above the sample when nitrite reacted Hb was added to the membrane or nitrite reacted with Hb bound to the membrane. NO was not released unless both Hb and membranes were present