Nitrite enhances RBC hypoxic ATP synthesis and the release of ATP into the vasculature: a new mechanism for nitrite-induced vasodilation

Zeling Cao,1 Jeffrey B. Bell,2 Joy G. Mohanty,1 Enika Nagababu,1 and Joseph M. Rifkind1

1Molecular Dynamics Section and 2Comparative Medicine Section, National Institute on Aging, National Institutes of Health, Baltimore, Maryland

Submitted 24 November 2008; accepted in final form 18 August 2009

Nitrite enhances RBC hypoxic ATP synthesis and the release of ATP into the vasculature: a new mechanism for nitrite-induced vasodilation. Am J Physiol Heart Circ Physiol 297: H1494–H1503, 2009. First published August 21, 2009; doi:10.1152/ajpheart.01233.2008.—A role for nitric oxide (NO) produced during the reduction of nitrite by deoxygenated red blood cells (RBCs) in regulating vascular dilation has been proposed. It has not, however, been satisfactorily explained how this NO is released from the RBC without first reacting with the large pools of oxyhemoglobin and deoxyhemoglobin in the cell. In this study, we have delineated a mechanism for nitrite-induced RBC vasodilation that does not require that NO be released from the cell. Instead, we show that nitrite enhances the ATP release from RBCs, which is known to produce vasodilation by several different methods including the interaction with purinergic receptors on the endothelium that stimulate the synthesis of NO by endothelial NO synthase. This mechanism was established in vivo by measuring the decrease in blood pressure when injecting nitrite-reacted RBCs into rats. The observed decrease in blood pressure was not observed if endothelial NO synthase was inhibited by N^ω-nitro-L-arginine methyl ester (L-NAME) or when any released ATP was degraded by apyrase. The NO-associated vasodilation under hypoxic conditions: a new mechanism for nitrite-induced vasodilation.

Nitrite reduction; nitric oxide; hypoxia; red blood cell; adenosine 5′-triphosphate

NITRIC OXIDE (NO) as a vasodilator plays a major role in regulating blood flow and vascular tone (19, 20). A role for red blood cell (RBC) deoxygenation in the delivery of NO has been proposed (22). RBC-delivered NO has been considered as contributing to normal physiological hypoxic vasodilatation (43), as well as a source for the additional NO required under various pathological conditions (12, 18, 24, 40). It, however, needs to be explained how the RBC increases the availability of NO. Two pathways have been proposed for RBC-induced NO-associated vasodilation under hypoxic conditions: 1) the release of some of the adenosine 5′-triphosphate (ATP) from RBCs under hypoxic conditions (10, 49) and 2) the interaction of this ATP with purinergic receptors (4) stimulate the synthesis of NO by endothelial NO synthase (eNOS) (2, 10, 49). The direct release of NO from RBCs under hypoxic conditions (8, 22, 26, 33, 42) has a vasodilatory effect. The source of the RBC-derived NO has been extensively studied. RBCs have been reported to have NO synthase activity (26), although the role of this enzyme has not been established. The original hypothesis for the accumulation of RBC NO (22) involved NO released from the endothelium being taken up by the RBCs and then transferred to a thiol group forming S-nitrosohemoglobin (SNOHb) that can release its NO under hypoxic conditions when the Hb quaternary conformation shifts from the R-oxygenated conformation to the T-deoxygenated conformation. More recently, an alternative hypothesis has been proposed involving the reduction of nitrite, formed partly by the reaction of NO with oxygen, back to NO by a reaction of nitrite with deoxygenated Hb (deoxyHb) (8, 33, 42). During this reaction, NO, N₂O₃, SNOHb, and various forms of heme-NO including heme nitrosylated Hb [Hb(II)NO], heme nitrosylated methemoglobin [Hb(III)NO], and a localized heme-NO intermediate are formed (9, 22, 34, 35).

The reaction involving nitrite is particularly attractive because 50–350 nM nitrite is present in the circulation (25, 36) and is taken up by RBCs (36) and reacts with Hb, producing NO. The major challenge for the nitrite hypothesis is, however, to explain how NO formed in the RBC is able to be released without first reacting with the large excess of oxyhemoglobin (oxyHb) and deoxyHb, both of which rapidly react with NO (11, 13).

In this study, we investigate the potential coupling of the reaction of RBCs with nitrite and the release of ATP from RBCs. By the demonstration that nitrite enhances the release of ATP and that this released ATP can act as a vasodilator, a potential mechanism for nitrite-induced vasodilation that does not require the release of NO from the RBCs is presented.

MATERIALS AND METHODS

Animals. Male Wistar rats 250–350 g (3 to 4 mo old) were housed and studied in accord with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (manual 3040-2, 1999) and approved by the Institutional Animal Care and Use Committee (protocol no. 369-MDS-2010). Rats were anesthetized by breathing isoflurane (2 to 3% mixed with oxygen). All injections, including RBCs, nitrite, ATP, N^ω-nitro-L-arginine methyl ester (L-NAME), and apyrase, were injected directly into the femoral vein without any catheter, using syringes with 27-gauge needles. However, for blood withdrawal the left carotid artery was cannulated with a catheter (Catheter Abbocath, No. AJB215449) with blood samples collected into a syringe and immediately injected into 4-ml heparin vacutainers (Becton Dickinson).

A total of over 60 rats were used with different rats generally used for different experiments. However, for the experiments where the time course of a reaction was followed, the blood samples for all the time points were obtained from the same rat. For these experiments where multiple blood samples were being drawn from a single rat,
only 0.6 ml of blood were withdrawn for each time point. However, in other cases, the volume drawn was not critical.

Preparation of RBCs. Blood was centrifuged at 1,125 g for 10 min at 4°C using an Allegra 21R centrifuge (Beckman Coulter, Fullerton, CA). The plasma and buffy coat were removed by aspiration. For samples used for the determination of ATP, the RBCs were resuspended in cold Tris-buffered saline (TBS; pH 7.4) composed of 140.5 mM NaCl, 21.0 mM Tris·HCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.2 mM MgSO₄, 0.1% dextrose, and 0.5% bovine serum albumin (39). This suspension was centrifuged to wash the cells. This washing process was repeated two more times. For heme-NO measurements, the cells were resuspended and washed in phosphate-buffered saline (PBS; pH 7.4) containing 100 μM disodium EDTA (34).

Deoxygenation of RBCs. RBCs were transferred into an anaerobic Coy glove box (Coy Laboratory), which uses a gas mixture of 5% hydrogen and 95% nitrogen together with a palladium catalyst to remove any residual oxygen (35). RBCs suspended in PBS or TBS (pH 7.4) were rocked for 2 h at 37°C to completely deoxygenate the cell suspension.

Determination of ATP by luminescence. ATP levels were determined by the luciferin-luciferase technique using Invitrogen kits (Invitrogen, Carlsbad, CA) with a reported sensitivity of 0.1 pmol. The assay for ATP was performed according to the manual provided with the kit. Samples (10 μl) were added to a 96-well plate, followed by the addition of 100 μl of the standard reaction solution containing dithiothreitol, luciferin, and firefly luciferase. The light emitted from the reaction of ATP was measured at 565 nm using a luminometer (Wallac 1420 VICTOR2). A standard curve with known concentrations of ATP was used to determine the ATP concentration. All samples were corrected for the background luminescence signal obtained with TBS without added ATP. For all samples, the average of three measurements were used. ATP was measured in the absence and presence of RBCs to check that RBCs did not interfere with the ATP assay.

Chemiluminescence determination of Hb-associated NO. An analysis of heme-NO was performed using a model 280 Nitric Oxide Analyzer (Sievers Instruments). To determine the total heme-associated NO, but not free nitrite or nitrate, 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) in water. Before sample injection, the reagents in the purge vessel were completely deoxygenated by flushing with argon. The sample was injected (25 μl) into the bottom of the purge vessel using a syringe with an 8-inch-long needle. The reagents in the purge vessel were changed after every 12–16 injections (33). 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.

Determination of blood pressure. Before their blood pressure was measured, the rats were anesthetized with isoflurane (2–3% mixed with oxygen) and the body temperature was maintained at 37°C by a warming pad. Blood pressure was measured using CODA-6 pressure cuffs (Kent Scientific), which were placed around the tail. Animals were given 10 min to stabilize, followed by 5 min of the monitoring of the basal level of blood pressure. After basal blood pressure was measured, reagents were injected into the femoral vein of the rat. Basal blood pressure was continuously monitored for 15 min.

The effect of nitrite on RBC total ATP. Total RBC ATP was measured by the luminescence method on an aliquot of the cells lysed in three volumes of deionized water. For the in vitro ATP determinations, a suspension of RBCs (1% Hct) in TBS buffer (pH 7.4) was preequilibrated under normoxic or anoxic conditions for 2 h at 37°C. Nitrite was then added to this suspension and rocked to initiate a reaction of nitrite with the RBC Hb. The resultant 50 μM nitrite concentration corresponded to 4.1 heme:nitrite molar ratio. To follow the time dependence, aliquots of cells were lysed and analyzed before the nitrite was added and 1, 10, 30, and 60 min after the nitrite was added.

For the in vivo determination, ~0.25 ml of 12 mM nitrite were injected directly into the femoral vein, resulting in a concentration of nitrite equivalent to 10 μmol/kg body wt. Blood (0.6 ml) was drawn from a catheter placed in the carotid artery before nitrite injection and 1, 10, 30, and 60 min after nitrite injection. The RBCs were washed, and an aliquot of 1% Hct was lysed in three volumes of deionized water.

The effect of nitrite on the release of ATP from RBCs. RBCs were adjusted to 1% Hct with TBS buffer (pH 7.4) and preequilibrated under anoxic conditions or normoxic conditions for 2 h at 37°C. Nitrite was then added to this suspension to initiate a reaction of nitrite with the 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 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. The in vivo process was monitored by measuring the effect of nitrite injection (see Animals) on the level of plasma ATP.

Formation of RBC-NO. To determine the basal RBC-NO, 1.5 ml blood were drawn from a catheter in the carotid artery. The blood was immediately processed to remove the plasma and buffy coat and washed with PBS three times. The Hct was adjusted to 50% with PBS. Hb-associated NO was determined by integrating the chemiluminescence signal obtained with RBCs lysed with three volumes of deionized water. The increase in RBC-NO due to the nitrite reaction in vivo was studied by comparing the basal level of RBC-NO with the level obtained 60 min after 10 μmol/kg body wt of nitrite was injected directly into the femoral vein.

In vivo determination of changes in blood pressure. For the study of the effect of ATP, 0.33 μmol/kg body wt of ATP was injected into the femoral vein. For the determination of the effect of nitrite-reacted RBCs, the change in blood pressure after 30 min when directly injecting 0.8 ml of RBCs into the femoral vein of the rat was compared with the injection of ~0.25 ml of 12 mM nitrite (see Animals) and the injection of 0.8 ml of RBCs reacted with 0.5 μmol of nitrite in a sealed RBC vial.

The effect of t-NAME was investigated by injecting into the femoral vein of the rat ~0.2 ml of t-NAME, resulting in an t-NAME concentration of 25 μmol/kg body wt before injecting nitrite-reacted RBCs. The effect of apyrase was investigated by adding 40 units apyrase/ml of 50% Hct RBC to the RBCs before reacting with nitrite. RBCs (0.8 ml) were then directly injected into the femoral vein of the rat.

Statistical analysis. Origin 6.1 (Microcal Software, Northampton, MA) was used for analysis of the data. Data are presented as means ± SE. ANOVA was used in analyzing the decrease in blood pressure when ATP was injected. For other experiments the paired Student’s t-test was used for comparing groups of samples with P ≤ 0.05 considered as statistically significant.

RESULTS

Nitrite enhances ATP synthesis in RBCs. RBCs (1% Hct) were preincubated under anoxic or normoxic conditions for 2 h to set the oxygenation status of the samples. Aliquots of the same blood sample were used for the anoxic and normoxic experiment. After preincubation, the RBCs were reacted with
Nitrite (50 μM) and the time-dependent increase in total ATP was monitored (Fig. 1, A and B). The total RBC ATP was determined by lysing aliquots of the reaction mixture before nitrite addition and 1, 10, 30, and 60 min after the nitrite reaction was initiated. The lag in the time-dependent increase in ATP synthesis under hypoxic conditions that is not observed under normoxic conditions is not statistically significant. However, the trend can be related to the requirement for the nitrite reacted Hbs to bind to the membrane, displacing both glycolytic enzymes (GEs) and Hb. The higher affinity of deoxyHb than oxyHb for the membrane can slow this process down.

Sixty minutes after the nitrite reaction was initiated, there was a 54.5% significant increase in the ATP concentration in the anoxic RBCs ($P < 0.01, n = 4$) from 367.2 ± 57.4 to 567.3 ± 76.4 μM (Fig. 1A). Under normoxic conditions, the ATP also underwent a 42.9% increase ($n = 4$) from 371.1 ± 56.5 to 530 ± 90.7 μM (Fig. 1B). Because of the larger variance between samples, attributed in part to the long lag time for the reaction of nitrite with oxyHb (23), the increase under normoxic conditions was not statistically significant.

An in vivo experiment to determine whether nitrite increases ATP synthesis was performed by directly injecting sodium nitrite dissolved in PBS (10 μmol/kg body wt) into the femoral vein. Before nitrite injection and at various times after the injection (Fig. 1C), 0.6 ml of blood were removed from a catheter placed in the carotid artery and the total RBC-ATP was determined by washing the cells and then determining the ATP in lysed cells. The initial sample drawn before nitrite injection was also used to study the in vitro increase in ATP synthesis induced by nitrite under anoxic and normoxic conditions (Fig. 1, A and B). Sixty minutes after nitrite injection, the average total RBC ATP (Fig. 1C) increased significantly by 136.7% from 163.2 ± 43.1 to 386.3 ± 60.3 μM ($P < 0.02, n = 4$). The average percent increase for these samples was 172 ± 48%.

A comparison of the in vitro and in vivo results indicate a greater more rapid increase in ATP in vivo (Fig. 1C) than in vitro (Fig. 1, A and B). In fact, under in vivo conditions, an appreciable fraction of the 60-min increase takes place within 1 min, during which time no in vitro effect is observed. This difference can perhaps be attributed to the preincubation required to adjust the oxygenation conditions. This effect is even more pronounced under anoxic conditions, which stimulate ATP synthesis (21), than under normoxic conditions (compare Fig. 1A and Fig. 1B).

Nitrite facilitates release of RBC ATP. RBCs (1% Hct) were preincubated under anoxic or normoxic conditions for 2 h to set the oxygenation status of the samples. The RBCs were then reacted with nitrite (50 μM). To study the nitrite-induced release of ATP, we used 30 min, which corresponded to the shortest time that gave a significant increase in total ATP (Fig. 1).

Fig. 1. Increase in total ATP in red blood cells (RBCs) induced by nitrite. A: in vitro anoxic condition. A suspension of RBCs (1% Hct) in TBS buffer (pH 7.4) was preequilibrated under anoxic conditions in an anaerobic glove box for 2 h at 37°C. Nitrite was then added to this suspension, resulting in a 50 μM nitrite concentration. RBC ATP was measured by the luminescence method on an aliquot of the cells lysed in 3 volumes of deoxygenated deionized water before the nitrite was added and 1, 10, 30, and 60 min after the nitrite was added. The error bars indicate the SE obtained by averaging 4 independent experiments. The increase in ATP after 60 min was statistically significant ($P < 0.01$). B: in vitro normoxic condition. The same experiment was performed under normoxic conditions at atmospheric Po2. To be able to compare the normoxic and hypoxic results, the normoxic sample was also preequilibrated for 2 h at 37°C before the addition of nitrite. Nitrite was then added to this suspension, resulting in a 50 μM nitrite concentration. RBC ATP was measured by the luminescence method on an aliquot of the cells lysed in 3 volumes of deoxygenated deionized water before the nitrite was added and 1, 10, 30, and 60 min after the nitrite was added. The error bars indicate the SE obtained by averaging 4 independent experiments. The increase in ATP after 60 min was not statistically significant ($P < 0.01$). C: in vivo. Nitrite (10 μmol/kg) was injected directly into the femoral vein of rats (250–350 g). Blood samples (0.6 ml) were drawn from a catheter placed in the carotid artery before nitrite injection and 1, 10, 30, and 60 min after nitrite injection. The RBCs were washed, and an aliquot of 1% Hct was lysed in 3 volumes of deionized water. The lysate was used to determine the concentration of ATP by the luminescence method (see MATERIALS AND METHODS). The error bars indicate the SE obtained by averaging 4 independent experiments. The increase in total RBC ATP after 60 min was statistically significant ($P < 0.02$).
Glycolysis and, therefore, ATP synthesis in the RBC is known to be inhibited by the binding of GEs to the RBC membrane (30, 51). deoxyHb competes with GEs for the same binding sites on band 3 of the RBC membrane (5) and increases glycolysis (30). It has, furthermore, been shown (21) that the deoxygenation of the RBC, which increases the concentration of deoxyHb, also increases the release of ATP. To determine whether the same mechanism is responsible for the effects of nitrite (see above), we investigated the interaction of nitrite-reacted Hb with the membrane under both hypoxic and normoxic conditions.

1, A and B). To correct for any ATP released during the preincubation and/or the ATP released by the lysis of RBCs during sample preparation (see below), we determined the increase in released ATP instead of the absolute level of ATP during the 30 min nitrite reaction (Fig. 2). As shown in Fig. 2, both the anoxic and normoxic reaction of 1% RBCs with 50 µM nitrite not only increased ATP synthesis but also resulted in an increase in the ATP released from RBCs. The increase was greater under anoxic conditions than normoxic conditions, although the difference was not statistically significant.

The lysis, before and after the nitrite reaction, was determined by the absorption of Hb in the region of the highly sensitive 415-nm oxyHb peak in the supernatant from the centrifuged sample used for luminescence. In some cases, low levels of Hb (~0.15 OD at 415 nm) attributed to lysis caused by the washing of the RBCs was observed. A multicomponent fitting of the spectrum in this region with spectra of oxyHb, methemoglobin (metHb), and deoxyHb indicated that >90% of the Hb in these samples was oxyHb. However, this level of Hb did not increase during the nitrite reaction. When the increase in ATP during the nitrite reaction is plotted, instead of the actual values of ATP determined (Fig. 2), any contribution of ATP released because of lysis is eliminated.

To determine whether the reaction of nitrite with RBCs can increase the release of ATP in vivo, an aliquot of 12 mM nitrite in PBS was injected into the femoral vein of rats, resulting in a nitrite level of 10 µmol/kg body wt. No effect was observed when injecting vehicle (see Fig. 6B). This level of nitrite was previously shown (42) to result in an increase in RBC heme-NO and a decrease in blood pressure without any significant change in the respiratory rate or other noticeable deleterious effects on the rat. As shown in Fig. 3, 60 min after the injection of nitrite, there was a significant (*P < 0.05, n = 9) increase in plasma ATP from 4.8 ± 0.8 to 9.1 ± 2.5 nM. To ascertain that the increase in released ATP was not due to increased lysis, absorption spectra were run on the plasma (46). Any samples with an observable level of Hb based on the absorption in the region of the highly sensitive 415-nm oxyHb peak were discarded.

Nitrite injection (10 µmol/kg body wt) was also shown (Fig. 4) to produce a significant (*P < 0.05, n = 4) increase in RBC-NO from 0.60 ± 0.19 to 1.63 ± 0.37 µM after 60 min. This increase is attributed to the uptake of nitrite by RBCs, which can then react with deoxyHb chains formed when the RBCs pass through the microcirculation and the venous circulation. The increase in RBC-NO indicates that at least some of the added nitrite is getting into the RBCs. An effect of nitrite on the RBC is also indicated by the increase in RBC ATP (Fig. 1C). These changes in the RBC result in an increase in the release of ATP (Fig. 2) that contributes to the observed increase in plasma ATP.

Nitrite increases Hb binding to RBCs. Glycolysis and, therefore, ATP synthesis in the RBC is known to be inhibited by the binding of GEs to the RBC membrane (30, 51). deoxyHb competes with GEs for the same binding sites on band 3 of the RBC membrane (5) and increases glycolysis (30). It has, furthermore, been shown (21) that the deoxygenation of the RBC, which increases the concentration of deoxyHb, also increases the release of ATP. To determine whether the same mechanism is responsible for the effects of nitrite (see above), we investigated the interaction of nitrite-reacted Hb with the membrane under both hypoxic and normoxic conditions.
Deoxygenated RBCs were reacted for 60 min with low levels of nitrite corresponding to a Hb to nitrite ratio between 40 and 100. These cells were washed and then lysed. The chemiluminescence method was used to determine the heme-NO. Determinations were made on both the total hemolysate and an aliquot of hemolysate, which was anaerobically centrifuged to prepare membrane-free hemolysate. The difference between the heme-NO in the noncentrifuged hemolysate and the centrifuged membrane-free hemolysate was used to determine the fraction of nitrite-reacted Hb associated with the membrane. As shown in Fig. 5, the fraction of nitrite-reacted Hb on the membrane decreases when the excess deoxyHb increases. However, even with a 100-fold excess of deoxyHb, the fraction associated with the membrane was > 0.3 indicating that nitrite reacted Hb has a significantly higher affinity for the membrane than deoxyHb.

Under normoxic conditions, the reaction of nitrite does not result in the formation of a nitrite/NO complex. Instead, oxidation of the Hb takes place (23). We, therefore, compared the levels of metHb in the hemolysate before and after centrifugation, as well as the metHb levels in the dispersed pellet. This study was performed on normoxic samples of 5% Hct without the prior addition of nitrite and after nitrite was added to the sample resulting in a final nitrite concentration of 0.05 mM and a heme:nitrite molar ratio of 20:1. After a 30-min reaction time and before centrifugation, the samples were diluted so that the visible spectrum could be measured and analyzed. For these experiments, Triton was added to the hemolysate and the resuspended pellet before running the spectra to eliminate any turbidity, which interferes with the proper fitting of the data, generally resulting in elevated levels of metHb. As shown in Table 1, the metHb is found preferentially on the membrane even though there is a large excess of oxyHb. This indicates that metHb has a significantly higher affinity for the membrane than oxyHb.

The vasodilatory effect of ATP injection. Plasma ATP has been reported (50) to stimulate NO synthesis and has a vasodilatory effect. To determine whether the increase in ATP observed when cells are treated with nitrite has a vasodilatory effect, we measured the effect that directly injecting ATP had on blood pressure. For this purpose we used 0.33 μmol/kg body wt ATP, which provides ATP in the nanomolar range similar to that released from RBCs by nitrite (Fig. 2) and which has been shown to reduce vascular resistance (50). Figure 6A shows the time course for the decrease in blood pressure observed in all three animals studied when ATP (the disodium salt) dissolved in PBS was injected into the femoral vein. The significance of this effect, despite individual variability, is demonstrated in Fig. 6B by plotting the average percent decrease in blood pressure after ATP injection. With the use of ANOVA to analyze this decrease, the 17.7 ± 2.9% drop in blood pressure after 15 min was statistically significant (P < 0.05, n = 3). For a comparison, the insignificant change in blood pressure when the same volume of PBS was injected is shown in Fig. 6B.

Basal RBC-NO contributes to plasma ATP. We have shown that, both in vivo (Fig. 3) and in vitro (Fig. 2), the reaction of nitrite with Hb in RBCs results in an increase in released ATP that can induce a decrease in blood pressure (Fig. 6). The physiological relevance of this process is demonstrated by comparing the basal level of RBC-NO and the basal level of plasma ATP before any injections into the rat. Figure 7 shows the relationship between RBC-NO and plasma ATP. The second order polynomial fit of the data (r = 0.58, P < 0.05) indicates a significant correlation with the increase in ATP becoming more pronounced for higher levels of RBC-NO. At low levels of RBC-NO, contributions to plasma ATP from hypoxia and mechanical deformation (21, 48) should dominate. However, at higher values of RBC-NO, the contribution of the nitrite-induced release of ATP from RBCs provides a significant fraction of plasma ATP, resulting in the upward curvature seen in Fig. 7.

The decrease in blood pressure induced by nitrite and nitrite-reacted RBCs. The results in Nitrite facilitates release of RBC ATP indicate that nitrite injection increases the level of plasma ATP (Fig. 3) and that ATP injection decreases blood pressure (Fig. 6). Figure 8 further shows that 15 min after the injection of 10 μmol/kg body wt nitrite, a significant decrease in blood pressure is observed. Whereas the injection of 0.8 ml of 50% Hct RBCs had no significant effect on the blood pressure, a significant decrease in blood pressure was observed after 15 min if the RBCs were first reacted with nitrite to

---

Table 1. Comparison of the binding of metHb and oxyHb to RBC membranes

<table>
<thead>
<tr>
<th></th>
<th>metHb, μM</th>
<th>oxyHb, μM</th>
<th>Total Hb, μM</th>
<th>metHb, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemolysate*</td>
<td>4.1 ±3.2</td>
<td>17.3 ±1.1</td>
<td>21.4 ±4.3</td>
<td>19.2</td>
</tr>
<tr>
<td>Supernatant†</td>
<td>−0.21 ±0.15</td>
<td>16.6 ±0.2</td>
<td>16.6 ±0.2</td>
<td>undetectable</td>
</tr>
<tr>
<td>Pellet‡</td>
<td>0.36 ±0.03</td>
<td>1.6 ±0.3</td>
<td>2.0 ±0.6</td>
<td>18.0</td>
</tr>
<tr>
<td>RBC + nitrite‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemolysate*</td>
<td>2.3 ±0.5</td>
<td>16.2 ±0.9</td>
<td>18.5 ±1.2</td>
<td>12.4</td>
</tr>
<tr>
<td>Supernatant†</td>
<td>0.04 ±0.01</td>
<td>13.5 ±1.3</td>
<td>13.5 ±1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Pellet‡</td>
<td>1.8 ±0.2</td>
<td>8.1 ±4.6</td>
<td>9.9 ±0.2</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. metHb, methemoglobin; oxyHb, oxygenated Hb; RBC, red blood cell.*0.1% Triton was added to the hemolysate to eliminate scattering. †The pellet was suspended in the same volume of PBS as the initial sample, and 0.1% Triton was added. ‡The Hb:nitrite molar ratio was 20:1.
increase the level of RBC-NO. For this experiment, we used a Hb:nitrite ratio of 16.6, resulting in the injection of 0.5 μmol of nitrite, which is approaching the physiological levels of 50–350 nM nitrite. The observation that the effect of nitrite-reacted RBCs, which correspond to an appreciably lower level of nitrite than the 10 μmol/kg body wt used for the nitrite injection, demonstrates that the vasodilatory effect is mediated by the RBC and is not a direct effect of nitrite.

To characterize the source for this decrease in blood pressure, we first treated the rats with L-NAME, which inhibits eNOS. As shown in Fig. 9A, the injection directly into the femoral vein of 0.2 ml of L-NAME, resulting in a concentration of L-NAME in the rat of 25 mg/kg, produces an increase in blood pressure. As shown in Fig. 9A, the subsequent injection of 0.8 ml of a 50% Hct solution of nitrite-reacted RBCs (see above) directly into the femoral vein did not produce a decrease in blood pressure, consistent with a process that involves the activation of eNOS.

In Fig. 9B, we further show that the decrease in blood pressure is essentially eliminated, even without prior treatment with L-NAME when apyrase (40 units) is added together with the RBCs. Apyrase degrades any ATP released, demonstrating that the decrease in blood pressure caused by RBCs involves the release of ATP.

**DISCUSSION**

The reaction of nitrite with RBCs and the associated release of ATP. It has been shown that nitrite is reduced during the reaction of nitrite with deoxygenated RBCs (8, 33, 42). During this reaction, NO, N2O3, SNOHb, and various forms of heme-NO including heme Hb(II)NO, heme Hb(III)NO, and a delocalized heme-NO intermediate are formed (9, 22, 35). The formation of NO, as well as other species such as SNOHb that can have vasodilatory effects, implies that the RBC nitrite reaction can produce vascular dilation. There have, in fact, been a number of subsequent studies demonstrating the vascular dilatory effects of nitrite that have been shown to be associated with the reaction of nitrite with deoxygenated RBCs (8). These studies have been the basis for the proposals (8, 33) that the hypoxic nitrite reductase activity of RBCs supplies NO to the vasculature without requiring the actual transport of NO by the RBC. The reaction involving nitrite is particularly attractive because 50–350 nM nitrite is present in the circulation (25) and is taken up by RBCs (36) where it readily reacts with Hb-producing NO.

The major unresolved issue with respect to this mechanism is understanding how NO generated in the RBC can be released.
to the vasculature without first being quenched by the very rapid and efficient reaction of NO with deoxyHb (13), which produces Hb(II)NO that is not expected to release its NO, and the equally rapid reaction with oxyHb (11, 13) that produces nitrate which has no vascular effects.

The Gladwin group (16) originally suggested that the release of NO by nitrite reduction involves a metabolite that is part of the RBC membrane and that this NO is released without ever entering the RBC. In a more recent article (1), they proposed a reaction where excess nitrite reacts with the low levels of metHb present in the RBC and that the reaction of NO with this nitrite-metHb complex is assumed to be able to compete with deoxyHb and oxyHb for the NO formed, producing N2O3 that can either react with thiol groups or be released from the cell.

On the other hand, we have emphasized (34, 35, 44) the role of a delocalized intermediate that retains potential NO bioactivity without reacting with Hb. It is, however, still necessary to explain how the NO can be released from the RBC after it is released from the intermediate.

Our current studies on the effect of nitrite on the release of ATP bypass the difficulty of releasing NO from the RBC by showing that the nitrite reaction enhances the release of ATP (Fig. 2).

As shown in Fig. 2, an increase in the release of ATP by nitrite was unexpectedly observed for normoxic RBCs as well as hypoxic RBCs. The normoxic effect of nitrite could not be attributed to the nitrite reductase reaction of Hb, since oxyHb does not reduce nitrite, but should be related to the increase in metHb formed when nitrite reacts with oxyHb (see The mechanism for nitrite-induced release of ATP). Both of these reactions can occur in vivo using the 50 – 350 nM plasma nitrite (25, 36) as the RBCs are continuously being oxygenated and deoxygenated. However, studies on the effect of Hb oxygen saturation on nitrite uptake (3) indicate that, because of a more rapid reaction of nitrite with deoxyHb than oxyHb (45), it is primarily the hypoxic reaction involving deoxyHb chains of partially oxyHb which is responsible for most of the nitrite uptake by RBCs in vivo.

As soon as the RBCs become partially hypoxic, the level of the NO/nitrite Hb species is expected to increase, triggering an immediate increase in the released ATP. Such a process would predict that, at any time, the level of RBC-NO in venous blood should be correlated with the level of plasma ATP. Figure 7 shows the expected correlation. At low levels of RBC-NO, other contributions to plasma ATP including the hypoxic and mechanical release of ATP from RBCs may dominate. However, at higher values of RBC-NO, the RBC provides a significant fraction of plasma ATP, resulting in the upward curvature seen in Fig. 7. These results are consistent with our previously reported observation (33) that as much as 75% of RBC-NO originates from the reaction of nitrite with the deoxyHb in the cell.

Vasodilation associated with the release of ATP. A role for plasma ATP in the regulation of vascular tone (10, 29, 49) has been reported. This process has been shown to involve an interaction with P2X purinergic receptors on endothelial cells, resulting in the stimulation of NO synthesis as well as the formation of prostacyclin, both of which mediate local vasodilation (14, 29, 32). Consistent with these earlier studies, we observed that the administration of ATP, in the range found to be released in our experiments in response to nitrite (Figs. 2 and 3), caused a decrease in blood pressure (Fig. 6), suggesting a generalized vasodilatory effect.

The potency of the vasodilatory effect of nitrite-reacted RBCs is demonstrated by the results of Fig. 8 where the effect of nitrite-reacted RBCs is compared with the direct injection of nitrite. In this experiment, essentially the same decrease in blood pressure was observed even though the 0.5 μmol of nitrite injected with the RBCs is more than an order of magnitude lower than the 10 μmol/kg body wt used when nitrite is directly injected.

The role of ATP-induced eNOS synthesis of NO in this process is demonstrated by the inhibition of the RBC-induced decrease in blood pressure by L-NAME, which inhibits eNOS (Fig. 9A) and apyrase, which degrades any ATP released (Fig. 9B).

These data demonstrate a new mechanism for nitrite-induced vasodilation, which retains a role for RBCs, unlike the proposed mechanisms for nitrite-induced NO involving reactions with mitochondria or xanthine oxidase. This mechanism, however, does not require that NO be released from RBCs. Instead, the nitrite reaction increases the level of ATP within the releasable pool of ATP, which is released under hypoxic conditions when the signal transduction pathway for ATP...
release is activated. This process further stimulates eNOS to synthesize more NO, increasing vasodilation.

In contrast to our results indicating an increase in ATP release associated with nitrite reduction by RBCs, it has been reported that NO in the RBC inhibits the release of ATP from RBCs (37, 39). The inhibitory effect has been attributed to the inactivation of the heterotrimeric G protein G_{i}, which is involved in the signal transduction pathway responsible for ATP release by NO (39). This inhibition requires NO. Since we see an increase of ATP release instead of the decrease observed in the NO studies, the nitrite-induced effect presumably does not involve NO and instead can be attributed to the metastable intermediates that are formed before the production of NO (35, 44).

While we have shown that RBC-NO contributes to the hypoxic release of ATP, our results do not rule out the possibility that other RBC-associated factors also contribute to hypoxic vasodilation. These can include a direct release of NO, N_{2}O_{3}, and/or nitrosothiols.

**The mechanism for nitrite-induced release of ATP.** An understanding of how the reaction of nitrite increases the release of ATP depends on the factors that control the enhanced release of ATP from RBCs under hypoxic conditions. The release of ATP from RBCs involves a signal transduction pathway via its action on the heterotrimeric G protein G_{i} (38). This pathway involves adenyl cyclase synthesis of cyclic AMP and protein kinase A (48), as well as the cysitic fibrosis transmembrane conductance regulator.

The triggering of this process by hypoxia has been shown to involve (21) the deoxygenation of Hb, which undergoes a conformational change that results in a higher affinity for the cytoplasmic end of membrane band 3 than oxyHb. The increased binding of deoxyHb displaces GE s that bind to the same region of band 3 (51), resulting in an increase in anaerobic glycolysis and the associated synthesis of ATP.

When our results on nitrite-reacted RBCs (Figs. 2–4) are compared with the results of Jagger et al. (21), it can be suggested that the reaction of nitrite with Hb results in a further increase in the affinity of Hb for the membrane and greater displacement of GE s from the membrane and more ATP synthesis.

This increased affinity for the membrane of nitrite-reacted deoxyHb was demonstrated by adding low levels of nitrite to deoxygenated RBCs and determining the fraction of nitrite-reacted Hb associated with the membrane (Fig. 5). For this purpose, the concentration of nitrite relative to deoxyHb was kept low with a Hb:nitrite ratio in the range of 40–100 so that the limited number of membrane sites would not be saturated with nitrite-reacted Hb and so we could compare the affinity for deoxyHb and nitrite-reacted deoxyHb. The finding that, even with a 100-fold excess of deoxyHb, a significant fraction of the nitrite-reacted Hb (>0.30) is bound confirms an appreciable increase in affinity when deoxyHb reacts with nitrite.

For normoxic RBCs, the nitrite-reacted intermediates (33–35) are not formed. Instead, the reaction of nitrite results in the oxidation of Hb to metHb and the formation of nitrite. Earlier studies have shown that deoxyHb has a higher affinity for the membrane than oxyHb (52) and that hemichromes (53) formed from oxidized Hb have a higher affinity than deoxyHb. Our results (Table 1), however, indicate that metHb found in fresh RBCs and metHb formed when nitrite reacts with RBCs under normoxic conditions have appreciably higher affinity for the membrane than oxyHb. These results imply that the nitrite-induced increase in ATP synthesis and the subsequent release in ATP are also coupled to an increase in the binding of Hb to the membrane, even though RBC-NO is not formed.

It is still necessary to explain why the inhibition of glycolysis affects the hypoxic release of ATP when only a small fraction of the approximate millimolar ATP in the RBC is released (28). This has been explained by the newly synthesized ATP forming a pool of releasable ATP located in the region of the RBC membrane, which is preferentially released during hypoxic glycolytic stimulation (21).

The formation of a distinct pool of releasable ATP is consistent with an earlier proposal of ATP compartmentation in human RBCs (17). The buildup of this pool with the newly synthesized ATP can be explained in terms of the complex of GE s shown to form around the cytoplasmic end of band 3 (5, 15). The assembly of this GE complex is thought to provide efficient channeling of substrates between sequential enzymes in the glycolytic pathway. Thus, although the actual rates of glycolysis decrease when GE s are bound to band 3, the role of band 3 in forming this GE complex can perhaps prime the RBCs for more efficient glycolysis in the region of the membrane when the complex is displaced from band 3 by deoxyHb. This compartmentalization thus explains the observation that the increased ATP synthesis when Hb displaces the GE s results in an increase in the release of ATP both under anoxic and normoxic conditions, even though there is only a small fractional increase in total intracellular ATP (21).

Further support for this hypothesis is provided by a recent study (31) that shows that the amyloid peptide [Aβ (1–42)] inhibits the release of ATP from the human erythrocyte. This same peptide activates caspase 3 in the RBC (7, 27), which cleaves the NH2-terminal end of band 3 (27). The inhibition of ATP release does not involve a decrease in glycolysis or the inhibition of the signal transduction pathway responsible for the release of ATP. Instead, it is coupled to the cleavage of band 3, which seems to be required for the formation of a membrane-associated releasable pool of ATP. Thus, even though glycolysis and ATP synthesis continues, the ATP produced is not released.

**Physiological relevance.** Our in vitro and in vivo studies demonstrate that nitrite taken up by the RBC increases the level of ATP within the releasable pool of ATP, which is released under hypoxic or anoxic conditions when the signal transduction pathway for ATP release is activated. A vasodilatory effect of this released ATP is demonstrated by a decrease in blood pressure. The involvement of nitrite-reacted RBCs in the in vivo release of ATP is demonstrated by the effect of infusing nitrite-reacted RBCs instead of nitrite (Fig. 8). It is particularly significant that RBCs have the same effect on blood pressure even though the level of nitrite injected with the RBCs corresponds to more than a 10-fold lower level of nitrite than that directly injected (Fig. 8).

The role of eNOS activation by released ATP is demonstrated by the inhibition of the decrease in blood pressure when L-NAME, which inhibits eNOS, is injected before the nitrite-reacted RBCs (Fig. 9A) and when apyrase (Fig. 9B), which degrades any released ATP, is injected with the nitrite-reacted RBCs.

In the evaluation of the physiological relevance of our findings, it is, however, necessary to consider the higher
nonphysiological levels of nitrite used for many of our experiments, as well as the slow time course for many of the reactions studied.

For the in vitro experiments (Figs. 1, A and B, and 2) where we investigated the effects of nitrite added to RBCs, we used concentrations of nitrite appreciably higher than the normal 50–350-nM levels of nitrite (25, 36). For these experiments we used the 1:4 nitrite:heme molar ratio for which we have carefully studied the nitrite reaction (44). As long as we have an excess of heme, the reaction is thought to be analogous to the physiological reaction with a much larger heme excess, and we were able to obtain measureable reproducible increases in ATP synthesis and ATP release.

For the in vivo experiment where we directly injected nitrite (Figs. 1C, 3, 4, and 8), we used an appreciably lower nitrite level of ∼30 μM and obtained an analogous increase in ATP synthesis (Fig. 1C), ATP release (Fig. 3), and RBC-NO (Fig. 4) and a decrease in blood pressure (Fig. 8). The most definitive experiment that demonstrates an effect at physiological concentrations of nitrite is the nitrite-reacted RBCs where we only injected 0.5 μmol of nitrite.

The time course of the in vitro experiments is affected by the relatively slow uptake of nitrite into RBCs (36) and the subsequent reaction of nitrite with deoxyHb chains (35). In addition, for the in vitro experiments, a 2-h preincubation was required to adjust the sample to the required oxygen level. During this time, ATP is being synthesized and released, limiting the subsequent effect of the nitrite reaction. This difference is seen by the relatively rapid in vivo reaction with a major fraction of the reaction over in <1 min (Fig. 1C) compared with the gradual slow reaction observed for the preincubated in vitro reactions (Fig. 1, A and B).

These results establish the physiological effect of nitrite and nitrite-reacted RBCs that are approaching physiological conditions for the in vivo reactions. However, further support for the contention that the nitrite-induced release of ATP does take place under physiological conditions is provided by the results of Fig. 7 where we compared the basal levels of RBC-NO with the level of plasma ATP. In vivo, the 50–350 nM nitrite (36) is continuously being taken up by RBCs and interacting with RBC Hb. These interactions result in a RBC heme-associated NO of 282 ± 108 nM in arterial blood and 456 ± 93 nM in venous blood (33). Our results indicate that this pool of RBC-NO already present in the RBC plays a crucial role in fine tuning the physiological release of ATP and its subsequent vasodilatory effects (Fig. 7). Furthermore, the time for nitrite uptake (36) and the reaction of nitrite with Hb (35) is bypassed under these in vivo conditions, because we are dealing with a pool of nitrite-reacted Hb already present. The time frame for a vasodilatory effect of this pool of RBC-NO only depends on the time required to release ATP from RBCs.

However, from our studies, we cannot address the question of whether the nitrite reaction contributes to hypoxic vasodilation that controls the flow of blood through the microcirculation. Under normal conditions, the role of the nitrite reaction may be to help maintain the pool of releasable ATP and/or to help set the level of vascular tone in the circulation. Alternatively, the nitrite reaction may play a role in regulating the vascular response under conditions of increased NO demand and under pathological conditions where the supply of endothelial NO needs to be augmented. While our results do not address the specific physiological role of the enhanced release of ATP by nitrite, it establishes a role for nitrite-reacted Hb in enhancing the release of ATP by RBCs and the vasodilatory processes that result from enhanced plasma ATP.

ACKNOWLEDGMENTS

We thank Donna Tignor for help in setting up animal experiments, Mohamed R Mughal for technical assistance with the animal experiments, and Dana Spence for input regarding the release of ATP from RBCs.

GRANTS

This research was supported by the Intramural Research Program of the National Institute on Aging.

REFERENCES


NITRITE ENHANCES THE RELEASE OF ATP FROM RBCs