Role of erythrocyte in regulating local O2 delivery mediated by hemoglobin oxygenation

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The primary role of the red blood cell (RBC) in the circulation is to deliver O2 to the periphery while transporting CO2 and other wastes generated by metabolizing tissues to the lungs. Yet, despite decades of research, the precise mechanisms that are responsible for matching the O2 demand of the tissues to the supply of O2 by the blood remain unclear. The O2 sensor locus has typically been hypothesized to be located in one of two regions: 1) in association with the walls of the vasculature itself (either in the endothelium or in the vascular smooth muscle) (7, 18, 19, 31) or 2) in a specific region of tissue or the parenchyma (14, 15). Recently, a third model has been presented (10, 20), which proposes that the erythrocyte, in addition to its role as an O2 carrier, is the sensor and effector of the local regulation of O2 delivery. In this scenario, as the erythrocyte enters the arteriolar tree, it experiences an O2 gradient with a magnitude that is proportional to the level of metabolic activity of the nearby tissue. In addition to the offloading of O2 from the hemoglobin molecule, a signal is released by the RBC, which triggers the increase of blood flow to the region through the vasodilation of the feeding arterioles. One mechanism that has been proposed by Stamler et al. (36) implicates low-molecular-weight nitrosothiol as the signal molecule (36). The nitroso group is carried on a cysteine residue, Cysβ93, of the hemoglobin molecule with the binding affinity dependent on the O2 saturation (SO2). Measurements of the concentration of S-nitrosohemoglobin (SNO-Hb) in arterial and venous blood showed that SNO-Hb was higher in arterial blood than in venous blood, implying that SNO-Hb is picked up in the lungs and released in the periphery in response to changes in SO2.

Release of signaling molecules from erythrocytes. A second mechanism is that the signal is generated by the O2-dependent release of ATP from the RBC (3, 6, 10, 27). It has been demonstrated that ATP is released from the RBC in response to hypoxia in the presence of hypercapnia (1), in response to hypoxia alone (10), as well as in response to mechanical deformation (35). Isolated rat cerebral arterioles have been shown to vasodilate under low extraluminal O2 only in the presence of RBC. This vascular response was accompanied by a concomitant increase of ATP in the vessel effluent (5). ATP has also been shown to bind to the purinergic P2y1, P2y2, and P2x receptors, eliciting a vasodilatory response on P2y1 and P2y2, whereas the converse is true on P2x (21, 32). More recent experiments (4, 27) demonstrated that ATP (but not adenosine), when injected into the lumen of an arteriole or venule, triggers a conducted vasodilatory response in the feeding arteriolar tree in the hamster cheek pouch retractor muscle. A conducted vasodilatory response, defined by Ellsworth et al. (10) as a vascular response that extends well beyond the region of initiation, provides a mechanism for coordination of microvascular change and is essential for the variation in vessel diameter to have any significant impact on blood flow and O2 supply to the tissue (23).
Focus of study. This study focuses on possible mechanisms responsible for the initiation of the signal transduction pathway involved in the release of ATP from the erythrocyte in response to a fall in SO2. We hypothesized that ATP efflux from the RBC is triggered by the conformational change of the hemoglobin molecule from its fully relaxed (liganded) state (R state) to its deoxygenated (tense) state (T state). We tested this hypothesis by reducing the total saturation of the hemoglobin molecule both in the presence and absence of small concentrations of CO. The high-affinity binding of CO to hemoglobin prevented the hemoglobin molecule from changing conformation to the deoxygenated state and, as we expected, also inhibited the release of ATP from the RBC. Furthermore, because deoxyhemoglobin has also been implicated as a regulator of ATP production in the erythrocyte through glycolysis (29, 33), we hypothesized that this would be a key stage in the SO2-dependent release of ATP from the RBC; that is, ATP release in response to an increased concentration of deoxyhemoglobin must be preceded by ATP production through glycolysis. We tested whether ATP efflux would be impaired when glycolysis is inhibited by incubating RBC with citrate [an inhibitor of the glycolytic regulatory enzyme, phosphofructokinase (PFK)] and fluoride (an inhibitor of the glycolytic enzyme, enolase) and by exposing the treated cells to different levels of PO2 to evaluate their ATP response.

A growing body of literature (1, 4, 10, 27, 35) suggests that the O2-dependent release of ATP from the erythrocyte may be an important regulatory mechanism in the matching of local O2 supply and demand in the microcirculation. This paper reflects the first attempts at elucidating the initiation events in its associated signal transduction pathway.

METHODS

Blood collection. Male Sprague-Dawley rats (450–600 g) were anesthetized with pentobarbital sodium (0.1 ml/100 g body wt ip) and exsanguinated via cardiac puncture into Vacutainer blood collection tubes containing heparin (control), citrate, or fluoride. Arterial and venous blood was also collected from four rats. The rats were anesthetized according to the same procedure and the carotid artery and jugular vein were cannulated. The blood (2–3 ml) was removed from each animal in this manner and analyzed for plasma [ATP].

Gas equilibration chamber. Well-mixed blood was injected into a 1-m long Silastic tubing (0.10 mm in diameter) pre-treated with heparinized saline to prevent RBC lysis. The tubing was mounted inside a humidified chamber, where the gas composition and temperature were regulated. The blood was maintained at a temperature of 37°C throughout the experiments; the %O2 of the sample chamber was measured continuously with a Criticon OxyChek O2 sensor calibrated by exposing the sensor to 100% N2 and 100% O2 environments.

Plasma ATP measurements. ATP levels were determined via the luciferin-luciferase technique (37). This chemiluminescence assay was chosen for its high degree of sensitivity and specificity for ATP. The assay cocktail was made by adding 250 μl of firefly extract (FLE-50, Sigma; St. Louis, MO) to 250 μl of purified synthetic D-luciferin (50 mg/100 ml distilled water, Sigma) and the cocktail was placed in a photocuvette inside a light-tight chamber. During ATP assay, 10 μl of whole blood were removed from the sample chamber and diluted into 10 ml of physiological salt solution (pH 7.4, 300 mosM) kept at 37°C and composed of (in mM) 140.5 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, 21.0 tris(hydroxymethyl)aminomethane with 1 g/l glucose, and 5 g/l albumin. Diluted blood samples (0.5 ml) were introduced into the light-tight chamber via an injection port positioned above the photocuvette. An attached photomultiplier tube (model HC135–01, Hamamatsu) collected the light. To reduce the effect of background noise, a photon-counting method was used to collect the data and the digital pulses were sent via a serial interface to a personal computer. Custom-designed software was used to automate the collection, storage, and analysis of the kinetic curve. Calibrations were performed on the day of the experiment by using the same physiological salt solution as specified above spiked with known quantities of ATP. Collected experimental light intensity values were then converted to plasma [ATP]. All of the measurements were performed in duplicate; variability between individual measurements was within 10%.

Blood gases. The blood (150 μl) was placed into a sealed blood gas collection tube (model ADL-520, Radiometer) and analyzed for blood gases and hemoglobin saturation (model OSM3, Radiometer). All of the blood samples were kept on ice and analyzed within 3 h of collection. The parameters measured were the percentage of saturated hemoglobin (rHb); the percentage of saturation of hemoglobin with O2 and CO; i.e., the percentage of hemoglobin in the R state, 95% confidence interval ±0.33%), the percentage of desaturated hemoglobin (tHb), the percentage of saturation of hemoglobin without O2 or CO; i.e., the percentage of hemoglobin in the T state, ±0.33%), PO2 (±0.4 mmHg), PCO2 (±0.5 mmHg), pH (±0.002), carboxyhemoglobin (±0.2%), and HCO3 (±0.5 mM). Plasma hemoglobin was also measured via a colorimetric technique where the rate of 3,3’,5,5’-tetramethylbenzidine oxidation by H2O2 is proportional to the hemoglobin concentration (sensitivity 0.5 μM; 527-A, Sigma). This was performed as a control to eliminate RBC lysis as a potential source of extracellular ATP.

Experiment 1: measurement of ATP efflux versus SO2. Blood was collected from four male Sprague-Dawley rats as outlined above. Heparinized whole blood (1.5 ml) from each rat was added to the sample chamber and permitted to equilibrate under normoxia (21% O2-5% CO2-balance N2) for 5 min. Blood samples were removed for baseline plasma ATP levels and SO2. The gas composition of the chamber was then changed to 5% CO2-balance N2 to deoxygenate the hemoglobin over time. Sample blood (150 μl) was removed every 5 min for 20 min and analyzed for erythrocyte SO2 and plasma ATP levels. All of the measurements were made in triplicate.

Experiment 2: CO treatments. Blood from five male Sprague-Dawley rats was collected as described above. The blood from each rat was divided into two aliquots; one 1.5 ml aliquot was injected into the sample chamber kept at 37°C, whereas the other was kept at room temperature. The blood in the sample chamber was equilibrated to a high SO2 (95% O2-5% CO2) for 5 min and blood gases and plasma ATP measurements were made. The gas composition was then changed to 5% CO2-balance N2 for 5 min to deoxygenate the hemoglobin molecule and blood gases and plasma ATP were assayed again. The chamber was then flushed as described above and the second aliquot of blood was injected. This sample was permitted to equilibrate for 5 min to high SO2 (95% O2-5% CO2) and plasma ATP measurements and blood gases were assayed. Finally, the gas composition was
changed to 5% CO2-8% CO-balanced N2 to deoxygenate the hemoglobin molecule (reduce SO2), while leaving rHb high (as the CO simply displaces the O2 on the hemoglobin molecule). Blood gases and plasma ATP measurements were made after 5 min. The 8% CO level was chosen so that an SO2 of 65%, an rHb of >95%, and a PO2 between 40-44 mmHg could be attained at the end of the 5-min treatment. The order of measurement of the different treatments was reversed for each animal to account for any effects of sample timing.

Experiment 3: glycolytic inhibitor treatments. Blood was collected from five male Sprague-Dawley rats into blood collection tubes containing heparin (100 USP), sodium citrate (0.105 mM), or sodium fluoride (7.5 g/l). Heparin was not included in the citrate or fluoride samples because these compounds have anticoagulative properties. Whole blood (1.5 ml) from each animal was injected into the tubing of the gas exchange chamber. Initially, the gas composition of the chamber was 21% O2-5% CO2-balance N2 and the blood was permitted to equilibrate at these conditions for 5 min. This was immediately followed by a blood gas analysis and plasma [ATP] measurement. The blood was then exposed to 5 min of 5% CO2-balanced N2 to reduce the SO2. Blood gases and plasma ATP measurements were made. The above procedure was then repeated for the second and third aliquots of blood incubated with the alternate inhibitors with thorough rinsing of the sample chamber between aliquots. The sample order was changed for each experiment to account for differences in timing. The total length of time from exsanguination to completion of all three groups was <1 h.

Statistics. Student’s paired t-tests were used to compare ATP efflux values between those blood samples exposed to low PO2 with and without the presence of CO. A paired t-test was also used to compare the arterial and venous levels of plasma [ATP] from the rats. A linear regression analysis was performed on the plot of ATP efflux vs. SO2 and PO2, whereas a one-way ANOVA was used to compare the ATP efflux values between blood samples exposed to low PO2 with and without the presence of glycolytic inhibitors. Significance was assigned at P < 0.05. All statistics were performed with the use of SPSS software, version 9.0. Results are means ± SE.

RESULTS

Characteristics of gas exchange chamber. Five minutes of exposure to normoxia results in a blood PO2 of 130 ± 4 mmHg and a SO2 of 98 ± 2%. Switching to the O2-free gas (5% CO2-balance N2) for 5 min reduces the blood PO2 to 44 ± 2 mmHg and an SO2 of 64 ± 2%. This corresponds approximately to the O2 levels of RBC entering the capillary bed of skeletal muscle (11). Plasma hemoglobin levels remained constant with CO treatment or incubation with citrate or fluoride; there was no evidence of hemolysis in the samples.

Plasma [ATP] as function of rHb. Plasma [ATP] was plotted as a function of the percentage of total rHb and the resultant graph is shown in Fig. 1 (n = 4 rats). A regression analysis performed on the data yielded a correlation coefficient of 0.98, whereas the analogous analysis performed on PO2 yielded a correlation coefficient of only 0.54.

CO treatment. Figure 2 is a plot illustrating the change in plasma [ATP] levels with and without the presence of CO (n = 5 rats). CO had no effect on the PO2 of the erythrocytes after a 5-min exposure to the O2-free gas (44 ± 1 and 42 ± 2 mmHg with and without CO, respectively). However, the total hemoglobin saturation of the blood differed dramatically with an rHb of 92% in the presence of CO and 64% in its absence. The total percentage of tHb was only 6% under CO and 35% without it. There was no difference in pH, PCO2 and bicarbonate levels between the two groups (7.44 ± 0.01, 36.3 ± 0.6 mmHg, and 24.8 ± 0.9 mEq/L, respectively, without CO and 7.42 ± 0.02, 36.4 ± 0.5 mmHg and 23.4 ± 1.0 mEq/L with CO present, P > 0.05 for all groups). The plasma [ATP] increased in the low O2 case by 1.3 μM or 12% in the absence of CO (consistent with the earlier experiments with heparinized whole blood) but decreased 0.3 μM or 12% when 8% CO was present in the chamber (significantly different from the control behavior, P < 0.05).

Glycolytic inhibitor treatments. A plot illustrating the O2-sensitive change in plasma [ATP], with (citrate or fluoride) and without (heparin) the inhibition of
glycolysis, is shown in Fig. 3 (n = 5). Five minutes of exposure to the CO2/N2 mixture increased plasma [ATP] in the sample with heparin by 1.0 μM or 45% on average. This response was dramatically reduced after citrate incubation (an increase of only 0.1 μM or 12%) and was actually reversed under fluoride treatment (a decrease of plasma [ATP] by 0.2 μM or −23%). *P < 0.05.

Arterial versus venous plasma [ATP] levels. Arterial blood was found to have a mean plasma [ATP] of 3.4 ± 0.4 μM at an SO2 of 80.3% compared with 5.0 ± 0.5 μM at an SO2 of 25.5% on the venous side. This corresponded to a mean increase of 1.7 ± 0.4 μM or 53 ± 12% (P < 0.05). These measured values compare well with the predicted values of 3.1 and 5.2 μM calculated by using the in vitro regression analysis of Fig. 1. The direct comparison with Fig. 1 can be made because carboxyhemoglobin is a negligible component of the total hemoglobin concentration in vivo; i.e., SO2 ≈ rHb. Figure 4 shows a plot of the in vivo experimental data.

DISCUSSION

How ATP crosses the erythrocyte membrane. In the past decade, there has been increased research focused...
on the precise mechanisms of ATP release from the erythrocyte. In 1992, Bergfeld and Forrester (1) showed that ATP is released from human erythrocytes in response to brief periods of hypoxia in the presence of hypercapnia. They demonstrated how ATP release may be prevented by blocking band 3 with the use of micromolar concentrations of a translocation inhibitor (niflumic acid), a transport site inhibitor (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid), or a channel blocker (dipyridamole). ATP efflux was also strongly affected by the presence of nanomolar concentrations of nitrobenzylthioinosine, a blocker of the nucleoside transporter band 4.5. More recently, one member of the ATP-binding cassette, the cystic fibrosis transmembrane conductance regulator (CFTR), has been shown to act as a mediator of deformation-induced ATP release from the RBC (34). What is unknown is through what mechanism does a change in PO2 stimulate ATP release from the RBC (34). What is unknown is through what mechanism does a change in PO2 stimulate ATP release from the RBC (34). What is unknown is through what mechanism does a change in PO2 stimulate ATP release from the RBC.

**ATP efflux correlates with hemoglobin SO2.** If ATP release is determined by the oxygenation status of the hemoglobin molecule, then ATP efflux should correlate more closely with SO2 than with PO2. This hypothesis is supported by Fig. 1, which illustrates the correlation of plasma ATP with SO2 and the corresponding plot with PO2. A higher correlation ($r^2 = 0.88$ vs. $r^2 = 0.54$) with SO2 than with PO2 is suggestive (but not conclusive evidence) of the involvement of the hemoglobin molecule in the release of ATP. This is consistent with our hypothesis that it is the oxygenation status of hemoglobin that is directly linked with ATP efflux from the RBC rather than the PO2.

**CO inhibits ATP efflux.** The role of the oxygenation status of the hemoglobin molecule was tested directly by quantifying the release of ATP under conditions of low O2 in the presence and absence of small concentrations of CO. CO and O2 bind competitively on the hemoglobin molecule. However, the affinity of CO for the hemoglobin molecule is 200 times that of O2 (17). Therefore, exposure to relatively low levels of CO will still result in a substantial displacement of O2 from hemoglobin. This displacement does not result in an increase of deoxyhemoglobin because the tightly bound CO molecule forces the hemoglobin to remain in the R state.

In Fig. 2, we see the results of exposing RBC to a low PO2 environment with and without the presence of CO. In both cases, the PO2 was lowered to levels consistent with PO2 levels found in the rat venous system. Without CO the rHb fell to 65%, but with 8% CO present, the rHb remained high at 92%. In both cases, PCO2 and pH were maintained. As shown in Fig. 2, the reduction of PO2 in and of itself is insufficient to stimulate ATP efflux. There must be an increase in the concentration of deoxyhemoglobin for ATP efflux to be triggered.

When the increase in deoxyhemoglobin is prevented by CO binding to hemoglobin, ATP release from the erythrocyte is inhibited. This experiment supports our hypothesis that ATP release from the erythrocyte must be preceded by a change in the oxygenation status of hemoglobin.

**Deoxyhemoglobin and glycolysis.** It is known that hemoglobin binds to the cytoplasmic domain of band 3 (CDB3) (25). This binding affinity between hemoglobin and CDB3 is much higher in the deoxygenated state than the oxygenated one (39). In addition, it is known that CDB3 is the site for numerous other ligands, including PFK, glyceraldehyde-3-phosphate dehydrogenase and aldolase (26). The binding sites for all these molecules, including deoxyhemoglobin, occur near the N-terminus of CDB3 (30). There is some debate in the literature about the degree of overlap among these binding sites but it has become clear that certainly PFK and deoxyhemoglobin, sharing very similar topology including the key central cavity, do compete at CDB3 (24, 38). This competition implies a link between ATP production (glycolysis) and the oxygenation state of the RBC (via the relative concentration of deoxyhemoglobin present) (13, 33).

The close proximity of the key elements at the inner membrane (deoxygenated hemoglobin, PFK, CDB3, CFTR, bands 3 and 4.5), coupled with the evidence of SO2-dependent release of ATP (Fig. 1 and 2), lead us to postulate the existence of a linkage between ATP efflux and ATP production. We hypothesized that as the local plasma PO2 drops (due to increased tissue metabolic demand), O2 is offloaded from hemoglobin, resulting in an increase in the concentration of deoxyhemoglobin in the cytoplasm. This increase in deoxyhemoglobin results in the displacement of the key regulatory glycolytic enzyme PFK from CDB3, stimulating glycolysis and the subsequent accumulation of ATP at the membrane. We propose that stimulating glycolysis triggers a signal transduction pathway, resulting in the release of ATP from the RBC via one of the previously proposed mechanisms [band 4.5 under the mediation of band 3 and/or CFTR (1, 34)].

**Glycolytic inhibitors impair ATP release.** Figure 3 illustrates the effect of preincubating RBC with different glycolytic inhibitors on ATP efflux in response to low SO2. Both the citrate and fluoride reduced or eliminated the ability of the erythrocytes to release ATP in response to exposure to low PO2, although the magnitude of the effect differed between the two treatments. Citrate did not eliminate the increased ATP efflux due to low SO2 but fluoride did. This difference possibly reflects the different mechanism of action each molecule has on the glycolytic pathway. Citrate inhibits PFK, the enzyme that catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate by enhancing the inhibitory effect of ATP on PFK. Thus ATP production must be stimulated before citrate can exert its effect. Fluoride, on the other hand, inhibits enolase, the enzyme responsible for catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate, by binding with free inorganic phosphate to form flu-
of microvascular O$_2$ transport.

The question may be asked whether the observed increase of plasma ATP on the order of 1–2 μM would be sufficient to alter vascular tone in vivo. In 1995, Ellsworth et al. (10) demonstrated that the intraluminal application of 1 μM ATP to the arterioles of the cheek pouch retractor muscle in the hamster stimulated an increase in arteriolar diameter 8–10%, while the cheek pouch retractor muscle in the hamster stimulated an increase in vessel diameter 8–10%, while McCullough et al. (27) reported that the dose-dependent increase in arteriolar diameter to ATP (0.01–100 μM) in the same model was conducted (100–200 μm) upstream from the site of administration. Collins et al. (4) also showed a conducted vasodilatory response in the hamster cheek pouch retractor muscle in response to 1 μM ATP 450 μm upstream from the site of application with a concomitant doubling of capillary supply rates. The magnitude of the hypoxic response observed in our present studies is consistent with the levels of ATP used in these in vivo experiments.

In conclusion, evidence has been presented that supports the novel role of the RBC in regulating its own flow to the tissue. We have shown that the ATP efflux is SO$_2$ dependent and that the mechanism for ATP release is likely via deoxyhemoglobin stimulating glycolysis within the RBC. We still need to determine the signal transduction pathway from the stage of turning on glycolysis to ATP efflux through a specific channel. However, if the pathways are the same for deformation and SO$_2$-dependent release of ATP then G$_i$ protein seems a likely link in the chain of events. Whether the mechanism proposed here or the one proposed by Stamler et al. (30) is the primary regulator of O$_2$ delivery must still be determined. It is likely that they both function as complementary rather than competing systems.

In either case, this new paradigm should make us view the RBC in a very different way. When the RBC was only thought of as a deformable sac filled with hemoglobin, its role was viewed primarily as a simple carrier of O$_2$. The only impact injury to the RBC (or genetic defects) would have on O$_2$ delivery would be through increased viscosity and loss of deformability, a shift of the hemoglobin SO$_2$ curve, or an increased fragility and loss of circulating RBC, leading to anemia. Recognition of the RBC as a sensor and effector of local O$_2$ delivery raises the possibility that RBC injury may impair local O$_2$ autoregulation. Because there are multiple redundant systems that regulate microvascular blood flow, the loss of the RBC as a sensor may not have immediate life-threatening effects. However, the ability to respond to increased metabolic demand and to properly redistribute flow within an organ on the basis of local O$_2$ demand may be impaired, thus leading to fatigue and possibly local tissue injury.

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