Lactate interferes with ATP release from red blood cells

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Submitted 10 November 2006; accepted in final form 9 February 2007

Rozier MD, Zata VJ, Ellsworth ML. Lactate interferes with ATP release from red blood cells. Am J Physiol Heart Circ Physiol 292: H3038–H3042, 2007. First published February 16, 2007; doi:10.1152/ajpheart.01238.2006.—Upon exposure to low PO2, the red blood cells of most species, including humans, release increased amounts of ATP that ultimately serves as a regulator of vascular tone and thereby regulate blood flow to meet the needs of the tissue for oxygen and other metabolic substrates. Mortensen et al. (13) reported that in humans during maximal exercise, systemic oxygen delivery became limited due to a plateau or decline in oxygen extraction defect occurs in the short term. If such an extraction defect is present and maintained over a long period of time, a similar failure to supply the tissue appropriately could contribute to the significant morbidity and mortality associated with malaria, sepsis, and other pathological conditions.

The red blood cell is classically considered to be the major supplier of oxygen to tissue. Ellsworth et al. (4) proposed that beyond its being a carrier of oxygen, the red blood cell itself is involved in the regulation of oxygen supply. When the red blood cell is transiently exposed to low PO2, low pH, or mechanical deformation, ATP release increases (4, 19). Following release, the ATP binds to purinergic receptors initiating a conducted vasomotor response that increases blood flow to the tissue (3, 12). In hamster striated muscle, a component of this response is the production of nitric oxide, although other mediators have been reported for other tissues and species (3, 12). In a previous study evaluating ATP release from human red blood cells infected with the malaria parasite, it was observed that a Plasmodium falciparum infection rate of 15% to 20% was adequate to reduce the responsiveness of red blood cells to reduced oxygen levels (5). Since 80% to 85% of the cells were not infected, it was unlikely that it was the parasite itself that was causing the effect. Thus some other factor had to be involved. The malaria parasite is known to produce a large amount of lactate (9), and lactic acidosis has been shown to be a contributing factor and sometimes the sole explanation for death in malaria patients (11). We hypothesized that lactate interferes with the production of ATP within the red blood cell and/or its release from red blood cells in response to oxygen demand. Furthermore, if the effect of lactate exposure could be reversed by sodium dichloroacetate (DCA), a drug used clinically to lower lactate levels (10, 21), we anticipated being able to restore the ability of red blood cells to release ATP in response to exposure to low PO2. These studies would additionally provide insights into the connection between ATP production and release and would further support the role of the erythrocyte as a sensor of oxygen need and regulator of perfusion. In addition, it would help explain why increasing systemic oxygen levels is not alone sufficient to improve the clinical outcome of patients with elevated lactate levels, including those with malaria and sepsis.

METHODS

Blood was obtained from New Zealand White rabbits (random sex, 2–3 kg body wt, n = 22) on the day of the experiment. The animals...
were anesthetized with ketamine (12.5 mg/kg im) and xylazine (1 mg/kg im) followed by pentobarbital sodium (10 mg/kg iv). After tracheal intubation, the animals were mechanically ventilated with room air (tidal volume, 10 ml/kg, rate 20–25 breaths/min). A catheter was placed into the carotid artery for administration of heparin (50 units). Ten minutes after the administration of heparin, the animals were exsanguinated via the carotid catheter. The protocol used to obtain the rabbit blood was approved by the Institutional Animal Care and Use Committee of Saint Louis University.

The whole blood was immediately centrifuged at 500 g for 10 min at 4°C. Following centrifugation, the plasma and buffy coat were removed by aspiration and discarded along with the uppermost layer of red blood cells. The remaining packed red blood cells were subsequently resuspended and washed three times in tris(hydroxymethyl)aminomethane (Tris)-buffered Ringer solution (pH 7.4, 300 mosM) containing (in mM): 140.5 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, and 21.0 Tris to which 1 g/l dextrose and 5 mg/l albumin had been added.

The red blood cells were divided into two aliquots. Initially, 1 ml of red blood cells was diluted 1:9 in the standard Tris-buffered Ringer solution as described above with the second in a 5% lactated Tris-buffered Ringer solution (3.1 g/l sodium lactate). The diluted cells were allowed to sit for 1 h before determination of ATP.

Following incubation with lactate or its vehicle, 950 μl of the resuspended red blood cells were pipetted into one of four temperature-controlled tissue baths, each containing 95 ml Tris-buffered Ringer solution (as above without albumin). Two of the baths were equilibrated with 12% O2-5% CO2-balance N2 (normoxia; P O2, ~85 mmHg), and two were equilibrated with 0% O2-5% CO2-balance N2 (low P O2; P O2, ~30 mmHg). In each bath, P CO2 was ~34 mmHg and pH ~7.4 at 37°C. After the addition of the cells, the tissue baths were covered and the gas was turned off immediately. This procedure allowed for sufficient mixing of the red blood cells. One minute after the addition of the cells, a 200-μl sample of the bath contents was removed for analysis of ATP concentration (see ATP assay). Since the assay system only measures extracellular ATP, it was not necessary to separate the cells from the supernatant before ATP measurement. PO2, P CO2, pH, and temperature were determined after the determination of the ATP values using a blood gas analyzer (Radiometer, BMS3-MK2). A cell count was obtained for each of the red blood cell suspensions assayed.

In an effort to ascertain the mechanism by which lactate was exerting its effect, we subsequently repeated the experiment (n = 13) with an additional aliquot similarly resuspended for 1 h in 5% lactated Tris-buffered Ringer solution and then subsequently exposed for 1 h to DCA at a dose (0.34 g/l), calculated to correspond with a dose of DCA used clinically (48 mg/kg iv) (10, 21). Using an enzymatic, colorometric assay (Sigma 735-10), we determined that DCA significantly reduced lactate levels within 5 min. However, for technical reasons, we waited an hour before determining whether that drug restored ATP levels in our red blood cells.

Intracellular ATP. At the conclusion of the protocol, a 50-μl sample of each of the resuspended red blood cells was lysed with 1 ml of distilled water. A 25-μl sample of these lysed cells was diluted to 10 ml with the Tris-buffered Ringer stock. Each solution was then assayed and corrected to an individually determined cell count to provide an estimate of intracellular ATP concentration for each of the three conditions.

ATP assay. The method employed is a modification of the technique developed by Strehler and McElory (22) in 1957, which utilizes the ATP concentration dependence of light efflux induced by the reaction of ATP with firefly tail extract. In this system, the sensitivity of the assay was augmented by the addition of synthetic α-luciferin to the crude firefly tail extract that decreases threshold levels to nanomolar levels. A 200-μl sample of the red blood cell suspension fluid was injected into a cuvette containing 100 μl of crude firefly tail extract (FLE 50, Sigma) and 100 μl of a solution of synthetic α-luciferin (50 mg/100 ml distilled water, Sigma). The signal was measured using a luminometer (TD20/20, Turner Designs) that provides a digital output of both the peak signal and the area under the curve. A standard curve was obtained on the day of the experiment just before the sample assay. A cell count was determined from each sample, and the ATP measured was normalized to a cell count of 200,000 cells based on that cell count.

Data analysis. ATP data are presented as means ± SE. Data were analyzed using an analysis of variance followed by either a Wilcoxon matched pairs test or Tukey-Kramer multiple comparisons test. All data analysis was done using a statistical software package (Instat, GraphPad Software, San Diego, CA). Significance was assigned at P < 0.05.

RESULTS

Release of ATP from red blood cells exposed to lactate. Under normoxic conditions, the amount of ATP released by control cells was not significantly different from that released by cells exposed to lactate (Fig. 1). Control values were 8.8 ± 4.25 and 5.75 ± 2.27 × 10−7 mol for the control and lactate-treated cells, respectively. However, when these cells were exposed to a P O2 of ~28 mmHg, control cells had a significant 32 ± 10% increase in ATP release, which is consistent with previous results (4). However, the lactate-treated cells did not show any increase in ATP release with the average change −8 ± 14%. To establish that inhibition of ATP release was the result of the direct effect of the lactate, we repeated the original experiment with the addition of an extra aliquot of cells that we exposed to lactate and then DCA. We found that DCA restored the ability of the lactate-treated red blood cells to release ATP in response to exposure to low P O2 (Fig. 2). Indeed, levels of ATP released were not different from those of untreated control cells.

Intracellular levels of ATP. Since the ATP released by the red blood cell is produced by glycolysis and since lactate inhibits this process, we ascertained whether the intracellular levels of ATP were affected by incubation with lactate (Fig. 3). We found that the intracellular levels of ATP for lactate-treated
cells were significantly lower than the levels for control cells (2.23 ± 0.21 and 1.64 ± 0.18 mmol for control and lactate-treated cells, respectively). Reducing the level of lactate with DCA did not restore intracellular ATP to control values even after 1 h. Indeed the value was not different from the level seen following incubation with lactate alone (1.54 ± 0.15 mmol).

**DISCUSSION**

This study confirms that rabbit red blood cells release ATP when exposed to an environment of low PO2. This release is inhibited when the red blood cells are incubated in a solution with lactate in which pH is normal (Fig. 1). Importantly, the increased release in response to low PO2 is restored by DCA, a drug used clinically to lower blood lactate levels. Since pH was normal in all cases, lactate itself must interfere directly with the mechanism that links exposure of red blood cells to low PO2 with ATP release from these cells.

Clinically, it has been reported that patients with malaria present with a compromised microcirculation as a result of parasite adhesion to vascular cells, anemia, and inflammation (18). Coincident with these effects is a consistent finding of elevated blood lactate levels. Indeed, the magnitude of the increase is directly correlated with both the severity of infection and clinical outcome (1, 10, 11). Since, in vivo, increased lactate is generally considered to reflect a lack of oxygen supply, an increased supply of oxygen should ameliorate their conditions. However, increasing tissue oxygen availability by increasing oxygen content does not impact survival in humans with malaria (6), suggesting that there is a more complex defect possibly related to an impairment in the ability of the erythrocyte to either sense or respond to low PO2. Even though the computed levels of lactate used in this study (21.17 mmol/l) are at the high end of the reported clinical values for septic and malaria patients, the data showing that reducing lactate levels with DCA restores the erythrocytes responsiveness confirm the association between lactate and impaired ATP release.

Since mature mammalian red blood cells lack mitochondria, ATP production is the result of the activity of a group of glycolytic enzymes associated with the membrane most likely at band 3 (2). Exposure of the red blood cell to a low PO2 environment induces a conformational change in the hemoglobin molecule converting oxyhemoglobin to deoxyhemoglobin. Jagger et al. (8) suggested that this conversion to deoxyhemoglobin dislodges the glycolytic complex from band 3, activating phosphofructokinase, accelerating glycolysis (2) and increasing ATP production. In their study they demonstrated that incubating rat red blood cells with citrate, an inhibitor of the glycolytic regulatory enzyme phosphofructokinase, or fluoride, an inhibitor of the glycolytic enzyme, significantly attenuated ATP release in response to a low PO2. They concluded that the release of ATP is directly linked to its production or that production is governed by the oxygenation state of the hemoglobin molecule. Since lactate is an inhibitor of glycolysis, exposure of red blood cells to high levels of lactate should result in a decrease in intracellular levels of ATP. In this study, we observed that following a 1-h incubation with lactate, intracellular ATP levels were significantly lower than control although they remained at millimolar levels. What is especially increased amounts of ATP in response to low PO2 (5). However, this failure to release could not be attributed to the presence of the parasite itself since there was only a 15–20% infection rate. However, the well-documented consequence of a malaria parasite infection is an elevation of circulating lactate. Here we demonstrate that, in the presence of an elevated lactate, there is impairment in red blood cell ATP release similar to that seen in malaria-infected cells, suggesting that it is lactate that is responsible for the impairment in the ability of the erythrocyte to either sense or respond to low PO2. Even though the computed levels of lactate used in this study (21.17 mmol/l) are at the high end of the reported clinical values for septic and malaria patients, the data showing that reducing lactate levels with DCA restores the erythrocytes responsiveness confirm the association between lactate and impaired ATP release.
interesting is that although incubating the cells with DCA restored the ability of the red blood cells to release ATP, it did not restore intracellular levels to control values. If the glycolytic capacity in the red blood cell is linked to the oxygenation state of the hemoglobin molecule, as suggested by Jagger et al. (8), then incubating our samples under deoxygenated conditions might have restored the levels of intracellular ATP to control levels. However, our observation that DCA restored release in our study would lend support to the idea that there are compartments for ATP (7) associated with different regions of the membrane possibly associated with a unique glycolytic machinery that serves to control ATP within a localized region such as a transport protein. In our case, we suggest that it is associated with a “releasable pool”. 

Sprague and his colleagues (15, 16, 19) have described a signal transduction pathway within the red blood cell that regulates ATP release in response to low PO2 and mechanical deformation. This pathway is initiated by the activation of the membrane-bound G protein, Gi. We have speculated that exposure to low PO2 causes a conformational change in the membrane-bound hemoglobin molecules that cause stress on the membrane activating Gi, although this has yet to be confirmed. Oleareczyk et al. (16) reported that pertussis toxin, an inhibitor of Gi, inhibited ATP release from rabbit red blood cells in response to low PO2, which would support the involvement of Gi in this signaling pathway with the means of its actual activation still to be determined. Our results support the idea that the defect in ATP release observed by Jagger et al. (8) is not related to the actual release mechanism itself but rather to the availability of ATP within the “releasable pool” that can be depleted and replenished independently. Such an independent subset of glycolytic enzymes would coincide with the compartmentation suggested by Hoffman (7).

The mechanism by which DCA lowers circulating lactate levels is generally considered to involve stimulation of pyruvate dehydrogenase (PDH), an enzyme only reported to exist in mitochondria (6, 20) and thus not likely present in the mature mammalian red blood cell. However, within the red blood cell, cytosol is another enzyme, lactate dehydrogenase, an enzyme that converts lactate to pyruvate in a manner similar to the conversion of pyruvate to acetyl CoA by PDH (23). Therefore, it is quite possible that DCA may have some role in accelerating this process (as it does in the well-documented mitochondrial process). As a consequence, cells incubated in the lactate solution are not able to equilibrate the intracellular lactate-to-pyruvate ratio, but those incubated subsequently with DCA can reestablish an appropriate equilibrium. Another possibility is that PDH does exist in the mature red blood cell, possibly as a remnant of the mitochondria expelled during maturation, and DCA acts by increasing its activity directly.

Although our data suggest that lactate inhibits ATP release by directly affecting its production, there are other possible mechanisms. It is conceivable that lactate either affects the ability of the red blood cell to sense conditions of low PO2 or inhibits some component of the signal transduction pathway for its release. The significant decrease in intracellular ATP levels implies that there was a direct effect of the lactate on ATP production which the addition of DCA was unable to completely reverse. We have no evidence to either support or refute either of these possibilities, but they should be considered as potential contributory mechanisms.

The results of these studies may not dramatically change the clinical procedures by which malaria or sepsis patients are treated, but they may elucidate why DCA has shown some promising results for these diseases. Clearly, parasite-induced lactate levels are just one complication of malaria. Although treatment with DCA has been shown to lower blood lactate levels, it has not been effective at reducing mortality rates (10), suggesting that there are other serious components of the disease, including anemia, pulmonary edema, and renal failure. However, if DCA does restore the ability of the red blood cell to serve as a controller of vascular perfusion, then the clinical outcome may improve and the other complications may be ameliorated. The results presented here demonstrate that the red blood cell may actually be the site at which complications associated with hyperlactataemia occur. Further investigating the mechanism by which lactate is able to prevent ATP release will add to the effectiveness of treatment for malaria and other diseases in which hyperlactataemia is a component. Clearly, the results of this study suggest that defects in the ability of the erythrocyte to serve as a vascular controller in addition to its role as an oxygen carrier may play a role in diseases in which oxygen supply relative to demand is impaired.

ACKNOWLEDGMENTS

The blood used for these studies was obtained from the laboratory of a colleague in our department (Dr. Randy Sprague), and we thank him for sharing it with us and for useful discussions related to this study.

GRANTS

This work was supported by a National Heart, Lung, and Blood Institute Grant HL-056249. Support for V. J. Zata was provided by the School of Medicine of St. Louis University as part of a summer program for medical students.

REFERENCES


AJP-Heart Circ Physiol • VOL 292 • JUNE 2007 • www.ajpheart.org


