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Erythrocytic ATP release in the presence of modified cell-free hemoglobin

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ABSTRACT

The red blood cell (RBC) has been proposed as an O_2 sensor through a direct link between the desaturation of intracellular hemoglobin (Hb) and ATP release, leading to vasodilation. We hypothesized that the addition of cell-free Hb to the extracellular space provides a supplementary O_2 source that reduces RBC desaturation and, consequently, ATP release. In this study, the saturation of RBC suspensions was lowered by additions of deoxygenated hemoglobin-based oxygen carrier (HBOC) and then assayed for extracellular ATP. When an acellular human Hb intramolecularly cross-linked between α subunits (ααHb, $p50 = 33$ mmHg) was added to the red cell suspension, ATP production was significantly less than that in the presence of a lower $p50$ HBOC (Hb cross-linked between β subunits, ββHb, $p50 = 8$ mmHg). These results provide a potential mechanism for the O_2 affinity of HBOCs to interfere with a vasodilatory signal.

1. Introduction

Local regulation of microvascular flow is a complex process evolved to match the intravascular oxygen supply to the metabolic demands of the tissue. The release of ATP, a vasodilator, from deoxygenated erythrocytes has been studied as a link between the O_2 content of flowing blood and tissue metabolic demand.[1] The release of ATP by RBCs under brief conditions of hypoxia and hypercapnia was first described by Bergfeld and Forrester,[2] and subsequently under conditions of hypoxia and reduced pH by Ellsworth et al.[3]. Investigators have described ATP release from RBCs of several different animal species,[3–6] ATP release from RBCs under mechanical deformation,[5,7,8] and have characterized the inhibition of ATP release due the presence of nitric oxide [9,10].

It is reported that a reduction in intracellular pO_2 initiates a pathway, including activation of the heterodimeric G proteins, G_s and G_i, and the subsequent production of adenyl cyclase by protein kinase A, which activates the cystic fibrosis transmembrane conductance regulator [5,8,9,11]. Intracellular ATP produced by glycolysis is released into the plasma space, where it diffuses to the vessel wall and activates P_2 purinergic receptors and increases production of NO and other vasodilators [12]. Jagger et al. showed ATP release to be a linear function with RBC Hb saturation, which suggests that the ATP signaling pathway is linked to O_2 content of the blood [4].

The infusion of Hb-based O_2 carriers (HBOCs) generally causes vasoconstriction and hypertension due to their ability to efficiently scavenge endothelial-derived nitric oxide (NO) and attenuate the vasodilatory NO signal to the vascular smooth muscle [13]. In data compiled from several experiments, Olson et al. showed that the hypertensive response to a 10% topload of HBOC correlates to the HBOC's rate of NO binding and not HBOC O_2 affinity [13]. However, experiments performed with HBOCs with similar NO binding rates and a significant blood volume exchange (50%) showed a larger hypertensive response for high $p50$ HBOCs compared to low $p50$ HBOCs [14,15]. These results indicate that given the appropriate conditions, the $p50$ of a HBOC can be a significant effector of blood pressure.

When an HBOC is added to the bloodstream, the additional O_2 supplied by the HBOC helps maintain the intravascular pO_2 and RBC Hb saturation. We hypothesize that the maintenance of RBC O_2 saturation by HBOCs reduces the vasodilatory ATP signal (Fig. 1). According to this concept, the infusion of any HBOC will reduce the release of ATP from RBCs, but the reduction will be larger for an HBOC with low O_2 affinity that unloads O_2 at higher pO_2 than RBCs. *In vivo*, the interactions of HBOCs with RBC ATP release likely include other interactions unrelated to O_2. NO produced by the endothelium can inhibit ATP release, yet this inhibition may be blocked by the efficient scavenging of NO by HBOCs. Furthermore, hemodynamic changes due to HBOC infusion have the potential to alter RBC deformation, another effector of ATP release.

This study describes an experiment in which a concentrated suspension of RBCs is desaturated in the presence of two intramolecularly cross-linked tetrameric Hbs, differing only in O_2 affinity ($p50 = 8$ mmHg versus $p50 = 33$ mmHg). This experiment was performed to test our hypothesis that RBC ATP production is positively correlated with HBOC O_2 affinity. Because it is impractical to remove large amounts of O_2 from an oxygenated concentrated RBC/HBOC suspension without significant pH changes, we performed a virtual O_2 transport experiment, where oxygenated RBCs were mixed with desaturated HBOC to give a suspension with O_2 bound to half of the heme sites. We found a statistically significant difference between the ATP released from RBCs in the presence of differing O_2 affinity HBOCs,

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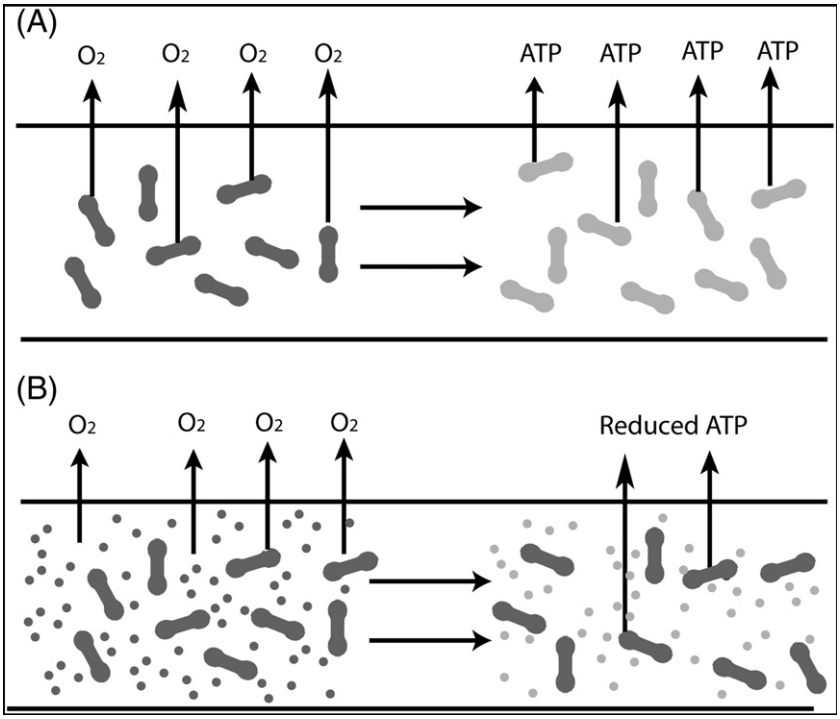

Fig. 1. Schematic of ATP-mediated vascular control. (A) RBCs offload O_2 and release ATP as they desaturate, causing vasodilation. (B) RBC/low-affinity HBOC suspensions releases O_2 from both RBCs and HBOC, allowing RBCs to maintain a higher level of fractional saturation and reducing ATP release.

thus confirming the hypothesis. To our knowledge, this is the first experimental investigation of the interactions of between cell-free Hb and RBC ATP release.

2. Methods

2.1. RBC and Hb preparation

Fresh human erythrocytes were collected from a volunteer on the day of the experiment. Whole blood was centrifuged, and the plasma and buffy coat were removed. The packed red cells were washed and centrifuged three times (5 min at 4 × *g*) with a buffer composed of 77.5 mM NaCl, 46.0 mM Na_2HPO_4, 8.5 mM NaH_2PO_4, 10 μM $CaCl_2$ with 1 g/l dextrose and 5 mg/L human serum albumin, and then resuspended to a final concentration of 8.75 mM of heme. The oxygen equilibrium curves for RBCs were not measured; instead values for the pO_2 at half saturation ($p50$) and Hill number (n_H) from the literature were used for calculations ($p50 = 29$ mmHg, $n_H = 2.6$) [16]. This study used modified Hbs that were prepared in house for preclinical studies. αα-Hb is tetrameric human Hb internally cross-linked between α subunits at the Lsy99 residues [17]. αα-Hb has O_2 affinity similar to RBCs ($p50 = 33$ mmHg, $n_H = 2.4$) (Table 1). ββ-Hb is tetrameric human Hb internally cross-linked between β subunits at Lys82 residues, [18] with O_2 affinity much higher than αα-Hb or RBCs ($p50 = 8$ mmHg, $n_H = 1.7$). Unmodified human Hb was not used because the difference in ATP release between it and ββ-Hb was not likely to be significant. The procedures used to produce αα-Hb and ββ-Hb were the same as those given in the literature, [17,18] and both Hb solutions were formulated at concentrations of 2.5 mM (heme) in Ringer's lactate. The oxygen equilibrium curves were analyzed using a Hemox analyzer (TCS Scientific Corp.; New Hope, PA) (Fig. 2). For experiments, the Hb solutions were diluted to 675 μM in the phosphate buffer mentioned above at a final pH = 7.4. The final experimental conditions of 625 μM Hb and 625 μM RBC (heme) were chosen because they were near the concentration of Hb available to us, and because the use of high Hb concentrations minimizes the importance of small O_2 leaks and other systematic experimental errors.

Table 1
Properties of Hbs at 37 °C.

Hemoglobin	$p50$ (mmHg)	n_H	[Hb] (μM)	Description
RBC	29	2.6	8750	Human red cells
αα-Hb	33	2.4	675	Human Hb cross-linked between α subunits
ββ-Hb	8	1.7	675	Human Hb cross-linked between β subunits

2.2. Experimental protocol

Cell-free Hb at a concentration of 675 μM was deoxygenated for 20 min in a 37 °C tonometer (Tonometer 237, Instrumentation Laboratories; Lexington, MA) flushed with humidified N_2 gas. The tonometer consists of a rotating glass cup that creates a thin fluid layer to facilitate gas transport. The pO_2 of the Hb was measured to be

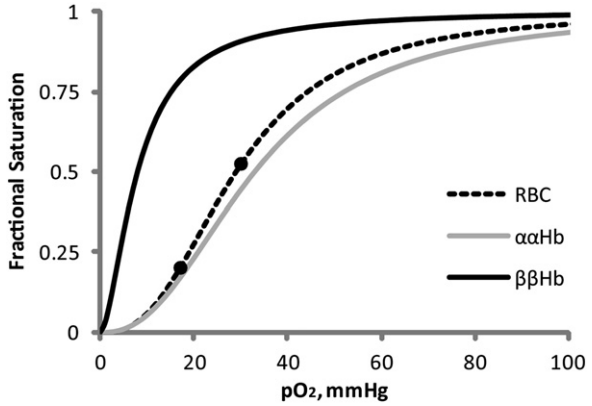

Fig. 2. Oxygen equilibrium curves of RBCs and Hbs. Filled circles represent the predicted RBC saturations in the experiment when RBCs are mixed with αα-Hb ($pO_2 = 30$ mmHg) and ββ-Hb ($pO_2 = 17$ mmHg).

<2 mmHg using an O_2 sensor probe (Foxy, Ocean Optics; Dunedin, FL) to ensure significant desaturation. A gas-tight syringe (Hamilton; Reno, NV) was evacuated with N_2 and filled with 650 µL Hb and 50 µL RBCs, with a heme concentration of 8.75 mM. A 20-µL bubble of N_2 gas was injected into the syringe and used for mixing the Hb and RBCs by gently inverting the syringe five times. The syringe was then placed in a 37 °C water bath for 2 min for thermal equilibration. The Hb/RBC suspension was tested immediately for pO_2 and pH in a blood gas analyzer (Rapidlab 248, Siemens; Deerfield, IL). The suspension was then centrifuged, and the supernatant was diluted by a factor of 10 and assayed for extracellular ATP concentration using a standard luciferin–luciferase bioluminescence assay kit (Sigma-Aldrich; St. Louis, MO) in a single tube luminometer (Optocomp-I, MGM Instruments; Hamden, CT). Standard curves were calculated on the same day as the experiments on solutions with the same [Hb] as the experiment to account for Hb light absorbance. Each experiment was repeated three times ($n=3$), and each sample was assayed for ATP in triplicate.

Both RBC lysis and RBC deformation during the post-experiment centrifugation would also increase the extracellular ATP concentration in our experiments. "Baseline" experiments were therefore performed, where the protocol was repeated with an Hb sample that was not deoxygenated, thus providing an estimate of the combined effects of lysis, RBC deformation, and non-O_2 related ATP release. Because of this, the absolute numbers cannot be compared directly to results by previous investigators.

2.3. Theoretical O_2 release

The exchange of O_2 between oxygenated RBCs and deoxygenated cell-free Hb can be predicted based on the equilibrium O_2 binding behavior of each. We used the Hill equation to describe the fractional saturation of Hb or RBCs (Eq. (1)).

$$Y = \frac{(pO_2/p50)^{n_H}}{1 + (pO_2/p50)^{n_H}} \quad (1)$$

The total O_2 content of the RBC/Hb suspension was then calculated as the sum of the dissolved, RBC-bound, and extracellular Hb-bound components (Eq. (2)), where α is the solubility of O_2 in plasma (1.33 µM/mm Hg [19]) and [RBC] and [Hb] are heme concentrations due to RBCs and extracellular Hb, averaged over the solution volume.

$$[O_2]_{total} = \alpha pO_2 + [RBC]Y_{RBC} + [Hb]Y_{Hb} \quad (2)$$

Fig. 3 shows total O_2 plotted versus pO_2, based on Eq. (1) and (2). In this experiment, the total O_2 concentration was 640 µM, which is equal to the sum of the dissolved and bound O_2 in 50 µL oxyRBCs diluted to a total volume of 700 µL. These calculations predict equilibrium $pO_2=30$ mmHg for RBC/ααHb and $pO_2=17$ mmHg for RBC/ββHb. According to the Eq. (1), RBCs will be ~20% saturated in the presence of ββHb and ~50% saturated in the presence of ααHb (shown as filled circles in Fig. 2). The literature on RBC ATP release at reduced pO_2 suggests that the lower RBC saturation for RBC/ββHb will correlate with a larger amount of ATP to be released by the RBCs.

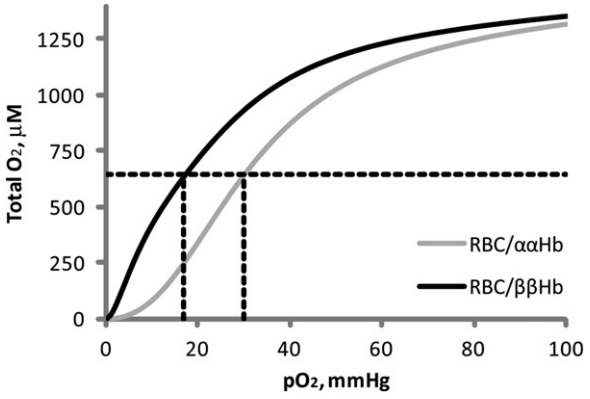

Fig. 3. Total O_2 content for suspensions with 625 µM RBCs and 625 µM extracellular Hb as a function of equilibrium pO_2. The horizontal line gives the total O_2 content in the experiment (~640 µM). Vertical lines show the estimated final pO_2 for RBC/ααHb and RBC/ββHb ($pO_2=30$ mmHg and $pO_2=17$ mmHg).

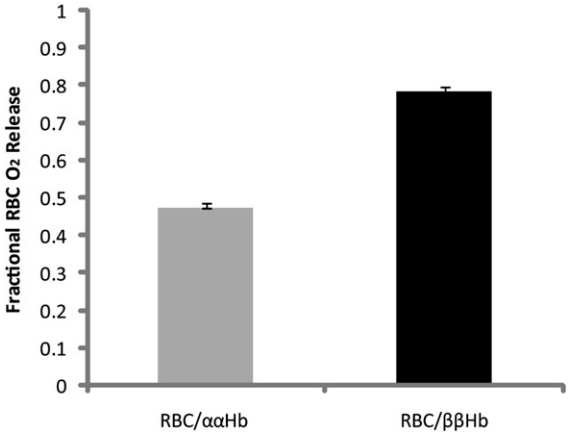

Fig. 4. Change in RBC fractional saturation calculated from the measured experimental pO_2s and the O_2 equilibrium curves for RBCs. Error bars are calculated as the standard deviation of pO_2 input into Hill equation.

3. Results

3.1. Blood gas measurements

The pO_2 values measured in the blood gas analyzer were similar to the predicted values, $pO_2=30.3\pm0.7$ mmHg for RBC/ααHb, and $pO_2=17.8\pm0.4$ mmHg for RBC/ββHb. The pH of the suspensions was 7.35 ± 0.2. Based on the final pO_2 and the O_2 equilibrium curves (Fig. 2), the fractional O_2 release from RBCs was calculated as $47\pm2\%$ for RBC/ααHb and $78\pm1\%$ for RBC/ββHb (Fig. 4).

3.2. ATP Release

Fig. 5 shows the assayed RBC ATP release. These results are reported as an increase above RBC baseline ATP release, which in our protocol also contains some contribution due to RBC lysis and RBC deformation. Given a RBC volume as 7×10^{-14} L from the literature, [20] our "baseline" ATP production is $5\pm0.7\times10^5$ molecules ATP/RBC. The increase in the assayed ATP concentration above baseline is 65 ±

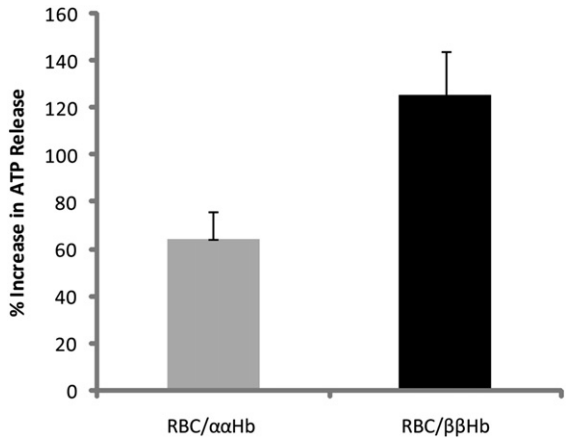

Fig. 5. Increases in RBC ATP release above baseline. Results are statistically significant ($p<0.02$, based on two-tailed, unequal variance t-test).

11% for ααHb and 126 ± 19% for ββHb, a difference that is statistically significant ($p<0.02$).

4. Discussion

The experimental results confirm the hypothesis that ATP release by RBCs in the presence of extracellular Hb is dependent on the O_2 affinity of the Hb, providing a possible mechanism for high *p*50 HBOCs to modulate the vasodilatory ATP signal. Although the theory of erythrocytic-derived ATP as a mediator of vascular tone was initially described by Bergfeld and Forrester in 1992 and subject to extensive research since then, we believe that this report is the first to consider the phenomenon of RBC ATP release in the context of HBOCs with varying *p*50. Reducing the *p*50 of HBOC to below that of RBCs appears to be a prudent choice to maximize the ATP signal to the vascular walls. Increasing the O_2 affinity to a point that it can no longer deliver sufficient O_2 to tissue is an obvious concern, although an extremely low *p*50 HBOC ($p50 = 5$ mmHg) has been shown to be able to release O_2 in the capillaries if the blood is sufficiently hemodiluted and the tissue is hypoxic [21,22].

Clearly, the simple *in vitro* experiment described here is insufficient to determine if there is an effect of HBOC-ATP signaling interference on microvascular flow regulation. The primacy of NO scavenging in producing HBOC-induced vasoconstriction is well established, but it is possible that conditions exist where secondary factors, such as O_2 affinity and plasma viscosity, also play important roles. The functionality of an O_2 sensing mechanism associated with the RBCs will be altered when O_2 is supplied from a non-erythrocytic source. Our data demonstrate a possible mechanism for high *p*50 HBOCs to reduce the vasodilatory ATP signal more than low *p*50 HBOCs, although it should be noted that the high affinity of HBOCs for NO could interfere with this affect by removing NO inhibition of RBC ATP release.

Here, we have described a mechanism that relates the *p*50 of a HBOC to the production of a vasodilatory signal. Further studies by qualified investigators are merited to ascertain the physiological importance of this effect, particularly of the relative importance of ATP and NO on microvascular hemodynamics when HBOCs are present in the bloodstream.

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