THESIS

A NOVEL APPROACH TO REAL-TIME MONITORING OF ERYTHROCYTE ATP RELEASE AS A FUNCTION OF HYPOXIA

Submitted by

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ABSTRACT

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Background: Matching blood flow to tissue oxygen demand is essential for maintaining metabolic homeostasis and sustaining human life. Recent studies suggest that red blood cells (RBCs) play a role in local vasodilatory signaling by releasing ATP in response to hypoxia. RBC ATP release and overall function are impaired with age and disease. Until now, luciferin/luciferase bioluminescence is the only method described to quantify ATP release from RBCs. Here, we describe a novel approach where ATP release is measured as a function of hypoxia continuously and in real time using an Oroboros Oxygraph O_2K respirometer. *Purpose:* This report describes the development and application of this new approach. *Methods:* We obtained blood samples from 10 healthy, young adults (18-35y) via venipuncture. Washed RBCs were diluted to 5% hematocrit and added to the glass chamber of a calibrated Oxyfluorimeter along with 5μM Mg-G. Nitrogen gas was constantly injected into the chamber at 1 ml/min to decrease PO_2. An LED-based fluorescence detection device monitored Mg-G fluorescence, which was used to calculate extracellular [ATP]. *Results:* When 5% HCT RBCs were exposed to 30 minutes of hypoxia, Mg-G fluorescence (V) continuously increased. During 30 minutes of progressive hypoxia, PO_2 in the chamber decreased from 121.9 ± 1.3 to 9.8 ± 0.8 mmHg, and Δ extracellular [ATP] from normoxia (μM) increases from 0 to 6,985.0 ± 793.6 μM. Extracellular [ATP] accumulates markedly when PO_2 in the chamber reaches 50.60 ± 1.52 mmHg. *Conclusion:* Using this novel method, we identified a PO_2 threshold at which extracellular ATP accumulates rapidly, which is consistent with the range of PO_2 that elicits Hb

desaturation in RBCs. This approach may allow for detailed mechanistic studies into the relationship between hypoxia, Hb desaturation, and RBC ATP release.

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TABLE OF CONTENTS

ABSTRACT……ii
ACKNOWLEDGEMENTS……iv
CHAPTER 1 – LITERATURE REVIEW……..1
Red blood cells: Biochemical properties and clinical relevance
Section 1: Techniques for measuring and imaging ATP……2
Section 2: Methods of eliciting ATP release from red blood cells……..…5
Conclusion……9
REFERENCES……..……11
CHAPTER 2 – INTRODUCTION……16
CHAPTER 3 – METHODS……..19
Research environment
Human subjects
RBC collection
Hypoxia studies
Instrumentation……20
Data analysis and statistics
CHAPTER 4 – RESULTS……22
Using fluo-oximetry to detect change in Mg-G fluorescence elicited by hypoxia
Quantifying [ATP] from raw Mg-G fluorescence……23
Determining the PO_2 that elicits marked increase in extracellular [ATP]……24
CHAPTER 5 – DISCUSSION……26
REFERENCES……31

CHAPTER 1 – LITERATURE REVIEW

Red blood cells: Biochemical properties and clinical relevance

Matching tissue oxygen supply to demand is essential for sustaining metabolic activity. The erythrocyte, or red blood cell (RBC), primarily functions as a vehicle for oxygen in mammalian blood. Due to several unique traits, RBCs are ideally suited for gas exchange. The RBC's capacity to carry oxygen is due to a protein that comprises 97% of its volume, known as hemoglobin (Hb). Hb binds easily and reversibly with oxygen, which diffuses from the RBC to areas of low partial pressure of oxygen (PO_2). RBCs maintain a unique biconcave-disc shape. Because of their structure, they have 30% more surface area than spherical cells of the same size. [1,2] This shape is maintained by a complex network of cytoskeletal proteins, which also allow the membrane to deform and temporarily change shape. The diameter of a red cell (~8 μm) is larger than that of the average capillary (~3-5 μm), therefore membrane deformation is essential for circulation.

Recent studies indicate that RBCs play a critical role in local vasodilatory signaling by releasing adenosine triphosphate (ATP), a potent vasodilator, in response to hypoxia and membrane deformation.[3–6] Upon release, ATP binds to purinergic receptors on the vascular endothelium to evoke vasodilation, thereby increasing tissue blood flow and oxygen delivery.[7,8] The signaling mechanisms for RBC ATP release have yet to be fully elucidated. One predominant theory is that RBCs release ATP in proportion to the number of unoccupied oxygen binding cites on the hemoglobin molecule.[9–11] This implies that the RBC is not only a vehicle for oxygen, but a sensor of hypoxia and an effector in the response to increase delivery by virtue of ATP release.[3–5,10,12] Low PO_2 in the microvasculature stimulates the offloading of O_2 from Hb, which elicits a conformational change in the Hb molecule from its relaxed (rHb) to tense (tHb)

state. This change in shape of Hb is thought to cause membrane deformation of the RBC. Evidence suggests that this hypoxia-induced membrane deformation elicits the release of ATP.[13,14] The exact mechanisms by which this occurs are still being investigated. In vivo studies suggest that RBCs release ATP in response to deformation without a hypoxic stimulus. For example, when perfused through differently sized pores, rabbit RBCs release more ATP travelling through the smaller openings.[12] In other words, RBCs release ATP in proportion to the deforming stimulus. Mechano-sensing mechanisms are often studied in the context of deformation alone. Interestingly, when RBCs from young healthy adults are treated with Diamide, a membrane-stiffening drug, ATP release in response to hypoxia is blunted.[15] This suggests a critical link between hypoxia and deformation-induced ATP release.

As a hypoxia-sensing cell, the RBC plays a critical role in locally regulating blood flow.[9,16] Importantly, RBC ATP release is impaired with advancing age in healthy humans, as well as in patients with Type II diabetes and pulmonary hypertension, and is hypothesized to contribute to impaired tissue perfusion and ischemic vascular disease.[17] Due to its clinical relevance, RBC ATP release has been studied for decades. The following review will explore well-established and new methods used to study the properties of RBCs and related ATP release.

Section 1: Techniques for measuring and imaging ATP

Luciferin-luciferase assay for RBC ATP release

To date, luciferin/luciferase bioluminescence has been the only method described to quantify ATP release from RBCs.[15,18–23] Bioluminescence, or light produced during biochemical reactions, was first studied by Dubois in 1885 when he discovered that fireflies cease to produce light when immersed in both hot and cold water. When he added sample of the hot water extract to the cold-water extract, luminescence returned.[24] Dubois hypothesized that there was a heat-stable substance in the hot water extract that could be stimulated to luminesce in the presence of an enzyme in the cold-water extract. He called the heat stable substance

"lucifèrine" and the enzyme "luciferase." In 1947, William McElroy found that adding ATP to non-light-emitting firefly extracts could restore presence of light in proportion to the amount of ATP added. In this sense, ATP is firefly luciferin. The first bioluminescent assay for measuring ATP content in solution was developed by Strehler and McElroy in 1957.[24] When RBC ATP release is measured using this assay, either plasma or isolated RBCs are diluted in physiological buffer in an Eppendorf tube. The tube is placed in a luminometer device, which is completely sealed to outside light. Luciferin and $MgCl_2$ are introduced to the sample via automatic injection, and a photomultiplier tube measures light output from the sample via automatic photon-counting. The sample emits light in proportion to ATP, or luciferase, in the sample. Light output is reported in relative light units (RLU).[25] These can be extrapolated to ATP values based on standard curve calibrations, where known concentrations of ATP are added to the same physiological buffer used during studies. Light output is correlated with the known [ATP] to generate a standard curve. In the following experiments, RLUs are automatically converted to [ATP]. In most research settings, RBC ATP release is measured via luciferin-luciferase bioluminescence. This assay has been adapted to work for a range of methodologies which will be discussed in section 2.

Measuring ATP in other contexts

Nearly all biological functions are governed by ATP's synthesis and consumption. The study of temporal and spatial ATP dynamics is vital to understanding cellular metabolism and human physiology. While this review focuses on ATP primarily in the context of RBC signaling, ATP detection techniques are employed in several areas of study. We will briefly discuss these techniques, as they may be applicable to studying RBC physiology in the future.[26]

Electrophysiology is used to measure the movement of ATP across ion gradients. Membrane P2X receptors are ATP-gated cation channels with ATP-dependent gradients.[26–28] In PC12 and HEK-293 cells, which express P2X on their membranes, patch-clamp electrophysiology has been used to detect ATP in proximity to the membrane based on the electrophysiological current. Hazama and colleagues have measured ATP release from pancreatic β cells by measuring P2X current during a whole-cell patch clamp.[29] Similarly, ATP-

mediated potassium (K_{ATP}) channels have been used to study submembrane ATP concentrations in COSm6 monkey kidney cells and *Xenopus* oocytes.[30]

Magnetic resonance spectroscopy (MRS) is a common imaging technique that can directly and non-invasively quantify ATP in vivo through phosphorous magnetization. Due to the impracticality of MRS, most researchers opt to image ATP using some combination of optical microscopy and molecular probing. A molecular probe is an atom(s) or molecule(s) used to study other molecules. Most commonly in this context, fluorescent molecules are used to detect molecules of ATP, light-detecting microscopy captures fluorescence. Magnesium green (Mg-G) is a typical fluorophore used to indirectly measure ATP hydrolysis.[31] Mg-G has higher affinity for ATP than ADP. Intracellularly, ATP is usually bound to Mg. When Mg-ATP hydrolysis occurs, Mg-G binds to the newly freed Mg ions, which increases the Mg-G fluorescent signal.[26] In isolated mitochondria, Mg-G fluorescence is used to reliably measure ATP-ADP exchange rate.[32]

ATP hydrolysis can be detected using synthetic ATP analogs, which utilize Förster-type resonance energy transfer (FRET). FRET occurs between a donor fluorophore covalently attached to a y-phosphate group and an acceptor fluorophore which is attached to the base.[33] When ATP hydrolysis occurs, the phosphodiester bond is broken and the donor and acceptor drift away from each other, which decreases FRET and results in an increase in donor fluorescence.[26] It is important to consider the molecular weight of fluorescent ATP analogues as they can be heavier than regular ATP, causing them to behave slightly differently. Assessment of ATP with this method should be accompanied by rigorous validation.[26]

Exogenous luciferin, previously discussed for measurement of RBC ATP release, is used measure ATP in other contexts. Luciferin is obtained from firefly extract and emits light in an ATP-dependent fashion.[34,35] Numerous cell types have been transfected with luciferin on their outer extracellular membrane to detect ATP leaving the cell.[27] Pellagatti and colleagues used this method to detect ATP in the extracellular tumor environment of HEK-293 cells.[36] Luciferin-luciferase assays are not recommended for temporal imaging of ATP because the luminescence signal degrades quickly. Furuya and colleagues however have improved upon

this limitation by using a cooled electron multiplying charge coupled device camera and image intensification to make up for low light output.[37]

Section 2: Methods of eliciting ATP release from RBCs

Red blood cells release ATP in response to deformation and hypoxia. In vivo, these stimuli are linked and often simultaneous. In order to oxygenate tissue, RBCs squish to travel through capillaries with smaller diameters. Additionally, the PO_2 of systemic capillaries is low enough to elicit offloading of O_2 from RBCs. Several approaches are used to stimulate and measure ATP release. Such techniques are being innovated to better mimic physiological conditions. This section will include well-established and emerging methods for eliciting ATP release from RBCs.

Hypoxia

The most common way to expose RBCs to hypoxia in vitro is to suspend washed RBCs in buffer in a rotating bulb tonometer in which the composition of air can be tightly controlled. The glass bulb tonometer is connected to gas tanks via tubing, and the composition of gas flowing into the bulb can be regulated and manipulated by the researchers. Typically, RBCs are diluted to 20% hematocrit and equilibrated with normoxic gas (16% O_2, 6% CO_2, balance N_2) for 15 minutes. A small sample from this control condition is extracted, and extracellular ATP is measured using the luciferin-luciferase assay, which will be described later. For hypoxic conditions, the gas mixture is manipulated to contain less O_2, which is balanced with N_2. After a period of gas equilibration, another sample is extracted for measurement of extracellular ATP. It is important to note that these measurements are made serially, after the cells have been exposed to hypoxia and reached equilibration.

Deformation

Most laboratory research has focused on deformation-induced ATP release. Deformability depends on cytoskeletal proteins spectrin, actin, and ankyrin among others.[38,39] Membrane

protein band 3 is also thought to play a role in mechano-signaling.[40] Dysfunction in deformability results in shortened lifespan for the RBC, and has been observed in malaria, sickle cell anemia, diabetes, heart attack, and paroxysmal nocturnal hemoglobinuria (PNH). Because of its clinical relevance, RBC deformability is studied by researchers across a range of interests.

RBC deformability is determined by 1) surface area-to-volume ratio of the cell. Due to the biconcave disc shape of these cells, this ratio is high with mean cell volume of ~90 μm^3 and mean surface area of ~135 μm^2. The typical cell containing ~90 μm^3 has a surface area of 97 μm^2.[41] 2)Cytoplasmic viscosity, which depends on the abundance of Hb. 3) visco-elastic properties of the RBC membrane. The complex membrane network consists of three layers, an outer carbohydrate-rich glycocalyx surface, a middle lipid bilayer, and the inner protein network. This protein network is responsible for deformability of the cell and recovery of the biconcave disc shape after deforming stimuli. The integrity of this skeletal network is a marker of cell function[42] and is often measured as surface roughness. The skeletal network or surface roughness changes during diseased states or under the influence of pharmacology.[43,44]

Increase in RBC rigidity can be correlated with any of the three aforementioned factors.[1,45] Membrane behavior can be assessed and expressed based on the following characteristics: 1) shear elastic modulus; 2) area compressibility modulus; and 3) bending modulus. Shear elasticity is associated with a constant area of elongation or shear along the membrane. Area compressibility is a measure of resistance to a compression or expansion in area. This indicates surface dilation independent of shear or bending. The bending modulus represents the curvature of the RBC membrane and drives shape change independent of shear or expansion.[46,47] For an example of how these moduli can be used, it's possible to measure the time a deformed RBC takes to recover its shape. This is represented by the ratio of the elastic

shear modulus to membrane surface viscosity.[1,47,48]

The most challenging aspect of these studies is simulating physiological conditions of circulation. RBCs are subject to intense physiological change during circulation, where the diameter of vasculature and O2 tension are both constantly changing. Biochemical properties of RBCs in isolation or whole blood can be studied using a rheoscope, a combination of a rheometer and microscope. A rheometer is a device used to measure the way a liquid or suspension responds to force.[49] With this technique, RBCs or whole blood samples are placed in a rotating rheometer plate and observed using microscopy to estimate their relaxation time constant.[50] Steady state RBC elongation can be measured as the cells are subjected to different levels of shear force. When the shear force is stopped, relaxation time constant, or time to

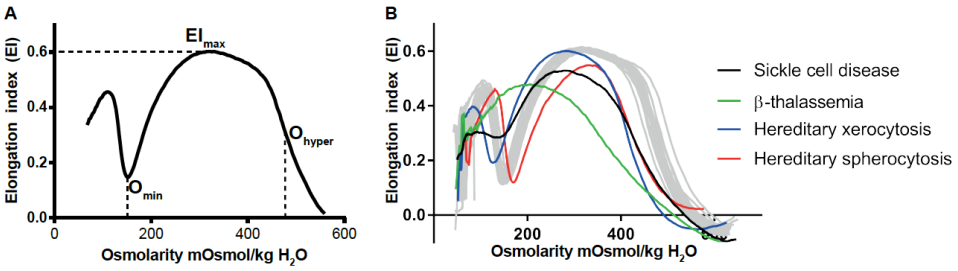

FIGURE 1 | Osmotic gradient ektacytometry as a tool to measure red blood cell (RBC) deformability. The technique is discussed in detail by Clark et al. (1983); Lazarova et al. (2017), and Da Costa et al. (2016). Briefly, during osmotic gradient ektacytometry, the RBC is subjected to an osmotic gradient (range ≈50 mOsmol/kg H_2O – 650 mOsmol/kg H_2O) under constant shear stress, while the elongation index (EI) is measured. The EI corresponds to the deformability at various osmotic conditions. **(A)** the left graph depicts an osmotic gradient ektacytometry curve from a healthy control with various intersection points: the EI_{max} reflects the maximal deformability of the RBC, O_{min} reflects the osmotic fragility and O_{hyper} reflects the (de)hydration state (or intracellular viscosity) of the RBC. **(B)** In the right graph osmotic gradient curves of patients with sickle cell disease (black line), β-thalassemia (green line), hereditary xerocytosis (blue line) and hereditary spherocytosis (red line) and several individual healthy controls (gray lines, $n = 20$) are depicted.

Figure 1. Adapted from Huisjes *et al* 2018

resumption of discoid shape, can also be measured.[1]

Another approach, termed ektacytometry, RBC deformability is measured using a laser diffraction viscometer.[51,52] This technique generates a deformability index (DI; also referred to as elongation index) curve. The curve provides information about surface area, surface to volume ratio, and internal viscosity. Using this curve, it is possible to minimum and maximum DI. The DI max, or peak of the curve, indicates equilibrium between the surface to volume ratio and the intracellular viscosity. When extracellular osmolality decreases, DI decreases. Minimum DI represents the greatest volume an RBC can reach before hemolysis.[1,53] It's possible to

determine RBC pathology by how the DI vs osmolality curve shape differs from normal (Figure 1).

Deformability can also be assessed for a single cell, rather than a group or sample. Single cell experiments allow direct measurement of viscoelastic properties and response to membrane perturbation. In larger samples, these things can only be estimated. Examples of single cell techniques include micropipette aspiration,[54] optical tweezers,[55,56] flickering analysis,[57,58] atomic force microscopy (AFM),[59] microfluidics,[1,60] and ultrasound.[61] The disadvantage of single cell methods such as micropippetting or tweezing is that membrane disruption is applied in a non-physiological manner. Recently, microcapillary flow and microfluidic device techniques were developed to increase physiological relevance. These techniques mirror *in vivo* conditions more closely.[60] Microfluidic devices combine video microscopy with transparent machinery which mimics circulatory structures such as capillaries.[62] The structure of these devices varies based on the experimental question, and several examples are shown in Figure 2 below.

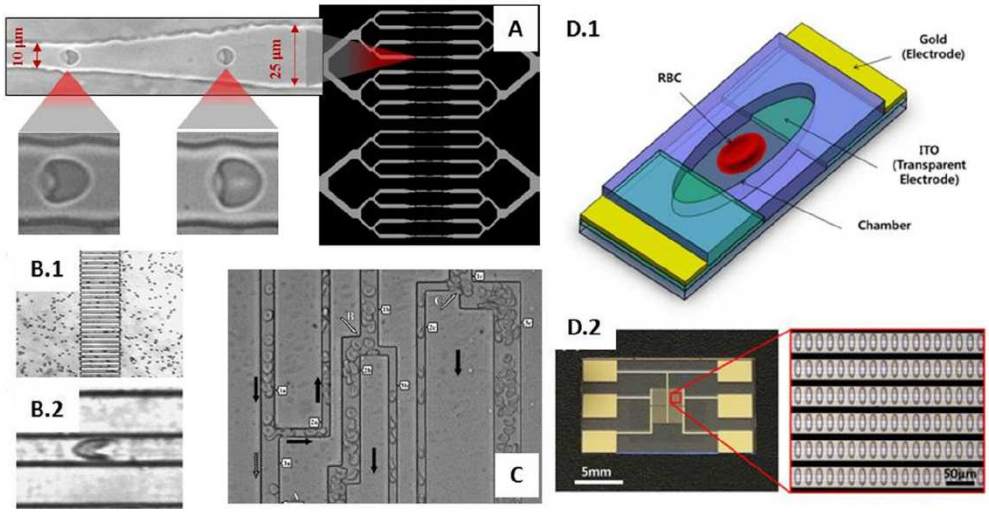

FIG. 3. Microfluidic experimental studies of RBC flow using microcirculation-mimicking models. (A) RBC membrane elastic modulus and surface viscosity are measured by using a converging-diverging geometry. Adapted from Ref. 65 and Reproduced by permission of The Royal Society of Chemistry. (B1) Low- and (B2) high-magnification images of deformed erythrocytes in glass microchannels. Adapted from Ref. 83. (C) Flowing of RBCs in channels of different widths.[89] (D1) Schematic diagram of a chamber unit for RBC deformability measurement (SiCMA chip), and (D2) Fabricated SiCMA chip with an enlarged view of the chamber array.[86]

Figure 2. Adapted from Tomaiuolo *et al* 2014

For a 2002 study by Robert Sprung, Randy Sprague, and Dana Spence, microbore tubing was engineered to mimic human resistance vessels. In this study, a luciferin-luciferase solution is perfused through the tubing, followed by isolated RBCs. Constant perfusion pressure is maintained by an infusion pump. A section of the tubing travels through a photomultiplier tube (PMT), which measures luminescence (Fig 3).[63]

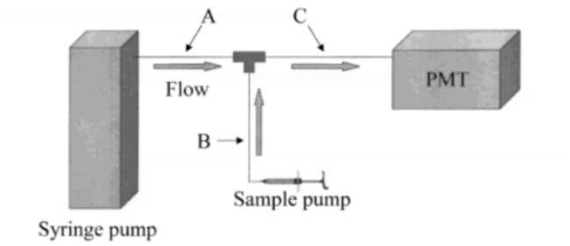

Figure 1. Schematic representation of instrumentation used to study stress induced ATP release from RBCs. A luciferin/luciferase mixture is pumped through a section of tubing (A) while RBC samples are pumped from the sample pump through a section of tubing (B), where RBCs are deformed. The sample mixes with the luciferin/luciferase solution from the syringe pump at a mixing tee and is propelled through a third section of tubing (C) to the PMT for detection.

Figure 3. Adapted from Sprung *et al* 2002

As described above, light output can be extrapolated to [ATP]. Interestingly this study suggests that RBC ATP release is negatively correlated with size of the vessel; smaller tubing elicits greater ATP release. Additionally, there was a positive correlation between RBC ATP release and length of the microbore tubing. This indicates that longer time under stress elicits greater ATP release. These data demonstrate the possibility that RBCs release ATP continuously during membrane deformation.

Conclusion

The role of red blood cells in vasodilatory signaling has yet to be fully elucidated, mainly due to the infeasibility of performing *in vivo* study. Although luciferin-luciferase bioluminescence has been adapted to work in various settings, exploration of more dynamic methodologies could benefit this field of research. Additionally, most studies focus on deformation-induced ATP release. In the microvasculature, deformation and shear stimuli are always accompanied by hypoxia. As demonstrated by Sridharan and colleagues, cell membrane deformability and response to hypoxia are critically linked. Studying the interaction between hypoxia- and deformation-induced signaling cascades may reveal relevant therapeutic targets for RBC and

vascular dysfunction. Collaborative innovation by scientists in many disciplines from physiology, to chemistry, to physics and engineering will advance our understanding of red blood cell function and its impairment with age and disease.

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CHAPTER 2 – INTRODUCTION

Matching tissue oxygen supply to demand is essential for maintaining metabolic homeostasis and sustaining human life. Erythrocytes (red blood cells, RBCs) are responsible for delivering oxygen throughout the body. Recent studies establish that RBCs play a critical role in vasodilatory signaling by releasing adenosine triphosphate (ATP), a potent vasodilator, in response to hypoxia.[3–6] Upon release, ATP binds to purinergic receptors on the vascular endothelium to evoke vasodilation, thereby increasing tissue blood flow and oxygen delivery.[7,8] One predominant theory is that RBCs release ATP in proportion to the number of unoccupied oxygen binding cites on the hemoglobin molecule.[5,9–11] This implies that the RBC is not only a vehicle for oxygen, but a sensor of hypoxia and an effector in the response to increase delivery by virtue of ATP release.[3–5,10,64] As a hypoxia-sensing cell, the RBC plays a critical role in locally regulating blood flow.[11,18,65] Importantly, RBC ATP release is impaired with advancing age in healthy humans, as well as in patients with Type II diabetes and pulmonary hypertension, and is hypothesized to contribute to impaired tissue perfusion and ischemic vascular disease.[19,66,67,20]

Determining the mechanisms regulating ATP release from RBCs is essential to understanding age- and disease-related impairments in blood flow regulation. Until now, luciferin/luciferase bioluminescence has been the only method described to quantify ATP release from RBCs.[18,19,20–23,68] For this technique, RBC samples are exposed to static hypoxia levels in vitro, and extracellular ATP is measured serially in separate aliquots from each sample. Each measurement is conducted before and after the cells are exposed to hypoxia, and after ATP release has occurred, providing only a physiological snapshot of ATP release as a function of hypoxia. While this technique has provided valuable insights into RBC function,[12,14,16-20] it is limited in that ATP release is measured only at static time points before and after the cells are stimulated.

The signaling mechanisms for RBC release of ATP have yet to be fully elucidated. When PO_2 in the microvasculature falls below 60 mmHg, oxygen begins to rapidly dissociate from hemoglobin (Hb), which elicits a conformational change in the Hb molecule from a relaxed (rHb) to tense (tHb) state. This change in shape of Hb is thought to cause membrane deformation of the RBC, which may lead to ATP release.[69] The exact mechanisms by which this occurs are still being studied. RBCs are also forced to deform as they traverse capillary networks, with the average capillary diameter being smaller than the diameter of an erythrocyte.[1] *In vitro* studies suggest that RBCs release ATP in response to deformation without a hypoxic stimulus. For example, when perfused through differently sized pores, RBCs release more ATP traveling through the smaller openings. In other words, RBCs release ATP in proportion to the deforming stimulus.[12] Mechano-sensing mechanisms are often studied in the context of deformation alone. Interestingly, when RBCs from young healthy adults are treated with Diamide, a membrane-stiffening drug, ATP release in response to hypoxia is blunted. This suggests a critical link between hypoxia and deformation-induced ATP release. Furthermore, several studies suggest that RBC membrane protein band 3 binds reversibly with deoxy-Hb.[69–72] Transgenic mice lacking this binding site display reduced RBC ATP release.[69] In vivo data suggest that mechanical compressions of human forearm muscle significantly increases plasma [ATP] and ATP effluent from resting conditions.[73] This indicates that mechanical deformation stimulates RBC ATP release independent of increased metabolic demand. In the same study, Crecelius et al. measured plasma [ATP] and ATP effluent when mechanical compressions were superimposed on rhythmic forearm exercise. ATP effluent from the working muscle was significantly higher with combined compressions and exercise than with either performed alone. This supports in vitro findings that hypoxia and deformation can stimulate ATP release independently and in combination.[73–75]

Here, we describe a novel approach where ATP release is measured as a function of hypoxia continuously and in real time using an Oroboros Oxygraph O_2K respirometer/ fluorometer. Traditionally used for high-resolution mitochondrial respirometry, we utilize this instrument as a “fluo-oximeter” to monitor PO2 and extracellular ATP during progressive hypoxia. Using fluorometry, we monitor RBC ATP release with magnesium green (Mg-G), an established fluorescent ATP sensor.[76] This method may provide the opportunity to study the kinetics of ATP release in response to hypoxia in real-time in the context of age, disease, and therapeutic intervention. This report describes the development and application of this novel approach. We hypothesized that we could induce progressive hypoxia in RBCs via injection of N_2 gas into a closed respirometry chamber containing suspended RBCs and Mg-G, eliciting ATP release from RBCs that is detectable with Mg-G fluorescence. We further hypothesized that the rate of ATP release will increase linearly as PO_2 in the chamber decreases.

CHAPTER 3 – METHODS

Research environment

All experiments were performed in the Human Performance Clinical Research Laboratory at Colorado State University under the supervision of Drs. Dinenno and Lark with IRB approval for protocol #20-10068H.

Human subjects

Young healthy subjects, aged 18-35, of both genders and all races and ethnic backgrounds were recruited from Fort Collins and the surrounding communities. Based on our previous studies on this topic[12,18], we studied isolated RBCs collected from 10 subjects.

RBC collection

Blood was obtained by venipuncture of the antecubital and collected into Vacutainer tubes containing sodium heparin (158 USP units) after a 4 h fast and 12 h abstention from caffeine, alcohol and exercise. RBCs were isolated by centrifugation of the collected whole blood (500 g, 4°C, 10 min) followed by removal of the plasma and buffy coat by aspiration. Packed RBCs were resuspended and washed three times in a cell wash buffer (CWB) containing (in mM) 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21.0 Tris-base, 5.5 glucose and 0.5% BSA, with pH adjusted to 7.4 at room temperature.

Hypoxia studies

All studies were performed immediately after blood collection and RBC isolation. Washed RBCs were diluted to 5% hematocrit with a warmed (37°C) bicarbonate-based buffer containing (in mM) 4.7 KCl, 0.3 $CaCl_2$, 2.2 $MgSO_4$, 140.5 NaCl, 11.1 glucose, 23.8 NaHCO3 and 0.5% BSA. 1.7 ml of buffer or buffer + RBCs were added to the glass

chamber of a calibrated Oxygluorimeter (Oroboros Instruments, Innsbruck Austria) with a stir bar rotating at 300 RPM. When RBCs were completely saturated with oxygen, magnesium green (Mg-G) (5 μm) was added and the chamber then sealed with a volumetric stopper to restrict gas exchange with ambient air. A light emitting diode coupled with an optical fiber was inserted into the O_2K viewing window to detect Mg-G fluorescence.

Instrumentation

All experiments were performed in an Oroboros O_2k fluo-respirometer. 60 ml syringes were filled with 100% N_2 gas and then placed in a constant-infusion syringe pump (Harvard Apparatus) to deliver N_2 gas via silastic tubing into the O_2K chambers through a blunted 51mm spinal needle. This allows for the nitrogen to be injected into the air pocket between the 5% HCT RBC mixture and tops of the chamber stoppers. This air pocket is known as the gas phase. After injected N_2 fills the gas phase, N_2 slowly replaces O_2 in the RBC mixture, decreasing PO_2 in the chamber. PO_2 is sensed at the bottom of the chamber (in the liquid phase) by a polarigraphic O_2 sensor. Fluorescence excited by an LED was measured through the glass window of the O_2K chamber. Our experiments utilized oximetry to monitor PO_2 and fluorometry to measure extracellular [ATP] using Mg-G.

Data analysis and statistics

PO_2 and fluorescence data were collected simultaneously every 2 seconds during each experiment and stored on a computer for analysis. Absolute ATP values were *quantified* from fluorescence data using a standard curve which was established prior to each experiment. The standard curve was performed by adding known concentrations of ATP to the chamber, which contained Mg-G and 5% HCT RBC mixture for background fluorescence comparable to the experimental setup. [ATP] of each addition was correlated with the resulting raw fluorescence output (V) (Figure 3A). Standard curves with an R^2

value below 0.95 were repeated. Analyses were performed using Prism8 statistical software (GraphPad). To calculate the degree of hypoxia required to elicit an increase in ATP release, [ATP] during normoxia was calculated as the average [ATP] for one minute before hypoxia is initiated. Δ [ATP] was calculated by averaging [ATP] in 1-minute bins starting at the initiation of hypoxia. The first point where 1-minute average [ATP] was more than two standard deviations higher from normoxia was defined as the PO_2 required to elicit RBC ATP release.

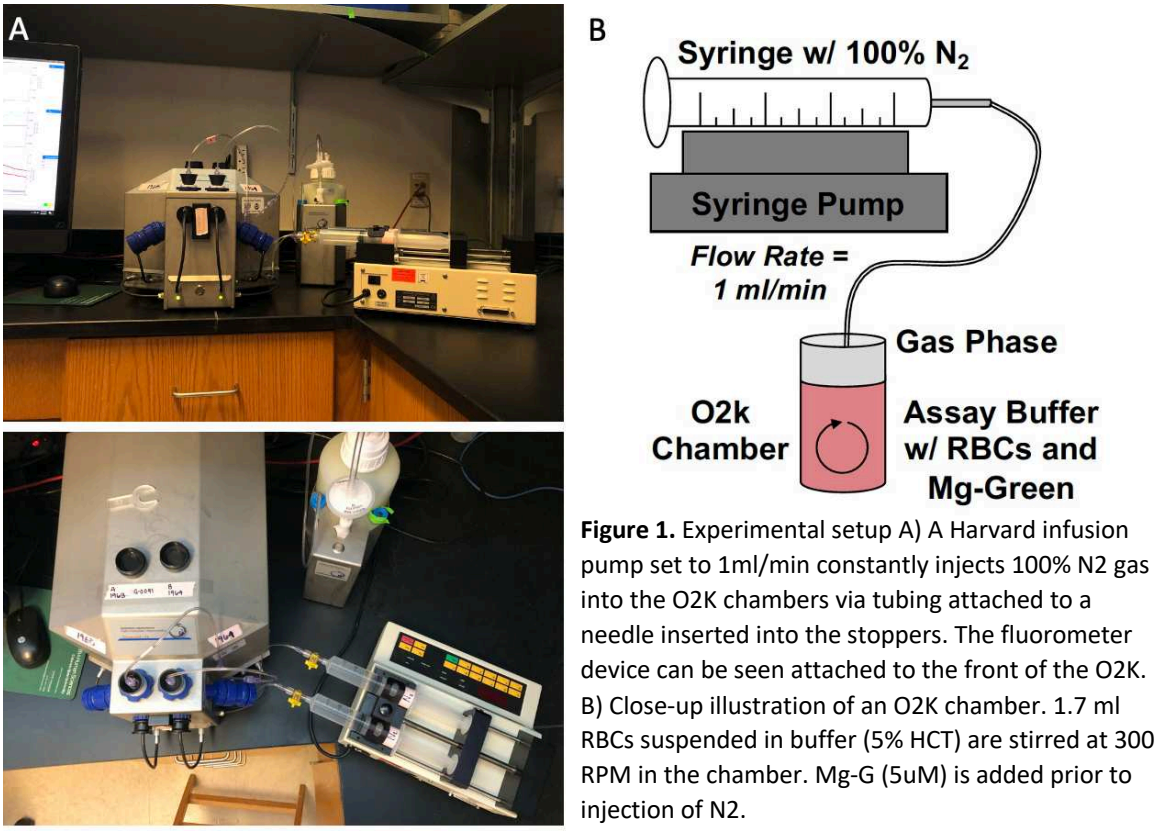

Figure 1. Experimental setup A) A Harvard infusion pump set to 1ml/min constantly injects 100% N2 gas into the O2K chambers via tubing attached to a needle inserted into the stoppers. The fluorometer device can be seen attached to the front of the O2K. B) Close-up illustration of an O2K chamber. 1.7 ml RBCs suspended in buffer (5% HCT) are stirred at 300 RPM in the chamber. Mg-G (5uM) is added prior to injection of N2.

Using fluo-oximetry to detect change in Mg-G fluorescence elicited by hypoxia

To establish the device's ability to detect change in fluorescence in response to hypoxia, we conducted a series of control experiments. First, we injected N_2 into the chamber for 15 minutes, with only buffer and Mg-G present (Figure 2A). PO_2 decreased gradually to 0mmHg and Δ fluorescence (-mV/s) remained zero throughout the trial. We next determined whether RBCs alter Mg-G fluorescence during normoxia. (Figure 2B). For 15 minutes, PO_2 remained steady at 122 mmHg, while Δ fluorescence (-mV/s) fluctuated around zero. This is consistent with a lack of ATP release from RBCs when fully oxygenated. These experiments were conducted to rule out change in fluorescence resulting from nitrogen injection alone, or influence of RBC metabolism on fluorescence

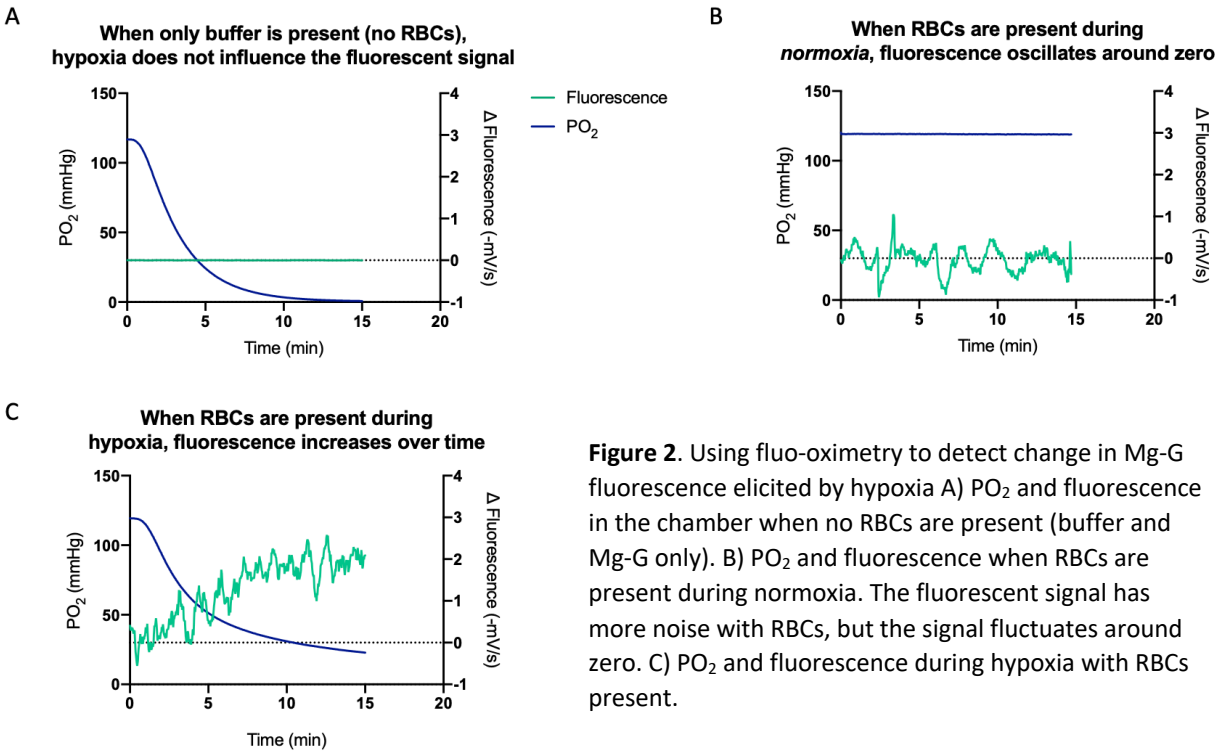

Figure 2. Using fluo-oximetry to detect change in Mg-G fluorescence elicited by hypoxia A) PO_2 and fluorescence in the chamber when no RBCs are present (buffer and Mg-G only). B) PO_2 and fluorescence when RBCs are present during normoxia. The fluorescent signal has more noise with RBCs, but the signal fluctuates around zero. C) PO_2 and fluorescence during hypoxia with RBCs present.

during normoxia. Finally, we injected N_2 into the chamber for 15 minutes with RBCs present (Figure 2C). PO_2 gradually decreases while raw fluorescence (V) increases over time. This is consistent with the idea that RBCs release ATP in the presence of hypoxia.

Quantifying [ATP] from raw Mg-G fluorescence

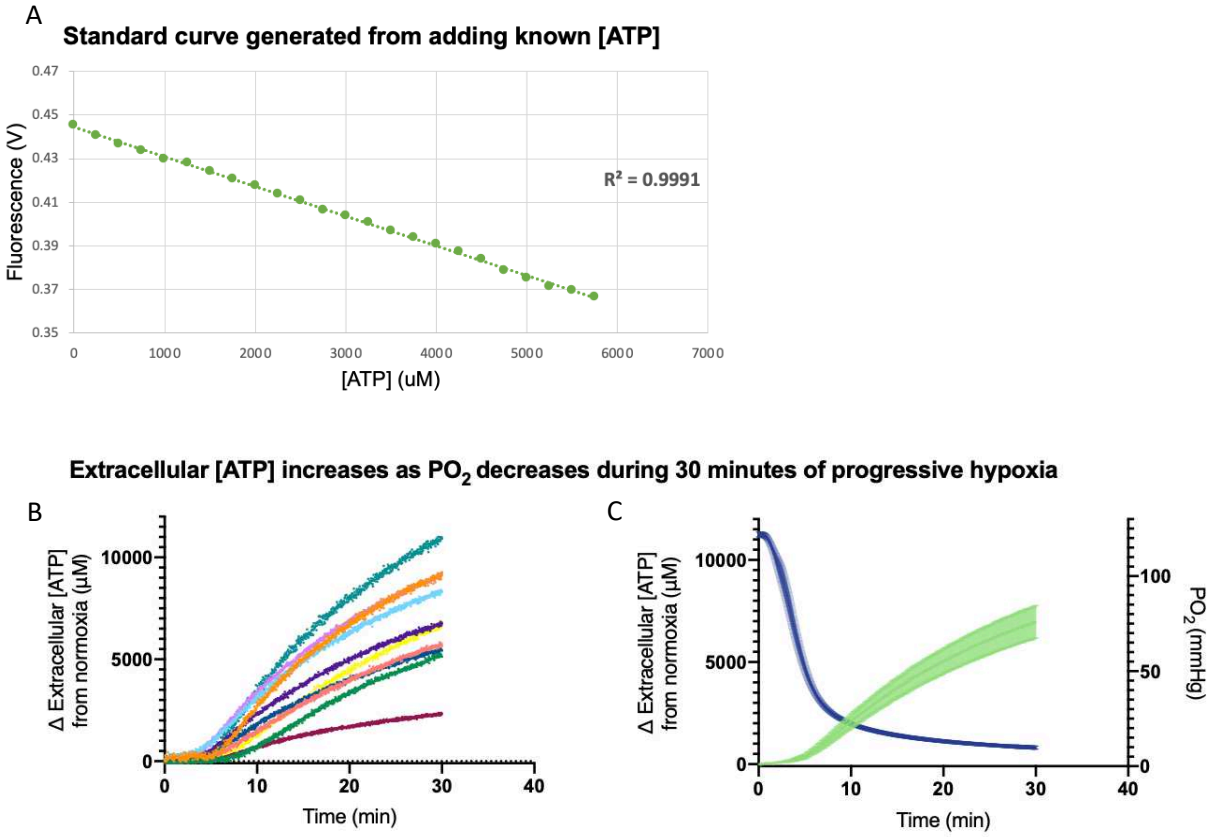

Figure 3. Quantifying [ATP] from raw Mg-G fluorescence A) Example standard curve for generating [ATP] (μM) from the Mg-G signal (V). B) Change in extracellular [ATP] from normoxia (μM) during 30 minutes of progressive hypoxia for each participant. C) Mean change in extracellular [ATP] from normoxia (μM) and PO_2 (mmHg) during 30 minutes of progressive hypoxia.

Absolute ATP values were *quantified* from fluorescence data using a standard curve which was established prior to each experiment. The standard curve was performed by adding known concentrations of ATP to the chamber, which contained Mg-G and 5%

HCT RBC mixture for background fluorescence comparable to the experimental setup. [ATP] of each addition was correlated with the resulting raw fluorescence output (V). The O_2K automatically extrapolated fluorescence to [ATP] after calibration. Figure 3A is an example of one pre-experimental standard curve. Standard curves with an R^2 value below 0.95 were repeated.

After establishing that [ATP] can be quantified, we performed hypoxia experiments on RBCs collected from 10 subjects. ATP release from RBCs is presented as Δ extracellular [ATP] from normoxia (μM). Figure 3B depicts the mean change in extracellular [ATP] (μM) during progressive hypoxia for each of the 10 subjects. Figure 3C represents mean ± SEM Δ extracellular [ATP] from normoxia (μM).

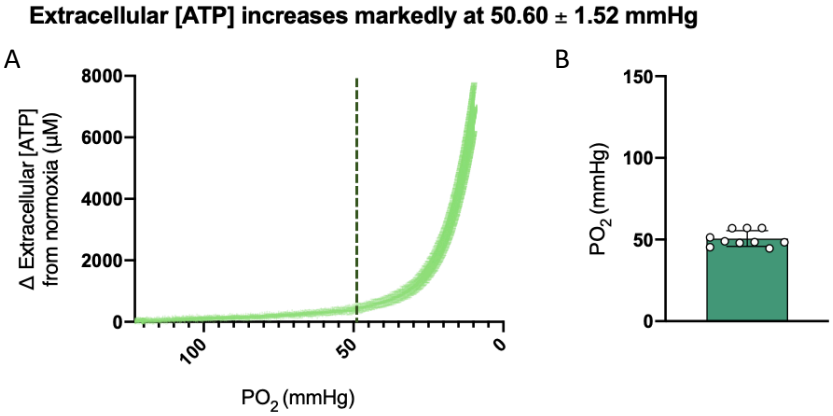

Figure 4. A) Change in extracellular [ATP] from normoxia (uM) plotted against PO_2 (mmHg). The dark green dotted line indicates the PO_2 value at which [ATP] begins to accumulate extracellularly. B) Extracellular ATP begins to significantly accumulate at 50.60 +/- 1.52 mmHg.

Determining the PO_2 that elicits marked increase in extracellular [ATP]

Figure 4A depicts change in extracellular [ATP] from normoxia (μM) plotted against PO_2 of the chamber (mmHg). [ATP] during normoxia was calculated as the average [ATP] for one minute before hypoxia is initiated. Δ [ATP] was calculated by averaging [ATP] in 1-minute bins starting at the initiation of hypoxia. The first point where 1-minute average

[ATP] is more than two standard deviations higher from normoxia is 50.60 ± 1.52 mmHg (indicated by the dark green dotted line). Each participant's PO_2 value is plotted in Figure 4B. This critical PO2 indicates the point when extracellular ATP accumulates rapidly, and is consistent with the range of PO_2 that elicits Hb desaturation in RBCs.

CHAPTER 5 – DISCUSSION

The goal of this project was to begin developing and assessing the feasibility of a new method. Many steps were taken to repurpose a mitochondrial respirometer for the measurement of ATP release from red blood cells (Figures 1A and B). First, we determined whether ATP release could be detected with Mg-G fluorescence. Mg-G is commonly used in the study of mitochondrial respiration to measure ATP hydrolysis.[32] In this sense, measurement of Mg-G fluorescence in the Oroboros Oxygraph O_2K is well-established. In the context of this study, RBCs are suspended in physiological buffer, and Mg-G binds to magnesium in that buffer when it's added to the chamber during normoxia. As ATP is released and accumulates in the buffer, Mg-G preferentially binds ATP, decreasing the fluorescence signal. We present the fluorescence signal as -mV/s in figure 2 so the figures are more intuitive. An increase in extracellular [ATP] elicits a decrease in fluorescence, so we flipped the signal to more clearly depict ATP release patterns.

To pilot the use of Mg-G, we first added it to the chamber with only buffer, and injected nitrogen to progressively decrease PO_2 (Figure 2A). PO_2 decreases to zero and during this time, the fluorescent signal does not change. This indicates that hypoxia alone does not affect Mg-G's affinity for Mg in buffer. Next, we tested the "opposite" scenario, where RBCs were suspended in buffer and PO_2 is constant at room air (122 mmHg) (Figure 2B). In this scenario, the presence of RBCs creates noise in the fluorescence signal but change in fluorescence oscillates around zero during the 15 minute time period. This indicates that Mg-G fluorescence does not change with RBCs during normoxia the way we expect it to during hypoxia. Next, we performed hypoxia trials with RBCs present in the chamber. In this trial, we observe the change in fluorescence increasing over time (Figure 2C). Collectively, the observations in Figure 2 indicate that change in fluorescence may be attributed to accumulating extracellular ATP released from RBCs.

We then generated standard curves to convert Mg-G fluorescence signal to [ATP] (Figure 3A). The standard curve is generated by adding known concentrations of ATP to the chamber which contains buffer and RBCs (normoxia). Decrease in the raw fluorescent signal is strongly correlated with ATP additions, and [ATP] can be determined. After generating the data in figures 2A-C and 3A, we determined that Mg-G was worth pursuing as a fluorescent indicator of ATP release.

To our knowledge, Mg-G has not previously been used to measure ATP released from RBCs. Luciferin-luciferase bioluminescence is the only method used to quantify ATP in this context. Bioluminescence provides static measurements of extracellular ATP, before and after hypoxia or other stimuli such as deformation. Using our proposed method, you can see that extracellular [ATP] increased over time during 30 minutes of hypoxia for all participants (Figure 3B). Extracellular [ATP] at the end of 30 minutes varies by participant, but the ATP release profile of each participant follows a similar pattern. Group data for 30 minutes of hypoxia is presented in Figure 3C. As PO_2 decreases from 121.9 ± 1.3 to 9.8 ± 0.8 mmHg, Δ extracellular [ATP] from normoxia (μM) increases from 0 to 6,985.0 ± 793.6 μM. Notably, [ATP] increases at a gradual slope, and then more rapidly during the latter half of the experiment. To our knowledge, the kinetic profile of ATP release from RBCs has not been depicted previously.

Oxy-hemoglobin dissociation is a dynamic process. Hb's affinity for oxygen decreases as PO_2 decreases. At PO_2 levels below 60 mmHg, oxygen begins to dissociate rapidly. Hemoglobin is 50% saturated around 26.6 mmHg; this point in time is known as the P_{50}. Our data suggest that extracellular [ATP] accumulates markedly when PO_2 in the chamber reaches 50.60 ± 1.52 mmHg (Figures 4A and B). This PO_2 is associated with marked oxy-hemoglobin dissociation. When oxygen binds or dissociates, hemoglobin's structure changes. The kinetics of ATP release during this process have not been studied, but evidence suggests that this hypoxia-induced change causes the RBC membrane to deform, eliciting ATP release.[70–72]

Specifically, membrane protein band 3 reacts with Hb in an oxygen-dependent fashion. Deoxy-Hb binds reversibly with band 3 at an 8-fold higher rate than oxy-Hb. Walder and colleagues describe the binding site for band 3 being deep within the deoxy-Hb tetramer, and this binding site is inaccessible to band 3 when Hb is oxygenated.[77] The Hb-O_2 saturation curve experiences a rightward shift when RBCs are prepared with cytoplasmic domain of band 3. Chu and colleague describe that band 3-Hb association occurs dynamically over a physiological range of PO2s,[71] and they continued to study this phenomenon by breeding transgenic mice to lack a deoxy-Hb binding site on band 3. These mice displayed lower ATP release in response to hypoxia than did mice with the normal human deoxy-Hb binding characteristics intact.[69] ATP release was not completely abolished, however, and there may be redundant mechanisms at play. For this reason, we believe analysis of temporal ATP release patterns will help dissect the signaling mechanisms for ATP release and associated change in cell behavior over a range of physiological PO_2.

Hemolysis is always a concern when working with RBCs. *In vivo*, RBCs lyse for a variety of reasons. Lysis can occur when RBCs are being handled outside of the body, especially if the temperature, pH, or tonicity of their medium is altered significantly from physiological conditions. Our proposed method for studying RBCs is compatible with previously described analysis of free Hb to determine hemolysis.[18,69,78] Before and after experimentation, the 5% hematocrit solution is centrifuged for 10 minutes at 500g and 4 degrees C. The supernatant is analyzed for free Hb by measuring absorbance at 405nm. We did not measure hemolysis for all subject samples, but this will certainly need to be done for future experiments. For the samples we did analyze for hemolysis, % free Hb was less than or near 0.1% after 30 minutes of normoxia or hypoxia. Traditionally, > 0.1% free Hb is considered detectable lysis and these samples are excluded from analysis. For future studies, we will take precautions to minimize lysis, such as

shortening the time period in which RBCs equilibrate in the chambers before experimentation. RBC lysis will be measured pre and post hypoxia for all participants in future studies.

The next step is to validate this device against the standard luciferin-luciferase bioluminescence assay. Although our proposed method provides more information, RBCs from the same participant should be run simultaneously through both protocols. Static bioluminescence does not provide a temporal ATP release profile, but we can compare change in extracellular [ATP] from normoxia (%) after a certain level of hypoxia is attained. This would provide a comparison to the gold standard.

This approach may provide novel opportunity for studying age- and disease-related RBC dysfunction and pharmacological manipulation of RBC signaling pathways. In the microvasculature, deformation and shear stimuli are accompanied by hypoxia. As demonstrated by Sridharan and colleagues, cell membrane deformability and response to hypoxia are critically linked. RBC membranes stiffen with age, which decreases their deformability.[1,2,20] In one participant treated with diamide, we found that the critical PO_2 at the onset of marked ATP accumulation was lower than in controls. This would indicate that a greater hypoxic stimulus is necessary to overcome membrane stiffening and ATP release is delayed, consistent with the aforementioned studies. We plan to run more diamide trials in the future to determine whether this change in critical PO_2 is significant, and whether stiffened cells release less ATP in a given time period during progressive hypoxia.

ATP is considered a potent vasodilator, increasing local oxygen delivery to match tissue demand. Our data suggest that RBCs begin releasing ATP around 50 mmHg, when Hb is roughly 80% saturated depending on physiological conditions. This result is not surprising considering previous studies of RBC biology, but it does provide a marker of cell function that can be used to study age- and disease-related changes and potential therapeutic targets for

RBC dysfunction. We believe this approach will allow for detailed mechanistic studies into the relationship between hypoxia, Hb desaturation, and RBC ATP release.

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