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INVESTIGATING THE MECHANISM OF HYPOXIA-INDUCED RELEASE OF ATP FROM ERYTHROCYTES AND ITS ROLE IN NITRIC OXIDE PRODUCTION

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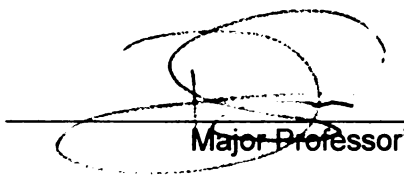

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Date

INVESTIGATING THE MECHANISM OF HYPOXIA-INDUCED RELEASE OF ATP FROM ERYTHROCYTES AND ITS ROLE IN NITRIC OXIDE PRODUCTION

By

Andrea Nicole Faris

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ABSTRACT

INVESTIGATING THE MECHANISM OF HYPOXIA-INDUCED RELEASE OF ATP FROM ERYTHROCYTES AND ITS ROLE IN NITRIC OXIDE PRODUCTION

By

Andrea Faris

Since the discovery of nitric oxide (NO) as the endothelium derived relaxing factor (EDRF), its role in the circulation has been extensively studied; most importantly, NO can elicit relaxation in smooth muscle cells underlying the endothelial cells in blood vessels such as arteries and arterioles. One stimulant for NO production is red blood cell (RBC)-derived adenosine triphosphate (ATP). Through a series of mechanisms, ATP stimulates NO production in endothelial cells, where it diffuses to smooth muscle cells resulting in dilation. There have been many reported stimuli for the release of ATP from RBCs, such as, deformation, c-peptide and hypoxia. In hypoxic conditions, RBCs release an increased level of ATP; however, the cause of hypoxia-induced ATP release is still unknown.

Hemoglobin changes conformations from the R (relaxed) state to the T (tense) state when exposed to a hypoxic environment. Moreover, hemoglobin is attached to the RBC membrane through a band 3 protein. Through the differing conformations hemoglobin undergoes, it is hypothesized a deformation is induced on the RBC membrane, therefore, resulting in ATP release. A relationship between deformation and hypoxia was studied using a flow-through setup. The data from these results suggested a non-additive relationship. This led to the idea that hypoxia and deformation-induced ATP release may be following the same signaling pathway.

A widely accepted signaling pathway for deformation-induced ATP release has been previously reported. It starts with the activation of a G-protein and ends with the ATP traversing the membrane through a CFTR-mediated mechanism. Through the inhibition of each protein in this pathway and subsequent exposure to hypoxia, a decrease in ATP release was measured. Furthermore, upon stiffening of the RBCs with a known cell stiffener, ATP release decreased in a dose dependent manner. These data suggest, along with those previously mentioned, hypoxia-induced ATP release is a form of deformation.

Two theories for hypoxic vasodilation are the conversion of nitrite to NO through deoxyhemoglobin or the transnitrosylation of hemoglobin for delivery to respiring tissue. While these theories are extensive in their research, they lack many details. For example, the NO released from RBCs must traverse the blood stream, endothelium and sub-endothelium to elicit relaxation in the smooth muscle cells. This mechanism is physiologically unlikely. From studies performed, RBCs donated NO when placed in a hypoxic buffer; however, the NO donated is not NOS-mediated and not ATP-dependant. Through the use of a microfluidic device and fluorescence microscopy, measurable NO was detected from endothelial cells, and the concentration of measurable NO was dependant on RBC-derived ATP.

Through the use of flow-through analysis techniques a non-additive relationship was discovered for hypoxia and deformation. Furthermore, through the inhibition of the deformation-induced signaling pathway and exposure to hypoxia, a pathway for hypoxia-induced ATP release was elucidated. Finally, with the use of microfluidic devices, a mechanism for hypoxic vasodilation was demonstrated.

~ I dedicate this thesis to my dad and mom, Nabeel and Ilene…my two truest blessings in life…I love you more than you will ever know.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Dana Spence. At a time when I did not have a group, you took me into your lab. From day one you pushed me to do my best and without your encouragement I could not have made it. In all the years I have been in grad school and all the years to come in my career, I am positive I will never come across a better advisor. Thank you for everything. I would also like to thank my committee members for their time, suggestions and questions.

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CHAPTER 1

1.1 AN INTRODUCTION TO BLOOD AND BLOOD FLOW

The circulatory system is an organ system that passes nutrients, gases, hormones and blood cells, to and from the body in order to maintain homeostasis. The main components of the circulatory system are the heart, blood, and blood vessels. A blood vessel consists of the adventitia, the media, the elastica interna, and the endothelium. The last two layers comprise a layer called the intima. Most layers consist of connective tissue; however, the media layer contains smooth muscle cells. A schematic of a blood vessel is displayed in Figure 1. The circulation is also sometimes classified as the pulmonary circulation, where blood is oxygenated, and the systemic circulation, where oxygenated blood is provided to organs. An average adult body contains about five liters of blood, which is comprised of plasma, white blood cells, platelets and red blood cells (RBCs).

Plasma is the yellow liquid component of whole blood in which the RBCs are suspended and makes up about 55% of whole blood. Its contents are mostly water, dissolved proteins, gases, glucose and hormones.

White blood cells in the circulatory system aid against infection. There are two main types of white blood cells distinguishable by their granules: granulocytes and agranulocytes and are often a marker for infection. A liter of adult blood contains between 4×10^9 and 1.1×10^{10} white blood cells. Platelets are small, anucleated cells measuring about 2-3 μm in diameter that have an average lifespan of about 8 to 12 days. The main function of platelets in the blood is

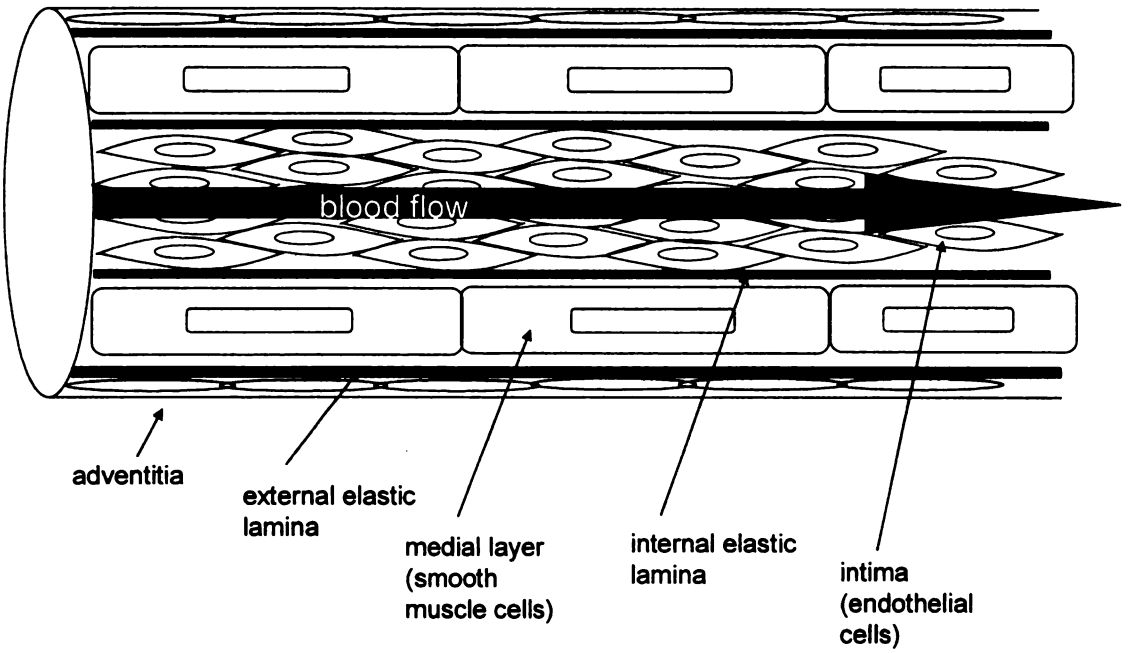

Figure 1. Schematic of a human blood vessel. The adventitia is comprised of mostly connective tissue. The external elastic lamina is comprised of elastic connective tissue. The medial layer is comprised of smooth muscle cells, the site of NO-induced vasodilation. The internal elastic lamina is comprised of elastic connective tissue. The intima is comprised of endothelial cells, the site of NO production. RBCs flowing through the vessel come in direct contact with this layer.

hemostasis (clot formation). A decrease in platelet count could lead to excessive bleeding, while activation is thought to play a role in vessel blockage.

RBCs, the most abundant cells in blood, are anucleated and are responsible for oxygen delivery. They measure 8 – 10 μm in diameter and are about 90 fL in volume. Adult humans have roughly 2×10^{13} RBCs with men producing slightly more than women. RBCs are synthesized in the bone marrow at a rate of about 2×10^{6} every second. While RBCs are developing they are known as reticulocytes and stay in this form for about 7 days until they transform into erythrocytes. The average lifespan of an RBC in the bloodstream is about 120 days. RBCs lack a nucleus, therefore, they cannot synthesize their own proteins and also rely on anaerobic glycolysis for energy production.

The RBC membrane is an ordered array of lipids and proteins. The two main proteins found on an RBC membrane are band 3 and the glycoproteins, the latter being responsible for a mammals' blood type. The ordered array of lipids and proteins affects RBCs deformability; however, while these proteins aid the RBC is deformability and regulating immune recognition, hemoglobin is the most important component of an RBC.

Hemoglobin comprises 97% of an RBCs dry content and 35% including water. It was first discovered by Hunefeld in 1840 and the structure was determined in 1959 by Perutz,[1] who used three-dimensional x-ray crystallography to resolve a structure of horse oxyhemoglobin. It is with this knowledge, and those previously reported on myoglobin[2], prior to Perutz, that the hemoglobin structure was derived.

On a cellular level, hemoglobin is a metalloprotein responsible for ensuring aerobic respiration in cells of vertebrates. A figure of hemoglobin's structure is shown in Figure 2. Each of the four globular subunits of hemoglobin consist of a heme group. The Fe atom in these heme groups is responsible for the uptake of oxygen throughout the body and its subsequent delivery. The Fe atom must be in the Fe^{2+} state to bind oxygen, which will temporarily oxidize it to the Fe^{3+} state. While Fe can exist in the ferric state, it cannot bind oxygen and is referred to as methemoglobin. Once oxygen is collected by hemoglobin, it traverses the body in search for respiring tissue where it is then exchanged through a system of arterioles, capillaries, and venules infused into tissue. Once metabolic demand is met, hemoglobin is carried back to the lungs where the partial pressure of oxygen is around 100 mm Hg, a pressure sufficient for hemoglobin to uptake oxygen; in other words, the hemoglobin is reoxygenated in the lung bed. While the heme groups are most responsible for oxygen transport, other structural characteristics of hemoglobin allow for improved transport and more efficient oxygenation of vascular reperfusion.

Currently, there are two models to explain the allosteric changes accompanying oxygen uploading that leads to cooperative binding. The KNF model, named for the founders, Koshland, Nemethy and Filmer,[3] explains the sequential model, meaning the subunits can change their conformation to increase the binding of oxygen to other subunits. The other model, the MWC model,[4] named after its founders, Monod, Wyman and Changeux, describes hemoglobin as existing in two states, the R or "relaxed" state and the T or "tense" state. The R state is the

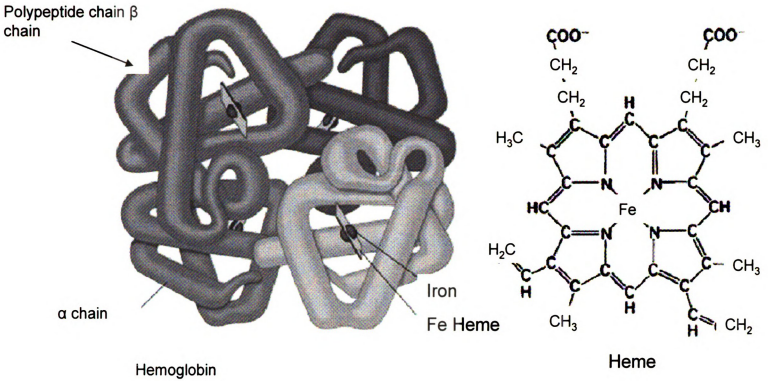

Figure 2. Structure of hemoglobin and molecular drawing of heme structure.[130] This schematic displays the placement of the four heme groups on hemoglobin. Each chain (alpha and beta) have a heme moiety. To the right, the molecular structure of heme is shown. This includes the Fe atom and the porphyrin ring surrounding it. O_2 binds to iron atom and takes on a "bent" conformation with one oxygen atom protruding out.

oxygenated state, while the T state is the deoxygenated state. Equilibrium exists in these two states such that partial binding of oxygen shifts the equilibrium to the R state and partial unloading of oxygen shifts back to the T state. An understanding of cooperative binding is still not clear, however, the fact that it does occur is imperative in oxygen transport. If hemoglobin acted like myoglobin, a globular protein which stores oxygen, tissues would perish due to a lack of oxygenation.

1.2 Nitric Oxide as a Major Vasodilator in the Circulatory System

Oxygenated heme can only get to respiring tissue due to the RBC. The RBC must flow through small resistance vessels, therefore, a mode of opening these vessels is required. Dilation from a substance such as nitric oxide (NO), was first discovered in 1987 by Ignarro, Palmer and Furchgott as the endothelium-derived relaxing factor (EDRF) as one means of opening these vessels.[34, 35] In the discovery of NO as the EDRF, segments of vessels were prepared from bovine intrapulmonary artery and vein and were perfused with an oxygenated Krebs-bicarbonate solution at 37 °C. NO released from these segments after pharmacological stimuli was measured by the diazotization of sulfanilic acid and subsequent coupling with *N*-(1 naphthyl)-ethylenediamine to produce a highly colored product that can be measured spectrophotometrically. The EDRF and NO from artery and vein segments stimulated cyclic GMP levels. Furthermore, the cyclic GMP accumulation elicited by the EDRF and NO was inhibited by methylene blue, oxyhemoglobin, pyrogallol (a powerful reducing agent) and potassium. Both EDRF and NO elicited the same responses in

both artery and vein segments. This led the authors to conclude that the EDRF was indeed NO.

Shear stress is one proposed stimulant of NO production by the endothelium. In studies performed on resting muscle, shear stress resulted in dilation of the arteriole. Moreover, as long as shear stress was present, dilation was produced. Upon administration of a nitric oxide synthase (NOS, an enzyme responsible for NO production) inhibitor, L-NMMA, arteriole dilation decreased by 85%, but remained the same diameter at basal conditions.[5]

Studies performed by Sprague *et. al.* revealed shear stress alone did not induce NO production, but rather the presence of RBCs was needed.[6] Specifically, through the administration of L-NAME, another NOS inhibitor, and measurement of transpulmonary pressure (TPP), it was discovered that NO was a determinant of vascular resistance; however, the inhibitory effect of L-NAME was only seen when the lungs were perfused with RBCs. These data suggest a dependence of NO production in the endothelium on RBCs, a discovery that potentially could modify the aforementioned findings involving shear stress.

Due to the importance of NO in the vasculature, it may also be a determinant in cardiovascular disease. Atherosclerosis, a condition in which the artery wall thickens due to build up of fatty materials such as cholesterol, is associated with compromised NO activity.[7] This loss in NO activity is thought to occur in the early stages of the disease and inflicts considerable effects on its development, such as leukocyte diapedesis (movement of leukocytes out of the circulatory system) and

further thickening of the arterial wall through smooth muscle cell proliferation. The origin of the decreased production of NO in atherosclerosis has not been determined. However, one mechanism proposes that atherosclerotic lesions have a decreased NOS expression.[8]

1.3 Proposed Mechanisms of Vasodilation in Response to Hypoxia

Due to the demand of oxygen for aerobic respiration, it is reasonable to conclude that blood flow must change in some way to ensure oxygen delivery to tissues in need. During an ischemic attack, blood flow is inhibited to organs for a certain amount of time. In this time frame, the respiring tissue is undergoing metabolic processes, many of which are to ensure reperfusion (flow of blood), while others further insult the already injured tissue, such as the production of reactive oxygen species (ROS) in hypoxic injury.[9] The mechanisms leading to reperfusion have been intensely studied and can be subdivided into three categories. First, the vesicles themselves somehow sense oxygen concentration, and dilate when this oxygen tension is low. The second mechanism proposes that the production of ROS can help relax vessel tone and lead to improved flow. The third proposed mechanism suggests that a metabolite is released in respiring tissue that calls for an increase in vascular tone. The following three paragraphs provide further information on these mechanisms.

In vitro and *in vivo* studies have been performed to determine whether the arteriole itself acts as an oxygen sensor. The work of Pittman and Duling in 1974

demonstrated that in porcine isolated carotid artery strips, the mechanical tension declined in accordance with strip thickness and oxygen tensions, implying oxygen tensions and arteriole diameter plays a role in vessel dilation.[10] Accordingly, through *in vivo* measurements performed by Jackson in 1984, it was found the arterioles themselves do not sense changes in oxygen tensions unless the arterioles are downstream from capillaries, or venules. Furthermore, only global changes in oxygen tension (global changes referring to a change in oxygen tension in the superfusate flowing over the entire preparation) elicited a response in vascular diameter. In 1994, Messina *et. al.* demonstrated the dependence of vascular constriction in increased oxygen tensions on endothelium.[11] Once the endothelium was eliminated or incubated with a prostaglandin inhibitor, indomethacin, rat cremaster arterioles stopped constricting in oxygen tensions from 20 to 150 mm Hg and 150 to 660 mm Hg.

As mentioned above, ROS production is the second mechanism of reperfusion. ROS are released upon hypoxic insult.[9] One source of this release may come from the NADP(H) oxidases (NOX) NOX 1,2 and 4. These enzymes are found in the vasculature wall and are believed to increase ROS, including superoxide and peroxide.[8, 9] In this construct, ROS generation varies with oxygen tension, although this idea has been disputed.[12]

The third mechanism mentioned that is a plausible cause for reperfusion is the release of a metabolite to surrounding tissue. Such a metabolite can be identified as

adenosine triphosphate (ATP). The first discovery of extracellular ATP in a perfusate was reported by Holton in 1959.[13]

1.4 ATP as a Stimulant for the Vasodilator NO

In 1972, it was reported by Burnstock that ATP acts as an extracellular signaling molecule in non-adrenergic (non adrenaline mediated) and non-cholinergic (non acetylcholine mediated) neuromuscular transmission in the gut and urinary bladder.[33] In 1978, specific receptors mediated by adenyl nucleotides called P_2 were discovered.[14] P_2 receptors were then categorized on the basis of molecular structure and function: P_{2X}, ligand – gated ion channels, and P_{2Y}, G protein – coupled receptors.[35] P_{2Y} receptors respond to both purine (ATP|ADP) and pyrimidine (UTP|UDP) nucleotides; more specifically, P_{2Y2}, P_{2Y11} and P_{2Y13} are all specific to ATP.[28]

When present in and around the vasculature, ATP can either activate P_{2X} receptors on the outside of vessels resulting in vasoconstriction,[14] attenuate sympathetic (nervous system responsible for "flight or fight", i.e. raising blood pressure) vasoconstriction,[29] or activate P_{2Y} receptors on the vessel lumen resulting in dilation.[14] Once ATP activates a P_{2Y} receptor on endothelial cells, intracellular [Ca^{2+}] is increased through the phospholipase-C/inositol-1,4,5-triphosphate (IP_3)

pathway.[31, 32] NOS enzymes responsible for NO production are located in the endothelial cells and their activity is increased by a Ca^{2+}/calmodulin-dependent or kinase-dependent mechanism. Upon P_{2Y} activation, Ca^{2+} concentration increases and this influx of Ca^{2+} induces a conformational activation of eNOS, resulting in phosphorylation at Ser-1177.[15] Once eNOS is activated, it catalyzes the five electron oxidation of a guanidino nitrogen of L-arginine. The mechanism of this reaction is displayed in Figure 3. Through the conversion of L-arginine to L-citrulline, NO is produced per 2 mol of O_2 and 1.5 mol of NADPH consumed. After NO is produced, it binds to the soluble form of guanylyl cyclase, which then converts cyclic guanosine triphosphate to cyclic guanosine monophosphate (cGMP).[16] This cGMP can then activate K^+ channels leading to hyperpolarization and relaxation, increase intracellular Ca^{2+} entry into cells, or stimulate a cGMP-dependent protein kinase that activates myosin light chain phosphatase, an enzyme that dephosphorylates myosin light chains leading to smooth muscle relaxation. A schematic of the mechanism is displayed in Figure 4. Jankowski *et. al.* demonstrated that accumulation of cGMP increases in a parallel manner to ATP-induced glomeruli relaxation suggesting yet further evidence that ATP may be a determinant in NO production.[17]

1.5 RBC-Derived ATP Release

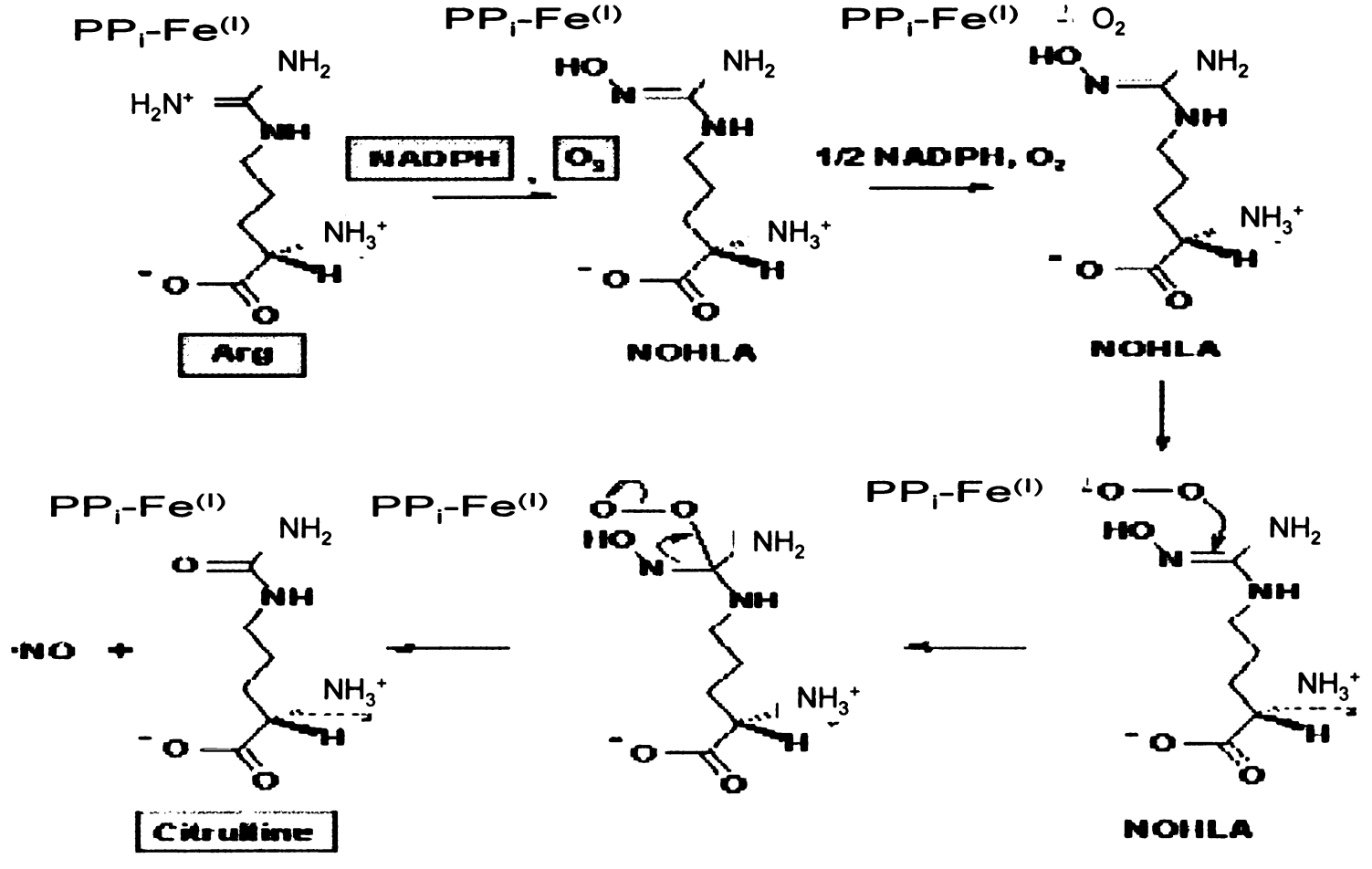

Figure 3. The five electron oxidation of a guanidino nitrogen of L-arginine. Through the conversion of L-arginine to L-citrulline, NO is produced per 2 mol of O_2 and 1.5 mole of NADPH consumed. This reaction takes place in the endothelium upon activation of eNOS.[135]

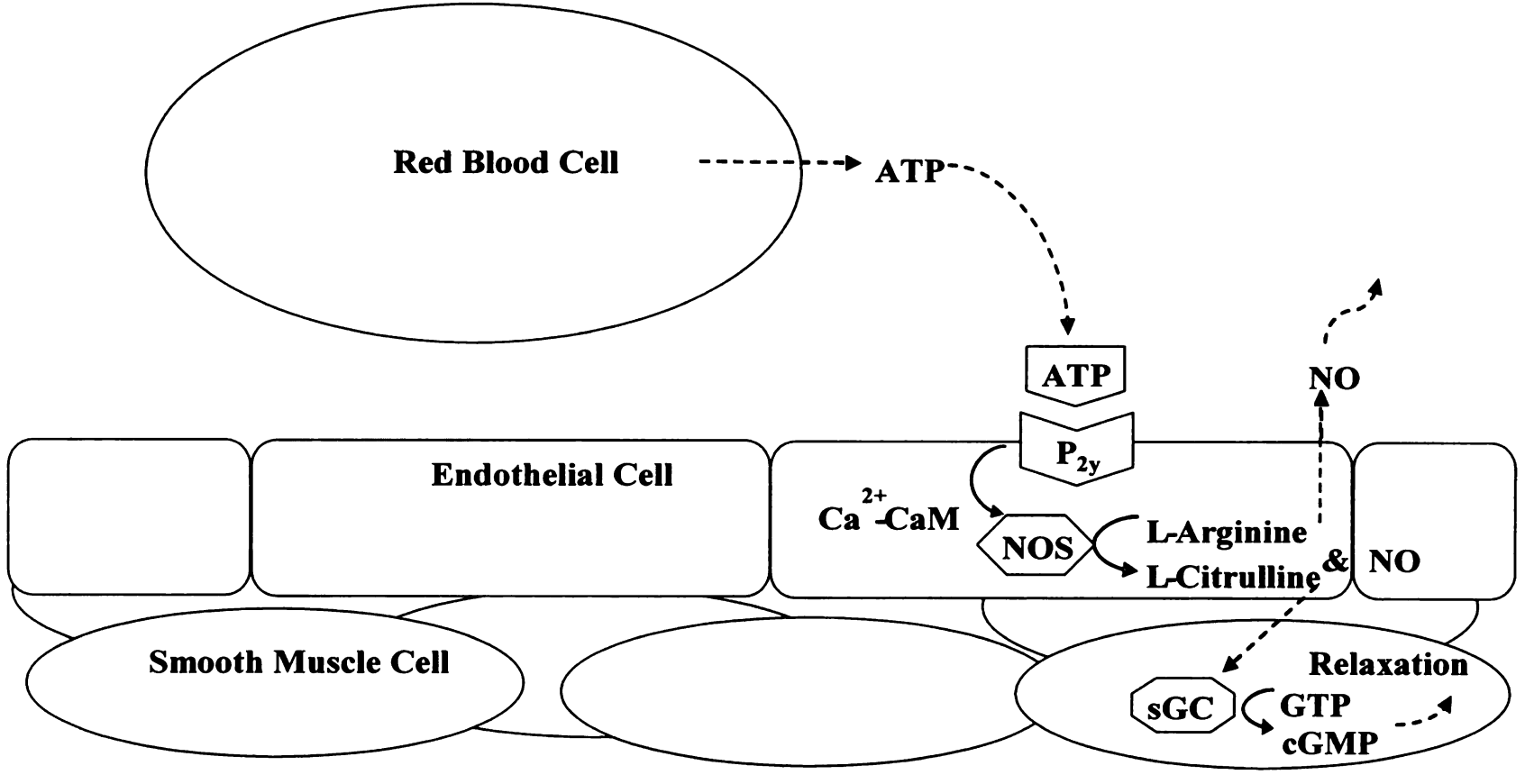

Figure 4. Diagram of the fate of ATP released from an RBC. RBC-derived ATP release stimulates NO production in the endothelium lining vessel walls. ATP is released from RBCs due to a number of different stimuli such as deformation and hypoxia. Once released, ATP activates a purinergic receptor on the endothelial cell, activating eNOS through calcium flux into the cell. eNOS catalyzes the production of the vasodilator NO which then diffuses to the smooth muscle cells causing them to relax, and allowing dilation of the vessel to occur.

ATP can be released from RBCs due to a number of different stimuli, one of which is mechanical deformation[18-21] and pharmacological agents[22-24] (iloprost). It was first reported by Sprague *et. al.* in 1996 that mechanical deformation of RBCs resulted in ATP release. A 10% hematocrit of RBCs were subjected to deformation by introduction into a blood filtrometer, where a polycarbonate membrane with pore sizes of 5, 8 or 12 μm represented the filter. Movement of RBCs through the membrane was induced by opening an outflow tap. If both average pore size and the hematocrit are controlled, the transit time of the RBCs is a measure of their deformability or ability to migrate through the pores. After flow, 250 μL of the RBC sample were then collected from the filter and placed into a cuvette with 250 μL of luciferin/luciferase. The mixture of both ATP and luciferin/luciferase results in a chemiluminescence reaction where the amount of light is proportional to the concentration of ATP in the sample. In rabbit RBCs, resting intracellular ATP concentration is ~ 1.6 $\pm$ 0.4 mM. Upon passage through the filter, both ATP release and RBC transit time increased significantly. In human RBCs, resting ATP concentrations were similar; however, human RBCs displayed a higher transit time reflecting the slightly larger diameter of human RBCs in comparison to those of rabbits. Importantly, the ATP release from human RBCs increased due to mechanical deformation.

In addition to deformation-induced release of ATP, there are also pharmacological reagants that will stimulate this release. For example, prostacyclin analogs have been used in drug therapy for patients with primary pulmonary hypertension; however, the mode of action of prostacyclin has not been elucidated.[25-28] More specifically, the active prostacyclin analog, iloprost, has been studied

extensively. Iloprost binds to the IP receptor (prostacyclin receptor) that is coupled to the G_S protein and adenylyl cyclase. The mechanism by which iloprost reduces vascular resistance and aids in treating primary pulmonary hypertension has been proposed to be via the G_S protein pathway. A signal transduction pathway involving the G_S protein for the release of ATP from RBCs under deformation and pharmacological stimuli has been described.[23] and involves the activation of adenylyl cyclase. The binding of iloprost to rabbit and human RBCs has been previously demonstrated;[29-31] however, the only measured affect of iloprost on RBCs has been an increase in the deformability.[32, 33]

As previously mentioned, deformability is one stimuli for the G_S signaling pathway for RBC-derived ATP release. This led the authors to suggest iloprost may decrease vascular resistance through the release of ATP, a stimulant for the potent vasodilator NO. In a study performed by Sprague *et. al.*, rabbit RBCs, incubated with iloprost displayed a 332 $\pm$ 72% increase in ATP release that was prevented in the presence of an IP receptor antagonist, CAY10441.[34] It was suggested the increase in ATP release is a result of the increased deformability of the iloprost influenced RBC. Moreover, iloprost results in ATP release from RBCs in the absence of flow.

Another stimulus for ATP release from RBCs is hypoxia or a lowering of oxygen levels in the environment. The first study of hypoxia-induced ATP was performed by Paddle and Burnstock.[35] They investigated whether ATP was released

from hypoxic myocardium in guinea pig hearts by switching the oxygen concentration of the perfusate.[35] ATP concentrations were found to be increased threefold by the end of the hypoxic exposure. Furthermore, perfusion pressure diminished, suggesting an increase in vessel dilation. Subsequently, other studies were performed that confirmed the results reported by Paddle and Burnstock.[36]

Since these discoveries, more evidence has accumulated suggesting that hypoxia results in ATP release. In 1992, Bergfeld *et. al.* measured the ATP release from human RBCs exposed to a brief period of hypoxia/hypercapnia (hypercapnia is a condition in which there are high levels of carbon dioxide in the blood).[37] The ATP release after exposure to 50 s of hypoxia/hypercapnia at 37 °C increased from 0.45 x $10^6 \pm 0.04$ to 2.67 x $10^6 \pm 0.27$ molecules/RBC released.

ATP is a highly charged molecule in comparison to other biomolecules that come into contact with the RBC membrane, therefore, Bergfeld and Forrester further investigated the mechanism by which ATP was leaving the RBC. ATP carries a charge of negative four; therefore, departure from the RBC results in a large potential difference across the membrane. Therefore, it is thought that an efflux of other charged molecules must be employed to balance the electrical equilibrium.

The band 3 protein on the erythrocyte membrane is an anionic channel responsible for the efflux of Cl- and HCO_3^-; however, it can also be responsible for the transfer of a large range of molecules from the RBC. Through inhibition of the band 3 protein, and subsequent exposure to hypoxia, the authors concluded the band 3

protein plays a role in the release of ATP from the RBC as indicated by a decrease in ATP release.

Coinciding with the results performed on the band 3 protein, were those obtained from the inhibition of a separate nucleoside transporter, band 4·5. It was also demonstrated that inhibition of band 4·5 protein, and successive exposure to hypoxia, also resulted in a decrease in ATP release. These data suggest both proteins play a role in the release of hypoxia-induced RBC-derived ATP release.

The authors proposed a model for such a situation. The ratio of band 3 to band 4·5 protein exists as 1.2×10^6 to 10^4, and assuming one channel per protein exists, only a small portion of the band 3 protein would be utilized in the actual release of ATP. This leaves the band 3 protein open for Cl-/ HCO_3^- exchange. Furthermore, the nucleoside portion of the band 4·5 protein is directly involved in the release of ATP. A schematic of the pathway proposed by Bergeld for hypoxia-induced ATP release is shown in Figure 5.

From this study, it was concluded that the release of hypoxia-induced ATP from human erythrocytes occurs through the band 4·5 protein. This study demonstrated one mechanism of ATP release; however, it fails to explore how hypoxia stimulates the ATP release.

1.6 Hypoxic Vasodilation

Many theories propose the RBC elicits vasodilation not only through the release of ATP, a stimulant for NO production, but rather through the release of NO

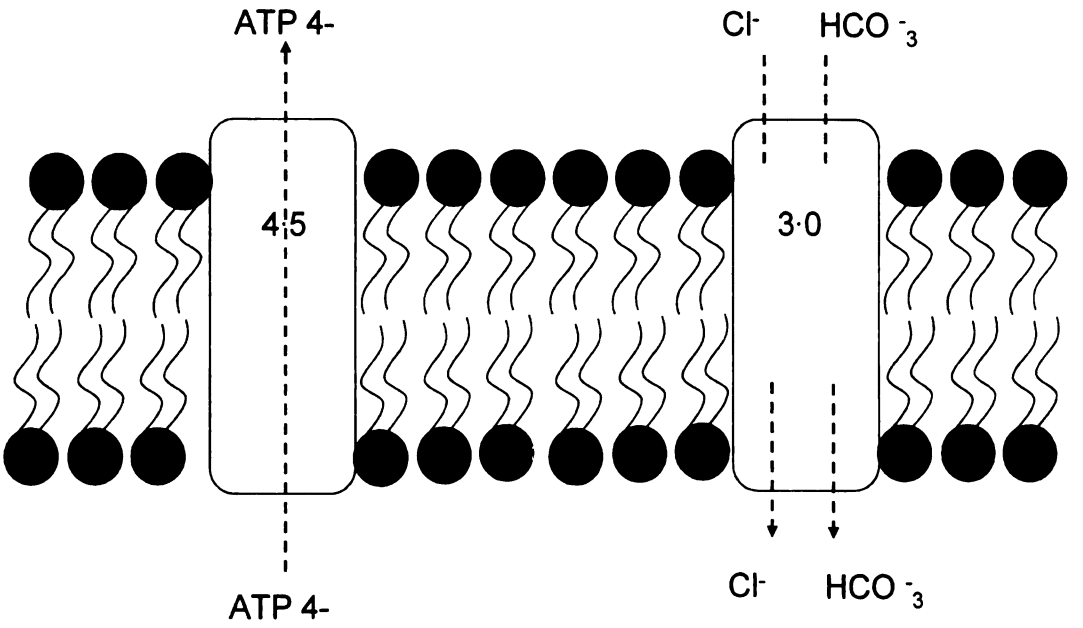

Figure 5. Mechanism for ATP release proposed by Bergfeld and Forrester.[37] ATP leaves the RBC through the band 4·5 protein. The efflux of the ATP causes an imbalance in the electrical potential of the cell. ATP has a charge of negative 4, and upon leaving the cell, takes a Mg^{2+} atom with it. This leaves a negative 2 charge on the RBC that needs to be compensated for to keep electrical balance. Band 3 imports a chloride ion and a bicarbonate ion to keep electro-homeostasis.

itself. Vasodilation in response to tissue hypoxia is a crucial and necessary step in regulating vascular homeostasis. Upon hypoxic insult, vasodilation ensures the delivery of oxygen and metabolites to respiring tissue.[38] Hemoglobin, the main carrier of oxygen throughout the body, is responsible for oxygen delivery and therefore the RBC itself mediates this response. The RBC as a component in this process has been made clear;[39] however, the mechanism has not been elucidated. Many proposed mechanisms exist, such as ATP release,[40, 41] nitrite reductase[42-44] and SNO-Hb.[45, 46]

In order for hypoxia-induced ATP release to be a determinant in hypoxic vasodilation it must be shown that ATP increases vascular caliber *in vivo*. Sprague *et. al.* demonstrated that concentrations of ATP as low as 300 nM resulted in a decrease in total pulmonary vascular resistance in the perfused rabbit lung. McCullough *et. al.* displayed a dependence of a vasodilator response on ATP using the hamster cheek pouch retractor muscle.[47] Addition of ATP ranging from a concentration of 10^{-8} M to 10^{-4} M, induced an increase in vessel diameter from 16 to 65% with the maximum change seen at ~150 μM. As previously demonstrated by Bergfeld, hypoxia induces ATP release in RBCs.[37] This shows a definitive relationship between hypoxia-induced ATP release and subsequent vasodilation. A schematic of hypoxia-induced RBC-derived ATP is displayed in Figure 6.

In addition to the theory that hypoxic vasodilation is mediated by the RBC, others believe that another RBC-mediated mechanism exists. Specifically, studies

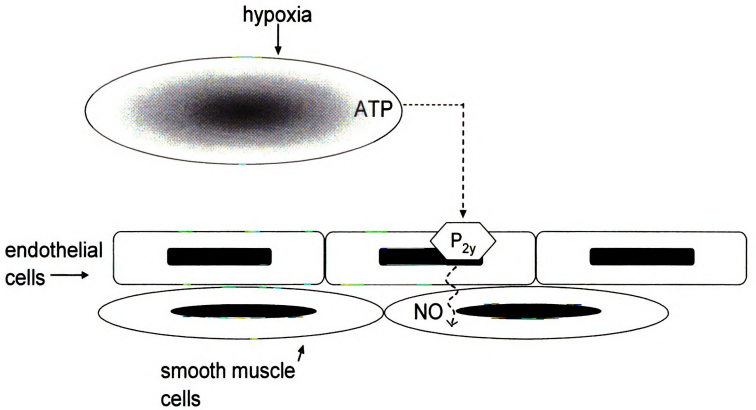

Figure 6. Schematic of hypoxia-induced ATP release. Once ATP is released from an RBC is activates the purinergic receptor, P_{2y}, on endothelial cells. This, in turn, activates eNOS which produces the potent vasodilator NO. NO diffuses to the smooth muscle cells where it elicits vasodilation.

have shown that hypoxic vasodilation include the role of nitrite reduction and deoxyhemoglobin for the production of NO. Nitrite concentrations in the plasma and RBC are reported to be 120 nM and 290 nM, respectively.[48] Moreover, nitrite has been recognized as a precursor for NO under acidic and ischemic conditions.[49, 50] At physiological pH, addition of nitrite dilates aortic rings through the activation of soluble guanylate cyclase (sGC).[51-53] These processes suggest a biological function for nitrite in the control of vascular homeostasis.

It has recently been reported that oxygen sensing by the RBC is controlled through the conversion of nitrite to NO through deoxyhemoglobin.[42, 43, 54] NO production from nitrite in cell-free hemoglobin or from RBCs has also been established.[43, 55-59] Crawford *et. al.* displayed a maximum in nitrite reductase activity when hemoglobin is at P_{50}; the largest quantity of NO produced at 50% – 60%.[60] The conflicting physiochemical requirements of open binding sites in the T state for nitrite to bind and the greater redox reaction rate of hemoglobin in the R state results in this optimal conformation. In addition to increases in NO production, deoxygenating RBCs and nitrite both stimulate various NO-mediated activities such as vasodilation, cGMP formation, and mitochondrial respiration inhibition. These effects were inhibited by the NO scavenger, C-PTIO. A mechanism for this process is displayed in Figure 7.

While this mechanism has sufficiently described a possible role for nitrite in hypoxic vasodilation, there exist weaknesses in its viability. For example, after

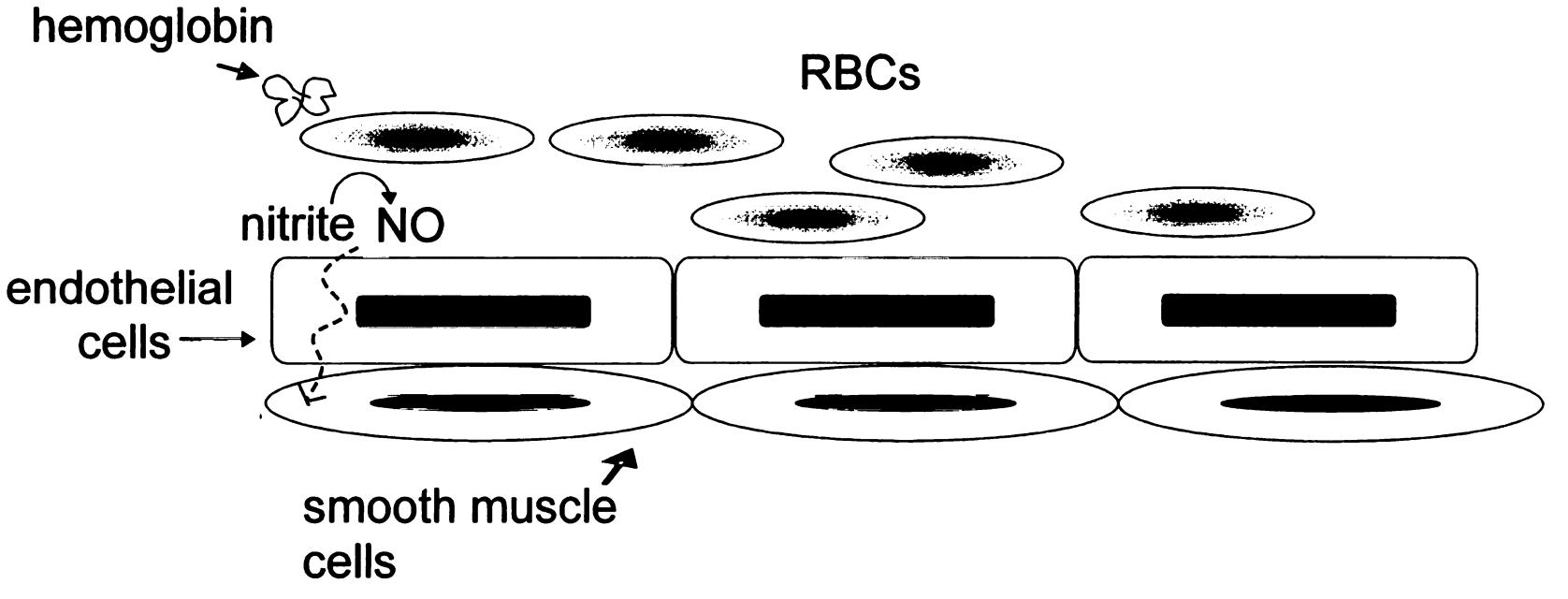

Figure 7. Mechanism of hypoxic vasodilation that proposes at 50% oxygen saturation hemoglobin acts as a nitrite reductase reducing bioavailable nitrite to NO. This NO then diffuses through the endothelium, and the sub-endothelium to the smooth muscle cells where it can bind to soluble guanylate cyclase and elicit vasodilation.

deoxygenated RBCs and nitrite are combined, vasodilation takes from 2 to 7 minutes until complete relaxation is achieved. When oxygen tension was decreased as a function of time the vasodilation occurred more quickly. These data suggest the discrepancy in time of relaxation is dependant upon the steady-state and non-steady state oxygen tensions.

In 1996, Stamler and co-workers suggested a SNO-Hb (S-nitrosylated hemoglobin) paradigm. In this construct, NO binds to oxyhemoglobin on the highly conserved $Cys^{\beta 93}$ and is released upon transition from the R (oxy, low spin) to the T (deoxy, high spin) state.[61] The position of NO on the hemes and thiols is redistributed in accordance with the position of the RBC in the circulation. More importantly, interactions of the cysteine residue in the hemoglobin-binding cytoplasmic domain of the anion exchanger AE1 promotes the deoxygenated structure which serves to relocate NO group transfer to the membrane.[62] Furthermore, the NO released from SNO-Hb has been shown to induce vasodilation in blood vessels +/- the endothelium.[46, 61, 63] A mechanism for this process is displayed in Figure 8.

While the idea of SNO-Hb inducing vessel dilation appears to be acceptable, there exists a number of problems with this theory. First, the reaction of a reduced thiol with NO requires a one electron oxidation; however, there is no obvious electron acceptor. The ferriheme is an unlikely source because of the slow rate of reaction.[64] A second problem arises in the measurements. Specifically, many groups have reported undetectable levels of SNO-Hb in the circulation.[46, 64-68] Moreover, the

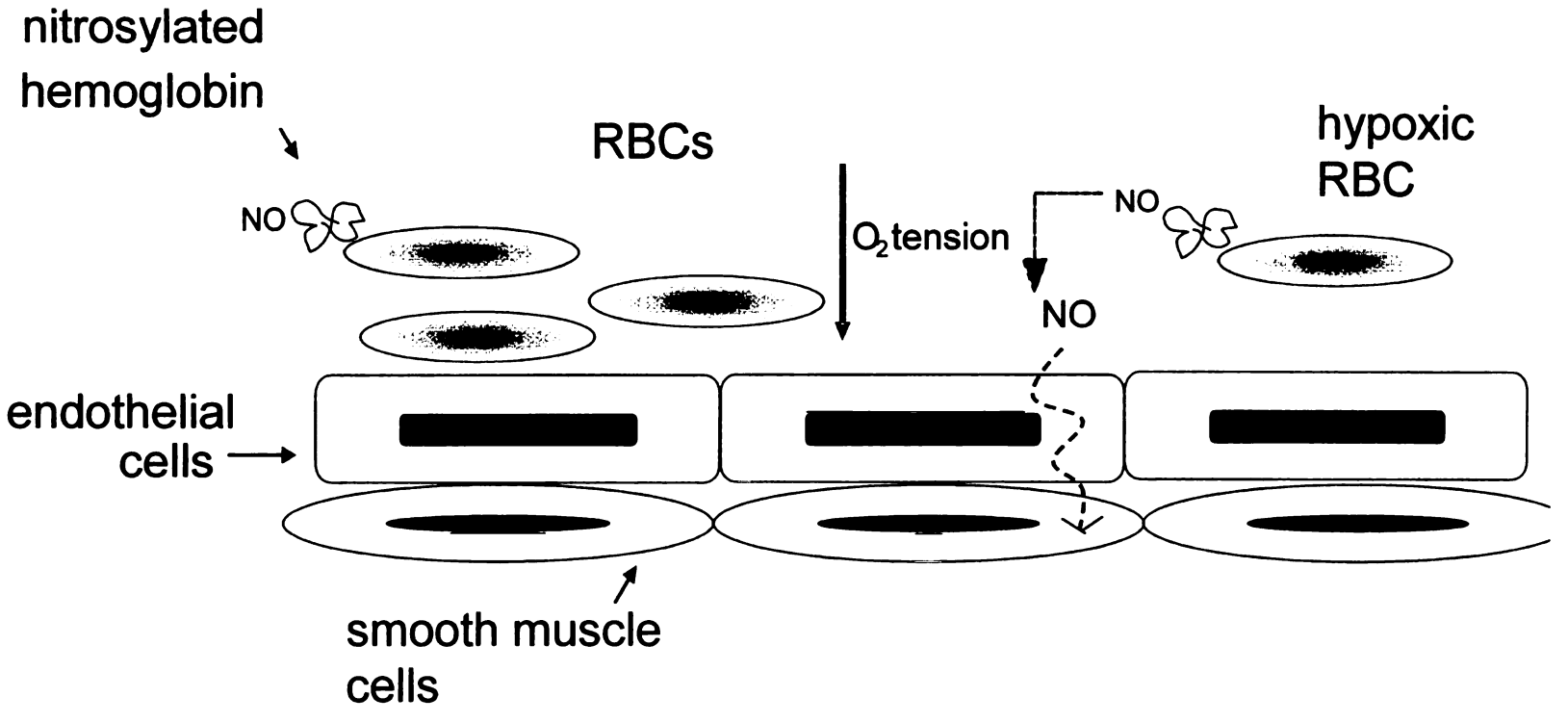

Figure 8. Mechanism of hypoxic vasodilation that proposes hemoglobin uptakes NO in the lungs and conserves it on the β93 cysteine residue. While traversing the circulation, the nitrosylated hemoglobin release NO when it enters an area of hypoxia. This NO then diffuses through the endothelium, and the sub-endothelium to the smooth muscle cells where it can bind to soluble guanylate cyclase and elicit vasodilation.

measurements used to quantify SNO-Hb involve the liberation of NO.[69-71] One method involves photolysis and chemiluminescence to detect liberated NO.[69] Another method involved Hg^{2+}, which breaks down nitrosothiols, and then subsequent chemiluminescence of the nitrogen oxide species; however results using the latter method were only consistent in rodent RBCs and not in human.[67, 70, 71] A third problem arises from the unclear mechanism of NOx transfer from SNO-Hb to the smooth muscle cell. As mentioned earlier, the AE1 membrane protein may be responsible for the transfer, however, because of the methodology of the experiments involved it is not clear whether NO is responsible or other nitroso species. More importantly, this proposed mechanism explains the transfer of NO to the intracellular surface of the RBC but fails to explain how the NO traverses the lumen, past the endothelial wall and elastica interna, to the smooth muscle cells. Furthermore, this assumption excludes the properties of blood flow presented by Fahreus-Lindquist, which states blood possesses turbulent flow in a vessel the size of an artery with a cell-free layer forming at the vessel wall. A fourth problem, coinciding with the second, results from a discrepancy in the values reported for SNO-Hb ranging from <1nM to 5μM.[66, 67, 72, 73]

Nagababu *et. al.* has attempted to establish a relationship between these three conflicting ideas. Through the intermediates formed from the nitrite reductase activity of hemoglobin, SNO-Hb is formed.[74] Hb(III)NO is seven times more effective at producing SNO-Hb than Hb(II)NO. While other investigators have seen a higher

production of SNO-Hb with Hb(III)NO; the idea of this forming SNO-Hb was ignored because only a negligible amount of the intermediate was assumed to be formed.[43, 66, 75] The authors had previously discovered the intermediate is stable through EPR and chemiluminescence.[42] The authors have also established the intermediate responsible for SNO-Hb formation is the nitrosonium cation NO^+.[42]

One proposed mechanism for the transfer of NO to β-93 cysteine is through a reaction of Hb(II)NO with H_2O to form $H_2NO_2^+$ or HNO_2 which can then diffuse through the hydrophobic globin to the cysteine;[76] however due to hydrophilic nature of these molecules this is not a likely mechanism. Another mechanism is through the use of nitrite as the nucleophile facilitating the release of N_2O_3.[77] N_2O_3 is a hydrophobic molecule and therefore can diffuse through the hydrophobic pocket leaving this as a plausible mechanism. A final mechanism presented by the authors leaves out the presence of transnitrosation but rather employs an electron transfer between the NO^+ and the β-93 cysteine.

1.7 The Effects of Hypoxia on the Vasculature

Along with the occurrence of vasodilation through a number of proposed mechanisms, hypoxia also exerts many more effects on the vasculature. Type 1 glomus cells (a cell located in the carotid body that helps regulatebreathing) are responsible for recognizing low oxygen tension.[78] The nerve receptor responsible for

innervating these cells is still debatable; however, [79] dopamine has been shown to modulate the sensitivity of the carotid body to hypoxia.[80] Acetylcholine has also been recognized as a possible neurotransmitter in the role of the carotid body in recognizing hypoxia.[81] After sensing hypoxia, many other changes occur in the body. The most immediate responses include inhibition of the O_2 sensitive K+ channel, membrane depolarization, and an influx of calcium and release of neurotransmitters from the synaptic vesicles. However, there are other physiological events in response to hypoxia.

One major response to hypoxia is the increase in blood volume, or polycythemia, regulated by erythropoietin, a glycoprotein synthesized and released from the kidney during hypoxia.[82] It regulates erythropoiesis, the production of red blood cells. Under normoxic conditions, erythropoietin levels are normal; however, under hypoxia, the levels increase. Unlike the carotid body which activates at oxygen levels around 15%, erythropoietin levels don't increase until oxygen saturation is below 10%.[83]

Another major response to hypoxia is angiogenesis, or the growth of new blood vessels. In tumor cells, angiogenesis occurs quickly supplying the cancerous cells with nutrients and metabolites. Evidence shows this increase in angiogenesis is due to an increase in the vascular endothelial growth factor (VEGF).[84] VEGF is regulated by hypoxia at the level of gene expression and is increased after approximately 6 h of hypoxic conditions.[85]

The Pasteur effect is the change in metabolism due to a low oxygen saturation. Aerobic respiration switches to anaerobic glycolysis, producing less ATP. Within the first hours of hypoxia, glycolysis increases. Because anaerobic glycolysis produces substantially less energy, its rate increases to compensate.[86] This occurs by two mechanisms: the increase in enzymatic activity and gene expression of the glycolytic enzymes, and an increase in glucose uptake through increased activity of GLUT 1 glucose transporter.[87]

As the cells that line vessel walls, endothelial cells are unmistakably one of the most important aspects of the vasculature. Eukaryotic cells produce energy in aerobic conditions. This environment yields the greatest output of energy; however, in many diseases such as cancer oxygen levels remain low. This requires an adaptive response of endothelials cells to a drop in oxygen tension. Not surprisingly, endothelials cells are relatively resilient to hypoxic conditions.[88] Under conditions of hypoxia for 48 h, endothelials cells still maintain about 70% of their ATP production and metabolism.[89]

Endothelial activation is a coined response to the different signaling pathways that become activated under hypoxic conditions. For example, in conditions of hypoxia, cAMP levels are decreased,[90] which may be due to a decrease in adenylyl cyclase activity.[90] Moreover, a decrease in phosphodiesterases may also contribute to lower cAMP levels.[91-93] Following closely, another cyclic nucleotide with decreased levels in endothelial dysfunction is cGMP.[94, 95] cGMP is a product of activation of guanylate cyclases by NO, therefore, it is a major component of vessel dilation.[96]

cAMP and cGMP decrease in endothelial cells are just one of many processes that occur in response to hypoxia. Other processes such as gene transcription regulation become altered. One major change is the membrane of endothelial cells after exposure to 24 h hypoxic conditions. A diagram of the processes of endothelial activation is displayed in Figure 9.

The membranes of endothelial cells permit molecules to pass through by a process called restricted diffusion.[97] This type of diffusion allows for small molecules to pass while larger molecules need transport molecules to aid in their crossing. In conditions of 24 h hypoxia, the membrane of endothelial cells loses its selectivity process and allows for the passage of molecules, large or small to diffuse through.[98] This change in membrane permeability highly contributes to the procoagulant state as exposure of the endothelial substrata, rich in collagen and tissue factor, is exposed.[99] These processes are reversible, with a return to normal restrictive diffusion 24 h after exposure to normal oxygen tension.[99]

Adhesion receptors are surface proteins present on endothelial cells and leukocytes. They work to maintain the proper position of these two cell types. Hypoxic conditions lead to an increased expression of adhesion receptors, which leads to further expression of additional adhesion receptors.[100] More specifically, selectin E is expressed on both endothelial cells and leukocytes and is involved in leukocyte adherence to the endothelial wall.[101] Once adhered, leukocytes are able to migrate

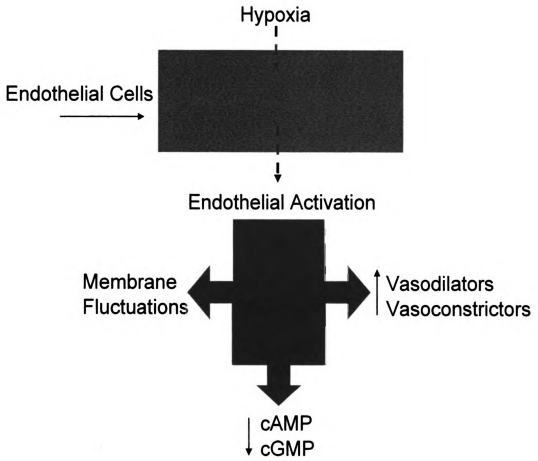

Figure 9. Cascade of events stemming from endothelial activation, the activation of the endothelium in response to hypoxia. Upon endothelial activation, membrane fluctuations occur, (i.e. the junction between endothelial cells open wider), cAMP and cGMP, components for the signaling pathway for the production of NO, levels are decreased and vasodilator and vasoconstrictor levels

through the endothelial membrane by the adhesion molecule vascular endothelial (VE)-cadherin.[102, 103]

It has been established the endothelium plays a role in the regulation of vascular tone. Through the release of such molecules as NO, endothelial cells can induce vasodilation on underlying smooth muscle cells.[52, 104] Conversely, under conditions of hypoxia, the endothelium can elicit a vasoconstrictive response. The most potent vasoconstrictor in the endothelium is endothelin-1.[105] Hypoxia induces the expression and secretion of endothelin-1.[106] Upon secretion, endothelin-1 binds to receptors on vascular smooth muscle cells, increases calcium influx and activates protein kinase C which phosphorylates the myosin leading to vasoconstriction.[107] It has been shown by the same group that NO suppresses this effect.[108]

One major protein in hypoxia pathway is hypoxia-inducible factor (HIF), a transcriptional factor composed of a HIF1β subunit and one of the HIFα subunits. Of these, HIF-1α is the most extensively studied.[109-111] HIF-1 α is regulated by an O_2-dependent pathway: the hydroxylation of proline or asparagine residues.[112-114] In well-oxygenated cells, HIFα is short lived (< 5 min).[115] HIF-1α is synthesized continuously but is degraded quickly by the ubiquitin-proteasome system through a process that requires two O_2-dependent domains.[116-118] Conversely, in hypoxic conditions, HIFα accumulates.[119, 120] HIF-1α is unique to HIF-1 and is the primary determinant in DNA binding and transcriptional activity whereas HIF-1β is unaffected

by hypoxia.[116, 121, 122] One process controlled by HIF-1α is the negative regulation of hepcidin, the key hormonal regulator of iron homoeostasis.[123] Hepcidin is synthesized in the liver and inhibits active transmembrane export of iron by blocking ferroportin, an iron transporter.[124] Through the stabilization of HIF-1α, both hypoxia and iron deficiency suppress hepicidin and therefore increase bioavailable iron.[123]

Reactive oxygen species (ROS) are formed as a result of oxidative reactions. Enzymes exist to protect cells from the deleterious effects of ROS, such as catalase, superoxide dismutase and glutathione peroxidase.[125] ROS becomes harmful when released in large amounts and in the presence of catalytic iron ions, which are released under hypoxia.[126] In the presence of hypoxia, the antioxidant system may not counteract ROS this would lead to lipid peroxidation in RBCs and cell membrane damage.[127] In a present study done by Devi *et. al.* the effect of hypobaric hypoxia and antioxidants in improving oxidative stress in the RBCs of rats was tested.[128] This was accomplished by evaluating the antioxidant defense enzymes, assaying the lipid peroxidation, and determining the status of protein oxidation. The antioxidant enzymes evaluated were superoxide dismutase, catalase and glutathione peroxidase. The markers of oxidative stress assayed were hemolysis, osmotic fragility and membrane malondialdehyde, a marker of lipid peroxidation. Finally, to assess protein oxidation, membrane protein carbonyl content was measured, advanced oxidized protein products and membrane sulphydryl groups. Results indicate an increase in the activities of superoxide dismutase and catalase under intermittent hypoxia. Hemolysis

caused by free radicals can be characterized by lipid peroxidation and redistribution of oxidized band 3 within the membrane.[129] The authors noted an increase in hemolysis, induced by hydrogen peroxide, that may be due, in part, to the higher occurrence of these events in hypobaric-hypoxia rats. In conclusion, oxidative stress was higher in rats with intermittent hypobaric hypoxia which was offset with supplementation of antioxidants such as vitamin C, vitamin E and L-carnitine.

1.8 Thesis Objective

From the binding of oxygen to hemoglobin in the lungs to the distribution in respiring tissue, it is abundantly clear the processes involved in hypoxia are very complex. Findings from 150 years ago, such as NO binding to heme, are still being elucidated. Many processes occur in the vasculature to ensure correct dilation and vasoconstriction of blood vessels. NO is a major determinant of these processes in the vasculature. Through the binding of NO to soluble guanylate cyclase, smooth muscle cells that underlie the endothelium dilate. NO can be induced by shear stress or by a metabolite released into the blood stream, such as ATP, which can activate purinergic receptors on endothelial cells that, in turn, activate eNOS, the enzyme responsible for NO production in the endothelium. Through various stimuli, such as mechanical deformation, or hypoxia, RBCs release ATP; however, the mechanism due to hypoxia remains incomplete. The release of ATP from RBCs under hypoxic conditions has been demonstrated; however, the mechanism is still under speculation.

This dissertation will provide evidence that the different conformational changes hemoglobin undergoes in differing oxygen tensions induces a deformation on the RBC membrane, which is the origin of ATP release. In chapter 2, the ATP release from both hypoxia and deformation were tested simultaneously to establish whether an additive or non-additive relationship exists. The data obtained provided the hypothesis for the research in chapter 3. Through the inhibition of the deformation-induced signaling pathway and subsequent hypoxia and the addition of a cell stiffening agent, it was concluded hypoxia-induced ATP release is really a form of deformation on the RBC membrane. Furthermore, the mechanism of hypoxic vasodilation is described in chapter 4 starting from the RBC and ending with endothelium-derived NO.

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CHAPTER 2

2.1 MEASURING TWO STIMULI OF ATP RELEASE SIMULTANEOUSLY

Hypoxia is a condition in which a region of the body, or the body as a whole, is deprived of a sufficient oxygen supply. Oxygen transport in mammalian species is carried out by hemoglobin, a metalloprotein connected to the acidic amino-terminal segment of the band 3 membrane protein of RBCs by weak binding.[1] The hemoglobin molecule itself is comprised of four subunits: two α-chains of 141 amino acid residues each and two β-chains, each containing 146 residues.[2] Each chain carries a heme group that is held in pockets formed by several helical and non-helical segments. In its deoxygenated state, hemoglobin is in a (T) tense conformation. Upon oxygenation, hemoglobin changes to a relaxed (R) conformation. The change in conformations is responsible for its cooperative effect that ensures uptake and release of O_2 over the range of partial O_2 pressures between the lungs and tissues.[3]

Related to its role in oxygen transport, Ellsworth *et. al.* have reported that the RBC may actually serve as an oxygen sensor.[4] One mechanism to explain the RBC's internal oxygen sensory is through the release of sub-micromolar amounts of ATP upon exposure to brief periods of hypoxia.[5] It has been shown that ATP has the ability to stimulate NO production in both cultured endothelial cells and the pulmonary endothelium.[6-8] Importantly, NO is known to be a potent vasodilator and, as such, relaxes the smooth muscle cells surrounding the blood vessel enabling an

increase in blood flow to organs and tissue.[8] It is in this construct that the RBC is not only a deliverer of oxygen, but is also a determinant of blood flow due to the ability of hypoxia-induced ATP release to stimulate NO production.

In addition to hypoxia, there exist other methods of stimulating ATP release from RBCs. For example, it is well-established that mechanical deformation of RBCs results in the release of ATP. Sprague *et. al.* have demonstrated that deformation of the RBC occurs when RBCs traverse porous membranes having diameters similar to those of resistance vessels *in vivo*.[9] Moreover, it has also been demonstrated in an indirect manner (through the monitoring of vessel relaxation) that the introduction of RBCs through the isolated rabbit lung also results in ATP release.[10] Finally, by using microbore tubing or the channels in a microfluidic device to approximate the dimensions of resistance vessels *in vivo*, we have demonstrated the ability to measure deformation-induced ATP release from the RBC.[11, 12]

While hypoxia and deformation, as well as other methods (e.g. pharmacological),[13, 14] are known to induce ATP release from the RBC, none of these stimuli have been examined in combination. Here, quantitative data are reported from a determination in which RBCs were pumped through a microflow system similar to those studies previously reported.[15] However, in the studies reported here, the RBCs were also subjected to a brief period of hypoxia prior to their introduction into the microbore tubing. Such a protocol enabled the determination of ATP release from RBCs due to deformation and/or varying levels of hypoxia. Therefore, the work

described here is the first attempt to differentiate ATP release from RBCs due to hypoxia from that due to deformation. This conclusion would prove useful in the attempt to elicit the pathway resulting in hypoxia-induced ATP release. These studies should impact such diseases as diabetes where the RBCs do not respond as well to hypoxic conditions as those RBCs obtained from healthy controls.

However, measuring these effects simultaneously required the development of a system to determine both ATP release due to hypoxia and deformation. As previously mentioned, microbore tubing and microfluidic devices have been employed to mimic resistance vessels *in vivo*. Fluid dynamics are crucial in measurements as they replicate the circulation and introduce many variables not seen in a static system, such as shear stress, the formation of a cell-free layer and pressure changes. Previous work in the Spence group has resulted in instrumentation that includes flow and mimics the diameter of vessels *in vivo*.

Since the early '90's, microfluidic technology has flourished. In microfluidics, small volumes of samples traverse micron size channels embedded in a chip. Microfluidic devices, initially, were designed with simple channel layouts; however, they have developed to allow for much more complicated processes such as sample and reagent mixing, and purification.[16, 17] Microfluidic devices can be fabricated from many different substrates such as glass and silicon or polymers, more specifically, polydimethylsiloxane (PDMS). These devices will be more prevalent in studies described in chapter 4.

Here, an instrumental setup that mimics resistance vessels *in vivo* is described that includes the use of fused silica microbore tubing. A set up described by Edwards

et. al. used a temperature-controlled high pressure syringe pump to introduce reagants.[18] Luicferin/luciferase was pumped through fused silica microbore tubing with an outside diameter of 365 μm. The capillary tubing ended at a mixing tee with an internal volume of 29 nL. PSS was pumped through fused silica microbore tubing also having an inside diameter of 365 μm. The capillary tubing containing the PSS ended at a 4-port injection valve where RBCs were injected. The RBCs were then flowed through capillary tubing of varying lengths and diameters (25-75 μM). These inside diameters were utilized in order to induce a deformation on the RBCs. RBCs then mixed with luciferin/luciferase in the mixing tee and a chemiluminescence reaction occurred. The sample was then flowed through another piece of microbore tubing with an inside diameter of 50 μm. The polyimide coating was burned off the tubing with a flame leaving a window open for the light to be detected by a photomultiplier tube (PMT). The light was converted to voltage and measured on a computer. RBC-derived ATP release decreased with an increase in the capillary's inside diameter when the flow rate was held constant at 15 μL/min. This setup not only displays deformation-induced ATP release, but is an excellent mimic of physiological blood flow.

Through the use of flow-through instrumentation, we were able to induce deformation and hypoxia simultaneously on RBCs. The novelty of this system is the simultaneous measurement of both stimuli. Previous work measured deformation and hypoxia-induced ATP release in a static system after the stimuli was applied. This allowed us to determine an additive or non-additive relationship between the two

stimuli. This would further suggest if hypoxia and deformation release ATP through the same signaling pathway and if hypoxia is really a form of deformation.

2.2 EXPERIMENTAL

2.2.1 Collection of RBCs

Male New Zealand White rabbits (2.0-2.5 kg) were anesthetized with ketamine (8 mg kg^{-1}, intramuscular injection) and xylazine (1 mg kg^{-1}, i.m.) followed by pentobarbital sodium (15 mg kg^{-1} , intravenous injection). A cannula was placed in the trachea and the animals were ventilated with room air at 20 breaths min^{-1} and a tidal volume of 20 mL kg^{-1}. A catheter was placed into a carotid artery for administration of heparin and for phlebotomy. After heparin (500 units, i.v.) animals were exsanguinated. Blood was centrifuged at 500 *g* at 4 ° C for 10 min. The plasma and buffy coat were removed for other experiments. RBCs were then resuspended and washed three times in a physiological salt solution (PSS). The PSS was made by combining 25 mL of TRIS buffer [prepared by mixing 50.9 g of TRIS in 1 L of distilled and deionized water (DDW)] and 25 mL of Ringer's solution (164.2 g NaCl, 7.0 g KCl, 5.9 g $CaCl_2 \cdot 2H_2O$ and 2.83 g $MgSO_4$ in 1 L of DDW). After the addition of 0.50 g of dextrose and 2.50 g of albumin bovine fraction V (fatty-acid free) to the TRIS-Ringer's mixture, the entire solution was diluted to 500 mL with DDW and the

pH was adjusted to 7.35-7.45. The PSS was then filtered three times using a 0.45 μm filter.

2.2.2 Preparation of Reagants

Diamide, a known cell stiffening agent, was prepared for incubation with RBCs to render them less deformable. A 2 mM solution was prepared by dissolving 0.009 g of diamide in 25 mL of PSS.

Glibenclamide inhibits the cystic fibrosis transmembrane regulator, the last protein in the pathway for deformation-induced ATP release. It was prepared by adding 0.049 g of glibenclamide to 2 mL of 0.1 M NaOH. 8 mL of a solution containing 1 g dextrose and 20 mL DDW was added to the glibenclamide and NaOH solution, heated to 50 °C and stirred until dissolved, resulting in a 0.1 M solution of glibenclamide.

A 100 μM stock solution of ATP was prepared by adding 0.0551 g of ATP to 1000 mL of DDW. ATP standards with concentrations ranging between 0 and 1.5 μM were then prepared in PSS from the stock.

To prepare the luciferin-luciferase mixture involved in the measurement of ATP by chemiluminescence, 5 mL of DDW were added to a vial containing luciferase and luciferin. In order to enhance the sensitivity of the assay, 2 mg of luciferin were added to the vial. All reagants were purchased from Sigma Chemical (St. Louis, MO) The chemiluminescence reaction of lucierfin/lucierfase with ATP is displayed in Figure 10.

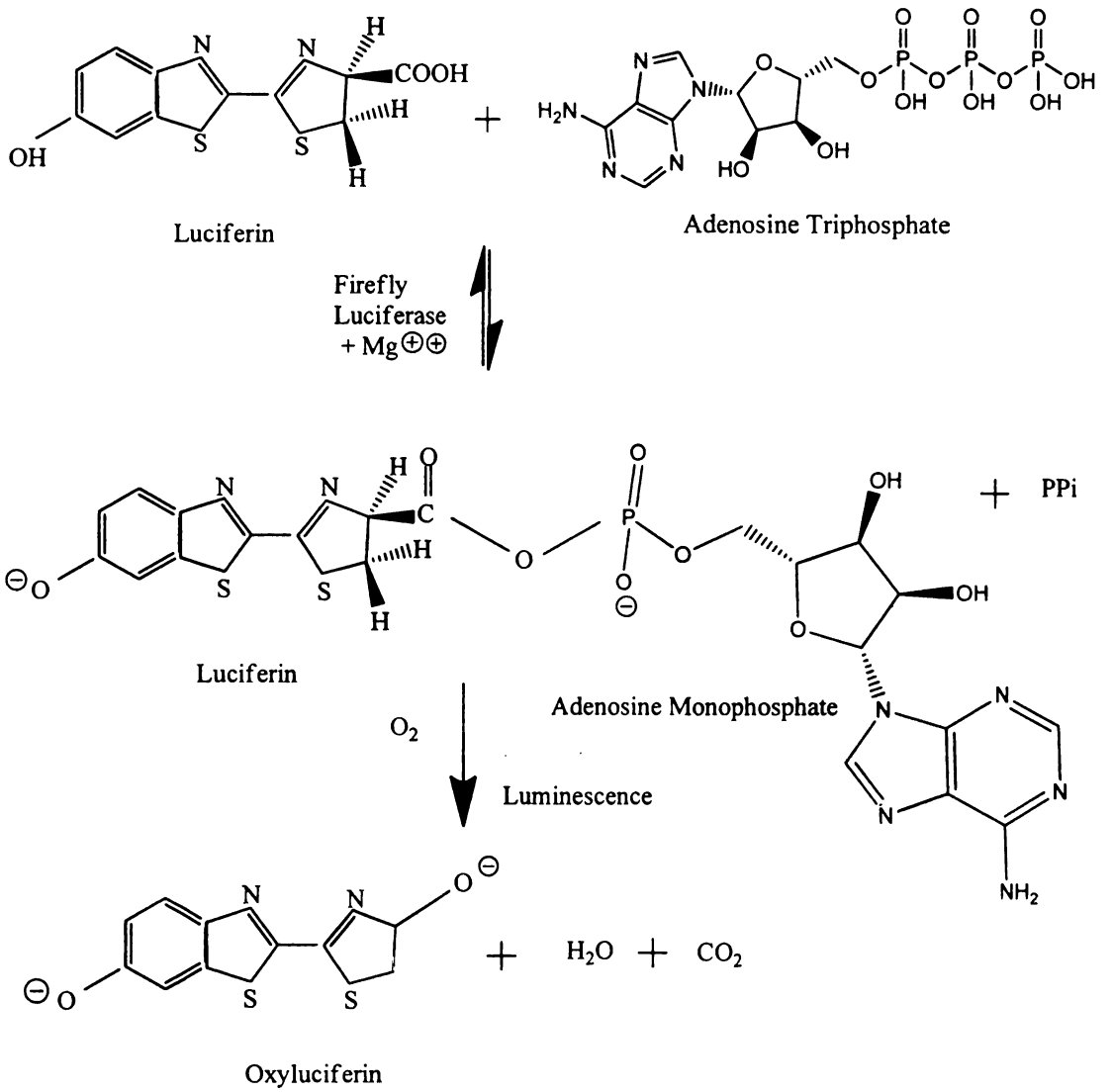

Figure 10. The two-step bioluminescence reaction between luciferin/luciferase and ATP.

2.3 METHODS

2.3.1 Measurement of ATP Release

For ATP measurements involving RBCs, all samples were diluted to a 7% hematocrit in PSS. In order to determine the ATP release due to deformation, the luciferin-luciferase mixture was placed in a 500 μL syringe. Either the ATP standards or the RBCs were placed in another 500 μL syringe next to the luciferin-luciferase mixture. The syringes were loaded on to a syringe pump and were connected to 50 cm of microbore tubing with an internal diameter of 50 μm and an outer diameter of 362 μm. The RBCs or ATP standards were then pumped through the microbore tubing at a rate of 6.7 μL min^{-1}. The two separate microbore tubes containing either the luciferin-luciferase mixture or the RBCs/ATP standards were combined at a T-junction. The now combined contents flowed through a 10 cm section of microbore tubing having an inside diameter of 75 μm, where the resultant chemiluminescence was detected by a photomultiplier tube (PMT). The PMT was housed in a light-excluding box to minimize ambient light. The tubing over the PMT had its polyimide coating removed in order to create a quartz window, through which the chemiluminescence was detected.

2.3.2 Flow-based Determination of Hypoxic RBCs

RBCs were deoxygenated by purging N_2 gas through 5 mL of a 7% RBC sample at 30 kPa for varying times resulting in oxygen saturations from 6.2% to 66.3%. The hypoxic RBCs were then placed into a 500 μL syringe and loaded on to the syringe pump next to a 500 μL syringe filled with the luciferin-luciferase mixture. The sample and reagent met at the T-junction and produced a chemiluminescence reaction, from which the emission which was measured by the PMT. A schematic of the instrumental setup is displayed in Figure 11.

2.3.3 Non-flow Measurement of Hypoxic RBCs

A setup similar to the flow technique described above was used for studies involving non-flow techniques. A PMT served as the transducer; however, the sample container was a plastic cuvette placed over the PMT. Samples were prepared using addition of RBCs and ATP standards of concentrations 0.25-1.5 μM. To perform the assay, 100 μL of ATP standard and 100 μL of luciferin/luciferase were pipetted into a plastic cuvette. Upon mixing of the ATP and luciferin/luciferase solutions, 15 s elapsed prior to measurement with the PMT setup. This allowed for improved reproducibility and helped to ensure that all measurements were performed at the same time interval. A schematic of the instrumental setup is displayed in Figure 11.

Next, 5 mL of 7% RBCs were then deoxygenated with N_2 gas at 30 kPa for varying times. 100 μL of the hypoxic RBCs were pipetted into the cuvette with 100 μL of the luciferin/luciferase solution prior to measurement of the chemiluminescence by the PMT.

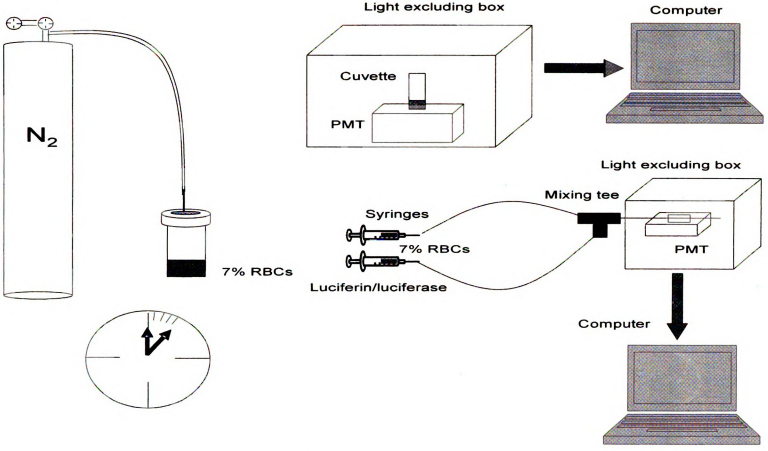

Figure 11. Schematic of deoxygenation technique. The top right schematic in the non-flow setup utilizing a PMT as the transducer. In this construct, only ATP release due to hypoxia is measured. There is no deformation of the RBCs. The bottom setup is the flow-through system using microbore tubing to induce deformation-derived ATP release. In this setup, both hypoxia and deformation-induced ATP release is measured simultaneously. RBCs were deoxygenated by gas purging with N_2 for 4 min.

2.3.4 Measurement of Oxygen Saturation

In order to construct the oxygen saturation curve, a 7% solution of RBCs was prepared. A Clark-type oxygen electrode was immersed into the solution with constant stirring. A Clark-type oxygen electrode has four main components: a teflon membrane, a platinum cathode, a silver anode and an electrolyte solution (KCl solution). Oxygen
diffuses through the teflon membrane to the platinum cathode where it is reduced. The equation for this reduction is displayed below:

$$O_2 + 2H_2O + 2e^- \rightarrow H_2O_2 + 2OH^-$$

Electrons for the platinum cathode are provided by oxidation of the silver anode after a −700 mV potential is applied. The current from the electrons reducing the oxygen is measured and this reading is the indicative of oxygen in solution. The buffer solution is used as a conductive species in between the two electrodes.

2.4 RESULTS

It has been established that RBCs, due to brief periods of hypoxia, release ATP.[4, 5, 19] It is also known that RBCs subjected to mechanical deformation release ATP;[9] however, to date there has been no attempt to investigate these two stimuli simultaneously. By mimicking resistance vessels *in vivo* and inducing both hypoxia and deformation it could be determined if these stimuli of ATP release were additive.

Times for deoxygenation were determined from the oxygen saturation plot as displayed in Figure 12. The largest decrease in oxygen saturation occurs within the first 15 s with a decrease in oxygen saturation from 66.3 to 22.3%. Figure 13 shows the ATP release over the same time span as the oxygenation saturation curve in a non-flow setup (no flow-induced deformation). Hypoxia was induced with N_2 gas at 30 kPa for varying times up to 4 min. The ATP release displayed in Figure 13 is inversely proportional to the oxygen saturation in Figure 12. As the percent oxygen saturation decreased, the ATP release from the RBCs due to hypoxia increased. Not surprisingly, the largest difference in oxygen saturation (between 66.3 and 22.3%) results in the largest incremental change in ATP release (from 0.107 $\pm$ 0.005 to 0.387 $\pm$ 0.016 μM). Upon cell lysis, intracellular ATP is released from the RBCs, greatly increasing the chemiluminescent signal. In order to demonstrate that ATP release is indeed due to hypoxia and deformation, and not cell lysis, glibenclamide was added to the RBCs prior to exposure to hypoxia. Glibenclamide inhibits the cystic fibrosis transmembrane regulator (CFTR) protein located in the RBC membrane. Sprague and co-workers have shown that this protein is involved in the signalling pathway of RBC-derived ATP release.[20] By inhibiting this protein, the ATP release in response to stimuli should decrease if cell lysis has not occurred. Figure 14 A. shows raw data obtained from RBCs, hypoxic RBCs and RBCs incubated with 100 μM glibenclamide for 30 min. Figure 14 B. shows the average release of ATP from the RBCs of n=3 rabbits. In accordance with expected results, the ATP does decrease after incubation with glibenclamide providing evidence that the measured ATP release is not a result of cell lysis.

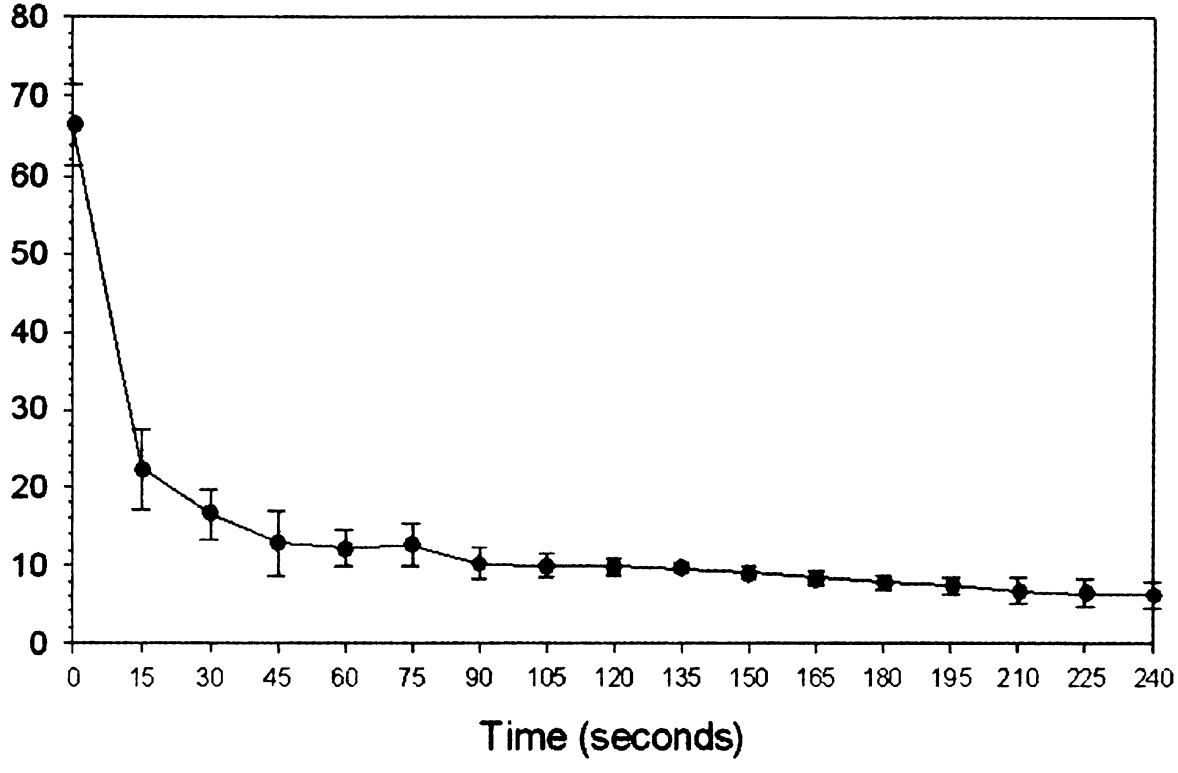

Figure 12. Time study of percent oxygen saturation upon deoxygenation with N_2 gas at 30 kPa for 4 min with measurements taken every 15 s. A Clark-type oxygen sensor was placed into the RBC sample during gas displacement. A thick layer of Parafilm® was placed on top of the tube holding the sensor in place along with inhibiting any interference from the atmosphere. Results displayed are averages from the RBCs of n=3 rabbits. All error bars are $\pm$ SEM.

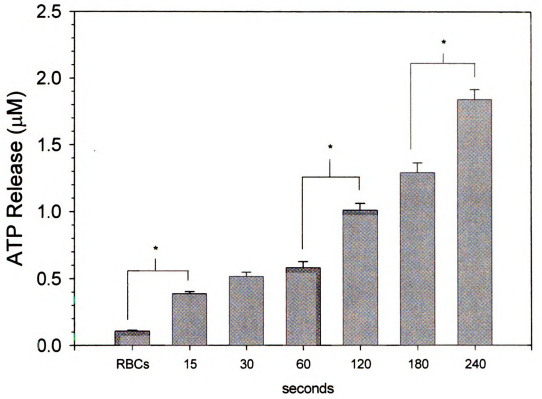

Figure 13. ATP release from RBCs over time in a non-flow system. The initial ATP release is from RBCs in the absence of hypoxia. Upon induction of hypoxia, the RBC-derived ATP is determined at different time intervals up to and including 4 min. A fresh vial of RBCs was prepared for each measurement. All error bars are $\pm$ SEM. Students t-test was performed on data to ensure the means are statistically different ($p<0.05$, $n=3$)

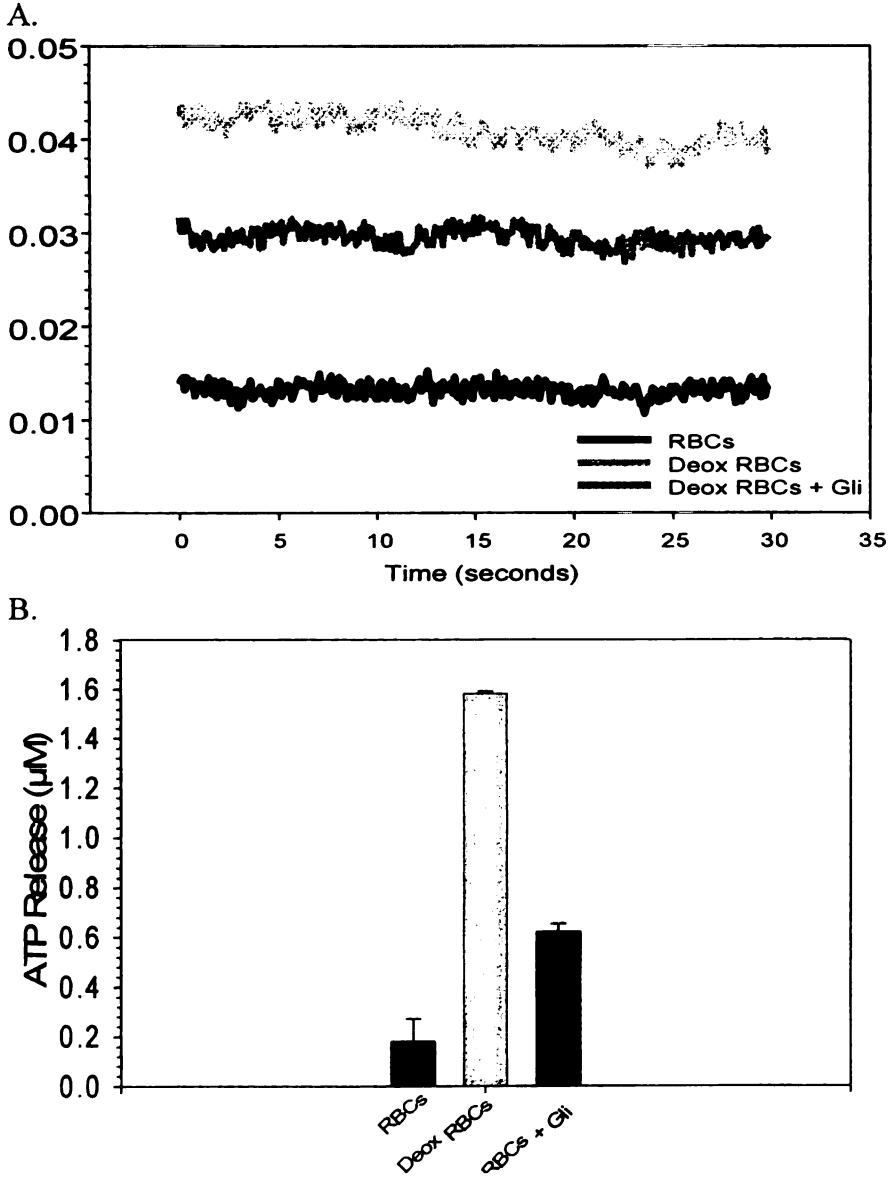

Figure 14. A. Chemiluminescent signals from different RBC samples. The bottom trace represents a 7% hematocrit RBC sample pumped through microbore tubing having an inside diameter of 50 μm. The middle trace represents a 7% hematocrit RBC sample incubated for 30 min with 100 μM glibenclamide (a CFTR inhibitor) then deoxygenated for 4 min with 30 kPa of N2 gas. This sample was then pumped through microbore tubing having an inside diameter of 50 μm. The top trace is a 7% hematocrit RBC sample deoxygenated for 4 min with 30 kPa of N2 gas prior to pumping through the tubing. **B.** Summary of the data shown in A. From the left as shown, the black bar represents ATP release from a 7% RBC sample flowed through microbore tubing having an inside diameter of 50 μm. The light gray bar represents ATP release from a 7% RBC sample deoxygenated with N2 gas at 30 kPa for 4 min prior to introduction to the tubing. The dark gray bar represents a 7% RBC sample incubated with 100 μM glibenclamide for 30 min then deoxygenated with N2 gas at 30 kPa for 4 min. All error bars are ± SEM. Students t-test was performed on data to ensure the means are statistically different ($p<0.001$, $n=3$).

As mentioned previously, both hypoxia and deformation were applied to the RBCs to determine if an additive relationship exists between the two stimuli. In order to provide both stimuli, RBCs were pumped through microbore tubing having an inside diameter of 50 μm after hypoxia was induced by gas exchange with N_2 at 30 kPa for varying times. As stated above, the window of oxygen saturation studies is in agreement with the largest incremental change (66.3-22.3%) in ATP release.

The non-flow set up described above was used to measure the effect of RBC derived ATP release due to hypoxia alone. This ensures that there is no deformation due to flow acting o the RBCs. Figure 15 shows the results of both hypoxia and deformation, and hypoxia alone on ATP release. Most notably, at around 25.0% oxygen saturation (15 s of N_2) the total ATP release does not appear to be additive. If an additive relationship did exist, the ATP release due to deformation and hypoxia (black bars) would always be larger than the corresponding gray bar (ATP release due to hypoxia alone). The data in Figure 15 suggests that hypoxia dominates as the main source of RBC-derived ATP release when oxygen saturation is less than 25%. Furthermore, the apparent non-additive feature also suggests that hypoxia and deformation may release ATP through the same mechanism.

Hemoglobin, the main oxygen carrier *in vivo*, is a component of the RBC membrane. To examine the possibility that hypoxia induces deformation of the RBCs, the cells were stiffened with diamide $[(CH_3)_2NC(O)N=NC(O)N(CH_3)_2]$[21] and then exposed to hypoxic conditions for 15 s. The conformational changes that hemoglobin undergoes from its oxygenated to deoxygenated state may be responsible for hypoxia-

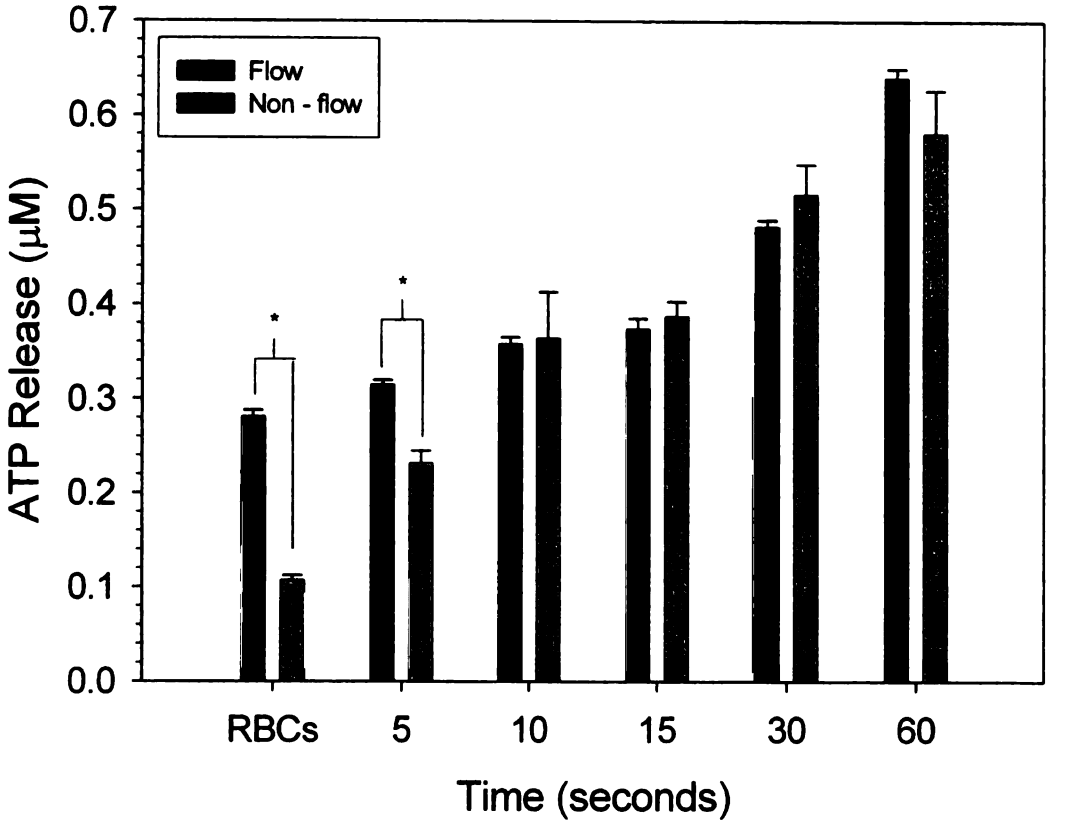

Figure 15. The ability of RBCs to release ATP in the presence of two stimuli: hypoxia and deformation. The black bars represent RBC-derived ATP release from a 5 mL sample of RBCs with a 7% hematocrit deoxygenated for varying times at 30 kPa. The sample was then pumped through microbore tubing having an inside diameter of 50 μm. The gray bar represents RBC-derived ATP release of a 5mL sample with a 7% hematocrit deoxygenated for varying times at 30 kPa. The sample was placed into a cuvette that was placed on top of a PMT and measured in the absence of flow. All error bars are $\pm$ SEM. Students t-test was performed on data to ensure the means are statistically different ($p<0.05$, $n=3$).

induced ATP release through deformation of the membrane. When incubated with RBCs, diamide oxidizes glutathione (the most abundant non-enzymatic antioxidant) to its dimer form. This results in a decrease in antioxidant defenses rendering the underlying spectrin more susceptible to oxidant attack. Moreover, an oxidant attack on the spectrin causes disulfide linkages, leaving the cell stiff. Diamide is favored over other cell stiffeners because it can oxidize the spectrin without affecting the cytosol of the RBCs.[21]

The non-flow setup used for these measurements involving diamide ensures that hypoxia is the only stimulant of ATP release. The data in Figure 16 shows that when hypoxic RBCs are incubated for 18 min with 40 μM diamide, ATP release decreased from 0.225 ± 0.040 μM to 0.102 ± 0.010 μM, thereby suggesting that hypoxia evokes less ATP release from stiffened RBCs. These results suggest that hypoxia-induced ATP release from RBCs may actually be a type of deformation on the RBC membrane.

2.5 DISCUSSION

Mechanical deformation is known to stimulate ATP release from RBCs through a G-protein mediated pathway.[9] When RBCs traverse the circulatory system, this ATP is released due, in part, to the shear stress imparted to the RBC. It is also known that hypoxia induces ATP release.[5] ATP is a stimulus of the endothelial synthesis of NO, which diffuses to the smooth muscle cells lining the outer vascular

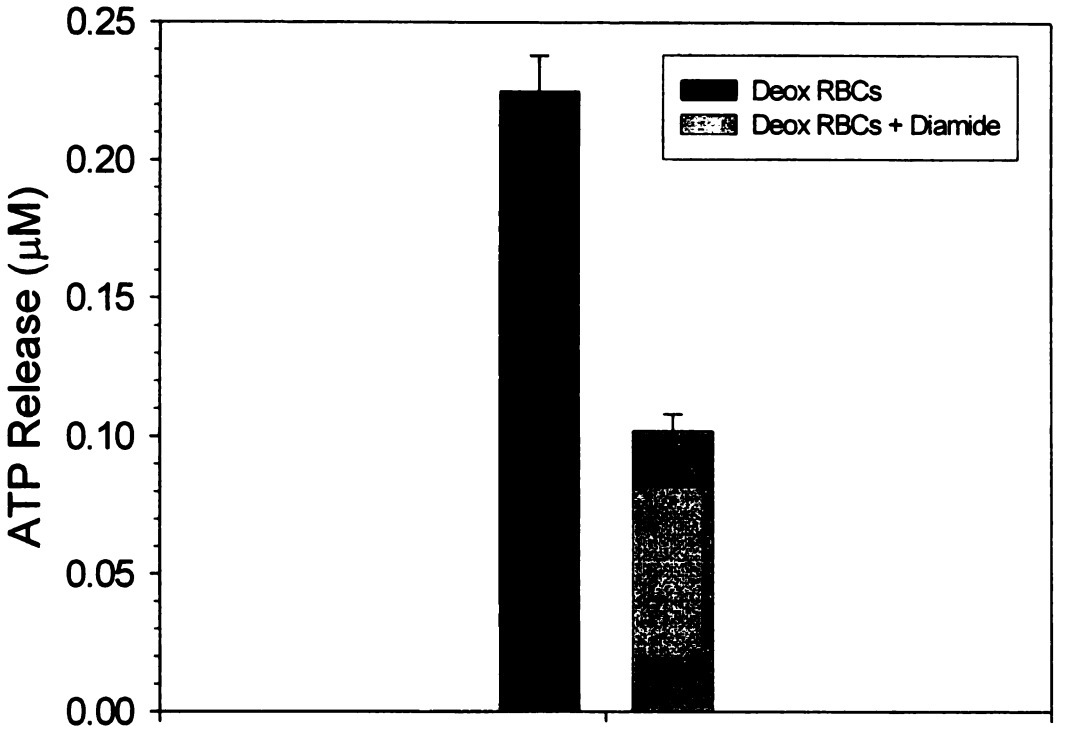

Figure 16. The ability of hypoxic RBCs to release ATP after incubation with 40 μM diamide, a cell stiffener. The black bar represents the ATP release of 5 mL of 3.5% hypoxic RBCs. The gray bar represents 5 mL of 3.5% hypoxic RBCs incubated for 18 min with 40 μM diamide. Deoxygenation of all RBC samples occurred for 15 s with N_2 gas at 30 kPa. The dosage of diamide was kept constant at 200 μL. All error bars are $\pm$ SEM. Students t-test was performed on data to ensure the means are statistically different ($p<0.05$, $n=3$).

wall. This initiates a signalling pathway involving cyclic guanine monophosphate (cGMP) that ultimately results in vasodilation.[6, 8-10] This effect of hypoxia-induced vasodilation ensures uptake of oxygen and other metabolites to deprived portions of tissue, for example, post-ischemic tissue.

Here, a non-additive relationship between hypoxia-induced ATP release and deformation-induced ATP release is established. Due this relationship, ATP released from both stimuli may follow the same signalling pathway for release from the RBC. By using a flow-through system that mimics vessels *in vivo*, both deformation- and hypoxia-induced ATP release from the RBC sample could be determined simultaneously. It was determined that at percent oxygen saturations greater than around 25.0%, deformation due to flow-induced shear is a primary stimulant of ATP release from the RBC. However, below 25.0%, the RBC-derived ATP seems to be primarily due to hypoxia. Therefore, our results suggest that, not only are the two factors non-additive, but that they also may have similar mechanisms of release.

As a possible explanation of this result, it is important to note that haemoglobin is attached to the RBC membrane and undergoes conformational changes due to differing oxygenation states. It is possible that these changes in conformational states induce a deformation on the RBC membrane, therefore, resulting in ATP release. The data in Figure 16 suggest that this is a possibility, although further studies are requires to confirm such a speculation.

Hypoxia-induced ATP release may be a determinant in such diseases as hypertension and diabetes. It has been shown that the RBCs of diabetic and pulmonary hypertension patients release less ATP than those of healthy controls.[15,

[22-24] As previously mentioned, RBC-derived ATP release stimulates NO production ultimately resulting in vasodilation. The decrease in ATP release from RBCs of patients with these conditions may be a factor in why diabetic patients are twice as likely to have a stroke and why their post-stroke complications are often increased by a factor of four.[25] As reported by Ellsworth and co-workers, if the RBC is an oxygen sensor (in addition to delivering oxygen to tissues), then the inability of certain RBCs to respond to hypoxia may impede RBC flow to tissue deprived of needed oxygen.

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CHAPTER 3

3.1 FINDING THE LINK BETWEEN HYPOXIA AND DEFORMATION

Ischemic tissue in need of oxygen will be replenished by the RBC, the oxygen carrier *in vivo*. However, this role for the RBC (oxygen carrier) is not singular. As mentioned in chapter 1, the RBC is a determinant in blood flow in both the systemic and pulmonary circulation via its ability to release adenosine triphosphate (ATP).[1] Upon binding of ATP to the P_{2Y} purinergic receptor located on the endothelial cell surface, endothelial nitric oxide synthase (eNOS) is stimulated resulting in the increase of the vasodilator nitric oxide (NO) and subsequent increased vessel dilation.[2, 3]

The RBC has been shown to release ATP in response to various chemical and physical stimuli. For example, pharmacological agents such as iloprost have been shown to increase the release of ATP from the RBC due to activation of the prostacyclin (IP) receptor found on the RBC.[4-6] Other G-protein activators such as mastoporan have also been shown to stimulate the release of ATP from the RBC.[7] Recently, our group has demonstrated that metal-activated C-peptide, a substance co-released with insulin, also has the ability to stimulate ATP release from the RBC through an increase in cellular glycolysis.[8]

In addition to the stimuli listed above, it is well-established that hypoxia and deformation of the RBC will result in the release of ATP from these cells.[1, 5, 9-11] A mechanism describing the secretion of ATP from RBCs has been proposed for deformation-induced release.[5] In chapter 2, we reported a method for quantitatively measuring the effects of hypoxia- and deformation-induced RBC-derived ATP.[12] From these studies, it was concluded that ATP release from the RBC due to hypoxia and deformation were not additive, suggesting a possible synergy between the two stimuli. Subsequent studies demonstrated that a stiffened RBC released a decreased amount of ATP upon being subjected to a hypoxic environment.

Ellsworth reported over a decade ago that the RBC may serve as both an oxygen deliverer and oxygen sensor.[1] Such conclusions, coupled with our previous work involving hypoxia-induced ATP release from stiffened RBCs shown in the previous chapter, suggest that hemoglobin (the carrier of oxygen in the RBC) may be a determinant in the ATP release mechanism. Hemoglobin changes conformational states upon deoxygenation from the R (relaxed) state to the T (tense) state.[13] Due to its binding to the RBC membrane through the acidic amino-terminal segment of the band 3 protein, it may be possible that the conformational change of hemoglobin induces a temporary deformation of the RBC membrane.[14] These findings suggest that RBCs subjected to hypoxia undergo a deformation that results in G-protein activation and subsequent release of ATP via a pathway previously proposed by Sprague.[5]

In 1996, Sprague *et. al.* determined mechanical deformation results in ATP release from rabbit and human RBCs. Since that discovery, the signaling pathway or mechanism for the release of ATP through an RBC membrane has been extensively studied. It has been suggested that ATP leaves the RBC through an "ATP binding cassette",[15] with the first ATP binding cassette suggested to be CFTR.[16]

It was first discovered in 1995 that CFTR may play a role in the release of ATP from RBCs;[17] however, its role as either an ion channel regulating ATP efflux[18, 19] or having no involvement in ATP release[20-22] was incomplete. Regardless, through the use of varying filter pore sizes to induce deformation on the RBC and the inhibition of CFTR with pharmacological agents, Sprague *et. al.* determined that CFTR was required in the release of deformation-induced ATP release.[16]

CFTR is reported to be stimulated by an increase in cAMP.[23-25] cAMP is generated by adenylyl cyclase, which is regulated by the heterotrimeric G-proteins.[26-29] Therefore, a widely accepted signaling pathway was elucidated starting with the heterotrimeric G protein G_S, and ending with the transmembrane protein CFTR. Through the use of western blot analysis and various pharmacological agents, a complete signaling pathway for deformation-induced ATP release was reported.[5]

G-proteins, G_S and G_i, were identified as the first proteins in the pathway. This G-protein is activated through a G-protein coupled receptor (GPCR) mechanism. Upon activation, the GPCR and G-protein couple together activating the G-protein.[30]

Once activated, the G protein regulates adenylyl cyclase, which is responsible for the conversion of ATP to cAMP. The formed cAMP then binds to two regulatory sites on protein kinase A facilitating its release from the catalytic subunits.[31] This activates protein kinase A and the subsequent phosphorylation of CFTR. CFTR aids in the release of ATP through chloride exchange; however, the protein ATP uses to traverse the membrane is not known. A schematic of this pathway is displayed in Figure 17.

In chapter 2, we defined a non-additive relationship between the ability of hypoxia and deformation to stimulate ATP release. the two stimuli. Moreover, upon stiffening of the RBCs with diamide and subsequent exposure to hypoxia, the ATP release decreased. These data, along with the knowledge of hemoglobin attached to the

RBC membrane and changing conformations upon exposure to varying oxygen tensions, led to the hypothesis that hypoxia is a form of deformation on the RBC. Moreover, this leads to the assumption that hypoxia-induced ATP is released through the deformation-induced signaling pathway for ATP release.

Here we provide data suggesting hypoxia-induced ATP release from RBCs follows the same signaling pathway as deformation-induced ATP release. Moreover, the origin of this claim stems from the idea that hypoxia causes a deformation on the RBC membrane through the conformational changes hemoglobin undergoes during varying oxygen tensions.

3.2 EXPERIMENTAL

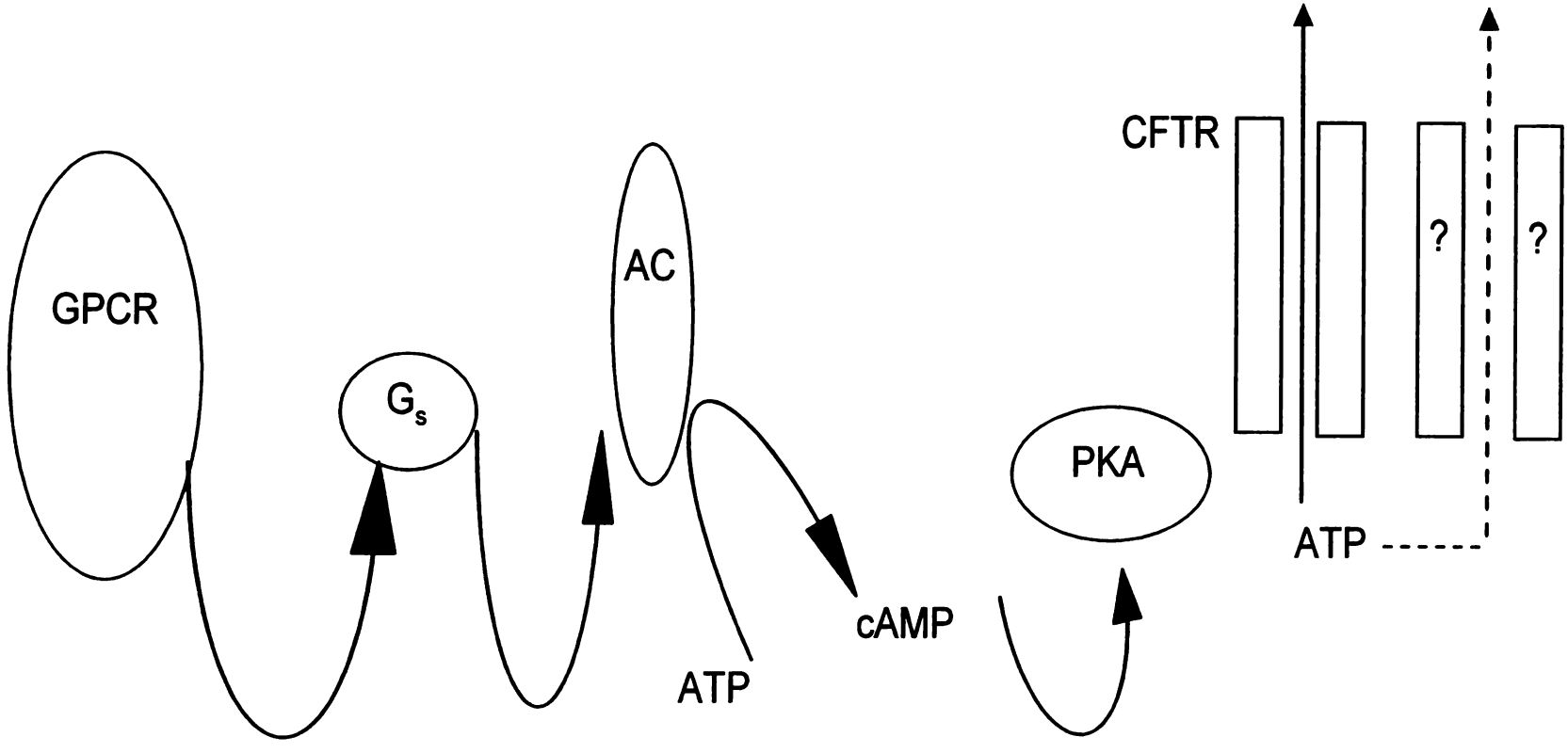

Figure 17. Schematic of the signaling pathway for deformation-induced ATP release. Deformation activates the G-protein coupled receptor which activates G_s or G_i through a coupling mechanism. Adenylyl cyclase becomes activated by the G protein and catalyzes the conversion of ATP to cAMP. cAMP then activates protein kinase A which phosphorylates CFTR by a double phosphorylation. Through the release of Cl^- ions from CFTR, ATP is released by another transmembrane protein that is unknown.

3.2.1 Collection of RBCs

Male New Zealand White rabbits (2.0-2.5 kg) were anesthetized with ketamine (8 mg kg^{-1}, intramuscular injection) and xylazine (1 mg kg^{-1}, i.m.) followed by pentobarbital sodium (15 mg kg^{-1}, intravenous injection). A cannula was placed in the trachea and the animals were ventilated with room air at 20 breaths min^{-1} and a tidal volume of 20 mL kg^{-1}. A catheter was placed into a carotid artery for administration of heparin and for phlebotomy. After heparin (500 units, i.v.) animals were exsanguinated. Blood was centrifuged at 500 *g* at 4 ° C for 10 min. The plasma and buffy coat were removed for other experiments. RBCs were then resuspended and washed three times in a physiological salt solution (PSS). The PSS was made by combining 25 mL of TRIS buffer [prepared by mixing 50.9 g of TRIS in 1 L of distilled and deionized water (DDW)] and 25 mL of Ringer's solution (164.2 g NaCl, 7.0 g KCl, 5.9 g $CaCl_2 \cdot 2H_2O$ and 2.83 g $MgSO_4$ in 1 L of DDW). After the addition of 0.50 g of dextrose and 2.50 g of albumin bovine fraction V (fatty-acid free) to the TRIS-Ringer's mixture, the entire solution was diluted to 500 mL with DDW and the pH was adjusted to 7.35-7.45. The PSS was then filtered three times using a 0.45 μm filter.

3.2.2 Preparation of Reagants

Diamide, a known cell stiffening agent, was prepared for incubation with RBCs to render them less deformable. A 2 mM solution was prepared by dissolving 0.009 g of diamide in 25 mL of PSS. Next, 100 μL of the 2 mM diamide was incubated with a 3.5% solution of RBCs. After 15 min of incubation, the RBCs were centrifuged at 500 *g* at 4 °C for 3 min and added to 5 mL of PSS with 1.5 mL of Oxyrase® that was incubated for 30 min at 37 °C. 100 μL of the RBC solution and 100 μL of luciferin/luciferase was added to a cuvette and 15 s was allowed before the measurement was taken using a PMT housed in a light excluding box.

G protein (G_S) inhibition was performed by incubation of the RBCs with pertussis toxin (PTX).[32] A concentration of 1 ng/mL of PTX was incubated with a 3.5% hematocrit of RBCs for 2 h. After 2 h, the PTX and RBC mixture was centrifuged at 500 *g* at 4 °C for 3 min. The supernatant was disposed of and the remaining solution of RBCs was added to 5 mL of PSS containing 1.5 mL of Oxyrase® that had incubated for 30 min at 37 °C . 100 μL of the hypoxic RBCs with PTX were pipetted into the cuvette to determine ATP release as described above.

Inhibition of CFTR was performed by incubating the RBCs with niflumic acid. Niflumic acid was prepared as a 2 mM solution by dissolving 0.141 g in 25 mL of DDW. It was further diluted such that the final concentration incubated with the 3.5% RBCs was 20 μM for 30 minutes after which it was centrifuged at 500 *g* at 4°C for 3 min. The supernatant was disposed of and the RBCs were added to a solution of 5 mL PSS with 1.5 mL Oxyrase® that was incubating for 30 min at 37 °C. The ATP release was then measured as described above.

H-89, a known protein kinase A inhibitor, was brought to a concentration of 1.8 mM by dissolving 5 mg in 5 mL DDW. 108 μL of H-89 was incubated with a 3.5% solution of RBCs for 2 h. The RBCs were centrifuged at 500 *g* at 4 °C for 3 min. The supernatant was disposed of and the RBCs were added to 5 mL of PSS with 1.5 mL of Oxyrase® that was incubated for 30 min at 37 °C. The ATP release was then measured as described above.

N-(Cis-2-phenyl-cyclopentyl)azacyclotridecan-2-imine-hydrochloride (MDL 12330A), a known adenylyl cyclase inhibitor, was prepared at a concentration of 400 μM by dissolving 2.5 mg in 66.3 mL of DDW. 190 μL of MDL was incubated with a 3.5% solution of RBCs for 20 min. The RBCs were centrifuged at 500 *g* at 4 °C for 3 min. The supernatant was disposed of and the RBCs were added to 5 mL of PSS with 1.5 mL of Oxyrase® that was incubated for 30 min at 37 °C. The ATP release was then measured as described above.

A 100 μM stock solution of ATP was prepared by adding 0.0551 g of ATP to 1000 mL of DDW. ATP standards with concentrations ranging between 0 and 1.5 μM were then prepared in PSS from the stock.

To prepare the luciferin-luciferase mixture used to measure the ATP by chemiluminescence, 5 mL of DDW were added to a vial containing luciferase and luciferin. In order to enhance the sensitivity of the assay, 2 mg of luciferin were added to the vial.

3.3 METHODS

3.3.1 Measurement of ATP Release

For ATP measurements involving RBCs, all RBC samples were diluted to a 7% hematocrit. In order to determine the ATP release due to deformation, the luciferin-luciferase mixture was placed in a 500 μL syringe. Either the ATP standards or the RBCs were placed in another 500 μL syringe next to the luciferin-luciferase mixture. The syringes were loaded on to a syringe pump and were connected to 50 cm of microbore tubing with an internal diameter of 50 μm and an outer diameter of 362 μm. The RBCs or ATP standards were then pumped through the microbore tubing at a rate of 6.7 μL min^{-1}. The two separate microbore tubes containing either the luciferin-luciferase mixture or the RBCs/ATP standards were combined at a T-junction. The now combined contents flowed through a 10 cm section of microbore tubing having an inside diameter of 75 μm, where the resultant chemiluminescence was detected by a photomultiplier tube (PMT). The PMT was housed in a light-excluding box to minimize ambient light. The tubing over the PMT had its polyimide coating removed in order to create a quartz window, through which the chemiluminescence was detected.

3.3.2 Non-flow Measurement of Hypoxic RBCs

A setup similar to the flow technique described above was used for studies involving non-flow techniques. A PMT served as the transducer; however, the sample container was a plastic cuvette placed over the PMT. Samples were prepared using

addition of RBCs and ATP standards of concentrations 0.25-1.5 μM. To perform the assay, 100 μL of ATP standard and 100 μL of luciferin/luciferase were pipetted into a plastic cuvette. Upon mixing of the ATP and luciferin/luciferase solutions, 15 s elapsed prior to measurement with the PMT setup. This allowed for improved reproducibility and helped to ensure that all measurements were performed at the same time interval.

Next, 5 mL of 7% RBCs were then deoxygenated with N_2 gas at 30 kPa for varying times. 100 μL of the hypoxic RBCs were pipetted into the cuvette with 100 μL of the luciferin/luciferase solution prior to measurement of the chemiluminescence by the PMT.

3.3.3 Absorption Measurements

Due to the different conformational changes hemoglobin undergoes as a result of differing oxygenation states, hemoglobin absorbs light at different wavelengths. By obtaining the absorbance spectrum of hemoglobin, its conformation state can be determined. Oxyhemoglobin's maximum absorbtion occurs at 542 nm and 578 nm while deoxyhemoglobin's maximum absorption occurs at 570 nm. Spectra were obtained for 0.07% RBCs, 0.07% deoxygenated RBCs, 0.07% RBCs incubated with diamide, 0.07% deoxygenated RBCs incubated with diamide.

3.4 RESULTS

To further investigate the possibility that hypoxia-induced release of ATP is actually a form of deformation, the ATP release from normal and stiffened RBCs after exposure to a hypoxic buffer was measured. The data in Figure 18 shows an increase of ATP release from RBCs from 15 nM to 492 nM when exposed to a hypoxic environment; however, when a separate aliquot of these RBCs were incubated in diamide, the stiffened RBCs decreased back to a value of 243 nM. While this value is still significantly higher than RBCs not exposed to hypoxia, the decrease in ATP release for the stiffened RBCs is also significant. The data in Figure 18 also demonstrates that RBCs stiffened with diamide release ATP in a dosage dependant manner, suggesting that RBC deformability may be a determinant in the cell's ability to release hypoxia-induced ATP.

The possibility exists that the stiffened RBC or diamide itself may prevent the release of oxygen from the RBC; therefore, absorbance spectra were obtained for aliquots of control and stiffened RBCs in normoxic and hypoxic buffers to determine if the hemoglobin in a stiffened RBC would release oxygen differently than a healthy, control RBC. Figure 19a-d display spectra obtained for a solution of 0.07% RBCs, 0.07% RBCs exposed to hypoxia, 0.07% RBCs stiffened with diamide, and 0.07% RBCS stiffened with diamide and then exposed to hypoxia. The spectra cleary demonstrate that the hemoglobin is able to release oxygen when the RBCs are exposed to a hypoxic buffer. The conformational change to the T state, indicative of oxygen loss, is evident by the single absorbance band at about 560 nm. These spectra also provide evidence that, whether or not an RBC membrane is in the normal state or stiffened, the exposure to hypoxia results in the

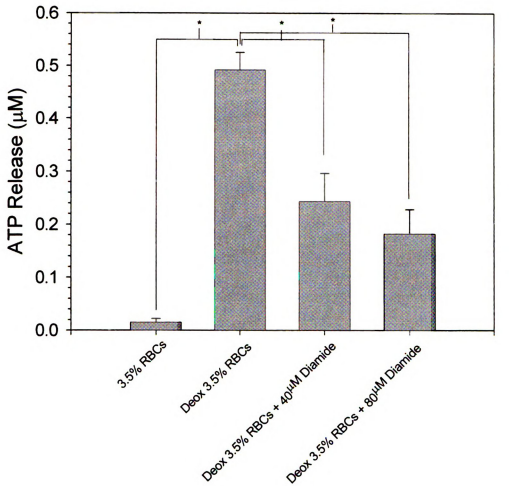

Figure 18. This graph represents the ability of hypoxic RBCs to release ATP after incubation with 40 μM diamide, a cell stiffener. All hypoxic RBCs were deoxygenated with a hypoxic buffer consisting of 1.5 mL of Oxyrase® enzyme system and 3.5 mL of PSS. The first bar represents ATP release of 3.5% normoxic RBCs. The second bar represents the ATP release of hypoxic 3.5% RBCs. The third bar represents hypoxic 3.5% RBCs incubated for 18 min with 40 μM diamide. The fourth bar represents hypoxic 3.5% RBCs incubated with 80 μM diamide. All error bars are ± SEM. Students t-test was performed on data to ensure the means are statistically different ($p<0.05$, $n=3$).

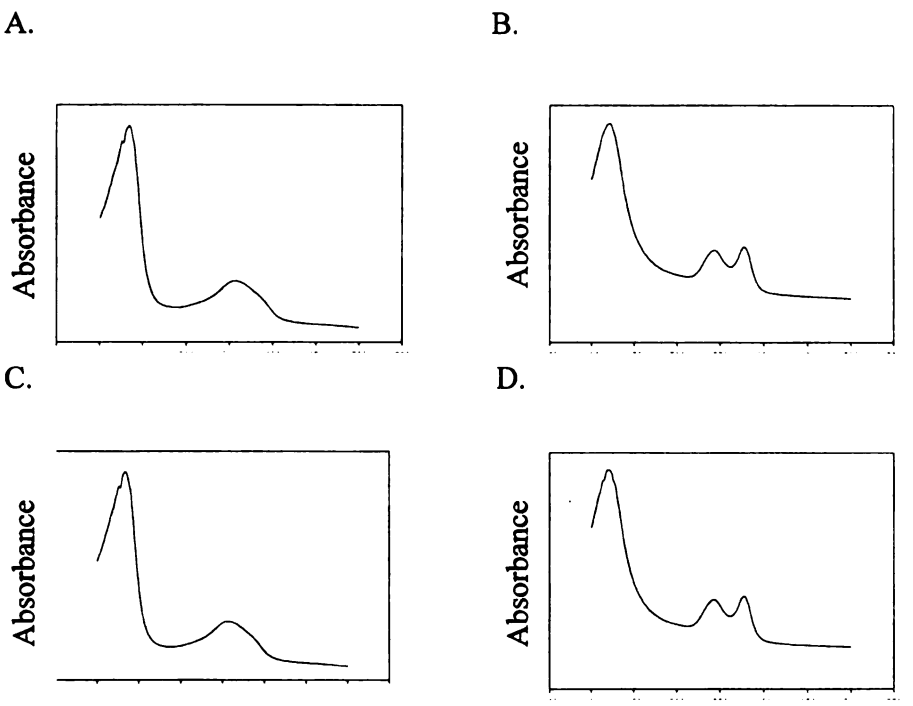

Figure 19. (A.) Visible spectra obtained from a 0.07% solution of normoxic RBCs. **(B.)** Visible spectra obtained from a 0.07% solution of hypoxic RBCs. RBCs were made hypoxic by introduction into a hypoxic buffer solution. **(C.)** Visible spectra obtained from a 0.07% solution of normoxic RBCs incubated with 100 μL of 2 mM diamide. **(D.)** Visible spectra obtained from a 0.07% solution of hypoxic RBCs incubated with 100 μL of 2 mM diamide. RBCs were made hypoxic by introduction into a hypoxic buffer solution after incubation with diamide. This data demonstrates that under hypoxic conditions, hemoglobin changes its conformation to the T state structure, visible by the difference in absorbance spectrum. Moreover, RBCs incubated with diamide and made hypoxic still release the O_2 to become deoxygenated; however, because the cell is rendered less deformable, the conformational change does not induce as much of a deformation; therefore, there is a decrease in ATP release as seen in Figure 18.

loss of oxygen from the RBC.

In order to determine if the pathway for hypoxia-induced ATP release is similar to that described for deformation-induced release, studies were performed to examine the role of G protein and CFTR, two proteins whose activities are required for ATP release from deformed RBCs. Hypoxic RBCs were incubated with 1 ng/mL PTX and the ATP release was measured. PTX inhibits G protein through an ADP ribosylation of the α subunit preventing the interaction of the G-protein coupled receptor with the G protein. The ATP release decreased from 0.850 ± 0.098 to 0.027 ± 0.014 μM, shown in Figure 20. This decrease suggests that G-protein activation is involved in the pathway by which hypoxia induces ATP release from the RBC.

Niflumic acid inhibits CFTR by plugging the channel pore.[33] Hypoxic RBCs were incubated for 30 min with 20 μM niflumic acid, The ATP release after incubation with niflumic acid decreased from 0.850 ± 0.098 to 0.511 ± 0.074 μM as displayed in Figure 20.

H-89 inhibits protein kinase A, the kinase responsible for the phosphorylation of CFTR. H-89 inhibits PKA by competitively binding to the ATP-binding site in a

1:1 ratio.[34] Hypoxic RBCs were incubated with 1.8 mM H-89 for 2 h. The ATP release after incubation with H-89 decreased from 0.850 ± 0.098 to 0.065 ± 0.033 μM as displayed in Figure 20.

Adenylyl cyclase is an enzyme regulated by G proteins. It catalyzes the conversion of ATP to cAMP; therefore supplying the cAMP for protein kinase A.

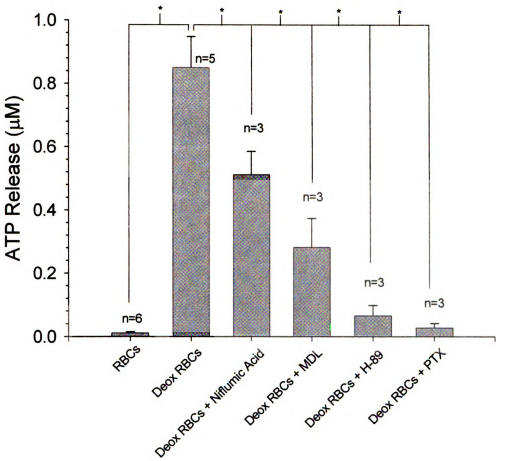

Figure 20. This figure displays the ATP release of the RBCs under the influence of different reagants. The first bar is the ATP release of 3.5% RBC under normoxic conditions. The second bar represents 3.5% RBCs deoxygenated with 1.5 mL Oxyrase® at 37 ºC for 30 min. All hypoxic RBCs were deoxygenated in this manner. The third bar represents hypoxic RBCs icubated with diamide, a known cell stiffener. The fourth bar represents hypoxic RBCs incubated with pertussis toxin, a G protein inhibitor. The fifth bar represents hypoxic RBCs incubated with niflumic acid, a CFTR inhibitor. The sixth bar represents hypoxic RBCs incubated with H-89, a PKA inhibitor. The last bar represents hypoxic RBCs incubated with MDL, an adenylyl cyclase inhibitor. All error bars are $\pm$ SEM. Students t-test was performed on all data to ensure the means are statistically different ($p<0.05$).

MDL inhibits adenylyl cyclase by binding to the hydrophobic region of the plasma membrane and altering the enzyme, more specifically, interacting with the catalytic site of adenylyl cyclase.[35] Hypoxic RBCs were incubated with 400 μM MDL for 20 min. The ATP release after incubation with MDL decreased from 0.850 ± 0.098 to 0.281 ± 0.092 μM as displayed in Figure 20.

3.5 DISCUSSION

Upon oxygenation and deoxygenation, the membrane - bound protein hemoglobin (the main oxygen carrier in the body) changes conformation. The structural difference hemoglobin undergoes in varying oxygen saturations may impact the RBC membrane. Interestingly, it has been reported both deformation and hypoxia induce ATP release.[1, 5, 9-11] It is believed that hypoxia-induced ATP release is a form of deformation-induced ATP release. This RBC-derived ATP serves as a source for NO production. After NO is produced it diffuses to underlying smooth muscle cells where a pathway occurs resulting in vasodilation and subsequent reperfusion of oxygen and metabolites. This response is especially important in oxygenating respiring tissue during stroke or ischemia.

In order to identify hypoxia-induced ATP release as that of deformation, proteins in the signalling pathway for deformation-induced ATP release were inhibited and the RBCs were then exposed to hypoxia. Upon inhibition of every RBC protein in the signalling pathway, the ATP release of hypoxic RBCs decreased significantly. These data suggest dependence for hypoxia-induced ATP release on the signaling

pathway proposed for deformation-induced ATP release. Importantly, these data suggest hypoxia-induced ATP release is a result of deformation of the RBC membrane due to different hemoglobin conformations in carrying oxygen tensions.

The results from this study have implications in the role of the RBC in diseases such as primary pulmonary hypertension and diabetes mellitus. It has been shown that the RBCs of patients with diabetes are less deformable when compared to those of a healthy.[36] Moreover, it has been shown that the RBCs of patients with diabetes and hypertension release less ATP.[37, 38] Interestingly, the occurrence of stroke in patients with diabetes is 1.5-3 times worse, with a greater reperfusion (flow of blood back to respiring tissue) injury.[39, 40] If hypoxia is a form of deformation and the RBCs of patients with diabetes are less deformable and release less ATP, the reason for increased stroke occurrence (or post-stroke complications) is so how could be due to the RBC not responding well (in terms of ATP release) in hypoxic conditions. When RBCs were made less deformable with diamide, less ATP was released in a hypoxic environment. A decrease in ATP release suggests a resultant decrease in NO production, a potent vasodilator. Without the dilation of the blood vessels, more oxygen and metabolites are not able to nourish respiring tissue, therefore, leading to higher reperfusion injury.

These findings also have an impact on the role of drug discovery for patients with diabetes. It was shown that drugs such as Trental and Iloprost increase the release of ATP.[4] Interestingly, Trental is administered to patients with diabetes to help with peripheral vascular disease; however, Trental has been shown to induce ATP

release only after deformation was induced.[4] If hypoxia-induced ATP release is a form of deformation, the administration of Trental to patients with a high risk of stroke may lower their chances of ischemic attack and decrease the reperfusion injury, because of the cell-softening ability of Trental.[4]

Other substances are also known to improve deformability. C-peptide is a 31 amino acid peptide co-released in equimolar amounts with insulin. In patients with type 1 diabetes (no insulin production) C-peptide is not bio-available. It has been previously demonstrated that C-peptide renders an RBC more deformable.[41] It has also been shown C-peptide increases ATP release;[8] therefore, in patients with type 1 diabetes, C-peptide therapy may produce more hypoxia-induced ATP by making the cell more deformable.

Another important drug used for the treatment of sickle cell disease is hydroxyurea. Sickle cell disease is a life long blood disorder in which the RBCs take on a sickle or rod-like shape. Interestingly, patients with sickle cell disease are more susceptible to stroke and hypertension.[42] Moreover, it has been suggested hydroxyurea renders RBCs more deformable.[43] By making the RBC less stiff, these cells may respond to hypoxia more efficiently, therefore, an increase in ATP release and the vasodilator NO.

Conversely, a stiffened RBC, like those of patients with hypertension, will not respond to a drug such as mastoparan 7, which activates the G protein independent of flow-induced shear. If the G protein is activated in a stiffened cell, the deformation

will not be as notable; this would mean the levels of ATP would not be increased. Such therapy would not be useful for patients with stiffened RBCs.

The data presented here demonstrates hypoxia-induced ATP release as a form of deformation-induced ATP release. Moreover, this finding has implications in diseases where the RBC has been found to be less deformable. Interestingly, the occurrence of stroke and post-stroke complications is greater in these diseases. With drug therapy that targets the RBC and renders it more deformable, hypoxia can have a more profound effect on the RBC increasing the ATP release and subsequently dilating vessels through NO production. This would decrease the occurrence of stroke and aid in reperfusion, limiting the injury of tissue post-ischemic attack.

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CHAPTER 4

4.1 CRITICISM OF CURRENT MECHANISMS OF HYPOXIC VASODILATION

Chapter 1 described two conflicting theories that exist to explain the mechanism of hypoxic vasodilation. The first theory orginates from Gladwin *et. al.* and describes the hemoglobin molecule as a nitrite reductase.[1] Under this construct, the hemoglobin molecule reduces nitrite, a bioavailable molecule (~290 nM in RBCs), to NO. In support of this theory, Nagababu and coworkers reported an NO formation when nitrite was added to deoxyhemoglobin and purged with argon; however,[2] it could not be deduced whether the NO formation was due to nitrite reduction. The authors believed that the NO has a weak complex with ferriheme and purging with argon displaced the NO, allowing it to be measured by chemiluminescence. Furthermore, Nagababu states that Gladwin's theory does not account for the high scavenging of NO by hemoglobin. NO has a lifetime of 0.65-1.5 s in the presence of hemoglobin;[3] therefore, the mechanism of NO leaving the RBC from ferriheme predicts it would merely re-attach to another deoxyheme.

There have been other studies suggesting a role for nitrite. Upon injection of nitrite into human forearm, blood flow increased according to a study performed by Cosby *et. al.*[4] A problem arises in the concentrations of nitrite used and if these concentrations are physiologically relevant. When concentrations used were 200 μM, Cosby found nitrite concentrations in the venous blood rose from 176 nM to 2.6 μM. The values found in venous blood are physiologically relevant; however, the

concentration at the site of injection is more concentrated as nitrite becomes diluted when combined with blood. Another study performed using the same parameters by Lauer and coworkers[5] did not observe the same effect; however, they measured blood velocity 1 min after injection while Cosby and coworkers waited 5 min after injection.

One major problem with the idea of nitrite reduction to NO from hemoglobin is the amount of NO that is actually released (not scavenged) and its ability to induce vasodilation. It has been reported by Cosby *et. al.*, the concentration of NO release from RBCs is 47 pM/s or (10^{-3}- 10^{-2} NO molecules $\mu m^{-2} \cdot s^{-1}$).[4] It has also been reported the amount of NO released by the endothelium is on the order of $10^{3} - 10^{4}$ NO molecules $\mu m^{-2} \cdot s^{-1}$.[6] These data suggest the NO released from RBCs, due to reduction from hemoglobin, is not sufficient enough to elucidate vasodilation. Moreover, along with being too small of a quantity, the NO released from RBCs must traverse the cell-free layer present in vessels, the endothelium, the sub-endothelium and make it to the underlying smooth muscle cells where it can elicit vasodilation. Physiologically, this seems highly improbable.

In addition to conversion of nitrite to NO, other theories exist involving the role of RBC and NO release. In 1996, Stamler and co-workers suggested the role of SNO-Hb (S-nitrosylated hemoglobin) in regulating vascular tone under conditions of hypoxia.[7] In short, hemoglobin uptakes NO in the lung along with O_2 and dispenses it in areas of low oxygen saturation. The NO is conserved on a βCys-93 residue and the NO is transnitrosated to the anion exchanger 1 protein (AE1) and then to glutathione or albumin for release from the RBC. It then diffuses to the smooth muscle cell where

it can bind to soluble guanylate cyclase and induce vasodilation. While SNO-Hb has been detected in concentrations of 2.5 μM in the human circulation,[7-10] there exist many disputes with the physiological feasibility of this model.

Based on the widely accepted kinetics of NO, it has been established that NO will react with dioxygen to form nitrate, and that it favors this reaction.[11, 12] In testing this theory, humans that inhaled NO exhibited a blood concentration of ~80 μM methemoglobin (a form of hemoglobin in which Fe is in the Fe^{3+} state not the Fe^{2+}, therefore, cannot bind oxygen) and nitrate, but only 1 μM iron-nitrosyl-hemoglobin and almost undetectable levels of SNO-Hb.[13] This is inconsistent with studies performed by Stamler and coworkers, which contested NO favored iron-nitrosyl heme and SNO-Hb.[14]

One major aspect of the SNO-Hb paradigm involves a role for glutathione (GSH) in the vasodilation effects of SNO-Hb potentiated by hypoxia.[10, 15] This current model is under dispute as recent research utilizing physiologically relevant concentrations of GSH displays an independence on oxygen tensions. In the presence of 1 mM GSH, SNO-Hb induced aortic ring relaxation under normal and hypoxic conditions.[16-19] This increase observed in vessel relaxation under hypoxic conditions[10] may be due to an increase in NO donors induced by hypoxia rather than the conformational changes trans- nitrosylated hemoglobin undergoes to release the NO.[20-23]

Finally, it has been previously mentioned that there exists a need for endothelial nitric oxide synthase (eNOS) in the production of NO. Upon injection with an NO synthase inhibitor, blood flow in human forearm decreased by 25-30%.[24-29] These data suggest a dependence of vasodilation on an NO synthase. In order for vasodilation to occur under Stamler's theory, the NO from SNO-Hb must be released and diffuse through the RBCs, the endothelium and smooth muscle cells to elicit vasodilation (because they contest it is not eNOS-mediated).

These two theories are very complex in their explanations and extensive in their reasoning; however, they still lack depth in many areas. For example, the trans-nitrosylation reported by Stamler *et. al.* requires the removal of one electron, although this mechanism has yet to be shown.[7, 8] In addition, the diffusion of NO through the RBCs, the endothelium and smooth muscle cells does not appear physiologically likely. NO is a reactive species and, as such, would be scavenged before this could occur. The lack of clarity in these areas suggests that another mechanism, such as, ATP-induced vasodilation, may be feasible. Through the use of microfluidic technology and fluorescence microscopy, combined with standard fluorescence measurements, a clear pathway has been revealed involving hypoxia-induced ATP release and subsequent endothelium-derived NO production.

In 2007, a microfluidic array was constructed that allowed for the interaction of multiple cell types, more specifically, between endothelial cells and red blood cells.[30] The array was fabricated with two individual PDMS molds irreversibly sealed together. One PDMS mold had 18 inlet holes punched through, 6 lines of 3 holes

each. A polycarbonate membrane available with varying pore sizes was situated underneath this layer. The second layer of PDMS was placed on the other side of the polycarbonate membrane so there were two layers of PDMS with a polycarbonate membrane embedded between them. The bottom PDMS layer had channels fabricated within and waste ports punched through. RBC flow was introduced into these channels. Bovine pulmonary arterial endothelial cells (bPAECS) were cultured on the polycarbonate membrane in the inlet holes punched through the top layer of PDMS. Flow was introduced with a displacement syringe pump through Tygon tubing. All channels had a width of 150 μm and a depth of 100 μm. 7% RBCs incubated with iloprost, a pharmacological agent that stimulates ATP release, were flowed in the bottom channels at a flow rate of 1.0 μL/min. ATP released from the RBCs diffused through the polycarbonate membrane and stimulated NO production from the endothelial cells cultured on the same membrane. Endothelial cells were pre-incubated with DAF-FM DA, an intracellular fluorescence probe for the detection of NO. Fluorescence intensity of the bPAECs increased from 16.5 $\pm$ 1.0 intensity units to 23.0 $\pm$ 3.5. This increase in fluorescence intensity can be attributed to an increase in NO production by the bPAECs in response to the increase in RBC-derived, iloprost-induced ATP release. While the fabrication of a vascular endothelium array is a novel finding, the use of this device in drug efficacy proves it all the more innovative. The contributions to a blood vessel mimic are indispensable as multiple cell types can be monitored simultaneously and with the attribution of flow.

4.2 EXPERIMENTAL

4.2.1 Collection of RBCs

Male New Zealand White rabbits (2.0-2.5 kg) were anesthetized with ketamine (8 mg kg^{-1}, intramuscular injection) and xylazine (1 mg kg^{-1}, i.m.) followed by pentobarbital sodium (15 mg kg^{-1} , intravenous injection). A cannula was placed in the trachea and the animals were ventilated with room air at 20 breaths min^{-1} and a tidal volume of 20 mL kg^{-1}. A catheter was placed into a carotid artery for administration of heparin and for phlebotomy. After heparin (500 units, i.v.) animals were exsanguinated. Blood was centrifuged at 500 *g* at 4 ° C for 10 min. The plasma and buffy coat were removed for other experiments. RBCs were then resuspended and washed three times in a physiological salt solution (PSS). The PSS was made by combining 25 mL of TRIS buffer [prepared by mixing 50.9 g of TRIS in 1 L of distilled and deionized water (DDW)] and 25 mL of Ringer's solution (164.2 g NaCl, 7.0 g KCl, 5.9 g $CaCl_2 \cdot 2H_2O$ and 2.83 g $MgSO_4$ in 1 L of DDW). After the addition of 0.50 g of dextrose and 2.50 g of albumin bovine fraction V (fatty-acid free) to the TRIS-Ringer's mixture, the entire solution was diluted to 500 mL with DDW and the pH was adjusted to 7.35-7.45. The PSS was then filtered three times using a 0.45 μm filter.

4.2.2 Preparation of Reagants

Diamide, a known cell stiffening agent, was prepared for incubation with RBCs to render them less deformable. A 2 mM solution was prepared by dissolving 0.009 g of diamide in 25 mL of PSS. Next, 250 μL of this 2 mM diamide was incubated with a 3.5% solution of RBCs. After 15 min of incubation, the RBCs were centrifuged at 500 *g* at 4 °C for 3 min and added to 5 mL of PSS containing 1.5 mL of Oxyrase® that was incubated for 30 min at 37 °C. The RBCs were then loaded into a gas-tight syringe and placed onto a syringe pump.

Inhibition of CFTR was performed by incubating the RBCs with niflumic acid. Niflumic acid was brought to a concentration of 2 mM by dissolving 0.141 g in 25 mL of DDW. It was further diluted so the final concentration incubated with 3.5% RBCs was 20 μM for 30 minutes and then centrifuged at 500 *g* at 4°C for 3 min. The supernatant was disposed of and the RBCs were added to a solution of 5 mL PSS with 1.5 mL Oxyrase® that was incubating for 30 min at 37 °C. The ATP release was then measured as described above.

10 mg of spermine NONOate was diluted in 0.01 M NaOH to a concentration of 380 μM. 10 μL of this solution was then added to 1 mL of phosphate buffered saline (PBS). Varying volumes of this solution were then added to Hank's Balanced Salt Solution (HBSS) to bring to a final volume of 1 mL for each standard. Standards were then incubated at 37 °C for 30 min to initiate the release of NO.

4.3 METHODS

4.3.1 Fluorometric Determination of NO

After incubation of standards, 100 μL of 10 μM 4-Amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) was added to each standard and incubated at 37 °C for another 15 min. 700 μL of each standard were then placed into a quartz cuvette and a standard curve was generated on a fluorometer with excitation at 485 nm and emmission at 510 nm. For NO measurements involving RBCs, the RBC samples were prepared as a hematocrit of 3.5%. Normoxic RBCs were centrifuged at 500 *g* at 4 °C for 3 min. 900 μL of supernatant were removed and incubated with 100 μL of 10 μM DAF-FM. 700 μL of the sample were then placed into a quartz cuvette and the NO was measured with the same parameters used for standardization. Deoxygenated RBCs were made hypoxic by incubation with a hypoxic buffer that contained 1.5 mL Oxyrase® enzyme system and 3.5 mL PSS. They were then incubated at 37 °C for 30 min. The hypoxic RBCs were centrifuged at 500 *x g* at 4 °C for 3 min. 900 μL of supernatant were removed and incubated with 100 μL of DAF-FM. 700 μL of the sample were then placed into a quartz cuvette and the NO was measured as described above.

4.3.2 Microfluidic Device Preparation and Measurement of NO

A six-channel microfludic array was fabriated using poly(dimethylsiloxane) (PDMS) to incorporate both flowing RBCs and cultured endothelial cells into the same device. Two individual layers of PDMS were thermocured around a track-etched polycarbonate membrane with pore diameters of 1.0 μm as previously described.[30] Briefly, each individual layer was obtained by baking 10 g of a degassed

20:1 elastomer base to curing agent mixture at 37 °C for 20 min on two seperate silicon wafer masters, one comprised of 6 parallel channels and one free from any raised features. The channels are approximately 200 μm in width by 100 μm high and 3 cm long and obtained using standard photolithographic techniques. A second 10 g degassed 5:1 mixture was poured ontop of each wafer and baked for an additional 20 min before being removed. At which time, inlet holes and waste ports were punched though the channel layer using a 20 gague luer stub adapter (Becton Dickinson, NJ) and a $^{1}/_{8}$" hole punch, respectively. An array of $^{1}/_{8}$" wells, 18 total, were punched through the blank layer of PDMS and after aligning the two layers, they were thermocured around the polycarbonate membrane by baking for an additional 30 min at 37 °C. A schematic of the production of a microfluidic device is displayed in Figure 21 along with a schematic of a microfluidic device displayed in Figure 22.

After completion of the microfluidic array, 10 μL of a 100 μg/mL solution of bovine plasma fibronectin (Invitrogen, CA) was pipetted into each well and incubated for 30 min then placed under UV light for an additional 15 min. before removing remaining liquid. Next, 10 μL of a bovine pulmonary artery endothelial cell (bPAEC) solution (averaging 2.16×10^{7} cells/mL) were pipetted into each well, then incubated at 5% CO_2 and 37 °C for 1 hr before removing and replacing the endothelial cell media (ECM). Endothelial cells were then allowed to incubate at 5% CO_2 and 37 °C for approximately 4 hrs before removing the ECM and replacing it with 10 μL of 5 mM L-arginine (4.4 mg in 5 mL phosphate-bufered (PBS) saline solution) and incubated for 1 hr at 5% CO_2 and 37 °C. The solution was replaced with 10 μL of a

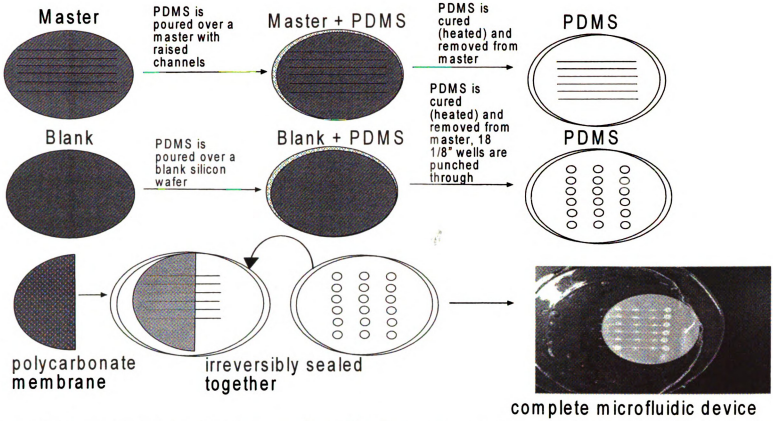

Figure 21. Production of a microfluidic array. First, PDMS is poured over a silicon wafer (master) with 6 channels raised. The master and PDMS are heated for 20 minutes for the PDMS to cure and polymerize. The PDMS is then removed from the master. PDMS is then poured over a second master, with no channels raised, and heated for the same amount of time, for the same reason. This PDMS layer is also removed from the master and 18 1/8" holes are punched through. A polycarbonate membrane is then placed over the PDMS layer with the channels and the PDMS layer with the holes punched through is placed on top. All three layers (PDMS with channels, polycarbonate membrane and PDMS with wells) are combined and heated for 30 minutes in order to irreversibly seal the two PDMS layers together. A photograph of the finished product is displayed after this last step.

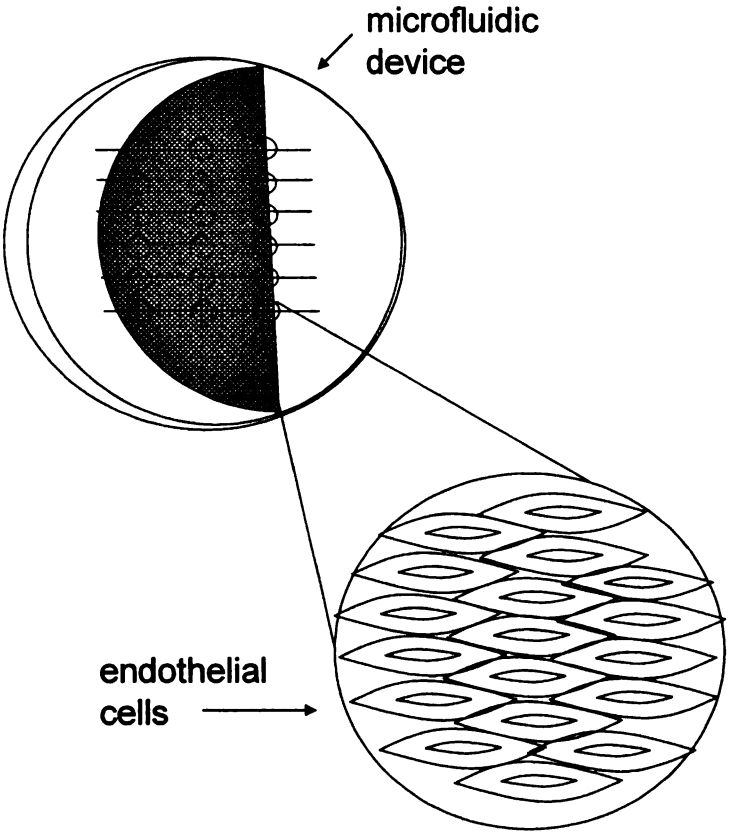

Figure 22. Schematic of a microfluidic device well with endothelial cells. Two layers of PDMS were irreversibly sealed with a polycarbonate membrane between. One layer of PDMS contained channels while the other had 18 wells punched through. Endothelial cells were cultured into the wells on the polycarbonate membrane. RBC solutions were flowed through the channels underlying the wells. Hypoxia-induced ATP release stimulated NO production in the endothelial cells cultured in the wells. The endothelial cells were pre-incubated with DAF-FM DA; therefore, any NO produced would be measured intracellularly with the fluorescence probe.

100 μM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) soltuion in PBS and allowed to incubate for 1 hr at 5% CO_2 and 37 °C. The DAF-FM DA was removed and the bPAECs were washed with PBS once, then 10 μL PBS was pipetted into each well.

Each RBC sample was placed in a separate 500 μL gas-tight syringe and a displacement syringe pump was used to pump each solution for 30 min at a rate of 1.0 μL/min through the underlying microfluidic channels. Fluorescence intensities relating to the intracellular NO producion were imaged using an Olympus MVX fluorescence macro stereomicroscope (Center Valley, PA), and each well was analyzed for pixel intensities. The MVX was fitted with a ET GFP Sputtered Filter Set (Chroma, VT) having λ_{max} excitation and emission wavelengths of 450 and 525 nm, respectively.

4.4 RESULTS

In order to clarify the role of the RBC as an NO donor, RBCs were made hypoxic and NO was measured spectrofluorometrically with DAF-FM as the probe. A standard curve of NO standards was generated and is displayed in Figure 23. The NO released from hypoxic 7% RBCs is displayed in Figure 24.

It was demonstrated in chapters 2 and 3 the RBC releases ATP in reponse to hypoxia. Moreover, it has recently been discovered the eNOS is expressed on RBCs; therefore, the release of ATP from hypoxic RBCs could activate eNOS on

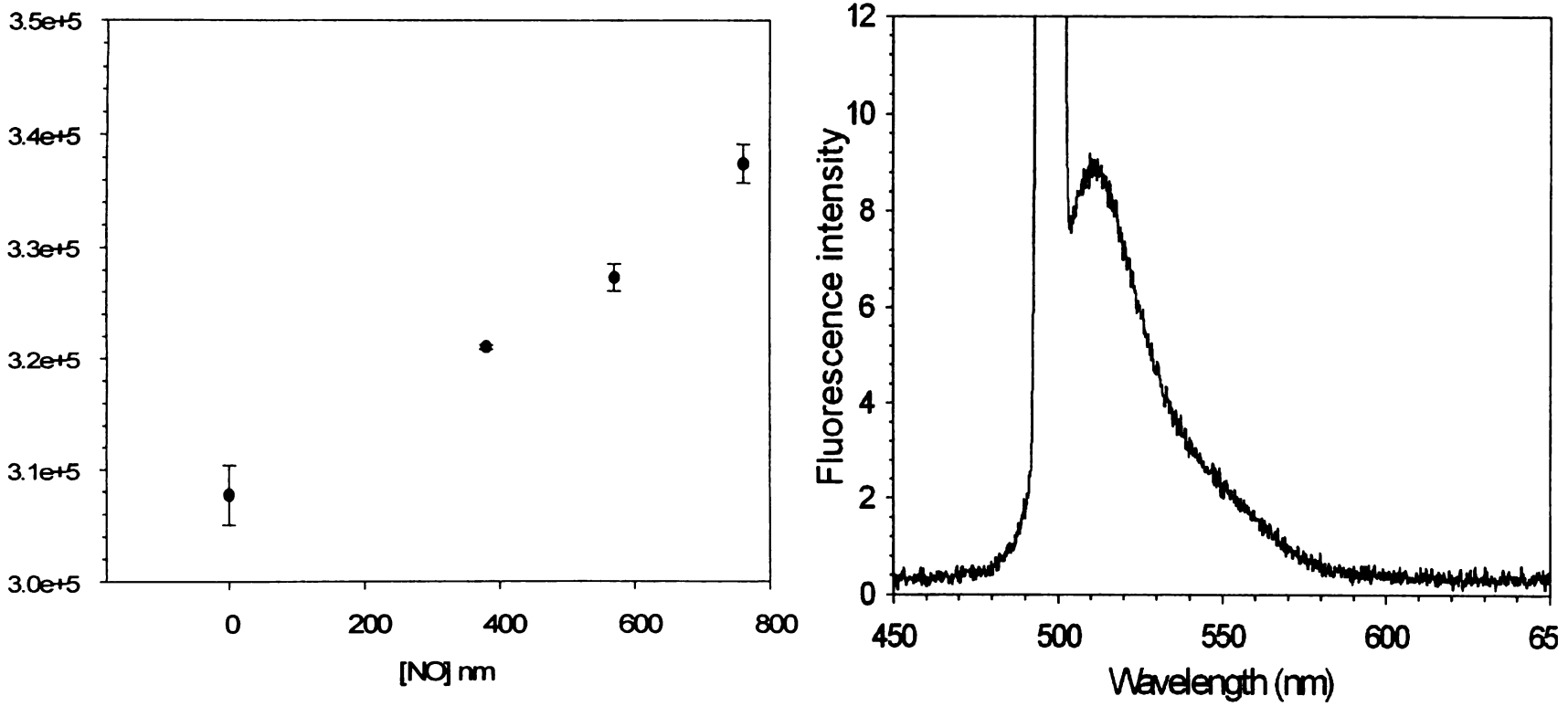

Figure 23. A. Standard curve for NO standards made from Spermine NONOate, 10 μM DAF-FM was incubated with the standards for 15 min prior to measurement. Excitation wavelength: 485 nm. Emission wavelength: 510 nm. **B.** Fluorescence spectrum of DAF-FM and NO.

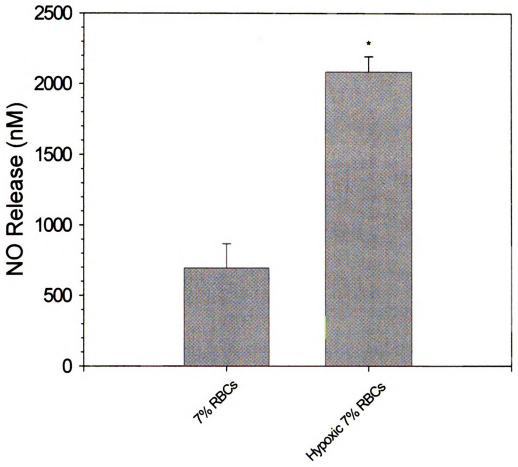

Figure 24. This figure displays the NO release from 7% RBCs under normoxic and hypoxic conditions. 10 μM DAF-FM was incubated with the supernatant of the RBC samples for 15 min prior to measurement. The increase in fluorescence intensity is indicative of an increase in NO release from RBCs under hypoxic conditions. Excitation wavelength: 485 nm Emission wavelength: 510 nm. All error bars are $\pm$ SEM. Students t-test was performed on data to ensure the means are statistically different ($p<0.05$, $n=5$).

other RBCs in the blood stream resulting in NO production from the RBC. Figure 25 displays hypoxic RBCs incubated with L-NAME, an eNOS inhibitor. Upon inhibition of eNOS, NO production is not statistically different from hypoxic RBCs. Moreover, incubation with various inhibitors of hypoxia-induced ATP release, such as diamide and niflumic acid, did not signigicantly alter NO release when compared to hypoxic RBCs. These data suggest an independence of NO release from RBC eNOS and further displays NO is simply released by the RBC rather than produced due to stimulation by ATP.

One of the main objectives of this work was to demonstrate that hypoxia-induced release of ATP was capable of stimulating endothelium-derived NO. A microfluidic device fabricated from soft-lithographic technology, and similar in design to that previously reported by our group,[30] was employed to investigate the intercellular communication between the RBCs and an immobilized endothelium. The images and summarized data in Figures 26 and 27 indicate that endothelium-derived NO is increased in the presence of hypoxic RBCs. Specifically, a 3.5% RBC sample incubated in hypoxic buffer stimulated greater than a 2-fold increase in fluorescence intensity (which is a measure of intracellular NO production within the endothelial cells) compared to RBCs alone. The fluorescence intensity resulting from the RBCs hypoxic environment is decreased by 28.6% and 40.4% when RBCs are first incubated with 100 μM diamide or 20 μM niflumic acid prior to introduction to the hypoxic buffer. This trend was dose dependent as the higher concentrations of diamide and niflumic acid resulted in a greater decrease in fluorecence intensity. Illustrating that the intracellular endothelial NO is

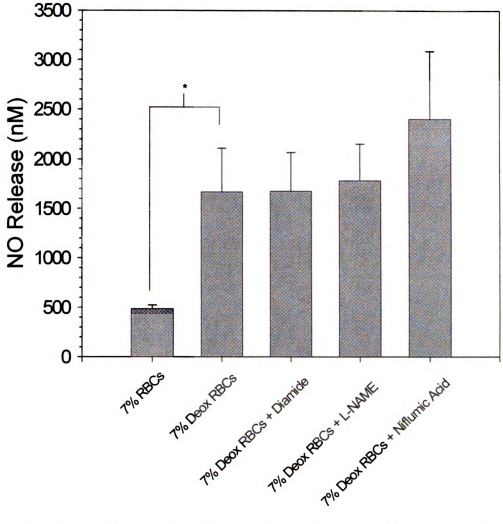

Figure 25. Measured NO release of 7% RBCs in normoxic and hypoxic conditions. Upon incubation with diamide, a cell stiffener, the NO release from RBCs was measured and found significantly similar to hypoxic RBCs alone. It has been established RBCs possess eNOS. Upon incubation with L-NAME, an eNOS inhibitor, the NO release did not decrease. The same trend was observed with niflumic acid, a CFTR inhibitor. These data suggest RBC eNOS does not play a role in the production of NO from RBCs. 10 μM DAF-FM was incubated with the supernatant of the RBCs samples for 15 min prior to measurement. Excitation: 485 nm Emission: 510 nm. All error bars are ± SEM. Students t-test was performed on data to ensure the means are statistically different ($p<0.05$, $n=3$).

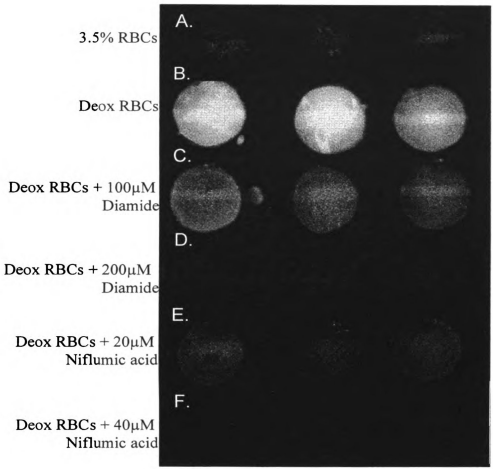

Figure 26. Fluorescence intensity of NO produced from bPAECs in response to hypoxia-induced ATP release. **A.** represents 3.5% normoxic RBCs. **B.** represents hypoxic 3.5% RBCs. **C.** represents hypoxic 3.5% RBCs incubated with 100 μM diamide, a cell stiffener. **D.** represents hypoxic 3.5% RBCs incubated with 200 μM diamide. **E.** represents hypoxic 3.5% RBCs incubated with 20 μM niflumic acid, a CFTR inhibitor. **F.** represents hypoxic 3.5% RBCs incubated with 40 μM niflumic acid. These data suggest the dependence of endothelial-derived NO production on hypoxia-induced ATP release. The NO released from the endothelial cells can then diffuse to the underlying smooth muscle cells and elicit vasodilation. 100 μM DAF-FM DA was incubated with the endothelial cells for 1 h. Data represents an n=3. All error bars are $\pm$ SEM. Students t-test was performed on data to ensure the means are statistically different.

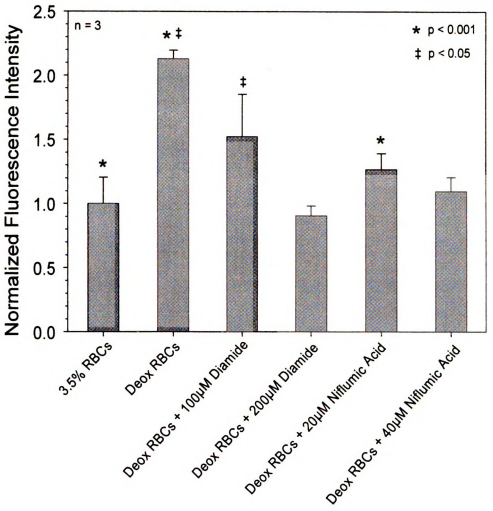

Figure 27. Graphical representation of the data in Figure 6. Endothelium-derived NO increased after upon stimulation of hypoxia-induced ATP release. The addition of diamide, a cell stiffener, decreased the measurable NO is a dose dependant manner. Furthermore, the addition of niflumic acid, a CFTR inhibitor, decreased the measurable NO, also in a dose dependant manner. All error bars are $\pm$ SEM. Students t-test was performed on data to ensure the means are statistically different.

dependent upon the RBC-derived ATP release, which is suggested here to be a function of the hypoxic environment. Moreover, the NO released from RBCs was not eNOS-mediated as NO concentration did not decrease after inhibition of eNOS with L-NAME.

4.5 DISCUSSION

The topic of hypoxic vasodilation has been disputed for many years. Two main, conflicting theories involve hemoglobin as a nitrite reductase[21] or the nitrosylation of NO to become SNO-Hb.[7] Both theories present valid reasoning for vasodilation in hypoxic conditions; however, both theories have yet to describe a full mechanism. For example, Stamler presents data using an aortic ring assay.[19] The aortic ring assay has been used to study vessel tone for many years. In 1998, Louis Ignarro won the Nobel Prize in medicine or physiology for his finding of NO as the EDRF. The aortic ring assay led to this discovery. It consists of a segment of a aortic ring mounted on a stirrup housed in tissue baths filled with a PSS. Reagents are added to the PSS containing the vessel and changes in isometric tension are measured with a pressure transducer and recorded with a polygraph. In many studies, the vessel can be modified to elude a certain effect of a reagant, for example, in Stamler's study the vessel was denuded of an endothelium and NO was pumped through the vessel to measure dilation and prove the endothelium is unnecessary in eliciting a dilatory response. However, in the absence of an endothelium the NO would have less interference when diffusing to the smooth muscle cells; therefore, an even greater

dilation would occur because all NO would be available for the smooth muscle cells to use for relaxation. In a sense, trying to disprove the theory of ATP-stimulated, NO-mediated hypoxic vasodilation, it was actually further concluded. Furthermore, the vessel is in a bath of PSS in which reagents are added. However, ATP released in the lumen of a vessel activates P_{2y}, which stimulates eNOS and ends in relaxation; however, ATP applied to the outside of a vessel activates P_{2x} which results in vasoconstriction. Such sensitivities to analytes and the location of their application must be carefully monitored in an aortic ring assay.

The data presented here suggests that ATP released from hypoxic RBCs stimulates NO production in endothelial cells. Through the use of a microfluidic array, endothelial cells were cultured on wells on polycarbonate membrane. 3.5% hypoxic RBCs were flowed in underlying channels. The ATP released from the RBCs due to hypoxia stimulated the P_{2y} receptor on the endothelial cells and subsequent NO production. The NO production was decreased in the presence of pharmacological agents that have been shown to decrease hypoxia-induced ATP release. This suggests a dependence of endothelial-derived NO on hypoxia-induced ATP release. As previously shown in Figure 24, RBCs release NO under hypoxic conditions, therefore, the NO released from the hypoxic RBCs pumped under the endothelial cells may have diffused through the endothelium, reacted with DAF-FM DA and was measured along with NO produced by the endothelium. This may be the case; however, we have also demonstrated inhibitors of hypoxia-induced ATP release (diamide, niflumic acid) do

not affect NO release from RBCs; however, they do affect the concentration of measurable NO in the endothelial cells.

The results shown here in chapter 4 do not validate the method by which RBCs release NO. It does, however, suggest another pathway for hypoxic vasodilation, specifically hypoxia-induced ATP release and subsequent production of NO. This also elucidates a pathway for hypoxic vasodilation as the NO produced from the endothelial cells then diffuses luminally and abluminally. Abluminally, it relaxes smooth muscle cells leading to vessel dilation. The establishment of a mechanism for hypoxia-induced vasodilation may help in the development of therapeutic agents for hypoxia-associated diseases such as sickle cell anemia, stroke and pulmonary hypertension, as discussed in the previous chapter.

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CHAPTER 5

5.1 CONCLUSIONS

For many years, the RBC has been viewed as a "sack of hemoglobin". Since then, its role in disease pathogenesis, as a therapeutic target, and as a regulator of blood flow has been extensively studied. It has been shown the RBC can control vascular caliber through a number of different mechanisms. One mechanism is the release of a metabolite into the blood stream, adenosine triphosphate (ATP). Multiple stimuli such as pharmacological agents (iloprost, mastoparan),[1-3] C-peptide[4] and deformation[5] have been reported to increase ATP release from the RBC. Here, the role of hypoxia as an important stimulant for ATP release from the RBC has been investigated.[6]

Hypoxia, an inadequate supply of oxygen to tissue or an organ as a whole, induces many different processes on the vasculature. For example, membrane integrity is compromised in endothelial cells subjected to hypoxia (leading to exposure of the collagen and fibrin rich under layer). Moreover, this compromised membrane integrity results in an increase in the procoagulant state.[7, 8]

Erythropoiesis, an increase in RBC production, has also been linked to low oxygen tensions.[9] When oxygen saturation is less than 10%, erythropoietin levels begin to increase, resulting in increased RBC production, of course this leads to more oxygen delivery.[10]

As mentioned previously, one major mechanism for reperfusion (blood flow) under hypoxic conditions is the release of ATP. Once ATP is released into the blood stream, it activates the purinergic receptor P_{2y} on the endothelial cells lining the vessel wall.[11] Upon activation, intracellular [Ca^{2+}] is increased through the phospholipase-C/inositol-1,4,5-triphosphate pathway.[12, 13] This increase in [Ca^{2+}] induces a conformational change on endothelial nitric oxide synthase (eNOS), an enzyme responsible for the production of NO. This NO can diffuse to the underlying smooth muscle cells, where it activates soluble guanylate cyclase, which converts cyclic triphosphate to cyclic guanosine monophosphate (cGMP).[14] Subsequently, the cGMP results in smooth muscle relaxation. It is under this construct that hypoxia-induced ATP release can elicit vessel relaxation, thereby rendering the RBC as both an oxygen sensor and oxygen delivery mechanism.[15]

A signaling pathway for deformation-induced ATP release has been proposed.[2] Through the activation of a G-protein, G_s or G_i, adenylyl cyclase is activated and converts ATP to cAMP. The cAMP then activates protein kinase A which phosphorylates the cystic fibrosis transmembrane regulator (CFTR), which is a required component for the release of ATP from the RBC. Since deformation releases ATP, and stiffened RBCs are associated with certain diseases where perfusion is also a problem, hypoxia seems more problematic with stiffened cells.

Under hypoxic conditions, hemoglobin changes conformations from the R (relaxed) to the T (tense) state. Hemoglobin is connected to the RBC membrane

through the band 3 protein. We hypothesize the change in conformational states of hemoglobin induces a deformation on the RBC membrane. This deformation is what results in the ATP release; therefore, we hypothesize hypoxia-induced ATP release is really a form of deformation.

5.1.1 A Non-Additive Relationship Between Hypoxia and Deformation-Induced ATP Release

To test the hypothesis if hypoxia and deformation are non-additive, we set up a microvessel mimic that induced both hypoxia and deformation simultaneously on the RBCs was used to investigate total ATP release. Through the use of fused silica microbore tubing having an inside diameter of 50 μm, ATP release was induced by deformation. Hypoxia was induced by gas exchange with N_2 gas for 4 min at 30 kPa. The RBCs were placed in a syringe that was placed on a syringe pump and flowed at a rate of 6.7 μL/min. Another syringe was filled with a luciferin/luciferase mixture and flowed along side the RBCs. Both mixtures met at a T-junction and a chemiluminescence reaction occurred between the released ATP and the luciferin/luciferase mixture. The mixed samples continued to flow from the T-junction over a PMT. A window was etched out of the microbore tubing so the light given off by the chemiluminescence reaction could be measured.

To determine the ATP release from hypoxia alone, RBCs were injected into a cuvette mounted over a PMT. An aliquot of the luciferin/luciferase mixture was injected into the cuvette along with the RBCs. After 15 s, the measurement was

obtained. The results from this study are displayed in chapter 2. Hypoxia and deformation displayed a non-additive relationship, suggesting that both stimuli follow the same signaling pathway.[16]

5.1.2 Defining the Signaling Pathway for Hypoxia-Induced ATP Release

As mentioned previously, a signaling pathway for deformation-induced ATP release has been proposed.[2] This signaling pathway was shown in chapter 3. To determine if this pathway is the same for hypoxia-induced ATP release, all proteins or enzymes in the pathway were inhibited and exposed to hypoxic conditions. If hypoxia appears to be a form of deformation as the ATP release decreased upon inhibition of any protein in the proposed deformation-induced pathway. As shown in chapter 3, inhibition of the signaling mechanism and subsequent exposure to hypoxia resulted in a decrease in ATP release. Moreover, upon stiffening of the RBCs with diamide, a known cell stiffener, and exposure to a hypoxic environment, the ATP release decreased in a dosage dependant manner. This data further confirms our hypothesis.

5.1.3 Hypoxic Vasodilation

Hypoxic vasodilation is a phenomenon that has been under intense investigation for the past 15 years. Two conflicting theories have been proposed. First, the theory of hemoglobin acting as a nitrite reductase has been proposed by Gladwin.[17] The authors report that deoxyhemoglobin, at p50, converts nitrite, already

in high concentrations in the blood, to NO. This NO then diffuses through the endothelium, sub-endothelium, and to the underlying smooth muscle cells. The second theory proposed by Stamler suggests hemoglobin becomes nitrosylated in the lungs and conserves the NO on a β93 cysteine residue. Upon entering a hypoxic environment, hemoglobin changes its conformation in order to induce the release of the NO. The NO then diffuses through the endothelium, the sub-endothelium and to the smooth muscle cells where it elicits vasodilation.[18] While both these theories are extensive in their reasoning, they raise some questions, physiologically.

Here, it was hypothesized that hypoxic vasodilation occurs due to an ATP release from the RBC as opposed to NO donation. To test this theory, microfluidic devices were utilized. Microfluidic devices have gained increasing popularity since their discovery in the early '90s. Due to their small size and the ability to mimic vessel diameters in a real time flow-through system, they are ideal for mimicking the vasculature.

A microfluidic array designed and fabricated by our lab was utilized. RBCs and hypoxic RBCs were delivered through channels underlying a porous polycarbonate membrane. Bovine pulmonary arterial endothelial cells (bPAECs) were cultured on the membrane. RBCs incubated with diamide and niflumic acid, a CFTR inhibitor, were also delivered in the underlying channels (chapter 3 showed these two reagants inhibit hypoxia-induced ATP release). Upon inhibition of ATP release, NO production was also decreased, suggesting that production of the vasodilator NO is dependant on ATP release from RBCs under hypoxia.

Summarily, this thesis demonstrates the pathway for hypoxia-induced ATP release is deformation-based and that hypoxic vasodilation is stimulated by this release, as opposed to RBC donation of NO.

5.2 FUTURE WORK

It has been previously demonstrated that the ATP release from RBCs obtained from patients with varying diseases increased or decreased. For example, the Spence group has measured the ATP release from patients with multiple sclerosis and found it increased when compared to RBCs of normal patients. Moreover, it has been established that patients with multiple sclerosis have higher NO levels in their cerebral spinal fluid that may lead to demyelination.[19]

Another disease where ATP plays a role is diabetes mellitus. It has been established the RBCs of patients with diabetes are less deformable[20] and release less ATP.[21, 22] This may decrease the production of NO and its vasodilatory effects and may be a reason for the higher occurrence of stroke and hypertension in patients with diabetes.[23] Based on these ATP release profiles, the RBC itself may be used as a biomarker for diabetes and related diseases such as cardiovascular disease.

Diabetes mellitus, literally translated to "sweet urine" is a syndrome of disordered metabolism. It is characterized by having a fasting blood glucose level of greater than 140 mg/dl and a non-fasting blood glucose level of greater than 200 mg/dl. In 2007, the Center for Disease Control and Prevention (CDC) estimated about

23.6 million people in the United States (about 7.8% of the population) had diabetes.[24] In patients with diabetes, blood glucose levels are higher than normal due to a decreased sensitivity to insulin or a complete deficiency of the hormone. In liver, muscle, and fat cells insulin is used to uptake glucose where it is stored in the form of glycogen or used in the production of energy through glycolysis. There are three classifications of diabetes: type 1 diabetes, type 2 diabetes, and gestational diabetes.

Type 1 diabetes mellitus (T1DM) results from immune-mediated destruction of the pancreatic islet beta cells. The cause of T1DM is not entirely known, although there are over 20 genes that influence susceptibility to type 1 diabetes, namely, the human leukocyte antigen (HLA) and the insulin gene locus. Therapy for T1DM includes injection of exogenous insulin.

Type 2 diabetes mellitus (T2DM) is described as an "insulin resistant" form of diabetes. Insulin resistance is defined as the inability of insulin to lower circulating glucose. It occurs in about 90% of patients with T2DM and gradually progresses and worsens over the years. Insulin resistance often arises from metabolic abnormalities known as the metabolic syndrome. Other conditions compose the metabolic syndrome including dyslipidemia, hypertension, abdominal obesity, and endothelial dysfunction to name a few. Although the role of insulin resistance to the origin of the metabolic syndrome is unclear, they are strongly associated. Currently, the World Health Organization (WHO) has approved two oral pharmaceutical therapies for T2DM: glibenclamide, a sulfonylurea, and metformin, a biguanide.[25]

Gestational diabetes mellitus (GDM) is a glucose intolerance that appears during pregnancy. Although insulin resistance is apparent in all pregnancies, to a

certain degree, some women cannot neutralize the insulin resistance and therefore develop GDM.[26] In order to diagnose GDM, a 1 hour, 50 g. glucose test is performed and if the resulting plasma glucose level is lower than 140 mg/dL the patient is not considered to have GDM. However, if the plasma glucose level is greater than 140 mg/dL, a second test is performed consisting of a 3 hour, 100 g. glucose test. After 1, 2, and 3 hours the plasma glucose levels are checked and if they are above 180, 155, and 140 mg/dL the patient is diagnosed with GDM. While this is the standard for GDM testing in the United States, the WHO uses a different method for diagnosis.[27-29]

It has been established that microvascular and macrovascular complications are present in prediabetic patients.[30-37] Early detection of diabetes allows for the finding of increased vascular disease.[9-13]

As previously mentioned, the occurrence of stroke in patients with diabetes is 1.5-3 times higher with a worse reperfusion injury.[23] We have demonstrated hypoxia-induced ATP release from the RBCs of diabetic rats is 39.3% less when compared to those of control rats. The results of this study are displayed in Figure 28. For future studies, testing the ATP release *in vivo* from animal models with diabetes after the occurrence of stroke would further elucidate if ATP release plays a role in their reperfusion injury and occurrence of ischemic attacks. Moreover, adding a pharmacological stimulant, such as pentoxyfilline, that increases ATP release could determine a drug therapy.

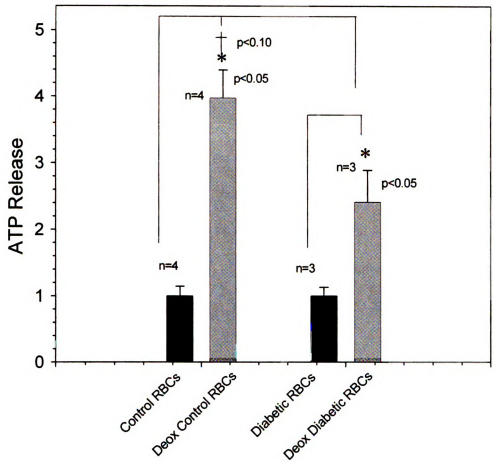

Figure 28. This graph displays the ATP release of control and BB/ZDR/Wor rats (Type 2 diabetic rats) exposed to a hypoxic environment. The ATP release from the RBCs of the diabetic rats decreased by 39.%, this demonstrates a lower ability of the RBCs to elicit vasodilation. Furthermore, this implies the reason for worse reperfusion injury in patients with diabetes.

Moreover, if the RBCs of patients with diabetes are not able to deliver oxygen, this would aid in their higher reperfusion injury. 2,3-DPG is an organic phosphate, produced by glycolysis, that is present on hemoglobin. 2,3-DPG allows hemoglobin to release bound oxygen to respiring tissue. Under conditions of hypoxia, glycolysis is increased; therefore, more 2.3-DPG.[38] If the RBCs of patients with diabetes do not respond as well to hypoxia with ATP release, it may be possible their glycolysis production doesn't increase either. Importantly, many of the glycolytic enzymes are attached to the band 3 protein, the same protein hemoglobin is attached to. Testing the 2,3-DPG levels in the RBCs of patients with diabetes could lead to a better understanding of where the oxygen is being delivered and if it is delivered prematurely in patients with diabetes.

Recent research has been focusing on the mechanism of hydroxyurea, a proven treatment in sickle cell disease. Sickle cell disease is one of the most common genetic diseases in the United States. It occurs from RBCs taking on an abnormal, rigid, sickle shape. Interestingly, this sickling event occurs only when hemoglobin is in the deoxygenated form.

The prevalence of sickle cell disease in the United States is about 1 in every 5,000 people, mostly affecting African-Americans. Due to a single amino acid mutation in hemoglobin, hemoglobin S is formed. This form of hemoglobin is the cause of the physiological conditions presented in this disease.[39] There are four types of sickle cell disease: sickle-cell anemia, sickle-hemoglobin C, sickle beta-plus-thalassemia and sickle-beta-zero thalassemia. The last three forms are very rare.

Previous studies report the mechanism of action for hydroxyurea is through an increase in production of fetal hemoglobin (HbF).[40] Fetal hemoglobin has a higher capacity for oxygen, therefore, more tissue is perfused and less sickling occurs. Moreover, an improvement in the deformability of RBCs in patients receiving hydroxyurea has been reported.[41] Due to the finding that hypoxia is a form of deformation, and hydroxyurea increases deformability, it was hypothesized that the ATP release from RBCs under the influence of both hypoxia and hydroxyurea will increase. Data obtained from this study suggested otherwise. The ATP release from RBCs due to hypoxia and hydroxyurea was at the same concentration as the ATP release from hypoxia alone. Interestingly, our lab has recently discovered an increase in NO production from RBCs pre-incubated with hydroxyurea (submitted data). This led to the idea that NO (which has a higher affinity for hemoglobin than oxygen) was attaching to the heme, releasing the oxygen atom. This would prevent hemoglobin from changes conformations and not allow the deformation to occur; thereby not releasing increased ATP. As shown in Figure 29, the depletion of oxygen after RBCs were preincubated with hydroxyurea occurs more readily. This suggests that NO is replacing the oxygen, leaving free oxygen in the buffer ready to be removed by my Oxyrase® enzyme system. These measurements were taken on a Clark-type electrode. Further studies can provide a more detailed mechanism for this hypothesis. For

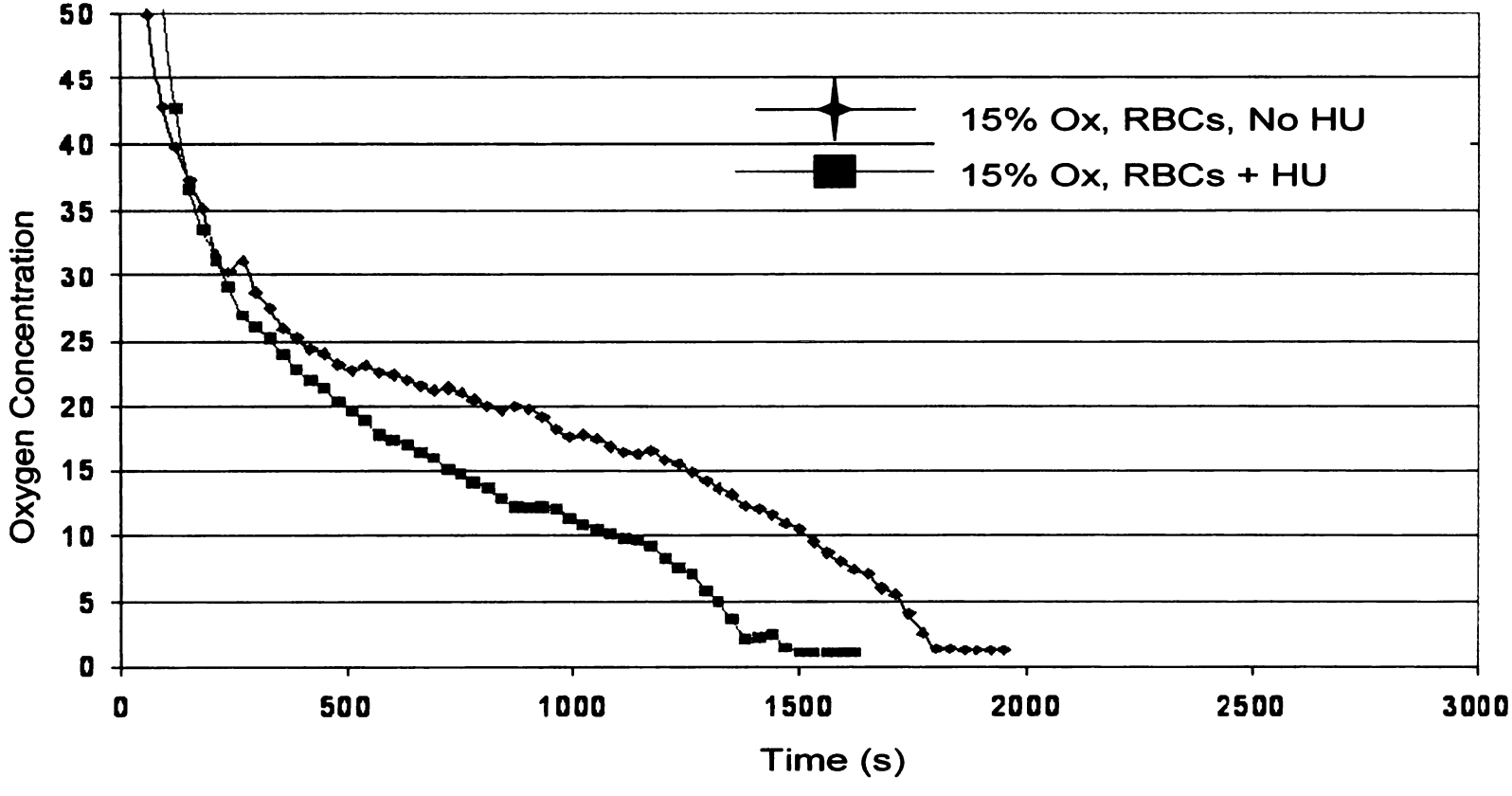

Figure 29. Oxygen saturation curve of hypoxic RBCs and hypoxic RBCs preincubated with 100 μM hydroxyurea. Oxygen was depleted from the RBCs preincubated with hydroxyurea more rapidly than compared to hypoxic RBCs with no reagent added. These data suggest oxygen is removed from hemoglobin at a more rapid pace, indicating the removal of oxygen and the binding of NO to hemoglobin.

example, measuring the concentration of NO on hemoglobin through EPR or IR, or creating a miniaturized oxygen electrode to better detect oxygen saturation.

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