Externalized ATP in intact erythrocyte suspensions: modulation and determination

Andrea Clarke

A Thesis

in

The Department

of

Chemistry and Biochemistry

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science (Biochemistry) at
Concordia University
Montreal, Quebec, Canada

August 2012

CONCORDIA UNIVERSITY
School of Graduate Studies

This is to certify that the thesis prepared
By: Andrea Clarke

Entitled:
Externalized ATP in intact erythrocyte suspensions: modulation and determination

and submitted in partial fulfillment of the requirements for the degree of
Master of Science (Biochemistry)

Complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

__Guillaume Lamoureux_____ Chair

__Xavier Ottenwaelder_____ Examiner

__Peter Pawelek_____ Examiner

__Ann English_____ Supervisor

Approved by _____

Chair of Department or Graduate Program Director _____

_____2012 Dean of Faculty

Abstract

Well recognized for its role as the energy currency of cells, ATP plays an altogether different role in the mammalian vascular system. Erythrocyte-derived extracellular ATP is fast gaining recognition as a vital vasodilator in blood; stimulating a rapid widening of blood vessels when released into the circulation. Currently, the primary technique employed to determine the concentration of ATP released from erythrocytes is the luciferin-luciferase chemiluminescent assay, which is both robust in signal and specific for ATP. However, the assay is subject to interferences inherent to working with erythrocytes, such as the presence of hemoglobin, which filters the emitted chemiluminescence signal.

At the outset, the current work examined the luciferin-luciferase chemiluminescent assay in the microplate format and evaluated the parameters necessary to determine ATP in suspensions of intact erythrocytes. Based on the methodological considerations which arose from the examination of the assay, the concentration of ATP in suspensions of intact erythrocytes was compared to that of their supernatants. It was determined that the ATP released into the supernatants of erythrocyte suspensions represents but a fraction of the ATP which is externalized and accessible for enzymatic reactions. Known stimuli of ATP release, including CO_2 and hypo-osmotic stress, were also used to compare the sensitivity of erythrocytes obtained from humans to those from rats, a common pharmacological model organism: human erythrocytes proved to be more sensitive to stimuli than rat erythrocytes.

Ultimately, a method for measuring ATP in suspensions of intact erythrocytes was developed, and the possibility of two modes of erythrocyte release was discovered. The erythrocyte has long been held as a sensor within the vasculature, and this work underscores the growing importance being placed on the erythrocyte's role as an effector through the regulation of the release of the vasodilator ATP.

Dedication

To Mom and Dad, for your unerring support and the wealth of knowledge and love that you have bequeathed me.

To Alison, my stalwart partner throughout the writing process, and the very best older sister that a person could ever want .

and

To Julien, for being the still, happy place that I can go to every time.

Thank you.

Contents

List of Figures

List of Tables

List of acronyms

ATP	Adenosine Triphosphate
CCAC	Canadian Council on Animal Care
eNOS	Endothelial Nitric Oxide Synthase
GTN	Nitroglycerin
Hb	Hemoglobin
Ht	Hematocrit
K_D	Dissociation constant
metHb	methemoglobin
mOsm	milliosmole
NO	Nitric Oxide
NO_2^-	Nitrite
PSS	Physiological saline solution
RLU	Relative light units
UAREC	University Animal Research Ethics Committee
UHREC	University Human Research Ethics Committee

1. Chapter 1. Introduction

1.1. Erythrocyte ATP and vasodilation

Erythrocytes, of which there are roughly 5 million per microlitre of whole blood (1), are non-nucleated biconcave discs whose major function is to ensure the supply of oxygen and nutrients to cells and tissues as well as the removal of carbon dioxide (CO_2) and metabolic waste products. In erythrocytes, the function of intramembrane cation pumps, maintaining membrane fluidity, and intracellular signalling all depend on adenosine triphosphate (ATP). Erythrocyte ATP is generated primarily through glycolysis, also known as the Embden-Meyerhof pathway and is rather abundant intracellularly, with reported concentrations as high as 2 mM (2). ATP is composed of adenine covalently bound to a ribose monomer which is in turn bound to three organic phosphate groups (Figure 1.1).

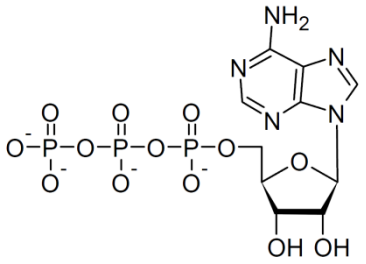

Figure 1.1 Structure of ATP (generated using ChemSketch)

In addition to being the source of energy driving many intracellular processes, ATP is also released into the circulation where it acts as an extracellular signalling molecule. It has been documented that isolated erythrocytes from humans (3) (4) (5)

and rats (5) (6) release ATP in response to low O_2 tension. In the vascular system, the application of low micromolar concentrations of ATP had been found to relax arterial tissues (7).

1.2. NO production via ATP

The control of blood flow in the peripheral circulation through the relaxation and constriction of blood vessels is regulated so as to ensure that the supply of oxygen adequately matches local demand. In working tissues where the demand for oxygen is considerably higher than when at rest, there is a concomitant an increase in blood flow (8). The increased flux of oxygen loaded erythrocytes from the arteries is local (as opposed to throughout the entire body) and occurs as a result of vasodilation (9). The vascular system's capacity to adjust the volume of blood vessels represents a key component of its ability to respond to the metabolic needs of organs and tissues. Vascular tissues (arteries and veins) are composed of several layers of different cell types working in concert to change the diameter of the vessel lumen. The endothelial cell layer (endothelium) lines the inside of blood vessels and as such is exposed to small molecules, such as ATP, that are in transit through the circulatory system. Receptors at the endothelium propagate the signal from circulating molecules to the smooth muscle cell layer, which in turn determines vascular tone. ATP binding to purinergic receptors at the endothelium activates endothelial Nitric Oxide Synthase (eNOS). When active, eNOS produces nitric oxide (NO) by catalyzing the two step oxidation of L-arginine to L-citrulline with oxygen and

NADPH serving as cofactors (Figure 1.2) (10). NO, formerly known as the endothelial derived relaxation factor, is a highly reactive free radical (10) which can readily diffuse to smooth muscle cells. NO initiates a signal cascade by activating guanylyl cyclase which leads to a decreased intracellular calcium concentration and results in smooth muscle relaxation (11).

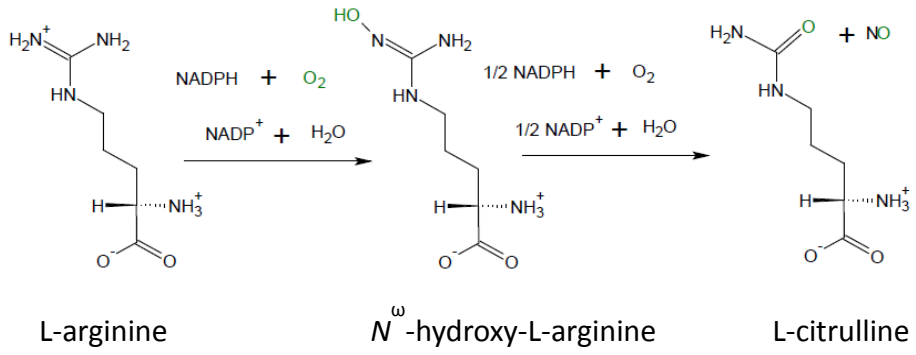

Figure 1.2 Schematization of the activity of endothelial nitric oxide synthase (eNOS). (generated using ChemSketch)

1.3. Nitrite and NO bioactivity

As a freely diffusing small molecule, NO is also able to diffuse out of the endothelium and into the vascular lumen where it is oxidized to nitrite (NO_2^-). Plasma NO_2^- levels reflect eNOS activity and are considered to be an indicator of vascular health (12). In mammals, the concentrations of plasma and erythrocyte NO_2^- are tightly regulated and have been found to be in the 10^2 nM NO_2^- (12). In fact, the erythrocyte has been described as a major storage site for NO_2^- in human blood (13). Recently NO_2^-, which was long considered to be an inert oxidation

product of NO has been found to be vasoactive and has been shown to be reduced back to NO by deoxyhemoglobin (deoxyHb) in erythrocytes (14). However, because of the elevated concentration of hemoglobin (Hb) in erythrocytes, NO produced within the erythrocyte could not be exported, but rather would be scavenged (2). Recently, the English lab group has reported the production of a vasoactive compound following the exposure of erythrocytes to physiologically relevant concentrations of NO_2^- (4). The export of ATP from erythrocytes in response to treatment with NO_2^- represents a potential conservation for NO vasoactivity which is not strictly NO derived.

1.4. Stimulated ATP release from erythrocytes

1.4.1. Deaeration and hypercapnia

The release of ATP from erythrocytes can be triggered by a number of physiological stimuli. The best documented stimulus of ATP release from erythrocyte is decreased oxygen saturation (3) (15) (16) (2). It is fairly common knowledge that Hb changes conformation upon binding and release ligands and in particular O_2 and interestingly, the release of ATP from deaerated erythrocytes has been found to be more closely correlated with the conformation of Hb than with the actual saturation (17). However, recent research has that deoxyHb has a well-defined binding site for the erythrocyte membrane as well as a greater affinity for the erythrocyte membrane than oxyHb (18). DeoxyHb readily competes with glycolytic enzymes which can also bind the membrane (19). This competition gives rise to a regulated compartmentalization of ATP production near the membrane. The export of ATP

from a membrane localized pool, rather than from bulk, cytosolic ATP, has been suggested recently (20). Hypercapnia, elevated CO_2 levels, as a stimulus of ATP release has been best documented when used in conjunction with hypoxia for erythrocytes (3), however, the release of ATP has been reported for other cells types following CO_2 exposure and vasodilation has ensued (21). In addition, CO_2 favors the release of O_2 by Hb through the Bohr effect, promoting the formation of deoxyHb (22). Given these findings, the potential that hypercapnia alone might stimulate ATP release from erythrocytes is high.

1.4.2. Mechanical deformation

Mechanical deformation, a process that erythrocytes undergo when passing through the narrow blood vessels of the microcirculation, has been reproduced experimentally using porous membranes (23) and microbore tubing (16). In addition, hypo-osmotic stress, which induces swelling in erythrocytes, induces ATP release from human (24) and mudpuppy erythrocytes (25). Interestingly, the binding of Hb to the erythrocyte membrane has been characterised as a form of mechanical deformation of the membrane (26).

1.5. Measuring ATP release

The primary method used to assay ATP is using chemiluminescence. Throughout the course of this work, we will use the firefly luciferin-luciferase assay system to quantify ATP. Firefly luciferase catalyzes a reaction between ATP, luciferin, and O_2 to form AMP, inorganic pyrophosphate, oxyluciferin, CO_2, and light (Figure 1.3).

Quantitative studies have determined that the quantum yield of the reaction is close to one photon per molecule of ATP which reacts with a molecule of luciferin (27).

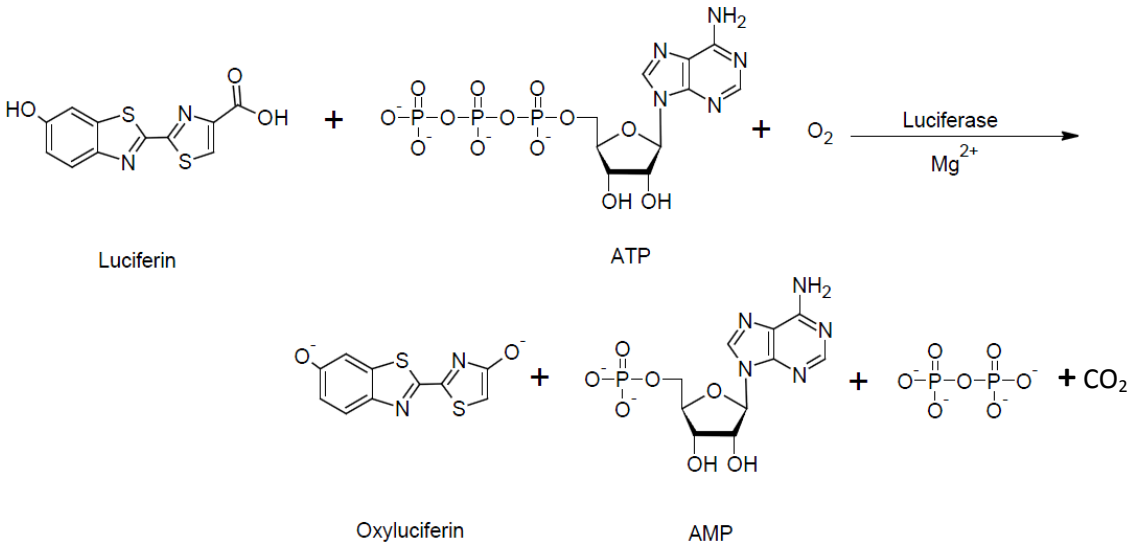

Figure 1.3 Chemiluminescence reaction catalyzed by the enzyme luciferase

D-Luciferin reacts with ATP and molecular oxygen in the presence of Mg^{2+} to produce an excited state oxyluciferin and release AMP, pyrophosphate and CO_2. The relaxation of excited state luciferin produces light in proportion to ATP concentration. (generated using ChemSketch)

This system is robust and very useful as there is a proportional relationship between the amount of ATP and the emitted light (λ_{max}=560 nm). Of twelve naturally occurring nucleoside triphosphates, it has been determined that only ATP is effective and highly specific as a substrate for the light producing reaction of firefly luciferase. The next most effective nucleoside yielded a signal that was less than 2% as that of ATP (28). While other nucleoside triphosphates do not interfere with the assay, Hb and whole blood have been reported to interfere with the assay as Hb absorbs strongly in the visible region in which luciferin emits (29) (Figure 1.4).

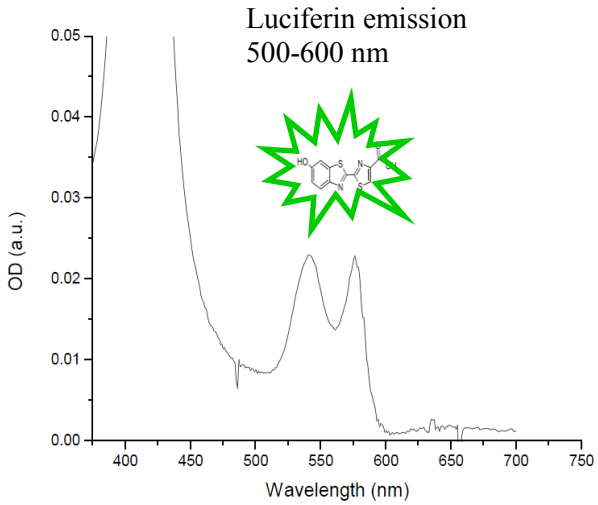

Figure 1.4 Overlap of emission spectrum of luciferin and the absorption spectrum of Hb. Visible spectrum of Hb overlayed with the range of luciferin emission. Adapted from Colin *et al* 2000 **(29)**.

The assay is sensitive to the ionic strength of the assay media (30). Most work which involves assaying ATP in erythrocytes uses a form of physiological saline solution (PSS) (15) (4), a viscous plasma mimic which is isotonic to erythrocytes due to a high salt content. The concentrations of cations and anions used to evaluate the quenching of the chemiluminescence did not exceed low millimolar, whereas the composition of PSS includes sodium and chloride in excess of 100 mM.

1.6. Relevance of measuring ATP

Impaired ability of erythrocytes to release ATP has been associated to the pathologies of several diseases. In cystic fibrosis, erythrocytes are less

deformable and have been found to release less ATP when exposed to hypoxia as well as pharmacological stimulants (31). Similarly, erythrocytes exhibit reduced capacity to release ATP when made hypoxic following treatment with insulin, an experimental condition which mimicks hyperinsulinemia encountered in pre-diabetes (2). Impaired ATP release from erythrocytes is also a characteristic of the pathology of primary pulmonary hypertension (32). Erythrocyte ATP release has the potential to be a metric of oxidative stress in the future, given the modulation of ATP release by reactive oxygen species (33).

1.7. Scope and outline of thesis

This manuscript based thesis details the work done measuring ATP in suspensions of intact erythrocytes by means of the luciferin-luciferase chemiluminescence assay. Chapter 2 reviews some of the initial challenges encountered in determining ATP concentrations in the suspensions of intact erythrocytes. The previous work undertaken by the English lab group with regards to NO_2^--stimulated ATP release from human erythrocyte suspensions was revisited and in addition, the effect on ATP release of metHb stabilizing anions was evaluated. Some preliminary work with rat erythrocyte suspensions were initiated in this chapter and brought to light certain considerations that are further explored in Chapter 3. In Chapter 3, we attempt to address some of the questions that arose from our preliminary work. This chapter is presented in the form of methodology-based publication which reviews the parameters necessary

to obtain consistent, accurate measurements of ATP from suspensions of intact erythrocytes. This chapter contains several complementary experiments which evaluate the interference that Hb, the primary erythrocyte protein, represents in the chemiluminescence assay for ATP and how this might be addressed. In Chapter 4, we present the experimental work that resulted from the methodology work covered in Chapter 3. In light of the interference that erythrocytes represent, we sought to compare the ATP concentrations in erythrocyte suspensions to that of their supernatants. In addition, we evaluate the accessibility of the ATP found in suspensions and the effect of different modulators of ATP release on the concentration determined in the suspensions and the supernatants. The totality of Chapter 4 is, likewise, presented in manuscript form. Finally Chapter 5 reviews the conclusions of the thesis and proposes future work.

2. Chapter 2 Preliminary work in modulation of ATP release from intact erythrocytes in suspension

2.1. Introduction

In low O_2 environments, erythrocytes release ATP into the circulation which stimulates vasodilation and improves O_2 delivery. As described in the introduction, ATP-stimulated vasodilation is mediated by nitric oxide (NO). In the circulation, NO bioactivity (as a vasodilator) is thought to be stored in the form of NO_2^- which is present in both erythrocytes and plasma (~300 nM and ~120 nM NO_2^- respectively) (13). There are a number of proposed pathways by which NO_2^- might act as a vasodilator including the production of NO_2^--modified intermediates (34) or the production of diffusible NO donors such as N_2O_3 (35). However, in most cases, NO_2^--derived vasodilation is assumed to involve the production of NO within the erythrocyte, and cannot account for the aggressive scavenging of NO by intracellular Hb (2). Recently, the English lab group proposed a mechanism for NO_2^- induced vasodilation that is not dependant on the production of NO within the erythrocyte. They reported that exposure to physiologically relevant concentrations of exogenous NO_2^- induced ATP release from erythrocytes *in vitro* (4), a phenomenon which has also been observed *in vivo* (15). Furthermore, they reported that the vasoactive compound nitroglycerin (GTN) also stimulates ATP release from erythrocytes (4). GTN is a common vasodilator in the clinical setting and is thought to be metabolized to NO_2^- (36) (37). In addition to stimulating ATP release from erythrocytes, both of these compounds have been shown to generate metHb (38) (39) (40). In fact, oral NO_2^- treatment in rats (41) (42), and NO_2^- infusion in rats (41) and humans (43) (40) elicited a concomitant increase in metHb.

Furthermore, an important side effect to GTN therapy is a clinically important increase in metHb concentrations, which leads to a condition known as methemoglobinemia. The release of ATP from erythrocytes following NO_2^- exposure could be related to the reactions of Hb with NO_2^-. It has been demonstrated that Hb binds to the erythrocyte membrane (18) and that a change in conformation of Hb was required for the release of ATP in low O_2 conditions (17). NO_2^--modified Hb has been found to have a higher affinity for the erythrocyte membrane than either deoxyHb or oxyHb (15). These findings compel us to consider whether metHb, which has high membrane affinity and is produced by the reaction of oxyHb with NO_2^-, might also play a role in vasodilation. There is a number of vasoactive, albeit potentially toxic, compounds which stabilize metHb. MetHb does not bind oxygen but can form bonds with a number of anionic compounds, including fluoride (F^-), azide (N_3^-) and cyanide (CN^-) (44). In isolated arterial tissues, fluoride (45) and azide (46) have a dilatory effect and systemic vasodilation has been documented following exposure to hydrogen cyanide (47) in the clinical setting. In order to determine whether the formation of metHb could stimulate the release of ATP, the effects of preincubation with the salts of stabilizers of metHb; KCN, NaF and NaN_3, were examined. Based on the dissociation constants for the ligands bound to (Table 1.1) and the fact that 100 nM NO_2^- elicited increased ATP release from erythrocytes (4), 100 nM of $NaNO_2$, KCN and NaN_3 and 1 mM NaF were selected for the preincubation of erythrocytes.

Table 2.1 Dissociation constant of metHb binding anions

	K_D (μM)
NO_2^-	5.1
F^-	12.6×10^3
N_3^-	3.98
CN^-	1.3×10^{-3}

Dissociation constants (K_D) of NaF, NaN_3 and KCN for metHb calculated from logK values **(48)**. The (K_D) for NO_2^- is that recently reported in a metHb study **(49)**

2.2. Materials and methods

Adenine 5-triphosphate disodium salt, bovine serum albumin, EGTA, $NaNO_2$, NaN_3, KCN, NaF, $MgCl_2$, and firefly lantern extract were purchased from Sigma–Aldrich (St. Louis, MO). The suppliers of the other chemicals were as follows: KCl from Fisher Scientific, Pittsburgh, PA; $CaCl_2$, $MgSO_4$ from ACP Chemicals, (Montreal, QC); NaCl; tris (hydroxymethyl) aminomethane from Bio Basic Inc, (Markham, ON), dextrose and Hepes from Bioshop (Burlington, ON); Distilled water was purified using a Milli-Q Simplicity 185 System (Millipore, Billerica, MA). White, non-sterile, polystyrene flat bottomed 96 well assay plates were purchased from Corning (Corning, NY). Physiological salt solution (PSS) was prepared fresh for each experiment with a final concentration (mM) of 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21 tris(hydroxymethyl)aminomethane, 5.5 D-glucose and 0.5% w/v bovine serum albumin. Sucrose buffer was made from 321.5 mM sucrose, 32.1 mM HEPES, 6.42 mM $MgCl_2$, 2.63 mM EGTA. The pH of PSS of both of the solutions was adjusted to 7.4. For human erythrocyte preparations, $NaNO_2$, NaN_3, KCN and NaF stock solutions were prepared as 50 fold stock solutions in 5% w/v D-glucose. For rat

erythrocyte preparations, a stock solution of 20 mM $NaNO_2$ in PSS was serially diluted to 200 nM.

The luciferase enzyme assay solution was prepared by dissolving 1.3 mg/mL firefly lantern extract in water and contained 2.6 mM $MgSO_4$ and 7.8 mM potassium phosphate (50). The assay solution was protected from light for the duration of the experiment and used within 4h of being prepared. A 2.5 mM ATP stock solution was prepared by dissolving 6.9 mg ATP in 5 mL PSS. ATP standard solutions (5 nM-20 μM) were prepared by diluting aliquots of the stock ATP solution in PSS. All solutions were prepared using reverse-osmosis water purified using a Milli-Q Simplicity 185 system.

2.2.1. Measurement of ATP by chemiluminescence

The assay was initiated by mixing 100 μL of the assay solution with 100 μL of ATP standard in a white 96-well plate in triplicate and then the chemiluminescence signal of the standards was recorded. Likewise, 100 μL aliquots of erythrocyte suspension were assayed by the addition of 100 μL of assay solution. The chemiluminescence intensity in the wells was measured immediately following 2 s of gentle mixing. The background chemiluminescence was determined by diluting a 100 μL aliquot of assay solution with 100 μL of PSS and this signal was subtracted from those of the standards and the erythrocyte samples. All readings were taken at ambient temperature (21-23°C).

2.2.2. Sample preparation

Whole blood was collected into 4 mL lithium heparin coated Vacutainer™ brand tubes obtained by venipuncture from healthy human female volunteers. The volunteers, aged 25-35 year old, of varying ethnicities and fitness levels had been duly consented in accordance with the University Human Research Ethics Committee (UHREC) of Concordia University. Female Wistar rats 375–450 g were housed and anesthetised in adherence with the research and teaching guidelines of the Canadian Council on Animal Care (CCAC) as per the University Animal Research Ethics Committee (UAREC) of Concordia University. The rats were anesthetised by 1-2 min of CO_2 inhalation and blood was obtained by cardiac puncture. Using a heparinised needle, a technician collected up to 3 mL of whole blood from each animal. The blood was then transferred to 4 mL lithium heparin coated Vacutainer™ brand tubes and kept on ice until centrifugation.

Within 2-4 h of collection, aliquots of blood were centrifuged at 1700 g at room temperature for 10 minutes. The plasma, platelets and white blood cells were removed by aspiration and the packed eyrthrocytes were then resuspended and washed three times in an equal volume of PSS. Packed human erythrocytes were suspended at 2% hematocrit (Ht) at 37°C in 10 mL of sucrose buffer with 6 mM glucose and either no ligand as a control, 100 nM (for samples treated with $NaNO_2$, NaN_3 and KCN) or 1 mM (NaF) of ligand final concentration. Following pre-incubation with a ligand, 1 mL aliquots of erythrocyte suspension were removed and centrifuged at 1700g for 45 seconds. The supernatant was removed by aspiration, the human erythrocytes resuspended at 1% Ht in

1980 μL of fresh PSS and then assayed as described above. Rat erythrocytes were suspended at 2% Ht and preincubated at 37˚C in either 100 nM $NaNO_2$ in PSS or untreated PSS as a control. Following pre-incubation, the erythrocytes were then spun down, the supernatant removed, the cells resuspended at 1% Ht and assayed as described above.

2.2.3. Data processing and statistical analysis

Data was processed using Microsoft Office Excel and plotted using Origin Pro 8.1 (OriginLab, Northampton, MA). Origin Pro 8.1 was also used for the ANOVA statistical analysis. The Spectra Max L luminometer used Molecular Devices' SoftMax Pro5.

2.3. Results

2.3.1. ATP assay results

Table 2.2. Blank-corrected chemiluminescence signal intensity of preincubated erythrocytes following resuspension at 1% Ht in PSS (RLU x10^5)

	<30 s	2 min	5 min	N
$Control_{NaNO2}$	7.05 ± 0.63	6.05 ± 0.80	5.29 ± 0.52	6
100 nM $NaNO_2$	7.38 ± 0.75	5.97 ± 1.31	4.91 ± 0.15	6
Control	6.90 ± 0.81	5.76 ± 0.67	4.95 ± 0.37	3
100 nM NaN_3	6.89 ± 0.19	5.85 ± 0.60	4.69 ± 0.70	3
100 nM KCN	6.25 ± 0.75	5.51 ± 0.67	4.92 ± 0.63	3
1 mM NaF	6.73 ± 0.39	5.46 ± 0.17	4.24 ± 0.10	3

Erythrocyte suspensions at 1% Ht were preincubated for <30 s-5 min in metHb stabilizing ligands $NaNO_2$, NaN_3, KCN or NaF in PSS. The suspensions were centrifuged and resuspended in fresh PSS, aliquoted into a 96 well plate and assayed as described in section 2.2.

Whether human erythrocytes were preincubated for up to 30 s, 2 min or 5 min with an anion, there was not a statistically significant change in the signal recorded as compared

to their time-matched controls (Table 2.2). The notable exception to this is the 5 min preincubation with NaF, wherein there was a significant decrease compared to the time matched control ($p < 0.05$). For all samples, whether treated or not, the signal intensity decreased as a function of preincubation time. These values were transformed to ATP concentrations (Table 2.3) using the calibration curves which were generated using ATP standards prepared as described in the methods. The concentrations determined for the erythrocyte samples ranged from 0.7 to 1.2 μM ATP. The decrease in signal intensity with time is equivalent to approximately 50 nM ATP /min. Throughout the course of this thesis, ATP values will often be presented as normalized extracellular ATP in order to best demonstrate the effect of a preincubation on the determined ATP concentration for erythrocytes. The ATP concentrations determined for each of the incubations is then divided by their respective controls to obtain a unit-less ratio of ATP concentrations. This final data transformation was performed on the determined ATP from Table 2.3 and is presented in Table 2.4.

Table 2.3 ATP concentration of preincubated erythrocytes following resuspension at 1% Ht in PSS calculated by linear regression (μM)

	<30 s	2 min	5 min	N
$Control_{NaNO2}$	1.13 ± 0.10	0.97 ± 0.13	0.85 ± 0.08	6
100 nM $NaNO_2$	1.18 ± 0.12	0.95 ± 0.21	0.78 ± 0.23	6
Control	1.10 ± 0.13	0.92 ± 0.11	0.79 ± 0.06	3
100 nM NaN_3	1.10 ± 0.03	0.94 ± 0.10	0.75 ± 0.11	3
100 nM KCN	1.00 ± 0.12	0.88 ± 0.11	0.79 ± 0.10	3
1 mM NaF	1.08 ± 0.06	0.87 ± 0.03	0.68 ± 0.02	3

The data from Table 2.1 was transformed using blank corrected calibration curves in order to determine the concentration of ATP in the 1% Ht suspensions in PSS following preincubation with metHb stabilizing ligands.

Table 2.4 Normalized extracellular ATP of preincubated erythrocytes following resuspension at 1% Ht in PSS

	<30 sec	2 min	5 min	N
100 nM $NaNO_2$	1.05 ± 0.04	0.98 ± 0.19	0.92 ± 0.24	6
100 nM NaN_3	1.01 ± 0.10	1.02 ± 0.04	0.94 ± 0.07	3
100 nM KCN	0.91 ± 0.11	0.96 ± 0.12	0.99 ± 0.08	3
1 mM NaF	0.98 ± 0.07	0.96 ± 0.12	0.86 ± 0.04	3

The data which was used to create data 2.2 was used to determine the fold change in ATP concentration of the 1% Ht erythrocyte suspensions in PSS following preincubation with metHb stabilizing ligands

The initial work performed by the English research group on NO_2^--stimulated ATP release from human erythrocytes pre-incubated at 2% Ht and assayed at 1% Ht, had recorded ATP concentrations in the low nanomolar range (4). Similarly, when using identical Ht and methodology we obtained ATP concentrations that were up to an order of magnitude higher. Given erythrocytes' millimolar intracellular ATP concentration, it was deemed possible that rupture of the erythrocytes, known as hemolysis, could have been a factor in producing these elevated ATP concentrations. Without centrifuging the erythrocytes and measuring the Hb content of their supernatants, it is not possible to obtain an accurate measure of sample hemolysis. As this had not been done, a theoretical index of hemolysis was determined for the samples.

2.3.2. Approximation of sample hemolysis

Bergfeld and Forrester (3) determined the extent of hemolysis by calculating the maximum number of molecules of ATP released per cell following 100% hemolysis. Based on their work, the degree of hemolysis of my samples was calculated as follows:

Approximation of the number of cells:

1% Ht = 50 000 cells*μl^{-1}

For 100 µL of erythrocytes at 1% Ht

$$\#\ \text{cells} = 50000\ \text{cell}\ \mu L^{-1} \times 100\ \mu L = 5\times10^{6}\ \text{cells}$$

$$\#\ \text{molecules ATP} = \left([ATP]M \times 6.022\times10^{23}\ \text{molecules} / \text{Moles} \times 2\times10^{-4} L\right)$$

$$\#\ \text{molecules ATP cell}^{-1} = \left([ATP]M \times 6.022\times10^{23}\ \text{molecules} / \text{Moles} \times 2\times10^{-4} L\right) / 5\times10^{6}\ \text{cells}$$

The maximum amount of ATP released by 100% hemolysis averaged 6.3 X 10^7molecules $cell^{-1}$ (3).

$$\%\ \text{hemolysis} = \#\ \text{molecules ATP cells}^{-1} / 6.3\times10^{7}\ \text{molecules ATP cells}^{-1}$$

Table 2.5 Approximation of the maximum possible % hemolysis of preincubation erythrocytes in presence and absence of 100 nM $NaNO_2$

	ATP (µM)		molecules ATP x10^{14}		molecules ATP $cell^{-1}$ x10^7		% hemolysis	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ctrl <0.5 min	1.13	0.10	1.36	0.12	2.72	0.24	43.16	3.84
ctrl 2 min	0.97	0.13	1.17	0.16	2.33	0.31	37.03	4.94
ctrl 5 min	0.85	0.08	1.02	0.10	2.04	0.20	32.34	3.20
$NaNO_2$<0.5min	1.18	0.12	1.42	0.15	2.85	0.29	45.20	4.63
$NaNO_2$ 2 min	0.95	0.21	1.15	0.25	2.30	0.51	36.52	8.06
$NaNO_2$ 5 min	0.78	0.23	0.946	0.28	1.89	0.56	30.03	8.88

The approximation of the maximum possible hemolysis following <30 s- 5 min preincubation of erythrocytes with 100 nM $NaNO_2$. Based on the ATP concentrations determined in Table 2.2 and the calculations used in previous work related to assay ATP from lysed erythrocytes.

Table 2.6 Approximation of the maximum possible % hemolysis of preincubation erythrocytes in presence and absence of 100 nM NaN_3, 100 nM KCN or 1 mM NaF.

	ATP (nM)		molecules ATP $x10^{14}$		molecules ATP $cell^{-1} \cdot x10^7$		% hemolysis	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ctrl <0.5 min	1.10	0.13	1.33	0.16	2.66	0.16	44.32	2.48
ctrl 2 min	0.92	0.11	1.11	0.13	4.32	0.13	35.27	2.05
ctrl 5 min	0.79	0.06	0.95	0.07	1.91	0.07	30.28	1.14
NaF <0.5 min	1.08	0.06	1.30	0.08	2.60	0.08	41.22	1.19
NaF 2 min	0.87	0.03	1.05	0.03	2.10	0.03	33.39	0.52
NaF 5 min	0.68	0.02	0.82	0.02	1.63	0.02	25.91	0.31
NaN_3 <0.5min	1.10	0.03	1.33	0.04	2.66	0.04	42.15	0.59
NaN_3 2 min	0.94	0.10	1.13	0.12	4.35	0.12	35.78	1.85
NaN_3 5 min	0.75	0.11	0.90	0.14	1.81	0.14	28.66	2.14
KCN<0.5 min	1.00	0.12	1.20	0.15	2.41	0.15	38.23	2.3
KCN 2 min	0.88	0.11	1.06	0.13	2.13	0.13	33.74	2.05
KCN 5 min	0.79	0.10	0.95	0.12	1.90	0.12	30.11	1.93

The approximation of the maximum possible hemolysis following <30 s- 5 min preincubation of erythrocytes with 1 mM NaF or 100 nM KCN or NaN_3. Based on the ATP concentrations determined in Table 2.2 and the calculations used in previous work related to assay ATP from lysed erythrocytes.

Using an approximation based on the methods of Bergfeld and Forrester (3), the human erythrocyte suspensions underwent a maximum of anywhere from 25-45% hemolysis (Table 2.5 and 2.6). However, we must yet explain how it would be possible for there to be a decrease in ATP with time if the erythrocytes were indeed undergoing lysis. It would stand to reason that we would observe an increase in ATP concentrations with time, as more and more cells would be lysed under the experimental conditions that were able to induce over a quarter of the cells to lyse. Erythrocytes have a number of enzymes which

consume ATP for various cell maintenance functions, such as pumping cations across the erythrocyte membrane. The activity of these ATPases could account for the decrease in ATP observed over time in the sample suspensions.

2.3.3. Rat erythrocytes

The opportunity to work with erythrocytes collected from Wistar rats presented itself. Given the importance of rodents as pharmacological models, we opted to take advantage of the availability of samples. Rat erythrocytes were preincubated and assayed without the use of a sucrose buffer, which originally served as an osmoprotectant for prolonged incubations (4). The signal intensities for the rat erythrocytes chemiluminescence (Table 2.7) and the corresponding ATP concentrations (Table 2.8) were an order of magnitude lower than those recorded for human erythrocytes. Furthermore, the $NaNO_2$-treated samples were consistently lower than the control samples, which would indicate that $NaNO_2$ was actually attenuating the release of ATP from rat erythrocyte suspensions by 20% to 40%, which was in direct contradiction of the increase in erythrocyte ATP release documented in recent literature ((4), (15)). However, there was a marked decrease in the triplicate values recorded for most of the rat erythrocytes suspensions which gave rise to standard errors of 19% to 42% of the average (CV values in Table 2.7). On average, the standard error for the signal intensities represented 27% of the average of the triplicate values.

Table 2.7. Effect of 30 s preincubation of erythrocyte suspensions to 100 nM $NaNO_2$ on signal intensity

$NaNO_2$	1st	2nd	3rd	Average
-	4.57	4.25	2.36	3.73
+	3.50	2.51	2.56	2.86
-	4.14	3.26	2.60	3.33
+	3.74	2.32	1.59	2.55
-	*4.95*	*3.03*	*3.11*	*3.70*
+	*3.93*	*2.57*	*2.78*	*3.09*
-	*3.45*	*4.38*	*2.36*	*2.70*
+	*2.02*	*1.52*	*1.27*	*1.61*

Rat erythrocytes obtained by cardiac puncture and prepared as described in section 2.2. Blank corrected chemiluminescence signal intensity of erythrocytes following 30 s exposure to PSS (-) or to 100 nM $NaNO_2$ (+) and resuspension at 1% Ht in PSS (RLU $x10^4$) Italicized samples assayed by D.Di Marco

Table 2.8. Effect of 30 s preincubation of erythrocyte suspensions to 100 nM $NaNO_2$ on ATP concentrations

$NaNO_2$	1st	2nd	3rd	Average	CV%
-	487	454	255	399	31%
+	375	271	277	307	19%
-	443	350	281	358	23%
+	400	251	174	275	42%
-	*528*	*326*	*334*	*396*	*29%*
+	*420*	*277*	*299*	*332*	*23%*
-	*370*	*246*	*255*	*290*	*24%*
+	*219*	*167*	*141*	*176*	*23%*

Rat erythrocytes obtained by cardiac puncture and prepared as described in section 2.2. ATP concentrations (nM) determined based on data from Table 2.6. for erythrocytes following 30 s exposure to PSS (-) or to 100 nM $NaNO_2$ (+) and resuspension at 1% Ht in PSS Italicized samples assayed by D.Di Marco

2.4. Discussion

The primary difference between the present material and methods and those published by this lab group in the past is the use of 0.1% dextrose in the PSS instead of 0.2%. The PSS was made in this manner following the recipes provided by the authors, which differed from the published concentrations. PSS, dextrose not withstanding has an osmolarity of 296.8 mOsm/L; 0.2% w/v dextrose brings the osmolarity to 307.8 mOsm/L. The use of 0.1% w/v dextrose would give the PSS an osmolarity of 302.3 mOsm/L, which is similar to the normal osmolality of erythrocytes - 300 mOsm/kg (51). If the elevated levels of ATP measured for the human blood samples were due to hemolysis, the source of said hemolysis remains undetermined. Originally, we posited that a reduced final glucose concentration might have contributed to sample hemolysis, but it could also be due to a number of factors including an error in the concentration of osmolytes in the physiological saline solution used to prepare the samples. It is important to note that the degree of hemolysis determined was in no way reflected in the visual aspect of the samples. In fact, in absence of visible spectroscopy of sample supernatants, all statements with regards to hemolysis remain highly speculative.

The amount of ATP released from human erythrocytes decreased over time for both the controls and the treated cells. The time dependant decrease in the amount of ATP measured is most likely the result of the activity of nucleases. In addition, the accumulation of ADP, an ATP degradation product, would occur over time, and ADP binding to the P2Y receptors on the erythrocyte could readily inhibit ATP release (52).

When repeating these manipulations, it will be essential to centrifugate the erythrocytes and measure the Hb content of the supernatants in order to more accurately evaluate the

degree of hemolysis of the samples. Using Bergfeld and Forrester's correlation between % hemolysis and OD560, we would expect an absorbance of ~0.09 at 560 nm for 40% hemolysis, and so roughly 0.9 in the soret region (3). Erythrocyte hemolysis is generally evident in the physical aspect of the erythrocyte suspensions. There is a marked transition from opacity to translucence with increased hemolysis, which was not observed. While the % hemolysis determined above is an approximation, the erythrocyte suspension and supernatant did not present any evident signs of over one third of the cells having been hemolyzed. In light of the extent of hemolysis, the decrease in ATP levels over time is more likely due to degradation of ATP than the inhibition of ATP release from the cells due to the products of said degradation.

The rat erythrocyte samples were prepared using a fresh stock of Ringer's and Tris base solutions and the ATP concentrations determined were in line with those documented. The downward trend in the ATP concentration with time is evocative of ATP degradation. That this also occurred with the rat erythrocyte samples brought this matter to the forefront of our experimental considerations. ATP degradation has been posited by several lab groups (53) (4) in light of the documented presence of vascular ATPases. However, the loss of signal could also be due to the transience of the light emitted during the reaction of oxyluciferin with ATP, whose decay can vary from very fast (<1 sec), to rather slow (> 5 min) (54). The decrease in signal within triplicate readings was indicative of the signal being recorded too long after the initiation of the assay. From this observation, the decay of the oxyluciferin signal became the first of a number of factors that were evaluated for the assay of ATP from intact erythrocytes by chemiluminescence.

3. Chapter 3 *Systematic Investigation of Chemiluminescence Determination of Extracellular ATP in Intact Erythrocyte Suspensions in Microplate Format*

Authors: Andrea Clarke, Marc Ouellet, and Ann.M.English

Affiliation: Concordia University

Short title: Assaying ATP released from intact erythrocytes

Person to whom correspondence should be addressed: Ann.M.English. Dept. of Chem. & Biochem., Concordia University Loyola campus, Renaud Science Complex, 7141 Sherbrooke St. W., Montreal QC, H4B 1R6

Appropriate table of contents header: enzymatic assays and analyses

3.1. Abstract

As erythrocyte-derived extracellular ATP gains recognition as an essential, local vasodilator in blood, improving the accuracy and simplicity of its determination in erythrocyte suspensions is increasingly necessary. However, the luciferin-luciferase chemiluminescence assay for ATP has not been systematically evaluated for intact erythrocyte suspensions. Whole blood and Hb contribute to matrix effects on the luciferin signal and we hypothesize that Ht constitutes the main matrix effect in the chemiluminescence quantitation of ATP in erythrocyte suspensions. ATP standard curves in presence of Hb, standard addition, and dilutions of erythrocyte suspensions were employed to evaluate matrix effects. We found that Hb attenuated the chemiluminescence signal of ATP standards in a dose-dependent manner. Moreover, using standard addition, we established the maximum Ht at which the chemiluminescence signal for added ATP was not affected by the presence of erythrocytes in the assay matrix. Finally, we report that the extracellular ATP-derived chemiluminescence signal from erythrocyte suspensions is bimodal as a function of Ht. Ultimately, erythrocytes' interference in the measurement of their extracellular ATP by chemiluminescence can be limited by working at very low Ht. This finding coupled with optimized assay parameters for a 96-well plate, increases the throughput of the assay while requiring mere micro-litres of blood.

3.2. Introduction

Erythrocytes act as effectors in the circulatory system through their release of ATP. In the lumen of blood vessels, ATP is an extracellular signalling molecule, which increases blood vessel diameter by stimulating endothelium-dependent vasodilation

(53). In vitro, erythrocyte-derived ATP has been measured in lysates (55) (56), supernatants (24), and suspensions (56) (16) (3), using the luciferin-luciferase chemiluminescence assay. The strength of this assay lies in the fact that the light producing reaction of ATP with oxyluciferin is catalyzed by luciferase and has a quantum yield of very close to 1 (54) (Figure 3.1).

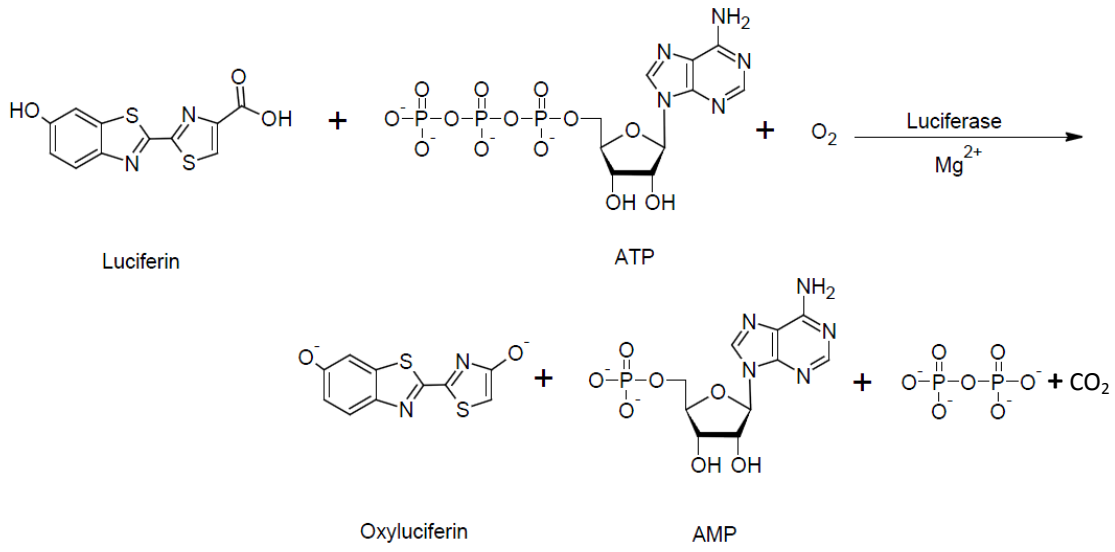

Figure 3.1. Chemiluminescence reaction catalyzed by the enzyme luciferase

D-Luciferin reacts with ATP and molecular oxygen in the presence of Mg^{2+} to produce an excited state oxyluciferin and release AMP, pyrophosphate and CO_2. The relaxation of excited state luciferin produces light in proportion to ATP concentration (generated using ChemSketch).

We sought to establish the limitations of this assay when using a standard chemiluminescence plate reader to measure erythrocyte-derived ATP. Therefore, we evaluated the thermal stability of luciferase, the decay of the signal emitted under typical assay conditions as well as the effect of various interfering molecules on the recorded signal intensity.

Ions such as Cl^-, Na^+, and Ca^{2+}, quench the signal emitted by oxyluciferin (57) (30) when present in the millimolar range (1-100 mM). The physiological saline solution (PSS) used to prepare an erythrocyte suspension contains these and other ions in the range of 1-140 mM. PSS is a necessity when working with intact erythrocytes because it has an osmolality comparable to that of the plasma, serum and whole blood of a large number of mammals, including rats and humans (~300 mOsm/kg) (51), hence keeping erythrocytes intact. As our work involves investigations of osmotic pressure-induced ATP release, we examined the effect of the percent (by volume) of PSS on the signal strength recorded for the chemiluminescence assay.

Both whole blood and Hb interfere with the assay due to Hb's strong absorption in the region of oxyluciferin emission (500-650 nm) (29). The percent volume of erythrocytes, or Ht, dictates its Hb concentration and is a key parameter when assaying erythrocyte ATP. The goal of this study was to determine extracellular ATP concentrations of intact erythrocytes in suspension quantified using the luciferin-luciferase chemiluminescence assay using a 96-well plate reader. We hypothesized that there would be significant, Ht-dependent absorption of the chemiluminescence by the erythrocytes due to their high Hb content. This inner-filter effect was evaluated and we examined different strategies to correct for the presence of erythrocytes in the sample suspensions. The accurate determination in erythrocyte suspensions revealed a few caveats. Surprisingly, the workhorse for ATP assays, the luciferin-luciferase base chemiluminescence assay had not been evaluated for intact erythrocytes.

3.3. Materials

Adenine 5-triphosphate disodium salt, bovine serum albumin, bovine metHb, and firefly lantern extract were purchased from Sigma–Aldrich (St. Louis, MO). The suppliers of the other chemicals were as follows: KCl from Fisher Scientific, Pittsburgh, PA; $CaCl_2$, $MgSO_4$ from ACP Chemicals, (Montreal, QC); NaCl; tris (hydroxymethyl) aminomethane from Bio Basic Inc, (Markham, ON); Hepalean 1,000 U/mL heparin Organon Canada (Scarborough, ON); dextrose from Bioshop (Burlington, ON); Distilled water was purified using a Milli-Q Simplicity 185 System (Millipore, Billerica, MA). Physiological salt solution (PSS) was prepared fresh for each experiment with a final concentration (mM) of 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21 tris(hydroxymethyl)aminomethane, 11 D-glucose and 0.5% w/v bovine serum albumin. The pH was adjusted to 7.4 with 10 N HCl. White, non-sterile, polystyrene flat bottomed 96 well assay plates were purchased from Corning (Corning, NY).

3.4. Methodology

3.4.1. Measurement of ATP by chemiluminescence

The luciferase assay solution used to quantify ATP was prepared just prior to use by dissolving 1.3 mg/mL firefly lantern extract, containing both luciferin and luciferase, in fresh Milli-Q water. In addition to luciferin and luciferase, the resulting assay solution also contained 7.8 mM potassium phosphate and 2.6 mM magnesium sulphate (58). The solution was mixed by gentle rocking and centrifuged at ambient temperature for 10 min at 3300 g using an IEC GP8R centrifuge. The supernatant was decanted into a foil wrapped Falcon tube and aliquots were poured into a foil covered reservoir for use with a

12-channel multichannel pipettor (Eppendorf). A 2.5 mM ATP stock solution was prepared by dissolving 6.9 mg of ATP (weighed on a Mettler Toledo MX5 microbalance) in 5 mL fresh PSS and stored at -20°C. Aliquots of the stock solution were thawed and diluted 1000-fold in PSS to give a 2.5 μM solution, which was used to prepare standards ranging from 10 to 1500 nM ATP in PSS. The ATP standards were kept on ice and equilibrated to 23°C in the plate reader. The assay was initiated by mixing 100 μL of the assay solution with 100 μL of ATP standard in a white 96-well plate in quadruplicate. The plates were kept in the dark prior to the experiments to minimize plate phosphorescence from exposure to a combination of natural and fluorescent light. The chemiluminescence signal of the standards was recorded with a Spectra Max L plate reader at 23°C. A calibration curve in the nanomolar range was generated by plotting the signal intensities relative to a blank (in relative light units, RLU) of the standards vs. [ATP].

In our studies of stimulated ATP release, the erythrocytes are maintained at 37°C; it would be desirable to assay ATP in suspensions at this temperature. The stability of luciferase assay solution at 37°C was examined. The assay solution was prepared as described above, placed in a 37°C water bath for 5 to 30 min and then used to assay ATP standards which were kept on ice.

3.4.2. Decay of chemiluminescence vs. time

The chemiluminescence signal was monitored to establish the time frame in which the signal decay could be determined. A 100 μL aliquot of 400 nM ATP standard solution in PSS into a 96-well plate and the assay was initialized by the addition 100 μL of assay

solution. A total of 200 readings were taken every 0.1 s over 20 s and a total of 60 readings were taken every 5 s over 5 min.

3.4.3. Inner filter effect of Hb on chemiluminescence

Whole blood from female Wistar rats has a mean Ht of 50% and a mean Hb concentration of 2.63 mM (170 mg/mL) (12). A 1% Ht suspension is a 50-fold dilution of erythrocytes with a Hb concentration of ~53 μM (3.4 mg/mL). ATP standards with final concentrations of 0, 50, 500 or 5000 nM prepared in PSS, were assayed with metHb solutions of 0.51-256 μM (equivalent to approximately 0.01-5% Ht) in 96-well plates.

3.4.4. Blood collection, preparation and assay of erythrocyte samples

Female Wistar rats 375–450 g were housed and anesthetised in adherence with the research and teaching guidelines of the CCAC as per the UAREC of Concordia University. Rats were restrained, and a small portion (<1 cm) of the animal's tail was removed using a straight razor. Blood flow from the tail was ensured by gentle stroking the tail from the base to the tip. Up to 1 mL of whole blood was collected in lithium heparin coated BD microtubes by this method.

Informed written consent was obtained from healthy adult human female volunteers, ages 18-30 years of varying ethnicity and fitness levels. The collection and use of blood from human volunteers were in accordance with the UHREC of Concordia University. Blood was collected into 4 mL heparinised tubes by venipuncture from a tourniqueted antecubital vein and kept on ice until centrifugation.

Within 1 h of collection, an aliquot of whole rat or human blood was transferred into a 2-mL Eppendorf tube and centrifuged in a Micromax RF Thermo IEC at 1700 g for 5 min at 4°C. Following centrifugation, the plasma and the fraction containing white blood cells and platelets were removed by aspiration with a micropipettor. An equal volume of PSS was added to the packed erythrocyte sample. The cells were suspended by gently pipetting up and down and then centrifuged as above. The supernatant was replaced with fresh PSS kept at ambient temperature and the cells were washed in this manner two more times. During the wash steps, a small volume of packed erythrocytes was removed to ensure that none of the plasma and other cell types remained.

3.4.5. Standard Addition

To examine the chemiluminescence signal intensity of erythrocyte suspensions as a function of % Ht, a 1% Ht suspension of rat erythrocytes was prepared in PSS. The 1% Ht suspension was diluted with PSS to give a range of erythrocyte suspensions with Ht varying from 0.01 to 0.6% which were assayed for ATP as described above. Alternatively the method of standard addition was used to determine the amount of ATP in the suspensions. Aliquots of the ATP stock solution in PSS were added to suspensions to give final ATP concentrations of 0-500 nM ATP in 1 mL suspensions of 0.25%-0.0039% Ht. The signal intensities were plotted versus [ATP] and the x-intercept of extrapolated line should yield the unknown concentration of ATP in the erythrocyte suspensions.

3.5. Results

3.5.1. Time course of chemiluminescence decay

The chemiluminescence signal was measured within 10 s of adding 100 μL of luciferase assay solution to 100 μL of ATP standard in a 96-well plate. Figure 3.2 shows the time course of signal decay from a well containing 200 nM final concentration. Over the course of 5 min, we observed a 40% decrease in the signal but a strong chemiluminescence signal was present after 5 min. The decay of the emission from the relaxation of high energy oxyluciferin was fitted exponentially and the half-life of the signal was estimated to be 10.1 ± 0.9 min.

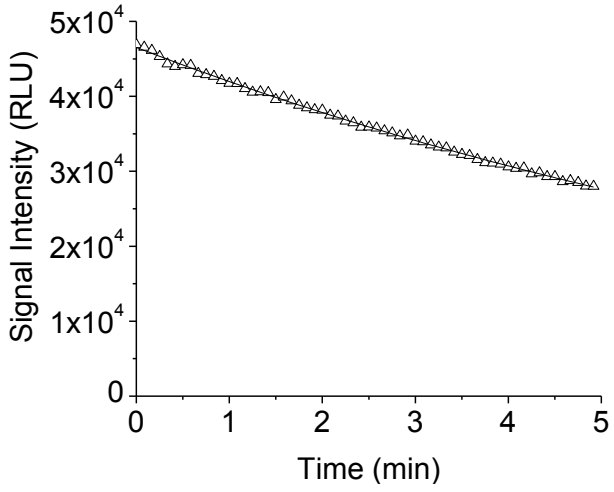

Figure 3.2 . Decay over 5 min of the chemiluminescence emission from oxyluciferin.
The ATP-driven luciferase-catalysed oxidation of luciferin was initiated in a 200 μL solution containing 200 nM ATP, pH 7.4 at 23°C. The signal intensity (relative light units, RLU) was integrated over 1 s and recorded every 5 s.

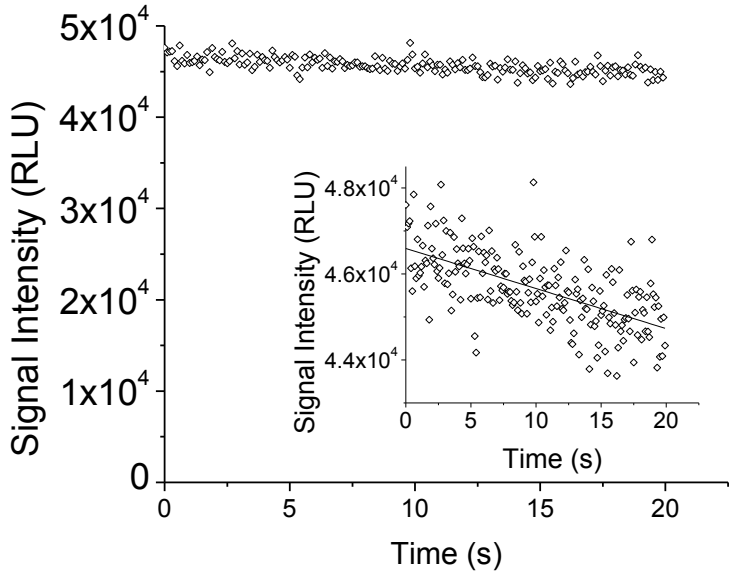

Figure 3.3. Decay over 20s of the chemiluminescence emission from oxyluciferin. The ATP-driven luciferase-catalysed oxidation of luciferin was initiated in a 200 μL solution containing 200 nM ATP, pH 7.4 at 23°C. The signal intensity (relative light units, RLU) recorded every 0.1 s for a total of 200 readings over 20 s. Inset data with y-axis expanded.

The emission was also measured over 20 s (Figure 3.3) since the time required for the luminometer to measured quadruplicate wells is 10 s. The negative slope corresponds to a decay of 4% within 20 s of the assay being initiated. Expansion of the y-axis reveals the distribution of the data points (inset Figure 3.3). A linear fit of the data shows an equal distribution of data points above and below the line ($y=-93.2x+4.6x10^4$; $R^2=0.3879$). The standard deviation of the 200 intensity readings that were recorded was 2%.

3.5.2. **Effect of preincubation of assay solution at 37°C**

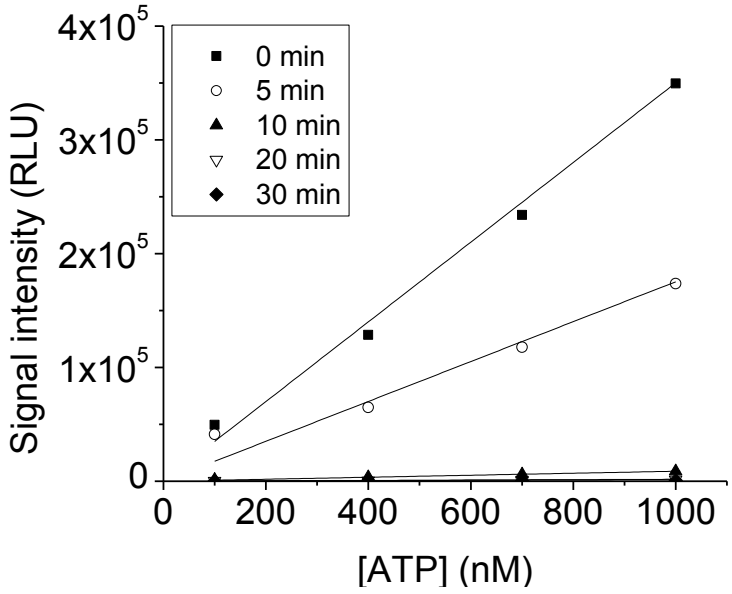

Figure 3.4. Preincubation of assay solution at 37°C decreases slope of the calibration curve. The luciferase assay solution was preincubated in a water bath at 37°C and 100 μL aliquots were added to 100 μL of ATP standards, pH 7.4 at 23°C following 0 (■), 5 (○), 10 (▲), 20 (▽), and 30 (◆) min preincubation. The signal intensity was recorded immediately after the initiation of the assay by the addition of the luciferase assay solution. Note that the data points at 10, 20 and 30 min overlap.

Table 3.1 Effect of pre-incubation of assay solution at 37°C on the calibration curve.

Time (min)	Slope (RLU/nM)	R^2
0	350	0.997
5	175	0.962
10	8.8	0.998
20	1.4	0.955
30	1.7	0.889

Slopes and R^2 values of data in Figure 3.4. The luciferase assay solution was preincubated in a water bath at 37°C and 100 μL aliquots were added to 100 μL of ATP standards, pH 7.4 at 23°C following 0, 5, 10, 20 and 30 min preincubation. The signal intensity was recorded immediately after the initiation of the assay by the addition of the luciferase assay solution.

Following a 5 min preincubation of the assay solution at 37°C, the signal recorded was 50% lower than t=0 (Figure 3.4); this is in good agreement with previous work reporting a 50% decrease after 4 min at 37°C (59). Following 10 min preincubation at 37°C, the signal decreased by >95%, however the standard curves remained linear for up to 30 min (Table 3.1).

3.5.3. Effect of total salt concentration on the chemiluminescence signal

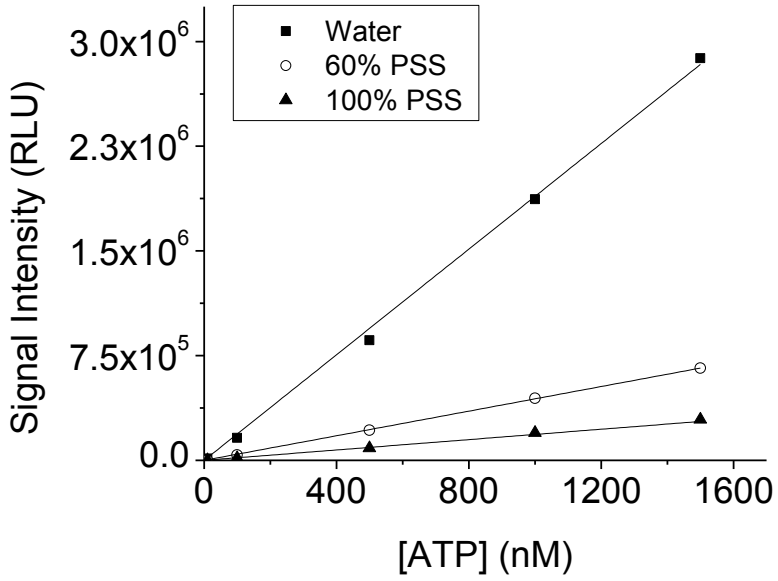

Figure 3.5. Reducing the salt concentration increases the chemiluminescence signal. ATP standards were prepared in PSS (▲), 60% PSS (60:40 v/v PSS:Milli-Q water) (○) or in Milli-Q water (■). In each case, 100 µL of the luciferase assay solution were added to 100 µL of ATP standards, pH 7.4 at 23°C. The signal intensity was recorded immediately after the initiation of the assay by the addition of the luciferase assay solution.

Compared to the 100% PSS control, diluting PSS to 60% increased the slope of the standard curve over 2 fold from 185 to 440 RLU per nM ATP (Figure 3.5). Preparation of

the standards in Milli-Q water resulted in a slope of 1891 RLU per nM ATP; 10 times higher than the slope of calibration curve prepared with 100% PSS. As reported previously, the assay is highly sensitive to the total salt concentration of the medium (30) (57). We also examined the calibration curve in PSS with 80.7 mM NaCl, (the main osmolyte) but with the same concentration of other solutes to probe for specific ion effects. The slope increased 1.7-fold, from 194 to 329 RLU per nM ATP compared to that obtained using 100% PSS (data not shown).

3.5.4. Inner-filter effect of Hb on the chemiluminescence signal

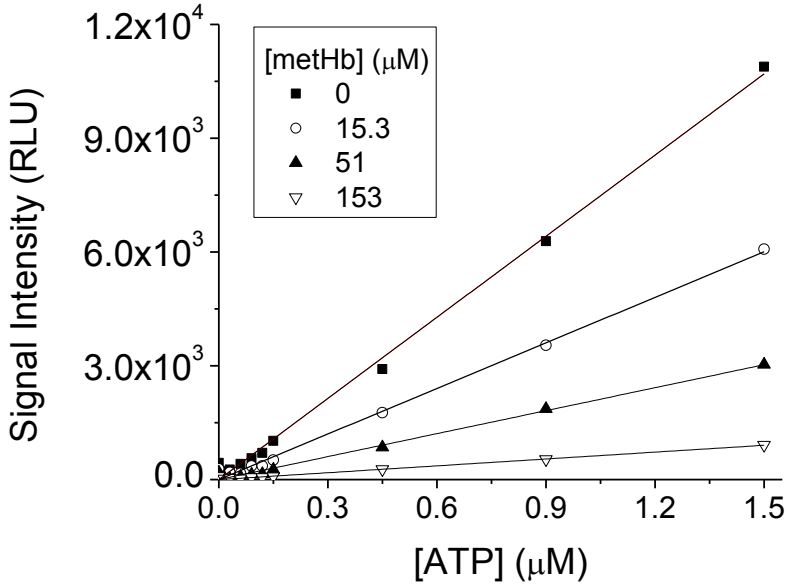

Figure 3.6 Inner-filter effect of metHb on the ATP calibration curve: ■ Control: standards prepared in PSS. ○ standards prepared in PSS containing 15.3 μM metHb.▲ metHb: standards prepared in PSS containing 51 μM metHb. ▽ metHb: standards prepared in PSS containing 153 μM metHb. In each case, 100 μL of the luciferase assay solution were added to 100 μL of ATP standards, pH 7.4 at 23°C. The signal intensity was recorded immediately after the initiation of the assay by the addition of the luciferase assay solution.

Table 3.2 Inner-filter effect of metHb on the calibration curve

[Hb] (μM)	Slope (RLU/nM)	R^2
0	7130	0.997
15.3	4004	0.997
51	2019	0.995
153	603	0.992

Slopes and R^2 values of data in Figure 3.6. The assay was initiated by the addition of 100 μL of luciferase assay solution to 100 μL of ATP standards prepared in metHb treated PSS, pH 7.4 at 23°C. The signal intensity was recorded immediately after the initiation of the assay. The signal recorded in the presence of metHb is significantly lower than with PSS alone (Figure 3.6).

The commercial metHb preparations decreased the slope of the calibration curve in a dose dependant manner (153 μM: y=603x, 51 μM: y=2162x, 15.3 μM y=4004x compared to the control y=7130x) (Figure 3.6). In Figure 3.7, varying metHb concentrations were assayed in the presence of a series of fixed concentrations of ATP. For any given concentration of ATP, there is a rapid, stoichiometric decrease in the chemiluminescence signal recorded up to 5.13 μM metHb; above 5.13 μM, the signal decrease slows considerably. It is important to note that 5.13 μM is equivalent to the Hb concentration of a 0.1% Ht erythrocyte suspension. At all concentrations of ATP, the chemiluminescence signal recorded as a function of the concentration of metHb present in solution was related via double exponential trend. As in Figure 3.6, for any given concentration of metHb, a plot of the chemiluminescence signal recorded as a function of ATP concentration was linear (data not shown).

3.5.5. Signal intensity as a function of [metHb]

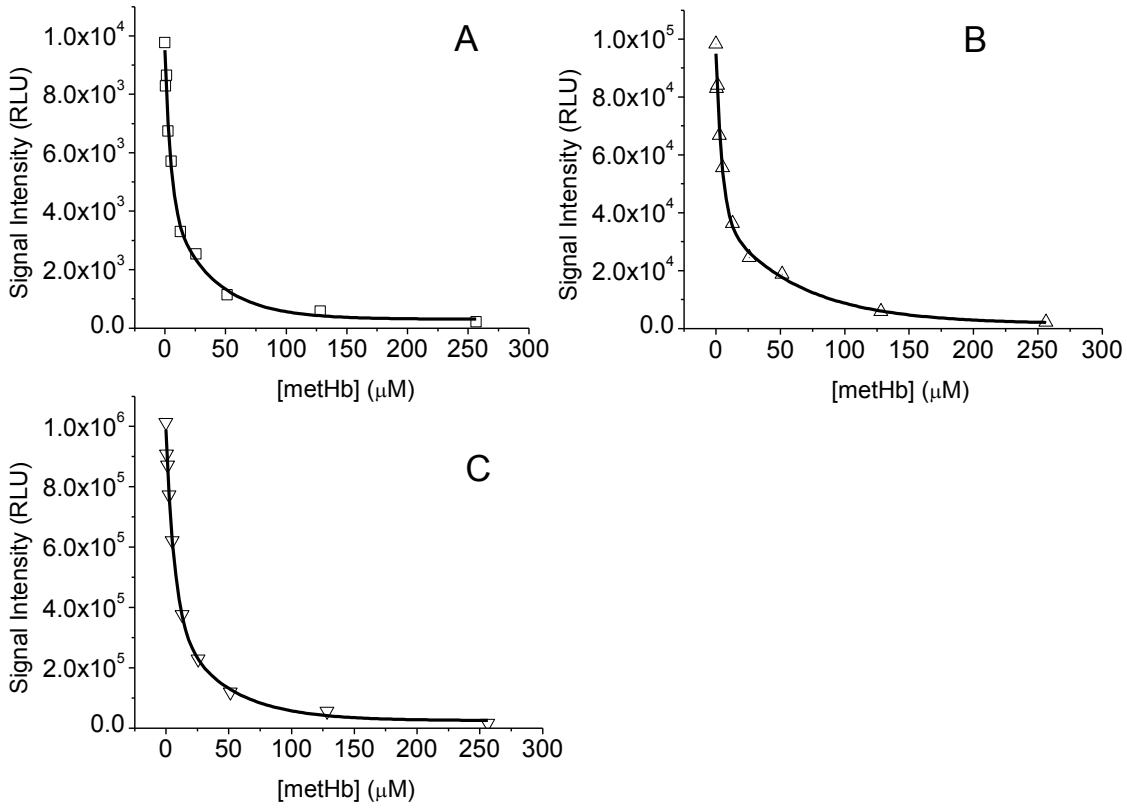

Figure 3.7 Signal intensity as a function of metHb concentration for fixed ATP concentrations: MetHb solutions of 0.51-256 μM were prepared with PSS or ATP standards (A:□50 nM; B:△500 nM; C:▽5000 nM). In each case, 100 μL of the luciferase assay solution were added to 100 μL of ATP standards, pH 7.4 at 23°C. The signal intensity was recorded immediately after the initiation of the assay by the addition of the luciferase assay solution.

3.5.6. Signal intensity as a function of Ht

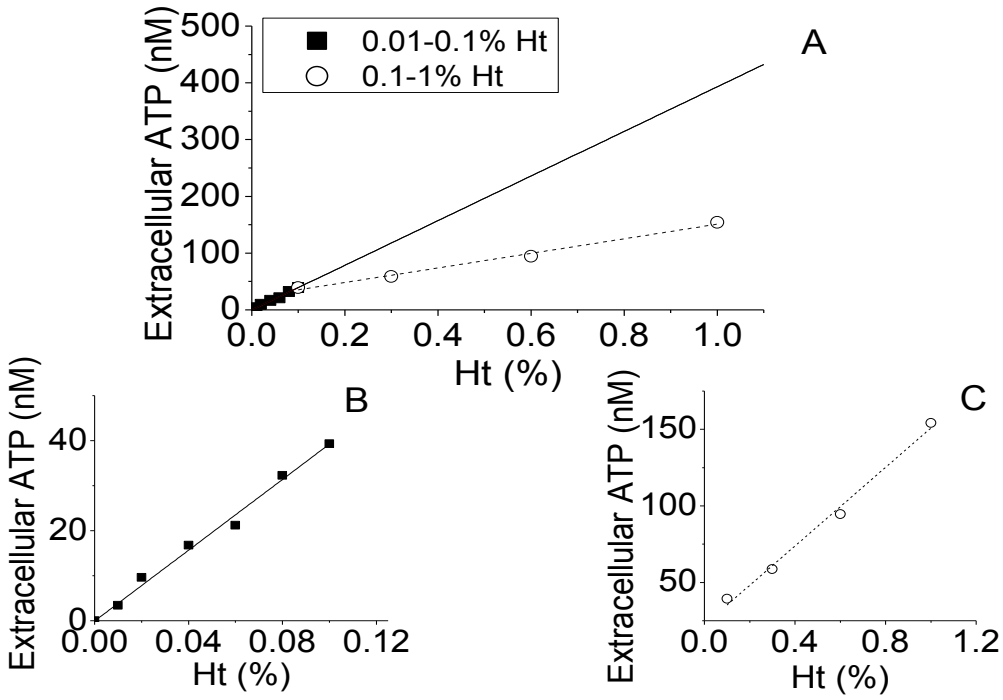

Figure 3.8. **Signal intensity as a function of % Ht.** A 1% Ht rat erythrocyte suspension in PSS was diluted with PSS to prepare suspensions ranging from 0.01 to 0.6% Ht. The luciferase assay solution was added to the suspensions in 96 well plates to initialise the assay. A. Linear regression of signal intensities of 0-0.1% (■) and 0.1-1% Ht (○). B. Linear regression of signal intensities of 0 to 0.1% Ht solutions. C. Linear regression of signal intensities of 0.1-1% Ht solutions. The linear regression of the 0 to 0.1% Ht suspensions in A was forcasted forward to 1% Ht. N=5

ATP concentrations increase with Ht but show a biphasic plot distribution (Figure 3.8a). Below 0.1% Ht, the inner-filter effect of % Ht is negligible as the plot is linear through 0%. A second linear phase is observed between 0.1 and 1.0% Ht (Figure 3.8c) with a lower slope, which suggests constant inner-filter effect in this % Ht region. To correct for this inner-filter effect, the signal of suspensions with Ht <0.1% was extrapolated to 1.0%

(Figure 3.8a). Based on the extrapolated signal intensity, the ATP concentration of a 1% suspension was estimated to be 2.6±0.2-fold higher than measured for rat erythrocytes.

3.5.7. Standard addition

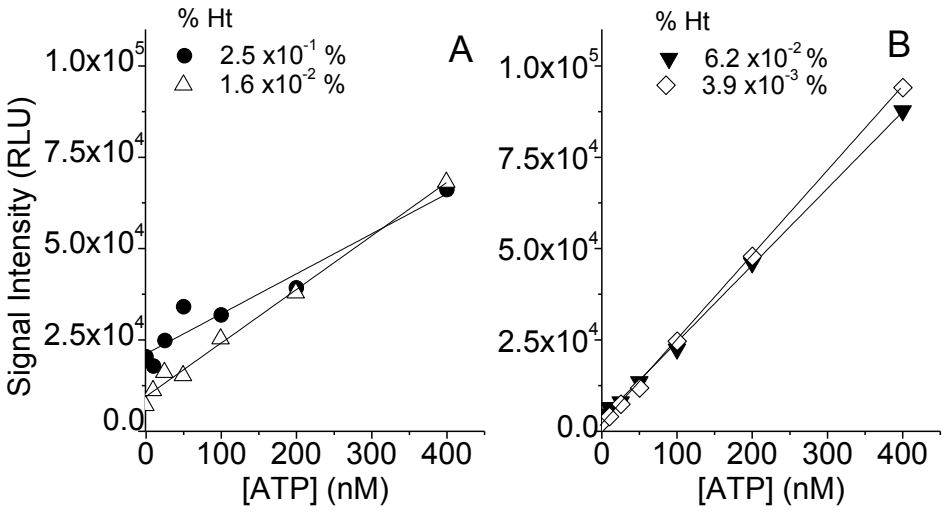

Figure 3.9. **Determination of ATP in human erythrocyte suspensions by standard addition.** A fixed volume of erythrocytes suspension in PSS (10 μL) was added to ATP standards in PSS to obtain a final Ht of 0.25% and 0-400 nM ATP (◆). The signal was recorded and the x-intercept determined using linear regression. The same procedure was repeated for 0.016% (△), 0.06.2% (▼), and 0.0039% (◇) Ht.

The signal increases in proportion with the concentration of ATP added to the suspensions; this proportionality improves as a function of decreasing Ht (Figure 3.9). However, at the Ht evaluated, the R^2 of the linear regressions curves were above well above 0.9 for every suspension (Table 3.3). The x-axis intercept for each Ht, which represents the initial concentration of ATP in the erythrocyte suspensions prior to the addition of exogenous ATP (60), was scaled to 1% Ht in order to compare them. The

initial ATP concentration at 1% Ht was determined to be 1147±205 nM (average±S.E.M).

Table 3.3. Approximation of [ATP] of a 1% Ht erythrocyte suspension from x-axis intercept of linear regression of standard addition data

%ht	Equation	R^2	x-intercept (nM)	[ATP] for 1% solution (nM)
0.25	109x +21219	0.9419	194	775
0.062	147x+9403	0.9929	64	1026
0.016	210x+3461	0.9987	16.5	1055
0.0039	231x+1564	0.9997	6.8	1733

3.6. Discussion

3.6.1. Time course of chemiluminescence decay

For 200 nM ATP, the signal half life was estimated to be 10 min (Figure 3.2). The decay of the light signal is characteristic of a *slow decay* signal, which can be used to accurately measure ATP concentrations in several wells consecutively without dramatic loss of signal (61). For each sample, we assay multiple aliquots for improved accuracy and add the luciferase assay solution manually to our samples and standards. These aspects of our assay compelled us to evaluate the transience of the signal in order to ensure the precision of our measurements. In the first 20 s following the initialization of the assay, we observed a signal decay of 4% (Figure 3.3), as our data is collected within 10 s of initialization, we lose less than 4% of the signal when assaying a sample in quadruplicate.

The indeterminate error associated with the assay was found to be 2%; this is significantly lower than the sample error which can arise with multiple readings of biological samples.

3.6.2. Thermal stability of assay solution

Less than 5% of the signal remained following 20 min preincubation of the assay solution at 37°C (Figure 3.4). Despite the significant decrease in signal intensity, the calibration curves are linear; it would be feasible to determine the concentration of ATP with assay solution pre-incubated at 37°C. An important caveat to this is the necessity to pre-incubate the assay solution in small aliquots for precisely the same amount of time. While it would be ideal to preincubate the luciferin assay solution at the same temperature as sample erythrocyte suspensions (37°C), there is a real risk of jeopardizing an experiment due to the unyielding time constraints imposed by the rapid, thermal inactivation of luciferase (59).

3.6.3. Effect of salt concentration on the chemiluminescence signal intensity

Decreasing the salt concentration relieves quenching of the chemiluminescence signal. We observed a 2-fold increase slope of the calibration curve with standards prepared in 60% PSS and a 10-fold increase for ATP standards prepared in Milli-Q water (Figure 3.5). Since ATP release from intact erythrocytes depends on the composition of the medium (O_2 saturation, osmotic strength, etc), it will be critical to ensure that factors altering ATP release are not masked by the effects of salt on the assay. Thus, calibration

curves must be prepared for every medium used otherwise the concentration of ATP measured for a given sample might be in error.

3.6.4. Inner filter effect of Hb on chemiluminescence

Free Hb interferes with the chemiluminescence signal recorded when assaying for ATP (29) and as a result, the true concentration of ATP in a Hb solution will be underestimated. The plot of signal intensity vs. metHb concentration is biphasic (Figure 3.7). We attribute the high interference in the low metHb range to signal absorption in the bulk solution. At higher metHb concentrations, light is only emitted from the surface, and therefore less is absorbed by metHb. However there is attenuation even at very low Hb concentrations, when the inner-filter effect should be negligible, which suggests that other quenching mechanisms are operative. Therefore the use of cell-free Hb to correct for inner-filter effects is not a viable option. Having

3.6.5. Signal intensity as a function of % Ht

The signal intensity of erythrocyte suspensions of varying Ht were converted to ATP concentrations using a standard ATP calibration curve. The signal emitted increased linearly with Ht to 0.1%. Above this value, a second linear region was observed with a decreased slope (Figure 3.8). As the chemiluminescence signal is robust at Ht at low as 0.01% Ht, the inner-filter and other matrix effects can be addressed by simply decreasing the Ht. Extrapolation of the 0-0.1% Ht plot showed that the ATP concentration of a 1% Ht s uspension will be underestimated by ~3 fold. Assaying ATP in suspensions at

higher % Ht would more closely mimic physiological conditions and the Ht of an erythrocyte suspension could influence cell to cell interactions. Correction factors such as these could be used to approximate the ATP concentration in suspensions at higher % Ht.

3.6.6. Standard addition

Untreated, washed erythrocytes in suspension have a basal ATP concentration that was measured using the luciferin-luciferase assay. Hb interference can be mitigated by diluting the suspensions below 0.1% Ht, and such, we used low % Ht suspensions to perform standard addition. Linear regression was used to determine the initial ATP concentration in the suspensions and then these were scaled to 1% Ht. The ATP concentration for the suspensions scaled to 1% Ht was determined to be in the low micromolar range. This raised the question if the ATP released into the medium represents all of the ATP present in suspension. In other words, is some of the ATP associated with the erythrocyte or is it all freely diffusing in the medium? This issue is addressed in Chapter 4.

3.7. Conclusions

Measuring ATP release from intact erythrocytes by chemiluminescence has been performed for well over 20 years and has provided critical insight into the role of erythrocytes as both sensors and effectors of the metabolic needs of tissues (62). While there are methodology papers that cover various aspects of the chemiluminescence of

ATP detection such as the kinetics of signal decay (61), to our knowledge, there has not been such a report for erythrocyte suspensions. The signal emitted by the reaction of ATP with a crude firefly lantern extract is both intense and sufficiently persistent to allow for replicate readings to be recorded for greater precision. We sought to account for the fact that Hb, the major protein component of erythrocytes absorbs strongly in the visible region overlapping the chemiluminescence produced by the activity of luciferase. We report that it is possible to correct for the filtering effect of erythrocytes simply by decreasing the Ht below 0.1% where the absorbance of the emitted signal does not affect the proportionality of the instrument response.

Given our findings, we recommend that the luciferase assay solution be at ambient temperature, that the assay be performed at 23°C, and that replicate measurements be taken for samples if the assay solution being used is a slow decay reagent. Furthermore, we recommend that the % Ht be between 0.06% and 0.004%, a range where the Ht is low enough to mitigate the interference, but still high enough for there to be a strong signal without the need to supplement the assay with synthetic luciferin. Another approach to mitigating the filtering effects of Hb on the assay could come from modifying elements of the assay system itself. Luciferin has been modified to produce a red-shifted emission. This has been accomplished by the addition of an amino-seleno moiety which produces an emission with a λ_{max} of 600 nm (63) or conversely by conjugation to the near IR dye Cy5 which has a λ_{max} of 670 nm (64). Either luciferin analogues could prove to be a valuable tool in assaying erythrocyte ATP, as Hb does not absorb as strongly in the region of emission of this luciferin.

3.8. Acknowledgements

We would like to acknowledge the efforts of Diana Di Marco. Additionally this work was funded by Concordia University and scholarships offered by Astra Zeneca and Hydro-Québec through the Québec Black Medical Association.

4. Chapter 4 *Externalized ATP is predominantly cell-associated and not freely diffusing in suspensions of intact erythrocytes*

Authors: Andrea Clarke, Marc Ouellet, and Ann.M.English

Affiliation: Concordia University

Short title: Assaying ATP released from intact erythrocytes

Person to whom correspondence should be addressed: Ann.M.English. Dept. of Chem. & Biochem., Concordia University Loyola campus, Renaud Science Complex, 7141 Sherbrooke St. W., Montreal QC, H4B 1R6

4.1. Abstract

The importance of extracellular ATP in the modulation of vascular tone is no longer a novel premise. However, the mechanisms for ATP release from circulating erythrocytes remain under investigation. It is difficult to accurately measure ATP in erythrocyte suspensions due to Hb's high absorption. To circumvent this problem, researchers measured ATP in erythrocytes at low Ht (<0.1%) or in supernatants. However, it has never been demonstrated that comparable results could be obtained working with suspensions and supernatants. To determine whether the ATP measured in the supernatants provides an accurate measure of the ATP exported from the erythrocytes, exposure to CO_2, N_2, hypo-osmotic stress and NO_2^- were used to stimulate erythrocyte ATP release. Baseline levels of ATP in the supernatant grossly underestimated the amount of externalized ATP. For example, the ATP concentration in rat erythrocyte suspensions was 10-50x higher than in the supernatants. Furthermore, the ATP concentrations determined for rat erythrocytes were consistently higher than for human erythrocytes, but human erythrocytes were found to be more sensitive to certain stimuli. While ATP can be measured in the supernatants of erythrocyte suspensions, a considerable portion remains unreleased and yet still accessible to enzymatic degradation. The major implication of this finding is the possibility of two modes of erythrocyte ATP release: one where ATP is freely released into the supernatant and another, where it remains largely accessible for cellular signaling, but is not consumed unless the erythrocyte interacts directly with a protein.

4.2. Introduction.

In addition to being sensitive local oxygen levels, erythrocytes also act as modulators of vascular tone through the release of adenosine triphosphate (ATP) into vessel lumen (65). When extracellular ATP interacts with endothelial receptors it acts as a potent vasodilator; increasing blood flow and consequently, oxygen saturation (66). Rather than coming from bulk, cytosolic ATP, the ATP that is released into the vasculature from erythrocytes is thought to come from discrete pools, such as those that are compartmentalized for use by membrane-spanning pumps (20). In fact, hypoxia-stimulated release of a fluorescent ATP-analog from these pools has been recently reported (67). Our recent examination of inteferents and assay parameters for the measurement of ATP in erythrocytes suspensions (Chapter 3), prompted us to question whether comparable results would be obtained working with suspensions and supernatants. In vitro, erythrocyte-derived ATP has been measured in supernatants (24) and suspensions (56) (16), using the luciferin-luciferase chemiluminescence assay but it is unknown whether all of the ATP that is externalized by erythrocytes is released and freely diffusing in the medium. In addition to comparing the concentration of ATP externalized in suspensions and supernatants, we examined the effects of known stimuli of ATP release on the concentrations recorded for erythrocyte suspensions and supernatants prepared from human and rat blood samples. The recovery of cells following removal of the stimulus was evaluated using sequential centrifugation. We report here that the ATP measured in erythrocyte suspensions and supernatants are not comparable, and in fact, represent two forms of externalized ATP. In the

supernatant, ATP is freely diffusing, while in suspensions, ATP remains associated with the erythrocyte.

4.3. Materials

Adenine triphosphate disodium salt, bovine serum albumin, potato apyrase, firefly lantern extract and $NaNO_2$ were purchased from Sigma–Aldrich (St. Louis, MO). The suppliers of the other chemicals were as follows: KCl from Fisher Scientific, (Pittsburgh, PA); $CaCl_2$, $MgSO_4$ from ACP Chemicals, (Montreal, QC); NaCl; tris (hydroxymethyl) aminomethane from Bio Basic Inc, (Markham, ON); Hepalean (1,000 U/mL heparin) from Organon (Canada), dextrose from Bioshop (Burlington, ON); ultrahigh purity nitrogen gas from Praxair (Danbury, CT). Distilled water was purified using a Milli-Q Simplicity 185 System from Millipore (Billerica, MA). A Mettler Toledo MX5 microbalance (Mississauga, On) was used to weighed out all compounds which were aliquoted in mg.

Physiological salt solution (PSS) was prepared as follows: 5% v/v tris (hydroxymethyl) aminomethane (420 mM), 5% v/v Ringers solution (94 mM KCl, 40 mM $CaCl_2$, 24 mM $MgSO_4$, 2810 mM NaCl), 0.2% w/v D-glucose and 0.5% w/v bovine serum albumin. The pH was adjusted to 7.4 with 10 N HCl. The PSS was prepared fresh at the beginning of each experimental day.

4.4. Methodology

4.4.1. Luciferin-luciferase assay – assay mixture and standards

The luciferase assay solution used to quantify ATP was prepared by dissolving firefly lantern extract in MilliQ H_2O. The solution of 1.3 mg/mL, was mixed by gentle rocking, and centrifuged at ambient temperature for 10 min at 3300 g using the IEC GP8R centrifuge. The supernatant was decanted into a foil wrapped 15 mL Falcon tube kept at room temperature. Aliquots of the assay mixture were poured into a foil covered reservoir for use with a multichannel pipettor. A 2.5 mM ATP stock solution was prepared by dissolving 6.9 mg of ATP in 5 mL of fresh PSS.

Aliquots of the stock solution were diluted 1000 fold in PSS to give a 2.5 μM solution which was then used to prepare standards ranging from 10 to 1.5 μM ATP in PSS. Once prepared, the ATP standards were kept on ice, and 100 μL was aliquoted in quadruplicate into white 96 well plates. The chemiluminescence reaction was initiated with the addition of 100 μL of the assay solution to these standards. The chemiluminescence signal of the standards was recorded with a Spectra Max L luminometer, Spectrafluor plus plate reader from Molecular Devices (Sunnyvale, California) and the calibration curve generated by plotting the signal intensity in relative light units (RLU) of the standards against their nanomolar ATP concentrations.

4.4.2. Preparation and assay of erythrocyte samples

Female Wistar rats 375–450 g were housed and anesthetised in adherence with the research and teaching guidelines of the CCAC as per the UAREC of Concordia

University. Rats were anesthetised by 1-2 min of inhalation of CO_2 (from sublimated dry ice) and blood was obtained by cardiac puncture. Using a heparinised needle, a technician collected up to 3 mL of whole blood from each animal. The blood was then transferred to 4 mL lithium heparin coated Vacutainer™ brand tubes and kept on ice until centrifugation. Rats were restrained, and a small portion (<1 cm) of the animal's tail was removed using a straight razor. Blood flow from the tail was ensured by gentle stroking the tail from the base to the tip. Up to 1 mL of whole blood was collected in lithium heparin coated BD microtubes by this method. Informed written consent was obtained from healthy adult female volunteers, ages 18-30 years of varying ethnicity and fitness levels. The collection and use of blood from human volunteers were in accordance with the UHREC of Concordia University. Blood was collected into 4 mL heparinised tubes by venipuncture from a tourniqueted antecubital vein and kept on ice until centrifugation. Within 1 h of blood collection, an aliquot of whole blood was transferred into a 2 mL Eppendorf tube and centrifuged in a Micromax RF Thermo IEC at 1700 g for 5 min at 4°C. Following centrifugation, the plasma and the fraction containing the white blood cells (leukocytes) and platelets were removed by aspiration. The packed erythrocytes were suspended in an equal volume of fresh PSS and then centrifuged as above; the cells were washed in this manner two more times. A small volume of packed erythrocytes was removed during the wash steps to ensure that all of the plasma and buffy coat was removed. Packed erythrocytes were kept on ice until the experiments were conducted.

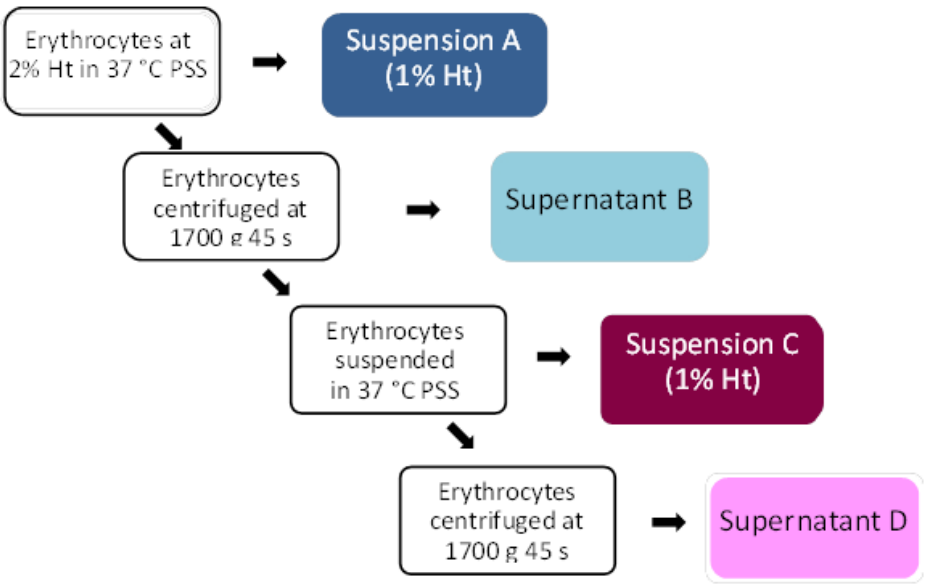

Figure 4.1. Scheme of sequential centrifugation in luciferin-luciferase assay

Sequential centrifugation was employed in order to examine the extent of the interference that erythrocytes represent. It also allows us to distinguish the effects of stimuli of ATP release on erythrocyte suspensions from the supernatants. The packed cell volume or Ht, is the percentage of the total volume that the erythrocytes represent.

As schematized in Figure 4.1, 1 mL of 2% Ht suspension was prepared by dispensing 20 µL of isolated erythrocytes into a dolphin tip microcentrifuge tube containing 980 µL of 37°C PSS. The erythrocytes were pre-incubated at this step. The microfuge tube was inverted to mix and then placed in the water bath at 37°C for 30 s. The 2% Ht suspension was then dispensed in the plate in four, 50 µL aliquots. To these aliquots, 50 µL of fresh 37°C, PSS was added to bring the Ht of the suspension to 1% in the wells (suspension A in Figure 4.1). The remaining 800 µL of original suspension was centrifuged at 1700g for 45 s at ambient temperature. Following centrifugation, 100 µL of the supernatant was aliquoted (supernatant B in Figure 4.1). The remaining supernatant was removed and the

packed erythrocytes were suspended in 1584 μL of fresh PSS to yield 1600 μL of a 1% Ht suspension. From this suspension, four 100 μL aliquots were dispensed (solution C in Figure 4.1). The remaining 1200 μL was centrifuged at 1700g for 45 s at ambient temperature. As with supernatant B, 100 μL of the supernatant was aliquoted (supernatant D in Figure 4.1). For all samples tested, chemiluminescence reaction was initiated as before, by the addition of 100 μL of assay solution. A portion of supernatant D was used to determine whether the samples had undergone hemolysis using the optical density in the visible region. All solutions were aliquoted in quadruplicate in 96 well plates.

4.4.3. Modulation of erythrocyte ATP

4.4.3.1. Apyrase

A 27 U/mL stock solution was prepared by dissolving 1 mg of 54 U/mg of apyrase in 2 mL PSS. Using this stock solution, a 2% Ht suspension was prepared in 1 mL of PSS with a final concentration of 10 U/mg apyrase. 1 U will produce 1.0 μmole Pi/min (from ADP or ATP) at pH 6.5 at 30 °C (68). Following a 30 min incubation at 37°C, the erythrocyte suspensions and supernatants were assayed as described in section 4.4.

4.4.3.2. Preincubation with $NaNO_2$

For the treatment of erythrocytes with $NaNO_2$, a 20 mM stock solution of $NaNO_2$ treated PSS was prepared by adding 1.38 mg $NaNO_2$ to 1 mL PSS. The $NaNO_2$ stock solution was then diluted serially to obtain 10 mL of a 200 nM $NaNO_2$. Aliquots of 500 μL of the resulting 200 nM solution were added to 480 μL of PSS in 2 mL dolphin tipped microfuge tubes and briefly vortexed at high speed. Through the addition of 20 μL of packed erythrocytes we obtained a 2% Ht in 1 mL containing 100 nM $NaNO_2$. The

sample was mixed by inversion and the microfuge tubes were placed in a 37°C water bath for 30 s assayed as described in section 4.4.

4.4.3.3. N_2 exposure of erythrocytes

A total volume of 3 mL of a 2% Ht erythrocyte suspension was prepared and dispensed equally into two 20 mL glass vials. One aliquot was left uncapped at room temperature and the other was capped with a syringe-fitted septum. A constant supply of N_2 at 5 psi was delivered through the syringe. A needle was passed through the septum to allow the gas in the vial to be expelled. Following 15 min of N_2 exposure, 1 mL of the erythrocyte suspension was removed, placed in a 2 mL Eppendorf tube and assayed for ATP as described in section 4.4. The time matched control was assayed in the same manner.

4.4.3.4. Pre-incubation of erythrocytes in hypo-osmotic media

Packed erythrocytes were submitted to hypotonic stress by suspending 20 μL of packed erythrocytes in 980 μL 60% v/v PSS at 37°C. Following the aliquoting of suspension A and supernatant B into the plate, suspension C and supernatant D were obtained following the suspension of erythrocytes in 100% PSS, as described in section 4.4.

4.4.4. Data processing and statistical analysis

Data was processed using Microsoft Office Excel and plotted using Origin Pro 8.1 (OriginLab, Northampton, MA). Origin Pro 8.1 was also used for the ANOVA statistical analysis. The Spectra Max L luminometer used Molecular Devices' SoftMax Pro5.

4.5. Results

4.5.1. Sequential centrifugation of erythrocytes

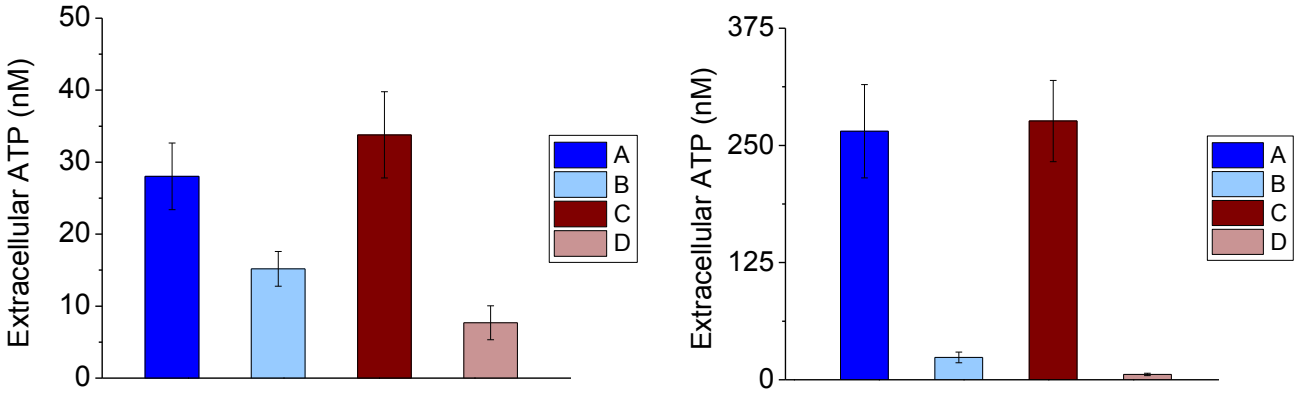

Figure 4.2. Distribution of extracellular ATP in erythrocyte suspensions and supernatants following 30 s incubation in PSS at 37°C. Erythrocyte suspensions A and C and supernatants B and D were prepared as described section 4.4. The error bars denote the standard error of the mean (± s.e.m.). Left panel: Female human erythrocytes obtained by venipuncture N=16. Right panel: Female Wistar rat erythrocytes obtained by tail bleed. N=14.

We measured ATP from erythrocytes following sequential centrifugation in order to determine whether suspensions and supernatants had comparable ATP concentrations and thus evaluate the interference of intact erythrocytes with the signal (Figure 4.2). Free of the interference from erythrocytes, the supernatants were expected to have a higher ATP concentration than their respective erythrocyte suspensions, providing a more accurate measure of the amount of ATP being released from the cells. The concentration of ATP in both human and rat erythrocyte suspensions were significantly higher than in their respective supernatants. Interestingly, while the ATP concentration of the supernatants of rat and human erythrocytes are similar in concentration (**human** B:15±2.4 nM, D:7.7±2.3 nM and **rat** B:23.9±5.7 nM, D:5.8±1.3 nM), the ATP concentration measured in rat

erythrocyte suspensions is 10 times higher than those measured in human cells (**human** A:28±4.6 nM, C:34±6.0 nM and **rat** A:265±50 nM, C:276±43 nM). This disparity gives rise to a marked difference in the ratio of measured ATP in the erythrocyte suspensions as compared to their respective supernatants; 2-4x in humans and 10-50x in rats. For each species, the concentration of ATP measured in suspensions A and C were the same, indicating that handling did not produce artefacts in ATP release.

4.5.2. Treatment of rat erythrocytes with 10 U apyrase

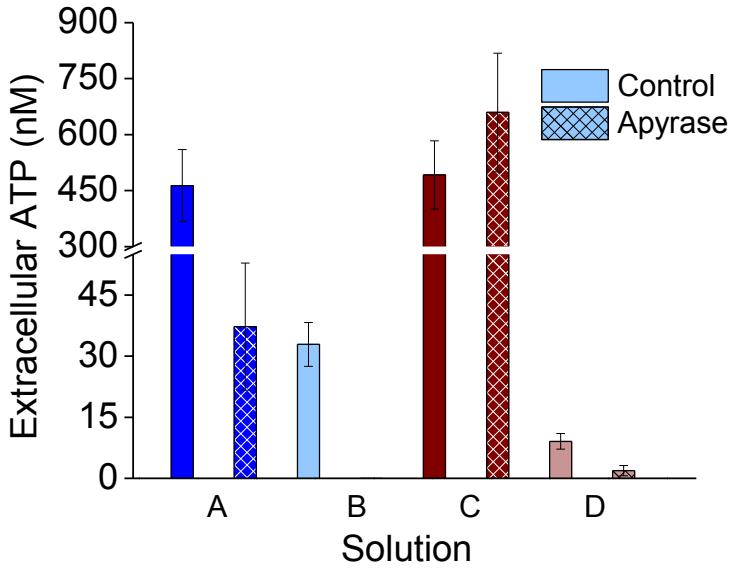

Figure 4.3. **Distribution of extracellular ATP in Wistar rat erythrocyte suspensions and supernatants following 30 min incubation with 10 U apyrase at 37°C.** Erythrocyte suspensions in A were exposed to 10 U apyrase in PSS; suspensions A and C and supernatants B and D were prepared as described section 4.4. Solutions A-D represent erythrocyte suspensions and supernatants as described in Figure 4.1. The error bars denote the standard error of the mean (± s.e.m.). N=4.

Erythrocytes were incubated with apyrase, an ATP-hydrolyzing enzyme which has been used to demonstrate the accessibility of ATP to reagents in solution (3). We also sought to determine whether removing the ATPase would restore the ATP concentrations. In

suspension A, a decrease of at least 80% was observed relative to the time matched control while in supernatant B, the signal was completely extinguished following apyrase treatment. When the apyrase treated PSS was removed and the erythrocytes suspended in fresh PSS, the ATP concentration was up to 40% higher than the control; however solution D was up to 80% lower. Our findings here demonstrate that ATP which is accessible to luciferase (without which it would be impossible to determine ATP concentrations), is also accessible to apyrase.

4.5.3. Effects of CO_2 inhalation on ATP release from erythrocytes

Erythrocytes were collected from Wistar rats by tail bleed and/or by cardiac puncture as described in section 4.4. Suspensions and supernatants prepared from hypercapnic erythrocytes display the same trend as those prepared from normoxic erythrocytes; the ATP concentrations measured in the suspensions were considerably higher than their supernatants. Also, the concentration in suspensions A and C are not significantly different from one another following in vivo exposure to CO_2. However, the administration of CO_2 to Wistar rats prior to blood collection yields extracellular ATP concentrations that are close to double those found in blood obtained by tail bleed (A: 2.36±0.21, B: 2.43±0.26, C: 2.00±0.18, D: 1.85±0.25 Figure 4.4 bottom panel).

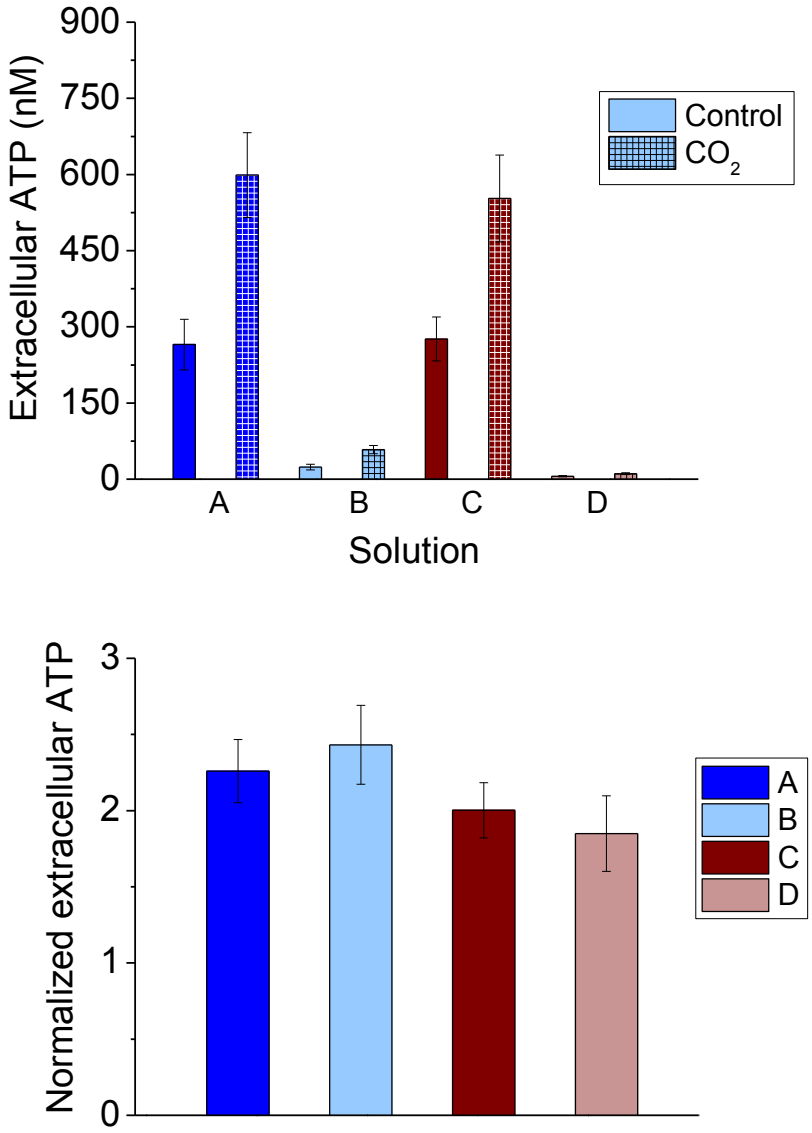

Figure 4.4. ATP release from Wistar rat erythrocytes exposed to hypercapnia. Erythrocytes from Wistar rats collected by tail bleed (control) or by cardiac puncture following hypercapnic euthanasia (CO_2) as described in section 4.4. Upper panel. Effects of 2-3 min *in vivo* exposure to hypercapnia on the extracellular ATP concentrations recorded in erythrocyte supernatants and suspensions. Lower panel. Normalized ATP concentration of hypercapnic erythrocytes compared to erythrocytes collected by tail bleed. A-D represents erythrocyte suspensions and supernatants as described in Figure 4.1. The error bars denote the standard error of the mean (± s.e.m.). N=13 (hypercapnia), N=14 (tail bleed).

In 2008, Faris and Spence determined that two stimuli of ATP release could elicit a greater response when used simultaneously (16). Similarly we sought to determine whether further ATP release could be stimulated from rat erythrocytes collected following CO_2 exposure. The addition of $NaNO_2$ to the medium had no effect on the extracellular ATP concentrations determined for suspensions and supernatants of cells that were exposed to hypercapnia (A: 1.11±0.22, B: 0.90±0.17, C: 0.93±0.14, D: 1.13±0.27) (data not shown). In addition to hypercapnic erythrocytes, normoxic erythrocytes were exposed to 100 nM $NaNO_2$. The erythrocyte suspensions are still 10-50x higher than their respective supernatants, the same trends that have been observed so far with rat erythrocyte samples (data not shown).

4.5.4. Effects of NO_2^- preincubation on ATP release from erythrocytes

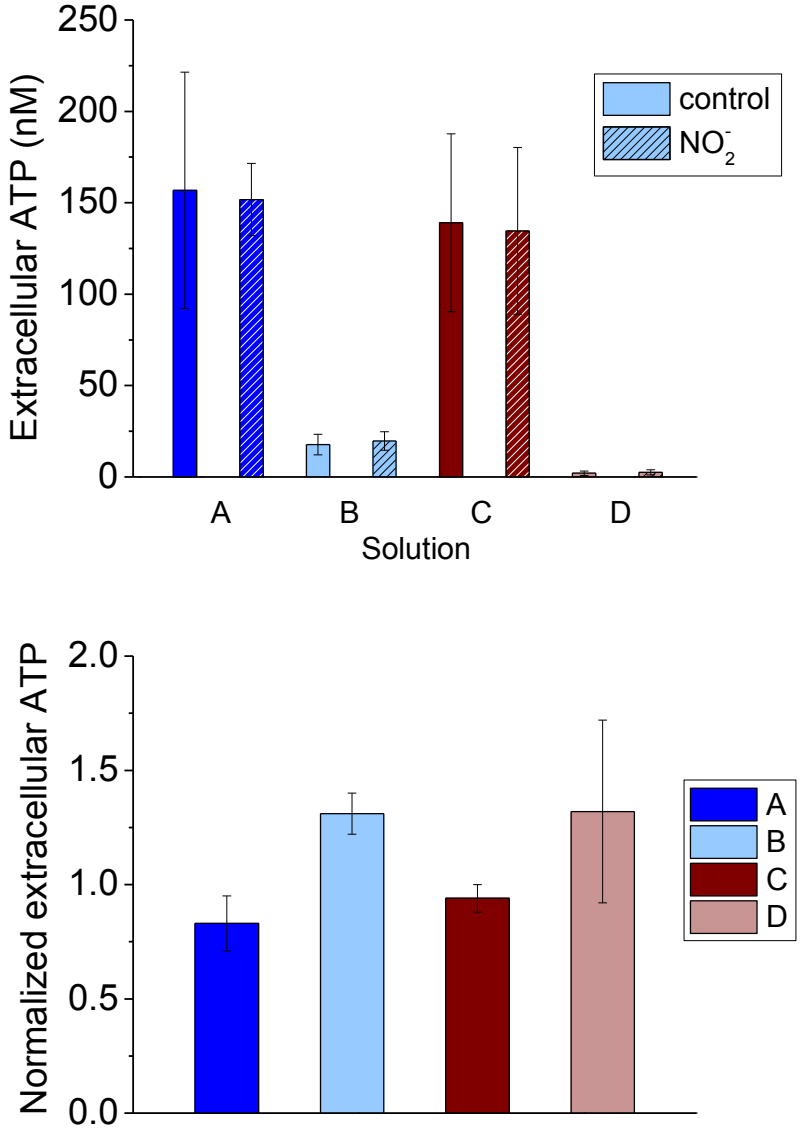

Figure 4.5 Variation in extracellular ATP following 30 s exposure of Wistar rat erythrocytes obtained by tail bleed to $NaNO_2$. Erythrocytes collected from Wistar rats by tail bleed, as described in section 4.4, were either incubated with 100 nM $NaNO_2$ (NO_2^-) or left untreated (control). Upper panel: Effects of incubation with 100 nM $NaNO_2$ on the recorded extracellular ATP concentrations of suspensions and supernatants prepared from erythrocytes collected by tail bleed. Lower panel: Normalized extracellular ATP concentration of $NaNO_2$ treated erythrocytes samples compared to untreated erythrocyte samples. A-D represent erythrocyte suspensions and supernatants as described in Figure 4.1. The error bars denote the standard error of the mean (± s.e.m.). **N=5**

Previous work with human erythrocytes undertaken by our group measured prompt ATP release from erythrocytes following treatment with 100 nM $NaNO_2$. Cells were centrifuged and re-suspended in untreated PSS prior to measuring ATP (suspension C) and a 1.5 fold increase in the extracellular ATP concentration was reported for human erythrocytes (4). There was no additional response to the treatment of hypercapnic rat erythrocytes with 100 nM $NaNO_2$ and so we evaluated the effect of 100 nM $NaNO_2$ with normoxic rat erythrocytes. The relative distribution of ATP between the supernatants and suspensions is similar to what we have observed so far (Figure 4.5 top panel). $NaNO_2$ exposure did not elicit a substantial change in the normalized ATP concentrations measured in solutions A,C and D, however a 30% increase is observed in solution B (Figure 4.5 bottom panel. A:0.83±0.12, B; 1.31±0.09, C: 0.94±0.06, D: 1.32±0.40).

4.5.5. Effect of Deaeration on ATP release from erythrocytes

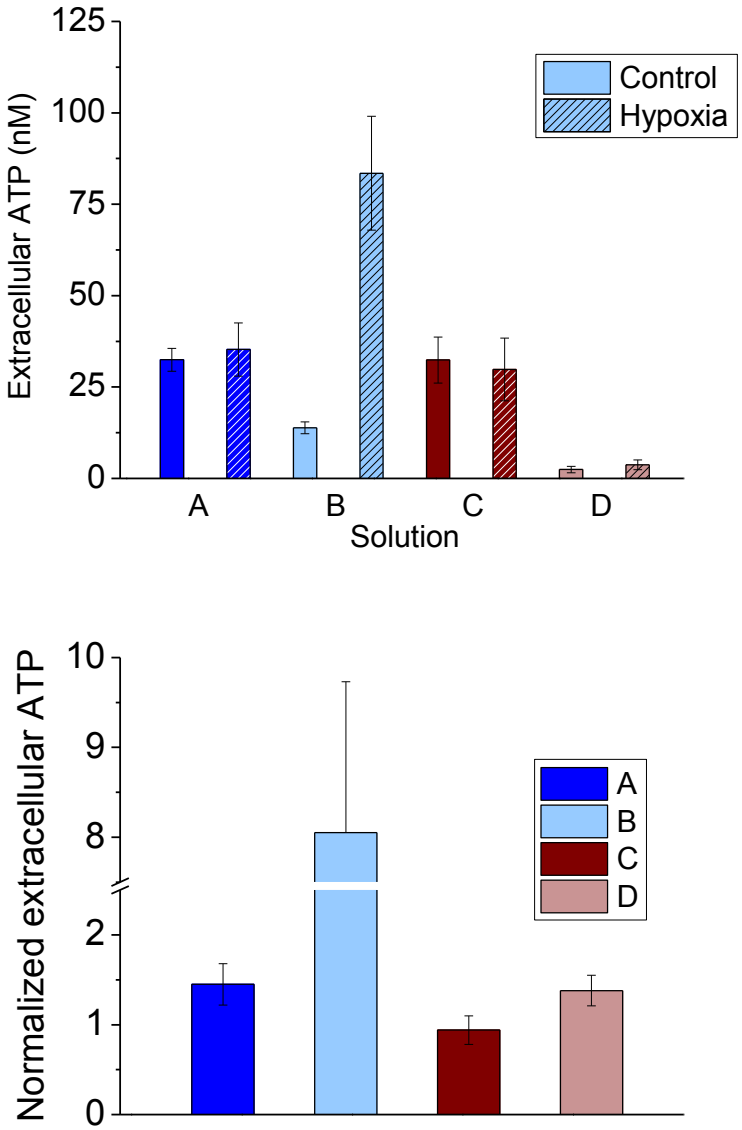

Figure 4.6. Hypoxia induced ATP release from human erythrocytes. Erythrocytes collected by venipuncture from female human volunteers as described in section 4.4. were stored for 15 min, either under air (control) or 5 psi N_2 gas (hypoxia). Upper panel: Effects of hypoxia on the recorded extracellular ATP concentrations of suspensions and supernatants prepared from erythrocytes. Lower panel: Normalized extracellular ATP concentration of suspensions and supernatants prepared from hypoxic erythrocytes compared to normoxic controls. A-D represents erythrocyte suspensions and supernatants as described in Figure 4.1. The error bars denote the standard error of the mean (± s.e.m.). **N=9 replicates from 6 volunteers**

Hypoxic conditions generated by deaerating erythrocytes is a well-documented means of stimulating ATP release in mammalian erythrocyte suspensions (3). When human erythrocyte suspensions were dearated, there was a 40% increase in suspension A and supernatant D and an 600% increase in supernatant B (**N_2**:A: 35.26±7.29 nM, B: 83.48±15.60 nM, C: 29.79±8.57 nM, D: 3.72±1.35 nM; **control**: A: 32.47±8.12 nM, B: 13.83±4.64 nM, C: 32.38±6.30 nM, D: 2.43±0.90 nM). The bottom panel of Figure 4.6 highlights the significant fold increase in ATP release was observed immediately following treatment with N_2, but not following centrifugation and suspension in fresh media (A: 1.45±0.23; C: 0.94±0.16). The increase in ATP that we observed in suspension A is congruent with findings for the hypoxic release of ATP from human erythrocytes (4). Supernatant B showed a considerable increase in measured ATP compared to its time matched control. In the case of supernatant D, the increase is 4x less than that observed in solution B (B: 8.05±1.68, D: 1.38±0.17). In the research regarding ATP release from erythrocytes, the effects of hypoxia have primarily been examined in human (3), or New Zealand rabbit erythrocytes (16). We had found the scarcity of data on rat erythrocytes curious given their ubiquity in drug development and research. The exposure of Wistar rat erythrocytes to 15 min of N_2 gas at 5 psi did not affect the trend seen with erythrocyte suspensions and supernatants, with the notable exception of supernatant B (**N_2**: A: 40.2±7.6, B: 29.3±1.9, C: 50.5±6.1, D: 0.53±0.30; **control**: A: 45.0±11.1, B: 4.7±0.8 C: 42.1±6.9, D: 0.41±0.21). Normalization reveals that deaeration did not significantly increase ATP concentrations in the suspensions (A:1.13±0.40, C:1.34±0.33). As with human erythrocytes, only supernatants B showed a considerable fold increase in normalized ATP concentrations (6.66±0.80).

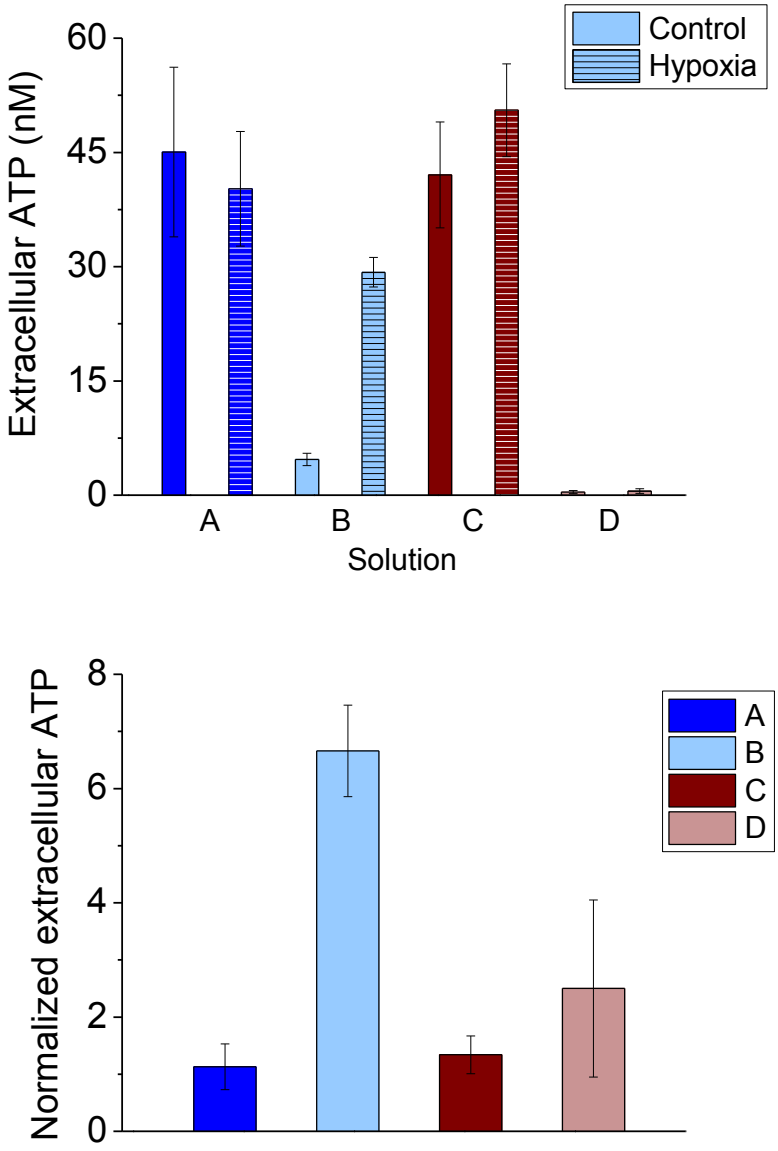

Figure 4.7 Hypoxia induced ATP release from Wistar rat erythrocytes. Erythrocytes from Wistar rats were collected by tail bleed as described in section 4.4. were stored for 15 min, either under air (control) or 5 psi N_2 gas (hypoxia). Upper panel: Effects of hypoxia on the recorded extracellular ATP concentrations of suspensions and supernatants prepared from erythrocytes. Lower panel: Normalized extracellular ATP concentration of suspensions and supernatants prepared from hypoxic erythrocytes compared to normoxic controls. A-D represents erythrocyte suspensions and supernatants as described in Figure 4.1. The error bars denote the standard error of the mean (± s.e.m.). **N=4**

We observed an increase in extracellular ATP concentrations measured in suspension A and supernatants B and D prepared from hypoxic human cells, compared to control. From the rat preparations, only in supernatant B did we observe a significant increase in measured ATP. Human erythrocytes released more ATP into supernatant B than did erythrocytes obtained from rat. Human cells appear to be more sensitive to hypoxia that rat cells which might be related to the disparities between the two mammals' circulatory systems.

4.5.6. The effects of hypo-osmotic stress (40% osmotic downshift) on ATP release from erythrocytes

Experimentally, osmotic stress has been used as a model for shear stress, a known stimulus of ATP release from erythrocytes (24). A 40% downshift in osmolarity was used as a means of stimulating ATP release in suspension A and supernatant B, and the erythrocytes were suspended in an isotonic solution in suspension C and supernatant D, as described in the section 4.3.3. Osmotic downshifts, which induce swelling, a form of mechanical deformation, expose erythrocytes to compression.

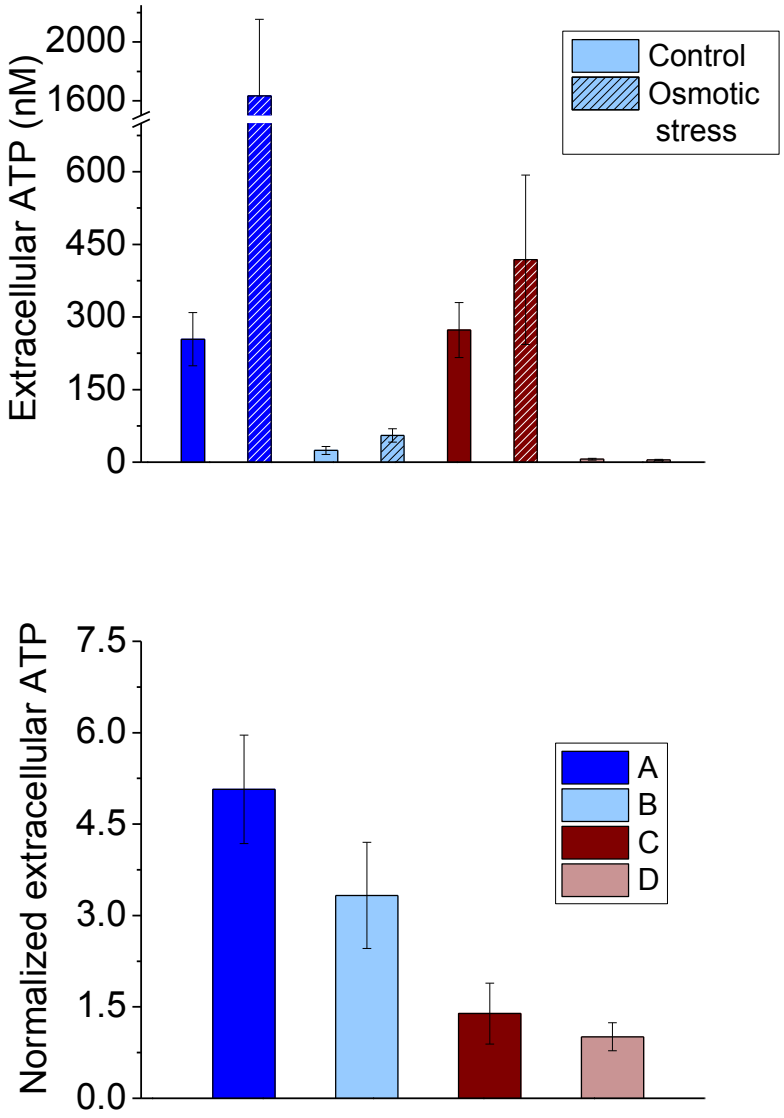

Figure 4.8. Variation in extracellular ATP following 30 s exposure to a 40% osmotic down shift of Wistar rat erythrocytes Erythrocytes from Wistar rats were collected by tail bleed as described in section 4.4. Upper panel: Effects of a 40% osmotic downshift on the recorded extracellular ATP concentrations of suspensions and supernatants prepared from Wistar rat erythrocytes. Lower panel: Normalized extracellular ATP concentration of suspensions and supernatants prepared from Erythrocytes in solution A were suspended in 60% PSS. Solutions A-D represent erythrocyte suspensions and supernatants as described in Figure 4.1. The error bars denote the standard error of the mean (± s.e.m.). N=9

A 40% downshift caused a spike in ATP in both suspension A and supernatant B, which is not observed in suspension C and supernatant D following a return to isotonic media (Figure 4.8 top panel. **Control:**A: 253.91±54.7 nM, B: 24.2±8.4 nM, C: 274.3±56.8 nM, D: 6.2±1.7 nM, **osmotic stress**: A: 1633.5±520.5 nM, B: 55.2±13.8 nM, C: 418.32±175.0 nM, D: 4.7±1.3 nM). The fold difference in ATP concentrations measured in solution A and B following osmotic downshift was significantly higher than the control (Figure 4.8 bottom panel. A: 5.1±0.9; B: 3.3±0.9). The increase in the latter solution is in line with results obtained under similar conditions by the Locovei lab group (24). When these erythrocytes were then suspended in isotonic PSS, there was not a significant fold increase in measured ATP concentrations (Figure 4.8 bottom panel. C: 1.39±0.50; D: 1.01±0.23).

As with the rat erythrocytes, in suspension A and supernatant B of the downshifted human erythrocyte, an immediate, significant increase in measured ATP concentrations was observed, while in solution C and D, the concentrations of ATP determined return to control levels (Figure 4.9 top panel. **control**:A: 13.83±1.23 nM, B: 7.2±2.8 nM, C: 27.2±2.6 nM, D: 1.56±0.44 nM. **osmotic stress**: A: 1733.0±213.5 nM, B: 25.7±11.9 nM, C: 40.1±16.5 nM, D: 1.9±1.1 nM). The trend in ATP for solution A-D from human erythrocytes is similar to rats, even if the fold increase is not (Figure 4.9 bottom panel. A: 132.5±22.1, B: 6.2±2.7, C: 1.6±0.7, D: 1.3±0.38). Human erythrocyte solution A released comparable levels of externalized ATP than rat erythrocytes. However, while the increase in suspension A for rat erythrocytes is ~5 fold, it is 100 fold in human erythrocytes. Like rat erythrocytes, the increase in ATP release observed in humans erythrocytes for supernatant B is congruent with previously reported findings (24). Following

centrifugation and suspension in fresh media, the extracellular ATP concentrations are not significantly different than the control values. It is important to note that two standard curves were used to quantify ATP from suspensions and supernatants exposure to an osmotic downshift. For suspension A and supernatant B, the standards were prepared in 60% PSS; for suspension C and supernatant D, the standards were prepared in 100% PSS. Two standard curves were used because the ion concentration of the media influences the signal intensity recorded (Chapter 3).

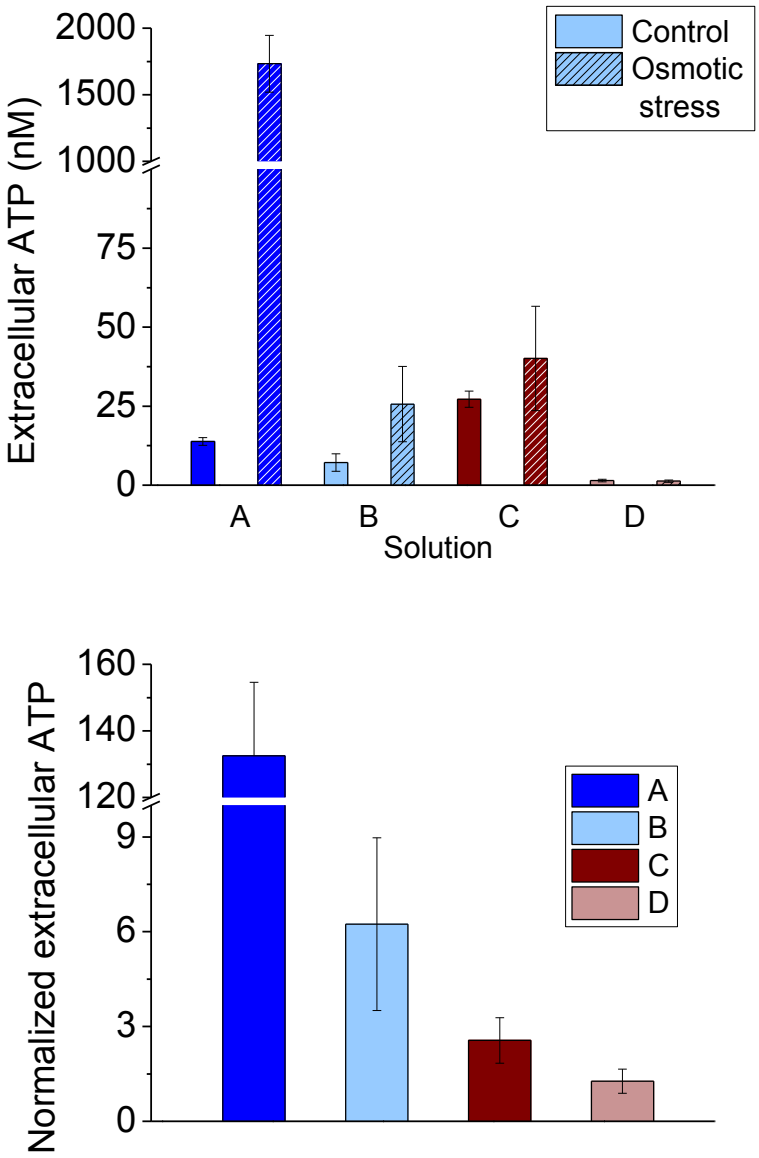

Figure 4.9. Variation in extracellular ATP following 30 second exposure to a 40% osmotic down shift of human erythrocytes obtained by venipuncture. Erythrocytes from female human volunteers were collected by venipuncture as described in section 4.4. Upper panel: Effects of a 40% osmotic downshift on the recorded extracellular ATP concentrations of suspensions and supernatants prepared from Wistar rat erythrocytes. Lower panel: Normalized extracellular ATP concentration of suspensions and supernatants prepared from Erythrocytes in solution A were suspended in 60% PSS. Solutions A-D represent erythrocyte suspensions and supernatants as described in Figure 4.1. The error bars denote the standard error of the mean (± s.e.m.). N=4 replicates from 2 volunteers.

4.6. **Discussion**

We had anticipated that the ATP concentrations in the supernatants would be higher than in the suspensions due to the inner-filter effect observed by our group previously (Chapter 3). The elevated concentration of ATP in the suspensions as compared to their supernatants is indicative of ATP being externalized without being released into the supernatant (Figure 4.1). However, this ATP remains accessible to react with extracellular proteins including luciferase, without which there would not have been a measurable signal. This externalized ATP is also accessible to apyrase, an exogenous ATPase which was used to further probe the extracellular nature of the ATP measured in suspensions and supernatants. An incubation with 10 U of apyrase completely abolished the signal measured in suspension A and supernatant B and following its removal the signal was restored to control values in suspension C and supernatant D (

Figure **4.2**). The release of ATP from erythrocytes into the vascular lumen has to be carefully controlled due to its potency as a vasodilator. It is possible that the ATP measured in erythrocyte suspensions is associated with the membrane through the proteins that have been reported to be involved with its release such as pannexin-1 (24) or the cystic fibrosis transmembrane conductance regulator (CFTR) (31) (23) in order to regulate access to it. The disparity between erythrocyte suspensions and supernatants reveals that both solutions can be highly informative and that there might be distinct mechanisms regulating ATP's externalization to the erythrocyte as oppose to the circulation. Sequential centrifugation brought to light an intriguing aspect of the localisation of ATP in erythrocyte suspensions and established the importance of

continuing to evaluate the ATP concentration of erythrocyte suspensions, in spite of their inherent filtering effect. It is important to note that the maximum hemolysis observed throughout the course of this work was 2.1% (A_{410}=0.35), which is congruent with a literature value for spontaneous hemolysis of 1% (3).

The extensive loss of signal in suspension A and solution B in the presence of apyrase demonstrates that it is possible to hydrolyse both the ATP that is associated with erythrocytes as well as the ATP in the supernatant with apyrase. When treated with apyrase, treatment of the suspensions with apyrase led to 80% loss of their ATP signal and no ATP was detected in their supernatants (Figure 4.3). We have also observed a novel phenomenon by which the ATP that is measured in the erythrocyte solutions is restored following hydrolysis by apyrase and the removal of the ATPase. This observation highlights the importance of the erythrocyte-associated ATP in rat erythrocytes: ATP concentrations in supernatant C were consistently restored to concentrations equal or greater to those initially observed in the control. Apyrase has been used previously to establish whether the ATP measured in the erythrocyte solutions was actually extracellular (3). We confirm that the ATP measured in the suspensions is externalized and accessible to apyrase. The treatment of erythrocytes with apyrase should most definitely be done with human cells. We know that humans have lower levels of ATP in their suspensions, and this could probe the relative importance of ATP from suspensions as compared to from supernatants.

Bergfeld and Forrester, examined the effects of hypoxia and hypercapnia on ATP release from human erythrocytes (3). The opportunity presented itself in our study to examine the

effects of hypercapnia alone on the modulation of ATP release from erythrocytes since CO_2 was used as a means of euthanizing Wistar rats. Hypercapnia along with hypoxia has been demonstrated to elicit ATP release from human erythrocytes (3). We evaluated the effect of hypercapnic asphyxiation of Wistar rats by CO_2 inhalation and observed a 2 fold increase in all of the suspensions and supernatants that were assayed compared to the control samples obtained by tail bleed (Figure 4.4). CO_2, which is a metabolic waste product, is also a potent vasodilator (69). The response that we observe is in line with CO_2's role as a vasodilator which has been documented as potentiating dilation of vessels in the presence of oxygen (70). The precise mechanisms of ATP release in response to either NO_2^- or hypercapnia are unknown.

The addition of NO_2^- *ex vivo* to erythrocytes that were exposed to hypercapnia *in vivo* did not increase the amount of ATP released from erythrocytes and only stimulated ATP release from supernatant B in normoxic erythrocytes (Figure 4.5). Previous work involving the stimulation of ATP from erythrocytes by NO_2^- used elevated concentrations and prolonged (>30 min) exposure times (72) (15). Similar lengthy incubations of human erythrocytes were shown to inhibit ATP release from erythrocytes (4). When experimental conditions similar to our own were used with human erythrocytes, the nanomolar ATP concentration of suspension C was 1.5 fold higher following 30 s exposure to 100 nM NO_2^- (4). Mammalian plasma NO_2^- concentrations, irrespective of whether collected from the venous or arterial circulation, are in a comparable range (ie.10^2 nanomolar) (12). This tight regulation of plasma NO_2^- concentrations in mammals is why we opted to use the same concentration of NO_2^- that had been effective with human erythrocytes for our ex vivo incubations in a physiologically relevant timeframe.

The limited effect of NO_2^- on rat erythrocyte suspensions might be due to the difference between rat and human Hbs (72). Namely, it has been shown that rat erythrocye Hb has two cysteine residues that serve to detoxify the cell that are not present in human Hb (72). It is a distinct possibility that the additional detoxifying capacity of rat Hb is the reason that supraphysiological conditions of NO_2^- have been required to observe a significant increase in the ATP concentrations measured in erythrocyte suspensions in other work (15).

Given that CO_2 stabilizes deoxyHb and can drive O_2 away from Hb (22) it is curious that hypoxia, which increases the relative concentration of deoxyHb, did not yield increases in ATP concentrations from rat erythrocytes suspensions comparable to those observed following hypercapnic asphyxiation. It has been demonstrated that the conformation of Hb is more directly correlated with ATP release than oxygenation state (with the T state favouring ATP release) (73), however it is worth noting that those experiments were performed in the presence of 5% CO_2. Our observations are indicative of a role for CO_2 in potentiating ATP release and this would be congruent with findings in the medical profession, where it has been recognized that carbogen, oxygen containing 5% of CO_2, increases blood flow and is more effective in oxygenating tissues than oxygen alone (70).

The same order of magnitude of extracellular ATP following osmotic downshift was measured in suspension A for human and rat erythrocytes (Human: 1733.0±213.5 nM, rat: 1633.5±520.5 nM) (top panel of Figure 4.8 and Figure 4.9). However, the relative amount of ATP in response to the 40% osmotic downshift compared to control values is significantly higher for suspension A in humans than in rats (human: 132.53±22.10, rat: 5.07±0.19). Similarly, the fold increase in supernatant B is greater in human than in rats

(human: 6.24±2.73, rat 3.33±0.87), despite rat erythrocytes releasing more ATP (human: 25.66±11.92, rat: 55.25±13.82) (top panel of Figure 4.8 and Figure 4.9). Extracellular ATP has been shown to play a role in the recovery of cell volume in response to an osmotic downshift. This response is in part mediated by increasing the permeability of chloride of the membrane (74). Furthermore, the presence of apyrase was shown to limit the recovery of cell volume in rat hepatocytes (74). Humans cells appear to be more sensitive to osmotic downshifts despite human and rat erythrocytes having similar osmolalities (300 mOsm/kg) (51).

4.7. **Conclusion**

Overall, the concentration of ATP measured in the erythrocyte suspensions A and C tended to be far greater than in their respective supernatants B and D, and this regardless of species. These findings are indicative the efficiency of the erythrocytes in managing ATP, which is a vital metabolite. In absence of stimuli of ATP release, the majority of ATP is externalized without being released into the vessel lumen and lost. Instead, only a small portion of the ATP is released to serve as signalling molecule. When the erythrocytes are exposed to stimuli of ATP release, the concentration of ATP available for release or for interaction with purinergic receptors is increased. Further investigation is required to distinguish the role of ATP that can be released into the supernatant (ie. plasma) as opposed to ATP that is measurable in erythrocyte suspensions.

4.8. Acknowledgements

This work was funded by Concordia University and scholarships offered by Astra Zeneca and Hydro-Québec through the Québec Black Medical Association

5. General Conclusions and Suggestions for Future Work

5.1. Conclusions

5.1.1. Chapter 2

To better understand the mechanism of stimulated ATP release from erythrocytes, we tested metHb stabilizing ligands. The rationale being that given metHb's high affinity for the erythrocyte membrane and that membrane bound Hb can signal ATP release, these ligands should stimulate ATP release from erythrocytes. The ATP concentrations of human erythrocyte suspensions were determined to be in the low micromolar range, for all ligands, as well as for the control samples. This finding is not in agreement with current literature with regards to stimulated ATP release from human erythrocytes, which places the ATP concentration for human erythrocyte suspensions in the low nanomolar range (75) (4). While it is possible that hemolysis played a role in these elevated values, our findings in Chapter 4 have brought to light other factors which might have contributed to elevated ATP concentrations: osmotic stress.

Given the availability of an abundance of rodent blood, we sought to simplify the incubation process for the $NaNO_2$ stimulated release of ATP from erythrocytes as well as troubleshoot the assay as required. Exposure of rat erythrocytes to $NaNO_2$ appeared to depress ATP release and there was a notable downward trend in replicate aliquots. This downward trend had raised concerns with regards to the kinetics of the

assay and prompted a more complete evaluation of the luciferin-luciferase assay for ATP as used for intact erythrocyte suspensions detailed in Chapter 3.

5.1.2. Chapter 3

The linearity of the ATP standard curves was not affected by a change in ion concentration or the presence of Hb. However, the signal intensity was decreased as a function of both increasing ion and Hb concentrations. The chemiluminescence signal from erythrocyte suspensions and ATP treated Hb are bimodal as a function of Ht and Hb concentration respectively. The shape of the curves are indicative of a filtering effect which might be mitigated in part by the encapsulation of Hb by the erythrocyte membrane. Ultimately, working at low Ht can help limit the erythrocytes' interference in the measurement of their extracellular ATP by chemiluminescence, but a certain degree of attenuation still occurs. Even with attenuation, the ATP concentration determined for erythrocyte suspensions increased as a function of Ht, which led to the hypothesis that some of the ATP may be associated with the erythrocytes.

5.1.3. Chapter 4

The ATP concentration determined for erythrocyte suspensions was found to be considerably higher than for their supernatants in both human and rat samples. Untreated human erythrocytes had lower ATP concentrations in their suspensions and similar ATP concentrations in their supernatants as compared to rat erythrocytes. The ATP in suspensions and supernatants were accessible to both luciferase, without which the assay would not be possible, and apyrase. In addition, apyrase provided the

first example of the strength of sequential centrifugation as a tool to probe the recovery of the erythrocytes following treatment. In the presence of apyrase, the signal from ATP was significantly decreased, if not extinguished, whereas when apyrase was removed, the levels ATP were restored. Hypercapnia was the only stimulus that affected all of the suspensions and the supernatants for rat erythrocytes. Otherwise, we found that rat erythrocyte suspensions are not sensitive to physiologically relevant concentrations of $NaNO_2$. Also, erythrocytes externalize ATP in response to hypo-osmotic stress. In the end, the difference between ATP concentrations measured in suspensions and supernatants was surprising given that we expected the presence of erythrocytes to attenuate the signal as compared to the supernatant. This finding indicates that of the ATP externalized by the erythrocyte, only a portion would be released into the circulation, effectively conserving one of the cells valuable metabolites.

5.2. Future work

5.2.1. Chapter 2

The effect of metHb stabilizing ligands on ATP release from erythrocytes should be revisited. Understanding whether metHb can play a role in vasodilation could help inform the development of vasoactive compounds that can improve oxygen delivery by stabilizing metHb. While elevated metHb concentrations can cause cyanosis, in certain emergency medical situations, there can be a trade-off between low-grade cyanosis and improved blood-flow and circulation.

5.2.2. Chapter 3

Standard addition and the signal intensity as a function of %Ht were performed with rat and human erythrocytes respectively. The experiments should be reproduced with erythrocytes from both species. Furthermore, the Hb used in the experiments was bovine in origin and given that human and bovine Hb do not have the same affinity for ATP (76), it could be of interest to obtain human Hb for the evaluation of signal intensity as a function of Hb concentration for fixed ATP concentrations. The importance of the erythrocyte membrane in limiting Hb's quenching of the chemiluminescence signal should also be investigated using erythrocyte ghosts to encapsulate Hb in solution.

5.2.3. Chapter 4

There remains a broad range of potential stimuli that can be examined using sequential centrifugation. Including hyper-osmotic stress, which causes cell shrinkage, in addition to using a physiological range of osmotic shifts (<230 or >350 mOsm/L) would allow us to examine the sensitivity of erythrocytes the different ion concentration that could be encountered in individuals with varying degrees of renal problems, providing potential insight into the role that erythrocyte ATP might play in co-morbidities (77). That being said, I believe that the next step for this work would be to determine whether the same measurements can be taken using whole blood. Erythrocyte ATP is a known vasodilator and impaired ATP release from erythrocytes

has been associated with the vascular pathologies of diseases such as primary pulmonary hypertension (32). Being able to streamline the ATP assay using whole blood would facilitate the use of ATP as a metric of vascular health. The assay would then require a smaller volume of blood, ideally that could be obtained via a simple pinprick as opposed to venous collection.

6. Bibliography

1. **U.S. National Library of Medicine.** RBC count. *MedlinePlus.* [Online] 01 25, 2011. [Cited: August 11, 2012.] http://www.nlm.nih.gov/medlineplus/ency/article/003644.htm#Normal%20Values.

2. *Insulin Inhibits Low Oxygen-Induced ATP Release from Human Erythrocytes: Implication for Vascular Control.* **Hanson, M S., et al.** 2009, Microcirculation, Vol. 16, pp. 424-433.

3. *Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia.* **Bergfeld, G R. and Forrester, T.** 1992, Cardiovascular Research, Vol. 26, pp. 40-47.

4. *Nitrite and nitroglycerin induce rapid release of the vasodilator ATP from erythrocytes.* **Garcia, J I., et al.** 2010, Journal of Inorganic Biochemistry, Vol. 104, pp. 289-296.

5. *The red blood cell as an oxygen sensor: what is the evidence?* **Ellsworth, M L.** 2000, Acta Physiologica Scandinavica, Vol. 168, pp. 551-559.

6. *Red blood cell regulation of microvascular tone through adenosine triphosphate.* **Dietrich, H H., et al.** 2000, American Journal of Physiology Heart and Circulatory Physiology, Vol. 278, pp. H1294-H1298.

7. *Conducted vascular responses: communication across the capillary bed.* **Collins, D M., McCullough, W T. and Ellsworth, M L.** 1998, Microvascukar Research, Vol. 56, pp. 43-53.

8. *Ideas about control of skeletal and cardiac muscle blood flow (1876-2003): cycles of revision and new vision.* **Rowell, L B.** Journal of Applied Physiology, Vol. 97, pp. 384-392.

9. *Maximal perfusion of skeletal muscle in man.* **Andersen, P. and Saltin, B.** 1985, Journal of Physiology , Vol. 366, pp. 233-249.

10. *The Effects of Nitric Oxide on Red Cell Development and Phenotype.* **Čokić, V P. and Schechter, A N.** Current Topics in Developmental Biology. 2008, Vol. 82, pp. 169-215.

11. **Klabunde, R E.** Nitric Oxide. *Cardiovascular physiology concepts.* [Online] 09 23, 2008. [Cited: August 11 2012.] http://www.cvphysiology.com/Blood%20Flow/BF011.htm.

12. *Plasma nitrite reflects consitutive nitric oxide activity in mammals.* **Kleinbongard, P., et al.** 2003, Free Radical Biology & Medicine, Vol. 35, pp. 790-796.

13. *Erythrocytes are the major intravascular storage sites of nitrite in human blood.* **Dejam, A., et al.** 2005, Blood, Vol. 106, pp. 734-739.

14. *Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation.* **Cosby, K., et al.** 2003, Nature Medicine, Vol. 9, pp. 1498-1505.

15. *Nitrite enhances RBC hypoxic ATP synthesis and the release of ATP into the vasculature: a new mechanism for nitrite-induced vasodilation.* **Cao, Z., et al.** 2009, American Journal Physiology Heart and Circulatory Physiology, Vol. 297, pp. 1494-1503.

16. *Measuring the simultaneous effects of hypoxia and deformation on ATP release from erythrocytes.* **Faris, A. and Spence, D M.** 2008, The Analyst, Vol. 133, pp. 678-682.

17. *Role of erythrocyte in regulating local O2 delivery mediated by hemoglobin oxygenation.* **Jagger, J E., et al.** 2001, American Journal of Physiology Heart and Circulatory Physiology, Vol. 28, pp. H2833-H2839.

18. *Characterization of the deoxyhemoglobin binding site on human erythrocyte band 3: implications for O_2 regulation of erythrocyte properties.* **Chu, H., et al.** 2008, Blood, Vol. 111, pp. 932-938.

19. *Hypoxia limits antioxidant capacity in red blood cells by altering glycolytic pathway dominance.* **Rogers, Stephen C., et al.** 2009, The FASEB Journal, Vol. 23, pp. 1-12.

20. *On the functional use of the membrane compartmentalized pool of ATP by the Na+ and Ca++ pumps in human red blood cell ghosts.* **Hoffman, J F., Dodson, A. and Proverbio, F.** 2009, Journal of General Physiology, Vol. 134, pp. 4351-4361.

21. *On the peripheral and central chemoreception and control of breathing: an emerging role of ATP.* **Gourine, A V.** 2005, Journal of Physiology, Vol. 568, pp. 715-724.

22. *Effect of carbon dioxide on human hemoglobin.* **Salhany, J M.** 1972 , The Journal of Biological Chemistry, Vol. 257, pp. 3799-3801.

23. *Deformation Induced ATP release from red blood cells requires CFTR activation.* **Sprague, R S., et al.** 1998, American Journal of Physiology Heart and Circulatory Physiology, Vol. 275, pp. H1726–H1732.

24. *Pannexin 1 in erythrocytes: Function without a gap.* **Locovei, S., Bao, L. and Dahl, G.** 2006, Proceedings Of The National Academy Of Sciences, Vol. 103, pp. 7655–7659.

25. *Extracellular ATP stimulates volume decrease in Necturus red blood cells.* **Light, D B., et al.** 1999, American Journal of Physiology, Vol. 3, pp. 480-491.

26. *Prostacyclin Receptor-Mediated ATP Release from Erythrocytes Requires the Voltage-Dependent Anion Channel (VDAC).* **Sridharan, M., et al.** 2011, American Journal of Physiology Heart and Circulatory Physiology, p. In press.

27. *Spectral emission and quantum yield of firefly bioluminescence.* **Seliger, H H and McElroy, W D.** 1960, Archives of Biochemistry and Biophysics, Vol. 88, p. 136.

28. *Nucleoside triphosphate specificity of firefly luciferase* . **Moyer, J D. and Henderson, J F.** 1983, Analytical Biochemistry, Vol. 131, pp. 187-189 .

29. *Haemoglobin interferes with the ex vivo luciferase luminescence assay.* **Colin, M., et al.** 2000, Gene Therapy, Vol. 7, pp. 1333–1336.

30. *Assessment of ionic quenching onsoil ATP bioluminescence reaction.* **Wen, G., et al.** 2001, Soil Biology and Biochemistry, Vol. 33.

31. *Cystic Fibrosis Transmembrane Conductance Regulator Facilitates ATP Release by Stimulating a Separate ATP Release Channel for Autocrine Control of Cell Volume Regulation.* **Braunstein, G M., et al.** 2001, Journal of Biological Chemistry, Vol. 276, pp. 6621-6630.

32. *Impaired release of ATP from red blood cells of humans with primary pulmonary hypertension.* **Sprague, R S., et al.** 2001, Experimental Biology and Medicine, Vol. 226, pp. 434-439.

33. *An altered oxidant defense system in red blood cells affects their ability to release nitric oxide-stimulating ATP.* **Carroll, J., et al.** 2006, Molecular Biosystems, Vol. 2, pp. 305-311.

34. *Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and s-nitrosohemoglobin.* **Singel, D J. and Stamler, J S.** 2005, Annual Review of Physiology, Vol. 67, pp. 99-145.

35. *Catalytic generation of N_2O_3 by the concerted nitrite reducatase and anhydrase activity of hemoglobin.* **Basu, S., et al.** 2007, Nature Chemical Biology, Vol. 3, pp. 785-794.

36. *Mitochondrial aldehyde dehydrogenase mediates vasodilator responses of glyceryl trinitrate and sodium nitrite in the pulmonary vascular bed of the rat.* **Badejo Jr, A M., et al.** 2010, American Journal of Physiology Heart and Circulatory Physiology l, Vol. 299, pp. H819-826.

37. *A hypothesis on the metabolism of glyceryl trinitrate in vascular endothelial cells.* **Tian, Y., et al.** 2001, Clinica Chimica Acta, Vol. 313, pp. 51-57.

38. *Methemoglobinemia from intravenous nitroglycerin: a word of caution.* **Bojar, R M., et al.** 1987, Annals of Thoracic Surgery, Vol. 43, pp. 332-334.

39. *Association of methemoglobinemia and intravenous nitroglycerin adminstration.* **Kaplan, K J., et al.** 1985, American Journal of Cardiology, Vol. 55, pp. 181-183.

40. *Nitrite uptake and metabolism and oxidant stress in erythrocytes.* **May, J M., et al.** 2000, American Journal of Physiology - Cell Physiology, Vol. 279, pp. 1946-1954.

41. *In vivo studies on methemoglobin formation by sodium nitrite.* **Imaizumi, K., et al.** 1980, International Archives of Occupational and Environmental Health, Vol. 45, pp. 97-104.

42. *Pharmacokinetics of sodium nitrite-induced methemoglobinemia in the rat.* **Kohn, M C., et al.** 2002, Drug metabolism and disposition, Vol. 30, pp. 676-683.

43. *Characterisation of erythrocytic uptake and release and disposition pathways of nitrite, nitrate, methemoglobin and iron-nitrosyl hemoglobin in the human circulation.* **Hon, Y Y., et al.** 2010, Drug metabolism and disposition, Vol. 38, pp. 1707-1713.

44. *The magnetic properties and structure of ferrihemoglobin (methemoglobin) and some of its compounds.* **Coryell, C D., Stitt, F. and Pauling, L.** 1937, Journal of the American Chemical Society, Vol. 59, pp. 633-642.

45. *Fluoride produces endothelium-dependent relaxation and endothelium-independent contraction in coronary arteries.* **Cushing, D J., et al.** 1990, Journal of Pharmacology and Experimental Therapy, Vol. 254, pp. 28-32.

46. *Sodium azide dilates coronary arterioles via activation of inward rectifier K+ channels and Na^+-K^+-ATPase.* **Qamirani, E., et al.** 2006, American Journal of Physiology Heart and Circulatory Physiology, Vol. 290, pp. H1617-1623.

47. **National Institute for Occupational Safety and Health.** Emergency Response Card. *Centers of Disease Control.* [Online] May 12, 2011. [Cited: July 23, 2012.] http://www.cdc.gov/niosh/ershdb/PrintableERC_29750038.html.

48. **Antonini, E and Brunori, M.** *Hemoglobin And Myoglobin In Their Reactions With Ligands.* New York : Elsevier, 1971. p. 18.

49. *An electron paramagnetic resonance study of the affinity of nitrite for methemoglobin.* **Goetz, Bradley I., et al.** 2010, Nitric Oxide, Vol. 22, pp. 149-154.

50. Sigma-Aldrich - Catalog Product Detail. *Sigma-Aldrich Canada.* [Online] [Cited: August 11, 2012.] http://www.sigmaaldrich.com.

51. *Osmolality of mammalian blood and of media for culture of mammalian cells.* **Waymouth, C.** 1970, In Vitro, Vol. 2, pp. 109-127.

52. *P2 receptors in cardiovascular regulation and disease.* **Erlinge, D and Burnstock, G.** 2008, Purinergic Signalling, Vol. 4, pp. 1-20.

53. *Local control of blood pressure by purines.* **Burnstock, G.** 1987, Blood Vessels, Vol. 24, pp. 156-160.

54. *The energy source for bioluminescence in isolated systems.* **McElroy, W D.** 1947, Proceedings Of The National Academy Of Sciences, Vol. 342.

55. *Membrane Proteins of Incubated Erythrocytes: Effects of Sulphydryl Inhibition.* **Zail, S S. and Van Den Hoek, A K.** 1977, British Journal of Haematology, Vol. 37, pp. 353-361.

56. *Lactate interferes with ATP release from red blood cells.* **Rozier, M D., Zata, V J. and Ellsworth, M L.** 2007, American Journal of Physiology Heart and Circulatory Physiology , Vol. 292, pp. H3038–H3042.

57. *Ionic Effects on Firefly Bioluminescence Assay of Red Blood Cell ATP.* **Aledort, L M., Weed, R I. and Troup, S B.** 1966, Analytical Biochemistry, Vol. 17, pp. 268-277.

58. **Sigma Aldrich.** Firefly lantern extract Catalog Product listing. *Sigma-Aldrich Canada.* [Online] [Cited: August 14, 2012.] http://www.sigmaaldrich.com/catalog/product/sigma/fle250?lang=en&region=CA.

59. *The bioluminescent detection of platelet released ATP, collagen-induced release and potential errors.* **Soslau, G. and Parker, J.** 1991, Thrombosis, Vol. 66, pp. 15-21.

60. **Harris, D C.** *Quantitative Chemical Analysis 7th Edition.* China Lake : W.H. Freeman & Company, 2006. p. 1008.

61. *Use of Firefly Luciferase in ATP-Related Assays of Biomass, Enzymes, and Metabolites.* **Lundin, A.** *Methods in Enzymology.* 2000, Vol. 305, pp. 346-370.

62. *Erythrocytes: Oxygen Sensors and Modulators of Vascular Tone*. **Ellsworth, M L., et al.** 2009, Physiology, Vol. 24, pp. 107-116.

63. *A Selenium Analogue of Firefly d-Luciferin with Red-Shifted Bioluminescence Emission.* **Conley, N R., et al.** 2012, Angewandte Chemie International Edition, Vol. 51.

64. *Aminoluciferins as functional bioluminogenic substrates of firefly luciferase.* **Takakura, H., et al.** 2011, Chemistry, Vol. 6, pp. 1800-1810.

65. *Erythrocytes: Oxygen Sensors and Modulators of Vascular Tone.* **Ellsworth, M L., et al.** 2009, Physiology, Vol. 24, pp. 107-116.

66. *P2 receptors in cardiovascular regulation and disease.* **Erlinge, D. and Burnstock, G.** 2008, Purinergic Signalling, Vol. 4, pp. 1-20.

67. *Identification of cytoskeletal elements enclosing the ATP pools that fuel human red blood cell membrane cation pumps.* **Chu, H., et al.** 2012, Proceedings Of The National Academy Of Sciences. Vol 109, pp 12794-12799.

68. **Sigma Aldrich.** Potato Apyrase Product Catalog listing. *Sigma Aldrich.* [Online] Sigma Aldrich, 2012. [Cited: July 10, 2012.] http://www.sigmaaldrich.com/catalog/product/sigma/a6535?lang=en&region=CA.

69. *Enhanced catheter propagation with hypercapnia during superselective cerebral catherisaton.* **Djurberg, H G., Tjan, G T. and Al Moutaery, K R.** 1998, Neuroradiology, Vol. 40, pp. 466-468.

70. *Carbogen inhalation increases oxygen transport to hypoperfused brain tissue in patients with occlusive carotid artery disease: increased oxygen transport to hypoperfused br.* **Ashkanian, M., et al.** 2009, Brain Research, Vol. 1304, pp. 90-95.

71. *Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation.* **Crawford, J H., et al.** 2006, Blood, Vol. 107, pp. 566-574.

72. *Highly Reactive Cysteine Residues in Rodent Hemoglobins.* **Miranda, J J.** 2000, Biochemical and Biophysical Research Communications , Vol. 275, pp. 517–523.

73. *Role of erythrocyte in regulating local O2 delivery mediated by hemoglobin oxygenation.* **Jagger, J E., et al.** 2001, American Journal of Physiology Heart Circulatory Physiology, Vol. 28, pp. H2833-H2839.

74. *Autocrine signaling through ATP release represents a novel mechanism for cell volume regulation.* **Wang, Y., et al.** 1996, Proceedings Of The National Academy Of Sciences, Vol. 93, pp. 12020-12025.

75. *Metal-activated C-peptide facilitates glucose clearance and the release of a nitric oxide stimulus via the GLUT1 transporter.* **Meyer, J., et al.** 2008, Diabetologia, Vol. 51, pp. 175-182.

76. *Interaction of Organic Phosphates with Bovine Hemoglobin.* **Breepoel, P M., Kreuzer, F. and Hazevoet, M.** 1981, Pflugers Archives, Vol. 389, pp. 219-225.

77. **Jewish General Hospital.** In-patient critical values. *Department of Biochemsitry*. [Cited: August 13, 2012.]
http://www.jgh.ca/uploads/Diagnostic%20Medicine/JGH%20Lab%20Handbook.pdf