I. My Voyage through the World of Science

Since an early age, I have always had a great interest for the science of the microscopic. From the atom, the basic building block of matter, to the living cell, the fundamental unit of life, I realized from a young age just how much we can learn about our vast universe from observing its smallest components. This passion for science grew as I got older and culminated in applying for a research position at Wayne State University in Detroit, Michigan. The mentor I worked with, Dr. Jin-Sook Lee, was studying cellular structure-function at the time, and I would read through papers and journals in the lab in between cleaning dishes and filling buckets of ice. Eventually, I was allowed to pursue my own research projects, by which time I had a considerable understanding of, and interest in cellular transport, specifically the movement of important and vital molecules into and out of the cell, through the plasma membrane.

My interest in cardiovascular health stems from a range of factors. There is currently a health epidemic in the United States: our largely unhealthy lifestyles, fatty and high-cholesterol diet, and lacking exercise, combine with genetic factors, contribute to some of the highest levels of obesity, diabetes, and heart disease. In fact heart disease is prevalent in most developed and some developing countries, contributing to more deaths then cancer and HIV combined, world-wide. Heart disease causes are often misleading; for instance, obesity has been linked to heart disease for decades, yet many who suffer from cardiovascular ailments are slim and hardly fit this profile. In the past several decades, elevated blood cholesterol das been linked to heart disease. Though cholesterol is essential for numerous physiological functions, it is well documented that the long-term effects of elevated levels of plasma cholesterol pose a significant

health risk and is causal to diseases including angina, cardiovascular disorders, and diabetes. However, the short-term effect of elevated plasma cholesterol was unknown, and this is what I set out to determine. After doing some literature survey, I found that the majority of these "healthy" individuals in fact have very high levels of cholesterol (hypercholesterolemia). The accumulation of this fatty substance in the arteries, a condition known as atherosclerosis, causes difficulties in blood circulation, and therefore the impairment in body distribution of gases (oxygen and carbon dioxide) resulting in dizziness, and fatigue with the mildest of physical exertion. Additionally, I found a study dating back to the early 1970's in which doctors found that increasing the concentration of plasma cholesterol in humans had an adverse effect on oxygen transport into the red blood cell. Based on these studies, I wanted to determine the effect of elevated cholesterol at the plasma membrane of red blood cells on both water and gas transport into the cell.

My project was unique due to several reasons. First of all, the study was the first that set out to determine the role of elevated cell plasma membrane cholesterol on water and gas transport into the red blood cell. Second, the study was designed to determine the molecular underpinnings of the impairments of elevated plasma membrane cholesterol, and furthermore, new and novel approaches were used to conduct the study. Because of this, I had to incorporate statistics into my studies. This included many significance tests to see whether results were significantly higher or lower in comparison to each other. I had not taken a statistics class when I began this project, however, it was fairly simple to pick up on the necessary calculations and get an understanding of how statistics is essential and used in science. This project definitely connected science and mathematics for me as I progressed in my research, not because it was

intensely mathematical or computational but rather because there was such a consistent need for analysis and the establishment of significance of the results throughout the project. I was able to make connections between calculations and apply what I had learned as a middle and high schooler, and this was one of the most satisfying experience of the research process.

For students interested in pursuing their own research projects, whether they be in biology, physics, chemistry, mathematics, or engineering, specifically projects that combine mathematics and science, my chief piece of advice would be to find a topic or specific subject that they enjoy immensely and to actively seek out professors and researchers in the field. Establishing contact with a mentor is often the most difficult aspect of student research, but if a student demonstrates their interest in and passion for science, such mentors are usually more than willing to let the student into their lab to get a feel of what hands-on research is like.

II. My Research

A. Introduction

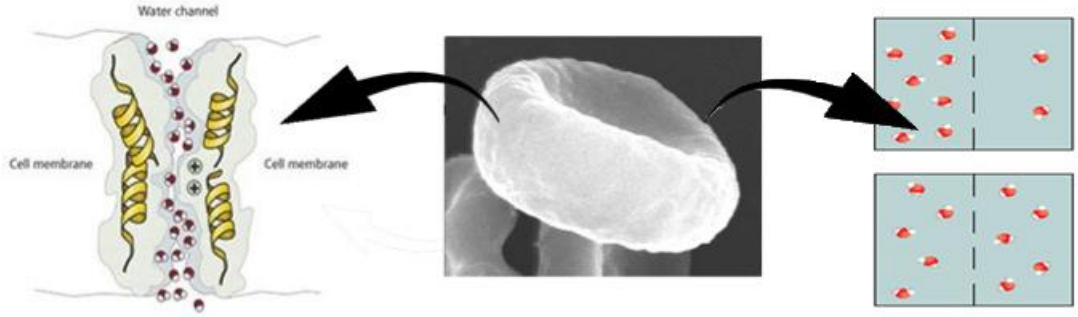
Elevated blood cholesterol is a rapidly growing threat to global health (1). Caused by many factors, including lack of physical activity, gender, age, diet, or genetic predisposition, elevated cholesterol in the long-term can manifest itself in numerous ailments, including heart attack, stroke, and angina. However, the short term effects of elevated blood cholesterol are considerably less known. According to the American Heart Association, a third of the U.S. population has ”high risk” cholesterol levels. Due to the large number of individuals living with high cholesterol, resulting in cardiovascular diseases that claim nearly a million lives each year, a molecular understanding of the

immediate effect of elevated cholesterol on human health is critical for the prevention, management, and treatment of cardiovascular disorders resulting from elevated blood cholesterol.

B. Background

Red blood cells (RBC), or erythrocytes, are collectively perhaps the most important component of blood, and serve important roles in ferrying life-sustaining molecules such as water, carbon dioxide, and oxygen throughout the human body. This highly vital cellular transport begins with the uptake of these molecules into the RBC through its plasma membrane, which can occur via two possible transport mechanisms:

Fig. 1. Schematic drawing of the water channel AQP1 at the RBC plasma membrane as well as the process of osmosis via the plasma membrane (AQP1 cartoon from *Chemistry World, Nov 2003*).



Passive transport involving the movement of water and gases through diffusion via osmosis at the RBC plasma membrane as a result of concentration gradients.

Active transport: In contrast to diffusion, active transport occurs via channels. In 1991 a more rapid mode of transportation for water into RBC through a water channel called Aquaporin-1 protein (AQP1) was discovered (2). Subsequent studies demonstrated AQP1 to be capable of transporting gases as well (3-5). AQP1 has a tetramer conformation in the plasma membrane of RBC and rapidly channels water, carbon dioxide, and oxygen

into and out of the cell. The effect of cholesterol on the molecular transport of O_2 has been suggested as early as the 1970s (6), when elevated plasma cholesterol levels were found to decrease the oxygen diffusion rates in RBC. Therefore, it was hypothesized in the present study that elevated blood cholesterol would be a detriment to both water and gas transport in RBC.

C. Objective

The goal of the present study was threefold:

-**Identify** the molecular players at the RBC membrane that regulates and transports both water and gases.

-**Determine** the effect of elevated plasma membrane cholesterol on the water and gas transport in RBC, and

-**Identify** therapeutic agents to ameliorate the detrimental effects of elevated plasma membrane cholesterol on RBC function.

D. Red Blood Cell Preparation

To investigate the immediate detrimental effect of high cholesterol on water and CO_2 transport in RBC, rat blood was used to isolate RBC and used in the present study. RBC purity was determined by both light and scanning electron microscopy (Fig.2).

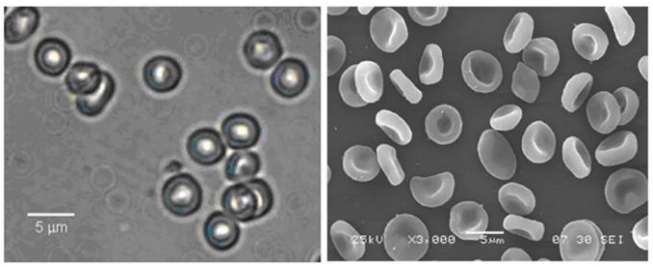


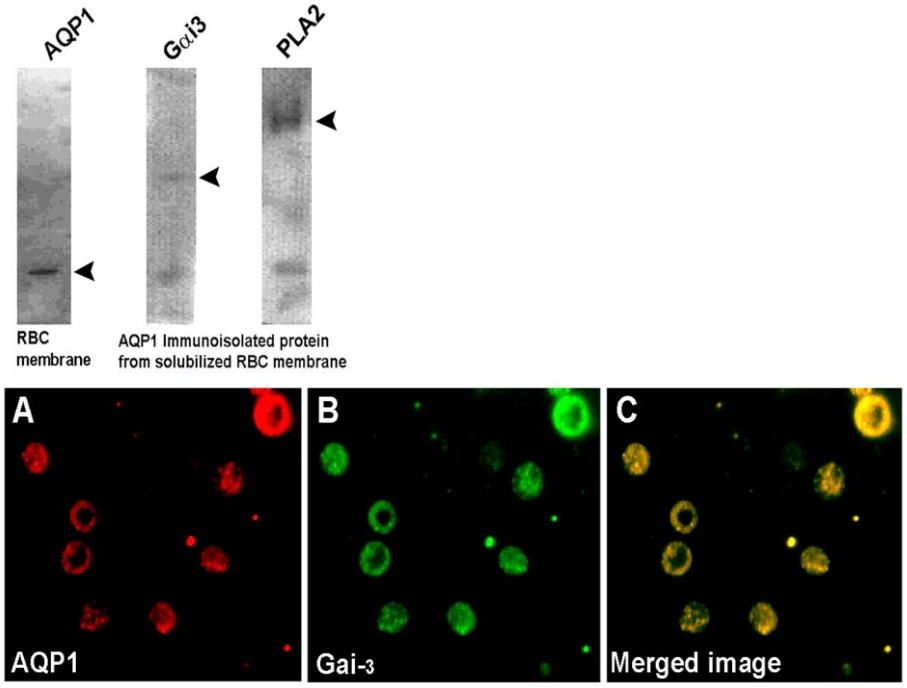
Fig. 2. Light (left) and electron (right) micrographs of isolated red blood cells (RBC) from rat blood. Note the purity of the intact 4-5 µm RBC. Bar = 5 µm.

Since the GTP-binding Gai3 protein and phospholipase A2 (PLA2) have previously been

reported to regulate AQP1 function (7), Western Blots, immunoprecipitation, and immunocytochemistry were performed to determine the presence of AQP1 and its association with Gai3 and PLA2 at the RBC membrane (Fig.3,4).

Fig. 3. One dimensionally resolved 10 μg of solubilized RBC membrane probed with AQP1 specific antibody (far left), and AQP1-immunoisolates from 200 μg of solubilized RBC membrane preparations (two right lanes) probed using $G_{\alpha i3}$, and PLA2 specific antibody.

Fig. 4. (A) Immuno-red fluorescent distribution of AQP1, and (B) immuno-green fluorescent distribution of $G_{\alpha i3}$ protein in isolated RBC. (C) Note the co-localization of the two proteins (yellow) when the images are merged, confirming the co-immunolocalization of AQP1 and $G_{\alpha i3}$ proteins in the RBC plasma membrane.



E. Water Transport Measurements

Real-time changes in RBC size were determined by dynamic light scattering (DLS) studies using a spectrofluorimeter (Fig.5 schematic drawing Below Left), and a schematic drawing of the RBC plasma membrane showing the major players in AQP1 regulation at the RBC membrane (Fig.5 Below).

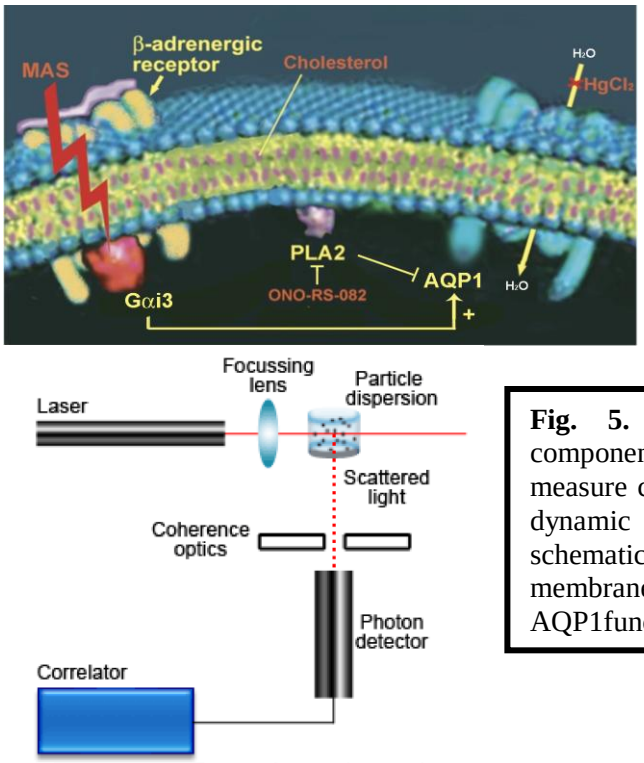


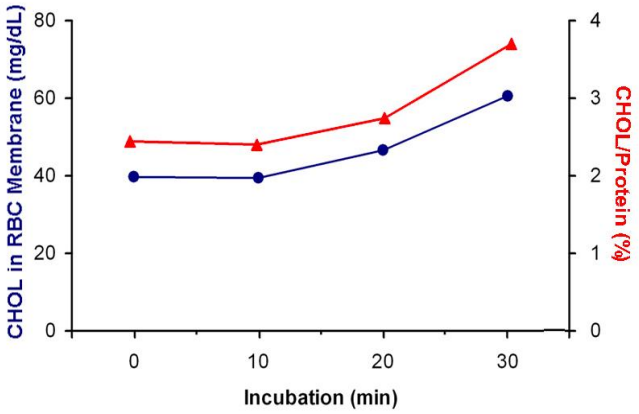
Fig. 5. Schematic diagrams of the key components in a spectrofluorimeter (Left), to measure changes in RBC size in real-time using dynamic light scattering (DLS). (Above) schematic drawing of the RBC plasma membrane showing the molecules regulating AQP1function.

Besides AQP1, the two other key proteins depicted in Fig. 5 are PLA2 and Gai3. These two proteins were found to co-localize with the AQP1 protein at the RBC membrane, as demonstrated from immunoprecipitation and immunolabeling studies (Fig. 3, 4). Since the GTP-binding Gai3 protein and PLA2 is associated with AQP1 function (7), and PLA2 is elevated in areas of plaques (8) and statins significantly reduce blood PLA2 levels, the effect of the PLA2 protein on RBC function was investigated using a PLA2 inhibitor ONO-RS-O82. Mastoparan was used to stimulate the water channel AQP1 via the Gai3 protein at the RBC membrane.

Time-dependent incorporation of cholesterol into RBC membrane (Fig.6) was estimated using a cholesterol assay kit, and the optimal concentration of regulatory molecules influencing water intake into RBC (Fig.7), was determined from dose response experiments using DLS. The role of cholesterol on AQP1-induced rapid gating of water into RBC was determined in isolated RBC preparations, and following addition of cholesterol and/or its depletion from RBC membrane

using β-cyclodextran (βCD), prior to Mastoparan-induced RBC water entry.

Fig. 6. Reconstitution of cholesterol into RBC plasma membrane. Note the time-dependent increase in cholesterol content in RBC plasma membrane following incubation of isolated RBC with 40 μM cholesterol. This study demonstrates the incorporation of exogenous cholesterol into RBC membrane, with maximal incorporation following 30 min of incubation.



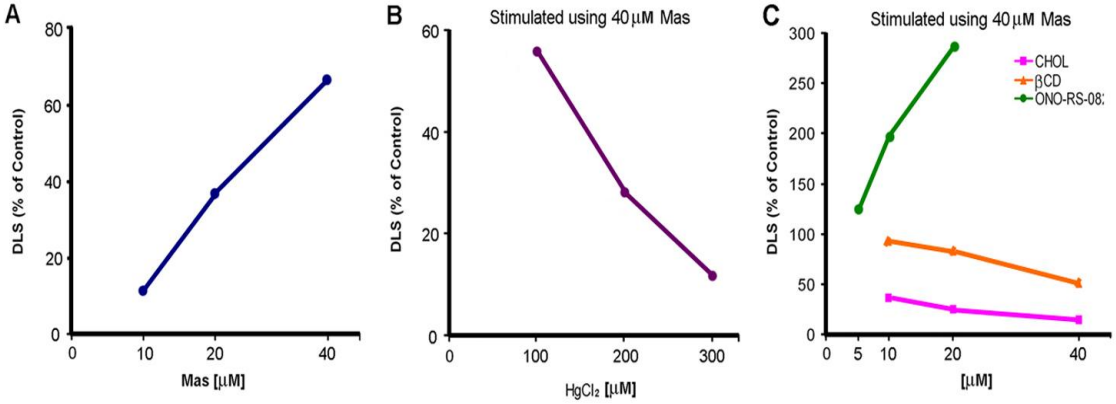


Fig. 7. Determination of the optimal dose of various compounds used in the study.

In order to determine if elevated cholesterol had any effect on the passive transport of water by osmosis into RBC , (Fig.8), experiments were carried our on RBC preincubated in 40 mM cholesterol or 300 mM $HgCl_2$, followed by incubation in water. RBC size changes were then determined in real-time using DLS.

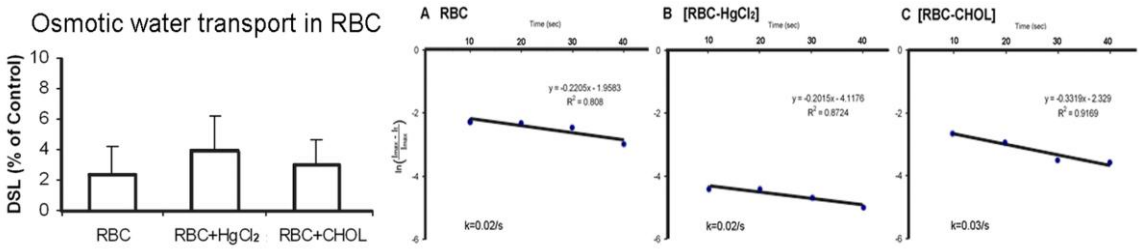
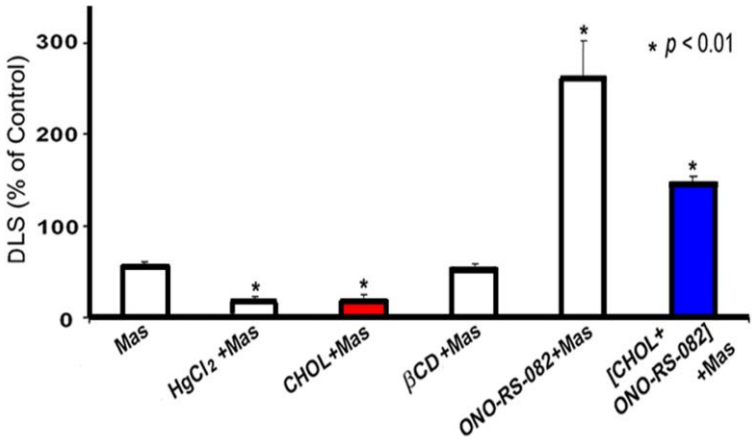


Fig. 8. Water transport into RBC via osmosis or passive transport. Note no significant effect of $HgCl_2$ or cholesterol on osmosis-driven water transport into RBC is observed. Both the potency and efficacy of water entry into RBC is greatly lower in passive transport, than water entry via active transport through the AQP1 channel following exposure to Mastoparan (Fig.7, 9-11).

Having established that elevated cholesterol at the RBC membrane does not influence the passive transport of water into RBC, the effect of cholesterol as well as the effect of removing cholesterol using β-cyclodextrin and the effect of blocking the phospholipase A2 protein using ONO-RS-082 was tested on active water transport through the AQP1 channel (Fig. 9).

Fig. 9. Rapid water gating and consequent swelling of isolated RBC, impaired by high cholesterol, and ameliorated by the PLA2 inhibitor ONO-RS-082. Data represents percent of control from dynamic light scattering (DLS) experiments on isolated RBC in presence of various modulators. Data is obtained from 2-3 experiments with 8-15 value points/experiment (* $P < 0.01$).



The kinetics of active water transport into the RBC plasma membrane were also measured using DLS, and it was found that the presence of elevated cholesterol resulted in a decrease in the rate of active water transport, and was even more detrimental than the AQP1 blocker $HgCl_2$. The results of the DLS studies were further confirmed using light microscopy, where

changes in RBC size were observed (Fig. 10). Surprisingly, ONO-RS-082 was ineffective in restoring the efficacy or rate of water transport into RBC that had been pre-exposed to elevated cholesterol (Fig. 11).

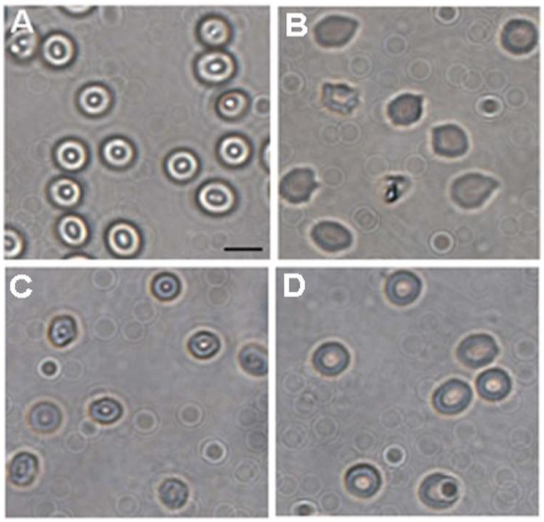


Fig. 10. Mastoparan-induced water transport into RBC is inhibited in the presence of $HgCl_2$ and cholesterol. (A) Isolated rat RBC. Bar = 5 μm. (B) RBC swell following exposure to 40 μM Mastoparan. (C) Isolated rat RBC preincubated in 300 μM $HgCl_2$ and followed by stimulation using 40 μM Mastoparan. Note the abrogation of Mastoparan-induced swelling. (D) Similarly, isolated rat RBC preincubated in 40 μM cholesterol followed by stimulation using 40 μM Mastoparan, exhibits significant inhibition of Mastoparan-induced swelling.

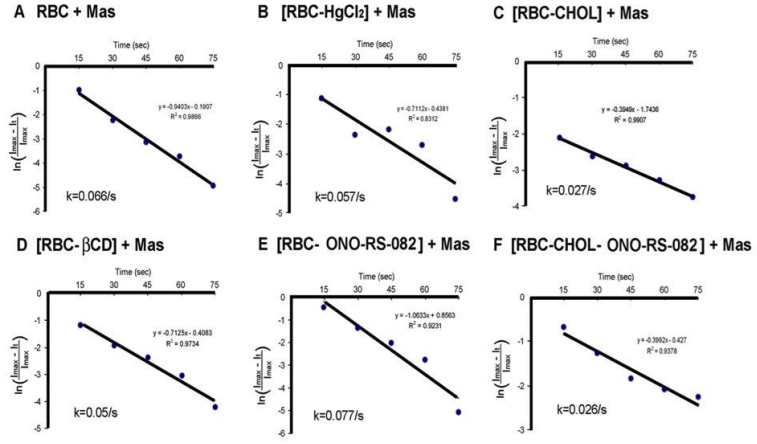


Fig. 11. The efficacy (rate) of Mastoparan-stimulable RBC swelling following exposure to various channel blockers, to elevated cholesterol, or the removal of cholesterol. Exposure of RBC to cholesterol, demonstrates a 50% loss (k = 0.066/s to 0.027/s) in the rate of RBC swelling following exposure to Mastoparan. Data represents one of 2-3 similar experiments.

Based on earlier studies that the potassium and chloride ion channels as well as the vH-ATPase play a role in regulation of AQP1 function (7), the presence and interactions between them in RBC was studied using immunoprecipitation and Western Blots, and their effects on AQP1 facilitated water transport were measured using optimal doses of their respective blockers.

Fig. 12. Potassium and chloride channels, and vH-ATPase are associated with AQP1 at the RBC membrane. Ten micrograms of total RBC membrane (RBCM) protein solubilized in 1% Triton-Lubrol, and 100 μg of RBCM immunoprecipitated using AQP1-specific antibody (RBCM-IP-AQP1), was resolved using 10% SDS-PAGE, followed by electrotransfer to nitrocellulose membrane, and probed using vH-ATPase, KiR6.1 (potassium channel), and CLC3 (chloride channel) specific antibody. Note the association of vH-ATPase, potassium, and chloride channels with AQP1 at the RBC membrane.

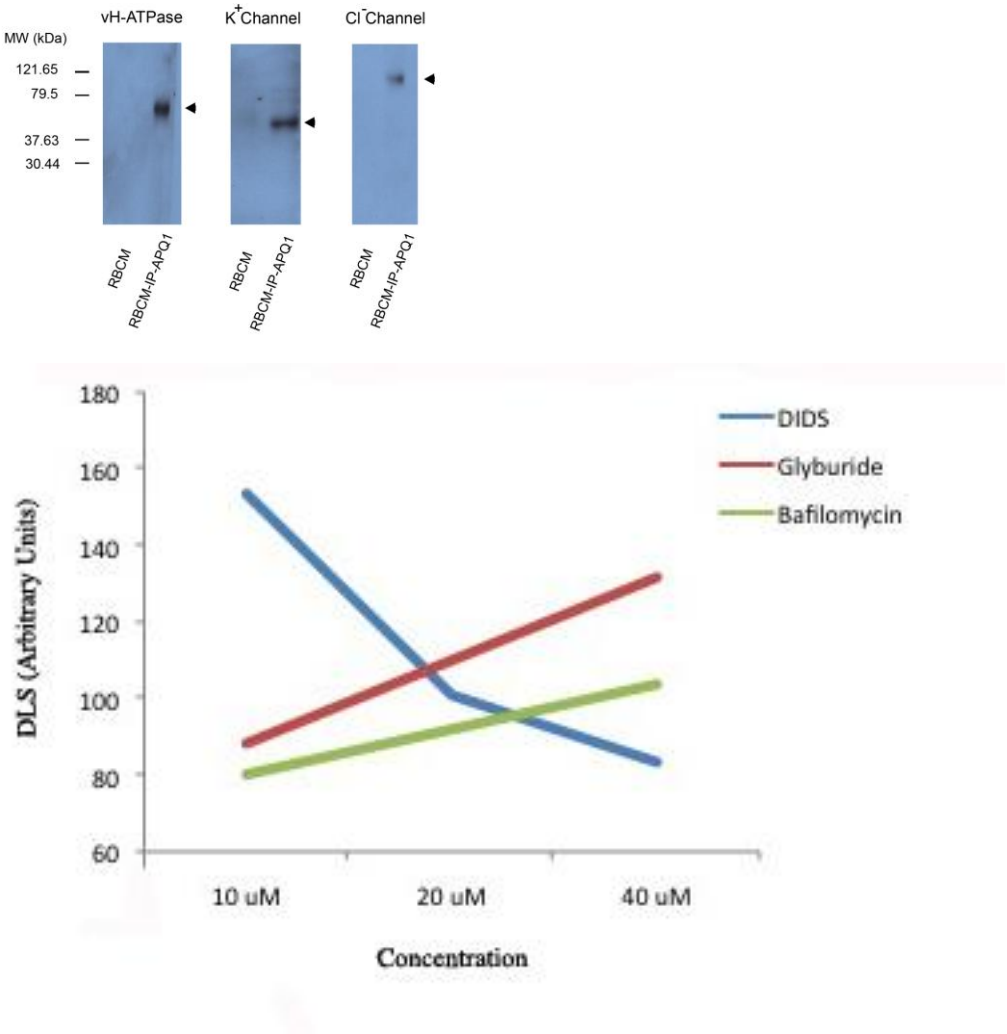


Fig. 13. Dose response of the chloride channel inhibitor diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), the potassium channel inhibitor (Glyburide), and the vH-ATPase inhibitor (Bafilomycin), on Mastoparan-induced water entry into RBC. Note a dose-dependent inhibition of water entry in presence of DIDS, as opposed to a dose-dependent stimulation of RBC water entry in the presence of Glyburide and Bafilomycin. The study further confirms the presence of vH-ATPase, potassium, and chloride channels at the RBC membrane, and their participation in the regulation of AQP1 function.

Having established the stimulatory role of the chloride channel as well as the inhibitory roles of the potassium channel and vH-ATPase, the effect of cholesterol on the activity of these ion channels was determined (Fig. 14).

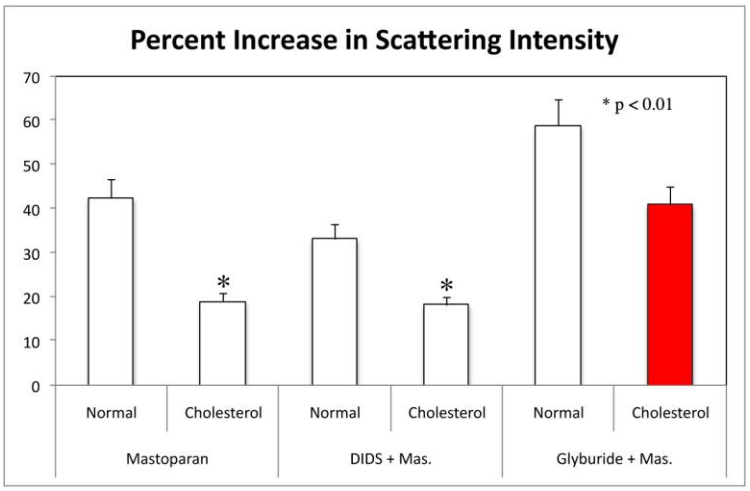


Fig. 14. Rapid water gating and consequent swelling of isolated RBC is impaired by high cholesterol, and ameliorated by the potassium channel inhibitor Glyburide. Note inhibition of rapid water entry following exposure to the chloride channel blocker DIDS (diisothiocyanatostilbene-2,2'-disulfonic acid), and stimulation in presence of the potassium channel blocker Glyburide. Data represents percent increase in dynamic light scattering (DLS) intensity of experiments on isolated RBC in presence of various modulators. Data is obtained from 3 experiments with 8-15 value points/experiment (* $P < 0.01$).

Finally, the response of RBC to ion channel blockers applied in different orders was used to investigate and determine the molecular pathway involved in active water transport into the cell.

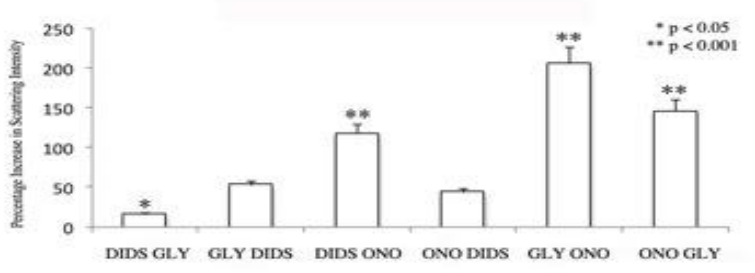


Fig. 15. Effect of the various sequential combinations of 40 μM of either Cl^-, K^+, or PLA2 inhibitor (ONO-RS-082, abbreviated as ONO), on Mastoparan-induced RBC water entry. Results suggest that in the signaling cascade involved in the G-protein mediated AQP1 stimulation, the chloride channel precedes the potassium channel, followed by PLA2. Data represents one of 3 separate experiments, with 8-15 value points/experiment that were averaged and is presented with standard deviation. (*$P < 0.05$ and **$P < 0.001$).

F. Gas Transport Measurements

To measure carbon dioxide transport into RBC, a novel method was designed and employed, based upon the biochemical and biophysical properties of the RBC, as detailed in Fig. 16.

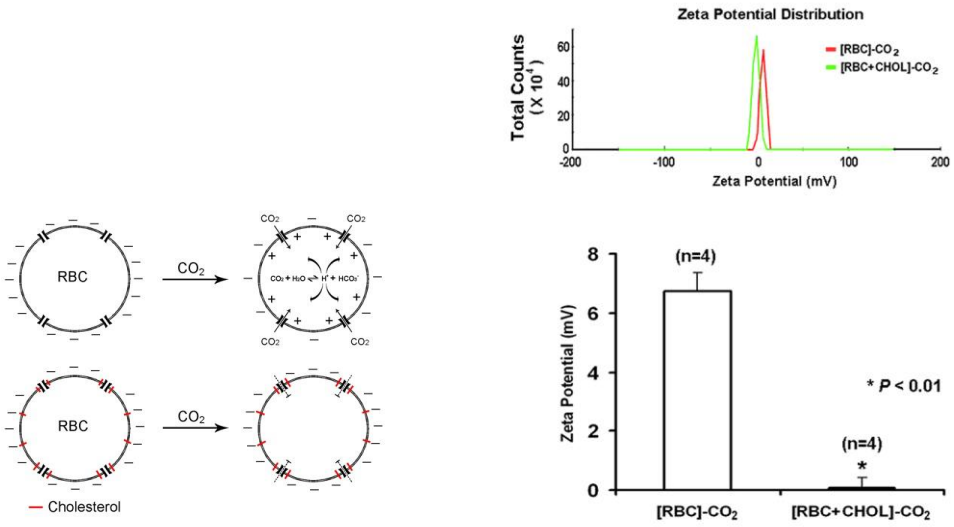
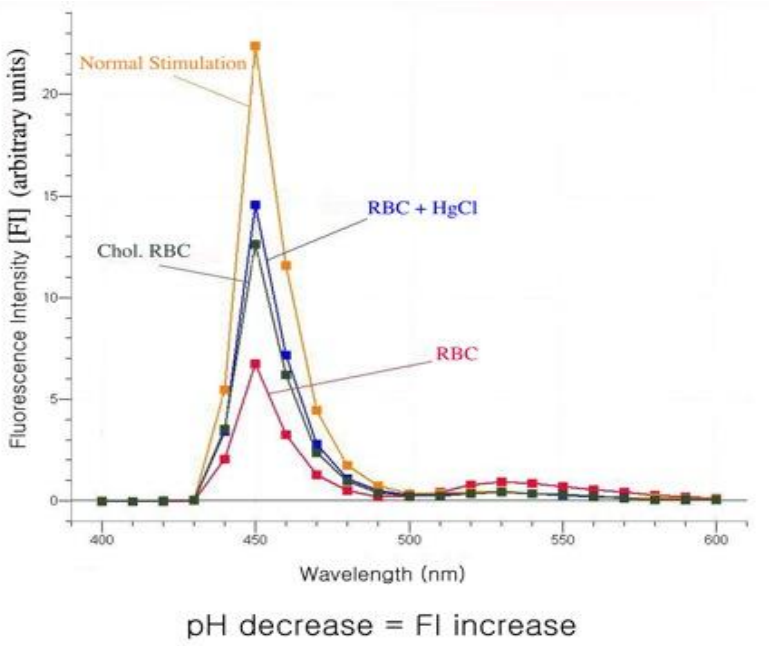


Fig. 16. (Above) The possible mechanism of RBC membrane charge neutralization following CO_2 entry, based upon the formation of carbonic acid from CO_2 and H_2O within the cell.

Fig. 17. (Above Right) Zeta potential measurements of RBC surface charge, before and following exposure to cholesterol and carbon dioxide. Note the detrimental effect of cholesterol on CO_2 entry into RBC, as demonstrated in the near abrogation of RBC membrane charge neutralization following exposure to CO_2.

These results were further validated using a pH-sensitive fluorescence dye experiments, as displayed in Fig. 18.

Fig. 18. The pH sensitive dye (BCESF:2',7'-bis-2-carboxyethyl-5-6-carboxyfluorescein), demonstrates CO_2 transport through AQP1 at the RBC membrane. The AQP1 inhibitor $HgCl_2$ blocks CO_2 entry, preventing RBC acidification. Similarly, cholesterol blocks the entry of CO_2 into RBC.



G. Conclusions

These studies demonstrate for the first time that elevated cholesterol has an extremely detrimental effect on active H_2O and CO_2 transport in RBC, specifically through the AQP1 channel located at the RBC plasma membrane. This detriment of elevated cholesterol to RBC function can be ameliorated by the K^+ ion channel blocker glyburide, and the PLA2 inhibitor ONO-RS-082. This new understanding will help in the early treatment and management of patients with elevated blood cholesterol levels. Future studies will provide critical information on the precise role of all molecular players involved in H_2O and gas transport in human RBC, enabling the fine-tuning of therapy to overcome health detriments.

H. References

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