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Liposomal delivery of PDE5 inhibitors and UT-15C to human erythrocytes

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LIPOSOMAL DELIVERY OF PDE 5 INHIBITORS AND UT-15C TO HUMAN ERYTHROCYTES

by

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ABSTRACT

Previous studies have shown that the controlled release of adenosine triphosphate (ATP) from human erythrocytes is an important mechanism for the regulation of vascular caliber. However, erythrocytes from patients with pulmonary arterial hypertension (PAH) fail to release ATP in response to the physiological stimuli of exposure to low oxygen tension or mechanical deformation of a magnitude these cells would encounter in the pulmonary circulation. This defect could be a significant contributor to the increased pulmonary vascular resistance (PVR) that is the cause of the pathological increase in vascular pressures in humans with PAH.

One important approach to the treatment of PAH is to reduce PVR with the administration of drugs such as prostacyclin or its analogs and phosphodiesterase 5 (PDE5) inhibitors. These medications can be used alone or in combination and may have serious unwanted side effects that are additive when used in combination.

Here an alternative drug delivery technique using drug-loaded liposomes is investigated that may allow for increased drug efficacy and, possibly, reduced unwanted side effects. Liposomes can encapsulate drugs and deliver them directly to specific cells. This research describes the successful incorporation and delivery of a clinically-used PDE5 inhibitor, tadalafil, via liposomes, to human erythrocytes to increase ATP release from erythrocytes exposed to the prostacyclin analog, UT-15C. This demonstrates the effectiveness of this technique and forms the basis for future *in vivo* trials to improve drug delivery and patient quality of life. Liposomal delivery, currently underutilized clinically, could represent a new treatment paradigm for patients with circulation issues.

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

NOMENCLATURE

Symbol	Description
AC	adenylyl cyclase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
DM2	diabetes mellitus type 2
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
EDRF	endothelium-derived relaxing factor (nitric oxide, NO)
FOQELS	Fiber Optic Quasi Electric Light Scattering particle size analyzer
GDP	guanosine diphosphate
GMP	guanosine monophosphate
GTP	guanosine triphosphate
IPR	prostacyclin receptor
L-NAME	N^G-nitro-L-arginine methylester
N_2	nitrogen
NO	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen
PAH	pulmonary arterial hypertension
PDE	phosphodiesterase
PGI_2	prostacyclin I_2
PS	1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine
PSS	physiological salt solution
PVR	pulmonary vascular resistance
RBCs	red blood cells, erythrocytes

sGC	soluble guanylyl cyclase
UT-15C	United Therapeutics' prostacyclin analog (the oral form of treprostinil)
VDAC	voltage dependent anion channel

1. INTRODUCTION

1.1. PULMONARY CIRCULATION

The circulation of blood within the body is necessary for life. A vital function of the circulation is oxygen delivery and waste removal. However, the system cannot operate at maximum capacity at all times. It is necessary for flow to be directed away from over-supplied tissues and delivered to regions of need. This is achieved in a variety of ways. Although blood flow could be stopped entirely in some areas in order for the output of the heart to be directed to other areas, this would be highly detrimental to the cells no longer receiving oxygen and nutrients. Alternatively, flow could be reduced in certain areas and increased in others. One way to achieve this is to increase the diameter of blood vessels in the under-supplied region leading to selective increases in blood flow to these areas. However, there must be highly regulated and selective mechanisms that control this selective blood vessel dilation.

Investigations to explore what controls these vascular changes include those of Dr. Persson et al. This group explored the function of endogenous nitric oxide (NO), also known as the endothelium-derived relaxing factor (EDRF) formed in the vascular endothelium from L-arginine via the activity of the enzyme nitric oxide synthase (NOS). NO induces vascular smooth muscle relaxation resulting in increases in vascular diameter (decreases in vascular resistance) in the pulmonary circulation [1].

Pulmonary vascular resistance (PVR) increases substantially in response to decreases in the oxygen tension in the alveoli of the lungs (hypoxic pulmonary vasoconstriction). This vasoconstriction is potentiated by the application of N^G-nitro-L-arginine methylester (L-NAME), an inhibitor of NOS [2,3]. These findings suggest that NO plays a critical role in the determination of vascular resistance in the lung. However, these studies did not reveal what stimulated the production of NO in the pulmonary circulation under these conditions. The data in support of the role of erythrocyte-derived adenosine triphosphate (ATP) as a stimulus for NO synthesis in the pulmonary circulation is described in the next sections.

1.1.1. A Critical Effector of Vascular Dilation in the Pulmonary Circulation. Experiments using isolated, blood-perfused rabbit lungs treated with N^G-nitro-L arginine methyl ester (L-NAME), an inhibitor of NO synthesis, or its vehicle showed a significant increase in pressure in response to increases in flow rate when NO synthesis was prevented [3]. This demonstrates that NO is important in the regulation of vascular resistance.

In separate studies, it was shown that inhibition of NO synthesis resulted in a reduction in blood flow to portions of the lung that were hypoxic. One interpretation of this result is that NO was synthesized in areas of increased pulmonary vascular resistance and was opposing vasoconstriction [2].

1.1.2. Role of Blood Components in the Synthesis of NO in the Pulmonary Circulation. Blood consists of red blood cells (erythrocytes, RBCs), white blood cells (leukocytes), platelets, and plasma. Each component serves a specific function. The research presented in this dissertation focuses on the element that affects vasodilation. The contribution of blood components to pulmonary vascular resistance was investigated in studies in isolated rabbit lungs, perfused with whole blood, plasma, erythrocytes, or a dextran-containing physiological salt solution (PSS). When lungs were perfused with blood or isolated RBCs suspended in PSS (at a hematocrit of 20% or greater) inhibition of NO synthesis with L-NAME resulted in significant increases in pulmonary vascular resistance when flow rates were increased [4]. In contrast, when the lungs were perfused with either plasma containing normal numbers of platelets and white blood cells but without erythrocytes or PSS containing dextran to increase viscosity to that of whole blood, L-NAME had no effect on flow-induced increases in vascular pressure. These studies demonstrated that RBCs were required for flow-induced increases in NO synthesis in the pulmonary circulation.

1.1.3. Mechanism of Erythrocyte-induced Increases in NO Synthesis in the Pulmonary Circulation. To investigate what property of the RBC is necessary to stimulate the synthesis of NO under the conditions described above, experiments were performed by Sprague et al. using human or dog RBCs perfused through isolated rabbit lungs. L-NAME increased pulmonary vascular resistance only in lungs perfused with human RBCs [5].

In order to determine why dog RBCs failed to stimulate NO synthesis under these conditions, RBCs from both species were subjected to mechanical deformation as would be encountered as these cells traverse the pulmonary circulation using a St. George's Blood Filtrometer. This device contains a filter with pores of 12, 8, or 5 μm. Human erythrocytes are approximately 7-8 μm [6]. Passage through smaller pores force the RBCs to flex to pass through when pulled by hydrodynamic forces. The amount of ATP that was released as the cells were deformed was then measured in the filter effluent. ATP was only found in the effluent when human RBCs were passed through the filters. These findings are consistent with the hypothesis that the ability of RBCs to release ATP when they pass through the pulmonary circulation plays an important role determining vascular diameter in that vascular bed.

Importantly, in addition to deformation, ATP is released from human erythrocytes via additional mechanisms including activation of the prostacyclin receptor (IPR) that is present in these cells [7]. The signaling pathways involved in ATP release in response to these stimuli have been characterized and have important differences. For example, in these pathways, the initial step is the activation of a different heterotrimeric G protein and the final conduit for ATP release differs [8-11].

1.2. PULMONARY CIRCULATION CONTROL

The pathway shown in Figure 1.1 illustrates the proposed method of signal transduction within the erythrocyte to initiate ATP release in response to prostacyclin stimulation [7]. Prostacyclin (PGI_2) or its analogs (such as UT-15C) bind to the prostacyclin receptor (IPR) [7] which activates the alpha subunit of the heterotrimeric G-protein G_s [9] via hydrolysis of guanosine triphosphate (GTP) into guanosine diphosphate (GDP) [12]. This active form of the alpha subunit then activates adenylyl cyclase (AC) [13] to hydrolyze ATP into cyclic adenosine monophosphate (cAMP) [14]. In this pathway, cAMP then activates the cAMP-dependent protein kinase (PKA) [15] which in turn activates the cystic fibrosis transmembrane conductance regulator (CFTR) [16]. ATP release is through the voltage dependent anion channel (VDAC) [10]. In the pulmonary vasculature, the released ATP binds to the purinurgic receptor 2y (P2Y) that stimulates NO production and leads to vascular relaxation [17].

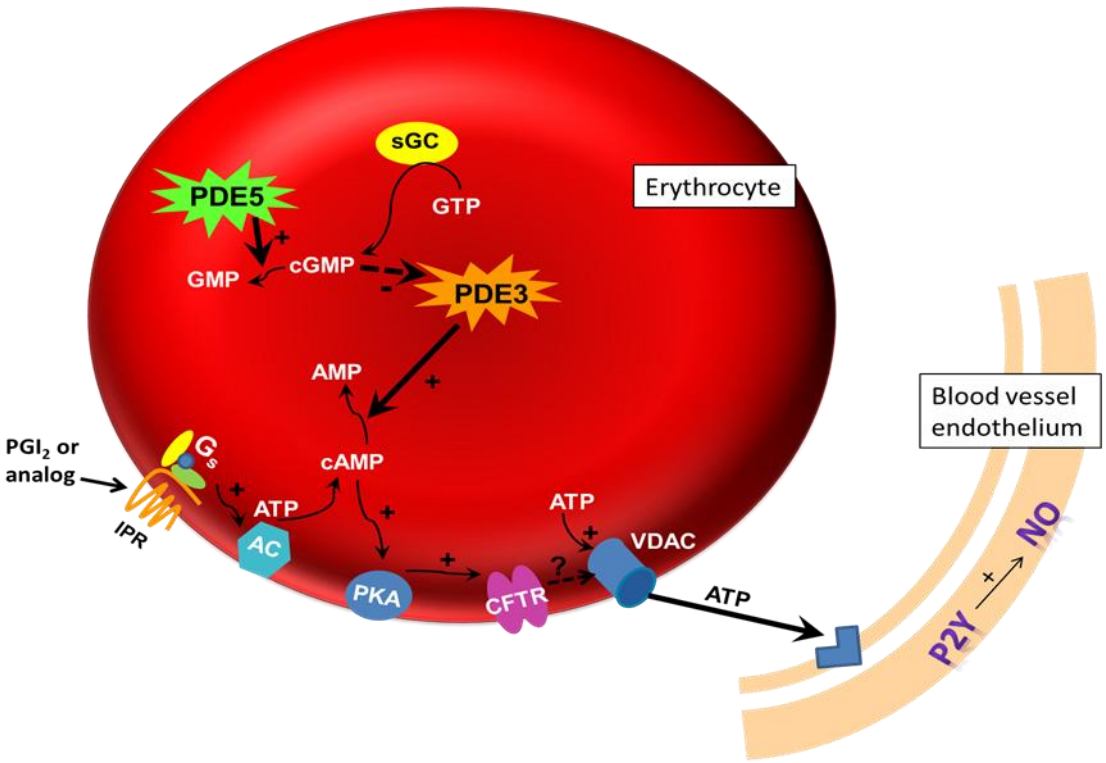

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Figure 1.1. Erythrocyte: Proposed Prostacyclin Signal Transduction Pathway. Abbreviations: IPR = PGI_2 receptor; Gs = heterotrimeric G protein, Gs; AC = adenylyl cyclase; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate; AMP = adenosine monophosphate; PKA = protein kinase A; CFTR = cystic fibrosis transmembrane conductance regulator; VDAC = voltage-dependent anion channel; GTP = guanosine triphosphate; cGMP = cyclic guanosine monophosphate; GMP = guanosine monophosphate; sGC = soluble guanylyl cyclase; PDE3 = phosphodiesterase 3; PDE5 = phosphodiesterase 5; (+) = activation and (−) = inhibition

In all cellular signaling pathways, there are components that act to regulate the magnitude and duration of activity. In the IPR pathway in erythrocytes, increases in cAMP are critical for ATP to be released, making the control of the levels of this cyclic nucleotide an ideal point for regulation of the pathway [7,18]. The level of cAMP present in the erythrocyte when this pathway is activated is a product of its synthesis by AC and its hydrolysis by phosphodiesterase 3 (PDE3) [19]. PDE3 can, in turn, be inhibited by increases in cyclic guanosine monophosphate (cGMP). Erythrocyte levels of cGMP are regulated by phosphodiesterase 5 (PDE5) [20]. Thus, amounts of ATP

released from erythrocytes following activation of the IPR are regulated by the activity of both PDE3 and PDE5.

The pathway for deformation-induced ATP release is similar to the IPR pathway but differs in two important ways (Figure 1.2) [13,21,22]. In contrast to the IPR pathway, this pathway requires activation of the heterotrimeric G-protein G_i instead of G_s and the final conduit is pannexin 1 [22-24]. These two pathways allow the RBC to respond to different stimuli from the environment.

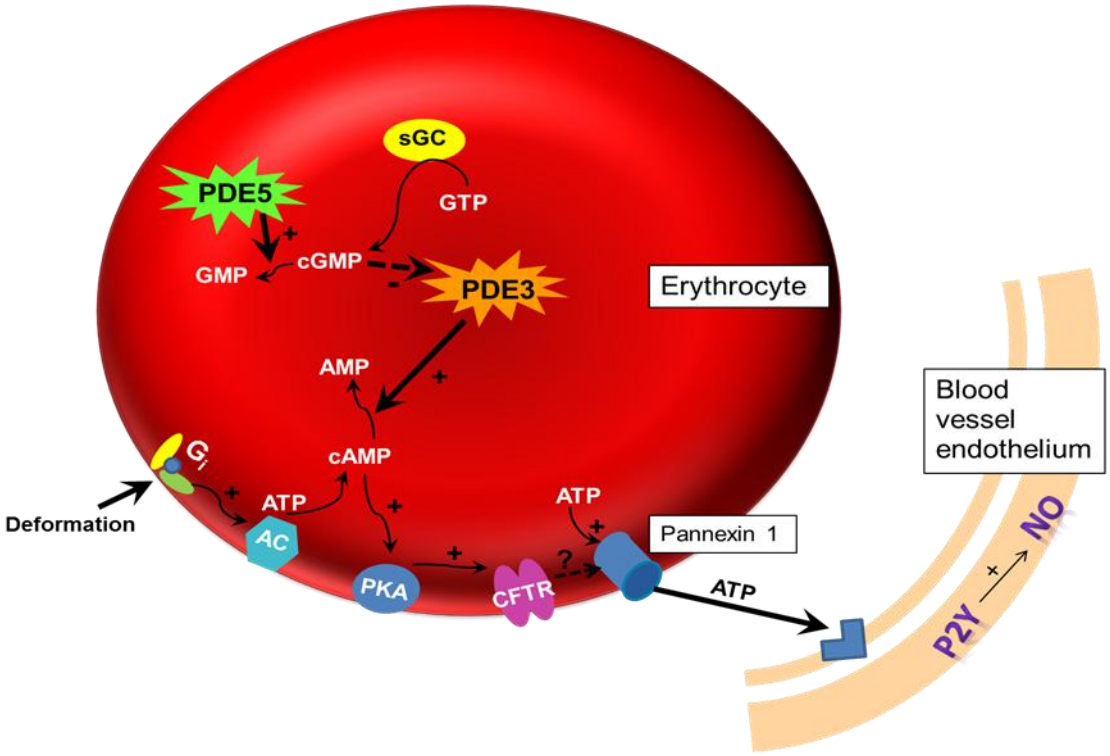

Figure 1.2. Erythrocyte: Proposed Deformation Signal Transduction Pathway. Abbreviations: IPR = PGI_2 receptor; Gi = heterotrimeric G protein, Gi; AC = adenylyl cyclase; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate; AMP = adenosine monophosphate; PKA = protein kinase A; CFTR = cystic fibrosis transmembrane conductance regulator; VDAC = voltage-dependent anion channel; GTP = guanosine triphosphate; cGMP = cyclic guanosine monophosphate; GMP = guanosine monophosphate; sGC = soluble guanylyl cyclase; PDE3 = phosphodiesterase 3; PDE5 = phosphodiesterase 5; (+) = activation and (−) = inhibition.

1.2.1. Prostacyclin. To increase ATP release from RBCs, prostacyclin or one of its analogs can be administered clinically. ATP release occurs via the pathway presented in Figure 1.1. A minimal amount of UT-15C is necessary to stimulate ATP release [7]. Unfortunately, in PAH patients, prostacyclin and its analogs have been reported to have significant side effects that can increase with increased dosage [25]. Partly in an effort to reduce some of these side effects, phosphodiesterase (PDE) 5 inhibitors can be prescribed to reduce the amount of UT-15C that is administered but maintain the desired decrease in PVR [26-28]. Importantly, this combination can stimulate increased ATP release from human RBCs [26,29]. However, a minimal amount of prostacylin or prostacyclin analog is necessary even in the presence of a PDE5 inhibitor.

1.2.2. Deformation-induced ATP Release. Similar to prostacyclin stimulation, deformation also stimulates human erythrocytes to release ATP [16,30]. The associated signaling pathway is illustrated in Figure 1.2.

1.3. LIPOSOMES

Unfortunately, PDE inhibitors have significant unwanted side effects [31-33]. For this reason, it is advantageous to minimize undesired interactions. Directed delivery of drugs to specific cells could reduce side effects. One such method is the liposome, a small lipid vesicle that encapsulates a drug and targets delivery to specific cells [34-36]. For PAH patients, it would be beneficial to deliver drugs, in particular, PDE5 inhibitors, directly to RBCs. This method has been shown to increase drug efficacy and tolerability [37-41]. In fact, liposomal delivery of the PDE3 inhibitor, cilostazol, has recently been shown to potentiate ATP release from erythrocytes from patients with DM2 [42].

2. POTENTIAL ROLE OF LIPOSOMES FOR THE DELIVERY OF PHOSPHODIESTERASE INHIBITORS TO ERYTHROCYTES FOR THE TREATMENT OF TYPE 2 DIABETES

It is estimated that there are more than 300 million individuals with type 2 diabetes (DM2) world-wide making this disease a major public health challenge [43]. Impaired vascular function is a significant complication of DM2 with cardiovascular disease accounting for nearly half of the deaths in humans with this condition [44,45]. Individuals with DM2 have a four-fold increased risk for claudication [46] and as much as a sixteen-fold increased risk for lower limb amputation [47-49]. Although this vascular disease is, in part, the result of an increased incidence of atherosclerosis in large conduit vessels [16], there is also extensive evidence that microvascular circulatory control is abnormal in humans with DM2 [50-53].

Patients with DM2 have diminished muscle blood flow both at rest [51] and with exercise [53]. Although direct studies of the skeletal muscle microcirculation are not possible in humans, such studies have been undertaken in several animal models of diabetes [54-56]. These studies demonstrate marked reductions in: 1) oxygen delivery [54,56], 2) capillary erythrocyte flux [55] and 3) convective oxygen delivery and diffusive oxygen transport [54]. Taken together, these reports indicate that, in DM2, oxygen delivery to skeletal muscle in amounts required to appropriately meet metabolic need is impaired.

It has been suggested that both endothelium-dependent and endothelium-independent vasodilation is impaired in humans with DM2 [57-61]. It has also been suggested that there is reduced nitric oxide (NO) synthesis [58], increased NO degradation [59] and/or abnormalities in the vascular smooth muscle [60] in these individuals. These reports demonstrate that, although vasodilation in response to both pharmacological and physiological stimuli is impaired in humans with DM2, the mechanisms responsible for this impairment have not been fully characterized.

2.1. ROLE OF ERYTHROCYTES IN THE CONTROL OF THE DISTRIBUTION OF PERFUSION IN THE MICROCIRCULATION

Although the erythrocyte is often considered to be primarily a cell dedicated to the transport and delivery of oxygen to the tissues, this cell has also been shown to participate in the regulation of vascular caliber [22,57,62-66]. In skeletal muscle, a critical stimulus for local dilation of blood vessels is the release of the vasodilator, ATP from erythrocytes exposed to low oxygen tension [22,57,62-66]. Indeed, this property of erythrocytes to stimulate vasodilation specifically in areas of decreased oxygen tension (increased oxygen utilization relative to supply) can influence the distribution of blood flow in the microcirculation of skeletal muscle resulting in optimal matching of the delivery of oxygen with need [65-67]. In humans with type 2 diabetes (DM2), the ability of erythrocytes to release ATP in response to exposure to low oxygen tension is severely impaired [68-70].

2.2. A SIGNALING PATHWAY FOR LOW OXYGEN-INDUCED ATP RELEASE FROM ERYTHROCYTES

ATP is a highly charged molecule that does not freely cross cell membranes. Therefore, the regulated release of ATP from erythrocytes requires the presence of signaling pathways that respond to discrete stimuli (Figure 1.2). Low oxygen tension-induced ATP release requires activation of the heterotrimeric G-protein G_i [22]. In this signaling pathway, the next steps require sequential activation of adenylyl cyclase (AC) [13,18], protein kinase A (PKA) [15] and the cystic fibrosis transmembrane conductance regulator (CFTR) [16,71]. The final ATP conduit in this signaling pathway is pannexin 1[11]. Importantly, it has been shown that expression of a single G_i isoform (G_{i2}) is reduced in erythrocytes of humans with DM2 [69,70] and is associated with markedly reduced ATP release in response to exposure of these cells to low oxygen tension [68,70]. Although no mechanism to increase G_{i2} expression in DM2 erythrocytes has been proposed, it has been reported that pharmacological approaches can increase the activity of the low oxygen signaling pathway for ATP release from these cells.

Cyclic AMP is a critical second messenger in pathways for ATP release from erythrocytes [13,15]. In all cells, cAMP levels must be tightly regulated for activation of signaling pathways and associated cellular responses discrete. In the low oxygen

pathway for ATP release from erythrocytes, levels of cAMP are regulated by PDE3 [14,20] (Figure 1.2). Importantly, inhibitors of PDE3 activity have been shown to potentiate cAMP levels and increase ATP release in response to low oxygen tension in erythrocytes of humans with DM2 [72]. However, in clinical use, systemic administration of PDE3 inhibitors has been reported to have adverse cardiovascular effects that limit the use of such drugs in humans with DM2 [73]. If PDE3 inhibitors could be delivered selectively to erythrocytes, it is possible that such adverse effects could be minimized. One approach for the selective delivery of drugs to erythrocytes is via the use of liposomes [74,75].

2.3. LIPOSOME CONSTRUCTION

Liposomes may be composed of one or many bilayer membranes (unilamellar or multilamellar). They can range in size from a few nanometers to several micrometers in diameter. Measurement of liposomal size can be determined by several methods including light scatter [76], flow cytometry [77], and electron microscopy [78]. In addition to size, the number of membranes in a liposome can affect its ability to release its contents into a cell once it fuses with the membrane of the target cell [79].

When constructing liposomes, different phospholipids can be selected on the basis of their charge to result in desired surface properties of the liposomal membrane. The electrical charge of a liposome can affect its binding affinity for different cell types [79-81]. Specifically, negative charges appear to be beneficial for the fusion of liposomes with erythrocytes [79,80]. Although positive charges have also been used, the incidence of hemolysis of erythrocytes was increased under these conditions [78,81].

In addition to total charge, different lipids used to construct liposomes contain different numbers and/or arrangements of atoms as well as single or double bonds which allow these molecules to be even more individualized [80]. Cholesterol and other components are often added to liposomes to stabilize their membranes, to more closely model cellular membranes, and/or to alter binding or fusion of liposomes to cells [82,83]. These alterations can affect the fluidity of the liposomal membrane. Membrane fluidity strongly affects the likelihood of fusion between liposomes and cells [34]. Importantly, liposomes with increased membrane fluidity were reported to display enhanced binding

with erythrocytes [84]. Other components that may be added to liposomes to enhance selective binding of liposomes to specific cell types or tissues include selective antibodies [39,85,86] or other molecules to decrease recognition of liposomes by the immune system [76]. In addition to the molecular constituents of liposomes, the medium in which liposomes and cells are incubated as well as the length of time, concentration, and temperature can also strongly influence the effectiveness of subsequent liposome-cell interactions and fusion events [37,82]. Thus, liposome-erythrocyte interactions are complex and not all liposome compositions are "erythrocyte-friendly" [37,81,87].

3. LIPOSOMAL-DELIVERY OF PHOSPHODIESTERASE 5 INHIBITORS AUGMENTS UT-15C-INDUCED ATP RELEASE FROM HUMAN ERYTHROCYTES

Erythrocytes have been shown to participate in the regulation of vascular caliber via the release of the potent vasodilator, ATP [61,22,65,88]. ATP is released from erythrocytes in response to both physiological and pharmacological stimuli [5,7]. Erythrocytes of humans with pulmonary arterial hypertension (PAH) fail to release ATP when exposed to one physiological stimulus, namely, mechanical deformation as would be encountered when these cells traverse the microcvasculature [13]. In contrast, PAH erythrocytes do release ATP when exposed to prostacyclin (PGI_2) analogs via a well-characterized signaling pathway that requires increases in intracellular cAMP (Figure 1.1) [7,26,29]. In this pathway, levels of cAMP are regulated by PDE3, a PDE that is inhibited by local increases in cyclic guanosine monophosphate (cGMP) [14]. Erythrocytes make cGMP; its levels are the product of synthesis by soluble guanylyl cyclase (sGC) and hydrolysis by PDE5 [20]. Importantly, inhibitors of PDE5 have been shown to augment PGI_2 analog-induced ATP release from both healthy human and PAH erythrocytes [26,29]. Although both PGI_2 analogs and PDE5 inhibitors are used in the treatment of PAH, the effectiveness of these drugs alone or in combination is sometimes limited by untoward systemic side effects including hypotension, flushing and headache [25,33]. If PDE5 inhibitors could be targeted to erythrocytes, it is possible that some of these side effects could be minimized while augmentation of PGI_2 analog-induced release of ATP from these cells would be preserved.

Liposomes can target drug delivery to specific cells, including erythrocytes [35,40]. However, the construction of liposomes that are both compatible with human erythrocytes and capable of carrying and delivering a PDE5 inhibitor has not been previously reported. Here it is reported that liposomes loaded with PDE5 inhibitors augment ATP release from human erythrocytes exposed to UT-15C, an oral form of the prostacyclin analog, treprostinil. These findings demonstrate that encapsulation of PDE5 inhibitors within liposomes is a viable approach to the delivery of these drugs to human erythrocytes. Delivery of PDE5 inhibitors to erythrocytes via liposomes could benefit

PAH patients by reducing the off-target drug effects while maintaining the therapeutic effect of a reduction in pulmonary vascular resistance.

3.1. MATERIALS AND METHODS

The materials and methods used for these experiments are defined in the following subsections. All experiments were conducted at the Missouri University of Science and Technology.

3.1.1. Isolation of Human Erythrocytes. Blood obtained from healthy human volunteers was collected into heparinized tubes at the Phelps County Regional Medical Center or Saint Louis University and transported to the Missouri University of Science and Technology where it was centrifuged at 500*g* at 4°C for 10 min. The plasma, buffy coat, and uppermost layer of erythrocytes were removed by aspiration [5,7,13,26]. Care was taken to remove the fewest erythrocytes possible. The remaining erythrocytes were resuspended and washed in a physiological salt solution (PSS) containing (in mM): 21.0 tris (hydroxymethyl) aminomethane, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl, and 1.2 MgSO4. For erythrocyte washing and purification, 5.5 mM glucose and 0.5% bovine serum albumin fraction V were added and the pH adjusted to 7.4. Erythrocytes were prepared on the day of use. Informed consent was obtained from all volunteers. The protocol for blood removal was approved by the Institutional Review Boards of the Phelps County Regional Medical Center, the Missouri University of Science and Technology, and Saint Louis University. All record keeping was in compliance with regulations of the Health Insurance Portability and Accountability Act.

3.1.2. Generation of DMPC Liposomes. Unilamellar liposomes were prepared by the extrusion method [89]. DMPC (10mg) was added to a mixture of methanol/chloroform (50/50, v/v) in the absence (blank, control) or presence of the PDE3 inhibitor, cilostazol (CILO, 2mM; Sigma-Aldrich, St. Louis, MO, USA), or either of two PDE5 inhibitors, zaprinast (ZAP, 1mM; Sigma-Aldrich) or tadalafil (TAD, 1mM; Eli Lily, Indianapolis, IN, USA). A stream of purified argon was used to evaporate the solvents to form a lipid/drug film on the walls of a microcentrifuge tube spun at 300 rpm in a Heidolph RZR stirrer (Heidolph Instruments, Cinnaminson, NJ, USA). The film was further dried under vacuum to remove traces of chloroform and methanol. The dried film

was rehydrated with 0.5 mL of PSS (without glucose or albumin) creating a dispersion of multilamellar vesicles. The solution was then extruded through two polycarbonate membranes with 100 nm pores eleven times using an Avanti Mini-extruder (Avanti Polar Lipids, Alabaster, Alabama, USA).

3.1.2.1. Generation of fluorophore-containing liposomes. The fluorophore-containing molecule, 25-NBD cholesterol (Avanti Polar Lipids) in chloroform, was added as the chloroform volume to the 50/50 v/v methanol/chloroform DMPC mixture prior to evaporation, rehydration with PSS, and extrusion. The sample was protected from exposure to light [90,91].

3.1.2.2. Generation of cholesterol-containing liposomes. Cholesterol (4.3mg, Avanti Polar Lipids) was added to 10mg DMPC and mixed in 60/40 v/v methanol/chloroform in the absence (blank, control) and presence of TAD prior to evaporation under a stream of argon during low speed centrifugation. After further drying under vacuum, 0.5 mL PSS was used to rehydrate the lipid film prior to extrusion [42,92].

3.1.2.3. Generation of phospho-L-serine-containing liposomes. 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (14:0, PS; Avanti Polar Lipids), 30% w/w, was added to 70% w/w DMPC in 50/50 v/v methanol/chloroform prior to evaporation and rehydration with 0.5mL PSS before extrusion [87,93].

3.1.3. Measurement of ATP. Washed erythrocytes were diluted with PSS containing glucose and albumin to a hematocrit of 20%. The luciferin-luciferase technique [5,94] was used to measure ATP. Briefly, a 0.2 mL sample of an erythrocyte suspension (0.04% hematocrit) was injected into a cuvette containing 0.1 mL of 10 mg/mL crude firefly lantern extract (Sigma) and 0.1 mL of 0.5 mg/mL D-luciferin (Research Products International, Mt. Prospect, IL, USA). The emitted light was detected with a luminometer (Turner Biosystems, Sunnyvale, CA, USA). An ATP standard curve was generated on the day of each study. ATP levels were measured at baseline and 5, 10 and 15 min after administration of UT-15C (United Therapeutics, Silver Spring, MD, USA). The peak value obtained is reported as nanomoles normalized to 4×10^8 erythrocytes. Cells were counted using a hemocytometer.

3.1.4. Measurement of Free Hemoglobin. Erythrocyte suspensions were centrifuged at 500*g* for 10 min at 4°C. The presence of free hemoglobin in the supernatant was measured using light absorption at 405 nm [16,95]. This method was used to detect and eliminate studies in which free hemoglobin levels increased indicating the presence of significant hemolysis. No studies were excluded due to increased hemoglobin levels.

3.1.5. Determination of the Effect of Various Concentrations of UT-15C on ATP Release from Human Erythrocytes in the Absence or Presence of the PDE5 Inhibitor, ZAP. Erythrocytes were diluted to a hematocrit of 20% in PSS containing glucose and albumin. Samples were pretreated with ZAP (10µmol/L) or its vehicle, dimethyl formamide (DMF), for 30 minutes prior to the measurement of ATP (baseline) and 5, 10, and 15 min after addition of UT-15C at concentrations of 10, 30 or 100 nmol/L. The peak ATP release is reported.

3.1.6. Measurement of Liposomal Binding to Erythrocytes Using Flow Cytometry. Erythrocytes were isolated and diluted to 20% with PSS containing glucose and albumin. Aliquots of the mixture were then incubated with 10 µL/mL liposomes, which did or did not contain the fluorophore (25-NBD cholesterol), for 10 min at room temperature in the dark. Samples were read on a BD Accuri C6 Flow Cytometer (Becton, Dickenson, and Co., Franklin Lanes, NJ, USA) equipped with a solid state 50mW laser tuned to 488nm. A 200µm ID fused silica capillary flow cell was used at a flow rate of 10µL/min with no gating and a threshold of 10,000 events. Forward and side scatter conditions remained unchanged through all tests. The measurable particle size range for this device is 1-40µm [77,96].

3.1.7. Investigation of Rheological Changes in Erythrocytes in the Absence or Presence of Liposomes. To determine if liposome-erythrocyte interactions resulted in any alterations in erythrocyte deformability, rheological studies were performed with the Anton Paar Modular Compact Rheometer 302 (Anton Paar, Ashland, VA, USA) at 20°C using parallel plate configuration (50mm diameter, 0.05mm gap) [97]. Isolated erythrocytes were diluted to a 40% hematocrit with PSS containing glucose and albumin then incubated with liposomes or saline prior to testing. Comparison of the apparent viscosity relative to shear rate via resistance measurements were made with increasing

oscillations over time. The presence of differences between treatment groups would demonstrate significant rheological alterations caused by the liposomes.

3.1.8. Determination of the Effect of UT-15C on ATP Release from Human Erythrocytes in the Presence of Blank Liposomes or Liposomes Containing Various PDE Inhibitors. Erythrocytes were diluted to a hematocrit of 20% in PSS containing glucose and albumin. Samples were pretreated with either blank liposomes or liposomes containing either the PDE3 inhibitor, CILO, or one of two PDE5 inhibitors, ZAP or TAD, for 30 min. ATP levels were then determined before and at 5, 10, and 15 min after the addition of UT-15C (1µmol/L). The peak ATP release is reported.

3.1.9. Data Analysis. Statistical differences among groups were determined using an analysis of variance followed by a Fisher's least significant difference (LSD) test. When possible, results are reported as mean ± the standard error (SE).

3.2. RESULTS

The following results were obtained using the methods above. The data is presented in the form of graphs or tables to best present the concept supported.

3.2.1. Effect of UT-15C on ATP Release from Erythrocytes in the Absence or Presence of ZAP. Erythrocytes were incubated with the PGI_2 analog, UT-15C, at concentrations of either 10 (n=3), 30 (n=7), or 100 (n=7) nmol/L. In the absence of ZAP, these concentrations of UT-15C did not stimulate ATP release (Figure 3.1). In contrast, in the presence of ZAP (10µmol/L), ATP release was stimulated by UT-15C at concentrations of 30 and 100 nmol/L (Figure 3.1).

3.2.2. Use of Flow Cytometry to Characterize Liposome-binding to Erythrocytes. Using flow cytometry, solutions of erythrocytes, liposomes, or both passed a laser emitting light of a specific wavelength (488nm). Detectors along the flow path recorded "events" (wavelengths of light) which were distinguished when excited molecular fluorophores emitted light at 523nm. Analysis of the locations of the detectors recording events estimated cell size. Larger cells exhibited greater forward scatter (FSC) whereas granular cells exhibited greater side scatter (SSC). Figure 3.2 provides paired plots of, respectively, erythrocytes, liposomes, and liposomes with erythrocytes.

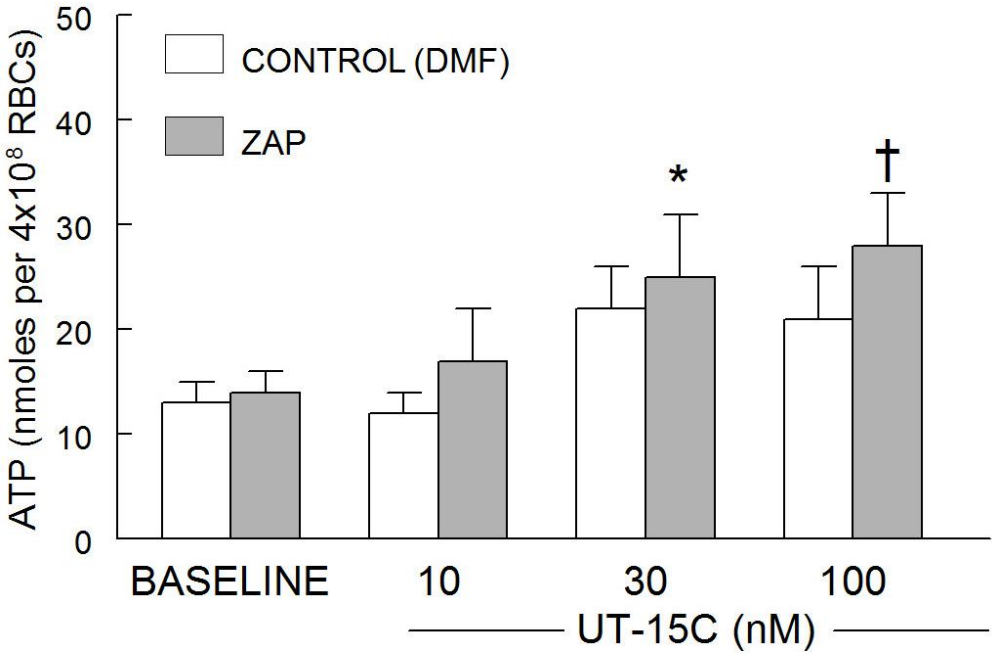

Figure 3.1. ATP Release without Liposomes. Effect of UT-15C (10, 30 or 100 nmol/L, n = 3, 7, and 7, respectively) on ATP Release from Human Erythrocytes in the Presence of Zaprinast (ZAP, 10 µmol/L) or Its Vehicle (dimethyl formamide, DMF).

Erythrocytes alone (Figures 3.2A and 3.2D) are relatively large (7-8µm, as shown in Figure 3.2A) and do not fluoresce, shown by the majority (95.6%) of events at channels below the marked threshold (Figure 3.2D). Liposomes made with DMPC alone (Figures 3.2B and 3.2E) are small or granular (~100nm, Figure 3.2B) and do fluoresce as shown by the greatest volume of events (76.9%) occurring at channels above the marked threshold (greater wavelength, Figure 3.2E). When erythrocytes were incubated with liposomes containing a fluorophore, the large cells (erythrocytes) exhibited fluorescence from binding with labeled liposomes (Figures 3.2C and 3.2F). Examination of the supernatant of erythrocytes that had been incubated with fluorophore-containing liposomes did not display fluorescence indicating that the vast majority of liposomes were bound by the erythrocytes. (Data not shown.)

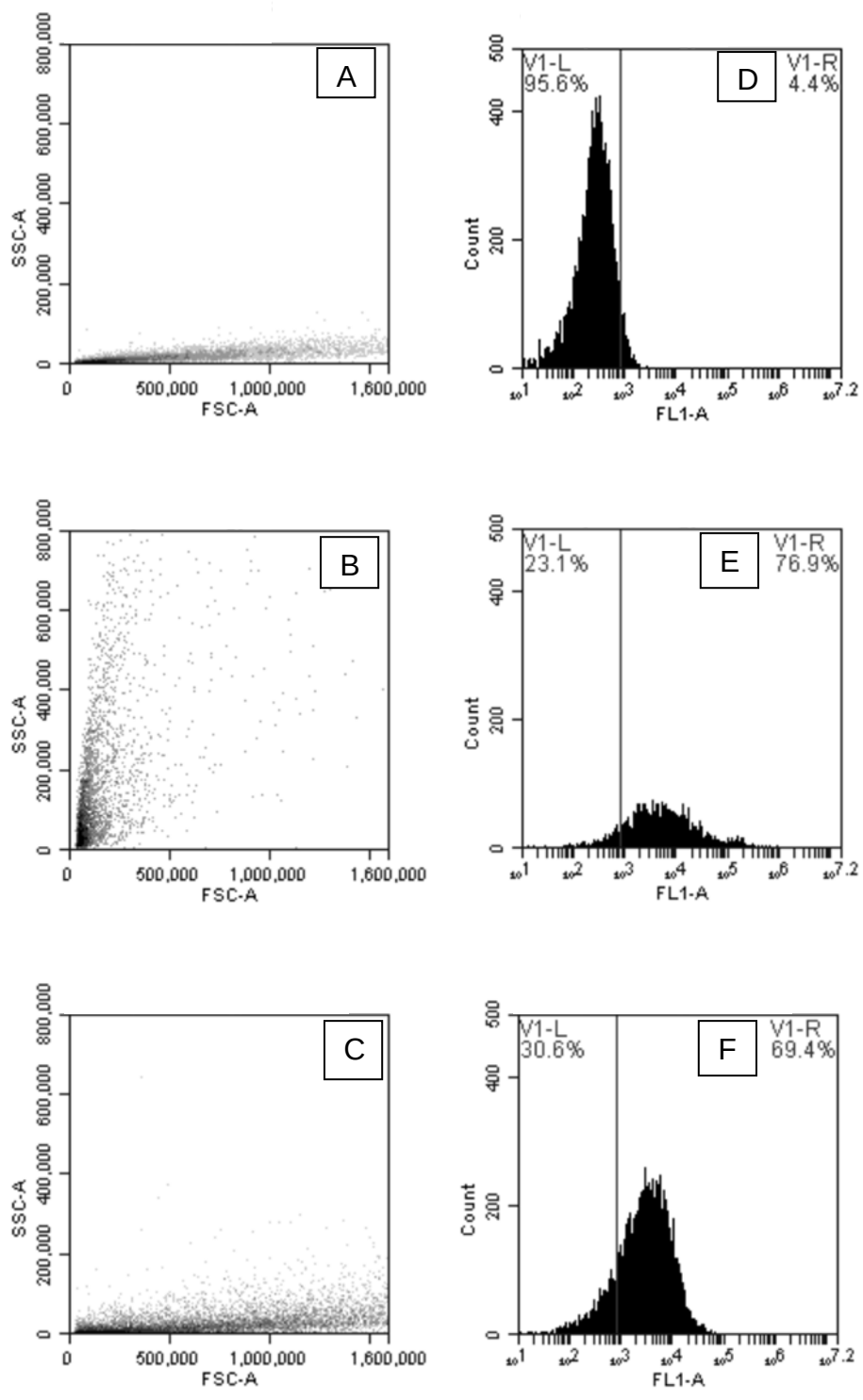

Figure 3.2. Flow Cytometry Graphs. Scatter Plots (A-C) and Histograms (D-F) of Erythrocytes (A,D), Liposomes (B,E), and the Combination (C,F).

3.2.3. Determination of Rheological Effects of DMPC Liposomes on Erythrocytes. Comparison of the apparent viscosity of erythrocyte suspensions at different applied shear rates revealed no differences between samples incubated with or without liposomes (Figure 3.3). As the concentration of erythrocytes and the suspending medium were kept constant, no measurable change in erythrocyte morphology or deformability was detected.

3.2.4. Effect of UT-15C on ATP Release from Erythrocytes in the Absence and Presence of DMPC-containing Liposomes without and with CILO or ZAP. Erythrocytes incubated with liposomes containing CILO failed to release ATP in response to UT-15C administration. In contrast, when erythrocytes were incubated with liposomes containing the PDE5 inhibitor, ZAP, the same concentration of UT-15C stimulated the release of ATP (Figure 3.4). These results demonstrate that although DMPC liposomes delivered the PDE5 inhibitor to human erythrocytes, this composition was not appropriate for the delivery of adequate amounts of the PDE3 inhibitor, CILO.

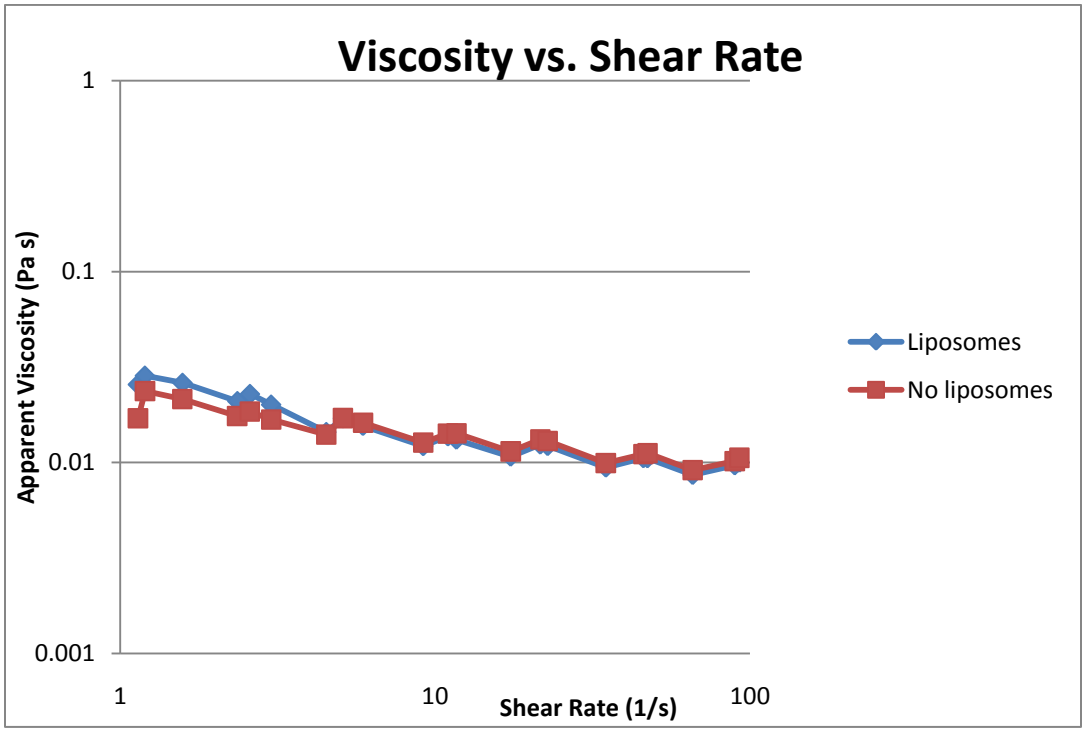

Figure 3.3. Rheometry. Rheometric Comparison of the Apparent Viscosity at Increasing Shear Rates of Erythrocyte Suspensions in the Absence or Presence of Liposomes (n=3).

3.2.5. Effect of UT-15C on ATP Release from Erythrocytes in the Absence and Presence of Cholesterol- or PS-containing Liposomes without and with the PDE5 Inhibitor, TAD. Figure 3.5 displays the influence on ATP release by different lipids. Incubation of erythrocytes with cholesterol-containing liposomes resulted in a significant increase in baseline ATP levels compared to those in other liposome preparations (Figure 3.5A). However, with these cholesterol-containing liposomes, an increase in ATP release following stimulation with UT-15C was seen in both the absence and presence of the PDE5 inhibitor, TAD (Figure 3.5A). Hemoglobin levels in the supernatant of erythrocytes treated with cholesterol-containing liposomes were not increased suggesting that the increased ATP levels were not the result of hemolysis.

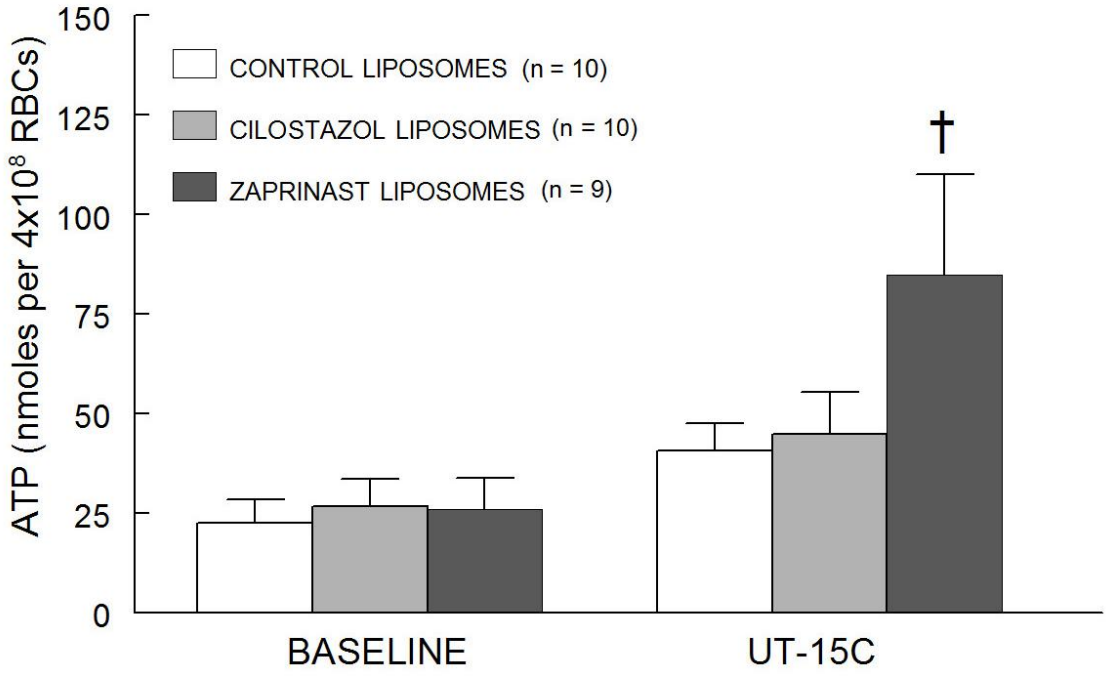

Figure 3.4. ATP Release Using Different PDE Inhibitors. Effect of UT-15C (1 µmol/L) on ATP Release from Human Erythrocytes in the Presence of Blank DMPC Liposomes (CONTROL) or Liposomes of the Same Composition Loaded with Either the PDE3 Inhibitor, Cilostazol, or the PDE5 Inhibitor, Zaprinast.

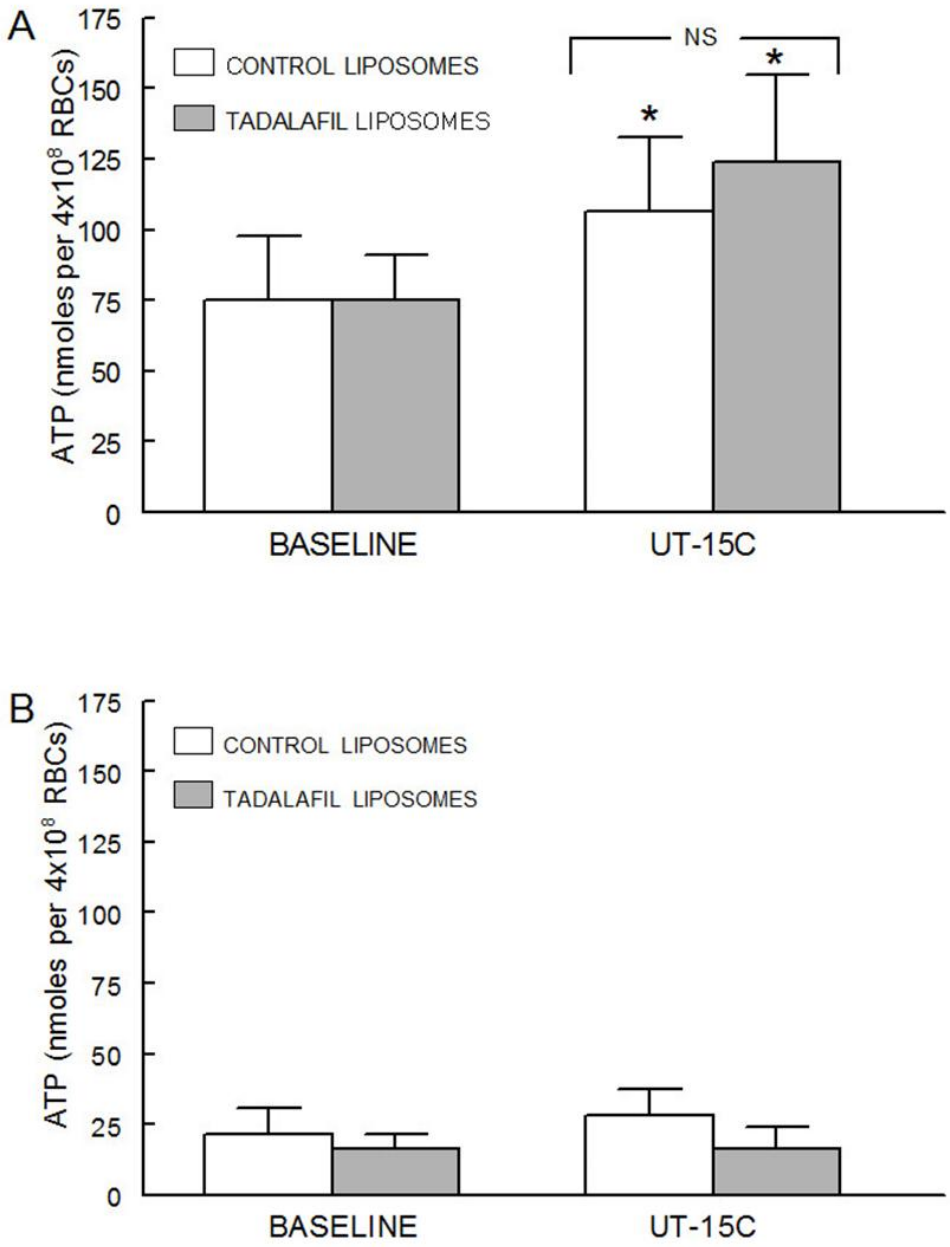

Figure 3.5. ATP Release Using Different Lipids. Effect of UT-15C (1 mol/L) on ATP Release from Human Erythrocytes in the Presence of Blank DMPC Liposomes (CONTROL) Containing Cholesterol (Panel A) or Phospho-serine (PS, Panel B) or Liposomes of the Same Compositions Containing the PDE5 Inhibitor, Tadalafil.

In contrast to the results with cholesterol-containing liposomes, erythrocytes exposed to liposomes containing PS had lower baseline values (Figure 3.5B) but failed to release ATP when exposed to UT-15C. Taken together, these studies demonstrate that liposome composition is of critical importance in delivering TAD to human erythrocytes.

3.2.6. Effect of UT-15C on ATP Release from Erythrocytes in the Absence and Presence of DMPC-containing Liposomes without and with TAD. ATP release was stimulated by UT-15C when DMPC liposomes were used to deliver TAD to erythrocytes (Figure 3.6). These findings demonstrate that when generating liposomal membranes for effective delivery of PDE5 inhibitors to human erythrocytes the phospholipid used must be carefully selected and that sterols are detrimental in this application.

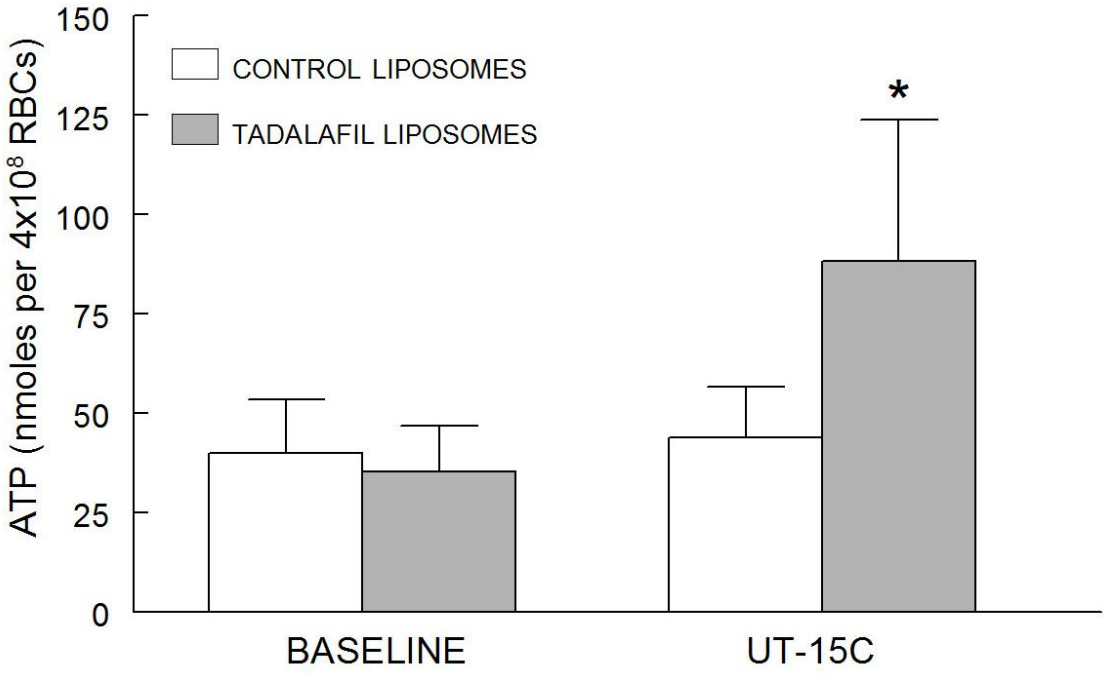

Figure 3.6. ATP Release Using DMPC. Effect of UT-15C (1 µmol/L) on ATP Release from Human Erythrocytes in the Presence of Blank DMPC Liposomes (CONTROL) or Liposomes of the Same Composition Loaded with the PDE5 Inhibitor, Tadalafil.

4. SUPPORTING EXPERIMENTATION

The previous two chapters contain information that has been submitted to scientific journals**.** This chapter presents information that was presented in poster format or has been used to help support experimental conclusions. Due to novelty (either in the experimental procedure or in methodological experience) or a lack of completeness, this data has not been submitted for inclusion in a scientific journal.

4.1. DETERMINATION OF LIPOSOME SIZE

It is important when utilizing liposomes to be cognizant of their size. Not only does size affect the volume of material that can be contained within the liposome [98.99] but it is also important to consider how these vesicles will compare and interact in the body and with the desired cells [41,42]. Two methods were employed to investigate liposomal size. Other methods also exist [77,78,100].

4.1.1. Density Light Scatter. One method to determine liposomal diameter is by dynamic light scatter (DLS) [77]. Although somewhat time consuming, this commonly accepted technique involves the measurement of the Doppler broadening of Rayleigh scattered light due to thermal (Brownian) motion [101]. Smaller particles move within the solution more rapidly than do large particles and therefore scatter light more frequently.

Using a Brookhaven Fiber Optic Quasi Electric Light Scattering (FOQELS) particle size analyzer at an angle of 135.8°, the data in Table 4.1 shows was generated. The dispersity index, previous called the polydispersity index (PDI), describes the heterogeneity of a solution: the greater the number, the greater the differences between particles. For these experiments, a 0.2μm syringe filter was used to reduce the diameters of the liposomes.

To understand the data from some of the samples, more discussion may be helpful. The second 1.0mg/mL sample was sonicated (another method to reduce liposomal diameter) [83]. The blank sample contained the buffer used to dilute the

Table 4.1. Raw FOQELS Data on Syringe-extruded Liposomes.

Concentration (mg/mL)	Dispersity Index	Diameter (nm)
0.5	0.168	846.9
0.75	0.430	620.2
1.0	0.105	588.4
1.0, sonicated	0.335	477.1
0.0, blank	0.005	17,249

liposomes. The value of the dispersity index of the blank is sensible but the diameter is extremely large. This is most likely due to the size of the water molecule, approximately 0.2nm, being too small to be accurately measured by the instrument [101].

Figures 4.1A and 4.1B display a wide range of liposomal diameters, larger than the syringe filter pore and desired sizes (0.2μm and 100nm, repsectively, possibly due to fusion of formed liposomes. Use of the Avanti Mini-extruder yielded smaller, more consistent liposomes as shown in Table 4.2. The exact concentrations are not listed due to losses in the syringes, filters, and filter supports. The difference in dispersity index between the lower concentration and the higher one is most likely due to the prevalence of liposomes. More liposomes made a higher concentration for more comparisons. The extruder was used to create the liposomes used in the experiments in the earlier chapters.

Table 4.2. FOQELS Measurements of Liposomes Formed with the Mini-extruder.

Concentration (mg/mL)	Dispersity Index	Diameter (nm)
Low	0.104	166.9
High	0.005	172.2

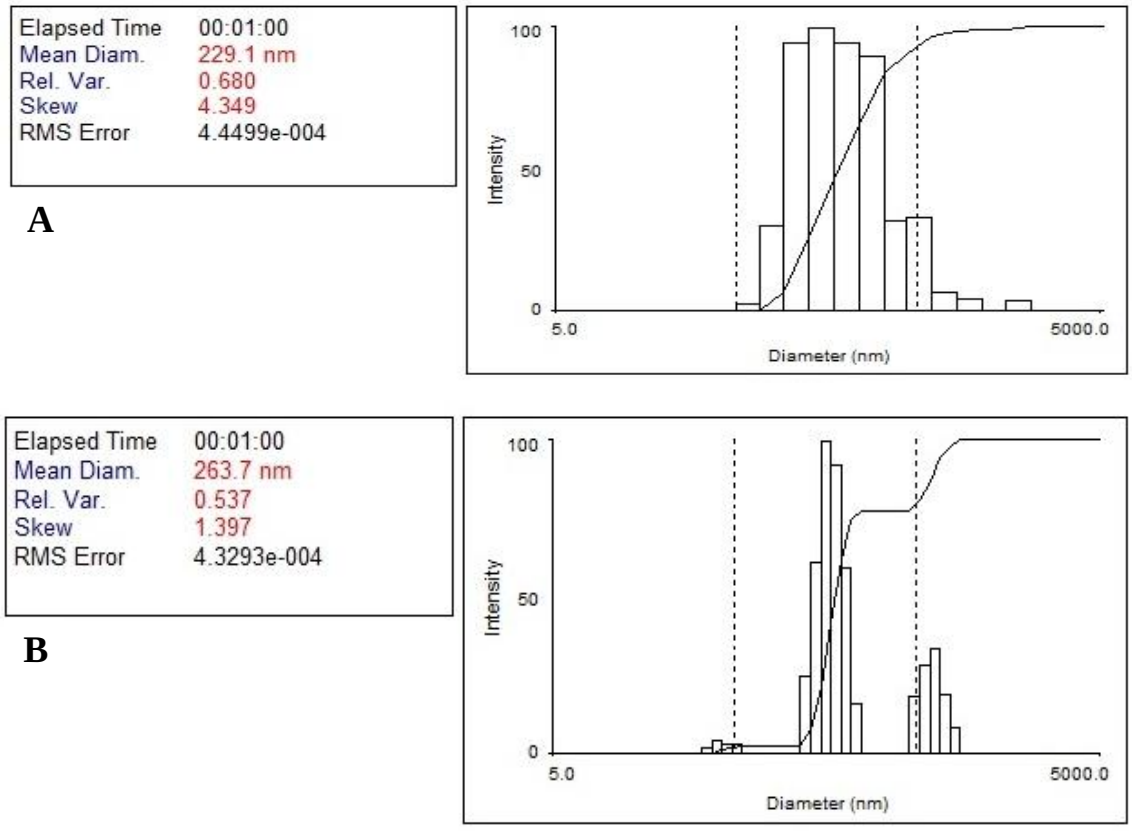

Figure 4.1. Size Ranges of Syringe-extruded Liposomes as Measured by FOQELS.

4.1.2. Diameters of Wet and Dry Liposomes by Particle Research. The following graphs, Figures 4.2 and 4.3, demonstrate the improvement achieved by the use of the mini-extruder and highlight some other important points. For example, the polar head groups of phospholipids form hydrogen bonds with water molecules in solution [102]. This allows the liposomes to appear larger when size is measured by light scatter via measurement of the hydrodynamic diameter [100]. When dried (thus the water is removed), the diameters of the liposomes are much smaller as shown in Figure 4.2. The liposomes for these tests appeared to have wet diameters around 120nm whereas the dry diameters were all less than 60nm.

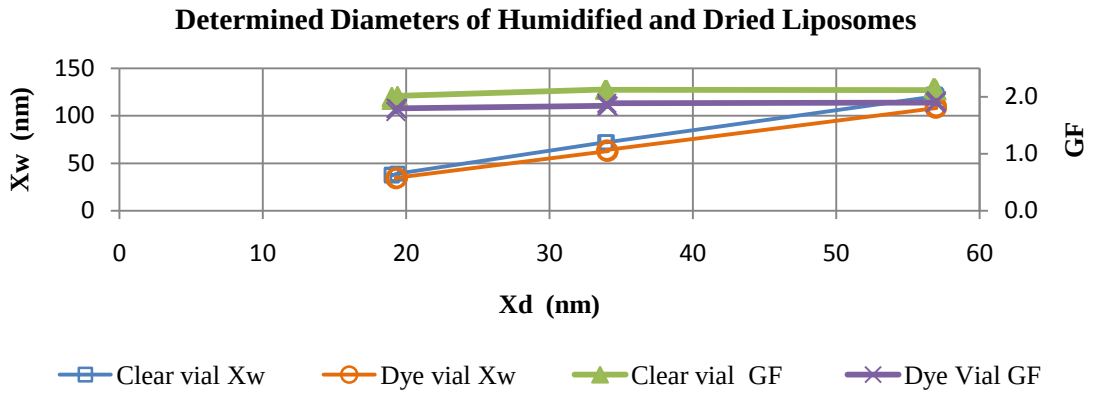

Figure 4.2. Determined Diameters of Humidified (w) and Dried (d) Liposomes with (Dye) and without (Clear) the Flurophore 25-NBD-Cholesterol Added.

Figure 4.3 shows the concentration of liposomes, both humidified (wet) and dried, at various diameters. The trends were very similar in the presence or absence of the fluorophore (dye). The majority of dried liposomes were small (about 25nm or less) whereas humidified liposomes were considerably larger (about 65nm).

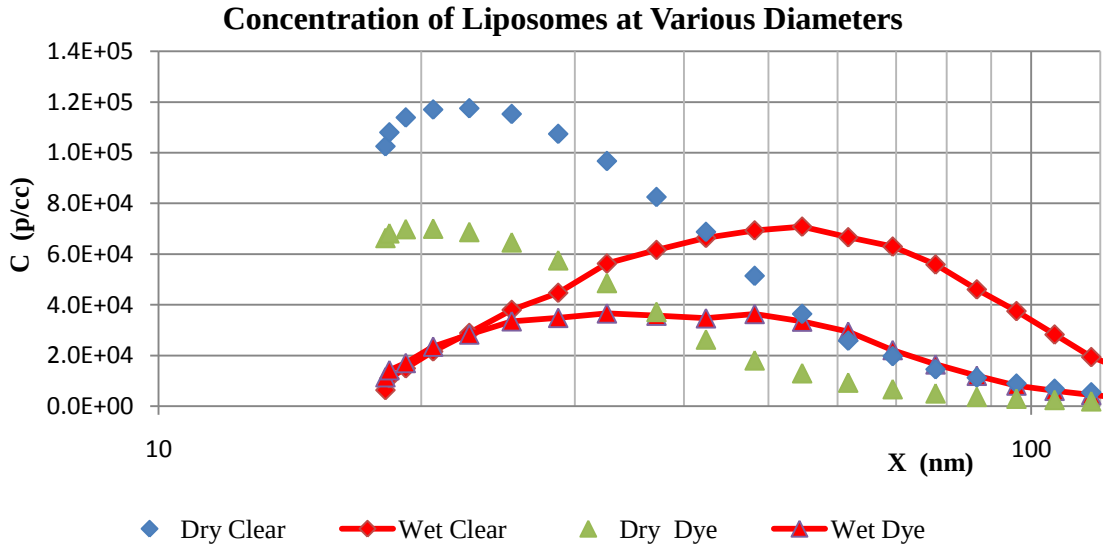

Figure 4.3. Concentration of Liposomes at Various Diameters, Wet or Dry.

One may consider that Figure 4.2 and 4.3 do not show liposomes of the same diameters. For example, the humidified liposomes (w) in Figure 4.2 remain relatively stable across the graph whereas the dried liposomes (d) increase to the right. This may be due to the infiltration of water molecules into the testing chamber which could bind to the DMPC polar head groups, distorting the light scatter and causing increased diameter readings. In Figure 4.3, on the other hand, the diameters all appear much smaller. In fact, the largest concentration of liposomes shows the smallest diameter. This makes sense when considering the fact that dried liposomes are smaller (not hydrated)—the same volume of solution would be much more concentrated. Likewise, the presence of water dilutes the number of liposomes in a volume. Therefore reducing the concentration. Regarding the concentration of dye-loaded liposomes, less of this sample of this variety was tested than sample without the flurophore (dye), thus samples with dye have lower concentrations due to less sample being available. These studies were conducted by the Center of Excellence for Aerospace Particulate Emission Reduction Research.

4.2. CHEMICAL ANALYSIS

Numerous chemical analyses have been employed over time to investigate different facets of liposomes [76,77,103]. The techniques employed with these experiments, beyond those described earlier, for determining the containment of TAD within liposomes included UV spectrum analysis, FTIR-ATR, low resolution NMR, and flow injection analysis.

4.2.1. UV Spectrum Analysis. UV spectrum analysis appeared to be a sensible investigation technique due to the wavelength of absorbance for tadalafil being 284nm [104,105]. Figure 4.4 displays the data obtained from the spectrophotometer. Unfortunately, no peaks are seen at this point. The first three samples are blank (empty, control) liposomes. The sample contained undiluted liposomes. The second sample was diluted 1:20 with buffer (PSS without glucose and albumin). The third sample was diluted 1:10. The tadalafil-loaded liposomes were also tested at a 1:10 dilution. It is possible that the tadalafil peak was not seen because the drug was contained within the liposomes.

4.2.2. FTIR-ATR and Low Resolution NMR. FTIR-ATR, Fourier transform infrared assay-attenuated total reflectance, and low resolution NMR produced no distinguishable results between liposomes containing TAD and those not containing TAD. (Data not shown.) Tadalafil should have been detectable in the absence of liposomes [104,106]. However, due to the closure of these facilities for renovation, continued testing and retrial were not possible.

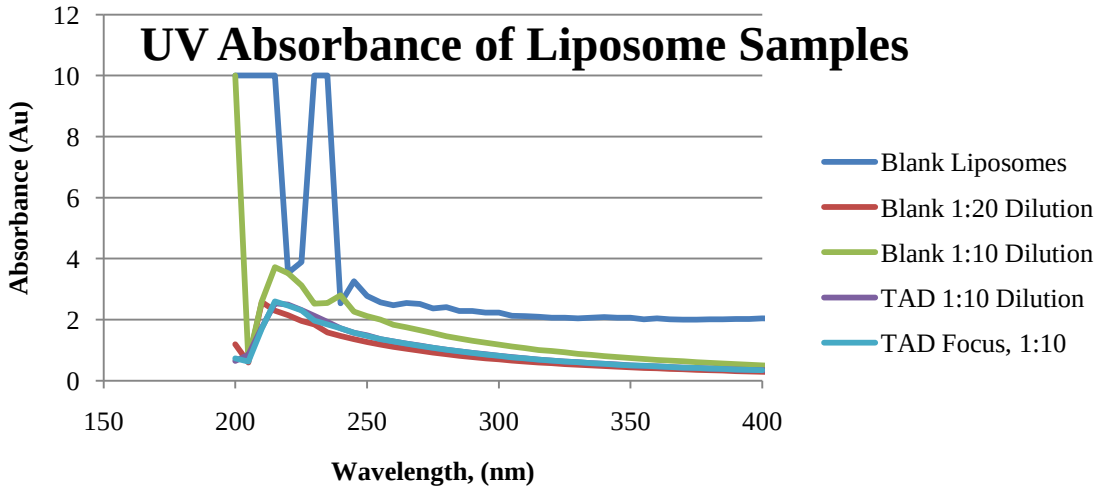

Figure 4.4. UV Spectrum Analysis of Liposomes with and without TAD.

4.2.3. Flow Injection Analysis. Lastly, flow injection analysis (HPLC without the column) was performed using the Hitachi LaChrom Elite with a flow rate of 0.5mL/min. This method was used because there was some concern that the liposomes could damage or be trapped within the HPLC column. The samples tested and peak results obtained are listed in Table 4.3. As can be seen, some samples produced more peaks than others. The diode array detector (L-2455) provided the results listed in the shaded columns. The data in the last three columns came from the refractive index detector (L-2490). Unfortunately, once again, the data was inconclusive. It may have been beneficial to have tested a variety of columns or to have tried a different HPLC. Or, perhaps a contaminant in the mobile phase (methanol) led to these results. More testing might have answered this.

Table 4.3. Peak Data from HPLC of Samples with and without Liposomes and TAD.

Sample	Diode	Array	Detector	Refractive	Index	Data
TAD in MeOH	0.67			1.15		
Blank liposomes	0.67			1.16		
TAD liposomes	0.62	1.03	1.67	1.54		
Supernatant of blank lip.	0.63	1.04	1.68	1.55		
Supernatant of TAD lip.	0.62	1.05	1.75	1.55		
Buffer	0.63	1.05	1.72	1.57		
Chloroform	0.65	1.07	1.48	1.57		
Chloroform w/blank lip.	0.61	0.7	0.79	0.99	1.46	9.2
Chloroform w/TAD lip.	0.61	0.7	0.79	0.99	1.46	9.89
TAD in chloroform	0.6	0.7	0.79	0.99	1.47	8.97

5. CONCLUSION

Although the pulmonary circulation must, at all times, accept the entire cardiac output, vascular pressure in the lung is vastly lower than in the systemic circulation. This is accomplished by virtue of the construction of the vascular bed in the lung such that increases in blood flow are accommodated by the recruitment of previously non- or under-perfused vessels and/or by distension of perfused vessels [107]. However, active vasodilation via the local synthesis and release of vasodilators including NO and PGI_2 is also a critical determinant of PVR [3,108]. In addition, it is now recognized that circulating erythrocytes contribute to levels of vascular resistance in the lung. These cells release ATP, a potent stimulus for NO synthesis, in response to increases in mechanical deformation as would occur as a result of pulmonary vasoconstriction or increases in flow velocity [16,109].

In humans with PAH, by definition, PVR is greater than normal. Although the underlying mechanisms responsible for the development and progression of PAH are not fully understood, it is clear that the ability of PAH erythrocytes fail to release ATP in response to mechanical deformation [13].

Two of the treatments for PAH that have resulted in reductions in PVR as well as prolongation of life are the administration of PGI_2 analogs and PDE5 inhibitors, alone or in combination [27,28]. It has been shown that human erythrocytes possess a PGI_2 receptor [7] as well as PDE5 [20] (Figure 1). Importantly, both healthy human and PAH erythrocytes release ATP in response to PGI_2 analogs and the magnitude of that release is increased in the presence of PDE5 inhibitors [26,29]. Here we extend that observation and report that the synergism between these drugs is potentially clinically important since concentrations of UT-15C that alone do not stimulate ATP release do so in the presence of PDE5 inhibitors (Figures 3.1 and 3.6).

Unfortunately, in the treatment of PAH, PGI_2 analogs and PDE5 inhibitors in combination can have unwanted side effects including headache, flushing and systemic hypotension that can limit the amounts of the drugs that can be administered [25,33]. In the present study we investigated the overall hypothesis that a PDE5 inhibitor delivered selectively to erythrocytes via liposomes would potentiate UT-15C-induced ATP release.

Such an approach could result in decreased side effects from both drugs without diminishing their effectiveness in the treatment of PAH.

Liposomes are small lipid vesicles that can target the delivery of drugs to specific cell types [37,85]. The feasibility of this approach for the delivery of PDE inhibitors to human erythrocytes is demonstrated by the report that a PDE3 inhibitor, CILO, could be incorporated into liposomes and effectively delivered to erythrocytes from humans with DM2 exposed to low oxygen tension to affect an increase in ATP release [42].

Here we report that in addition to CILO, liposomes constructed of DMPC are capable of delivering PDE5 inhibitors to human erythrocytes (Figures 3.2 and 3.6). It is of interest that, although liposomes containing the PDE3 inhibitor, CILO, restored low oxygen-induced ATP release from erythrocytes of humans with DM2 [42], this approach was not successful here (Figure 3.2). There are several possible explanations for this result. First, the signaling pathway stimulated by exposure of erythrocytes to low oxygen is not the same as that associated with activation of the IPR (Figures 1.1 and 1.2) [9,13,22]. Second, the amount of PDE3 inhibitor required may be greater in the IPR pathway. Finally, the IPR pathway is not defective in PAH erythrocytes while the low oxygen pathway is clearly compromised in erythrocytes of humans with DM2 [70]. Notwithstanding, the finding that in spite of the fact that PDE3 and 5 inhibitors were delivered with liposomes of the same composition, only PDE5 inhibitors were effective in augmenting UT-15C-induced ATP release. Thus, the response to PDE5 inhibitors cannot be attributed to non-specific liposome-erythrocyte interactions.

The extent of liposome binding to erythrocytes was evaluated using flow cytometry. A fluorophore was incorporated into DMPC liposomes. Fluorescence was detected when liposomes alone or erythrocytes that had been incubated with labeled liposomes were passed through the device. Based on the size of labeled particles detected when erythrocytes that had been incubated with labeled liposomes were studied, it can be concluded that few liposomes failed to interact with the erythrocyte membranes. This is confirmed by the finding that there was no flurophore detected in the supernatant of erythrocytes after interaction with labeled liposomes suggesting that the majority of the liposomes were bound to the erythrocytes. It was also important to establish that DMPC liposomes of this construction do not alter the rheological properties of human

erythrocytes. Here it is reported that there were no detectable rheological differences between erythrocytes incubated with or without liposomes.

The components that are used to construct liposomes for drug delivery must take into account the characteristics of the drug to be delivered and the cell that is the target for the liposome. Consequently, a number of different liposome formulations for different purposes have been reported [38,42,74]. It has been suggested that adding cholesterol [82,83,110] or a negative surface charge [111,112] may be beneficial in targeting liposomes to erythrocytes. Another potentially important issue in our study is that TAD has more aromatic rings than does CILO or ZAP. For that reason, the initial attempts to incorporate TAD into liposomes involved the use of formulations with added cholesterol or PS. Neither of these formulations proved satisfactory (Figure 3.5). Use of cholesterol-containing liposomes stimulated excessively high baseline ATP release (Figure 3.5A). In marked contrast, in the presence of PS-containing liposomes, UT-15C did not stimulate ATP release (Figure 3.5B). However, DMPC liposomes were loaded with TAD and, in the presence of these liposomes, UT-15C again stimulated erythrocyte ATP release (Figure 3.6). These studies illustrate the importance of selecting the optimal liposomal formulation for drug encapsulation and delivery.

In conclusion, these studies demonstrate that liposomal delivery of PDE5 inhibitors is feasible and that such an approach potentiates UT-15C induced ATP release. The findings are consistent with the hypothesis that directed delivery of this class of drugs to erythrocytes of humans with PAH could be a new and important method to augment PGI_2 analog-induced ATP release from these cells. Such an approach could significantly limit side effects of both drugs without compromise of their therapeutic effectiveness in PAH.

6. FUTURE DIRECTIONS

Future experiments that could aid in bringing this potential treatment method closer to fruition include the testing of liposome storage, the creation of liposomal formulations with erythrocyte-specific antibodies, and testing these liposomes *in vivo*. These efforts could be applied for both PDE3 and PDE5 inhibitors. Better chemical analyses should also be performed. It is important to determine that the supernatant does not contain the drug. Although some flow cytometry tests were performed, more robust investigation into the binding of liposomes to erythrocytes could utilize fluorescing antibodies that are quenched when absorbed into the cell. More in-depth and complete chemical analyses could be made. And, ATP tests attempting to increase ATP release from UT-15C-stimulated erythrocytes using the liposomal supernatant would be beneficial experiments to conduct. Successful tests using blood from patients with PAH are critical before clinical trials can be made with PAH patients to provide actual evidence of increased drug efficacy with reduced side effects. The use of liposomes is increasing growing but slowly. The potential to improve drug delivery with reduced negative consequences is significant. The variety of drugs that can be encapsulated within these lipid vesicles is practically endless.

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