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Liposomal-delivery of phosphodiesterase 5 inhibitors augments UT-15C-stimulated ATP release from human erythrocytes☆

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ABSTRACT

The use of liposomes to affect targeted delivery of pharmaceutical agents to specific sites may result in the reduction of side effects and an increase in drug efficacy. Since liposomes are delivered intravascularly, erythrocytes, which constitute almost half of the volume of blood, are ideal targets for liposomal drug delivery.

In vivo, erythrocytes serve not only in the role of oxygen transport but also as participants in the regulation of vascular diameter through the regulated release of the potent vasodilator, adenosine triphosphate (ATP). Unfortunately, erythrocytes of humans with pulmonary arterial hypertension (PAH) do not release ATP in response to the physiological stimulus of exposure to increases in mechanical deformation as would occur when these cells traverse the pulmonary circulation. This defect in erythrocyte physiology has been suggested to contribute to pulmonary hypertension in these individuals.

In contrast to deformation, both healthy human and PAH erythrocytes do release ATP in response to incubation with prostacyclin analogs via a well-characterized signaling pathway. Importantly, inhibitors of phosphodiesterase 5 (PDE5) have been shown to significantly increase prostacyclin analog-induced ATP release from human erythrocytes.

Here we investigate the hypothesis that targeted delivery of PDE5 inhibitors to human erythrocytes, using a liposomal delivery system, potentiates prostacyclin analog- induced ATP release. The findings are consistent with the hypothesis that directed delivery of this class of drugs to erythrocytes could be a new and important method to augment prostacyclin analog-induced ATP release from these cells. Such an approach could significantly limit side effects of both classes of drugs without compromising their therapeutic effectiveness in diseases such as PAH.

1. Introduction

Erythrocytes have been shown to participate in the regulation of vascular caliber via the release of the potent vasodilator, adenosine triphosphate (ATP) [1–4]. ATP is released from erythrocytes in response to both physiological and pharmacological stimuli [5,6]. 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 [7]. 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 cyclic adenosine monophosphate (cAMP) (Fig. 1) [6,8,9]. In this pathway, levels of cAMP are regulated by phosphodiesterase 3 (PDE3), a PDE that is inhibited by local increases in cyclic guanosine monophosphate (cGMP) [10]. Erythrocytes make cGMP; its levels are the product of synthesis by soluble guanylyl cyclase (sGC) and hydrolysis by PDE5 [11]. Importantly, incubation of both healthy human and PAH erythrocytes with inhibitors of PDE5 in solution have been shown to augment PGI_2 analog-induced ATP release [8,9]. 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

Abbreviations: ATP, (adenosine triphosphate); PAH, (pulmonary arterial hypertension); PDE, (phosphodiesterase); cGMP, (cyclic guanosine monophosphate); PGI_2, (prostacyclin); cAMP, (cyclic adenosine monophosphate); sGC, (soluble guanylyl cyclase); DMPC, (1,2-Dimyristoyl-sn-glycero-3-phosphocholine); ZAP, (zaprinast),; TAD, (tadalafil); PSS, (physiological salt solution); FSC, (forward scatter); SSC, (side scatter)

☆ The authors continue to have nothing to declare.

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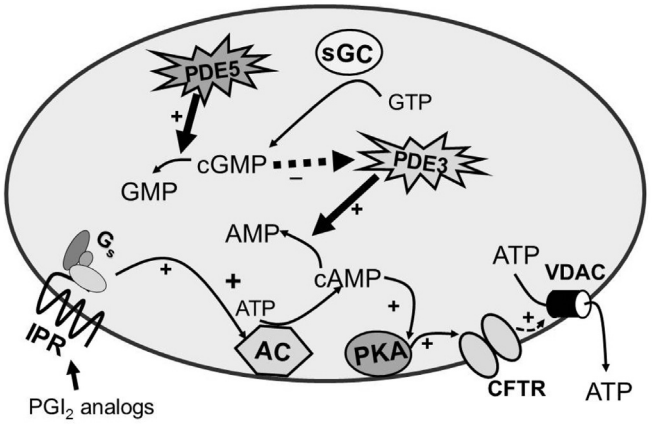

Fig. 1. Proposed signaling pathway for prostacyclin (PGI_2) receptor-mediated ATP release from erythrocytes. Exposure to PGI_2 or its analogs results in activation of the heterotrimeric G protein, Gs. This leads to activation of AC and an increase in cAMP that is regulated by PDE3 activity. Increases in cAMP activate PKA and, subsequently, CFTR. The final conduit for ATP release in this pathway is VDAC. 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.

by untoward systemic side effects including hypotension, flushing and headache [12,13]. 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 are small lipid vesicles that can target the delivery of drugs to specific cell types, including erythrocytes [14,15]. 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 we investigate the hypothesis that liposomes can be designed to deliver PDE5 inhibitors to human erythrocytes and that this approach will result in augmented ATP release when these cells are exposed to UT-15C, an oral form of the prostacyclin analog, treprostinil. The overall goal of this work is to demonstrate that encapsulation of PDE5 inhibitors within liposomes is a viable approach to the delivery of these drugs to human erythrocytes.

2. Materials and methods

2.1. Isolation of human erythrocytes

Blood obtained from 23 healthy human volunteers (13 females, 11 males; average age 56 years; range 27–78 years) 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 [5–8]. 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 HIPAA.

2.2. Preparation of DMPC liposomes

Unilamellar liposomes were prepared by the extrusion method [16]. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (10 mg) was added to a mixture of methanol/chloroform (1 mL, 50/50, v/v) in the absence (control) or presence of either of two chemically dissimilar PDE5 inhibitors, zaprinast (ZAP, 1 mM; Sigma-Aldrich) or tadalafil (TAD, 1 mM; 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). Liposomes were used up to seven days after preparation. No difference between the effects of liposomes on UT-15C-induced ATP release from erythrocytes was detected over this time period.

2.3. Preparation of fluorophore-containing liposomes

The fluorophore-containing molecule, 25-NBD cholesterol (Avanti Polar Lipids) in chloroform, was added as the chloroform volume (0.5 mL) 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 [17,18].

2.4. Measurement of ATP

Washed erythrocytes were diluted to a hematocrit of 20% with PSS containing glucose and albumin. ATP was measured using the luciferin-luciferase technique [5,19]. Briefly, a 0.2 mL sample of an erythrocyte suspension was injected into a cuvette containing 0.1 mL of 10 mg/mL crude firefly lantern extract (Sigma, St. Louis, MO, USA) 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.

2.5. 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 [20,21]. This method was used to detect and ensure that erythrocyte lysis did not contribute to any observed increases in ATP release. No studies were excluded due to increased hemoglobin levels.

2.6. 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 containing a fluorophore (25-NBD cholesterol) for 10 min at room temperature in the dark. Samples were anlyzed on a BD Accuri C6 Flow Cytometer (Becton, Dickenson, and Co., Franklin Lanes, NJ, USA) equipped with a solid state 50 mW laser tuned to 488 nm. A 200 μm ID fused silica capillary flow cell was used at a flow rate of 10 μL/min with no gating and a threshold of 10,000 events. Detectors

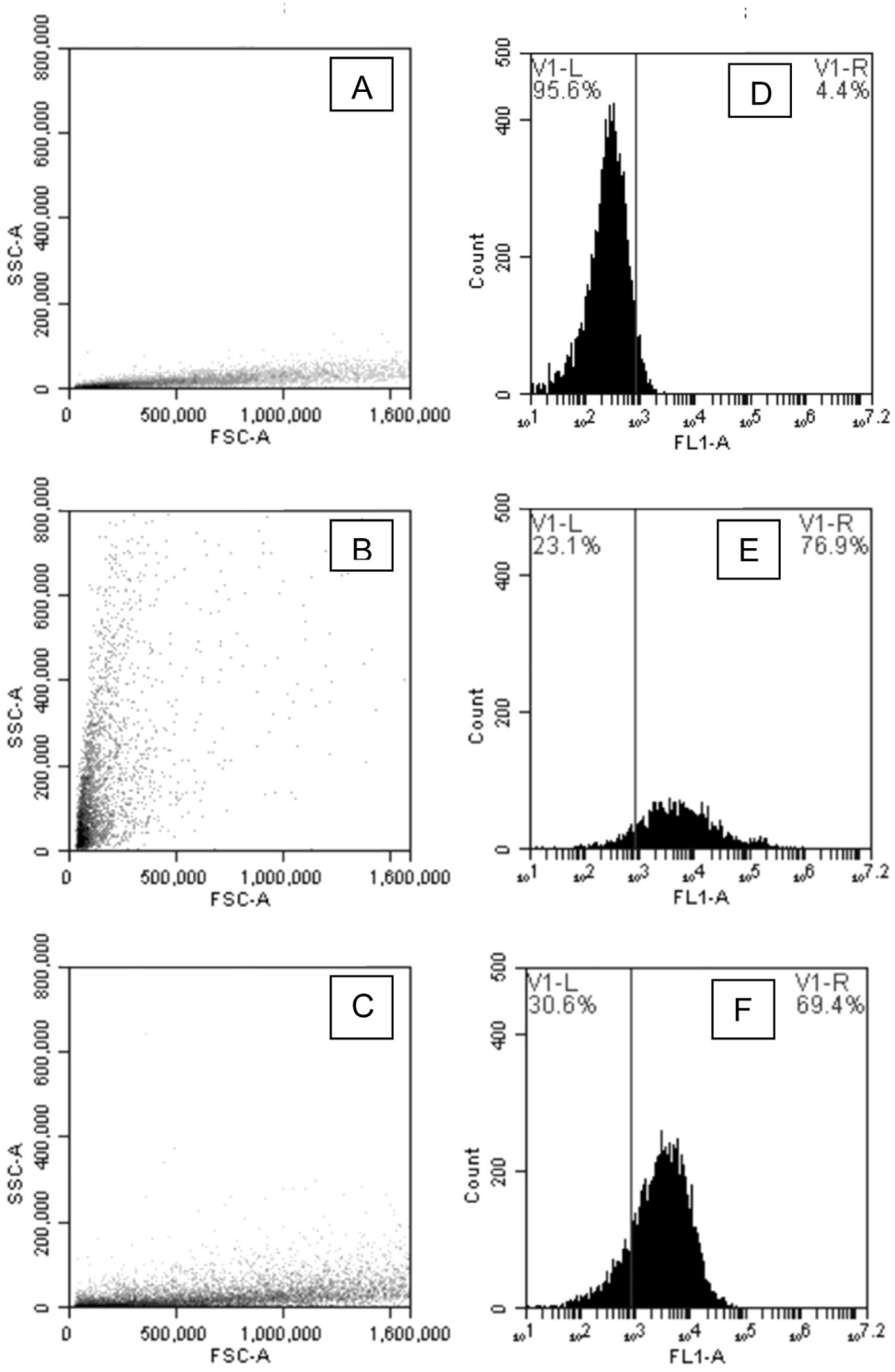

Fig. 2. Measurement of liposomal binding using flow cytometry. Larger cells (erythrocytes) exhibit greater forward scatter (FSC) whereas liposomes exhibit greater side scatter (SSC). Forward and side scatter conditions remained unchanged through all tests. Scatterplots A–C depict forward-scatter versus side scatter (indicating "cell" size) and histograms D–F demonstrate the presence or absence of a fluorescent label. Panel A demonstrates the presence of erythrocytes alone (7–8 μm in diameter). Panel B demonstrates the presence of liposomes alone (~ 100 nm in diameter). Panel C demonstrates that when erythrocytes and liposomes are co-incubated, only the erythrocytes signal is detected. Panels D, E and F display a threshold (the vertical line) between the absence (left) and presence (right) of fluorescence. Fluorescence was not seen in the erythrocytes alone (Panel D) but was present in labeled liposomes (Panel E). When the liposomes and erythrocytes were co-incubated, erythrocytes were shown to display the fluorescent label (Panel F). Percentages in the upper corners of the histograms (D–F) compare the events on the respective sides of the threshold.

along the flow path recorded "events" (wavelengths of light) which were distinguished when excited molecular fluorophores emitted light at 523 nm. Analysis of the locations of the detectors recording events estimated cell size. The measurable particle size range for this device is 1–40 μm [22,23].

2.7. Determination of the rheological properties of erythrocytes in the absence and presence of liposomes

To determine if liposome-erythrocyte interaction resulted in altered erythrocyte deformability, rheological studies were performed with the Anton Paar Modular Compact Rheometer 302 (Anton Paar, Ashland, VA, USA) at 20 °C using a parallel plate configuration (50 mm diameter with a 0.05 mm gap) [24]. Isolated erythrocytes were diluted to a 40% hematocrit with PSS containing glucose and albumin then incubated with liposomes or their vehicle, saline, prior to testing. Comparison of the apparent viscosity relative to shear rate via resistance measurements were made with increasing shear rate over time.

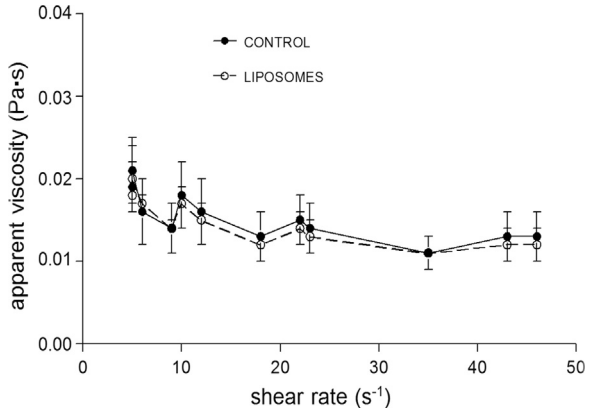

Fig. 3. Rheometric comparison between apparent viscosity (pascal second, Pa s) and shear rate (reciprocal seconds, s^{-1}) of erythrocyte suspensions in the presence or absence of DMPC liposomes. Erythrocytes alone (CONTROL, closed circles, n = 3) or erythrocytes incubated with liposomes (LIPOSOMES, open circles, n = 3) were suspended in PSS and subjected to increasing shear rates. Values are means ± SE.

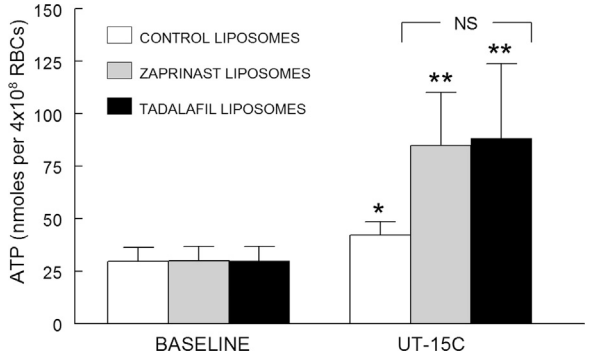

Fig. 4. : Effect of UT-15C (1 μmol/L) on ATP release from human erythrocytes in the presence of blank DMPC liposomes (CONTROL, n = 16, open bars) or liposomes of the same composition loaded with either of the PDE5 inhibitors, zaprinast (n = 9, grey bars) or taladafil (n = 7, black bars). Cells were treated for 30 min with liposomes. ATP was measured before and 5, 10 and 15 min after the addition of UT-15C. The peak ATP release is reported. Values are means ± SE. * = different from respective baseline ($P < 0.05$), ** = different from respective baseline and control liposomes after addition of UT-15C ($P < 0.05$), NS = not significantly different.

2.8. Determination of the effect of UT-15C on ATP release from human erythrocytes in the presence of blank liposomes or liposomes containing one of two chemically distinct PDE5 inhibitors

Erythrocytes were diluted to a hematocrit of 20% in PSS containing glucose and albumin. Samples were pretreated with either blank liposomes (n = 16) or liposomes containing either of two chemically distinct PDE5 inhibitors, zaprinast (n = 9) or tadalafil (n = 7) for 30 min. ATP levels were then determined before and at 5, 10, and 15 min after the addition of UT-15C (1 μmol/L). The peak ATP release is reported.

2.9. 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, the results are reported as the mean ± the standard error (SE).

3. Results

3.1. Use of flow cytometry to characterize liposome-binding to erythrocytes

Erythrocytes alone are large relative to liposomes (7–8 μm, Fig. 2A) and do not fluoresce. This is shown by the fact that in the presence of erythrocytes only, the majority (95.6%) of events are recorded at channels below the marked threshold, a point approximately half-way between the peaks displaying fluorescence and the peaks obtained in the absence of fluorescence (Fig. 2D). In contrast, liposomes made with DMPC and labeled with the fluorophore are small or granular (~ 100 nm, Fig. 2B) and fluoresce as shown by the greatest volume of events (76.9%) occurring at channels above the marked threshold wavelength (Fig. 2E). When erythrocytes were incubated with liposomes containing the fluorophore, the large cells (erythrocytes) exhibited fluorescence from binding with labeled liposomes (Figs. 2C and 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 is not shown.) The data presented are from one of four studies conducted on blood from different individuals and are representative of all results.

3.2. Determination of rheological effects of DMPC liposomes on erythrocytes

The comparison of the apparent viscosity of erythrocyte suspensions at different applied shear rates revealed no differences between samples incubated with or without liposomes (Fig. 3). This result demonstrates that the liposomes used in these studies did not cause significant changes in erythrocyte rheological properties. In addition, as reported previously [34], no change in erythrocyte morphology was detected.

3.3. Effect of UT-15C on ATP release from erythrocytes in the presence of blank liposomes or liposomes containing PDE5 inhibitors

The addition of UT-15C (1 μmol/L) to erythrocytes incubated with blank liposomes resulted in an increase in ATP release (n = 16) (Fig. 4). The same concentration of UT-15C added to erythrocytes that had been incubated with liposomes containing either zaprinast (n = 9) or tadalafil (n = 7) also stimulated ATP release (Fig. 4). Importantly, the amount of ATP released from erythrocytes exposed to liposomes containing the PDE5 inhibitors was significantly greater than that released from erythrocytes incubated with control liposomes (Fig. 4). There was no difference in ATP released in response to UT-15C in erythrocytes treated with either of the chemically dissimilar PDE5 inhibitors (Fig. 4).

4. Discussion

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 [25] as well as by active vasodilation resulting from the local synthesis and release of vasodilators including nitric oxide and PGI_2 [26,27]. In addition to the pulmonary vasculature itself, it is now recognized that circulating erythrocytes contribute to the low levels of vascular resistance present in the normal pulmonary circulation (5). These cells release ATP, a potent stimulus for NO synthesis in the lung, in response to increases in mechanical deformation as occurs when vascular diameter is decreased and/or the velocity of blood flow is increased in the pulmonary circulation [21,28].

In humans with PAH, by definition, pulmonary vascular resistance 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 to release ATP in response to mechanical deformation is severely impaired [7].

Two of the treatments for PAH that have resulted in reductions in pulmonary vascular resistance as well as the prolongation of life are the administration of PGI_2 analogs and PDE5 inhibitors, alone or in combination [29–31]. It has been shown that human erythrocytes possess: 1) a PGI_2 receptor [6], 2) PDE5 [10,11] and 3) a well-characterized

signaling pathway for ATP release in response to PGI_2 receptor activation [6,9–11] (Fig. 1). Both healthy human and PAH erythrocytes release ATP in response to PGI_2 analogs. Importantly the magnitude of that release is increased in the presence of PDE5 inhibitors [8,9]. 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 [12,13]. Liposomes can be used to deliver drugs to specific cells within the body [32,33]. In the present study, we investigated the hypothesis that a PDE5 inhibitor that is targeted to erythrocytes via liposomes would potentiate UT-15C-induced ATP release. Such an approach could result in decreased side effects without diminishing the effectiveness of these drugs in the treatment of PAH. The feasibility of this approach for the delivery of a PDE inhibitor to human erythrocytes is demonstrated by the report that a PDE3 inhibitor, cilostazol, could be incorporated into liposomes and effectively delivered to human erythrocytes [34].

Here we report that, in addition to a PDE3 inhibitor, PDE5 inhibitors can be targeted to human erythrocytes using liposomes composed of DMPC (Fig. 2). Importantly, the binding of these liposomes to erythrocytes did not alter erythrocyte rheology as measured by the response to increases in shear rate (Fig. 3). Although the binding of liposomes to erythrocytes is not a new finding, the delivery of PDE5 inhibitors via this method is.

We determined that although the concentration of UT-15C used in these studies was capable of stimulating ATP release from human erythrocytes, the magnitude of the response was significantly greater after erythrocytes were exposed to liposomes containing either of two chemically dissimilar PDE5 inhibitors (Fig. 4). The enhanced ATP release was independent of the PDE5 inhibitor used and there was no effect on baseline ATP levels indicating that the PDE5 inhibitors were only effective when the prostacyclin receptor-associated pathway for ATP release was activated. Although zaprinast is a PDE5 inhibitor, it is not used clinically. However, the second PDE5 inhibitor used in these studies, tadalafil, is currently employed in the treatment of PAH alone or in combination with prostacyclin analogs [13,29–31].

We also examined the extent of liposome binding to erythrocytes using flow cytometry. Based on the size of the labeled particles detected when erythrocytes were incubated with labeled liposomes, it can be concluded that few liposomes failed to interact with the erythrocyte membranes. This is confirmed by the finding that there was no fluorophore detected in the supernatant of erythrocytes after interaction with labeled liposomes supporting the hypothesis that the majority of the liposomes were bound to the erythrocytes.

5. Conclusions

In conclusion, these studies demonstrate that targeted delivery of a PDE5 inhibitor to human erythrocytes is feasible and that such an approach potentiates UT-15C induced ATP release. These findings are consistent with the hypothesis that targeted 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 without compromise of therapeutic effectiveness in these patients.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.09.002.

References

[1] M.L. Ellsworth, T. Forrester, C.G. Ellis, H.H. Dietrich, The erythrocyte as a regulator of vascular tone, Am. J. Physiol.-Heart C 269 (6) (1995) H2155–H2161.
[2] M.L. Ellsworth, Red blood cell-derived ATP as a regulator of skeletal muscle perfusion, Med. Sci. Sport Exerc. 36 (1) (2004) 35–41.
[3] M.L. Ellsworth, C.G. Ellis, D. Goldman, A.H. Stephenson, H.H. Dietrich, R.S. Sprague, Erythrocytes: oxygen sensors and modulators of vascular tone, Physiology 24 (2) (2009) 107–116.
[4] M.L. Ellsworth, R.S. Sprague, Regulation of blood flow distribution in skeletal muscle: role of erythrocyte-released ATP, J. Physiol. 590 (Pt 20) (2012) 4985–4991, http://dx.doi.org/10.1113/jphysiol.2012.233106.
[5] R.S. Sprague, M.L. Ellsworth, A.H. Stephenson, A.J. Lonigro, ATP: the red blood cell link to NO and local control of the pulmonary circulation, Am. J. Physiol. 271 (6 Pt 2) (1996) H2712–H2722.
[6] R.S. Sprague, E.A. Bowles, M.S. Hanson, E.A. DuFaux, M. Sridharan, S. Adderley, M.L. Ellsworth, A.H. Stephenson, Prostacyclin analogs stimulate receptor-mediated cAMP synthesis and ATP release from rabbit and human erythrocytes, Microcirculation 15 (5) (2008) 461–471, http://dx.doi.org/10.1080/10739680701833804.
[7] R.S. Sprague, A.H. Stephenson, M.L. Ellsworth, C. Keller, A.J. Lonigro, Impaired release of ATP from red blood cells of humans with primary pulmonary hypertension, Exp. Biol. Med. 226 (5) (2001) 434–439, http://dx.doi.org/10.1177/153537020122600507.
[8] E.A. Bowles, G.N. Moody, Y. Yeragunta, A.H. Stephenson, M.L. Ellsworth, R.S. Sprague, Phosphodiesterase 5 inhibitors augment UT-15C-stimulated ATP release from erythrocytes of humans with pulmonary arterial hypertension, Exp. Biol. Med. 240 (1) (2015) 121–127, http://dx.doi.org/10.1177/1535370214547155.
[9] S.M. Knebel, M.M. Elrick, E.A. Bowles, A.K. Zdanovec, A.H. Stephenson, M.L. Ellsworth, R.S. Sprague, Synergistic effects of prostacyclin analogs and phosphodiesterase inhibitors on cyclic 3',5'-monophosphate accumulation and adenosine 3',5'-triphosphate release from human erythrocytes, Exp. Biol. Med. 238 (9) (2013) 1069–1074, http://dx.doi.org/10.1177/1535370213498981.
[10] S.P. Adderley, R.S. Sprague, A.H. Stephenson, M.S. Hanson, Regulation of cAMP by phosphodiesterases in erythrocytes, Pharmacol. Rep. 62 (3) (2010) 475–482, http://dx.doi.org/10.1016/S1734-1140(10)70303-0.
[11] S.P. Adderley, K.M. Thuet, M. Sridharan, E.A. Bowles, A.H. Stephenson, M.L. Ellsworth, R.S. Sprague, Identification of cytosolic phosphodiesterases in the erythrocyte: a possible role for PDE5, Med. Sci. Monit. 17 (5) (2011) CR241–CR247, http://dx.doi.org/10.12659/MSM.881763.
[12] H. Pickles, J. O'Grady, Side effects occurring during administration of epoprostenol (prostacyclin, PGI_2), in man, Br. J. Clin. Pharmacol. 14 (2) (1982) 177–185 (PMCID: PMC1427755).
[13] P.J. Wright, Comparison of phosphodiesterase type 5 (PDE5) inhibitors, Int. J. Clin. Pract. 60 (8) (2006) 967–975, http://dx.doi.org/10.1111/j.1742-1241.2006.01049.x.
[14] A.K. Agrawal, A. Singhal, C.M. Gupta, Functional drug targeting to erythrocytes in vivo using antibody bearing liposomes as drug vehicles, Biochem. Biophys. Res. Comm. 148 (1) (1987) 357–361, http://dx.doi.org/10.1016/0006-291X(87)91118-1.
[15] M. Owais, G.C. Varshney, A. Choudhury, S. Chandra, C.M. Gupta, Chloroquine encapsulated in malaria-infected erythrocyte-specific antibody-bearing liposomes effectively controls chloroquine-resistant *Plasmodium berghei* in mice, Antimicrob. Agents Chemother. 39 (1) (1995) 180–184, http://dx.doi.org/10.1128/AAC.39.1.180.
[16] V. Torchilin, V. Weissig, Liposomes – a Practical Approach, 2nd ed., University Press, Oxford, 2003, pp. 3–29.
[17] S. Mukherjee, A. Chattopadhyay, Monitoring cholesterol organization in membranes at low concentrations utilizing the wavelength-selective fluorescence approach, Chem. Phys. Lipids 134 (2005) 79–84.
[18] T.J. Pucadyil, S. Mukherjee, A. Chattopadhyay, Organization of dynamics of NBD-labeled lipids in membranes analyzed by fluorescence recovery after photobleaching, J. Phys. Chem. B 111 (8) (2007) 1975–1983, http://dx.doi.org/10.1016/j.chemphyslip.2004.12.001.
[19] B.L. Strehler, W.C. McElroy, S.P. Colowick, N.O. Kaplan (Eds.), Assay of adenosine triphosphate in Methods in Enzymology, 3 Academic Press, New York, 1957, pp. 871–873.
[20] P. Holownia, E. Bishop, D.J. Newman, W.G. John, C.P. Price, Adaptation of latex-enhanced assay for percent glycohemoglobin to a Dade Dimension® analyzer, Clin. Chem. 43 (1) (1997) 76–84.
[21] R.S. Sprague, M.L. Ellsworth, A.H. Stephenson, M.E. Kleinhenz, A.J. Lonigro, Deformation-induced ATP release from red blood cells requires CFTR activity, Am. J. Physiol. 275 (5 Pt 2) (1998) H1726–H1732.
[22] K. Vorauer-Uhl, A. Wagner, N. Borth, H. Katinger, Determination of liposome size distribution by flow cytometry, Cytometry 39 (2) (2000) 166–171, http://dx.doi.org/10.1002/(SICI)1097-0320(20000201)39:2 < 166::AID-CYTO10 > 3.0.CO;2-M.
[23] N.K. Childers, S.M. Michalek, J.H. Eldridge, F.R. Denys, A.K. Berry, J.R. McGhee, Characterization of liposome suspensions by flow cytometry, J. Immunol. Methods 119 (1989) 135–143, http://dx.doi.org/10.1016/0022-1759(89)90390-6.
[24] P.A. Evans, K. Hawkins, P.R. Williams, R.L. Williams, Rheometrical detection of incipient blood clot formation by Fourier transform mechanical spectroscopy, J. Non-Newton. Fluid Mech. 148 (2008) 122–126, http://dx.doi.org/10.1016/j.jnnfm.2007.04.020.
[25] C.B. Wolff, D.J. Collier, M. Shah, M. Saxena, T.J. Brier, V. Kapil, D. Green, M. Lobo, A discussion on the regulation of blood flow and pressure, Adv. Exp. Med. Biol. 876

(2016) 129–135, http://dx.doi.org/10.1007/978-1-4939-3023-4_16.
[26] R.S. Sprague, A.H. Stephenson, R.A. Dimmitt, N.A. Weintraub, C.A. Branch, L. McMurdo, A.J. Lonigro, Inhibition of nitric oxide synthesis results in a selective increase in arterial resistance in rabbit lungs, Pol. J. Pharmacol. 46 (6) (1994) 579–585.
[27] R.S. Sprague, A.H. Stephenson, A.J. Lonigro, Prostaglandin I_2 supports blood flow to hypoxic alveoli in anesthetized dogs, J. Appl. Physiol.: Respir. Environ. Exerc. Physiol. 56 (5) (1984) 1246–1251.
[28] R.S. Sprague, M.L. Ellsworth, A.H. Stephenson, A.J. Lonigro, Increases in perfusate flow rate stimulate ATP release from red blood cells in isolated rabbit lungs, Exp. Clin. Cardiol. 3 (2) (1998) 73–77.
[29] H.A. Ghofrani, R. Widemann, F. Rose, H. Olschewski, R.T. Schermuly, N. Weissmann, W. Seeger, F. Grimminger, Combination therapy with oral sildenafil and inhaled iloprost for severe pulmonary hypertension, Ann. Intern. Med. 136 (2002) 515–522, http://dx.doi.org/10.7326/0003-4819-136-7-200204020-00008.
[30] H. Wilkens, A. Guth, J. König, N. Forestier, B. Cremers, B. Hennen, M. Böhm, G.W. Sybercht, Effect of inhaled iloprost plus oral sildenafil in patients with primary pulmonary hypertension, Circulation 104 (11) (2001) 1218–1222, http://dx.doi.org/10.1161/hc3601.096826.
[31] T. Itoh, N. Nagaya, T. Fujii, T. Iwase, N. Nakanishi, K. Hmada, K. Kangawa, H. Kimura, A combination of oral sildenafil and beraprost ameliorates pulmonary hypertension in rats, Am. J. Respir. Crit. Care Med. 169 (1) (2004) 34–38, http://dx.doi.org/10.1164/rccm.200303-346OC.
[32] S. Chandra, A.K. Agrawal, C.M. Gupt, Chloroquine delivery to erythrocytes in *Plasmodium berghei*-infected mice using antibody-bearing liposomes as drug vehicles, J. Biosci. 16 (3) (1991) 137–144, http://dx.doi.org/10.1007/BF02703366.
[33] V.N. Smirnov, S.P. Domogatsky, V.V. Dolgov, V.B. Hvatov, A.L. Klibanov, V.E. Koteliansky, V.R. Muzykantov, V.S. Repin, G.P. Samokhin, B.V. Shekhonin, M.D. Smirnov, D.D. Sviridov, V.P. Torchinilin, E.I. Chazov, Carrier-directed targeting of liposomes and erythrocytes to denuded areas of vessel wall, Proc. Natl. Acad. Sci. USA 83 (17) (1986) 6603–6607.
[34] S.A. Dergunov, E.A. Bowles, W. Gordon, M. Green, A. Bierman, M.L. Ellsworth, E. Pinkhassik, R.S. Sprague, Liposomal delivery of a phosphodiesterase 3 inhibitor resuces low oxygen-induced ATP release from erythrocytes of humans with type 2 diabetes, Biochem. Biophys. Rep. 2 (2015) 137–142, http://dx.doi.org/10.1016/j.bbrep.2015.05.011.