Potential Role of Liposomes for the Delivery of Phosphodiesterase Inhibitors to Erythrocytes for the Treatment of Type 2 Diabetes

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Abstract

Human erythrocytes participate in the regulation of vascular diameter in the microcirculation through the controlled release of the vasodilator adenosine triphosphate (ATP) in response to physiological stimuli, including exposure to low oxygen tension as occurs in the microcirculation of skeletal muscle. The localized release of this vasodilator has been suggested to be an important mechanism for matching perfusion (oxygen delivery) with need in this tissue. However, in certain diseases, such as type 2 diabetes (DM2), the ability of erythrocytes to release ATP in response to this stimulus is severely compromised. This defect in erythrocyte physiology could contribute to impairment of vasodilation in the peripheral circulation leading to vascular insufficiency and delayed wound healing. It has been shown that inhibitors of specific phosphodiesterases (PDEs) can augment low oxygen-induced ATP release from erythrocytes of humans with DM2. However, these drugs are associated with serious side effects that limit their use in clinical medicine.

Here we summarize the evidence in support of the hypothesis that delivery of PDE3 inhibitors encapsulated in liposomes to erythrocytes could provide a new approach for the treatment of DM2. In addition we report that inhibitors of PDE5 can also rescue low oxygen-induced ATP release from DM2 erythrocytes making this class of drugs another that could be targeted to erythrocytes.

Keywords: RBC, ATP, microcirculation, DM2, phospholipids

Introduction:

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 [1]. Impaired vascular function is a significant complication of DM2 with cardiovascular disease accounting for nearly half of the deaths in humans with this condition [2,3]. Individuals with DM2 have a four-fold increased risk for claudication [1] and as much as a sixteen-fold increased risk for lower limb amputation [3-6]. 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 [7-10].

Patients with DM2 have diminished muscle blood flow both at rest [9] and with exercise [10]. Although direct studies of the skeletal muscle microcirculation are not possible in humans, such studies have been undertaken in several animal models of diabetes [11-13]. These studies demonstrate marked reductions in: 1) oxygen delivery [11,12], 2) capillary erythrocyte flux [12] and 3) convective oxygen delivery and diffusive oxygen transport [11]. 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 [14-18]. It has also been suggested that there is reduced nitric oxide (NO) synthesis [19], increased NO degradation [20] and/or abnormalities in the vascular smooth muscle [21] 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.

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 [22-28]. In skeletal muscle, a critical stimulus for local dilation of blood vessels is the release of the vasodilator, adenosine triphosphate (ATP) from erythrocytes exposed to low oxygen tension [22-28].

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 [27-29]. 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 [30-33].

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). Low oxygen tension-induced ATP release requires activation of the heterotrimeric G-protein G_i [34]. In this signaling pathway, the next steps require sequential activation of adenylyl cyclase (AC) [35,36], protein kinase A (PKA) [37] and the cystic fibrosis transmembrane conductance regulator (CFTR) [38,39]. The final ATP conduit in this signaling pathway is pannexin 1[40]. Importantly, it has been shown that expression of a single G_i isoform (G_{i2}) is reduced in erythrocytes of humans with DM2 [30,31] and is associated with markedly reduced ATP release in response to exposure of these cells to low oxygen tension [31,32]. 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 [35,36]. In all cells, cAMP levels must be tightly regulated to keep 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 phosphodiesterase 3 (PDE3) [41,42] (Figure 1). 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 [43]. 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 [44]. 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 [45].

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 [46], flow cytometry [47], and electron microscopy [48]. 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 [49].

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 [49,50]. Specifically, negative charges appear to be beneficial for the fusion of liposomes with erythrocytes [48,49]. Although positive charges have also been used, the incidence of hemolysis of erythrocytes was increased under these conditions [48,51].

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 [49].

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 [52,53]. These alterations can affect the fluidity of the liposomal membrane. Membrane fluidity strongly affects the likelihood of fusion between liposomes and cells [54]. Importantly, liposomes with increased membrane fluidity were reported to display enhanced binding with erythrocytes [55]. Other components that may be added to liposomes to enhance selective binding of liposomes to specific cell types or tissues include selective antibodies [56-58] or other molecules to decrease recognition of liposomes by the immune system [46]. 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 [52,59]. Thus, liposome-

erythrocyte interactions are complex and not all liposome compositions are "erythrocyte-friendly" [51, 59, 60].

Liposomal delivery of a PDE inhibitor to human erythrocytes: Effect on low oxygen-induced ATP release:

Liposomes are capable of selectively delivering drugs to erythrocytes [59,61]. Recently, the ability to use liposomes to target a PDE3 inhibitor to human erythrocytes was shown by Dergunov, *et al.* [62]. In this study it was established that liposomes composed of dimyristoyl-phosphatidylcholine (DMPC): 1) have no adverse effect on erythrocyte morphology and 2) can transport the PDE3 inhibitor, cilostazol [62]. These investigators demonstrated that erythrocytes of patients with DM2 did not release ATP in response to exposure to low oxygen in the presence of liposomes that did not contain cilostazol. However, in contrast, incubation of DM2 erythrocytes with cilostazol-loaded liposomes restored the physiological release of ATP to these cells in response to low oxygen tension [62].

Potential role of PDE5 inhibitors in the regulation of low oxygen-induced ATP release from human erythrocytes:

It is important to note that, in addition to selective pharmacological agents, PDE3 is also inhibited endogenously by cGMP [63,64]. Human erythrocytes contain soluble guanylyl cyclase and generate cGMP [42,65]. In addition, these cells contain PDE5, a PDE that hydrolyzes cGMP [42] (Figure 1). Thus, inhibition of PDE5 that results in increased cGMP levels in the erythrocyte would, in turn, inhibit PDE3 activity and increase low oxygen-induced ATP release (Figure 1). Here we report that that treatment of DM2 erythrocytes with either of two chemically dissimilar PDE5 inhibitors (zaprinast or tadalafil) rescues the ability of these cells to release ATP when exposed to low oxygen tension (Figure 2). Studies are currently underway to determine if PDE5 inhibitors can be incorporated into liposomes and targeted to human erythrocytes.

Summary:

Liposomal drug delivery has been studied for decades. However, the challenge remains in finding the right combination of liposomal components to allow for the effective incorporation of specific drugs into

the liposome to provide selective delivery of the liposome-encapsulated drugs to erythrocytes. The feasibility of this approach is confirmed by studies described here demonstrating that selective delivery of PDE3 inhibitors to DM2 erythrocytes can be effective in rescuing the physiological response (ATP release) to the stimulus of low oxygen tension. We propose that liposomal delivery of drugs to the erythrocyte should be more seriously considered as a new therapeutic strategy for the treatment or management of the vascular disease associated with DM2.

Acknowledgements:

The authors would like to thank J. L. Sprague and J. E. Bowles for inspiration.

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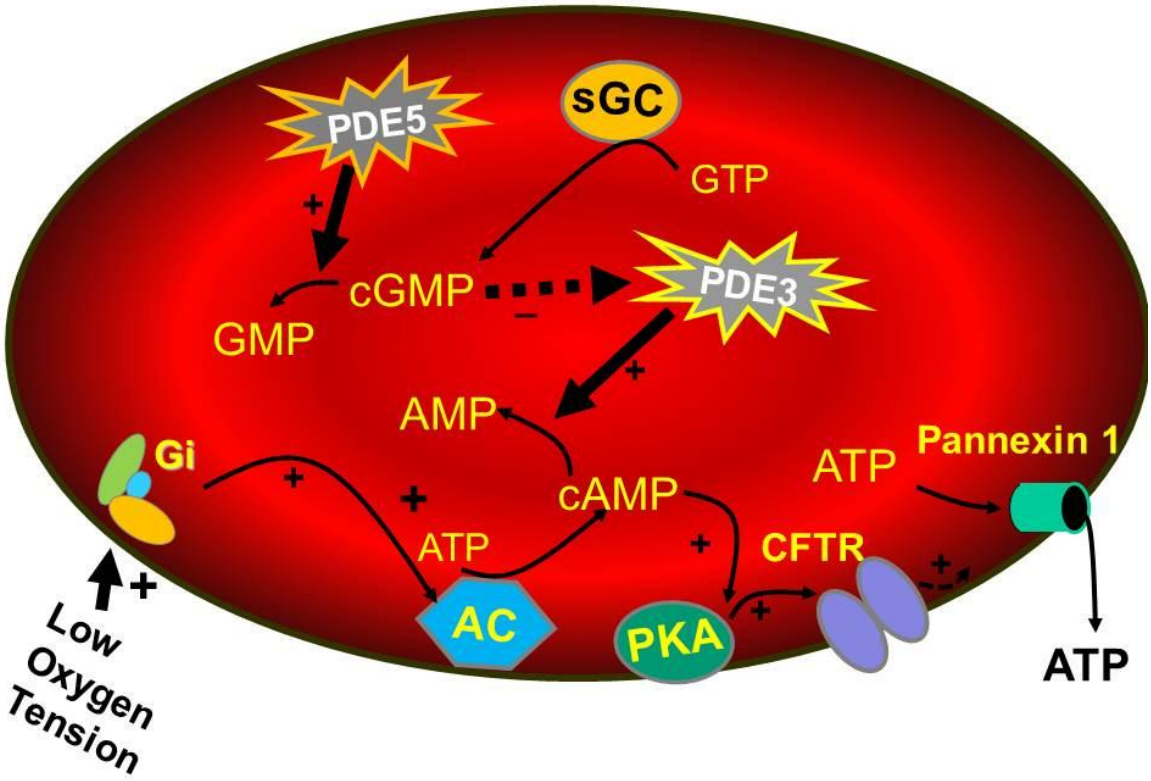

Figure 1: Proposed signaling pathway for low oxygen-induced ATP release from erythrocytes. Exposure to low oxygen results in activation of the heterotrimeric G protein, Gi. This leads to activation of AC and an increase in cAMP that is regulated by PDE3 activity. Increases in cAMP activate PKA and, subsequently, CFTR. The final conduit for ATP release in this pathway is pannexin 1.

Abbreviations: G_i = heterotrimeric G protein, G_i; AC = adenylyl cyclase; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate; AMP = adenosine monophosphate; PKA = protein kinase A; CFTR = cystic fibrosis transmembrane conductance regulator; GTP = guanosine triphosphate; cGMP = cyclic guanosine monophosphate; GMP = guanosine monophosphate; sGC = soluble guanylyl cyclase; PDE3 = phosphodiesterase 3; PDE5 = phosphodiesterase 5; (+) = activation and (−) = inhibition.

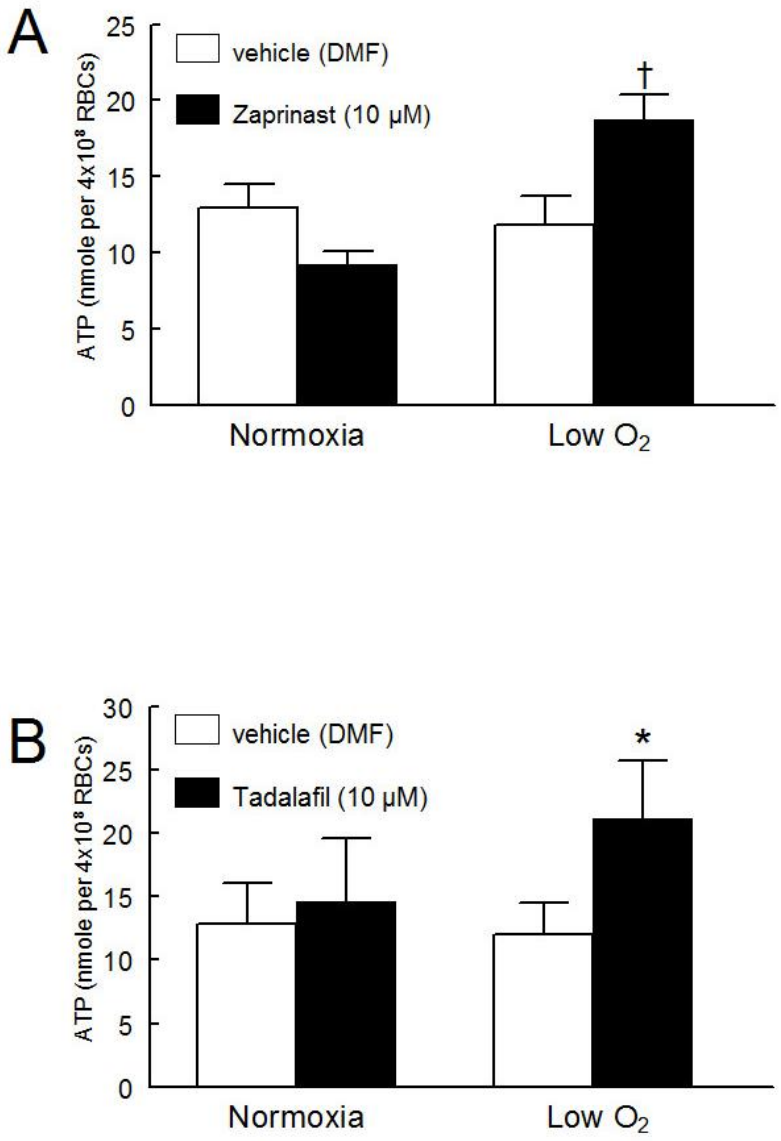

Figure 2: Effect of exposure to reduced oxygen (O_2) tension on ATP release from erythrocytes of humans with DM2 in the absence and presence of the PDE5 inhibitors zapranast (10 µM) (panel A, n=6) or tadalafil (10 µM) (panel B, n=4), or their vehicle, dimethylformamide (DMF). The methods have been described in detail previously (23, 32, 43) Briefly, washed erythrocytes were diluted to a 20% hematocrit in a Ringers buffer containing bicarbonate, in mM; 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl, 1.2 $MgSO_4$, 5.5 glucose, 21.4 $NaHCO_3$, 0.5% BSA, pH 7.4 at 37ºC (43). Cells were equilibrated for 30 min with a gas mixture containing 15% O_2, 6% CO_2, balance N_2 (pH = 7.41± 0.03, pCO_2 = 36 ± 2 mm Hg and pO_2 = 107 ± 5 mmHg; Normoxia) in a thin-film tonometer (model 237; Instrumentation Laboratory) in the absence and presence of blank liposomes (vehicle, black bars) of liposomes containing a PDE5 inhibitor (open bars). The gas mixture was then changed to one containing 0% O_2, 6% CO_2, balance N_2 (pH = 7.42± 0.02, pCO_2 = 38 ± 2 mm Hg and pO_2 = 10 ± 1 mm Hg; Low O_2). ATP release was determined 30 min after exposure to 15% O_2 and 10 min after exposure to 0% O_2 using the luciferin-luciferase method (23,32,43) . Values are the means ± SE. Greater than respective normoxia value (* = $p < 0.05$. † = $p <$ 0.01).

