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Use of the red blood cell as a simple drug target and diagnostic by manipulating and monitoring its ability to release adenosine triphosphate (ATP)*

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Abstract: Without question, one of the main tasks of the red blood cell (RBC) is to deliver oxygen to various tissues and organs in vivo. However, due to the lack of a nucleus and mitochondria, the RBC is typically not thought to be a determinant in many diseases or abnormal physiological conditions. Recent efforts by many labs world-wide are resulting in a body of evidence, suggesting that the RBC may serve many other roles in vivo besides that of an oxygen carrier. If so, the RBC may eventually emerge as one of the simplest drug targets and diagnostic tools available. Here, molecular evidence is provided, suggesting that the RBC, via its ability (or inability) to maintain proper levels of adenosine triphosphate (ATP) release in the circulation, may be a major factor in vascular regulation. Moreover, due to the RBC's response to slight modifications in its normal environment, the use of the RBC as an important diagnostic for early prediction of disease onset is discussed.

Keywords: ATP release; diagnostic tools; drug targets; red blood cells; vascular regulation.

THE RED BLOOD CELL: A SIMPLE INTRODUCTION

The red blood cell (RBC) has a diameter of approximately 6–8 μm, is non-nucleated, and any formed organelles, such as mitochondria, are absent. It has been estimated that the volume of a single RBC is about 87 fl [1] and approximately 40–45 % of the bloodstream is occupied by RBCs (also known as the RBC hematocrit). The lifespan of a typical RBC in the circulation has been reported to be a little over 100 days [2]. Based on the total volume of blood in an adult human (5 l) and the above information, the number of RBCs in vivo is approximately 2.6×10^{13}. The main function of RBCs in vivo is to deliver oxygen to various organs and tissue. However, while hemoglobin is probably the most well known constituent, the RBC is not without important proteins, receptors, transporters, and other molecular machinery that contribute to its overall metabolic status.

Throughout its lifespan in vivo, the typical RBC will make numerous trips through the vasculature, becoming re-oxygenated in the lungs prior to being pumped back through the circulation where it traverses through numerous vessel types, delivering oxygen to various sites. During this cycle, the RBC will flow through arteries (often classified as those vessels having diameters greater than 100 μm), arterioles (20–100 μm), and capillaries (below 20 μm). While RBCs travel through the capillaries, the

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hematocrit may drop significantly (fewer RBCs will pass through the vessels) and, quite often, the RBC itself may have to make some dramatic physical changes to pass through certain vessels and/or turns. Fortunately, a healthy RBC membrane is somewhat deformable and able to pass through such highly resistive vessels. In addition to its deformability properties, the RBC is thought to possess other means to facilitate its passing through resistance vessels, as discussed below.

THE RBC AS A PARTICIPANT IN THE CONTROL OF VASCULAR CALIBER THROUGH ATP RELEASE

It is well established that the endothelium-derived relaxing factor, nitric oxide (NO), is produced by endothelial cells and, when released, is capable of relaxing vascular smooth muscle [3–5]. Physiologically, an alteration in shear stress to which the endothelial lining of blood vessels is subjected has been suggested to be a major stimulus for NO release [4–7]. However, it has also been reported that, in the rabbit pulmonary circulation, and in the absence of RBCs, alterations in shear stress *alone* did not evoke release of NO in the pulmonary circulation [8]. In contrast, in lungs perfused with rabbit blood, NO is a determinant of vascular resistance, suggesting that the RBC is a determinant of NO production. Importantly, the component of blood responsible for the stimulation of endogenous NO synthesis was determined to be the RBC. In the absence of RBCs, the addition of dextran (a polysaccharide) to the perfusate of isolated lungs to increase viscosity and perfusion pressure to that of blood-perfused lungs did not stimulate NO synthesis. Taken collectively, these studies demonstrate that a property of the RBC not related to effects on viscosity or pressure contributes to endogenous NO synthesis.

It was reported that the viscosity- and pressure-independent effect of the RBC to stimulate endogenous NO synthesis in the pulmonary circulation occurred via the release of adenosine triphosphate (ATP) from the RBC [9]. Indeed, the application of ATP to endothelial cells results in increases in NO synthesis [10,11]. ATP is of particular interest because it is present in millimolar amounts in RBCs [8–10,12].

Multiple receptors for ATP have been identified and partially characterized [13–17]. In the vasculature, the P2X purinergic receptor is present primarily on vascular smooth muscle cells and its activation results in contraction of that cell [15,18,19]. Thus, ATP applied directly to the vascular smooth muscle of an intact vessel, e.g., that are released from nerve terminals, would be expected to produce vasoconstriction via activation of P2X receptors. In contrast, the P2Y receptor is found primarily on the endothelium [13,14,16,18,19]. The binding of ATP to the endothelial P2Y receptor results in the synthesis of NO and/or vasodilator arachidonic acid metabolites [20,21]. In contrast, ATP applied to the luminal side of a vessel, e.g., that released within the circulation from RBCs, would be expected to produce endothelium-dependent vasodilation through interaction with the P_{2y} receptor present on the endothelial cell and the subsequent release of NO [14,17,19].

If ATP is an important regulator of pulmonary NO synthesis, a hemodynamic response to ATP should be demonstrable in the intact circulation. Although previous studies have demonstrated that ATP administered into the lumen of the pulmonary blood vessels results in vasodilation and that NO may mediate this response, an endogenous source of ATP was not defined. Sprague et al. postulated that ATP released from the RBC was in response to mechanical deformation, which occurs with vasoconstriction or increased velocity of blood flow. This RBC-derived ATP acts on the endothelial cell to stimulate endogenous NO synthesis and enable the RBC to participate in local regulation of vascular caliber [8,9,22–27]. In support of this hypothesis, it was reported that ATP is released from RBCs in response to decreases in oxygen tension [22,25,26,28] acidosis [28], and, importantly, mechanical deformation [9,29,30]. These studies demonstrated that ATP is a determinant of endogenous NO synthesis solely in the presence of RBCs that release ATP in response to mechanical deformation (rabbits and humans) [8,9]. An overall schematic describing the potential role of the RBC in vasoregulation is shown in Fig. 1.

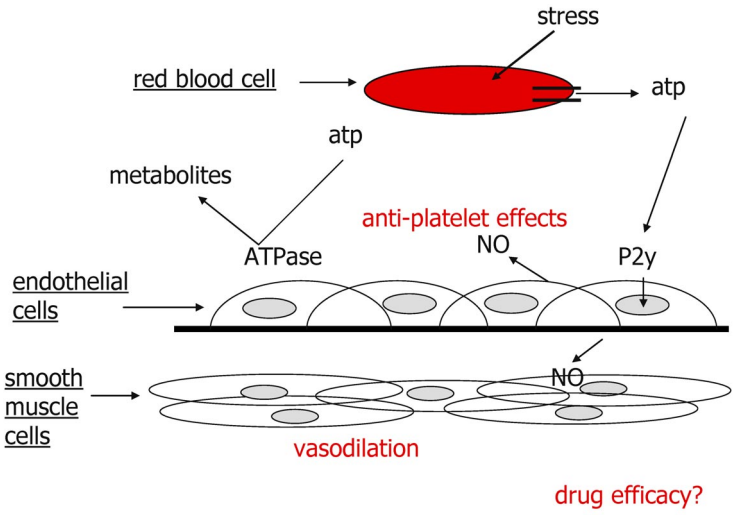

Fig. 1 Proposed involvement of the RBC in vascular regulation. ATP derived from the RBC is known to stimulate endothelium-derived NO and subsequent vessel relaxation [8]. Current efforts, some described here, are also attempting to provide evidence that the RBC may be a determinant in platelet function via ATP binding to platelet receptors [46]. Other results demonstrate that the RBC may be serving as a mediator of drug efficacy [44].

THE RBC AS A DETERMINANT OF PLATELET FUNCTION

Similar to RBCs, platelets are also non-nucleated, but do contain mitochondria. In addition, vesicles are also known to play a major role in platelet function. Platelets are smaller (2 μm) than RBCs and are known for a special function in the bloodstream; specifically, platelets are a key component in the blood-clotting process and vascular repair due to injury. Upon injury, platelets are exposed to sub-endothelial collagen, which is thought to activate the platelet, resulting in a shape change. The platelets become very adhesive and aggregate at the site of injury.

In addition to collagen, there are also other substances that are known to activate platelets such as fibrinogen, thrombin, adenosine diphosphate (ADP), and, to a lesser extent, ATP. Activation pathways exist for all of these substances and are fairly well understood. Unfortunately, the exact role of ATP in platelet function seems less clear. For example, the platelets have a receptor for ATP (the P2X receptor), and it is fairly well established that this receptor, upon binding to ATP, opens an ion channel for Ca^{2+} [31–34]. The influx of Ca^{2+} into the platelet is important due to its ability to bind calmodulin and participate in the production of NO, an inhibitor of platelet activation and adhesion. However, the P2X receptor is thought to become quickly desensitized to ATP and, as such, not to participate to a great extent in platelet activation or adhesion.

Clinically, there are interesting findings involving platelet activity, the RBC, and ATP. It is known that people who have such diseases as diabetes, cystic fibrosis (CF), and hypertension are all known to have hyperactive platelets [35–39]. Interestingly, all of these patient groups also reportedly have RBCs that release reduced levels of ATP (in comparison to healthy controls) in response to various stimuli [30,40–42]. These reports subsequently led our group to re-evaluate the role of ATP in platelet activity and, due to the ability of the RBC to release ATP, the role of this cell in platelet activity (summarized in Fig. 2). Our initial working hypothesis was that people with diabetes have hyperactive platelets due to a decrease in NO production, which may be the result of inadequate RBC-derived ATP.

There were "holes" in this hypothesis from the very beginning. First, as mentioned above, there are reports suggesting that the P2X receptor does not play a major role in platelet activation [43]. Secondly, there are also patient groups (e.g., people with sickle cell disease) who, during an RBC lysis event, may have higher than normal levels of ATP in the circulation (due to the release of the millimo-

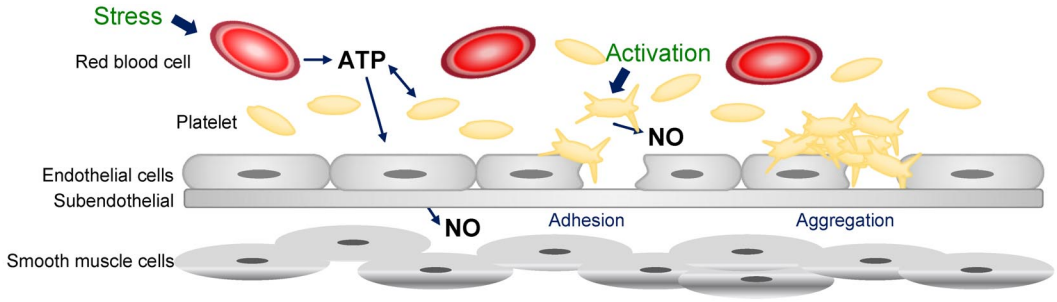

Fig. 2 Typically, the platelet flows through the bloodstream without significant adhesion to the vessel wall. Upon injury and exposure to subendothelial collagen, the platelets change shape and eventually aggregate to help overcome the vessel lesion. However, the involvement of RBC-derived ATP in helping to maintain non-activation events in the absence of lesions are not well understood. It is these types of cell-to-cell interactions that are currently under investigation by our laboratory.

lar levels of ATP in the RBC). Interestingly, people with sickle cell are also known to have hyperactive platelets. Thus, on the one hand, our hypothesis states that reduced ATP should result in platelet activation, yet the sickle cell scenario is in complete juxtaposition to this theory.

Regardless of the potential problems associated with the notion that RBC-derived ATP is a determinant in platelet function, a recent study by our group has demonstrated that when the platelet is deprived of exogenously added ATP, the platelet's tendency to aggregate is increased [44]. As ATP is added, the aggregation rates initially decrease only to begin increasing again as the levels of exogenous ATP continues increasing. Of course, these were in vitro studies that utilized only platelets and authentic ATP. To provide further evidence that ATP is a determinant in platelet activation, an improved model incorporating multiple cell types would be required.

To provide such a model, a microfluidic device was developed using soft lithographic procedures [45]. Microfluidic devices have proven very useful for biological analyses, especially those involving cells [46–50]. Cells can be patterned into the channels of these devices, and molecules that are either produced and/or secreted can be measured directly on the device. Our group and others have demonstrated that these devices can be used to investigate certain events that require flow such as ATP release from cells [51,52], platelet activation and adhesion [47,53], or matrix adhesion studies [54] that require flow. Recently, we demonstrated that multiple cell types can be investigated in a single device. For example, when these devices are patterned to contain channels that approximate vessels in vivo (e.g., 100 μm deep × 100 μm wide), endothelial cells can be immobilized in the channels. We have shown that when platelets are pumped over the immobilized endothelium cell layer, they adhere and can be quantitatively counted [47]. Interestingly, when platelets are pumped over the endothelium in the presence of RBCs, the number of platelets adhering to the endothelium decreases [44]. However, when the RBC's ability to release ATP is inhibited, the number of platelets adhering increased. A possible explanation for these results is that the ATP released from the RBCs is stimulating NO in both the platelets and endothelium (see Fig. 2). Freedman reported that NO produced by the platelets reduces platelet aggregation to other platelets, while NO produced by the endothelium prevents platelets adhering to the endothelium [55].

While the above results are interesting, they still do not explain how ATP can be interacting with the P2X receptor and stimulating Ca^{2+} influx into the platelet. A reflex-type answer would be suggesting that the ATP is degrading to ADP and, in fact, it is the ADP affecting the platelet activity. In fact, most people investigating ATP interactions with platelets utilize α-β-methyelene ATP [56], a form of ATP that is highly stable and does not degrade to ADP, even in the presence of apyrases. When using this stable form of ATP, there are very minimal biological effects on the platelet; for example, both ag-

gregation and Ca^{2+} influx into the platelet are minimally affected. Yet unpublished results from our laboratory suggest that the reason for the inactivity of the α-β-methyelene ATP is that, upon binding to the P2X receptor, it is not dephosphorylated to ADP and released. In this scenario, the P2X receptor would not be ready to accept another ATP molecule. This would explain the rapid desensitization and minimal effect on platelet function. Summarily, we believe that the P2X receptor is in fact, an apyrase that binds to ATP (allowing some Ca^{2+} into the platelet for NO production), but then breaks down that ATP to ADP where the ADP then further participates in platelet activation. If this proposed mechanism, shown in Fig. 3, is ultimately proven correct, it would have tremendous implications on drug design.

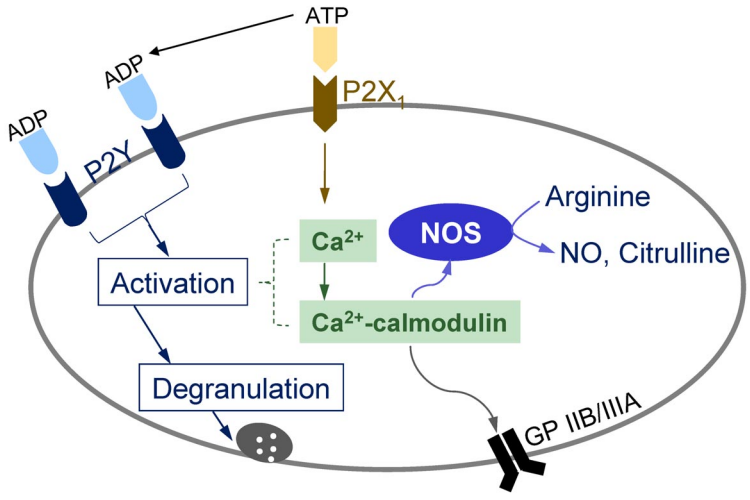

Fig. 3 A possible role for the P2X receptor in platelet function. Typically, when P2X is activated, Ca^{2+} is allowed to flow into the platelet where it is known to result in the stimulation of NO production via Ca^{2+}-calmodulin binding to eNOS. At present, most studies suggest that such an event has a limited role in platelet function due to the rapid desensitization of the P2X receptor. In contrast to current paradigms, it may be that the P2X receptor is actually an apyrase and, upon ATP binding to P2X, ADP is formed which continues to participate in the platelet aggregation process. In this construct, too little ATP would result in a reduction of platelet-inhibiting NO while an overabundance of ATP (as would occur in hemolysis-prone individuals) would saturate the local environment with both ATP and ADP, resulting in subsequent activation and aggregation of the platelets.

THE RBC AS A DRUG TARGET

If the RBC has the ability to stimulate NO production in certain cell types (e.g., endothelial cells and platelets), then manipulation of ATP release would seem to be a logical endeavor. For example, as mentioned above, ADP is a major determinant in platelet activation. If a certain, minimal level of ATP is required for NO production and platelet inhibition through the P2X receptor, a person with diabetes is already at a disadvantage because they release abnormally low levels of ATP. Assuming ATP is broken down by the P2X receptor to ADP and that ADP also participates in NO production via its stimulation of vesicular Ca^{2+} release, then blockage of the ADP receptor would worsen the already dangerous condition of a person with diabetes. Such an assumption would explain why clopidogrel, an anti-platelet drug that works by blocking the P2Y receptor, is often not prescribed to people with diabetes. While these patients often have hyperactive platelets, this hyperactivity may not be the result of overactivation of the P2Y receptor. Rather, it may be due to insufficient production of NO by the platelet and by blocking P2Y, whatever NO production is present will be reduced even further.

In the case of platelet hyperactivity and people with diabetes (or other RBCs that release reduced levels of ATP, such as people with CF), a more logical approach to reducing platelet activity would be to increase the release of RBC-derived ATP. Of course, the question then arises as to how to increase the release of ATP from the RBC. There are multiple stimuli for release of ATP from the RBC, including deformation of the cell, exposure of the cell to hypoxia, and incubation with various pharmacological agents.

Among the pharmacological agents that will increase ATP release from the RBCs are iloprost (a stable form of prostacyclin) [46] and trental (also known as pentoxyfilline, see results in Fig. 4). Interestingly, both of these drugs (which are not necessarily marketed as platelet inhibitors) have been shown to reduce platelet aggregation. We have also shown with the multicellular microfluidic device discussed above, that iloprost has the ability to stimulate endothelial NO production, but only in the presence of both RBCs and endothelial cells [46]. When the ATP release was inhibited in this same system, the iloprost had no effect on the NO production, suggesting that the NO production was mediated through the RBC.

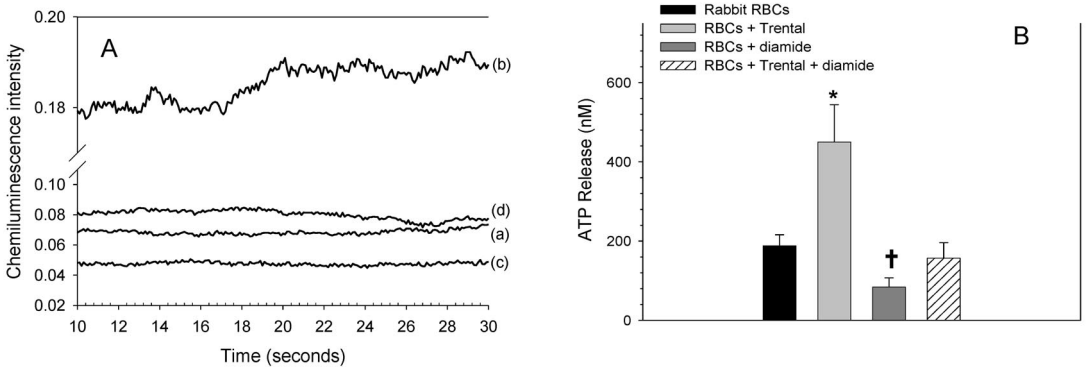

Fig. 4 Improved release of deformation-induced release of ATP from RBCs incubated in trental (pentoxyfilline). In A, representive chemiluminescence intensities of ATP release from (a) RBCs, (b) RBCs incubated with trental, (c) RBCs with diamide (a known cell-stiffening agent), and (d) RBCs with diamide and trental. The RBCs were prepared as a 7 % hematocrit prior to incubation with reagents and subsequent deformation in the microflow system comprised of microbore tubing having an inside diameter that approximates resistance vessels in vivo (50 μm). In B, the bars represent the average of normalized results with error bars representing the standard error of the mean. The asterisk (*) indicates a statistical difference between RBCs (n = 10) and RBCs incubated in trental (n = 10, $p < 0.01$). The dagger (†) indicates statistical difference between RBCs in diamide (n = 9) and RBCs alone ($p < 0.025$). There was no difference between the RBCs and RBCs with both trental and diamide (n = 4). These data suggest that trental is possibly softening the RBC membrane, thus resulting in the opportunity for increased levels of flow-induced ATP release. Statistical testing was performed using Student's t-test.

In the case of people with type 1 diabetes (who often suffer from complications associated with blood flow) the answer to these complications associated with blood flow (hypertension, hyperactive platelets) may be as simple as adding a substance to the patient's insulin that interacts with the RBC. Insulin is produced in the islets of Langerhans as part of a molecule known as proinsulin. C-peptide is a 31 amino acid peptide released from proinsulin during insulin production and, when released, is in equimolar amounts to insulin [57]. Since its discovery, C-peptide has been thought to play no significant physiological role in vivo. In fact, the function of C-peptide was seen as solely facilitating the formation of the disulfide bonds of the A- and B-chain of the proinsulin molecule. However, for the past decade the literature now suggests a functional, biological role for C-peptide. For example, in type I diabetes it has been established that C-peptide increases renal function [58–63] and increases the mi-

crovascular blood flow in the skin [64]. Other reports have demonstrated the ability of C-peptide to reduce diabetic complications associated with neuropathy [58,65–67]. Finally, it has been reported that human proinsulin C-peptide increases RBC deformability in patients with type I diabetes [68].

Inspired by previous reports linking C-peptide to blood flow, in addition to recent work from our laboratory describing the inability of RBCs obtained from people with type 2 diabetes to release normal amounts of ATP [40], we have examined the ability of the C-peptide to stimulate ATP release from the RBCs. When RBCs were incubated with C-peptide at physiological concentrations (1–10 nM), the RBC's ability to release ATP more than doubled over an 8 h incubation period. However, the activity of the C-peptide on the RBCs, measured by the amount of ATP released from the RBCs, was observed to deteriorate after about 24 h. We subsequently determined, by using electrospray ionization-mass spectrometry (ESI-MS) analysis, that the C-peptide was active only when bound to certain metals, such as zinc [69,70]. Since these studies, our group has found consistent success with C-peptide as a biologically active agent when a metal is added to the peptide in a controlled manner (i.e., the C-peptide is purified free of metals, characterized, and then a metal such as Zn^{2+} is added in known stoichiometric amounts). However, in the absence of Zn^{2+}, there is no activity. While studies are ongoing in our laboratory, we believe that improved and consistent results will be seen by other groups when such controlled solution preparation steps are implemented in their experimental protocols.

Once it was determined that metal activation of the C-peptide was required in order to maintain increased ATP release from RBCs, a more remarkable discovery was made. Specifically, metal-activated C-peptide has the ability to facilitate glucose transport into the RBC [69,70]. This finding is particularly important given that it is known that nearly 70–80 % of glucose clearance from the bloodstream during fasting times (i.e., between meals) is performed by non-insulin-dependent mechanisms that are not fully understood.

The ability of C-peptide, when metal-activated, to stimulate glucose uptake may also be of interest to patients with CF, since diabetes is now reported to be the most common comorbid disease in CF patients [71]. Diabetes affects about 20.8 million people in the United States, and it is estimated that by the year 2050, approximately 48.3 million people in the United States will have diabetes [72]. Diabetes is generally classified into three separate categories; type 1 diabetes (also known as juvenile diabetes), type 2 diabetes (also known as adult onset diabetes), and gestational diabetes. The latter form occurs during pregnancy and is often reversible at the end of the pregnancy. Most of the diagnosed cases of diabetes are of the type 2 form, and are now beginning to affect children as well as older adults. Though a complete understanding of the etiology of type 2 diabetes is lacking, it is often associated with phenomena such as hyperglycemia (indicated by an increased concentration of glucose in the bloodstream) and insulin resistance. The concept of insulin resistance is that while the beta cells of the islets of Langerhans are producing insulin, this insulin does not optimally perform its major function of facilitating glucose transport into such cells as muscle cells, fat cells (adipocytes), and endothelial cells.

In a recent study, it was reported that 64 % of patients with CF had fasting hyperglycemia [71]. Moreover, CF-related diabetes (CFRD) is unique in that it has features that are common to both type 1 and type 2 diabetes. For example, it is similar to type 1 diabetes in that ketoacidosis is rare and neuropathy is a common complication. However, it is similar to type 2 diabetes in that insulin is present and occurrence increases with patient age. Finally, insulin resistance is apparent.

Recent findings in our laboratory demonstrate that C-peptide-stimulated glucose transport into the RBC, and subsequent ATP release from these cells, is interesting when considering work performed independently by two other groups. Specifically, Sprague has shown that the RBCs obtained from people with CF fail to release ATP upon stimulation [30]. To date, it is well established that the CF transmembrane conductance regulator (CFTR) protein activity is a requirement for ATP release from the RBC to occur. Collectively, results from Sprague and our group suggest that, due to the inability of the CF RBC to release ATP, the intracellular concentrations of ATP may become slightly elevated and, subsequently, to maintain energy balance, the GLUT1 transporter does not allow glucose into the RBC. The reduction in glucose transport into the RBC may lead to higher levels of glucose in the bloodstream.

Indirect evidence that supports higher glucose levels in the RBC can be seen in those with CFRB, who often have HA1C values (glycated hemoglobin) that are normal [73]. Such a phenomenon could be interpreted as elevated glucose levels in the bloodstream, which is a result of reduced glucose uptake into the RBCs.

THE RBC AS A DRUG DIAGNOSTIC

A common theme to many of the potential uses of the RBC as a drug target is the abnormal release of ATP when these cells are exposed to various stimuli. Therefore, in addition to the RBC serving as a possible drug target, it may also have potential as a drug diagnostic. Of course, in some cases, the abnormal ATP release would probably not help in "diagnosing" a particular condition; for example, it is highly likely that a diagnosis of CF would not be performed by measuring a cell's ability to release ATP. However, in some select cases, the reduction in ATP release from the cell may precede disease onset.

As mentioned above, our group and others have shown that RBCs purified from the whole blood of people with diabetes release significantly less ATP in comparison to healthy controls [40,69,70,74]. Importantly, our group has taken RBCs from healthy rabbits and incubated them in buffers that contain hyperglycemic levels (10–25 mM) of glucose and found that the ATP release from these cells begins to decrease quite rapidly (hours). In this construct, the release of ATP may serve as another preliminary indicator to monitor in a person who may develop, or already have, diabetes.

In addition to ATP release, our group has also recently reported that C-peptide does not seem to interact as well with RBCs obtained from type 2 diabetic rats in comparison to controls [70]. Thus, it would stand to reason that more of the C-peptide may be left behind in the plasma. If so, this plasma C-peptide could then be used as a marker for potential onset of diabetes. Currently, these types of studies are being undertaken in our laboratories with promising results. Specifically, when exogenous C-peptide is added to solutions of RBCs that have incubated in buffers containing glucose levels ranging from 5 mM (healthy) to 20 mM (severe diabetic), the amount of C-peptide remaining in the plasma (indicative of not interacting with the RBC) begins to increase even at glucose levels of 7 or 8 mM. These types of results suggest that the RBC may actually serve as a rather sensitive drug diagnostic that can be measured within hours of the onset of diabetes conditions.

The RBC also has unique characteristics in other diseases where one would not think of the RBC as a factor. Recently, our group has performed measurements on the RBCs obtained from people with multiple sclerosis (MS) and found that the ATP release from these cells is almost three times higher than the release from RBCs of controls. This type of data alone could serve as a diagnostic tool in MS. However, our group is even more interested in the potential role of this RBC-derived ATP overstimulating NO production in the endothelium and potentially resulting in damage to the blood–brain barrier (Fig. 5). It has been reported by more than one group that high levels of NO exist in people with MS. Interestingly, there is other (circumstantial) evidence that makes this ATP release worthy of continued study. First, in studies by Feinstein's group, it was reported that murine models of MS did not have significant myelin damage when their P2X receptors were knocked out [75]. The blood–brain barrier damage was evident, but not the myelin damage. Studies are currently underway in our group to determine if the ATP release from the RBC is able to stimulate the endothelium NO (which is a P2Y-receptor-mediated activation), break down the barrier, but not damage the oligodendricytes (which have numerous P2X receptors to bind ATP) that are key to myelin sheath maintenance.

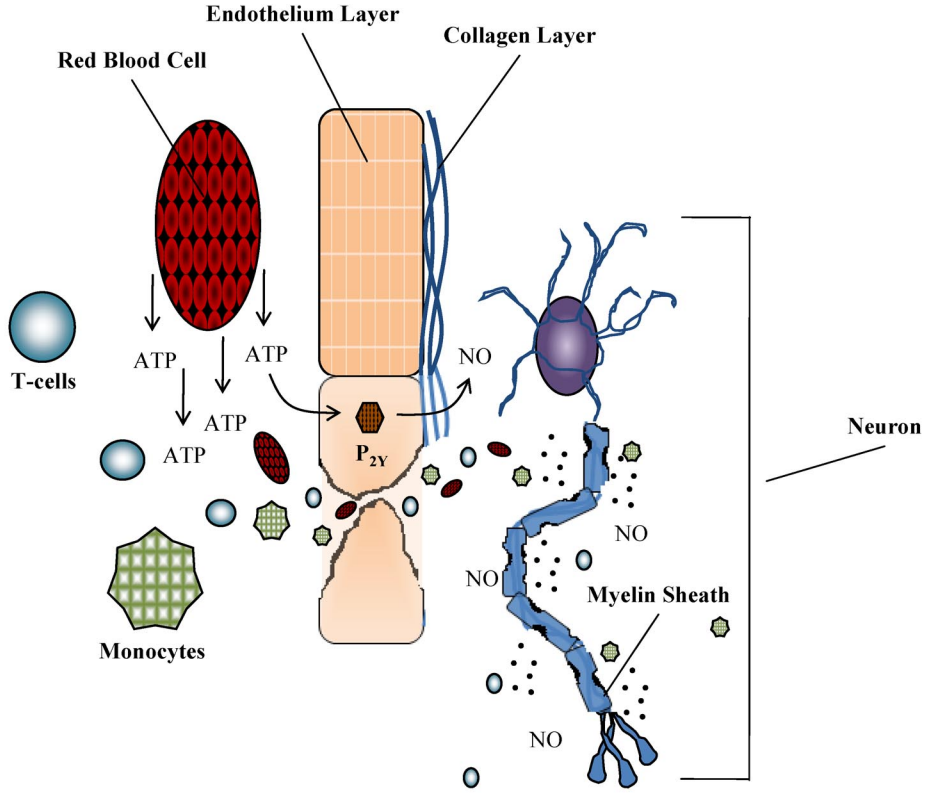

Fig. 5 Proposed role of the RBC and its release of ATP in the breakdown of the blood–brain barrier and subsequent damage to the myelin sheath. Abnormally high levels of ATP released from the RBCs of people with MS overstimulate endothelial cell-derived NO, resulting in the breakdown of the blood–brain barrier.

CONCLUDING THOUGHTS

Admittedly, there is a great deal of work to be performed if the RBC is going to be accepted as more than just a mechanism for the delivery of oxygen in vivo. However, there seems to be a great deal of evidence being accumulated, suggesting that the RBC may be a determinant in various diseases. If these various roles are validated, accompanying them will be a necessity to rethink the RBC's physiological duties and, in turn, reevaluate the pharmaceuticals that are employed to attenuate disease complications and/or progression.

Successfully validating these roles, however, will require novel biotechnological tools. For example, if the RBC does indeed affect other cell types in the circulation via its ability to release ATP, tools will be needed that enable real-time determinations of these interactions because many of the molecules participating in the communication (e.g., ATP and NO) are readily degraded in the bloodstream. Consider a study where RBCs are stimulated to release ATP followed by centrifugation of the cells, removal of supernatant containing ATP, addition of that supernatant to endothelial cells or platelets, and then monitoring of those cells for NO production. The 10–20 min required for all of these steps would not provide a very good indicator of the true communication between the cell types involved because (1) some of the ATP may have degraded during that time and (2) because the cells are not in the same device, any feedback mechanisms will not be available for monitoring.

Therefore, in the opinion of this reviewer, chemists must continue to develop molecular-based probes for monitoring these analytes of interest that are playing the role of communicators between dif-

ferent cell types. Moreover, chemists must also continue to consider techniques that will mimic in vivo conditions using controlled, in vitro platforms.

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