The Red Blood Cell and Nitric Oxide: Derived, Stimulated, or Both?

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Abstract: Following a brief introduction to properties of the red blood cell (RBC), this review will provide information pertaining to the role of the RBC as both a deliverer of nitric oxide (NO), and a stimulator of NO release *via* RBC-generated ATP. In addition, an evaluation of the potential downstream effects of RBC-derived ATP, *via* its ability to stimulate NO in other cell types, will be provided. Collectively, it is now apparent that the RBC is a determinant in functions other than oxygen delivery alone.

Keywords: Nitric oxide, adenosine triphosphate, red blood cell, endothelial cells, platelets, vasodilation, hypoxia, transendothelium electrical resistance (TEER), microfluidic technology.

1. INTRODUCTION

The average human red blood cell (RBC) has a volume of approximately 87 femtoliters, is about 6-7 μm in diameter, and spends, on average 110-120 days in the human body before being metabolized by such organs as the spleen. RBCs also represent about 40-45% of the total blood volume in an adult human (5 L); thus, the average adult has about 2 L of RBCs. The RBC also has many unique features in comparison to other cell types *in vivo* in that it lacks a nucleus and has no mitochondria. However, the RBC is not completely void of cell machinery; indeed, it has a very active cell bilayer, possesses a glucose transporter (GLUT1), has many of the same ion pumps as other cells, and is even thought to participate in the overall control of blood flow.

While the classical role of the RBC *in vivo* is to supply oxygen to demanding tissues and organs, it is now clear that the RBC itself participates in overall blood flow and, as such, increases the number of oxygen-carrying cells to the hypoxic tissue. Unfortunately, the mechanism by which the RBC facilitates blood flow is not unified, although the competing mechanisms that do exist have commonalities. In this review, the RBC's role as a participant in vasoregulation will be examined from two points of view; first, as a direct deliverer of NO and a stimulator of NO-release *via* RBC-generated ATP.

2. NITRIC OXIDE AS A VASOACTIVE MOLECULE AND DELIVERY BY THE RBC

Since the recognition of an endothelium derived relaxation factor (EDRF) [1] and subsequent identification as NO [2, 3], and the Nobel Prize in Physiology or Medicine in 1998, there has been some debate as to the physiological origin of vasoactive NO under hypoxic conditions. There are three general sources considered, two of which originate in the RBC: that as *S*-nitrosothiols from hemoglobin, and NO produced through nitrite reductase activity of hemoglobin. A further described potential mechanism is that of ATP induced NO production in the endothelium, through eNOS. These mechanisms are described pictorially in Fig. (**1**).

S-Nitrosothiols as Vasoactive NO

Stamler *et al* report that NO is carried in a stable form as a *S*-nitrosothiol at a highly conserved cysteine residue on the beta strand of hemoglobin [4, 5]. The vasoactivity of this NO species is then maintained and protected from the surrounding chemical environment in a more stable form. This is an important consideration as in the RBC and whole blood, there are many chemical species including heme, oxygen and small molecule thiols that can react with NO, eliminating its vasoactivity. While it has been shown the half-life of NO may be dependent on oxygen saturation [6], it is often estimated to be less than a few seconds [7, 8]; therefore a theory that presents a more stable form of NO, which would be more likely to reach the smooth muscle, is favorable. Furthermore, the formation of *S*-nitrosothiols is favorable at high oxygen saturations, and the release more favorable as the hemoglobin changes configuration at low oxygen saturations, offers a mechanism for release under hypoxic conditions [4, 9].

Nitrite Reductase Activity of Hemoglobin

Alternatively, other work provides evidence that the bioavaliable source of NO is through the nitrite reductase activity of hemoglobin, first investigated in the early 80's [10]. Much like the nitrosothiols mentioned above, nitrite is also proposed to be a stable form of storage for NO, and is present in the plasma at concentrations between 500 and 1000 nM [11]. A significant piece of supporting information for this hypothesis is an observable concentration gradient for nitrite between arterial and venous blood [11, 12]. While leaving questions to the specific mechanisms, this decrease in concentration as the blood is deoxygenated indicates the nitrite is being consumed, perhaps in the formation of NO [13]. Furthermore, in this study, an increase in blood flow is observed with the infusion of nitrite *in vivo*. While the authors acknowledge the challenges in NO escaping reaction with hemoglobin, it is known that the nitrite reductase

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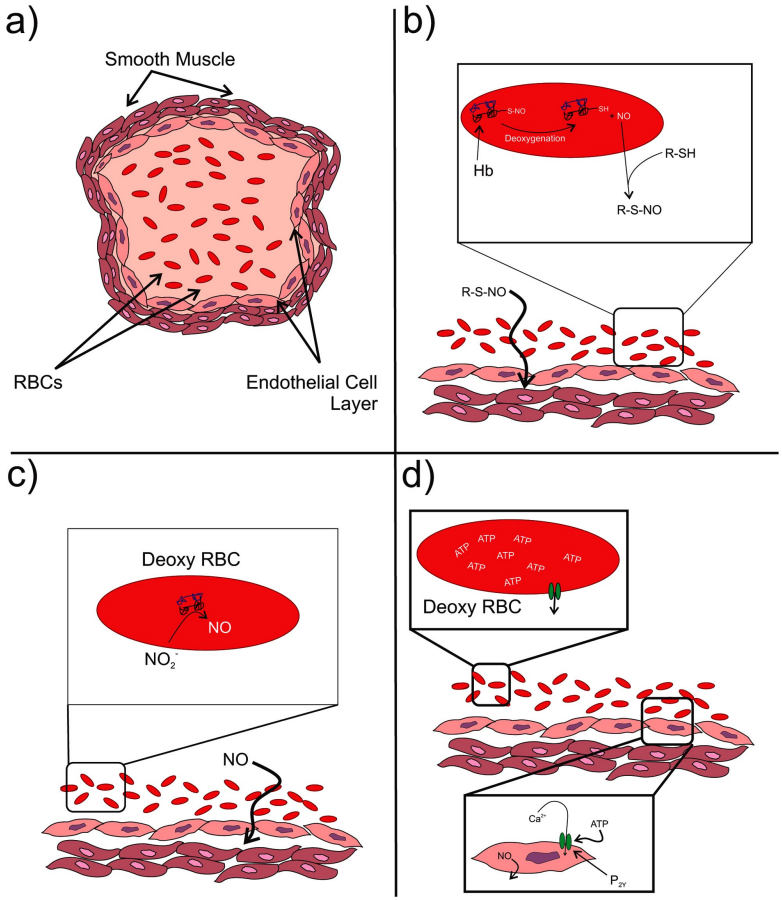

Fig. (1). Summary of Proposed Mechanisms for RBC Mediated Vasodilation: Shown in panel (a) is a typical blood vessel cross section. The center of this cross section contains the RBCs, plasma, and other blood components. The inner layer of the vessel wall consists of the endothelial cells, which are surrounded by the smooth muscle. The remaining panels are cross sections of (a). Shown in (b) is a pictorial representation of the SNO-Hb mechanism in which cysteine bound NO is released when Hb changes to the deoxygenated configuration. NO is then able to diffuse out of the cell, where it reacts with a free thiol species, such as glutathione or albumin. This species then carries it to the vessel wall where it must diffuse to the smooth muscle to induce vasodilation. Panel (c) summarizes the mechanism considering Hb as a nitrite reductase. In this case, nitrite diffuses into the RBC where deoxygenated Hb can act as a nitrite reductase, producing NO. This NO can then diffuse through the endothelium, to the smooth muscle, inducing vasodilation. Finally, in panel (d), the ATP mediated mechanism is summarized. ATP is actively released under hypoxic conditions, and then diffuses to the endothelium where it interacts with the P2Y receptor, inducing calcium flux into the cell. This activates eNOS, producing NO, which can then diffuse out of the cell and to the smooth muscle.

activity of hemoglobin is only seen in the deoxygenated state [10], offering a control mechanism for NO production.

3. STIMULATION OF NITRIC OXIDE BY THE RELEASE OF ATP FROM THE RBC

ATP and the Purinergic Receptors

Indeed, the application of ATP to endothelial cells results in increases in NO synthesis [14, 15]. ATP is of particular interest because it is present in millimolar amounts in RBCs [16-19]. Multiple receptors for ATP have been identified and partially characterized [14, 20-23]. In the vasculature, the P2x purinergic receptor is present primarily on vascular smooth muscle cells and its activation results in contraction of that cell [21, 24, 25]. In contrast, the P2y receptor is found primarily on the endothelium [14, 20, 22, 24]. The binding of ATP to the endothelial P2y receptor results in the synthesis of NO [14, 16] and/or vasodilator arachidonic acid metabolites [26, 27]. 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, 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 P2y receptor present on the endothelial cell and the subsequent release of NO [14, 25].

ATP Stimulation of NO in the Endothelium

Our group [28], along with others, have shown that when exposed to a hypoxic environment, RBCs release as high as 2 μM ATP, and that this ATP is able to activate eNOS [16, 29, 30]. Furthermore, upon reaching the endothelium, it is known this ATP can activate eNOS though P2y receptors

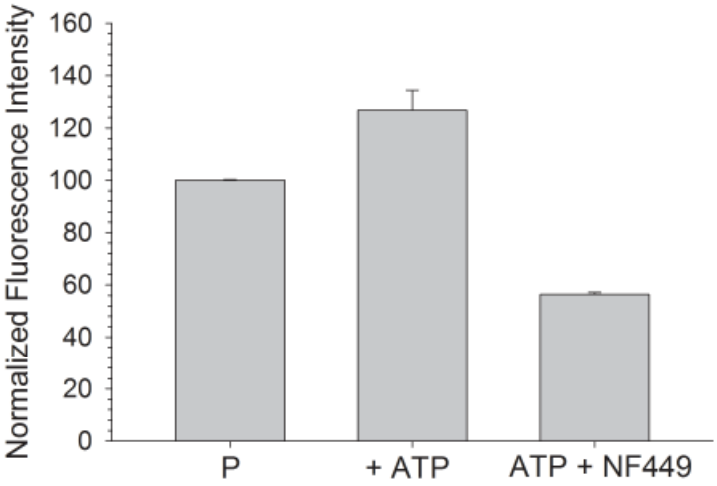

Fig. (2). Platelet NO production after the addition of ATP. The basal NO levels in the platelet were measured with a fluorescence probe (a) and compared to the fluorescence for another aliquot of the platelets that were stimulated with ATP (b). Finally, inhibition of the P2X receptor with NF449 (c) suggests that the ATP receptor on the platelet is active and participates in the ability of the platelet to produce NO.

[14, 31, 32]. This mechanism, like the above mentioned, utilizes the RBC as the mediator of vascular tone, and benefits from ATP being stable in the bloodstream over the relevant time scales. Furthermore, the vasoactive form of NO is formed closer to the muscle, in the endothelium as opposed to in the RBC, so there is less diffusional travel required for the unstable molecule, and it only needs to diffuse through a single cell membrane (the endothelial cell), as opposed to through the RBC, through the endothelial layer, and to the smooth muscle.

ATP Stimulation of NO Production in the Platelet

The primary function of platelets in the bloodstream involves hemostasis and the prevention of blood loss *via* clotting. Platelets normally circulate in the bloodstream without adhering to the endothelial cells lining the vessel walls however, when injury occurs and subendothelial collagen is exposed, platelets are activated. This activation is characterized by platelet shape change, which allows platelets to adhere to the vessel walls as well as recruit other circulating platelets to the growing thrombus [33, 34]. However, several other endogenous agonists also exist such as thrombin, ATP, ADP, thromboxane A_2 (TXA_2), serotonin and epinephrine, which also promote platelet activation [35]. Additionally NO, a platelet inhibitor has been widely shown to also mediate this process by activating soluble guanylate cyclase (GC) which initiates a protein kinase G (PKG) dependant pathway [36]. Bloodstream NO is not only produced by endothelial cells, but also by the platelets themselves [37].

Adenine receptors found on the platelet are major determinants in platelet function and include the $P2Y_1$ and $P2Y_{12}$ (both ADP receptors) as well as $P2X_1$ (ATP receptor) [38, 39]. Both types of receptors are thought to participate in platelet aggregation, but the evidence for role of $P2X_1$ in aggregation is not as clear. There are reports that show $P2X_1$ does not function in platelet aggregation[40].

Static measurements of Ca^{2+} influx [41] have shown that ATP can stimulate Ca^{2+} influx into the platelet *via* the $P2X_1$ receptor. These increased levels of Ca^{2+} can elevate the concentrations of bioavailable Ca^{2+}-calmodulin, thereby stimulating nitric oxide synthase and NO production. Fig. (**2**) shows NO production in platelets upon addition of ATP. Based on these reports, it would seem apparent that ATP plays a role in platelet NO production. Platelet aggregation studies have also been performed and showed increased platelet aggregation at low concentrations of ATP as well as high concentrations of ATP. It is possible that at low levels of ATP there is insufficient NO production to inhibit platelet activation, but at higher levels of ATP NOS may become saturated and the Ca^{2+}-calmodulin complex can activate the GPIIb-IIIa complex (membrane integrin involved in platelet activation/aggregation) on the platelet membrane [42]. Both of these situations would be dependent on the amount of ATP being released by the RBC.

4. DOES NITRIC OXIDE AFFECT THE ABILITY OF THE RBC TO RELEASE ATP?

The evidence in the literature strongly suggests that the RBC has the ability to release ATP in response to such stimuli as flow-induced mechanical deformation [43], hypoxia [28, 29], acidosis [16], as well as various molecular reagents [44-46] whose exact mechanisms are currently incomplete. Moreover, the RBCs ability to release NO in response to hypoxia is established [47]. Interestingly, in addition to these reports describing the release of these two molecules (ATP and NO) from the RBC, there are also studies suggesting that levels of NO or NO metabolites in the RBC may directly affect this cell's ability to release ATP.

There have been reports that NO added to RBCs can increase the cell's deformability [48, 50]. In this construct, one would then expect a resultant increase in ATP release from these cells upon exposure to deformation. In contrast, however, there are also reports [51, 52] suggesting that the addition of NO to the RBC can increase rigidity of the cell; in turn, such a decrease in deformability would be expected to reduce RBC-derived ATP upon exposure to deformation. In fact, it has been shown that the direct addition of NO to

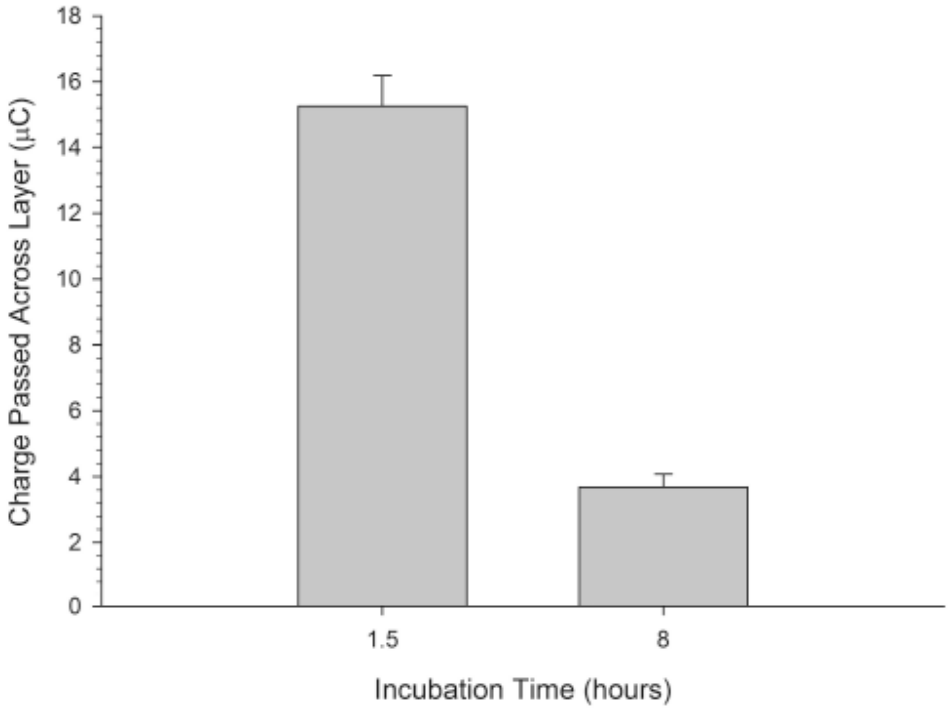

Fig. (3). Determination of Confluency of bPAEC in Microfluidic Devices. In order to determine the confluency of bPAECs in microfluidic devices, a square potential wave is applied across the cell layer, and the resulting current wave is integrated to determine charge passed across the layer. A lower charge results from a lower current, and therefore a more resistive and more confluent layer. These results suggest that proper incubation time of cell layers in devices is important.

the RBC can reduce this cell's ability to release ATP. Based on these reports, it would seem that there is inconclusive evidence regarding the effect of NO on ATP release from the RBC. However, a close reappraisal of these studies will reveal an interesting feature; specifically, it appears that the effect of NO on the RBC is concentration dependent.

Recently, it has been shown that either the addition of NO_2^- to RBCs results in either a decrease or increase in the ATP release from these cells [53]. It has been suggested that the addition of the nitrite to the RBCs results in the production of intracellular ATP due to an increase of activity in a membrane-bound glycolytic complex [54]. In addition to nitrite, it has also been shown that the direct addition of NO to RBCs resulted in an increase in ATP release when the cells were exposed to flow-induced deformation [55]. However, at higher levels of NO added to the RBCs the ATP release began to decrease. These studies suggest that the addition of NO or NO metabolites such as nitrite can indeed have an effect on ATP release from the RBC and, importantly, the effect seems to be dose dependent.

The above studies become important from a therapeutic point of view considering a recent study demonstrating that hydroxyurea (HU), the only proven therapy for people with sickle cell disease, seems to be stimulating eNOS in the RBC. The production of the NO by the RBC itself was concluded to be associated with the increased release of ATP from the RBCs after a brief incubation with the HU [55]. In accordance with this report, previous studies have linked some of HU's benefits as possibly due to NO [56, 57]; however, the results demonstrating that HU had a direct impact on NO production by the RBC itself and that this NO was subsequently facilitating ATP release from the RBC were without precedence.

5. ADVANCING BIOTECHNOLOGY FOR AN IMPROVED UNDERSTANDING OF THE ATP-NITRIC OXIDE RELATIONSHIP

To determine NO activities, it is important to consider the measurement technique. Being an unstable molecule, NO can be a challenge to measure in the presence of the complex matrices inherently present in these samples. Despite these challenges, electrochemical [58], fluorescence derivatization [32, 47], and chemiluminescence techniques are used in NO analysis. However, in many of the investigations involving the role of RBC-derived NO, a variety of bioassays are utilized to determine vasoactivity.

In initial investigations of EDRF [2], along with Stamler's studies above [59], immobilized sections of blood vessels attached to strain gauges are utilized to determine vascular tone. While this is a valuable experimental technique because it uses tissue in its proper geometry and function, it does not offer much in the way of selectivity in terms of identifying the specific identity of active species. However, some of this selectivity can be imparted through the use of enzymatic inhibitors, such as L-NAME or L-NMMA, or knockout animals [9]. Other studies performed by Gladwin's group utilize *in vivo* measurements of variables such as blood flow or blood pressure; however,

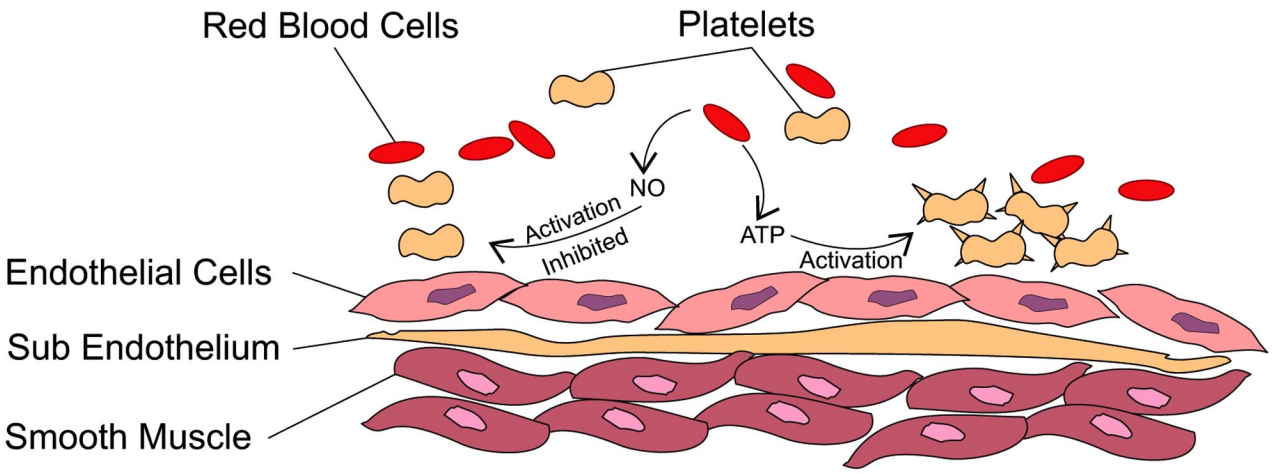

Fig. (4). Defining roles for the release of ATP and NO from the RBC. Under hypoxic conditions, the RBC is known to release both ATP and NO. While the ATP has been shown to participate in endothelial NO production, it may also have the ability to activate platelets (either through ATP or ADP activation of the platelet purinergic receptors); however, the NO that is released under hypoxic conditions would be able to inhibit the platelet activation that may occur due to the ATP release.

these methods lack the ability to specifically characterize the analytes of interest. However, these studies are still very informative due to the *in vivo* nature of the experiment.

To impart some selectivity to such investigations, our group utilizes microfluidic devices to simulate blood vessels. These devices offer the potential to couple most detection systems with functioning cells arranged in biologically relevant spatial locations. With these devices, we have been able to monitor RBC metabolism [60], monitor cell-cell communications including platelets, RBCs and endothelial cells [44, 61], and even electrochemically or through fluorescence derivatization [47, 58, 62], determine NO.

Furthermore, microfluidic systems provide improved control over the cells of interest, and offer an opportunity to monitor important variables that may affect overall results, such as endothelial layer confluence. While often considered critical for blood-brain-barrier investigations, cell confluence and the presence or absence of gaps between endothelial cells likely plays an important role in determining the levels of RBC derived NO capable of diffusing to the smooth muscle. Because the endothelial cells are cultured directly on the chip, and do not have to be removed or excised from an animal, cell layer integrity can be monitored.

Arguably, the most straightforward method for characterizing a vascular mimic is measuring transendothelial electrical resistance (TEER) across a barrier formed by a monolayer of endothelial cells. Since the cell layer restricts the movement of ions [63], TEER measurements have become the gold standard for monitoring endothelial cell monolayer integrity. The first reports of monitoring TEER across a cultured monolayer of brain endothelial cells were in 1987 [64, 65]. Since then, performing TEER measurements has become a common and simple technique and the instrumentation is now commercially available. Most of the commercially-based methods for measuring TEER across endothelial cell cultures adhered to microporous filters, or culture inserts, employ Volt-Ohm resistance meters equipped with "chopstick" electrodes, or chamber electrodes. While these instruments are capable of performing repeated fast measurements of various cell cultures with low TEER background, the same inadequacies in properly mimicking the true microvasculature that are prominent in diffusion cells also arise in these conventional TEER systems, such as a lack of shear stress and RBCs

Healthy endothelial cells form a fully confluent layer *in vivo*, which is also crucial to proper behavior. When an endothelial cell monolayer is prepared, it is generally characterized in one of two ways [66]: either by measuring TEER values or through the determination of permeability coefficients across the layer. As mentioned previously, these studies are generally performed using diffusion cell technology. Since the variability of models focusing on *in vitro* expression of the vasculature is so great, TEER measurements, or determination of permeability coefficients, should be, and often are prerequisite for publication. This stipulation should include vascular mimics involving microfluidics, which are becoming more widespread due to their ability to correct important shortcomings of the diffusion cell. Nevertheless, there are only two reports involving measuring barrier integrity across a monolayer of cells cultured on microfluidic devices [67, 68].

A more resistive, confluent monolayer of cells results in less total current observed as a result of decreased conductivity. Resultant data for TEER measurements performed across a monolayer of bPAECs in a microfluidic device is shown in Fig. (**3**); after 1 hour of growth, before confluence can be reached, and again after 8 hours after the cells have been allowed to become confluent. The use of microfluidic device technology for such measurements of cell layer integrity will increase the validity of cellular responses *in vitro* due to the ability to ensure endothelial cell monolayer cell confluence. Importantly, this *in vivo* mimic incorporates flow, thus providing another physical feature of the bloodstream that is not possible to investigate with current TEER-measurement systems.

6. FUTURE DIRECTIONS

Studies involving the potential role of the RBC as a participant in the regulation of blood flow are not new, although there has been a significant push since the early 1990's to explain the exact mechanism by which the RBC is a determinant. That said, there are still many questions involving the RBC, NO, and ATP that remain to be answered. For example, under hypoxic conditions, it is apparent that the RBC does release both ATP and NO. Do both of these molecules participate in smooth muscle relaxation? Or, is it possible that one of the molecules (ATP) is designed to stimulate NO in other tissue, such as the endothelium, while the NO derived directly from the RBC has another, very important function, namely, to inhibit platelet aggregation in the presence of such high levels of ATP being secreted by the RBC under hypoxic conditions? These possibilities are shown in Fig. (**4**).

Another area that will require further study is the clinical role of RBC-derived NO and ATP. Studies have shown that people with diabetes [69, 70], cystic fibrosis [43], and primary pulmonary hypertension all have RBCs that release less ATP in comparison to those RBCs harvested from the whole blood of controls. It is also known that these patient groups have hyperactive platelets, overall complications with blood flow, and, in the case of the CF patient, lower levels of NO bioavailability. The ability to modulate ATP release and NO bioavailability may prove beneficial to these groups. Studies have even been recently reported involving the importance of NO and the RBC in stored (banked) RBCs. In summary, while many strides have been made with respect to RBCs and NO, a complete understanding of their roles *in vivo* is lacking and continued efforts in this area are needed. Advances in molecular-level explanation of mechanisms and advances in biotechnological measurement schemes will facilitate this objective.

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CONFLICT OF INTERESTS

None.

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