Active modulation of human erythrocyte mechanics

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The classic view of the red blood cell (RBC) presents a biologically inert cell, that upon maturation, has limited capacity to alter its physical properties. This view developed largely due to the absence of translational machinery and inability to synthesise or repair proteins in circulating RBC. Recent developments have challenged this perspective, in light of observations supporting the importance of post-translational modifications, and greater understanding of ion movement in these cells, that each regulate a myriad of cellular properties. There is thus now sufficient evidence to induce a step-change in understanding of RBC; rather than passively responding to the surrounding environment, these cells have the capacity to actively regulate their physical properties and thus alter flow behaviour of blood. Specific evidence supports that the physical and rheological properties of RBC are subject to active modulation, primarily by the second-messenger molecules nitric oxide (NO) and calcium-ions (Ca\(^{2+}\)). Further, an isoform of nitric oxide synthase is expressed in RBC (RBC-NOS), which has been recently demonstrated to have an active role in regulating the physical properties of RBC. Mechanical stimulation of the cell membrane activates RBC-NOS leading to NO generation, which has several intracellular effects, including the S-nitrosylation of integral membrane components. Intracellular concentration of Ca\(^{2+}\) is increased upon mechanical stimulation via the recently identified mechano-sensitive cation channel, piezo1. Increased intracellular Ca\(^{2+}\) modifies the physical properties of RBC by regulating cell volume and potentially altering several important intracellular proteins. A synthesis of recent advances in understanding of molecular processes within RBC thus challenges the classic view of these cells, and rather indicates a highly active cell with self-regulated mechanical properties.
Role of cell mechanics in oxygen delivery

Cellular respiration is vital to sustained metabolism, and therefore life, and is only possible due to the integration of the cardiovascular, pulmonary, and circulatory systems. One of the challenges to oxygen loading, and subsequent transport, within the circulatory system is the extremely small apertures that blood needs to navigate: the smallest capillaries (2-3 µm) in the pulmonary and peripheral circulations are a fraction of the resting diameter (~8 µm) of red blood cells (RBC); consequently, the RBC is morphologically agile, with a unique ability among all mammalian cells to change shape and subsequently return to its resting state without any appreciable negative effects (e.g., hysteresis; 10). A pathologically reduced ability of RBC to deform has thus long been considered a precursor for heightened risk of tissue ischaemia at both micro- and macro-vascular scales, given rigid cells: i. have limited capability to perfuse the rich microvascular network of organs (47); and, ii. affect the bulk properties of blood through increasing the resistance to flow by contributing to hyperviscosity (4). Indeed, even the capacity for oxygen dissociation from haemoglobin during tissue perfusion has been reported to be limited by decreased cellular deformability (56). Further, many disease states associated with tissue necrosis, ischaemia, and/or hypoxia are characterised by poor cell mechanics of RBC (53). Thus, despite a vital role for optimal cellular mechanics being well established, the molecular regulation of these physical properties is only recently emerging.

Classic physical properties enable erythrocyte deformability
The properties of RBC that were classically recognised to confer their unique capacity to deform in response to mechanical stimulation included the excess surface area relative to cell volume, the intracellular viscosity, and a visco-elastic cellular membrane (10). The excessive surface area of RBC is clearly observed when the cell is compared with a perfect sphere: while the average volume of an RBC (~90 µm³) could be contained in a sphere with only 97 µm² of surface area, the surface area of an RBC is ~44% larger (~140 µm²) and thus yields a surface area-to-volume ratio that approaches 1.5 (10). The resultant excess surface area is provided by the bi-concave structure of the RBC membrane and facilitates significantly greater capacity to deform than a spherical shape. The intracellular viscosity of RBC is largely determined by haemoglobin concentration suspended in the cytosol. The mean intracellular viscosity of RBC is ~7 mPa·s at normal haemoglobin content, which is comparatively higher than the viscosity of the surrounding plasma (~1.2 mPa·s; 10). This disparity contributes to the deformation of RBC by facilitating an independent rotation of the cellular membrane around the cytoplasm, termed tank-treading (16). While the earliest interpretation of tank-treading indicated that the cell membrane was mostly independent of internal structures (16), advances over the last decades support that various entities complex with the intracellular surfaces of the cell membrane, which indeed may even serve to enhance the mechanical properties of the cell (36).

Further, the exceptionally deformable capacity of the RBC membrane has been long observed; this cell provides the template for lipid bilayer models of cell membranes and is noted for its exceptional capacity to deform upon exposure to shearing forces, subsequently reassuming its original bi-concave shape (4). The RBC membrane contains a cytoskeletal mesh-network with integral proteins establishing connections with the lipid bilayer, which stabilises the characteristic bi-concave shape of RBC. Collectively, the properties that permit a remarkable capacity of RBC to deform have been studied (Fig. 1), and until recently the
Recent advances challenge this perspective and support an ‘active’ regulation of mechanical properties that may acutely alter the physical properties of RBC.

The molecular structure of the erythrocyte membrane facilitates cellular deformability

The molecular composition of both the cytoskeletal and lipid bilayer components of RBC has been subject of extensive study during the past century (21, 25). The cytoskeleton consists of spectrin filaments (i.e., α- and β-subunits, which are antiparallel isomers), assembled in conjunction with filamentous actin to form hetero-tetramers in head-to-head manner (Fig. 2). These filaments are interconnected to the lipid bilayer: i. via complexation with integral membrane proteins band 3 and glycophorin; or ii. via actin and protein 4.1 (52). Smith et al., (49) recently demonstrated that the spectrin-actin cytoskeleton is stabilised by an isoform of non-muscular filamentous myosin (NMIIA). NMIIA is a hexameric actin-binding ATPase comprised of two heavy chains, two light chains, and two regulatory chains, and importantly is capable of contraction – which in other cell lines has been shown to be vitally important to cell function (46). Knowledge around the role that NMIIA exerts within RBC is still developing, although it has been recently demonstrated that via linkage to actin in the cytoskeletal component of the membrane, tension is regulated to maintain the cell’s unique bi-concave shape (49). It follows that the mechanical properties that are promoted by this unique shape are thus also integrally affected by NMIIA (49). Indeed, when NMIIA is inhibited using a NMIIA-specific inhibitor (i.e., blebbistatin), RBC exhibit elongated morphology at rest and lose their membrane curvature (49). How phosphorylation or other post-translational modifications may alter NMIIA-contractility and RBC mechanics,
however, remains to be resolved, although it is a valid area of interest given observations in other cell lines (9, 46, 58).

Collectively, although the molecular identity and structure of the RBC membrane has been largely determined, the interactive functions of numerous proteins remain unresolved, as is the dynamic re-assembly of membrane components during cellular deformation. Moreover, while classically the physical properties of RBC were viewed as the singular determinants of their extensive deformability, post-translational modifications of membrane proteins in particular have been suggested as mechanisms to acutely alter RBC mechanics (13, 24, 32).

Red blood cells are not sedentary: the role of nitrogen species

The concept that the physical properties of RBC may be modulated “actively” – that is, dynamically altered upon exposure to mechanotransductive stimuli – has emerged only over the past decade or so. While the presence of a process responsible for generating intracellular NO in RBC was speculated for some time (38), Kleinbongard et al., (31) only relatively recently employed independent and complimentary methods to demonstrate that RBC contain a NOS isoform that was distinct to the neuronal and inducible forms. Indeed, several key physical features were shown to reflect those of the endothelial-type NOS. The activity of this so-called “RBC-NOS” isoform was shown to be dependent on the substrate L-arginine, was calcium-sensitive, and also sensitive to phosphorylation via the PI3-kinase pathway (31) – all properties shared with endothelial NOS. Various groups subsequently confirmed that RBC carry a functional NOS-type enzyme, which facilitates endogenous production of nitric oxide (24, 39, 51, 54). Elucidating potential activation and down-stream targets mechanisms of RBC-NOS was thus of interest. Shear stress – induced either in vitro by exposing RBC to fluid shear in a microchannel or in vivo by increasing blood flow through exercise – may act
as an activator of RBC-NOS by inducing phosphorylation at its active residue serine 1177 (ser$^{1177}$) (51, 54). Increased phosphorylation of this residue has been reported by various groups to be closely associated with increased RBC deformability (22, 24, 32), while inhibition of RBC-NOS was previously shown to be associated with impaired cell deformability (24). The concrete activation mechanism of RBC-NOS is currently inferred from its alleged structural twin – endothelial NOS – although requires direct evidence. Given RBC and endothelial cells differ significantly in structure and intracellular composition (i.e., RBC lack a nucleus and Golgi apparatus, which are pivotal in activation of endothelial NOS; 44), further elucidation is required to examine the RBC-NOS isoform to discern its molecular mechanisms in higher resolution.

*Activation mechanism and down-stream targets of erythrocyte-derived nitric oxide synthase*

The primary mechanism currently proposed to elicit activation and mobilisation of RBC-NOS is mechanical stimulation (13, 54); that is, it is thought that physical forces deforming the cell membrane may be transduced into biochemical events that stimulate NO-generation directly, or alternatively via an independent method involving Ca$^{2+}$-signalling. Investigations aimed at discerning the effects of varying shear stress magnitudes on RBC deformability and NO-metabolism in flow led to a dose-response relationship being observed between shear stress and NO-production (27). Moreover, *in vitro* application of shear stress in the physiological range during laminar flow (i.e., comparable to forces present in the human circulatory system) was shown to elicit phosphorylation of RBC-NOS ser$^{1177}$, which occurred in concert with increased cell deformability (32). These data support that shear stress appears to activate RBC-NOS, and lead to predictable increases in intracellular NO production, that may impact cellular deformability. The mechanism for the shear-induced RBC-NOS activation remains elusive, although Ulker et al., (54) demonstrated that while shear exposure increased the phosphorylation of RBC-NOS at ser$^{1177}$, intracellular
concentrations of NO and intracellular Ca\textsuperscript{2+} concentration increased in tandem. Grau et al., (24) independently, demonstrated that NO produced during mechanical stimulation may reversibly incorporate into the cytoskeletal component of the cell membrane, as evidenced by increased S-nitrosylation of integral membrane proteins. Specifically, Grau et al (24) suggested that NO appears to bind the exposed sulphur atoms in α- and β-spectrin filaments forming nitrosylated residues, and they hypothesised that this process may explain the increased cellular deformability in the presence of increased intracellular NO concentration. Further observations to confirm the S-nitrosylation of the cell membrane remain elusive, although provide a tantalising suggestion of a mechanism for shear-mediated modulation of cell deformability with implications in vascular perfusion.

It collectively appears that shear stress activates RBC-NOS, and thus also increases intracellular availability of NO, which may impact the physical properties of the cell membrane via S-nitrosylation. How the flux of Ca\textsuperscript{2+} during mechanical stimulation of the cell membrane – such as when RBC pass narrow capillaries to facilitate gas exchange – is related to these events remains a topic of current investigation. While these characteristics infer striking similarities between endothelial NOS and RBC-NOS, it is stressed that these cells maintain fundamental biological differences, no less than the obvious differences in intracellular organelles. Several groups are thus currently exploring the function of RBC-NOS (24, 32, 54), although fundamental studies into the structure of RBC-NOS, and its potential localisation within RBC are ongoing, yet stand to deliver much greater understanding on the regulatory role of this protein.

*Modulation of erythrocyte mechanics by intracellular calcium*
Incubation of RBC with calcium was classically reported to induce membrane rigidification, and thus decreased cellular deformability; at least for experiments employing high concentrations of extracellular Ca\(^{2+}\) (45). The mechanism of cellular rigidification, however, has never been well understood, leading to inferences of a hypothetical ionic association of Ca\(^{2+}\) with some membrane components (41). It is clear that intracellular calcium of RBC is maintained at a comparatively low concentration (~40-60 nmol\(\cdot\)L\(^{-1}\); 6) relative to that of the surrounding plasma (~1.8 mmol\(\cdot\)L\(^{-1}\); 34), owing to the action of powerful calcium-ATPases (PMCA; 42). Given RBC do not possess mitochondria, and glycolysis is the sole pathway of endogenous ATP-generation, the capacity of RBC to maintain such a tightly regulated intracellular calcium concentration is thus dependent on the metabolic status of the cell.

O’Rear and colleagues (41) hypothesised calcium may impair the flexibility of the cell membrane due to electrostatic interaction of this cation with the membrane lipid bilayer – at least in the context of RBC being exposed to supraphysiological shears. Calcium depletes cellular ATP (35), which was initially thought to be essential for maintaining RBC deformability (57), by forcing high PMCA-activity. On the other hand, Clark et al., (11) demonstrated that ATP-depletion and calcium-accumulation have distinct effects on cellular deformability, hypothesising that the primary consequence of increased intracellular calcium is activation of the Gárdos channel (for a review, see 37) and subsequent loss of intracellular fluid. Briefly, the Gárdos channel is a calcium-activated potassium channel embedded in the RBC membrane (19) that facilitates transport of potassium-ions (K\(^{+}\)) to the extracellular space (33), and thus also water according to an osmotic shift, leading to a decrease in cell volume (i.e., Gárdos effect; Fig. 3). Of note, while the intracellular fluid leaving the RBC contains chloride ions (Cl\(^{-}\)) and water, haemoglobin is unable to leave the cell via this mechanism. It follows that intracellular viscosity increases due to the resultant higher relative abundance of haemoglobin, which has been experimentally validated and suggested to induce
impaired cellular mechanics (55). Recent developments have led to a paradigm shift indicating that the Gárdos effect may have valuable contributions to the maintenance of cellular mechanics, at least when shear stresses and/or calcium flux is maintained within physiological limits.

Mechanical stimulation, calcium and volume regulation

Early patch-clamp experiments performed on RBC to examine stretch-activated currents found evidence of a transient Ca^{2+}-flux that occurred in immediate response to mechanical stimulation. A negative pressure was placed onto single RBC using micropipettes to induce a reproducible deformation of the cell while isolating a membrane patch to record currents (14). It was observed that secondary to the Ca^{2+}-trace, K^{+} was transported in the opposite direction across the cell membrane (14). The authors thus hypothesised a possible interplay between a mechanically-activated calcium channel (that was yet to be identified) and the calcium-activated potassium channel, termed the Gárdos channel. Subsequent investigations into stretch-activated ion channels in the RBC membrane became more complicated given that the gold standard method – patch clamping – itself involves applying a mechanical stimulus to the membrane which could evoke currents from mechanically-activated channels (for a review, see 29).

Danielczok and colleagues (13) used in vivo and in vitro methods to assess transient calcium-increases within deformed RBC, visualised using a fluorescent probe. Upon cellular deformation of the RBC membrane, intracellular calcium transiently increased (13). While the RBC membrane is highly permeable to anions, it is poorly permeable to cations, and thus calcium flux involves transporters/pumps residing within the membrane. The hundreds of both selective, and non-selective transports are now known, with the vast majority of these
routes being voltage sensitive (for a review, see 2). Danielczok and colleagues (13) thus examined whether mechanosensitive cation-channels (i.e., piezo1), shown to be expressed on the RBC membrane (8), were critically involved in the transient calcium-response. Application of a specific piezo1-blocker, the peptide GsMTx-4 (1), abolished the expected flux in calcium-content caused by stretching of the RBC membrane. Rheological and biophysical measurements to assess the contribution of this mechanism to tissue perfusion more globally (e.g., quantification of flow velocities, shear stresses, transit times), however, were not performed in these experiments and would present a valuable addition to the observations of Danielczok and colleagues (13). Moreover, quantifying the alterations in cell volume as RBC pass narrow capillaries is required, in conjunction with investigations aimed to discern the reversibility of this phenomenon both on a cellular (e.g., return to normal cell size) and molecular level (e.g., ion-flux during states of reduced cell volume that facilitate a restoration of cellular homeostasis). Integrative studies investigating the interplay between mechanically-activated NO-generation and Ca²⁺-signalling pathways present ambitious albeit valuable directions for full understanding of the RBC active regulation of its mechanical properties. Given the calcium-dependency for activation of NOS observed in endothelial cells (17), in addition to existing evidence supporting a similar mechanism in the RBC (31, 54), it is plausible to suspect that cross-talk between these two signalling molecules exists.

Relevance in mechanical circulatory support and blood disorders

The mechanics of RBC are known to be impaired in a vast variety of pathologies (e.g., type 2 diabetes, coronary artery disease; 48, 53) which manifest in circulatory defects. Further, given these diseases may progress to require interventions facilitated by mechanical circulatory support (e.g., cardiopulmonary bypass; ventricular assist devices), which exert
Supraphysiological mechanical force on the blood (18), alterations in the sensitivity of second-messenger pathways may be particularly implicated in commonly observed post-surgical complications (30). Impaired transduction of NO- and Ca\(^{2+}\)-regulated signalling contributing to the observed rheological impairments in clinical conditions and after high-shear exposure presents a therapeutic opportunity. Stimulating or inhibiting mechanically-activated signalling pathways by pharmacological means could ameliorate the observed deficiencies in sustaining oxygen-delivery.

Sickle cell anaemia is caused by a single point mutation in exon I coding for beta-globin (12) which results in polymerisation of the haemoglobin beta-chain and thus ‘sickling’ of RBC under local hypoxia. Repeated collapses of RBC – such as through sickling when traversing the venous circulation – would provide a mechanical stimulus even stronger than shrinking induced by exposure to an hypotonic solution, which has been independently shown to induce piezo-1 activation and calcium-influx (13). The Gárdos effect has been associated with sickle cell pathology (i.e., exacerbating the ‘sickling’; 28) and inhibitors of the Gárdos channel (e.g., clotrimazole and senicapoc) have been therapeutically explored, although these agents proved ineffective in reducing vascular crisis events despite decreasing the relative abundance of ‘sickled’ cells and haemolysis (7, 50). Homozygous piezo1-mutations are expressed in 20% of the sickle cell anaemia population in contrast to 5% in the general population (40); however, preliminary clinical observations of piezo1-mutations in sickle cell anaemia patients did not significantly associate with disease severity (43). Independent studies established abnormal NO-metabolism in sickle cell anaemia patients; that is, sickle RBC present with increased NOS-activation and NO-production which are not associated with improved rheological characteristics (23). It appears, however, that NO may inhibit the Gárdos channel through interaction with cysteine residues on the extracellular side of the RBC membrane, thereby ameliorating the Gárdos effect (3, 5). Alternatively, ionic volume-
regulation via the Gárdos effect could offset NO-dependent increases in membrane flexibility; however, further studies investigating the concrete interplay between these pathways are required.

Mutations in the genes coding for piezo1 and the Gárdos channel have been reported in patients suffering from xerocytosis and other forms of rare anaemia (15, 20). The rheological properties and calcium-homeostasis of RBC were significantly altered, providing a potential mechanistic explanation for the premature haemolysis observed in these patients (26). Collectively, it appears that RBC-volume regulation via Ca$^{2+}$-homeostasis and endogenous NO-generation play pivotal roles in classic blood disorders rooted in abnormal properties of RBC. Given that only symptomatic treatments currently exist for these genetic blood disorders, pharmacological interventions to correct the acute regulation of Ca$^{2+}$ and NO within RBC is a promising approach to restore cellular mechanics and ensure tissue perfusion. For example, specific inhibitors of piezo1-channels (e.g., GsMTx4) may be titrated to prevent intracellular Ca$^{2+}$-overload, while stimulators of RBC-NOS (e.g., L-arginine) or up-stream kinases (e.g., 740 Y-P) could increase RBC-derived NO and thus improve cell mechanics which ultimately increase tissue perfusion.

Conclusion

Evidence has accumulated over the past decade establishing a significant role for second-messenger molecules, such as NO and Ca$^{2+}$, in contributing to the active regulation of the physical properties of RBC. While several interactions and mechanisms remain to be elucidated, it appears that tight control over the concentration of these messengers is pivotal for ensuring RBC homeostasis, cellular deformability, and ultimately tissue perfusion. Thus while the last decade has provided a new perspective of the active regulation of RBC, integration of molecular techniques, electrophysiology, and rheological assessment of blood
appears a fruitful avenue for improved therapeutic approaches for cardiometabolic and haematologic disorders, and further in the refinement of mechanical circulatory support devices that remain plagued by poor complication rates.

References


**FIGURE LEGENDS**

*Figure 1:* The physical properties classically known to facilitate the remarkable capacity of RBC to reversibly deform.

*Figure 2:* The molecular composition of the cytoskeletal component in the RBC membrane, that resides under the lipid bilayer; α- and β-spectrins self-associate head-to-head, while each dimer is anchored to the lipid bilayer via either ankyrin or actin-4.1 complexes. Myosin IIA connects actin molecules within the junctional complexes and thus stabilises the bi-concave shape of the RBC.

*Figure 3:* A synthesis of the observed regulatory pathways involving nitric oxide (NO) generation and calcium ion-movement (Ca\(^{2+}\)) within RBC yields a complex intracellular signaling network. Mechanical stimulation via exposure to fluid shear in blood promotes Ca\(^{2+}\)-influx via the mechanosensitive piezo1-channel (1). Complexation of Ca\(^{2+}\) with the carrier protein calmodulin also occurs, which then collectively bind RBC-NOS (2). RBC-NOS is subsequently activated, producing NO which appears to bind to α- and β-spectrins in close proximity via S-nitrosylation of free cysteine residues, leading to increased flexibility of the cytoskeleton and improved cellular deformability (3). Activation of the Gárdos channel occurs in response to sustained Ca\(^{2+}\)-influx (4), which facilitates export of potassium ions (K\(^+\)) and leads to a loss of intracellular fluid (5). Simultaneously, Ca\(^{2+}\) is slowly transported out of the RBC via the plasma membrane Ca\(^{2+}\)-ATPase (6).
Cytosolic haemoglobin concentration dictates intracellular viscosity

Viscoelastic membrane comprised of lipid bilayer and flexible membrane skeleton

Bi-concavity facilitates capacity to deform via high surface area to volume ratio
Fluid shear stress

1. piezo1
2. Akt kinase
3. NO
4. Ca\(^{2+}\)/calmodulin complex
5. Osmotic pressure
6. ATP

RBC-NOS

Spectrin filaments

Increased membrane flexibility

H\(_2\)O

K\(^+\)

Cell shrinkage and temporary loss of deformability