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REVIEW

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# Human erythrocytes, nuclear factor kappaB (NFκB) and hydrogen sulfide ($H_2S$) – from non-genomic to genomic research

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**ABSTRACT**

Enucleated mature human erythrocytes possess NFκBs and their upstream kinases. There is a negative correlation between eryptosis (cell death of erythrocytes) and the amount of NFκB subunits p50 and Rel A (p65). This finding is based on the fact that young erythrocytes have the highest levels of NFκBs and the lowest eryptosis rate, while in old erythrocytes the opposite ratio prevails. Human erythrocytes (hRBCs) effectively control the homeostasis of the cell membrane permeable anti-inflammatory signal molecule hydrogen sulfide ($H_2S$). They endogenously produce $H_2S$ via both non-enzymic (glutathione-dependent) and enzymic processes (mercaptopyruvate sulfur transferase-dependent). They uptake $H_2S$ from diverse tissues and very effectively degrade $H_2S$ via methemoglobin (Hb-$Fe^{3+}$)-catalyzed oxidation. Interestingly, a reciprocal correlation exists between the intensity of inflammatory diseases and endogenous levels of $H_2S$. $H_2S$ deficiency has been observed in patients with diabetes, psoriasis, obesity, and chronic kidney disease (CKD). Furthermore, endogenous $H_2S$ deficiency results in impaired renal erythropoietin (EPO) production and EPO-dependent erythropoiesis. In general we can say: dynamic reciprocal interaction between tumor suppressor and oncoproteins, orchestrated and sequential activation of pro-inflammatory NFκB heterodimers (RelA-p50) and the anti-inflammatory NFκB-p50 homodimers for optimal inflammation response, appropriate generation, subsequent degradation of $H_2S$ etc., are prerequisites for a functioning cell and organism. Diseases arise when the fragile balance between different signaling pathways that keep each other in check is permanently disturbed. This work deals with the intact anti-inflammatory hRBCs and their role as guarantors to maintain the redox status in the physiological range, a basis for general health and well-being.

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## Human erythrocytes (hRBCs) and their methemoglobin (metHb/$HbFe^{3+}$) as biological hydrogen sulfide ($H_2S$) carrier

Under physiological conditions, the auto-oxidation of about 1–3% of the total body hemoglobin (ferrous Hb/$HbFe^{2+}$) results in the generation of methemoglobin (metHb/$HbFe^{3+}$). Different organs such as liver, kidney, and brain produce the signal molecule hydrogen sulfide ($H_2S$). The cellular $H_2S$ biogenesis, that is, desulfuration of cysteine or homocysteine, is primarily accomplished by three enzymes: Cystathionine b-synthase (CBS), γ-cystathionase (CSE), and mercaptopyruvate sulfur transferase (MST) [1–3]. hRBCs does possess MST, but not the other two $H_2S$-producing enzymes [4]. While $H_2O$ flow through cell membrane is accelerated by aquaporins [5], the transmembrane diffusion of hydrophobic $H_2S$ requires no facilitator and its permeability coefficient is still 10.000 times higher than that of water [6]. Based on this property, $H_2S$ can exhibit broad toxicity effects or function as a signal molecule in a concentration-dependent manner. Besides the endogenously produced nitric oxide (NO) and carbon monoxide (CO), the cell membrane permeable $H_2S$ [7,8] plays an important role as a gaseous signaling molecule in biological and physiological processes. $H_2S$ regulates several biological and physiological processes, for instance: it shows anti-thrombotic effects [9], protects vascular tissues from atherogenic disease [10], enhances blood flow which protects against vascular ischemia [11] and inhibits glucose consumption and uptake. For reviews, see [12,13].

One hundred years ago, sophisticated experiment on animals provided the first evidence of fast $H_2S$ detoxification via its metabolism. The practice

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of a fast administered single dose of 10 ml of a 77 mmol/l $Na_2S$ solution was always lethal, whereas dogs that received a five-fold dose over a period of 20 minutes survived and showed no obvious damage [14]. Mammalian tissues (e.g. heart, kidney, brain, and intestine) as well as human erythrocytes are able to produce $H_2S$. Under physiological conditions, ~30% of the short-lived $H_2S$ exists in a non-dissociated form and ~70% in its hydrogen sulfide anion ($H_2S$ $HS^-$). It is important to note that: a) the completion of net acid efflux by the $H_2S/HS^-$ follows the same principle as that of $CO_2/HCO_3^-$ in the Jacobs–Stewart cycle (**see** Figure 1b) due to the lack of extracellular hydration and intracellular dehydration, the net acid efflux in $Cl^-/HS^-/H_2S$ cycle is faster than $Cl^-/HCO_3^-/H_2CO_3$ cycle, c) $HS^-$ is a very good substrate for the anion exchanger 1 (AE1) and d) $H_2S$ possesses a very high permeability coefficient in human erythrocytes [15]. The atomic structure of CO allows it to solely bind $Fe^{2+}$ with its two unpaired electrons [16]. Therefore, all of the spectral work with CO and heme proteins employs the native (reduced) forms. NO is able to bind both $Fe^{2+}$ and $Fe^{3+}$ in heme proteins. $H_2S$ binds rapidly to $Fe^{3+}$ in heme proteins, for example, methemoglobin ($HbFe^{3+}$) [4,17]. Vitvitsky et al. showed that hRBCs produce $H_2S$ via MST and in addition to this effectively clear sulfide via MetHb-catalyzed oxidation of $H_2S$ to thiosulfate and polysulfides [4], see also Fig. 1 and 2. Human erythrocytes are represented by ~5 billion per ml of blood, and each intact hRBC contains over 270 million hemoglobin molecules that are able to uptake $H_2S$ from diverse tissues and very effectively control its clearance. This ensures the maintenance of the physiological plasma and tissues concentration of free $H_2S$ in the range of 15 to 150 nM [18,19]. The very high lipid and water solubility of $H_2S$ allows quick passage through the alveolar membrane, which is the best condition for achieving an almost perfect equilibrium between blood and alveolar air. Human alveolar air measurements showed negligible free $H_2S$, indicating very low blood concentration [18]. The fundamental findings of Furne et al. also revealed the conventional experimental errors involved in $H_2S$ research. Considering that a) we have seen the work of Furne et al. 2008 as well as King et al. 2014, b) an adult healthy human has ~30 trillion ($3 \times 10^{13}$) circulating RBCs with a life span of 120 days [20], c) ~1% of the circulating hRBCs (~200–300 billion cells) are cleared per day and replaced by erythropoiesis, and d) that ~3.7 million ($3.7 \times 10^6$) cell-free, intact and respiratory competent mitochondria circulating per ml of blood plasma [21], those organelles apart from hRBCs contribute to the degradation of $H_2S$, maintain human plasma concentration of $H_2S$ clearly below a µM range under physiological conditions.

## Role of PKC-α and other $Ca^{2+}$-dependent pathways in IκB-α phosphorylation/degradation and NFκB activation

Ankyrin-containing proteins including IκBs act as specific protein–protein interactors [22–24]. The

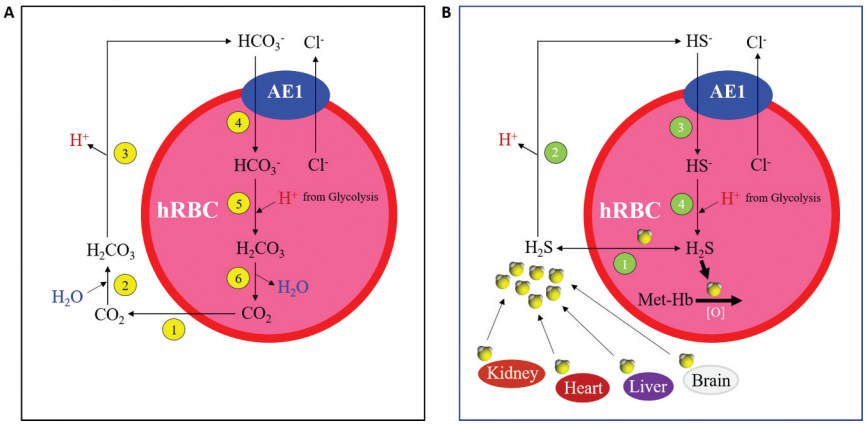


**Figure 1.** Role of human red blood cells (hRBCs) in uptake and degradation of $H_2S$. For more details see the main text.

prevailing opinion is that cellular activation via numerous stimuli initiates IκB-**α** phosphorylation, its subsequent dissociation from and abolition of its inhibitory effect on NFκB; and these events precede the IκB-**α** proteolytically degradation. However, this does not reflect the sequence of events. In fact, the IκB-**α** phosphorylation and its subsequent degradation enables NFκB release, which rapidly translocates into the nucleus [25] to drive the expression of genes, for eample, IL-8 expression [26]. The dual function of the chemokine IL-8 includes pathogen elimination by recruitment of neutrophils and being causative in several inflammatory diseases. Both IL-4 and human erythrocytes can curb IL-8 effects. IL-4 functions as an endogenous inhibitor of IL-8 expression [27] and hRBCs reduces the bioavailability of IL-8 substantially by acting as a sink for IL-8 [28–30]. Steffan et al. were the first to show a direct link between the synergistic effects of PKC- and $Ca^{2+}$-dependent phosphatase calcineurin on the regulation of IκB-**α** phosphorylation and pointed to the necessity of the existence of an IκB-**α** kinase (IKK) [25,31]. In the meantime, the existence of IKKs [32,33], but also of an IKK kinase, have been proven [34].

## Role of redox-sensitive canonical NFκB pathway in human erythrocytes (hRBCs). NFκBs in nucleated cells; the complexity of glutathione (GSH), NFκB, PKC, $Ca^{2+}$ and nitric oxide synthase (NOS) interactions

Human erythrocytes (hRBCs) possess the main members of the canonical NFκB pathway [35–37]. Virtually all publications on erythrocytes´ NFκBs available to date originate from our laboratory, which demonstrate a reciprocal relationship between age and abundance of NFκBs in hRBCs; the NFκB protein abundance is highest in young and lowest in aged erythrocytes. There is a positive correlation between cell volume, and a negative correlation between eryptosis (cell death of erythrocytes) and the amount of NFκB subunits p50 and p65. This finding is based on the fact that young erythrocytes have the highest cell volume and the lowest eryptosis rate, while in old erythrocytes the opposite ratio prevails [36].

Retrobulbarly collected whole blood, the subsequent isolation of erythrocytes from the homozygous NFκB-p50 deficient and congenic wild-type C57BL/6 and their subsequent incubation in Ringer solution enabled to demonstrate a direct correlation between NFκB-p50 deficiency and increased eryptosis [38]. Additional biological/physiological effects were: a) significant increase of white blood cell (WBCs) count and b) considerable weight loss in NFκB-p50 deficient mice. The former indicates systemic inflammation in NFκB-p50 deficient mice and the latter observation offers a possibility to treat obesity with NFκB inhibitors provided their bioavailability is sufficient [39,40]. It is known that NFκB-p50 homodimers are refractory to inflammation while NFκB heterodimers (e.g. RelA-p50 subunits) have an inflammatory function [41]. This is why impaired p50–p50 activation is associated with dysregulated inflammation and chronic inflammatory diseases.

The generation of a reduced form of glutathione (GSH), an intracellular antioxidant, is the result of two concerted ATP-consuming reactions conducted by 1) **γ**-glutamylcysteine synthetase (**γ**-GCS) and 2) GSH synthetase (GS) [42,43]. (Reaction 1): L-glutamate + L-cysteine + ATP → **γ**-L-glutamyl-L-cysteine + ADP + $P_i$ (Reaction 2): **γ**-glutamyl -L-cysteine + L-glycine + ATP → GSH + ADP + $P_i$

The first reaction is, however, feedback inhibited by GSH [44], see also Figure 2. In nucleated cells, GSH and NO, respectively, are able to inhibit IKK-b activity by reversible S-glutathionylation or S-nitrosylation, which ultimately impairs NFκB activation [45,46]. Protein kinase C-alpha (PKC-**α**) phosphorylates NFκB-p65 subunits [47] and this is associated with NFκB-dependent induction of **γ**-GCS and intracellular GSH de novo biosynthesis [48]. Interestingly, addition of exogenous NO donor DETA/NO results in NO-mediated release of "free" intracellular zinc, zinc-dependent increase of **γ**-GCS expression and GSH synthesis [49]. This could be a new IKK-NFκB--independent, NO-dependent pro-survival pathway connecting redox potential of a cell with intracellular "free" zinc concentration. Human erythrocytes (hRBCs) possess functional endothelial nitric oxide synthase (eNOS) (L-Arginine + $O_2$ + eNOS → L-Citrulline + NO) [50,51] and much more important is the fact that pro-survival NO and pro-eryptotic $Ca^{2+}$ keep each other in check [52]. Under physiological

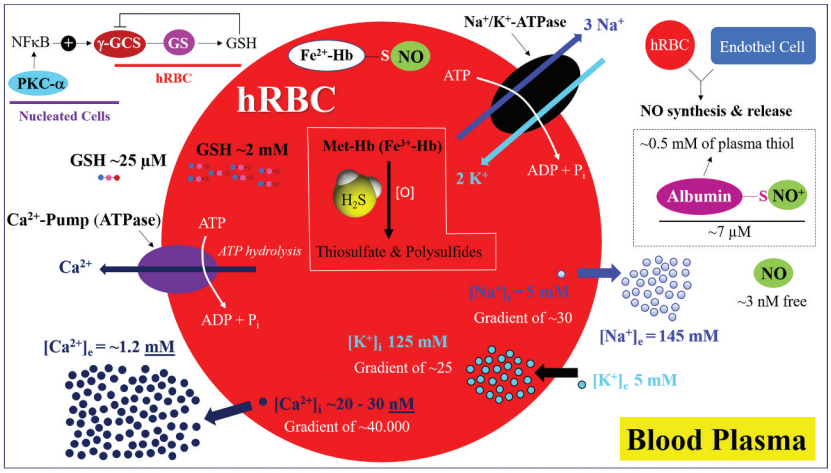


**Figure 2.** Illustration of ions distributions and the pro-survival role of nuclear factor kappaB (NFκB), nitric oxide (NO) and reduced form of glutathione (GSH) in human red blood cells (hRBCs). For more details see the main text.

conditions one portion of the abundant serum albumin binds to NO, forming a relatively long-lived albumin-NO-adduct (~7 µM S-nitrosothiol) and thus functioning as a sink for NO, while free NO with a plasma concentration of ~3 nM serves predominantly to maintain the vascular tone [53], see also Figure 2. For more details about the biological roles of NO we refer to the following reviews [54,55].

## Human erythrocytes (hRBCs), free and unbound Calcium ($Ca^{2+}$), $Ca^{2+}$ dependent Protein kinase C-alpha (PKC-α) and NFκB activation. The Bermuda Triangle $Ca^{2+}$-PKC-α-NFκB and its association with respiratory diseases

Intact hRBCs among mammalian cells have the lowest free, unbound intracellular concentration of calcium ions $[Ca^{2+}]_i$ under physiological conditions ranging between 20 and 30 nM [56]. In contrast to organelles-free mature hRBCs, other mammalian cells possess multiple organelles with a wide variety of free, unbound calcium distributions, for example, $[Ca^{2+}]_{cyt}$ ~50–100 nM, $[Ca^{2+}]_{mt}$ ~100 nM, $[Ca^{2+}]_{nucleus}$ ~100 nM, $[Ca^{2+}]_{ER}$ ~100–700 nM, for review see [57]. $[Ca^{2+}]_i$ directly impairs the transmembrane equilibrium distribution of the phospholipids, that is, their inward translocation from the outer to the inner leaflet of erythrocytes' plasma membrane. For instance, $[Ca^{2+}]_i$ of ~50 and ≥200 nM affect the inward translocation of phosphatidylethanolamine and acidic phosphatidylserine (PS), respectively [58], a process directly related to $Ca^{2+}$-dependent inhibition of aminophospholipid translocase (or flippase) activity. A sustained cytosolic calcium elevation $[Ca^{2+}]_i$ concomitantly promotes the activity of the phospholipid scramblase which then unspecifically initiates bidirectional PS translocation on both sides of the plasma membrane. In contrast to internalized PS, PS externalization or depletion is associated with a cell-type independent weakening of the plasma membrane $Ca^{2+}$-ATPase (PMCA)-mediated $Ca^{2+}$-efflux [59,60]. The following reviews illustrate $Ca^{2+}$ transporting systems, for example, PMCA and the $Ca^{2+}$-activated $K^+$ channel, known as Gardos channel [61–63]: In view of the antagonistic roles of NO and $Ca^{2+}$ [52] and association of early eryptosis with the removal/translocation of PS from the inner to the outer leaflet of the bilayer plasma membrane, it is not surprising that intact hRBCs maintain their $[Ca^{2+}]_i$ as low as possible and as much as necessary (see Figure 3).

It is undisputed that changes in $[Ca^{2+}]_i$ are associated with changes in cell functions [64]. $[Ca^{2+}]_i$ mediates both cell survival and apoptosis depending on its oscillation and excessive elevation, respectively [57,65,66]. The most prominent member of the protein kinase C (PKC) family is the $Ca^{2+}$-, acidic phospholipids (e.g. PS-) and diacylglycerol (DAG)-dependent conventional PKC-alpha (cPKC-**α**) [67–71]. According to structure and cofactor regulation, the PKC family is divided

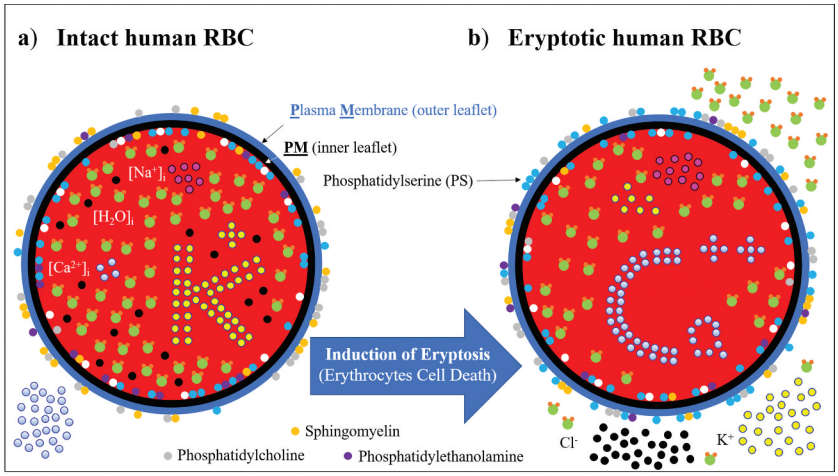


**Figure 3.** Qualitative illustration of four major plasma membrane phospholipids as well as intraerythrocytic distribution of potassium ($K^+$) and calcium ($Ca^{2+}$) ions in intact and eryptotic human erythrocyte.

into three classes: cPKCs (**α, β**I, **β**II, and **γ**), novel, that is, nPKCs (d, e, η, and θ), and atypical, that is, aPKCs (ζ and iota (i)). The first class being $Ca^{2+}$, PS, and DAG-dependent; the second being $Ca^{2+}$-independent but PS- and DAG-dependent, and the third class being $Ca^{2+}$- and DAG-independent but acidic phospholipids and ceramides dependent. hRBCs possess four cytosolic isoforms of PKCs: alpha, zeta, mu, and iota, of which only PKC-**α** with membrane translocation capability [72,73], that is, induction of eryptosis [74,75]. Using chelerythrine as a specific PKC-**α** inhibitor, we were able to show a direct correlation between the costunolide-induced GSH-depletion and PKC-**α** activation in hRBCs [76], a phenomenon also observed in nucleated mammalian cells. Thus, it is not astonishing that the capacity of hRBCs to synthesize ~2 mM of the pro-survival $[GSH]_i$ [77,78] exceeds the rate GSH turnover by 150-fold [79] to avoid a PKC-**α** mediated induction of erythrocytes death (eryptosis). Furthermore, $H_2S$ can be endogenously produced in the presence of GSH [80,81]. GSH is a linchpin of cellular defense protecting both prokaryotic [82] and eukaryotic cells [83,84] including hRBCs from biotic and abiotic stresses. In nucleated mammalian cells, PKC-**α** activation drives the pro-survival machinery [85–87], and its inhibition commonly triggers apoptosis in these cells [88,89]. In addition to this, respiratory diseases of viral [90] and bacterial [91] origin are associated with PKC-**α** activation. It is to note that intact hRBCs are actively involved in bacterial [92] and viral clearance from circulation [93–98], for reviews see [99,100]. The message is clear: specific PKC-α inhibitors, for exmaple, the bioactive molecule chelerythrine [101–104], as a natural product of plant origin can dose-dependently cause a pro-apoptotic effect in nucleated cells, thus creating a hostile environment for intracellular parasites including viruses and simultaneously can create a pro-survival effect in enucleated hRBCs [76]. Therefore, hRBCs in combination with PKC-**α** inhibitors (e.g., chelerythrine) should be a promising approach to treat COVID-19 [105]. PKC-**α** as the upstream kinase of the NFκB signaling pathway as well as NFκB itself, represent a link between nucleated and enucleated mammalian cells, which can be designated as: "NFκB, from non-genomic to genomic research".

## Inflammation vs. anti-inflammation. NFκB-p65, glycolysis, inflammatory diseases vs. hRBCs, $H_2S$, insulin

Obesity- and psoriasis-associated chronic low-grade inflammation and NFκB activation are two sides of the same coin that perpetuate each other. It is known that NFκB is a positive physiological regulator of glycolysis [106], for review see [107]. The following review clearly illustrates the relationship between the anti-inflammatory effects of insulin and the pro-inflammatory effects of glucose with NFκB as a common target [108]. Interestingly, glucose uptake is negatively

correlated with in adipose tissue up-regulation of $H_2S$ system. As already mentioned, a negative correlation exists between the intensity of inflammatory diseases and endogenous $H_2S$ levels. Psoriasis is directly associated with low serum $H_2S$ levels [109], for review, see [110]. Diminished adipose tissue $H_2S$ has been observed in obesity. $H_2S$ inhibits the expression of highly pro-inflammatory IL-8 in human keratinocytes and shows potential for psoriasis treatment [111]. In addition, hRBCs function as a sink for IL-8, thus minimizing the deleterious effects of NFκB-mediated IL-8 expression. Recently, Mezouari et al. demonstrated that $H_2S$ enhances the secretion of the glucoregulatory hormone glucagon-like peptide 1 and improves glucose clearance in mice [112], for review see [113]. In addition to these, endogenous $H_2S$ deficiency in patients with chronic kidney disease (CKD) is associated with impaired renal erythropoietin (EPO) production and EPO-dependent erythropoiesis [114]. Taken together, the role of anti-inflammatory hRBCs to regulate $H_2S$ homeostasis and to maintain its physiological concentration in the blood as well as to function as a sink for a many inflammatory cytokines and chemokines, is essential for maintaining cellular health as the basis for general health and well-being.

## $H_2S$-mediated regulation of biochemical pathways in human erythrocytes (hRBCs)

For adequate supply of the organism with molecular oxygen, hRBCs divert 20% of the uptaken glucose to Rapoport and Luebering glycolytic shunt [115], for review see [107]. In this process erythrocyte 2,3-bisphosphoglycerate (2,3-BPG) plays a central role. It negatively regulates hemoglobin oxygen ($O_2$) binding affinity, facilitates $O_2$ release from oxyhemoglobin [116] improving tissue oxygenation. $H_2S$ regulates 2,3-BPG production and it exists a reciprocal correlation between $H_2S$ concentration and 2,3-BPG production. $H_2S$ level increases during normoxic and decreases during hypoxic conditions [117]. This ensures maximum $O_2$ uptake in the lungs and maximum $O_2$ release in the peripheral tissues. It is to note that the reduced form of glutathione (GSH), glycolytic, and pentose phosphate pathways positively regulate $H_2S$ production in hRBCs [80]. hRBCs possess an active and functional endothelial nitric oxide synthase (eNOS) and are a major source of NO (hRBC-eNOS → NO production), contributing to the circulating NO pool [50,118]. The ability of hRBCs to take up endothelium-derived NO, thereby limiting NO available for vasodilation: $Fe^{2+}$-$HbO_2$ (oxy-Hb) + NO → $Fe^{3+}$-Hb (metHb) + $NO_3^-$, does not invalidate our statement just described. The localization of homodimeric hRBC-eNOS at the cytoplasm leaflet preferentially increase local metHb concentration which in turn acts like a shield to protect NO molecules – produced by hRBC-eNOS – from scavanging by oxyhemoglobin (oxy-Hb). This allows NO molecules not only to leave the erythrocytes but also to interact with their targets located in the immediate vicinity of hRBC-eNOS. Another important aspect is that metHb molecules generated in this process can now be used to clear sulfide via MetHb-catalyzed oxidation of $H_2S$ to thiosulfate and polysulfides. It is to note that high concentration of NO impairs dimer stability of eNOS as well as its activity and this loss of dimer (eNOS monomerization) can be reversed by thioredoxin/thioreductase system [119]. These sophisticated and coordinated processes curtail exuberant NO production in vivo. The following work illustrates in a very compact form the physical and chemical properties of NO and its physiological roles [120]. NO inhibits erythrocyte cell death (eryptosis) [52] and reduction of NO bioavailability has been observed in several diseases, for example, in sickle cell anemia [121]. Recently, we observed systemic inflammation and enhanced rate of eryptosis in NFκB-p50 (p50) deficient mice [38]. It is known that NFκB-p50 homodimers are refractory to inflammation while NFκB heterodimers (e.g. NFκB-p65-p50 subunits) have an inflammatory function [41]. NFκB-p65 (p65) activity is regulated by several reversible post-translational modification mechanisms. p65 is activated by phosphorylation [122] or acetylation [123] and inhibited by deacetylation [123,124]. To date, there is no single publication that has investigated the influence of $H_2S$ on NFκBs in hRBCs. We tend to believe that $H_2S$ with its anti-inflammatory properties exerts an inhibitory effect on p65 and positively regulates p50. We will clarify this experimentally in the near

future. In nucleated cells, publications on the influence of $H_2S$ on NFκBs are contradictory. According to several publications, $H_2S$-mediated p65 sulfhydration can lead to its activation and inhibition. These inconsistencies are rather due to a lack of standardized methods for determining $H_2S$ concentration.

## Conclusion and perspectives

Human erythrocytes (hRBCs) are a mobile organ that traverse our entire organism. They are involved in innumerable biological and physiological processes, are directly involved in virus and bacterial elimination from circulation, maintain the concentrations of many signaling molecules and antioxidants in physiological range, possess transcription factors such as NFκBs and their upstream kinases and act as a sink for many inflammatory cytokines and chemokines, thus minimizing their deleterious effects. Therefore, treatment of many pathological diseases without considering hRBCs, is myopic and not an adequate remedy.

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## Authors contributions

MG designed the project and mainly wrote the manuscript. All figures were made by MG. All authors read, discussed, improved, and approved the final version of the manuscript.

## Disclosure statement

The authors declare that no competing financial interests or otherwise exist.

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