



Review Article

Inborn defects in the antioxidant systems of human red blood cells

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ABSTRACT

Red blood cells (RBCs) contain large amounts of iron and operate in highly oxygenated tissues. As a result, these cells encounter a continuous oxidative stress. Protective mechanisms against oxidation include prevention of formation of reactive oxygen species (ROS), scavenging of various forms of ROS, and repair of oxidized cellular contents. In general, a partial defect in any of these systems can harm RBCs and promote senescence, but is without chronic hemolytic complaints. In this review we summarize the often rare inborn defects that interfere with the various protective mechanisms present in RBCs. NADPH is the main source of reduction equivalents in RBCs, used by most of the protective systems. When NADPH becomes limiting, red cells are prone to being damaged. In many of the severe RBC enzyme deficiencies, a lack of protective enzyme activity is frustrating erythropoiesis or is not restricted to RBCs. Common hereditary RBC disorders, such as thalassemia, sickle-cell trait, and unstable hemoglobins, give rise to increased oxidative stress caused by free heme and iron generated from hemoglobin. The beneficial effect of thalassemia minor, sickle-cell trait, and glucose-6-phosphate dehydrogenase deficiency on survival of malaria infection may well be due to the shared feature of enhanced oxidative stress. This may inhibit parasite growth, enhance uptake of infected RBCs by spleen macrophages, and/or cause less cytoadherence of the infected cells to capillary endothelium.

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Introduction

Red blood cells (RBCs) are specialized in transporting oxygen from the lungs to the tissues. For this purpose, RBCs contain large amounts of hemoglobin (Hb) and must be very flexible to pass the

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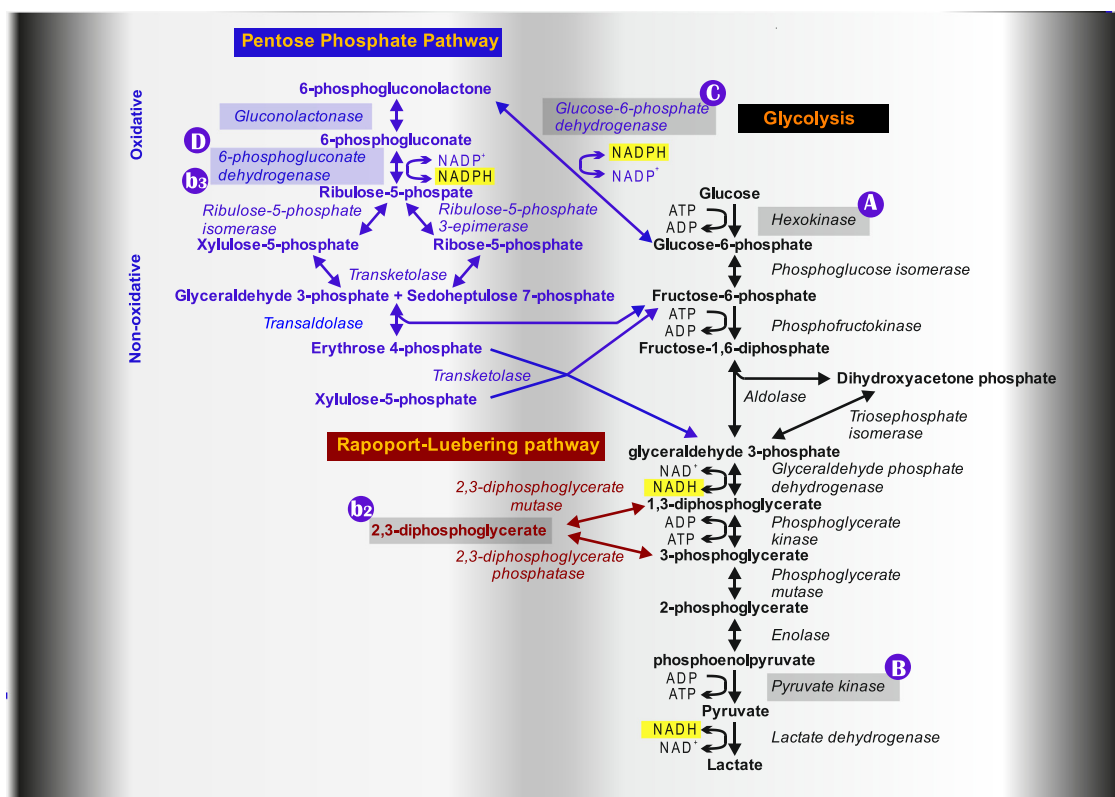


Fig. 1. Reaction scheme of glycolysis, pentose-phosphate pathway, and Rapoport-Luebering pathway. The rare deficiency of hexokinase (A) leads to diminished synthesis of ATP, NADH, and NADPH. Pyruvate kinase deficiency (B) frustrates ATP synthesis and causes high concentrations of 2,3-bisphosphoglycerate (b2). This last phenomenon results in inhibition of 6-phosphogluconate dehydrogenase (b3) and thus in low activity of the second step of NADPH synthesis. Glucose-6-phosphate dehydrogenase catalyzes the first step in the PPP (C) and deficiency results in low capacity to generate NADPH. Patients that are partly deficient in 6-phosphogluconate dehydrogenase activity (D) have diminished reduction potential because the second step in the PPP for the formation of NADPH is blocked (adapted with permission from Patrick Burger et al. [155]).

narrowest blood vessels. The unique rheological properties of RBCs are due to specialized cytoskeletal and membrane proteins and high concentrations of polyunsaturated fatty acids in the membrane. RBCs are devoid of mitochondria, so their energy is derived solely from the anaerobic degradation of glucose in the glycolytic pathway (Fig. 1). In addition, glucose-6-phosphate (G6P) can be shuttled into the pentose-phosphate pathway (PPP) to reduce NADP to NADPH, needed for protection against reactive oxygen species (ROS) and repair of oxidized proteins in the RBC [1].

The presence of high concentrations of molecular oxygen and iron (in the heme group of Hb) in RBCs carries the potential danger of ROS formation. Indeed, autooxidation of Hb (with the heme iron in the ferrous Fe^{2+} state) to methemoglobin (metHb, with Fe^{3+}) causes a continuous but limited intracellular production of superoxide ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) in these cells [2]. Oxidative damage to proteins and membrane lipids gradually impairs RBC function and is a major cause of cell aging [3,4].

RBCs not only lack mitochondria but also do not possess a nucleus, so their protein synthetic capacity is very limited. Nevertheless, these cells have a lifetime of about 120 days in the circulation. Protection against ROS and repair of oxidative damage must thus be very solid. Indeed, a diversity of antioxidant systems is known to protect and repair RBCs. First, there is the glutathione cycle, which can reduce oxidized proteins and ascorbate via glutaredoxins and H_2O_2 and lipid/alkyl peroxides via glutathione peroxidase (Fig. 2, right side) [5]. Glutathione can also detoxify xenobiotics via glutathione S-transferase (Fig. 2, top). Glutathione receives its reducing equivalents from NADPH, which—via thioredoxin—can also itself scavenge steady-state-produced hydrogen peroxide in a peroxiredoxin reaction (Fig. 2, bottom) [6]. In its turn, NADPH is kept in the reduced form via the G6P dehydrogenase (G6PD) and the

6-phosphogluconate dehydrogenase (6PGD) reactions of the PPP (Fig. 2, left side). Moreover, superoxide dismutase (SOD) can convert superoxide to hydrogen peroxide [7], and catalase can remove excess hydrogen peroxide (Fig. 2, bottom). Other, non-enzymatic reductants, such as the hydrophilic vitamin C and the lipophilic vitamin E, are taken up by RBCs and contribute to the protection against membrane damage [8,9], whereas vitamin C is also an important reductant for metHb [10]. RBCs take up significant amounts of oxidized vitamin C (ascorbate) via their Glut1 glucose transporter and regenerate the protective, reduced form of vitamin C (dehydroascorbate) to sustain high levels in RBCs (Fig. 2, top right) [11]. However, the main system for reducing metHb is the NADH/NADPH cytochrome b_5 reductase enzyme (not depicted in Fig. 2) [12,13].

RBCs use their high-capacity redox systems also to scavenge extracellular radicals [14] and thus provide a mobile protection system against radicals formed in the body as a whole [15]. In situations of moderate oxidative stress triggered by disease, or even in cases of mild enzyme deficiencies, sickle-cell trait, or β -thalassemia minor, limited hemolysis and subsequent radical formation can be dampened by the high-capacity antioxidant systems in intact RBCs. However, in situations with significant hemolysis, when the amount of plasma Hb saturates the protective capacity of haptoglobin- and hemopexin-mediated sequestration of Hb and heme, the consecutive ROS formation can cause serious vascular and organ damage [16].

In this review the inborn defects that frustrate proper ROS detoxification are discussed according to the antioxidative pathway involved (summarized in Table 1). We have also included the aspect of enhanced ROS formation in hemoglobinopathies and thalassemias (summarized in Table 2).

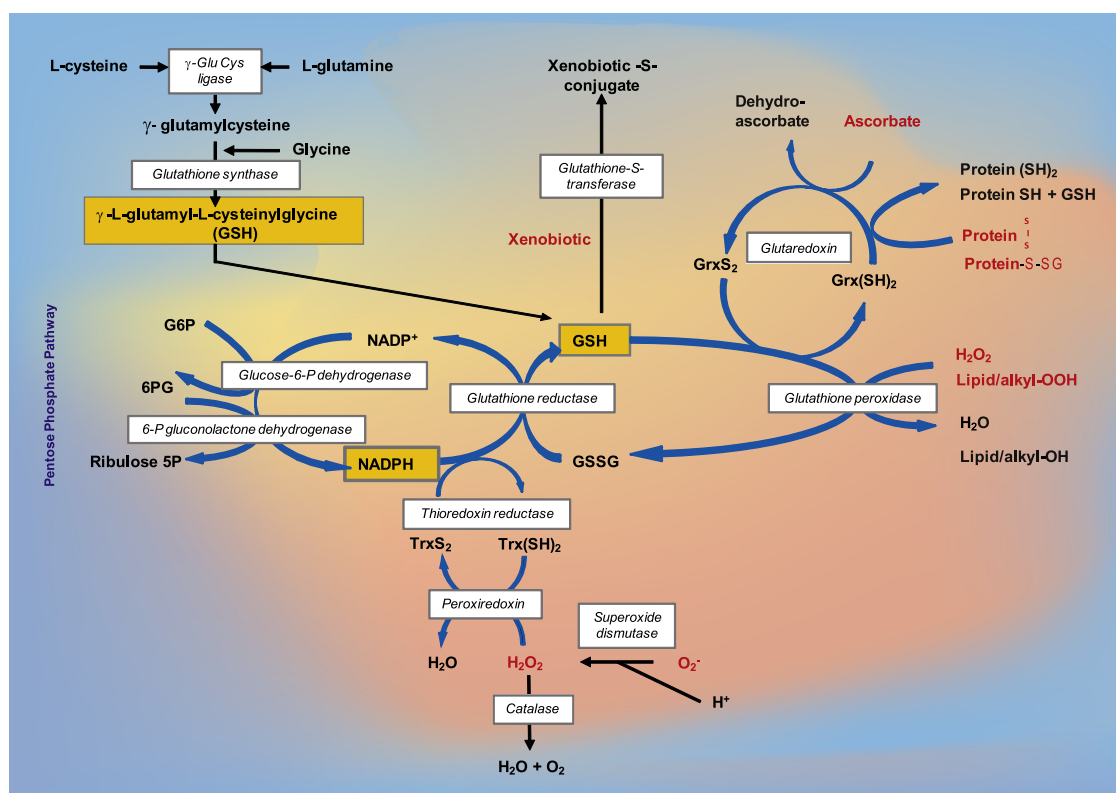


Fig. 2. Scheme of the major reducing pathways in erythrocytes. See the introduction for details.

Table 1

Cellular and clinical consequences of deficiencies in enzymes involved in ROS scavenging or in repair of ROS-inflicted damage to RBC proteins and lipids.

Pathway involved in ROS scavenging/gene deficiency (gene name)	Phenotype of homozygous/compound heterozygous patients	Reference
Glycolysis		
Hexokinase (<i>HK1</i>)	Chronic hemolytic anemia; low ATP, low NAD(P)H	[18,19]
Pyruvate kinase (<i>PKLR</i>)	Chronic hemolytic anemia; low ATP, low NAD(P)H; secondary to high 2,3-diphosphoglycerate is the inhibition of 6PGD	[22–25]
Pentose–phosphate pathway		
Glucose-6-phosphate dehydrogenase (<i>G6PD</i>)	Unstable variants; episodic hemolytic anemia (hemizygous and heterozygous), favism; low NADPH; protects against lethal malaria; frequent genetic abnormality (X-chromosome linked)	[36–43,45–51,56,57,129]
6-Phosphogluconate dehydrogenase (<i>6PGD</i>)	Episodic hemolytic anemia; low NADPH	[31–35]
Pyrimidine 5'-nucleotidase (<i>P5'N-1</i>)	Chronic anemia; secondary to high pyrimidines is the inhibition of G6PD by cytidine 5'-triphosphate, already in RBC progenitors	[52–55]
Glutathione (GSH) synthesis		
γ-Glutamyl–cysteine synthetase (<i>GCL</i>)	(Episodic) mild/moderate hemolytic anemia, favism; low GSH; occasionally systemic disease (mental retardation)	[81–87,107]
Glutathione synthetase (<i>GSS</i>)	(Episodic) mild/moderate hemolytic anemia, favism; low GSH; occasionally systemic disease (mental retardation)	[88–94]
Regeneration of GSH: glutathione reductase (<i>GSR</i>)	Episodic hemolytic anemia, favism; low GSH	[99–105,109]
Superoxide scavenging: superoxide dismutase (<i>SOD1</i>)	Heterozygous: amyotrophic lateral sclerosis, no anemia; complete deficiency not described in humans	[70,71]
Hydrogen peroxide scavenging		
Catalase (<i>CAT</i>)	No hemolytic anemia	[44,73–75]
Glutathione peroxidase (<i>GPX1</i>)	No hemolytic anemia	[72,80]
Methemoglobin reduction: cytochrome <i>b</i>₅ reductase (<i>CYB5R1</i>)	Type I, cyanosis, impaired oxygen delivery; type II, mental retardation and cyanosis	[13,110–113]

Generation of NADH in RBC: defects in glycolysis

NADH is the main reducing agent for keeping Hb in the ferrous state. Glucose breakdown via the glycolytic pathway is the only source of NADH (and ATP) in RBCs. When NADH is used for reduction of methHb, the total flux via glycolysis is enhanced, with pyruvate as the final product [17] (Fig. 1).

Hexokinase type 1 (HK1) is the first enzyme in the glycolytic pathway in RBCs (Fig. 1, point A). In the case of very low HK1 activity, both the generation of NADH and the supply of G6P to the PPP, and thereby the generation of NADPH, will be seriously hampered. HK1 deficiency is a very rare disorder [18,19]. Complete deficiency of HK1 has not been reported, and thus it is likely that some residual activity is vital for survival. Patients with diminished HK activity in their RBCs

Table 2

Cellular and clinical consequences of deficiencies in hemoglobin synthesis leading to increased ROS-inflicted damage to proteins and lipids.

Hemoglobinopathy involved in extra ROS formation	Phenotype	Reference
Thalassemia minor; partial defect in either α or β globin synthesis; iron from excess unpaired globin facilitates ROS generation	Mild microcytic hemolytic anemia; protects against lethal malaria	[115,131,153]
Thalassemia major; no ability to assemble hemoglobin A; unassembled globins denature and facilitate ROS generation	Life-threatening hemolytic anemia; transfusion dependency with danger of iron overload	[116,120,124–128]
Sickle-cell trait	Symptomless normocytic condition; protects against lethal malaria	[114,132,140,141,144,149,151,154]
Sickle-cell disease	Severe vaso-occlusive, hemolytic, and aplastic crisis and splenic sequestration; shortened life expectancy; transfusion dependency with danger of iron overload	[117,121–123,129,130,133–138,145,148]

suffer from mild to severe anemia [18]. Probably, the major cause of anemia is a diminished capacity to produce ATP needed for erythropoiesis and for vital cell functions.

Like HK1 deficiency, other enzyme deficiencies in the glycolytic pathway are rare. They all result in diminished energy supply, with moderate to severe anemia as a result [20]. Methemoglobinemia does not occur, because in these deficiencies generation of NADPH in the PPP is still possible, and thus NADPH cytochrome b_5 reductase activity—if it exists in RBCs [21]—or vitamin C may prevent significant methHb formation.

Pyruvate kinase (PK) deficiency [22,23] leads to an additional hemolytic effect. Because of the metabolic block caused by PK deficiency (Fig. 1, point B), the concentration of 2,3-diphosphoglycerate in the Rapoport–Luebering pathway (Fig. 1, point b2) is increased in the RBC, causing inhibition of 6PGD (Fig. 1, point b3) [24]. The resulting low PPP activity leads to decreased NADPH generation in PK-deficient RBC, which may then contribute to oxidation-related damage and even hemolysis [25].

In conclusion, diminished activity of enzymes in the glycolytic pathway results in variable chronic hemolytic anemia without obvious methHb formation.

Generation of NADPH in RBC: defects in the pentose–phosphate pathway

Continuous reduction of NADP^+ to NADPH is crucial for maintaining high concentrations in red cells of reduced glutathione (GSH) and peroxiredoxins, which serve as reducing agents in all peroxide- and thiol-reducing actions. Therefore, the NADPH-generating pathway is of utmost importance for the reduction capacity of RBCs. NADPH in RBCs can be generated in the PPP only by the transformation of G6P to 6-phosphogluconolactone, catalyzed by G6PD (Fig. 1, point C), and the successive reaction of 6-phosphogluconolactone to ribulose 5-phosphate, catalyzed by 6PGD (Fig. 1, point D). Under steady-state conditions in RBCs, the main flux of G6P is via glycolysis, and only a minor portion is metabolized via the PPP. However, under oxidative stress the flux of G6P through the PPP can be enhanced more than 20 times [26]. The activities of the glycolytic enzymes triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase are sensitive to oxidation and may act as the molecular switch for rerouting G6P to the PPP [27]. Also the availability of NADP^+ is a determinant for PPP activity.

At least four inborn errors in the PPP have been identified. The most common of these results from mutations in G6PD. Extremely rare deficiencies of 6PGD, ribose-5-phosphate isomerase [28], and transaldolase [29] have also been documented. These last two deficiencies affect the metabolism of the pentose sugars by the last part of the PPP in all tissues and lead to a severe clinical picture [30]. However, they have no effect on the formation of NADPH in RBCs and therefore have no hemolytic consequences.

Deficiencies in the activity of G6PD and 6PGD have a major impact on the protection against ROS. For 6PGD only partial deficiencies have been described [31–34], with well-compensated chronic hemolytic anemia and transient hemolytic periods as a result. Because there is still generation of NADPH possible via G6PD and residual 6PGD, these cases sometimes become apparent only in combination with another RBC defect [35].

G6PD deficiency is an X-chromosome-linked disorder, and more than 140 different mutations in the *G6PD* gene have been identified (HGMD: <http://www.hgmd.cf.ac.uk>). Most mutations lead to amino acid substitutions that destabilize the enzyme [36]. This instability of G6PD usually becomes apparent only in the long-living RBCs, which do not have the potential for protein synthesis. In other tissues, renewal of the enzyme can overcome its instability. Nonsense or frameshift mutations that totally prevent the synthesis of G6PD are not known, indicating that at least some G6PD activity is essential for life [37].

The relatively small decrease in lifetime of RBCs in G6PD deficiency [38] suggests that the reduction capacity is still sufficient to prevent the cells from early removal from the circulation under normal circumstances. In G6PD deficiency, as in most cases of hemolytic anemia, phosphatidyl serine (PS) exposure as a sign of early senescence is not detectable [39]. However, rheological studies of G6PD-deficient RBCs have shown a decreased RBC deformability in hemizygous male patients, but not in female carriers [40]. These findings are in concordance with an increased lipid peroxidation seen in G6PD-deficient subjects compared to carriers and controls [40] and indicate that important functions of RBCs can become impaired as a result of lack of protection against ROS.

G6PD deficiency can cause an acute hemolytic crisis during infection-related oxidative stress, after exhaustion of reduction capacity by oxidizing substances such as divicine, a product of fava beans [41] (the phenomenon is called favism), or after use of primaquine (an antimalarial drug) [42]. The lack of NADPH leads to concomitant loss of catalase activity, because catalase is protected from inactivation by bound NADPH [43,44]. Thus, all major scavenging systems are reduced in activity, and the hemolysis stops only when young RBCs, with more G6PD activity and a higher concentration of NADPH, come into the circulation. Hemolysis, jaundice, and kernicterus in the newborn are dangerous consequences of G6PD deficiency. These complications are strongly influenced by additional genetic variations in *AGT1A1* and *SLCO1B1*, which impair hepatic bilirubin clearance [45]. The sensitivity for hemolysis is dependent not only on the G6PD variant but also partly on as yet unknown modifiers [46]. Rare severe instability or decreased activity of G6PD leads to chronic nonspherocytic hemolytic anemia and—as a consequence of the lack of NADPH formation also in leukocytes—to immune disorders [46–51].

G6PD deficiency can also be the secondary result of pyrimidine 5′-nucleotidase (P5′N-1) deficiency, the third most common hereditary enzyme deficiency in RBCs [52,53]. The enzyme is involved

in the catabolism of RNA from young RBCs, and its deficiency leads to high concentrations of pyrimidines inside RBC. This results in marked inhibition of G6PD activity by cytidine 5'-triphosphate [54,55]. Because this process takes place in young RBCs, it may explain the chronic feature of the anemia seen in P5N-1 deficiency, unlike deficiencies that are caused by instability of enzymes, which become manifest later in the RBC life span.

For an extensive review of G6PD deficiency the reader is referred to Cappellini and Fiorelli [56], and for an excellent evidence-based review of medication that can cause hemolytic anemia in G6PD, to Youngster et al. [57]. For malaria resistance in individuals with G6PD deficiency, see Protection against malaria, below.

Reactive oxygen species causing RBC and tissue damage

ROS formation inside RBCs is almost entirely due to metHb formation, but under normal steady-state conditions, the RBC antioxidant systems can cope with this threat. The main ROS produced is $O_2^{\bullet -}$. Because of its charge, the superoxide anion radical itself is not particularly reactive and can even cross membranes via anion channels [14,58].

When $O_2^{\bullet -}$ is dismutated to H_2O_2 and this successively reacts with another $O_2^{\bullet -}$ molecule, the very reactive hydroxyl radical ($OH^{\bullet}$) can be generated via the Fenton reaction ($O_2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$). This reaction is strongly enhanced in the presence of free heme or iron, causing serious damage to cellular proteins and polyunsaturated lipids [59]. Also, $^{\bullet}NO$, which is normally present in RBC [60], can react with superoxide to yield peroxynitrite ($O_2^{\bullet -} + NO^{\bullet} \rightarrow ONO_2^{\bullet -}$), which is also a powerful oxidant. To limit excessive formation of these toxic molecules in times of oxidative stress caused by infectious disease, fever, or ingestion of oxidizing substances, the antioxidant system in RBCs is really challenged.

Protection is needed to prevent loss of cell deformability and preliminary cell aging. Aging in normal RBCs is related to the interaction of Hb with band 3 (the transmembrane chloride/bicarbonate ion-exchange protein) and the generation of free radical species in its vicinity. In RBCs with a higher internal ROS exposure, this aging process is likely to develop earlier. Oxidative modification of band 3 leads to changes in the Ca^{2+} and K^+ homeostasis [61], a less firm interaction of the membrane with the cytoskeleton, caspase activation, clustering of band 3, and formation of neoantigens [62]. Successive membrane loss by vesicle shedding is accompanied by PS exposure on the vesicles and changes in the ligand/receptor interactions of CD47 with SIRP α on spleen macrophages, by means of which the vesicles or damaged cells with impaired deformability are cleared from the circulation [63]. Such controlled removal of dysfunctional RBCs (eryptosis) is normally compensated for by enhanced synthesis of RBCs in the bone marrow and does not result in profound anemia. Only in the case of significantly reduced antioxidative capacity will RBCs become damaged to such an extent that appreciable amounts of Hb, heme, and iron are released into the circulation, which will enhance the formation of extracellular ROS and lead to vascular and RBC damage. This type of chain reaction can subsequently induce massive hemolysis.

Plasma Hb is kept largely in the ferrous (Fe^{2+}) redox state by reducing agents such as vitamin C and urate [16,64,65]. Plasma Hb (Fe^{2+}) facilitates the dioxygenation of $NO^{\bullet}$ to nitrate (NO_3^-), with concomitant formation of metHb(Fe^{3+}) [66]. The lack of $NO^{\bullet}$, subsequent vasoconstriction, and successive hypoxic conditions will enhance the redox cycling by the Fenton reaction [62] and thereby contribute to formation of significant amounts of hydroxyl radicals. Successive catalytic reactions of metHb, ferrylHb(Fe^{4+}), heme, and iron in the microcirculation and in the subendothelial

space may then produce excessive amounts of toxic peroxides and lipid peroxyl species that result in serious cellular injuries [67,68]. Adhesion of injured RBCs to vascular endothelium via the binding of PS presented on the outside of the RBC to PS-scavenging receptors on the endothelial cells also contributes to the vaso-occlusive effect in hemolytic patients [69]. These complex redox reactions of Hb and the effect of aging have recently been reviewed in detail [59].

Roles of SOD, catalase, and peroxiredoxins

Superoxide generated in RBCs is readily converted to hydrogen peroxide by the action of SOD (Fig. 2, bottom). More than 150 mutations in human cytosolic Cu/Zn SOD (SOD1) are known. Heterozygous patients suffer from late-onset, dominant amyotrophic lateral sclerosis, without obvious hemolytic complaints [70]. Fifty percent residual SOD activity can still cope with steady-state superoxide formation in RBCs, but total lack of SOD activity in humans is probably not compatible with life. SOD1 $^{-/-}$ knockout (KO) mice are viable, but they show a decreased RBC life span, reticulocytosis, and splenomegaly. SOD1 $^{+/-}$ mice are indistinguishable in these respects from their wild-type littermates [71].

Catalase is also abundantly present in RBCs. Cohen and Hochstein [72] already showed that under steady-state conditions catalase hardly participates in removing hydrogen peroxide in RBCs (Fig. 2, bottom). Acatlasemia is very rare. The 12 mutations described lead to diminished or absence of activity (HGMD: <http://www.hgmd.cf.ac.uk>). Although progressive gangrene formation due to infection with hydrogen peroxide-producing bacteria [73] in patients with very low catalase activity shows the significance of catalase in removing high doses of hydrogen peroxide, no hemolytic complaints have been documented [74]. Catalase-null mice are viable and show negligible differences in antioxidative functions compared to wild-type mice [75].

Peroxiredoxin 2 (Prdx2) is the major peroxiredoxin present in RBCs. The relatively high concentration of 0.24 mM of this protein, carrying two cysteines, makes it the third most abundant protein present in RBCs [76]. Prdx2 reacts stoichiometrically with low concentrations of peroxides, thus generating a disulfide-linked dimer (Fig. 2, bottom). In this way it acts as a quick sink of limited amounts of endogenously generated peroxides. Under normal conditions, the high level of reduced Prdx2 is maintained by involvement of thioredoxins, thioredoxin reductases, and NADPH as the electron donor. During extensive oxidative stress, the peroxiredoxin system soon becomes exhausted, and the cells are then dependent on the other antioxidative systems [6,77]. Deficiencies of Prdx2 have not been described in humans. However, the importance of the system is indicated by experiments with Prdx2 $^{-/-}$ KO mice [78]. These mice are anemic, and their RBCs have a short life span. Johnson et al. [6] have suggested an extra role for Prdx2 as a chaperone protein, to guide proper folding of Hb during erythropoiesis and to prevent Hb denaturation during the RBC life span [77,79].

Glutathione as the central element for scavenging ROS and repair of ROS-related damage

Glutathione synthesis

GSH is a tripeptide, synthesized from L-cysteine, L-glutamic acid, and glycine (Fig. 2, top left). In two successive steps, γ -glutamylcysteine is first synthesized from glutamate and cysteine by γ -glutamyl-cysteine synthetase (γ GCS), followed by attachment

of glycine to the C-terminus of the dipeptide, catalyzed by glutathione synthetase (GS).

GSH is present at high concentrations (2–10 mM) in RBCs and acts by itself or via glutathione peroxidase as a major reducing source to remove low concentrations of hydrogen peroxide and lipid/alkyl peroxides [2] (Fig. 2, right). Although glutathione peroxidase deficiencies were suggested some 40 years ago to result in hemolytic anemia and childhood seizure, the work of Beutler et al. [80] has made clear that these claims are not valid. Also in cases of deficiency of selenium, an essential cofactor for this enzyme, the resulting very low glutathione peroxidase activity is without hemolytic consequences.

A family with glutathione deficiency in the erythrocytes was first described in 1966 by Prins et al. [81]. The molecular defect in this same family was elucidated later as a γ GCS deficiency [82]. Today, only seven families with six different mutations are known [82–87] to carry this rare autosomal recessive disease, which is characterized by mild to moderate hemolytic anemia and episodes of jaundice. Occasionally, neurological symptoms have been reported.

For GS deficiency, more than 30 mutations in the gene for GS in more than 50 families have been described [88–93]. Depending on the residual enzyme activity, the clinical outcome of patients can vary considerably, from mild hemolytic anemia as the only complaint to moderate anemia with metabolic acidosis, immunologic impairment, and even serious neurological symptoms [94].

Glutathione reactions

In the reduced state, GSH is able to reduce other molecules, such as hydrogen peroxide, lipid peroxides, and disulfides. Because of its relatively high concentration, two molecules of glutathione that have donated a proton and an electron easily combine to form a homodimer of oxidized glutathione (GSSG) (Fig. 2, center). Glutathione can also bind to sulfhydryl groups on red cell proteins, e.g., β -globin [95], phosphofructokinase [96], and spectrin [97], thereby forming heterodimers that interfere with the function of these proteins. Several glutaredoxins are involved in maintaining the reduced state of protein sulfhydryl groups (Fig. 2, top right). Glutathione S-transferases are involved in protein glutathionylation, e.g., for detoxifying xenobiotics (Fig. 2, top). The role of RBCs in this process is not yet clear. Beutler et al. [98] have described a hemolytic patient with low glutathione S-transferase activity in the RBCs, but a direct relation between this deficiency and the hemolytic complaints was not proven.

Glutathione regeneration

Approximately 90% of the glutathione in RBCs is present as GSH. To keep the balance toward this reduced state, glutathione reductase (GR) catalyzes the formation of GSH from GSSG by means of NADPH (Fig. 2, center). GR is a flavonoid protein, and its activity relies on sufficient flavin intake. Alleged GR deficiencies are usually due to shortage of this cofactor and are easily corrected by supplementation of riboflavin in the food [99]. Intermediate to low GR activity poses no risk for developing chronic hemolytic anemia [100]. A high prevalence of an inherited flavin deficiency in RBCs in some areas in Italy where malaria was endemic is suggestive of evolutionary selection. The mechanism for this deficiency in flavin is probably a decreased conversion of dietary riboflavin to FMN, but not related to mutations in GR [101]. Low GR activity may give some protection against malaria [102,103] (see also Protection against malaria).

Hereditary GR deficiency was first described in 1976 in three children from a consanguineous marriage [104]. This family had a homozygous mutation in *GSR*, the gene for glutathione reductase.

Two other mutations in this gene were found in another patient with GR deficiency [105]. The first family [104] had a total lack of GR activity and was suffering from favism and juvenile cataract, but was without chronic anemia. The other patient, with some residual GR activity in the leukocytes, had severe neonatal jaundice.

The complete inability to regenerate reduced glutathione via GR activity in all tissues and the relatively mild clinical condition associated with it, as was the case in the first GR-deficient family, suggest redundancy in the formation of at least some reduced glutathione, e.g., by de novo synthesis or by recycling via the peroxiredoxin system [106]. In contrast, in the case of incapability to synthesize glutathione, the clinical outcome can be very severe and even lead to mental retardation [107], although the reported effect on RBCs is limited to moderate hemolytic anemia.

These conclusions are in agreement with the results obtained from mouse KO models for GSH deficiency. Such studies have revealed that enzymes involved in the biosynthesis of glutathione are nonredundant and that clinical severity is related to the residual activity of γ GCS and GS, more than to the cellular GSH content. A total deficit of activity of one of these enzymes is lethal in the early embryonic stage [108]. Genetic KO mice for GR, with a complete inability to regenerate reduced glutathione, had a normal viability [109].

Methemoglobin and regeneration of Fe^{2+} Hb by cytochrome *b*₅ reductase (NADH methemoglobin reductase)

Methemoglobinemia can be acquired by exposure to exogenous oxidizing compounds, e.g., nitrate in well water, even in individuals with uncompromised reduction capacity. Newborns are especially vulnerable because of their low cytochrome *b*₅ reductase activity [110]. Acquired methemoglobinemia is transient, because cytochrome *b*₅ reductase can reduce the metHb back to Fe^{2+} Hb as soon as the oxidizing source has been removed. However, in the rare situation of a genetic deficiency of cytochrome *b*₅ reductase, a constant high level of metHb exists. In these cases, exogenous oxidative challenge can lead to concentrations of metHb above 25% of all Hb, which seriously hampers oxygen delivery to the tissues. MetHb concentrations higher than 70% are life threatening [111].

To date 55 different mutations in the *CYB5R3* gene for cytochrome *b*₅ reductase have been described (HGMD: <http://www.hgmd.cf.ac.uk>), without signs of a founder effect [13,112]. The effect of mutations leading to an unstable enzyme is restricted to RBCs (type I), whereas mutations that lead to low expression or low activity affect functionality in all tissues (type II) and also give rise to mental retardation.

In addition to autosomal recessive inherited cyanosis due to mutations in *CYB5R*, rare M group variant hemoglobins also exist that spontaneously oxidize and cause methemoglobinemia in a dominant fashion [113]. Other than cyanosis no serious anemic complaints have been described for this patient group.

Hemoglobinopathies and thalassemias

Clinical consequences in people with the sickle-cell trait (heterozygous HbS) have rarely been reported, and its relation to mild disease is still under debate [114]. Carrier states for thalassemia (thalassemia minor) result in mild anemic conditions owing to impaired globin synthesis and ineffective erythropoiesis. The impact of enhanced ROS formation on erythropoiesis in these patients has not yet been fully elucidated [115,116]. The severe clinical conditions of sickle-cell disease (SCD; homozygous HbS) or thalassemia major (homozygous β -thalassemia or inactivity of

three or four α -globin genes) are obvious and often life threatening unless regular blood transfusions are given. The vaso-occlusion in SCD is the result not only of cell sickling, but also of oxidative stress-inflicted hemolysis, increased adhesion of RBCs to endothelial cells, vasoconstriction, increased coagulant activity, and successive immune responses by leukocytes [117,118]. Kidney insufficiency and successive end-stage renal failure in SCD is thought to be an example of a process in which cell-free ferrous Hb dioxigenates NO^* to nitrate. The subsequent vasoconstriction, due to the lack of NO^* , will then result in an ischemic condition, in which the free Hb can form the very reactive oxoferryl species that are harmful for the microvasculature and glomerular tissue [119].

Nutritional deficiencies and exhaustion of vitamins and trace minerals may contribute to aggravation of oxidative damage in hemoglobinopathies [10,120]. Clinical trials of oral glutamine therapy in SCD have demonstrated both improvement of redox state and clinical outcome [121], most likely by its preservation of NADPH levels [122]. The results of a pilot study in which SCD patients were treated with oral *N*-acetylcysteine suggest a reduced SCD-related oxidative stress [123].

In thalassemia, ROS formation mediated by iron originating from imbalanced globin production and regular blood transfusions can cause systemic tissue damage. Administration of iron chelators facilitates the excretion of the potentially harmful iron and has a strong beneficial effect [124]. Supplementation of vitamin E also improves the redox state of plasma and RBC contents [125] but does not significantly lower the need for transfusions [126]. Supplementation of thalassemia patients with *L*-carnitine, a quaternary ammonium compound with antioxidant properties and involved in intercellular lipid transport, has been reported to improve the hematologic parameters [127] as well as the RBC rheology and lipid reduction [128]. Although none of these antioxidants seems to be the magic bullet to cure SCD- or thalassemia-related disease, it is clear that they have beneficial effects by preventing oxidative damage due to iron-related ROS formation.

Surprisingly, patients with combined SCD and G6PD deficiency, in which radical formation may be expected to be even less manageable than in SCD, had clinical outcomes similar to those of patients with SCD alone [129,130]. Combined β -thalassemia minor and G6PD deficiency is seen quite often in some populations. Although this combination leads to very low NADPH levels in RBCs [131], an enhanced risk for severe hemolysis has not been reported. Apparently, changes in clinical severity are likely to be masked by enhanced erythropoiesis [131].

Protection against malaria

The most frequently encountered genetic disorders in humans are Hb mutants S, C, and E, together with α - and β -thalassemias, G6PD deficiency [45], and Southeast Asian ovalocytosis (caused by mutations in band 3). All of these mutated proteins are expressed in RBCs, and the mutations are prevalent in areas in which malaria is endemic. Most likely, therefore, these genetic disorders confer protection against parasite growth and development inside RBCs. In this way, these mutated genes are fixed at high frequency in the susceptible population because the advantage of protection against malaria in heterozygotes outweighs the disadvantage of RBC dysfunction in the homozygotes or compound heterozygotes. However, the mechanism of protection against malaria is still a matter of debate [132].

RBC membrane oxidation occurs in homozygous HbS (HbSS) RBCs, as evidenced by excessive lipid peroxidation and abnormal thiol oxidation [133–136]. This may be caused by the polymerization of HbS under low oxygen pressure, because heme and hemichrome as well as iron deposition are found in HbSS RBC membranes [137,138]. Infection of normal HbAA RBCs with *Plasmodium*

falciparum also leads to RBC membrane oxidation, as deduced from α -tocopherol and polyunsaturated fatty acid decreases in the membrane [139]. It is to be expected, therefore, that infection of HbAS or HbSS RBCs with *P. falciparum* leads to enhanced RBC membrane oxidation, because this infection causes increased HbS polymerization, probably due to increased oxygen consumption by the parasite [140–142]. This strong oxidative stress can either directly inhibit parasite growth and development or enhance uptake of infected RBCs by spleen macrophages, or both. Phagocytosis by macrophages is induced by a combination of hemichrome formation, aggregation of band 3 protein on the RBC surface, and opsonization by autologous IgG antibodies and complement factor 3 fragments [143].

Ferreira et al. [144] have shown in a model of sickle-cell trait in mice that upregulation of heme-degrading activity by heme oxygenase-1 (HO-1) in macrophages and endothelial cells, and the consecutive formation of carbon monoxide, constitutes an immunosuppressive mechanism preventing further formation of free heme [145,146]. It thereby protects against the lethal complications of malaria infection. Because HO-1 upregulation has been recognized as a general response to oxidative stress [147], it probably plays the same role in other cases of mild chronic hemolytic anemia, such as G6PD deficiency and thalassemia. The concept of protection against lethal malaria infection by HO-1 upregulation in plasma and successive immune suppression via carbon monoxide fits with the concept of anti-malaria prophylaxis by drugs that produce a mild oxidative stress: their effectiveness may also depend on the mechanism of HO-1 upregulation [147].

Parasitized RBCs adhere to the endothelial cells deep within postcapillary beds. This leads to obstruction of the microcirculation and results in dysfunction of multiple organs, typically the brain in cerebral malaria. *P. falciparum* induces RBC adherence to the endothelial cells of the blood vessels by means of a number of parasite-encoded proteins exported into the host cell. Some of these are exposed on the RBC surface and function as ligands for endothelial cytoadherence receptors, such as CD36 and ICAM-1 [148–150]. HbAS RBCs, but also HbAC RBCs and α -thalassemia RBCs, have reduced expression and uneven distribution of *P. falciparum* erythrocyte membrane protein-1, one of these ligand proteins, thus causing less cytoadherence [151–153]. Finally, also, microRNAs produced by *P. falciparum*-infected HbAA RBCs and in increased amounts by uninfected and infected HbSS and HbAS RBCs inhibit the growth rate of the parasite. These microRNA species are linearly integrated into key parasite mRNAs, thereby inhibiting their translation [154].

Concluding remarks

In general, a sustained but limited enhanced oxidative stress in RBCs (owing to either liberation of free heme and iron or low activity of protective mechanisms) has only a minor effect on RBC survival. However, such mild oxidative stress can irreversibly lead to functional changes, such as membrane loss and successive impaired rheologic properties. Heme degradation inside the cell will promote RBC repair and controlled removal of the RBC by spleen macrophages. Irreversibly damaged RBCs promote vascular injury through adhesion to microvascular endothelial cells and successive hemolysis. Depending on the extent of oxidative stress and the remaining capacity of the cellular reducing potential, such a hemolytic process can enhance its own ROS formation, bring harm to the surrounding tissue, and lead to anemic episodes. Mild RBC defects with limited hemolysis and not resulting in serious clinical complaints provide tolerance for lethal malaria infection and are relatively common in some populations. Most of the rare enzyme deficiencies discussed in this review interfere with protection mechanisms against oxidative damage and are not evolutionarily beneficial, because they also affect

the energy supply, are not restricted to RBCs, have an impact on erythropoiesis, or cause serious hemolytic complaints.

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