Review Article

Oxidative stress in β-thalassaemia and sickle cell disease

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ABSTRACT

Sickle cell disease and β-thalassaemia are inherited haemoglobinopathies resulting in structural and quantitative changes in the β-globin chain. These changes lead to instability of the generated haemoglobin or to globin chain imbalance, which in turn impact the oxidative environment both intracellularly and extracellularly. The ensuing oxidative stress and the inability of the body to adequately overcome it are, to a large extent, responsible for the pathophysiology of these diseases. This article provides an overview of the main players and control mechanisms involved in the establishment of oxidative stress in these haemoglobinopathies.

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Contents

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1. Introduction

Sickle cell disease (SCD) and β-thalassaemia are hereditary autosomal recessive red cell disorders and major causes of morbidity and mortality worldwide [200]. Their high prevalence is attributed to the resistance they provide, in the heterozygous state, to malaria infection [4]. It is estimated that 300,000–500,000 infants are born annually with a β-globin disorder and 50,000–100,000 affected children die each year in low and middle income countries [200].

In SCD a single amino acid substitution, from glutamic acid to valine at position 6 of the β-globin protein results in the formation of sickle haemoglobin (HbS), which has the propensity to polymerise under conditions of low oxygen saturation, such as in the microcirculation. This in turn leads to the deformation of erythrocytes containing the HbS polymers, which can block blood vessels in the microcirculation impairing oxygen delivery to tissues. A subsequent series of complications, such as pain crises, pulmonary hypertension and heart failure, comprise the characteristic symptoms of the disease [172].

β-thalassaemia is a highly heterogeneous group of genetic defects leading to decreased or absent β-globin production. More than 200 β-thalassaemia mutations have been currently identified affecting transcriptional and post-transcriptional processes (http://www.ithanet.eu) [1]. Insufficient β-globin production results in the accumulation and precipitation of unpaired α-globin chains, leading to haemolysis and ineffective erythropoiesis, which give rise to severe anaemia and a series of secondary complications, such as skeletal abnormalities, splenomegaly and growth defects [172].

Fig. 1. (A) Intracellular oxidative events. (1) Oxidative denaturation of Hb. It results in the production of ROS, free haeme and iron. Iron acts as a Fenton reagent in the Haber–Weiss reaction for the generation of hydroxyl radical. Haeme promotes oxidation reactions and a proinflammatory effect by activating NF-κB. (2) Enzymatic generation of superoxide by NADPH oxidase. NADPH oxidase is regulated by intra- (e.g. increased Ca^{2+}) and extra-cellular (pro-inflammatory cytokines) signals that are activated by ROS. (3) ROS and ROS-induced increase of intra-cellular Ca^{2+} activate caspase-3, which partially degrades band-3, affecting its interaction with the cytoskeleton. ROS and increased Ca^{2+} also activate the KCC and Gardos channel respectively, resulting in increased exit of K^+ from the cell. (4) Haemichromes mediate the oxidative crosslinking and phosphorylation of band-3 leading to band-3 clusterisation and dissociation from cytoskeletal proteins. This results in membrane blebbing and microparticle generation. Band-3/haemachrome clusters are recognised by anti-band-3-NAbs. (5) ROS promote oxidation of protein 4.1, actin and spectrin resulting in impaired interaction. (6) PS exposure results from ROS-induced disruption of normal membrane organisation. (B) Protective mechanisms in RBCs and the circulation. (1) Haptoglobin/Hb and haemopexin/haeme complexes are endocytosed by macrophages. Haeme is then degraded by HO-1 releasing biliverdin, CO and iron. Iron is then taken up by ferritin. (2) Antioxidant enzymes and molecules in RBCs. (3) Stress-response mechanisms in RBCs. FOXO3, HRI/eIF2α/ATF4 and NRF2 are oxidant-response pathways that regulate the expression of antioxidant genes. FOXO3 and HRI/eIF2α/ATF4 are important for terminal differentiation. PRDX2 is a chaperone of Hb and binds free haeme to prevent its oxidative actions. AHSP binds α-globin in the absence of β-globin and in the presence of oxidant insults. (4) Protein quality control pathways. Unpaired α-globins are selectively degraded via ubiquidin-mediated proteasomal degradation (UPS). When UPS becomes overwhelmed, α-globin aggregates are degraded via aggresome-mediated macroautophagy.

2. Sources of oxidant production in red blood cells

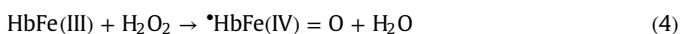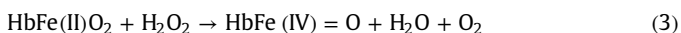### 2.1. Oxidative denaturation of haemoglobin

Although oxygenated haemoglobin [Hb(FeII)O$_2$] is considered a relatively stable molecule, it can physiologically autoxidize to methaemoglobin [Hb(FeIII)] (Eq. (1)) at a rate ∼0.5–3% per day (Fig. 1A) [193]. Autoxidation is most pronounced under hypoxic conditions (e.g. in the microcirculation) and for unstable haemoglobins (Hb) such as HbS or free α-globin chains [169]. Autoxidation is almost entirely responsible for reactive oxygen species (ROS) generation inside red blood cells (RBCs) [195]. In SCD and β-thalassaemia RBCs, Hb autoxidation is more pronounced as the Hbs molecules in these diseases are highly unstable [169].

$$HbFe(II)O_2 \rightarrow HbFe(III) + O_2^{2-} \tag{1}$$

The superoxide ions ($O_2^{\bullet-}$) produced by Hb autoxidation are rapidly converted to hydrogen peroxide (H$_2$O$_2$) either by spontaneous or enzyme-driven (superoxide dismutase) dismutation (Eq. (2)) [169,81].

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{2}$$

H$_2$O$_2$ can react with both oxyHb Eq. (3) and metHb (Eq. (4)) producing ferryHb [HbFe(IV)=O] (and oxyferryHb [$^{\bullet}$HbFe(IV)=O] respectively) [169,81].

$$HbFe(II)O_2 + H_2O_2 \rightarrow HbFe(IV) = O + H_2O + O_2 \tag{3}$$

$$HbFe(III) + H_2O_2 \rightarrow {}^{\bullet}HbFe(IV) = O + H_2O \tag{4}$$

FerryHb and oxyferryHb can subsequently react with H$_2$O$_2$ producing metHb (Eqs. (5) and (6)) [169,81].

$$HbFe(IV) = O + H_2O_2 \rightarrow HbFe(III) + O_2^{\bullet-} + H_2O \tag{5}$$

$${}^{\bullet}HbFe(IV) = O + H_2O_2 \rightarrow HbFe(III) + O_2 + H_2O \tag{6}$$
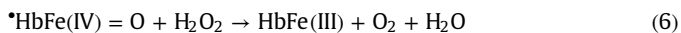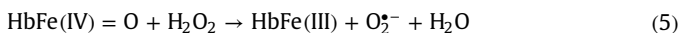
It has been noted that haeme has a lower affinity to metHb than oxyHb, and this results in increased dissociation of haeme [35]. In vitro studies have shown that H$_2$O$_2$ can react with free haeme with subsequent haeme degradation and iron release [130]. In addition, the reaction of ferryHb with H$_2$O$_2$ (Eq. (5)) results in the production of $O_2^{\bullet-}$ within the haeme pocket, which promotes haeme degradation and the subsequent release of haeme degradation products and iron [Fe(III)] [130].

2.2. Haeme and iron as oxidising molecules

Haeme and iron are highly oxidising agents. In fact iron, either in free form or bound to haeme and Hb, can act as a Fenton reagent in the Haber–Weiss cycle, which generates the highly reactive hydroxyl radical ($^{\bullet}$OH) (Eq. (7)) (Fig. 1A) [171]. Unlike $O_2^{\bullet-}$, hydroxyl radicals cannot be eliminated enzymatically and can, therefore, promote extensive oxidative damage [166]. Iron chelators have been shown to reduce lipid peroxidation in RBC membranes suggesting that ROS generation and oxidant damage are partially dependant on iron [178]. Reducing iron availability has also been shown to lessen the deposition of α-globin aggregates which leads to reduction of ineffective erythropoiesis in β-thalassaemia [105].

$$H_2O_2 + Fe(II) \rightarrow {}^{\bullet}OH + OH^- + Fe(III) \tag{7}$$
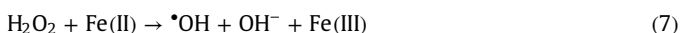
Haeme is a hydrophobic molecule that can interact with membrane lipids and proteins promoting oxidation reactions (Fig. 1A) [22]. Haeme has the ability to activate the redox sensitive transcription factor nuclear factor-κB (NF-κB) and promotes a

proinflammatory effect by binding to receptors, enzymes and transcription factors and alters cell function, metabolism and gene expression (Fig. 1A) [199]. Moreover, haeme can oxidatively denature DNA, activate caspases and cathepsins, impair mitochondrial function and affect the activity of antioxidant enzymes, such as glutathione reductase [132,169,191].

2.3. Enzymatic generation of ROS in SCD RBCs

Interestingly, a recent study by George et al. [63] has shown that in SCD RBC apart from Hb autoxidation and the Fenton reaction, ROS can also be generated enzymatically by NADPH oxidase (Fig. 1A). NADPH oxidase activity seems to be regulated by PKC, Rac GTPase and Ca^{2+} signalling inside RBCs, and is increased by TGFβ1 and endothelin1 cytokines, which show increased activity in SCD [63].

3. Intracellular oxidative events

3.1. Phospholipid asymmetry, phosphatidylserine exposure and eryptosis

Partial oxygenation of Hb in the microcirculation results in Hb conformational changes that are not only responsible for increased autoxidation [19] but also for increased affinity to the plasma membrane [37]. Therefore, partially oxygenated Hb, especially if it is unstable, is responsible for the majority of ROS generated at the RBC membrane [125]. However, ROS located at the plasma membrane are not readily accessible to the cytoplasmic antioxidant system [125] and can easily oxidise membrane lipids and proteins, causing extensive oxidative damage [24]. This can be further exacerbated in SCD and in β-thalasssemia RBC where increased and continuous production of ROS as a result of enhanced Hb autoxidation rapidly overwhelms the limited membrane antioxidants (e.g. such as vitamins E and C, glutathione peroxidase and peroxiredoxin-2) available [122,128,129,42].

Lipid peroxidation and protein oxidation result in loss of membrane lipid organisation and cellular deformability [24,95]. Cellular deformability is very important for RBC function as these have to deform and squeeze through the capillaries in the microcirculation to efficiently transport oxygen to tissues [158]. Additionally, the resulting disruption of the physiological asymmetry of phospholipids causes phosphatidylserine (PS) exposure to the outer membrane (Fig. 1A) [76]. PS is a signal for cell removal recognised by scavenging receptors in macrophages, which engulf and degrade the PS-exposed RBCs [88]. PS-exposed RBCs have been observed in both SCD and β-thalassaemia [46,76]. Another factor that leads to abnormal PS exposure in SCD is the repeated cycles of sickling and unsickling, which disturb the membrane phospholipid asymmetry and result in microvesicle formation [8].

PS exposure and subsequent macrophage degradation is the mechanism of RBC removal during eryptosis. Eryptosis is the suicidal RBC death, which occurs prior to senescence and after injury of RBCs. It is characterised by cell shrinkage and loss of membrane organisation and can be stimulated by oxidative stress, energy depletion, increased Ca^{2+} concentration, caspases and disrupted activity of several kinases like PKC and p38 MAPK [96], factors that are observed in SCD and β-thalassaemia.

3.2. Haemichrome formation, band-3 oxidation and senescence

Modifications in structure and function of membrane components are also caused by the direct precipitation of denatured haemoglobins or globin chains. SCD and β-thalassaemia are characterized by unstable haemoglobin molecules. In β-thalassaemia

instability is attributed to the unbalanced production of globin chains resulting in excess free α-globins, whereas in SCD the mutation that affects the tertiary and quaternary structure of HbS renders it easily denatured. Unstable haemoglobins tend to autoxidize, denature and precipitate as haemichromes [149]. Haemichromes are structures that consist of all the intermediate products of haemoglobin oxidative denaturation, starting from metHb until complete dissociation and release of haeme and iron [160].

Haemichromes have a high affinity for the cytoplasmic domain of band-3 and mediate the oxidative crosslinking through disulphide bonds (Fig. 1A) [116]. Upon band-3 oxidation tyrosine kinases, activated by oxidative stress, phosphorylate the cytoplasmic domain of band-3 [149,32]. This results in band-3 clusterization and dissociation from cytoskeletal proteins through disruption of ankyrin binding [116,59]. The resultant membrane blebbing and microparticle generation are important characteristics of SCD and β-thalassaemia [201].

A function of band-3 is to bind glycolytic enzymes and organise them on the membrane thus regulating the flux of glucose between glycolysis and the PPP. Therefore, the dislodgement of glycolytic enzymes from oxidised band-3 results in the activation of glycolysis and reduced NADPH production from the PPP [71]. NADPH is an important reducing agent of GSH and peroxiredoxin thus offering protection against oxidative stress [30]. Consequently, reduction in the levels of NADPH decreases the antioxidant capacity of RBCs and further exacerbates oxidative stress.

Moreover, naturally occurring anti-band-3 antibodies (NAbs) have a high affinity for the band-3/haemichrome clusters. Upon binding to the clusters they activate complement and trigger RBC clearance by macrophages (Fig. 1A) [111,116]. Anti-band-3 NAbs binding and complement activation are implicated in the removal of senescent RBCs and are also seen in haemolytic disorders such as SCD and β-thalassaemia [116,82].

3.3. Oxidative stress and the cytoskeleton

Oxidative stress has an effect not only on membrane but also on cytoskeletal proteins. Shinar et al. [180] demonstrated that in β-thalassaemia patients protein 4.1 exhibits a significant reduction both in its ability to increase the binding of spectrin to actin but also in its own ability to bind to spectrin. Spectrin is a major target of ROS and its oxidation results in disruption of its interaction with other cytoskeletal proteins like actin or with proteins that bridge the membrane and the cytoskeleton such as protein 4.1 (Fig. 1A) [180]. This leads to impaired stability in the interaction between membrane and cytoskeleton and thus increased fragility. In addition, when β-thalassaemia RBC membranes were resolved by gel chromatography, a-globin chains were co-purified with cytoskeletal proteins such as spectrin, actin and protein 4.1 [179]. Furthermore, α-globin aggregates were shown to colocalize with regions of defective association of spectrin and protein 4.1 in β-thalassaemia erythroblasts (Fig. 1A) [7]. This may result in interruption of the normal assembly of the cytoskeletal proteins and also in their oxidation as α-globin aggregates tend to easily autoxidise.

Oxidation of spectrin, actin and protein 4.1 has been observed in SCD RBCs [175,67]. Additionally, George et al. [64] have demonstrated increased phosphorylation of adducin at Ser-726, a target of PKC, in SCD RBCs [64]. The phosphorylation of adducin is known to result in dissociation of spectrin from F-actin. PKC, whose activity is increased in SCD regulates the activity of NADPH oxidase in SCD RBCs resulting in increased ROS production [63].

3.4. ROS as signalling molecules and their involvement in haemolysis

ROS not only function as oxidising molecules but are also able to modulate the activity of transcription factors, membrane channels, and metabolic enzymes and thus integrate into signalling pathways [104], possibly through oxidation of protein thiols [143]. Caspase-3 activation has been linked to increased ROS production in RBCs [119,43]. Caspase-3 is normally produced during erythropoiesis and cleaves GATA-1, Tal-1 and proteins involved in cytoskeletal and DNA integrity, initiating a cell death programme when erythropoietin (EPO) levels are low [212,51]. ROS-activated caspase-3 leads to partial degradation of band-3, affecting its interaction with cytosolic proteins and the cytoskeleton (Fig. 1A) [113]. The resulting impairment of membrane structure can lead to PS exposure [114]. In addition, oxidative stress has been shown to result in inhibition of the Ca^{2+}-ATPase pump in red blood cells [88] and the activation of Ca^{2+}-permeable unselective cation channels with subsequent increase in intracellular Ca^{2+} (Fig. 1A) [60]. This leads to activation of calpain, transglutaminase-2 and caspases, which can degrade cytoskeletal proteins and decrease cell stability [164]. Moreover, increased intracellular Ca^{2+} has been shown to inhibit phosphotyrosine phosphatase thus increasing band-3 phosphorylation [215], and also to reduce flippase activity leading to membrane scrambling and PS exposure [36]. Elevated Ca^{2+} has also been shown to result in activation of the Gardos channel with subsequent exit of K^+ (Fig. 1A). These changes in cation homoeostasis lead to water removal and therefore cell shrinkage [137,204,23], which is a characteristic of eryptosis [96]. Oxidative stress has also been found to affect the activity of the K–Cl co-transporter (KCC) in β-thalassaemia RBCs (Fig. 1A). The increased KCC activity results in enhanced exit of K^+ from RBCs [142,50]. However increased exit of K^+ can dissipate the K^+ gradient across the membrane and lead to membrane depolarisation. This results in entry of Cl^- with osmotically obliged water and subsequent cell swelling, cell membrane disruption and haemolysis [97].

As previously mentioned, in SCD and β-thalassaemia signs of increased senescence and eryptosis can be observed. However, in cases of extensive oxidative injury RBCs will become damaged to such an extent that they will not be removed by a controlled mechanism such as senescence or eryptosis but will be haemolysed [195]. Increased haemolysis has been seen in both SCD and β-thalassaemia [176,39].

3.5. Oxidative stress and ineffective erythropoiesis

Ineffective erythropoiesis is well documented in β-thalassaemia and is attributed to the presence of excess unpaired α-globins [168]. Unpaired α-globins have the tendency to precipitate and autoxidise producing high amounts of ROS, free haeme and iron [169]. As previously mentioned, these molecules can promote oxidation of membrane lipids and proteins resulting in band-3 clusterisation and PS exposure [116,76]. These are signs of senescence and eryptosis of RBCs, which are then removed by macrophages. Several studies have shown that there is an increased number of activated macrophages in the bone marrow of β-thalassaemia patients,indicative of enhanced senescence and eryptosis of erythroid precursors (Fig. 2) [11,38]. Interestingly, Wickramasinghe and Bush [203] have demonstrated the presence of α-globin precipitates in cells at the polychromatophilic erythroblast stage of maturation [203], which is the stage of increased haemoglobinisation and apoptosis in β-thalassaemia erythropoiesis (Fig. 2) [120].

Recently, Dussiot et al. [53] have shown that growth differentiation factor 11 (GDF11), a TGFβ superfamily ligand, blocks terminal differentiation of erythroid precursors in a mouse model of β-thalassaemia intermedia by promoting oxidative stress and α-globin precipitation. In addition, GDF11 induces apoptosis of early erythroblasts by inhibiting the Fas–Fas ligand pathway. More
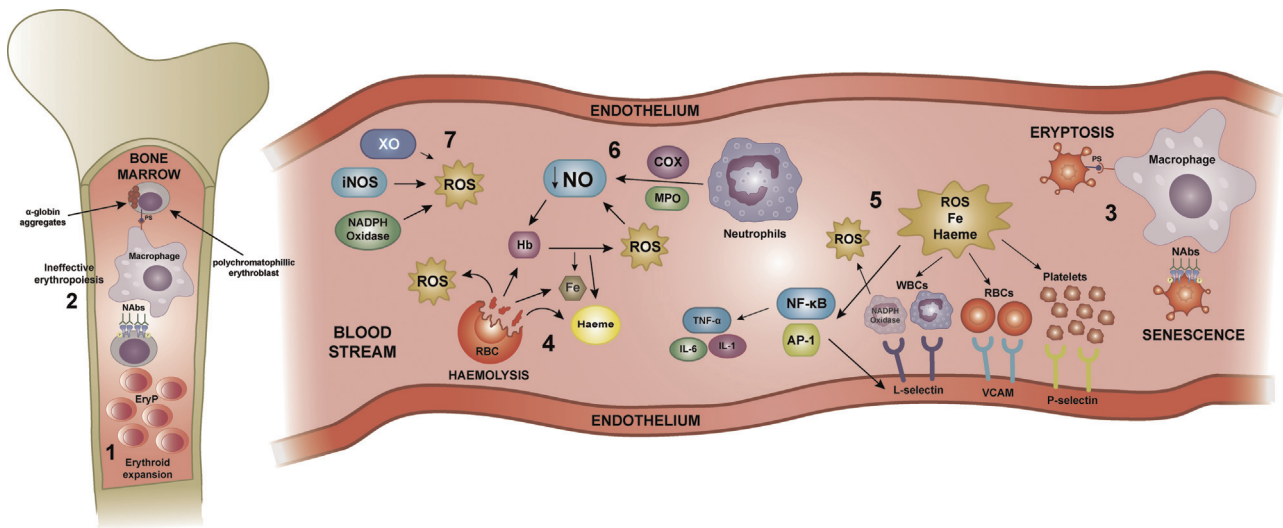
Fig. 2. Oxidative events in the bone marrow and circulation. (1) Erythroid expansion. (2) Ineffective erythropoiesis. (3) Endocytosis of RBCs by macrophages through two different mechanisms: eryptosis and senescence. (4) Haemolysis. It leads to Hb release in the plasma, which subsequently autoxidises producing ROS, free haeme and iron. (5) ROS, free haeme and iron intercalate into plasma membranes producing oxidative damage to the endothelium and haematopoietic cells. ROS and haeme activate NF-κB and AP-1, which increase the production of pro-inflammatory cytokines (IL-1, IL-6, TNFα) and adhesion molecules on the endothelium. This increases the adhesion of RBCs, leucocytes and platelets to the endothelium. Activated leucocytes generate more ROS by their NADPH oxidase, creating a vicious cycle of oxidative stress and inflammation. (6) Reduction of NO bioavailability due to free Hb, ROS, and neutrophil activation, (7) Increased expression of plasma and endothelial enzymes (e.g. xanthine oxidase, NADPH oxidase and iNOS) following I/R gives rise to more ROS.

importantly, GDF11 expression is induced by oxidative stress, indicating the presence of an autocrine amplification loop that promotes ineffective erythropoiesis [53].

Ineffective erythropoiesis has also been observed in human and mouse SCD [208,31]. Blouin et al. [31] have demonstrated that the late precursors (polychromatophilic normoblasts to reticulocytes) of a transgenic SCD mouse model presented altered morphology and increased fragmentation, which were attributed to the increased HbS polymerisation [31]. Interestingly, they suggested that the latter happens during medullary migration of reticulocytes to the periphery. Although there is little published information regarding the effects of oxidative stress on SCD ineffective erythropoiesis, the afore-mentioned study has attributed the observed ineffective erythropoiesis to the increased HbS polymerisation, which is known to be associated with enhanced autoxidation and ROS generation [169].

3.6. ROS implication in erythroid expansion and accelerated differentiation

In β-thalassaemia, ineffective erythropoiesis and haemolysis give rise to chronic anaemia, which results in tissue hypoxia and subsequent increase in EPO production. This in turn leads to enhanced extramedullary and bone marrow erythropoiesis, which is characterised by increase erythroid proliferation and accelerated differentiation (Fig. 2) [120,38]. A recent study has implicated ROS in the increased proliferation and differentiation of β•-thalassaemia/HbE erythroid precursors [103]. Leecharoenkiat et al. [103] suggested a model where an increase in aerobic respiration would result in increased levels of ATP. This could lead to an increase in Ca^{2+} intracellular levels possibly through activation of Ca^{2+} pumps. High Ca^{2+} levels stimulate oxidative phosphorylation resulting in further increase of ATP. ATP can directly or indirectly, through cAMP or Ca^{2+}, activate several ERK1/2 effectors such as PKA, PKB, and PKC. ROS, which are by-products of aerobic metabolism, are also able to activate these effectors, which in turn can increase proliferation and differentiation both directly or indirectly via ERK1/2 phosphorylation. Increased erythroid proliferation and differentiation lead to increased metabolic rates

creating a feedback loop. Therefore even a small initial imbalance in ROS could rapidly result in a significant negative outcome. This imbalance could be due to increased autoxidation of unstable Hbs or due to increased iron levels, both known to occur in β-thalassaemia [103].

Increased erythroid expansion has also been observed in SCD with spleen, rather than of bone marrow, being the predominant erythropoietic organ [85]. In fact, Kean et al. [85] have demonstrated that there is much higher erythroid hyperplasia in SCD mice compared to β-thalassaemic animals, having almost 3 fold more RBCs in their erythropoietic organs, mainly in the spleen. In addition, Blouin et al. [31] have observed in a transgenic SCD mouse model increased mobilisation of erythroid progenitors from the bone marrow to the periphery and subsequently to the spleen for immediate maturation [31]. Although, there are no publications on the effects of oxidative stress on the increased erythroid proliferation in SCD, the above model can also be supported by SCD, as enhanced autoxidation of HbS can promote excessive ROS production [169].

4. Protective mechanisms in rbcs

4.1. Antioxidant enzymes and molecules

Energy production in RBCs is dependant on the anaerobic degradation of glucose through the glycolytic pathway and subsequent lactate production as RBCs are devoid of mitochondria [30]. NADH produced by glycolysis is the main reducing agent of metHb, maintaining Hb in the ferrous state. The reaction is catalysed by the NADH-dependant cytochrome b_5 reductase enzyme [153]. The increased production of metHb in SCD and β-thalassaemia may thus result in enhanced glycolysis due to increased NADH demand [140].

Glucose-6-phosphate is used not only during glycolysis but also in the pentose phosphate pathway (PPP) for the reduction of $NADP^+$ to NADPH (Fig. 1B) [30]. NADPH is the reducing agent used by glutathione reductase for the reduction of oxidised glutathione (GSSG) to two molecules of glutathione (GSH). GSH is an

important antioxidant molecule of the glutathione cycle and it is used by several enzymes as a reducing agent (Fig. 1B). Glutathione peroxidase and peroxiredoxin generate GSSG during the reduction of peroxides (e.g. H_2O_2) and organic hydroperoxides (ROOH), whereas glutaredoxin uses GSH as a cofactor to reduce oxidised proteins and ascorbate. Additionally, glutathione-S-transferase utilises GSH to detoxify xenobiotics [115,195].

Thioredoxin is another antioxidant enzyme in RBCs, which reduces oxidised proteins and acts as electron donor to peroxiredoxin (Fig. 1B). It is kept in its reduced form by thioredoxin reductase, which uses NADPH as the reducing agent [12,195]. Superoxide dismutase is the enzyme that converts $O_2^{\bullet-}$ to H_2O_2, and subsequently catalase neutralises H_2O_2 to water and oxygen (Fig. 1B) [195]. Finally, RBCs can take up large amounts of vitamin C and vitamin E as they are used to protect against membrane oxidative damage [122,42].

In SCD and β–thalassaemia, the antioxidant enzymes and molecules are significantly decreased, although no defects in their expression and metabolism have been demonstrated. Instead, the increased and continuous production of ROS overwhelms the antioxidant machinery very rapidly [167].

4.2. Cytoprotective mechanisms during normal erythropoiesis and oxidative stress conditions

An interesting study by De Franceschi et al. [48] has proposed the presence of two stress-response cytoprotective mechanisms in β-thalassaemia erythropoiesis. ROS and haeme have been shown to reduce the activity of δ-aminolevulinate synthase-2, which is the rate limiting enzyme in the haeme production pathway. In addition, ROS seem to increase the expression of peroxiredoxin-2 (PRDX2) (Fig. 1B), which has a multifactorial role inside RBCs [48]. PRDX2 is mainly an antioxidant enzyme and reduces peroxides and thiols [141]. PRDX2 can translocate to the membrane through binding to the cytoplasmic domain of band-3 [121,154], probably to protect band-3 and its associated proteins from oxidative damage [49]. Haemichromes seem to prevent this translocation by masking the docking site for PRDX2 [49]. PRDX2 has also been shown to function as a molecular chaperone of Hb, both during erythropoiesis by maintaining proper Hb folding and after maturation by preventing Hb denaturation [109,184]. Moreover, it binds free haeme with high affinity possibly to prevent haeme's oxidative actions [48].

Alpha-stabilizing protein (AHSP) is another molecular chaperone that is present in RBCs (Fig. 1B). It binds free α-globin chains, maintaining their native structure en route to HbA synthesis [211]. More importantly, in the absence of β-globin and in the presence of oxidant insults it promotes structural changes to HbA so that the iron will not be able to catalyse redox reactions [214,56]. Decreased levels of AHSP exacerbate the phenotype of β-thalassaemia [91].

The haeme regulated inhibitor of protein translation (HRI) provides another cytoprotective mechanism (Fig. 1B). HRI represses globin translation, by phosphorylating the eukaryotic translation initiation factor 2α (eIF2α), during haeme deficiency [40], however, oxidative stress leads to activation of HRI independently of haeme levels [110]. Phosphorylated eIF2α inhibits general protein translation to avoid proteotoxicity and simultaneously increases the translation of selective mRNAs like ATF4 in order to initiate a stress response cascade [70]. Interestingly, the HRI/eIF2α/ATF4 signalling appears to be activated during erythropoiesis, possibly to reduce oxidative stress at the haemoglobinization stage. In fact, Hri knockdown foetal liver cells develop ineffective erythropoiesis during ex vivo differentiation [186], and the phenotypic severity of β-thalassaemia mice is exacerbated when Hri is deficient [69].

It appears that modulation of oxidative stress is significant during erythropoiesis since the production of Hb requires the uptake of haeme and iron, which are extremely oxidising molecules [65]. The importance of ROS modulation during erythroid maturation can be seen in G6pdΔES mouse cells where definitive erythrocytes apoptose at the stage of increased haemoglobinization. Importantly, their death is hindered by reducing agents [147]. Glucose 6-phosphate dehydrogenase (G6PD) is an enzyme of the PPP that produces NADPH, which is an important reducing agent [30]. This study indicates that in order to reach terminal differentiation and avoid apoptosis erythroid cells have to overcome oxidative stress.

HRI/eIF2α/ATF appears to be one of the pathways that combat ROS production during erythroid differentiation [186]. In addition, NRF2 and FOXO3α are two other well-known antioxidant protective signalling factors/pathways [117,84] (Fig. 1B). NRF2, an antioxidant transcription factor, induces the expression of haeme oxygenase-1 (HO-1), which is the rate limiting enzyme of haeme degradation [90]. Haeme appears to increase the stability of the NRF2 protein [5]. Interestingly, HRI/ATF4 is also implicated in HO-1 expression during ex vivo erythropoiesis or oxidative stress conditions [186]. Therefore, HRI seems not only to regulate globin synthesis according to haeme availability, but also alleviates excess, toxic levels of haeme by inducing HO-1 expression [186]. Additionally, NRF2 and ATF4 have been found to interact in order to promote HO-1 expression [72]. Moreover, NRF2 and HRI/ATF4 appear to have several other similar targets such as NQO1 (NADPH quinone oxidoreductase 1) and GST (glutathione-S-transferase), whose expression is increased in order to promote stress response signalling [146,70].

FOXO3α has been found to be activated at an intermediate stage of erythroid differentiation and remains active until terminal maturation. The signal for FOXO3α nuclear localisation and activation is provided by the downregulation of EPO/PI3K/AKT signalling at the stage of increased haemoglobinization in order to prevent ROS accumulation [117]. FOXO3α expression is also induced by oxidative stress and in response to that FOXO3α promotes cell cycle arrest in order to repair DNA damage or induces apoptosis [118,192,52,66]. FOXO3α upregulates the expression of several antioxidant enzymes such as SOD and catalase [136,93], and inhibits the proliferation of intermediate erythroid precursors in order to allow the erythroid differentiation programme to occur [117]. In fact FOXO3α seems to synchronise erythroid proliferation and maturation through an oxidative stress-mediated process. Mice deficient in FOXO3α die due to oxidative stress-induced ineffective erythropoiesis and this is possibly attributed to a hindered FOXO3α haematopoietic response [117].

4.3. Protein quality control pathways in β-thalassaemia

A novel yet little-studied field involves the role of protein quality control (PQC) pathways during erythropoiesis and their deregulation during β-thalassaemia. Khandros and Weiss [87] suggested that β-thalassaemia is a protein aggregation disorder that uses PQC pathways as defence mechanisms. During erythropoiesis the cell balances the production of individual globins, which are unstable and toxic in the absence of their partners. This supports the notion that the cell senses and regulates a possible imbalance. Additionally, β-thalassaemia trait individuals are relatively unaffected, which indicates that there is a threshold above which excess α-globin is detrimental [87].

Recent studies have shown that early erythroid precursors can balance globin levels through selective α-globin ubiquitin-mediated proteasomal (UPS) degradation (Fig. 1B) [177,196]. UPS has a physiological function during normal erythropoiesis, which is not only to degrade α-globin but also unnecessary proteins before the

enucleation process [55]. It was demonstrated that in β-tha-lassaemia, α-globin proteolysis was increased compared to healthy individuals or patients with other anaemias [196,33].

However, during late stages of erythropoiesis, either normal or thalassaemic, α-globins tend to accumulate and proteasomal capacity rapidly becomes overwhelmed [177,196]. Interestingly, Khandros et al. [86] have shown that although short-term proteasomal inhibition in β-thalassaemia cells prevented α-globin degradation, prolonged proteasomal inhibition in β-thalassaemia mice did not enhance α-globin accumulation but rather stimulated compensatory PQC pathways such as autophagy and heat shock chaperones. This indicates that multiple PQC pathways are coordinately activated in β-thalassaemia and that there is molecular cross-talk between them [86].

Autophagy is another physiological process that takes place during erythropoiesis. Reticulocytes degrade mitochondria and other organelles using macroautophagy [213], which is upregulated by GATA-1 [80]. Interestingly, electron micrographs of β-thalassaemia precursors have detected α-globin aggregates within lysosomes [203]. Additionally, autophagy appears to be upregulated in β-thalassaemia/HbE erythroblasts [108]. Khandros et al. [86] suggested that while moderate amounts of α-globin can be degraded by UPS, higher amounts tend to precipitate and are degraded via aggresome-mediated macroautophagy (Fig. 1B) [86]. Aggresomes are dynamic structures where insoluble proteins interact with the PQC machinery and provide a staging area where abnormal proteins can be sequestered for refolding, UPS degradation or autophagy [92].

Although the above studies have been performed in a β-thalassaemia setting, Basu et al. [25], using a 2D DIGE based proteomic approach, have demonstrated upregulation of proteasomal subunits and chaperones, such as AHSP and HSP70 in the cytosol of SCD RBCs compared to healthy RBCs [25].

5. Extracellular oxidative events

Upon haemolysis, Hb is released in the plasma and oxidative reactions take place that result in ROS generation, haeme and iron release (Fig. 2) [169,81]. These toxic molecules can intercalate into cell membranes and promote oxidative damage to the endothelium, haematopoietic cells and various organs (Fig. 2) [22,57]. In SCD and β-thalassaemia, signs of oxidative stress can be observed not just in RBCs but also in leucocytes, platelets, endothelial cells and several other tissues [10,29].

As previously mentioned, haeme and iron are considerably toxic molecules. Plasma free iron has been linked to carbonyl group production in plasma proteins, which is an indication of increased protein oxidation [44]. In addition, haeme can bind plasma proteins such as albumin and LDL [124,173]. Upon binding to LDL, haeme iron promotes its oxidation, which has a major effect on the generation of atherosclerotic plaques. Atherosclerotic plaques can promote lysis of RBCs and oxidation ofHb molecules. Free haeme and iron released by these oxidation reactions can further oxidise plaque components resulting in additional damage of the endothelium [131].

5.1. Protective mechanisms in the circulation

Several protective mechanisms function in order to avoid the detrimental effects of ROS, free haeme and iron. However, in cases of increased haemolysis these protective mechanisms become overwhelmed. Haptoglobin and haemopexin, whose levels are reduced in SCD and β-thalassaemia, scavenge Hb and haeme respectively (Fig. 1B). Haptoglobin-Hb complexes bind to CD163 receptors on macrophages, whereas the haemopexin–haeme complexes are recognised by receptors on hepatic parenchymal cells and macrophages. Following binding to receptors the complexes are endocytosed [182,34]. Upon entering the cell haeme drives expression of HO-1, biliverdin reductase and ferritin. Haeme is broken down to biliverdin, releasing carbon monoxide and iron (Fig. 1B) [41]. The latter can be taken up by ferritin in order to prevent oxidative reactions. Patients with SCD have increased cellular HO-1 protein levels in their polymorphonuclear cells (PMNs) compared to healthy volunteers [78]. Increased HO-1 expression was also observed in renal tubular epithelial cells and circulating endothelial cells of patients with SCD [133].

5.2. A vicious cycle of oxidative stress and inflammation

A major effect of haeme and ROS released into the circulation is to promote vascular inflammation by activating NF-κB and AP-1, which can then increase the expression of pro-inflammatory cytokines (IL1, IL6, TNFα) and adhesion molecules (L-selectin, P-selectin, VCAM-1, ICAM-1) on the endothelium (Fig. 2) [199]. This leads to increased adhesion of RBCs, leucocytes and platelets to the endothelium resulting in endothelial activation and dysfunction [62]. In addition, the produced pro-inflammatory cytokines promote the generation of additional ROS [63], while increased recruitment and adhesion of leucocytes to the endothelium can add to that effect [199]. Increased leucocyte influx is not only an important inflammatory marker but also a major source of ROS due to the superoxide producing enzyme NADPH oxidase (Fig. 2) [209,99]. The chronic oxidative stress in SCD and β-thalassaemia PMNs results in a reduced respiratory burst response, which is a mechanism of destroying engulfed bacteria, and thus leads to recurrent infections [9].

NADPH oxidase is not only responsible for ROS production in leucocytes but also in RBCs. As mentioned earlier, George et al. [63] have demonstrated that ROS production in SCD RBCs is not only generated by Hb autoxidation and Fenton reaction but also enzymatically by NADPH oxidase. Inside RBCs, NADPH oxidase activity seems to be regulated by PKC, Rac GTPase and Ca^{2+} signalling and is increased by TGFβ1 and endothelin1 cytokines, which have been shown to be enhanced in SCD (Fig. 1A) [63]. Therefore, it appears that NADPH oxidase-mediated ROS and systemic inflammation function within a vicious cycle since ROS are responsible for increased pro-inflammatory cytokine expression [199], and TGFβ1 and endothelin1 cytokines appear to increase ROS production by modulating NADPH oxidase activity [63].

5.3. Hypercoagulation in SCD and β-thalassaemia

SCD and β-thalassaemia are characterized by increased hypercoagulability as demonstrated by the increased markers of platelet activation and thrombin production [202,205,94]. Chronic oxidative damage of platelets [10] and adhesion to the endothelium can lead to their activation and aggregation (Fig. 2) [101,77]. Additionally, PS-exposed RBCs can adhere to the endothelium and activate the coagulation process. They provide a docking site for coagulation factor X and prothrombinase complexes, which can then be converted to activated factor X and thrombin respectively [216]. Another factor that adds to the hypercoagulation state observed in SCD and β-thalassaemia is the presence of tissue factor (TF) in endothelial cells. ROS can directly damage the endothelium by mediating oxidation reactions with membrane lipids and proteins [29,68]. This results in endothelial cell swelling and disengagement from the basement membrane, which eventually leads to TF exposure on the endothelial cell membrane [183]. Coagulation factor VII, the primary initiator of coagulation process, can bind to TF [134].

5.4. NO anti-thrombotic, anti-inflammatory and vasodilating properties

Reduction of nitric oxide (NO) bioavailability plays an important role in endothelial dysfunction, thrombosis and inflammation [14]. NO is a freely diffusible intercellular messenger, produced by a variety of mammalian cells including vascular endothelium, neurons, smooth muscle cells, macrophages, neutrophils, platelets and pulmonary epithelium [123]. Once formed, NO diffuses to nearby smooth muscle cells in which it reacts with the ferrous iron of the haeme group of guanylate cyclase, resulting in enhanced synthesis of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) [126]. Cyclic GMP then activates protein kinase G (PKG) which stimulates the Ca^{2+}-ATPase-dependant refilling of intracellular calcium stores and lowers intracellular calcium levels, thus mediating smooth muscle cell relaxation [106]. In addition, NO promotes vasodilation by up-regulating the endothelial vasodilator endothelin receptor B and down-regulating the vasoconstrictor endothelin-1 [185]. The cGMP/PKG-dependant signalling mechanism also promotes a potent inhibition of platelet aggregation [161]. NO's anti-thombotic properties also include inhibition of platelet activation and adhesion to the endothelium, TF expression and thrombin formation [58]. NO is also an important antagonist of inflammation [47]. It inhibits NF-κB activation through induction of the NF-κB inhibitor, IκBα expression and stabilisation of the NF-κBb/IκBα complex [152]. NF-κB regulates the expression of genes involved in inflammatory and immune responses such as VCAM-1, ICAM-1, P-selectin and E-selectin [2].

5.5. Reduced NO bioavailability

NO's vasodilating, anti-thrombotic, and anti-inflammatory properties are reduced in SCD and β-thalassaemia as a result of decreased NO bioavailability (Fig. 3) owing to increased haemolysis [165] and ROS generation (Fig. 2) [18]. NO binds very rapidly to free deoxyHb in the plasma and forms a stable Hb (Fe^{2+})-NO complex [74]. NO also reacts with oxyHb and leads to the formation of metHb and NO_3^- [74]. Additionally, superoxide binds to NO to produce peroxinitrite ($ONOO^-$) and thus not only contributes in the reduction of NO levels but also leads to further ROS generation [16,28]. The release of arginase during haemolysis is another factor that contributes to low NO levels. Arginase competes with nitric oxide synthase (NOS) for its substrate, L-arginine, which it uses to produce NO and L-citrulline [207]. NOS requires the cofactor tetrahydrobiopterin (BH4) to provide an electron for the reaction that hydroxylates L-arginine to N-hydroxy-L-arginine (NHA) [27]. Once NHA is formed, it is reduced to yield the final products NO and L-citrulline [6]. The absence of BH4, due to peroxinitrite production, or L-arginine leads to the decay of Fe^{2+}-dioxygen ($Fe^{2+}=O_2$) complex by forming $O_2^{\bullet-}$ and Fe^{3+}, an observable fact that is known as 'uncoupling' of NOS and results in further ROS production [157]. Another factor that leads to NOS uncoupling is asymmetric dimethyl-arginine (ADMA). ADMA is a NO synthase inhibitor, which competes with L-arginine for NOS [210,61] and its levels were shown to be increased in SCD patients [174].

Depletion of GSH in SCD neutrophils and significantly increased cyclooxygenase (COX) activity may also impact on rates of NO consumption and reflects increased oxidative stress associated with neutrophil activation [13]. Haeme-containing myeloperoxidase (MPO) localised within neutrophils catalytically consumes NO via the generation of radical intermediates and modulates NO bioactivity via adversely impacting on vasomotor function [54]. Nitrite is a substrate for MPO and becomes oxidised to the reactive oxidising and nitrating species nitrogen dioxide ($^\bullet NO_2$) which can cause nitration of protein tyrosine residues [20]. Notably, the diffuse immunoreactivity of MPO along the subendothelial space in tissues from SCD patients reinforces ex vivo observations describing interactions between endothelial cells and MPO [21]. There is elevated MPO immunoreactivity throughout the alveolar epithelium in lung tissues from patients with SCD, possibly contributing to the pulmonary hypertension and acute chest syndrome prevalent in SCD [20].
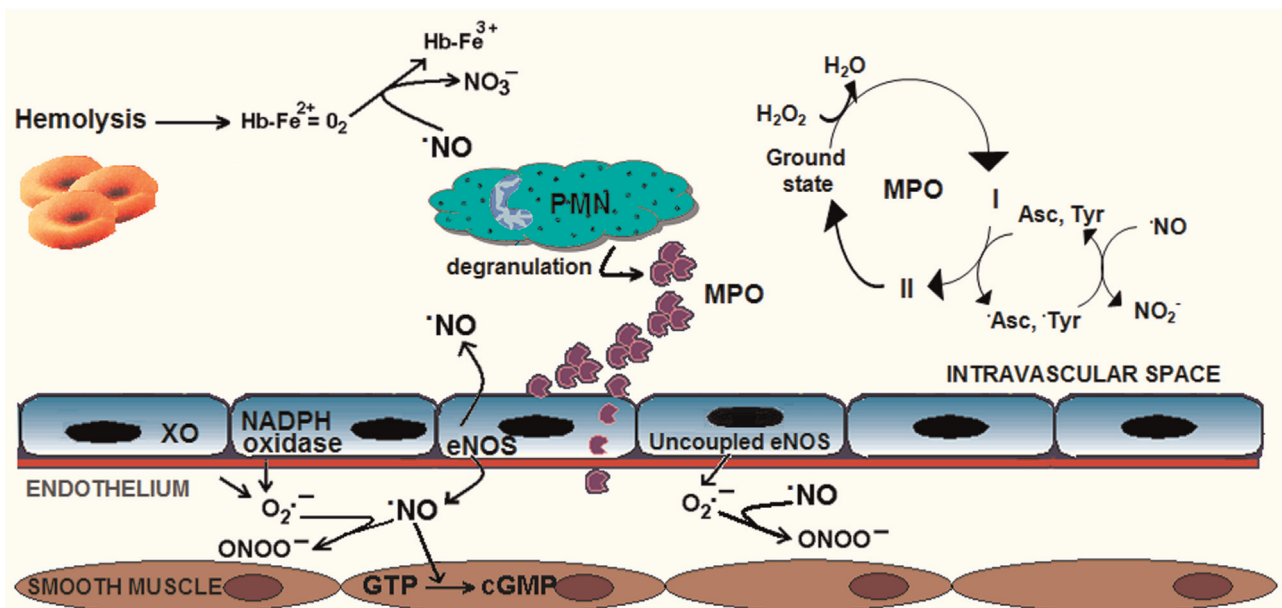

Fig. 3. Scheme of intravascular nitric oxide consumption in sickle cell disease. Superoxide ($O_2^{\bullet-}$) generated by uncoupled eNOS, xanthine oxidase, and NADPH oxidase reacts with NO to form peroxynitrite ($ONOO^-$). Nitric oxide is also consumed by plasma free haemoglobin, released by intravascular haemolysis and myeloperoxidase (MPO). The reaction between MPO and hydrogen peroxide (H_2O_2) results in the formation of compound I (I), the two electron oxidised form of the enzyme. Compound I can either oxidise halides in a single two-electron step or can oxidise multiple substrates through sequential one electron steps, forming compound II. Tyrosine (Tyr) and ascorbate (Asc) are abundant substrates that can undergo one-electron oxidation by MPO-compound I, forming tyrosyl ($^\bullet Tyr$) and ascorbyl ($^\bullet Asc$) radicals. The reaction of nitric ($^\bullet NO$) with both tyrosyl and ascorbyl radicals is diffusion limited and leads to the catalytic consumption of $^\bullet NO$.

5.6. Tissue ischaemia and reperfusion injury

Adherence of circulating blood elements with endothelial cells, coagulopathy, inflammatory responses by leucocytes, vasoconstriction due to decreased NO, and cell sickling in the case of SCD, are among a complex series of factors known to contribute to endothelial dysfunction and vascular obstruction [15,45]. The chronic activation and dysfunction of endothelial cells result in vascular damage in all organs [194,62,73]. Impaired cardiovascular function, pulmonary hypertension and renal failure are among the most significant consequences [100,159,188].

The initiation, progression and resolution of vaso-occlusion create an oxidative milieu and features common with ischaemia/reperfusion (I/R) injury [14]. Tissue damage in reperfusion injury can occur via xanthine oxidoreductase (XOR) which is an important source of oxygen free radical production [151]. During experimental hypoxia, XOR to xanthine oxidase (XO) conversion has been shown to be significantly greater in the liver and kidneys of SCD mice expressing a mixture of human HbS and murine Hb [144]. Extensive hepatocellular injury accompanied by increased plasma ALT levels, decreased liver XO immunoreactivity and catalytic activity was observed in knockout-transgenic SCD mice even under normoxic conditions [16]. Xanthine oxidase is elevated in SCD patient plasma and in the arterioles and venules of SCD mice [16,83]. Pro-inflammatory cytokines such as TNFα, IL-Iβ, and IFNγ, shown to be enhanced in SCD [199], have also been associated with increased xanthine oxidase in several tissues [79,98]. It has been shown that elevated levels of circulating and vessel wall XO in SCD lead to haemodynamic instability and contribute to the pathogenesis of extensive vascular dysfunction [16]. Interestingly, the interruption of blood flow results in tissue ischaemia and thus triggers the conversion of ATP to hypoxanthine and xanthine. After blood flow is restored hypoxanthine and xanthine oxidases, which are released from hepatic and other tissues, catalyse the reaction between oxygen and hypoxanthine and xanthine, respectively, generating uric acid, NADPH and $O^{\bullet -}$ (Fig. 2) [112,144,187].

Animal models of cardiac liver and kidney I/R all show increased expression of inducible NOS (iNOS or NOS2) and elevated tissue nitrite (NO_2^-) and nitrate (NO_3^-) levels (Fig. 2). NOS2 inhibitors significantly improve organ function in tissue I/R injury [102,107]. Increased NO production during I/R injury is cytotoxic via direct reactions of NO with ROS, generating secondary species capable of oxidation and nitration reactions. Enhanced immunoreactivity and selective nitration of liver and kidney actin is detected in both SCD mice and humans [17].

I/R appears to increase the expression of NADPH oxidase in PMNs, monocytes and endothelial cells (Fig. 2) [163,89]. Vascular endothelial cells contain all the phox proteins and the small molecular weight GTP binding protein of the multicomponent phagocytic NADPH oxidase system [26]. Endothelial cell NADPH oxidase is therefore an important source of $O_2^{\bullet -}$ and has been shown to mediate microvascular dysfunction in SCD transgenic mice [206]. NADPH oxidase inactivation was shown to be as effective as SOD overexpression in abolishing both the leucocyte and platelet adhesion responses that are normally observed in SCD mice [206].

5.7. Iron overload in β-thalassaemia

Iron overload is well documented in β-thalassaemia. It is caused by the increased intra- and extra-cellular oxidative denaturation of Hb and subsequent iron release, the recurrent blood transfusions, especially in β-thalassaemia major, and the enhanced absorption from the gastrointestinal (GI) tract [170]. The latter is attributed to the decreased hepsidin levels observed in β-thalassaemia.

Hepsidin is produced in the liver and binds to the iron exporter ferroprotein on the enterocytes, macrophages and liver cells,

mediating its internalisation and subsequent degradation preventing iron export from these cells [135,150]. Hepsidin is regulated by the iron status, inflammation, hypoxia and erythropoietic iron demand [138,155]. Its levels are decreased in β-thalasseamia due to the ineffective erythropoiesis and subsequent enhanced erythropoietic activity, which increases iron demand [139,3]. Oxidative stress has been implicated in both ineffective erythropoiesis [53] and erythroid expansion [103].

Recent findings support the role of an erythroid regulator on the reduction of hepsidin expression [148,198]. The erythroid regulator is possibly a factor that is secreted from proliferating or apoptotic precursors. TWSG1 (twisted gastrulation 1), isolated from immature precursors in β-thalassaemia mice, and GDF15 (growth differentiation factor 15), found in the plasma of patients with β-thalassaemia or other diseases characterised by ineffective erythropoiesis, are two of the factors considered to play a role in down-regulation of hepsidin expression [162,189,190]. Interestingly, another member of the TGF-β superfamily, GDF11, has recently been found to be associated with ineffective erythropoiesis in β-thalassaemia by inducing oxidative stress [53].

Iron overload in SCD appears at a later stage and has a lower occurrence [197]. This may be due to the lower ineffective erythropoiesis and bone marrow erythroid expansion, which result in reduced hepsidin suppression. As previously mentioned, increased erythroid hyperplasia in SCD in mainly seen in the spleen [85], moreover the fragmentation of erythroid precursors mostly occurs during their medullary migration to the periphery [31]. In addition, hepsidin levels may also be affected by the enhanced inflammation which increases hepsidin expression [156].

At high levels iron is an extremely toxic molecule. It can exit circulating RBCs and migrate from the plasma to haematopoietic and endothelial cells as well as various organs where it can promote oxidative reactions [57]. Myocardiopathy, liver cirrhosis, and endocrine complications are among the long term consequences of iron overload. Excess iron absorbed from the GI travels to the liver through the portal vein and thus liver fibrosis occurs as iron promotes collagen synthesis. Transfusional iron on the other hand first accumulates in the reticuloendothelial system and is then transferred to parenchymatous organs such as the heart and endocrine organs. The iron binding capacity of transferrin is overwhelmed by the continuous accumulation of iron and this result in the production of non-transferrin bound iron (NTBI) which is highly toxic. Interestingly, the transport of NTBI to organs is faster than that of transferrin bound iron leading to increased iron deposition and subsequent oxidative organ damage [75]. Moreover, the myocardium and endocrine tissues, unlike other tissues, have L-type voltage dependant calcium channels that transport NTBI and this possibly justifies the pattern of transfusional iron deposition in certain organs [145].

6. Conclusion: scd vs β-thalassaemia

SCD and β-thalassaemia are heritable haemoglobinopathies resulting from mutations affecting the structure or the level of expression of the β-globin chain. Although they share many similarities, the clinical manifestations of SCD are attributed to chronic haemolytic anaemia resulting in endothelial damage and vascular occlusion, whilst in β-thalassaemia ineffective erythropoiesis and subsequent iron overload are the main features of the disease.

6.1. Intracellular events

Kean et al. [85] have shown that SCD and β-thalassaemia mice are equally anaemic with excessive erythroid hyperplasia but for

different reasons. The half-life of SCD RBCs is more severely reduced compared to β-thalassaemia and the production of RBCs cannot sustain the excessive peripheral destruction. On the contrary, in β-thalassaemia mice there are more erythroid precursors than necessary to compensate for the reduced peripheral cell numbers. This indicates that if the erythroid progenitors were differentiating and transferred to the circulation efficiently, β-thalassaemia mice would have more than enough RBCs in the periphery and no anaemia [85]. This led to the conclusion that the increased haemolysis in SCD exceeds the enhanced erythropoietic activity, whereas in β-thalassemia ineffective erythropoiesis results in extremely low output of mature RBCs compared to the produced erythroid progenitors. Oxidative stress appears to be involved in both haemolysis and ineffective erythropoiesis.

Although the exact mechanisms and the differences between SCD and β-thalassaemia are not quite understood, increased Hb autoxidation seems to be the root of all evil. Within RBCs, autoxidation of Hbs, exacerbated by the unstable Hbs present in both diseases, generates ROS, which are responsible for oxidative damage of membrane and cytoskeletal components. Haeme and iron released by the oxidative denaturation of Hbs are strong oxidising agents themselves and can add to the oxidative stress. A recent study by George et al. [63] revealed that in addition to Hb autoxidation and Fenton reactions, NADPH oxidase can also contribute to ROS generation inside SCD RBCs. NADPH oxidase can be regulated by both intra- and extracellular signals. Interestingly, inflammatory cytokines, activated by ROS, can up-regulate NADPH oxidase, creating a vicious cycle of ROS and inflammation.

Oxidative damage can disrupt normal membrane organisation leading to PS exposure, which is a sign of eryptosis and a signal for cell removal by the macrophage system. Macrophages can also phagocytose senescent RBCs, which are recognised by the binding of anti-band-3 NAbs to band-3/hemichrome clusters. Signs of increased senescence and eryptosis can be observed in both SCD and β-thalassaemia. However, in cases of extensive oxidative injury RBCs will become damaged to such an extent that they will be haemolysed instead.

An important factor to consider is that β-thalassaemia precursors are mainly removed by macrophages in the bone marrow and extramedullary sites, following PS exposure or anti-band-3-Nabs binding, whereas SCD RBCs are mostly haemolysed in the periphery. In the case of β-thalassaemia, erythroid precursors cannot overcome oxidative stress in order to go through differentiation, whereas in SCD the problem begins when the RBCs reach the circulation. One factor that can explain these differences is the fact that HbS polymerisation, and thus increased autoxidation, takes place mainly in the microcirculation, where there is low oxygen saturation. In addition, α-globin chains may be more unstable than HbS and tend to aggregate, precipitate and autoxidise at an earlier stage. In fact, α-globin aggregates have been found in the polychromatophilic erythroblast stage, where increased haemoglobinisation takes place.

6.2. Extracellular events

Increased haemolysis leads to the release of Hb in the circulation, with subsequent oxidative denaturation and production of ROS, free haeme and iron. These oxidising molecules can damage plasma proteins and lipids and can also intercalate into plasma membranes promoting oxidative damage to the endothelium, haematopoietic cells and variousorgans. Vascular inflammation is one of the main consequences of haemolysis. It is characterised by increased production of pro-inflammatory cytokines and leucocyte influx, which can generate more ROS, creating a vicious cycle of oxidative stress and inflammation. Adhesion of RBCs, leucocytes and platelets to the endothelium is another sign of inflammation

and can also lead to hypercoagulation. Coagulation and inflammation are further exacerbated because the bioavailability of NO, an intercellular messenger with vasodilating, anti-thrombotic and anti-inflammatory properties, is reduced as a consequence of haemolysis and neutrophil activation.

All of the above can lead to extensive endothelial damage and are evident in both SCD and β-thalassaemia, as they both experience increased haemolysis of RBCs in the periphery [127,181]. However, in SCD greater levels of haemolysis compared to β-thalassaemia cause more severe problems. In addition, regular transfusions, which are common practice for β-thalassaemia major patients, can ameliorate the above pathologies. The excessive haemolysis together with the sickling of RBCs, they can lead to vaso-occlusive crises, which is a unique characteristic of SCD. Vasculopathy can also lead to pulmonary hypertension, stroke, leg ulcers and priapism, which are characteristic complications of SCD [127]. In addition, vaso-occlusion results in I/R injury via the increased expression of several plasma and endothelial enzymes (xanthine oxidase, NADPH oxidase and iNOS), which give rise to more ROS. It is therefore apparent that the oxidative stress in RBCs generates a feedback loop for more ROS production.

On the contrary, the pathogenesis of β-thalassaemia is mainly attributed to ineffective erythropoiesis and regular blood transfusions which result in iron overload. Oxidative stress has been implicated in ineffective erythropoiesis and the iron overload generated by this promotes additional oxidative damage to cells and tissues.

6.3. Protective mechanisms

Cytoprotective mechanisms are in place within cells to minimise damage to the maturing RBC from oxidative stress. These include PRDX2 (an antioxidant enzyme and chaperome for Hb and haeme), AHSP (a chaperone for α-globin), NRF2 (an antioxidant transcription factor regulating the expression of stress-response genes), FOXO3 (a transcription factor promoting antioxidant enzyme expression and inhibition of proliferation of erythroid precursors; important for terminal differentiation), HRI/eIF2α/ATF4 (inhibitor of protein translation and initiator of stress response cascade; important for terminal maturation) and PQC pathways (e.g. UPS, autophagy; important for the balance of α-globin levels). In addition, several antioxidant enzymes (e.g. SOD, catalase) and molecules (e.g. GSH, vitamin E and C) function within RBCs to neutralise the different reactive oxygen species. These mechanisms play a role in reducing oxidative stress during normal erythropoiesis but are deregulated or overwhelmed in disease.

Protective mechanisms are also functioning in the circulation. Haptoglobin and haemopexin can scavenge free Hb and haeme respectively and are subsequently endocytosed by macrophages. However, in the case of increased haemolysis, haptoglobin and haemopexin become rapidly overwhelmed. Upon entering the cell, haeme drives the expression of HO-1, which is the rate limiting enzyme for the degradation of haeme, and ferritin, which takes up the released iron to prevent its oxidative reactions.

Oxidative stress negatively impacts biological function at many levels in both SCD and β-thalassaemia and is responsible for the pathogenesis of these diseases. Better understanding of the main players and biological events will provide new avenues for the better management of these diseases.

Abbreviations: EryP: erythroid progenitors, WBCs: white blood cells. * Only abbreviations not already mentioned in the main body of the text are shown here.

Abbreviations: MPs: microparticles, TrxR: thioredoxin reductase, Trx-$(SH)_2$: reduced thioredoxin, Trx-S_2: oxidised thioredoxin, Prx-$(SH)_2$: reduced peroxiredoxin, Prx-S_2: oxidised peroxiredoxin, GPx: glutathione peroxidase, GR: glutathione

reductase, GRx: glutaredoxin, Ub: ubiquidin. * Only abbreviations not already mentioned in the main body of the text are shown here.

Acknowledgements

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