Published in final edited form as:

Curr Opin Physiol. 2019 June ; 9: 26–33. doi:10.1016/j.cophys.2019.04.022.

Redox Signaling in Sickle Cell Disease

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Abstract

Sickle cell disease (SCD) is characterized by chronic hemolysis and repeated episodes of vascular occlusion leading to progressive organ injury. SCD is characterized by unbalanced, simultaneous pro-oxidant and anti-oxidant processes at the molecular, cellular and tissue levels, with the majority of reactions tipped in favor of pro-oxidant pathways. In this brief review we discuss new findings regarding how oxidized hemin, hemolysis, mitochondrial dysfunction and the innate immune system generate oxidative stress while hemopexin, haptoglobin, heme oxygenase-1 (HO-1) and nuclear factor erythroid 2-related factor 2 (Nrf2) may provide protection in human and murine SCD. We will also describe recent clinical trials showing beneficial effects of antioxidant therapy in SCD.

Keywords

sickle cell disease; hemoglobin; oxidative stress; hemolysis; inflammation

Introduction

Sickle cell disease (SCD) is caused by a single point mutation in the hemoglobin beta chain that predisposes sickle hemoglobin (HbS) to polymerize upon deoxygenation, forming crystals that distort and injure the red blood cell (RBC) membrane (1). Consequently, fragile sickle RBCs are prone to lysis, releasing hemoglobin and other RBC cellular components into the circulation. This leads to endothelial oxidative injury, inflammation and increased adhesion of leukocytes, RBCs and platelets that results in repeated episodes of

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Declaration of Interests: None

microvascular stasis (“vaso-occlusion”), tissue ischemia and cumulative organ damage (2). Many features of SCD vaso-occlusion resemble the ischemia-reperfusion injury and vascular inflammation described in cardiovascular disease. Despite the many pathways by which oxidative stress exacerbates pathologies in SCD, there are few therapeutic interventions proven to be effective in reducing the pro-oxidant environment of SCD (Figures 1 and 2).

Hemoglobin, Heme and Hemin

Hemoglobin is comprised of 2 alpha globin chains, 2 beta globin chains and a heme cofactor that contains ferrous iron (Fe^{2+}); oxidation of iron to the ferric (Fe^{3+}) state results in methemoglobin. The heme cofactor is tightly bound to the globin proteins when iron is in the ferrous/oxy state; upon oxidation to the ferric form (hemin), methemoglobin is formed and the hemin cofactor is readily released from the protein. While the terms “heme” and “hemin” are frequently interchanged, in this review we refer to heme that is oxidized (Fe^{3+}) as hemin.

Sickle RBC, microparticles and hemolysis

HbS readily auto-oxidizes to form methemoglobin, hemin, and superoxide within the RBC. This impacts many cellular activities, including cytoskeletal oxidation, membrane lipid peroxidation and phosphatidylserine exposure, all culminating in decreased RBC deformability and premature cell death. Without mitochondria, RBCs rely on glycolysis for energy and produce NADH (nicotinamide adenine dinucleotide (NAD) + hydrogen (H)) that is a key antioxidant, reducing methHbS to its less dangerous ferrous state. Unfortunately nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is upregulated in sickle RBCs and depletes NADH. Glutathione, a scavenger of reactive oxygen species (ROS), is also depleted within sickle RBCs, further contributing to sickle RBC oxidative stress (3).

Repeated HbS polymerization (“sickling”) and RBC oxidative injury promote shedding of RBC microparticles (4). Sick RBC-derived microparticles induce ROS in cultured endothelial cell and acute vaso-occlusion in sickle mouse kidneys (5). Heme bound to externalized microparticle phosphatidylserine may drive this biologic effect (4, 6). Sick microparticles formed by shearing RBCs through a small gauge needle formed high levels of oxidized ferric and ferryl hemoglobin compared to control microparticles (7*). Furthermore, protein carbonylation and lipid hydroperoxides increased while antioxidants dropped in sick microparticles compared to minimal changes observed in healthy RBC microparticles. Interestingly, shear-generated or native circulating sick microparticles, but not control microparticles, formed oxidized βCys93 and, unexpectedly, ubiquitination of βLys96 and βLys145 in sick microparticle β globin, suggesting a novel link between oxidative stress and the ubiquitin pathways (7).

Chronic intravascular hemolysis releases a constant supply of cell-free hemoglobin and heme/hemin that exerts pro-oxidant effects in the circulation (8). Oxyhemoglobin (Fe^{2+}) scavenges nitric oxide (NO) to form nitrate (NO_3^-) and methemoglobin (Fe^{3+}), shunting NO away from endothelial cells and contributing to endothelial dysfunction (9). Release of arginase-1 from hemolyzed RBCs metabolizes l-arginine, the building block for NO, further

reduces NO bioavailability (10). Hemoglobin may also react with hydrogen peroxide (H_2O_2) through the Fenton reaction to form hydroxyl free radical ($OH^\bullet$) and methemoglobin. Methemoglobin can further degrade to hemein (Fe^{3+}), which is a major RBC damage-associated molecular pattern (DAMP). These hemolysis-associated ROS contribute to activation of inflammatory and adhesive pathways in endothelial cells, platelets and neutrophils that can lead to vaso-occlusion in SCD. In agreement, infusion of hemein directly induces acute vascular occlusion in sickle mouse lungs that is improved with inhibition of P-selectin mediated adhesion pathways (11*).

Protection against heme toxicity via haptoglobin, hemopexin and HO-1

Haptoglobin and hemopexin protect against heme toxicity by binding and removing cell-free hemoglobin and hemein, respectively. Unfortunately, both scavengers are rapidly cleared in the setting of hemolysis (8). Using mouse dorsal skinfold chambers to assess microvascular flow, infusion of hemoglobin or hemein causes acute vascular stasis in sickle, but not control mice, while the co-administration of haptoglobin or hemopexin restored the microvascular blood flow to control levels (12). Correspondingly, genetic overexpression of hemopexin in sickle mice prevents microvascular occlusion, upregulates protective liver nuclear factor erythroid 2-related factor 2 (Nrf2) expression and heme oxygenase-1 (HO-1) activity, and decreases pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation in the face of hemein infusion. Conversely, sickle cell hemopexin knockout mice exposed to hemein experience worse venostasis (13).

HO-1 is an inducible enzyme that catabolizes heme to biliverdin, carbon monoxide and ferrous iron and has multiple additional cytoprotective effects. In sickle mice, a single infusion of haptoglobin or hemopexin increased HO-1 expression and decreased NF- κ B activity in multiple tissues; the haptoglobin/hemopexin infusion also protected against vaso-occlusion for up to 48 hours (14). The effect of HO-1 on NF- κ B activity and venostasis was independent of plasma concentrations of hemoglobin or hemein, suggesting that the beneficial effects of haptoglobin and hemopexin are not wholly attributable to the clearance of hemoglobin and hemein from circulation (14). Administration of oral carbon monoxide to sickle mice increased Nrf2 and HO-1 expression while decreasing NF- κ B activation, soluble vascular cell adhesion molecule-1 (VCAM-1), and venostasis (15*)

Nrf2 antioxidant effects and fetal hemoglobin induction

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that protects cells from oxidative damage by promoting the expression of a wide variety of antioxidants (16-25). Kelch-like ECH-associated protein 1 (Keap1) regulates Nrf2 activity (20, 23, 24). Under normal physiological conditions, Keap1 sequesters Nrf2, which is then ubiquitinated by cullin-3 containing ubiquitin ligase and degraded. Under physiological stress, the interaction between Nrf2 and Keap1 is inhibited by reactive oxygen species or toxic xenobiotics, resulting in constitutive activation of Nrf2 (20, 23, 24). Nrf2 activation has been shown to have a protective role in oxidant mediated multi-organ injury associated with SCD by removing plasma heme caused by SCD induced hemolysis (16-19, 21, 25). Similarly, Nrf2 inducer CDDO-Im (2-cyano-3, 12 dioxooleana-1, 9 diene-28-

imidazolidine) also ameliorated inflammation and oxidative stress in liver and lung of SCD mice (16). Remarkably, in humans, Nrf2 gene silencing by miR-144 inhibited fetal hemoglobin (HbF) expression, which is used to reduce the disease severity of SCD. Blocking miR-144 reversed the silencing effect of Nrf2 (22*). In KU812 cells, hemin-induced oxidative stress (which triggers inflammation in SCD) also caused Nrf2 activation and HbF induction which was inhibited by miR-144 treatment (22). Chromatin immunoprecipitation assay, genome-wide miRNA microarray and primary erythroid progenitor data confirmed the binding of Nrf2 with γ -globin antioxidant response element, which is responsible for HbF production, and established its protective role in SCD regulation (22).

Mitochondrial dysfunction and oxidative stress in SCD platelets

Oxidative stress likely contributes to increased platelet activation observed in SCD and activated platelets are tightly integrated within the acute vaso-occlusive process (26*, 27). Compared to healthy controls, sickle platelets have decreased mitochondrial respiration, increased mitochondrial membrane potentials and produce higher levels of the mitochondrial oxidant product, H_2O_2 . The degree of mitochondrial hyperpolarization or H_2O_2 production correlates with the level of increased sickle platelet activation, which points toward mitochondrial complex V dysfunction. The degree of reduced complex V activity in sickle platelets correlates with measures of hemolytic rate, including the level of cell-free hemoglobin. Interestingly, treatment of healthy platelets with hemoglobin reduces complex V activity and mitochondrial oxygen consumption rates, while increasing mitochondrial membrane potential, H_2O_2 production, and platelet activation, similar to that seen in SCD. These hemoglobin-induced mitochondrial effects are comparable to the effects of treating platelets with oligomycin, a complex V inhibitor. This suggests that cell-free hemoglobin causes mitochondrial dysfunction through inhibition of complex V with resultant ROS production and platelet activation (28). However, mitochondrial dysfunction in SCD may extend beyond platelet complex V. Treatment of sickle mice with Coenzyme Q10, a vital electron carrier in the mitochondrial respiratory chain, decreases expression of ROS in dorsal horn of the spinal cord, suggesting that the presence of mitochondrial oxidative stress may also contribute to chronic pain signaling in SCD (29).

In the presence of H_2O_2 , ferrous hemoglobin oxidizes to ferryl hemoglobin plus a free radical, and then undergoes autoreduction to ferric methemoglobin. Mitochondria in any tissue may sustain oxidative damage as a result of ferryl hemoglobin and its radical. In cultured lung epithelial cells, exposure to ferryl HbS decreased mitochondrial maximal oxygen consumption rate and mitochondrial membrane potential. Incubation with haptoglobin prevented the decrease in mitochondrial oxygen consumption rate, implicating HbS (30).

Non-heme sources of oxidative stress

In addition to cell-free hemoglobin and hemin, NADPH oxidase, xanthine oxidase (XO), and uncoupled endothelial NO synthase (eNOS) generate oxygen free radicals that further promote endothelial dysfunction in SCD. Myeloperoxidase, released from activated

neutrophils, also generates potent oxidants that can scavenge nitric oxide and impair endothelial function in SCD (31, 32). Inhibition of myeloperoxidase in sickle mice dramatically improves endothelial-dependent vasodilation of aortas and reduced measures of oxidant stress and liver injury (32). Recent studies have confirmed increased levels of the oxidative stress markers myeloperoxidase (MPO), hydroxyl radical (HO•), lipid peroxidation and total thiols in SCD, while the antioxidants superoxide dismutase, glutathione and catalase were all reduced compared to controls (31, 33).

Innate immunity and vaso-occlusion

Innate immunity also plays a central role in modulating vascular and immune cell oxidative damage in SCD. Toll-like receptor 4 (TLR4) recognizes foreign pathogens and activates the innate immune system. In SCD, TLR4 likely engages a number of hemolysis- and inflammatory cell-derived ligands. High mobility group box protein 1 (HMGB1) is a DAMP that is released from activated immune cells or necrotic tissue. HMGB1 is increased in SCD plasma (34) and can synergize with products of hemolysis to activate toll-like receptors and RAGE (35, 36). Hemin induces TLR4 signaling in human lung microvascular endothelial cells, generating ROS, triggering apoptosis and endothelial barrier dysfunction (37). Hemin also activates NF- κ B phospho-p65 in a TLR4-dependent signaling cascade, which can upregulate endothelial adhesion molecule expression in the setting of SCD (12). TLR4 signaling also activates protein kinase C and NADPH oxidase with subsequent generation of additional ROS and oxidant stress (12). Furthermore, endothelial heme-TLR4 signaling appears to result in complement deposition that is mediated in part by P-selection expression (38).

There are strong inter-relationships between adhesion molecules, heme-mediated oxidative stress and the culmination of cell-cell interactions in sickle cell vaso-occlusion. Blockade or knock-out of endothelial P-selectin was protective against heme-induced acute lung injury in sickle mice (11). Furthermore, inhibition of neutrophil-platelet aggregates by blocking platelet P-selectin markedly improved lung vaso-occlusion in sickle mice, showing both the importance of platelet-neutrophil interactions in the vaso-occlusive process as well as the specific role of P-selectin (26). The complexity of neutrophil-platelet interactions is further evidenced by demonstrating that inhibiting neutrophil CD11b/CD18 (Mac-1) from binding to platelet glycoprotein Iba decreases neutrophil-platelet aggregations in SCD (27).

Clinical Therapeutic agents

Few medications are indicated for reducing the oxidative damage in SCD. It has also been challenging to determine the optimal biomarkers for measuring oxidant stress in SCD. Using N-ethylmaleimide to block free thiols during sample processing, followed by high-throughput mass spectrometry, Fu *et al* found that cysteine, rather than glutathione, was the highest thiol-containing compound in sickle plasma (39). This may be a novel method to assess the level of oxidative stress and the biologic effect of antioxidant therapies in SCD. Hydroxyurea is the best-established disease-modifying pharmacotherapy for SCD. In addition to fetal hemoglobin induction, hydroxyurea also acts as an NO donor. Treatment

with hydroxyurea increases RBC nitrite content, enhanced RBC deformability, and decreased reactive oxygen species levels in SCD (40).

Other therapeutic agents seek to protect RBC structural integrity from oxidative damage. The SCOT trial illustrated that omega-3 fatty acids have a protective effect on oxidative damage to sickle RBC membranes (41*). The study found increased total RBC membrane docosahexaenoic acid and eicosapentaenoic acid after just 4 weeks of therapy (41). GBT440 is an oral hemoglobin modifier that increases Hb oxygen affinity and reduces HgS polymerization in preclinical models, thereby preventing RBC sickling and improving RBC lifespan (42). Publication of a phase 1/2 clinical trial involving GBT440 (voxelotor) is forthcoming (43).

L-arginine, a substrate for NO production, has generated interest as a potential therapy to limit acute vaso-occlusive pain. Individuals with SCD patients treated with oral L-arginine plus hydroxyurea reported a reduction in pain episodes and had increased nitrite/nitrate levels compared to hydroxyurea alone (44).

Crizanlizumab is a monoclonal antibody that inhibits P-selectin. In the SUSTAIN trial, treatment with the higher dose of crizanlizumab reduced the annual rate of sickle cell-related acute painful episodes, but did not have an effect on the rate of other severe SCD complications such as sequestration or acute chest (45*).

Oral L-glutamine also reduced the median number of vaso-occlusive pain events over 48 weeks in individuals with SCD in a recent phase III trial (46). L-glutamine increases the level of reduced-NADs in sickle cell RBCs, which should relieve RBC oxidative stress. L-glutamine and others like it will undergo further study in the coming years to see what they add to SCD treatment (47).

Conclusions

SCD is characterized by a single-gene mutation with multi-system consequences (Figure 1). Mutated HbS leads to profound changes in RBC metabolism and physiology, endothelial signaling, sterile immune response, and the body's endogenous protections against hemolysis and hemolysis byproducts, all of which involve oxidative stress as either downstream or upstream mediators. Our knowledge of redox signaling in SCD is constantly growing as new signaling pathways are discovered. The therapeutic agents with the most promise for preventing oxidative damage in SCD exert their effects by decreasing ROS production or inhibiting pathways that respond to ROS (Figure 2). Improved understanding of oxidative stress in SCD will lead to targeted therapies that should improve outcomes for this underserved patient population.

Acknowledgements:

This work was supported by the National Institutes of Health grants HL128371 (C.A.H and K.A.P.), NS070711 (CAH), T32 HD071834 (D.N.D.), and support from the Vascular Medicine Institute, the Hemophilia Center of Western Pennsylvania, and the Institute for Transfusion Medicine.

Abbreviations:

DAMP	damage-associated molecular pattern
HbS	sickle hemoglobin
IVIG	intravenous immune globulin
HMGB1	High mobility group box protein 1
HO-1	heme oxygenase-1
H₂O₂	hydrogen peroxide
Keap1	Kelch-like ECH-associated protein 1
MEK	mitogen-activated protein kinase enzyme
MPO	myeloperoxidase
NADH	nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOS	nitric oxide synthase
Nrf2	Nuclear factor erythroid 2-related factor 2
RAGE	receptor for advanced glycation end products
RBC	red blood cell
ROS	reactive oxygen species
SCD	Sickle cell disease
TLR4	Toll-like receptor 4
VCAM-1	vascular cell adhesion molecule-1
XO	xanthine oxidase

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* of special interest

** of outstanding interest

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miR-144-5p. miR-144 can silence the NRF2 gene and prevent γ -globin transcription, but inhibiting miR-144 can reverse these effects. Treating KU812 cells with hemin induced NRF2 and HbF, and miR-144 inhibited that effect. The authors used chromatin immunoprecipitation to illustrate that NRF2 bound to the γ -globin antioxidant response element, and a miR-144-like molecule was able to inhibit this interaction.

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Figure 1: Redox Pathophysiology of Sickle Cell Disease (SCD).

Sickle RBC: A single point mutation in the beta globin (*HBB*) gene results in sickle hemoglobin (HbS), which reversibly polymerizes upon deoxygenation leading to red blood cell (RBC) sickling. Auto-oxidation of unstable HbS forms reactive oxygen species (ROS), upregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and depletion of glutathione, further contributing to RBC oxidative stress, cytoskeletal oxidation and lipid membrane peroxidation. Fragile sickle RBCs are prone to lysis, releasing intracellular components and microparticles into the circulation. **Cellular Redox Injury:** Cell-free Hb (Fe^{2+}) can be oxidized to MetHb (Fe^{3+}) by scavenging nitric oxide (NO) or reacting with H_2O_2 (Fenton reaction); once oxidized, hemin is readily released from MetHb. Increased circulating or cellular NADPH oxidase, myeloperoxidase (MPO), xanthine oxidase (XO) and uncoupled endothelial nitric oxide synthase (eNOS) also generate ROS in SCD. Immune cell and platelet activation release high mobility group box protein 1 (HMGB1) and cytokines. Hemin, ROS, HMGB1 and cytokines all promote sterile inflammation, endothelial dysfunction and increased expression of adhesion molecules, such as P-selectin and vascular cell adhesion molecule-1 (VCAM-1). **Vaso-occlusion:** RBC sickling and increased adhesion between the neutrophils, platelets, sickle RBCs and the vascular endothelium lead to stasis of blood flow, known as vaso-occlusion in SCD. Repeated episodes of vaso-occlusion produce ischemia/reperfusion injury that further contributes to cellular redox injury and sterile inflammation. Used with permission from Cheryl A. Hillery.

Figure 2: Protective Mechanisms and Therapeutic Targets.

Endogenous molecules and exogenous drugs target different pathways to protect against redox injury in SCD. ***Sickle RBC***: Fetal hemoglobin (HbF), glutathione, NADH, hydroxyurea, GBT440 (voxelotor), omega-3-fatty acids (SC411) and oral carbon monoxide help prevent RBC injury by reducing HbS polymerization or by stabilizing the RBC membrane. ***Cellular Redox Injury***: Haptoglobin, hemopexin, heme oxygenase-1 (HO-1), Nuclear factor erythroid 2-related factor 2 (Nrf2), L-glutamine, coenzyme Q10, a wide variety of antioxidants and inhibition of cytokines or leukotrienes are both natural protective mechanisms against redox injury and potential therapeutic strategies to reduce redox stress in SCD. ***Vaso-occlusion***: RBC transfusion, nitric oxide (NO), hydroxyurea, L-arginine, crizanlizumab (P-selectin inhibitor), rivipansel (pan-selection inhibitor), intravenous immune globulin (IVIG), CCP-224 (glycoprotein Iba inhibitor), mitogen-activated protein kinase enzyme (MEK) inhibitors and anti-platelet agents are aimed at improving endothelial dysfunction and preventing cell-cell interactions, which play vital roles in vaso-occlusion. Only hydroxyurea and l-glutamine are FDA approved for the treatment of SCD. Used with permission from Cheryl A. Hillery.

Table 1.

Selected clinical therapeutics proposed to reduce oxidative injury in SCD

Drug (Cited in Review)	Proposed Mechanism of Action
Oral carbon monoxide (14, 15)	Increases Nrf2 and HO-1 expression, decreases NF-κB activation and VCAM-1 levels
Oral L-arginine (44)	Increases nitric oxide production
Hydroxyurea (40)	Increases fetal Hb, RBC nitrite content, enhances RBC deformability, decreases ROS levels
Coenzyme Q10 (29)	Decreases ROS in dorsal horn of spinal cord
Oral L-glutamine (46)	Increases reduced form of nicotinamide adenine dinucleotides
SC411 (41)	Enhances DHA bioavailability
Crizanlizumab (45)	Inhibits p-selectin
Oral GBT440 (42, 43)	Increases O2 affinity for HgS; reduces HgS polymerization