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Review

Oxidative pathways in the sickle cell and beyond

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ABSTRACT

Polymerization of deoxy sickle cell hemoglobin (HbS) is well recognized as the primary event that triggers the classic cycles of sickling/unsickling of patients red blood cells (RBCs). RBCs are also subjected to continuous endogenous and exogenous oxidative onslaughts resulting in hemolytic rate increases which contribute to the evolution of vasculopathies associated with this disease. Compared to steady-state conditions, the occurrences of vaso-occlusive crises increase the levels of both RBC-derived microparticles as well as extracellular Hb in circulation. Common byproduct resulting from free Hb oxidation and from Hb-laden microparticles is heme (now recognized as damage associated molecular pattern (DAMP) molecule) which has been shown to initiate inflammatory responses. This review provides new insights into the interplay between microparticles, free Hb and heme focusing on Hb's pseudoperoxidative activity that drives RBC's cytosolic, membrane changes as well as oxidative toxicity towards the vascular system. Emerging antioxidative strategies that include the use of protein and heme scavengers in controlling Hb oxidative pathways are discussed.

1. Sickle cell anemia, a molecular disease of hemoglobin

Sickle cell disease (SCD) affects over 100,000 people in the United States and millions of people worldwide. Linus Pauling was first to coin the term “molecular disease” describing how this hemoglobinopathy originated from a single amino acid substitution [1]. This disease (caused by a mutation at the β6 position of Hb (β6Glu → Val)) results in the creation of a hydrophobic (sticky) patch (Val6) on the surface of one deoxy molecule in close proximity to another molecule with hydrophobic amino acids (e.g. Phe85 and Leu88). The primary molecular event is polymerization of these deoxyHbS molecules and subsequent aggregation into long fibers that leads to hemolytic anemia [2]. RBCs in hypoxic regions undergo the classic sickle cell shape change, and after several cycles of sickling and unsickling, they rupture releasing a mixture of Hb fibers and Hb molecules to circulation. SCD is characterized by chronic hemolysis, inflammation, vaso-occlusion, and ischemia-reperfusion injury leading to strokes and organ infarctions [3]. Vascular endothelial cell activation (a critical component of microvascular responses) plays a significant part in the development of the vaso-occlusive crises, the hallmarks of the disease. Ischemia reperfusion injury is characterized by intermittent cessation (and restoration) of blood flow and the production of reactive species (ROS); these ischemic events collectively contribute to the oxidative stress implicated in the SCD pathogenesis [4].

During its short life span, the SS RBC undergoes several cytosolic and membrane transformations that result in alterations to the proteome, metabolome, redox state, and rheological properties [5]. Altered cytosolic composition (in particular antioxidant enzymes) impacts the overall redox state of the cell. In addition to changes in the oxidative milieu, RBC hemolysis vasculopathy also results in the toxic accumulation of heme and Hb in the plasma [6]. It is well recognized that plasma levels of free Hb can be as high as 25 μM during sickle cell crisis, with basal Hb levels at 5–10 μM in sickle cell patients [7]. Free heme exerts multiple adverse effects, including leukocyte activation/migration, cytokine up-regulation, and oxidant production [8].

In spite of our increased knowledge of the molecular basis of SCD, no effective therapy has yet been found. Since the discovery of this condition, therapeutic efforts have primarily focused on preventing HbS polymerization (either directly or indirectly) and subsequent sickling of the RBC. It is difficult to separate the impact of the polymerization process from other alterations, including peroxidative stress and redox changes in the Hb molecule and within the RBCs [9].These changes bring about Hb instability which impacts the oxidative environment by triggering oxidative stress both intra- and extracellularly. The focus of this review is to describe SCD oxidative events (driven primarily by Hb) and their potential impact on the vascular system. Also reviewed in this article are recent attempts by many researchers to explore antioxidant strategies that are designed to control Hb-mediated redox activity in circulation.

Abbreviations: TLR4, Toll-like receptor; HO-1, heme oxygenase; SS RBCs, homozygous sickle cells; PS, phosphatidylserine; Nrf2, nuclear factor (erythroid-derived 2)-like 2

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2. Lessons learned from cell-free hemoglobin developed as blood substitutes

The development of Hb-based oxygen carriers (HBOCs) as viable oxygen therapeutics has been hampered by several safety concerns and adverse events associated with the infusion of HBOCs that include transient hypertension, gastrointestinal symptoms, pancreatic and liver enzyme, myocardial infarction, cardiac arrhythmias, renal injury and death in humans [10].

HBOCs are generally found in circulation (~250 μM–1000 μM (heme)) after infusion in humans and animals, with half-lives ranging from 8 to 17 h. This extended persistence in circulation is due to the fact that these Hbs have been chemically modified in a tetrameric or polymeric forms thus avoiding rapid clearance by kidneys [11].Three major biochemical mechanisms were put forward by researchers to explain the basis of free Hb-mediated toxicity that would otherwise have been suppressed inside RBCs. These are: (1) scavenging of endothelial derived nitric oxide (NO), a vasodilator; (2) oversupply of oxygen; and (3) heme-mediated oxidative reactions (for review see [12,13]). Hemodynamic imbalances (as manifested in blood pressure elevation) in response to HBOC infusion are primarily due to NO scavenging by Hb. An alternative mechanistic explanation to NO scavenging is the hypothesis of premature oversupply of oxygen to tissues. This results in autoregulatory vaso-constriction and/or through the formation of reactive oxygen species (ROS) and local destruction of NO [14]. Additionally, other less-studied enzymatic activities initiated by endogenous oxidants (as they react with the heme moiety of Hb) may have more lasting tissue-damaging effects than the other two mechanisms [11,15]. Hb oxidative toxicity and the consequences of these redox side reactions are difficult to study in living systems but animal studies have recently confirmed the involvement of oxidation reactions in the initiation of inflammatory responses [11,16]. Hb undergoes oxidation, in which the oxygen-bound ferrous (Fe^{II}) heme iron atom oxidizes non-enzymatically to the ferric/metHb (Fe^{III}) state (autooxidation), initially generating a mixture of protonated and anionic superoxide radicals (Fig. 1). H_2O_2 is produced during Hb autooxidation by spontaneous (k_{auto}) and enzymatic dismutation of superoxide (k_d). Hb autooxidation is associated with subsequent globin dysfunction and instability due to H_2O_2 generation resulting from dismutation of the initial superoxide products [17]. When H_2O_2 is in excess i.e., under oxidative stress conditions, a pseudoperoxidase catalytic cycle of Hb begins with three distinct steps in which H_2O_2 is ultimately and completely consumed (Fig. 1): (1) initial ferrous ($HbFe^{II}$) oxidation to a higher oxidation ferryl Hb($HbFe^{IV}$) (k1); (2) autoreduction of the ferryl intermediate to ferric ($HbFe^{III}$) (k2); and (3) reaction of $HbFe^{III}$(metHb) with an additional H_2O_2 molecule to regenerate the ferryl intermediate/ferryl protein radical (·$HbFe^{IV}$ = O) (k3) (Fig. 1)). This radical may migrate and further damage the protein, including the irreversible oxidation of βCys93 and other "hotspots" amino acids [18,19]. These internal reactions if not controlled also result in the modification of heme and its attachment to nearby amino acids. Irreversible oxidation of βCys93 to cysteic acid results in Hb dissociation into dimers, higher autooxidation rates, and rapid heme loss. Whereas, the Hb ferryl state and its associated radicals are more damaging than the ferric form, we have recently shown that heme loss from the ferric rather than the ferryl is at higher rates [20].

Due to the nature of chemical and/or genetic modifications employed in first-generation HBOCs, heme iron autooxidation and subsequent oxidative changes have been observed to occur at higher rates than those of unmodified Hb [21]. Lowered oxygen affinities (due to these modifications) have also been shown to enhance autooxidation rates [22], redox potential [23], and heme loss [24]. These issues have led many researchers to design countermeasures that can retard and/or control iron/heme oxidation in HBOCs. This ranged from either directly adding antioxidants (or reductants) to the HBOC solutions or even chemically crosslinking some of these antioxidants to the protein [21].

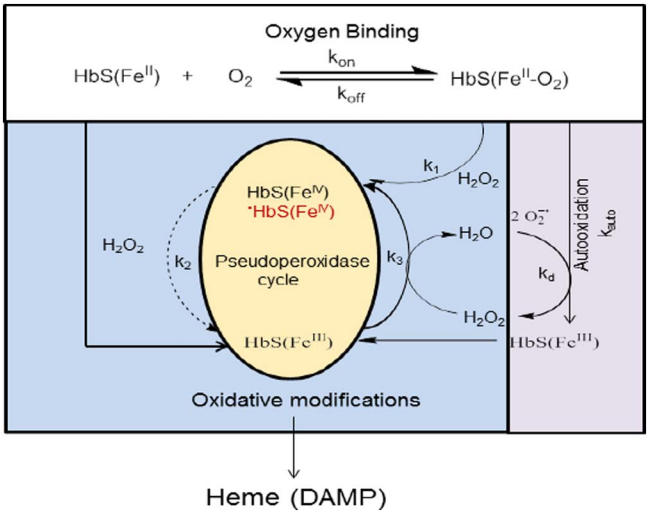

Fig. 1. Oxygenation and the pseudoperoxidase pathways in sickle cell hemoglobin. Hemoglobin ($HbFe^{II}$) reversibly binds oxygen (k_{off}/k_{on}), and spontaneously oxidizes at a slow rate (k_{auto}) to the non-functional ferric/metHb (HbFeIII) and superoxide ($O2\cdot^-$) that dismutate (k_d) to give peroxide (H_2O_2). In the presence of this and/or exogenous H_2O_2, a catalytic cycle between the ferric ($HbFe^{III}$) and the ferryl ($HbFe^{IV}$) hemes is initiated, in which H_2O_2 is eliminated in a peroxidase-like manner. However, in the case of HbS the autoreduction of ferryl back to ferric heme is slower (dotted line) than that of normal HbA, leading to a longer lived and more damaging ferrylHb [19]. If H_2O_2 reacts with the ferric form of Hb a protein radical is produce (.Hb^{IV}). The radical escapes through βCys93 resulting in its irreversible oxidation and the collapse of the β subunits. These oxidative changes then lead to unfolding and denaturation of the protein and heme loss. Heme is recognized as damage associated molecular pattern (DAMP) molecule able to trigger inflammation.

Measuring autooxidation and oxidative changes of HBOCs in circulation is difficult to monitor and is dependent on animal species used in these investigations. For example, the in vitro oxidation measurements of some HBOCs have been reported to be less of a predictor of the in vivo oxidation of Hb in rats (commonly used animal model) [25], whereas in sheep almost 30–40% metHb was accumulated after infusion of HBOCs and subsequent oxidation in circulation [26].

Guinea pigs, on the other hand, have been successfully used as a model for examining Hb oxidative processes because they (similar to humans and unlike rats) lack the enzymatic ability to produce ascorbate, a powerful reductant capable of controlling intravascular Hb oxidation [16]. It has also been demonstrated in this model that autooxidation after infusion of Oxyglobin™ (a bovine Hb polymerized with glutaraldehyde approved by the FDA for veterinary use) can compromise the ability of Hb to carry oxygen, as reflected by the suppression of hypoxia inducible factor (HIF-1α) (an oxygen sensor molecule) in kidney tissues for the first 4–6 after infusion [27]. Furthermore, renal HO-1 induction and L-ferritin expression were accompanied by significant iron deposition after Oxyglobin infusion. In a follow up experiment, evidence was presented to show that Oxyglobin transfusion suppressed renal antioxidant enzyme expression at the gene and protein level, possibly through epigenetic alterations involving DNA methylation [28]. In massive transfusion of stored blood (~10 units), it was also reported that Hb-driven pathologies as consequence of the RBC storage lesions were seen in guinea pigs that were attenuated by co-infusion of haptoglobin (Hp) [29].

A recent case of compassionate use of HBOC-201 (human analogue of Oxyglobin) was reported in a severely injured Jehovah's Witness patient, for whom survival was considered unlikely. Severe anemia and cardiac hypoxia were reversed after slow co-infusion of this Hb with ascorbic acid, a powerful reducing agent of Hb (1 g twice daily). No vasoactive side effects were associated with the treatment, possibly due to the slow infusion, and the patient survived [30].