Role of free radicals in the pathogenesis of acute chest syndrome in sickle cell disease
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Introduction
ACS is an important cause of morbidity and mortality in SCD, occurring in up to 45% of patients and recurring in up to 80% of those afflicted [1,2]. The hallmark pathologic event during ACS is vaso-occlusion, the etiology of which is probably multifactorial. One of the mechanisms responsible for vaso-occlusion is abnormal adherence of sickle RBCs, WBCs, and/or platelets to the vascular endothelium. Although the factors that lead to increased cellular adhesion and vascular damage are unclear, one possible explanation is that, during local vaso-occlusion, areas of ischemia/reperfusion develop. During periods of reperfusion, there is increased production of oxidizing molecules such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), peroxynitrite (ONOO$^-$), and the hydroxyl (•OH) radical. The present review summarizes the evidence for alterations in oxidant stress during ACS of SCD, and the potential contributions of RBCs, WBCs and the vascular endothelium to this process.

Keywords: acute chest syndrome (ACS), endothelium, hemoglobin, nitric oxide (NO), oxidant stress

Available online http://respiratory-research.com/content/2/5/280

ACS = acute chest syndrome; GPx = glutathione peroxidase; GSH = reduced glutathione; ICAM = intercellular adhesion molecule; NF-κB = nuclear factor-κB; NO = nitric oxide; NOS = nitric oxide synthase; PMN = polymorphonuclear leukocyte; RBC = red blood cell; SCD = sickle cell disease; TBARS = thiobarbituric acid-reactive substance; VCAM = vascular cell adhesion molecule; VOC = vaso-occlusive crisis; WBC = white blood cell.
In human studies [12,13], levels of thiobarbituric acid-reactive substances (TBARs) indicated that lipid peroxidation occurs in sickle erythrocytes at baseline. In addition, we observed a ninefold increase in the plasma levels of F$_2$ isoprostanes, a stable marker of lipid peroxidation, in the plasma of ACS patients as compared with that of normal volunteers (Klings ES et al, unpublished data). These findings suggest, both in humans and in mouse models of SCD, that there is increased oxidative burden and that alterations in the redox state may play a role in the development of vaso-occlusion. The source of oxygen radicals in these patients is probably multifactorial, because RBCs, WBCs, and the endothelium could each contribute to their aberrant metabolism. We review the role of each of these cell types in the generation of oxygen radicals, and the effects that these molecules have on cellular metabolism in SCD.

**Role of sickle red blood cells in oxidant production**

**Hemoglobin S as a source of oxidants**

The RBC is an important source of oxygen-related radicals in SCD. Hebbel et al [14], in 1982, demonstrated that sickle RBCs produce greater quantities of O$_2^\cdot$-H$_2$O$_2$ and •OH than do normal RBCs. Additionally, sickle RBCs at baseline exhibit increased levels of TBARs [12,13], suggesting that they are targets for oxidative stress. Although an evaluation of oxidant production by RBCs has not been conducted in SCD patients with ACS, data from mouse models of ACS [5,10] suggest that ischemia/reperfusion injury can occur in this setting.

Within RBCs, one of the mechanisms of O$_2^\cdot$- formation is via the deoxygenation of hemoglobin. During deoxygenation, there is a transfer of electrons between Fe and O$_2$, leading to the production of O$_2^\cdot$.- Auto-oxidation of hemoglobin, which occurs to a small extent physiologically, leads to the production of methemoglobin and O$_2^\cdot$- [15,16]. Because hemoglobin S auto-oxidizes at 1.7 times the rate of hemoglobin A, SCD patients may have a higher propensity for oxidant production [12]. Once hemoglobin is subjected to oxidant damage, it denatures and precipitates; these events increase its susceptibility to auto-oxidation [17]. Because of these findings, it has been hypothesized that the production of oxidants by RBCs would be greater than that observed at baseline.

**Effects of oxidant production on red blood cells**

Within the RBC, one of the targets of oxidant damage is the plasma membrane. In the presence of an O$_2^\cdot$-generating system Fe(III) is reduced to Fe(II), with subsequent formation of •OH from H$_2$O$_2$ [16]. The hydroxyl radical oxidizes unsaturated esterified membrane lipids, resulting in changes in fluidity of the bilayer. Additionally, there is increased ion permeability, inactivation of membrane enzymes and receptors, and covalent cross-linking of lipid and protein membrane constituents [18]. Membrane lipid peroxidation, measured by TBARs production, is elevated in sickle RBCs at baseline [13,19,20]. In addition to being markers for oxidative stress, lipid peroxidation products such as malondialdehyde have additional toxic effects because of their ability to react with proteins, nucleic acids, and lipids [16].

Once molecules such as O$_2^\cdot$- and H$_2$O$_2$ are formed, they are metabolized by antioxidant enzyme systems, such as superoxide dismutase (SOD), H$_2$O$_2$ is then metabolized to O$_2$ and H$_2$O by catalase and GPx. Deficiencies in SOD, catalase, and GPx in sickle RBCs lead to increased O$_2^\cdot$- and H$_2$O$_2$ production. GSSG, oxidized glutathione, and protein membrane constituents [18]. Membrane lipid peroxidation, measured by TBARs production, is elevated in sickle RBCs at baseline [13,19,20]. In addition to being markers for oxidative stress, lipid peroxidation products such as malondialdehyde have additional toxic effects because of their ability to react with proteins, nucleic acids, and lipids [16].

**Role of white blood cells in oxidant production in acute chest syndrome**

Although SCD is a genetic disorder of the hemoglobin molecule, there is a growing body of evidence that suggests...
that WBCs, particularly polymorphonuclear leukocytes (PMNs), are abnormal in this disease as well. Peripheral WBC counts are elevated in VOC and ACS, and WBC counts greater than 15,000 are associated with increased mortality [26,27]. Additionally, sickle PMNs at baseline have increased expression of the high-affinity Fc receptor CD64; this receptor is even more pronounced during crisis [28,29]. In addition to being a marker of PMN activation, CD64 appears to play a role in the adherence of sickle PMNs to the vascular endothelium [28]. When PMNs become activated or adherent to the endothelium, they can produce oxidants such as \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) through the activation of enzymes such as myeloperoxidase [30]. In a transgenic sickle cell mouse model, induction of acute lung injury was associated with increased myeloperoxidase activity as compared with wild-type mice, suggesting that the development of ACS in SCD may be accompanied by PMN infiltration into the lungs [31].

SCD patients have increased levels of the PMN chemokine IL-8 during VOC, suggesting that during crisis there is increased PMN recruitment [32]. Additionally, PMNs from SCD patients at baseline have increased myeloperoxidase activity [33]. Although not directly measured in human ACS, demonstration of increased myeloperoxidase activity in sickle WBCs at baseline and in transgenic mice during crisis suggests that this enzyme may play a role in oxidant generation by PMNs in ACS. In addition, sickle PMNs generate nitric oxide (NO) and \( \text{O}_2^- \) during crisis [34], and these molecules can react to form ONOO\(^-\). These data suggest that, in addition to increased activation, PMNs in SCD patients may have a greater propensity toward oxidant generation. Finally, when incubated with sickle RBCs, PMNs exhibit increased adherence to these RBCs with a resultant increase in production of oxidants, as measured by 2',7'-dichlorofluorescein diacetate fluorescence [35].

**Role of the endothelium in oxidant production during acute chest syndrome**

In addition to being a potential target for oxidative damage, the vascular endothelium may play a primary role in the generation of oxidants. Sultana et al. [6] demonstrated that coinubcation of sickle RBCs with human umbilical vein endothelial cells resulted in lipid peroxidation, as measured by TBARSs, and transendothelial migration of monocytes. Additionally, we demonstrated that coinubcation of plasma from ACS patients with bovine pulmonary artery endothelial cells resulted in formation of ONOO\(^-\) within the vascular endothelium [36]. We also demonstrated [36] that there are decreases in the antioxidant thiols and glutathione reductase system in bovine pulmonary artery endothelial cells after coinubcation with plasma from ACS patients. These findings suggest that the endothelium is more susceptible to oxidant-related damage during ACS.

These *in vitro* findings may be biologically relevant, because endothelial production of oxidants has been demonstrated *in vivo* using a transgenic mouse model of SCD [5]; exposure of these mice to hypoxia resulted in formation of oxidants within the vascular endothelium of the cremaster muscle. Although endothelial production of oxidants is difficult to measure directly in human disease, these *in vitro* and *in vivo* data suggest that the endothelium of the pulmonary arteries may be involved in oxidant production during ACS.

**Vascular endothelium as a target for oxidant damage**

Although there appears to be increased oxidant production and decreased antioxidant defense mechanisms in SCD, how this relates to the pathophysiology of ACS has not been elucidated. One mechanism responsible for vaso-occlusion is the adhesion of sickle RBCs to the vascular endothelium. It is hypothesized that, in part, this occurs secondary to endothelial damage and increased adhesion molecule expression resulting from increased oxidant burden. The vascular endothelium appears uniquely sensitive to damage from oxidizing molecules produced during VOC and ACS. One mechanism by which this may occur is via deactivation of NO (Fig. 2). Several studies have suggested that alterations in NO metabolism occur during SCD, particularly during VOC or ACS.

It was demonstrated that plasma NO levels are decreased in VOC and correlate with decreases in \( \text{L-arginine} \) [37] and increases in soluble VCAM-1 [38], a molecule that is implicated in adhesion of sickle RBCs to the endothelium. Rees et al. [39] found that, compared with control individuals, plasma nitrite (\( \text{NO}_2^- \)) levels are elevated in SCD patients during crisis; however, these levels were not significantly different from those in SCD patients at baseline. Lopez and coworkers [40,41] demonstrated that, although initial NO levels in VOC patients correlate inversely with pain score, sequential values are not predictive of clinical course. Additionally, interactions between sickle RBCs and the endothelium lead to abnormal vascular responses to NO in SCD. In two models of SCD, aortic vascular strips failed to relax when stimulated with acetylcholine [42] and infusion of the nitric oxide synthase (NOS) inhibitor \( \text{N}^3\)-nitro-L-arginine methyl ester resulted in decreased cerebral blood flow [43].

One possible explanation for the conflicting results regarding NO metabolism is that the NO that is produced is subsequently deactivated. NO, an endogenous vasodilator and inhibitor of platelet aggregation, is produced by a variety of cell types, including vascular endothelium, neurons, macrophages, and smooth muscle cells [44–46]. Additionally, by preventing metal catalyzed lipid oxidation, NO can act as an antioxidant [44]. It is formed from \( \text{L-arginine} \) by a family of enzymes termed the NOSs. Three iso-
forms of this enzyme exist [46,47]: constitutive neuronal NOS, endothelial NOS, and inducible NOS. Unfortunately, the beneficial actions of NO are often mitigated because of preferential shunting toward toxic metabolites such as NO$_2^-$, nitrate (NO$_3^-$), and ONOO$^-$ [47–49]. This occurs rapidly in the presence of oxygen and oxygen-related molecules such as O$_2^-$ and H$_2$O$_2$; specifically, NO reacts with O$_2^-$ rapidly to form ONOO$^-$, which exists in equilibrium with peroxynitrous acid (ONOOH). Because the half-life of ONOO$^-$ is very short (approximately one second), most of its toxic effects occur via reaction products of ONOOH, specifically OH and NO$_2^*$, molecules that are implicated in fatty acid oxidation and nitrosative stress via nitration of aromatic amino acids [50]. Production of oxygen radicals results in peroxidation of the lipid membrane, and may predispose the endothelial cell to apoptosis [9].

In addition to decreasing the bioavailability of NO, free radicals can contribute to vaso-occlusion through production of the potent vasoconstrictor endothelin-1. Exposure of cultured human endothelial cells to RBCs sickled in vitro results in a fourfold to eightfold induction of endothelin-1 mRNA [51]. Similarly, we demonstrated increased endothelin-1 mRNA and protein levels in endothelial cells exposed to plasma from ACS patients as compared with those exposed to plasma from SCD patients at baseline [52]. Endothelin-1 transcription may be induced by activation of the redox-sensitive NF-$\kappa$B or activating protein (AP)-1 by reactive oxygen radicals generated during ACS [12]. These findings suggest that, in addition to direct toxicity to the vascular endothelium, reactive oxygen species may contribute to vaso-occlusion through alteration in vascular tone.

Additionally, reactive oxygen species may act as second messengers to alter endothelial cell gene expression via activation of the redox-sensitive transcription factor NF-$\kappa$B. In unstimulated cells, NF-$\kappa$B is sequestered in an inactive state within the cytoplasm. When exposed to oxygen radicals NF-$\kappa$B is activated by phosphorylation and translocates to the nucleus, where it affects gene expression [53]. Among the genes that are upregulated by NF-$\kappa$B activity are those that encode the adhesion molecules VCAM-1 and ICAM-1, which can facilitate binding of sickle RBCs and WBCs to the endothelium. In this way, free radical generation in SCD may contribute to the propagation of vaso-occlusion.

Antioxidant defense mechanisms in sickle cell disease

Enzymatic antioxidants

There are several mechanisms by which aerobic organisms protect themselves from oxidative stress. On a cellular level, this occurs primarily through the actions of the enzymes superoxide dismutase, catalase, and GPx. As noted above, sickle RBCs at baseline are deficient in each of these enzyme systems [23–25]. Moreover, the reduced glutathione (GSH) level in sickle RBCs was approximately 50% lower than that observed in hemoglobin A RBCs [54]. Additionally, we demonstrated that, compared with plasma from normal volunteers, exposure to plasma from SCD patients at baseline decreases levels of GSH, which is an essential cofactor for GPx activity in cultured endothelial cells; there is a greater decrease in endothelial cell reduced GSH on exposure to plasma from ACS patients. Similarly, exposure to ACS plasma resulted in a decreased level of the endothelial cell antioxidant thiols [36]. These findings suggest that, in SCD patients, particularly during ACS, there is a decreased capacity to scavenge free radicals, making such persons more susceptible to oxidant-related damage.

Vitamins A, C, and E

Other cellular antioxidants include $\alpha$-tocopherol (vitamin E), ascorbic acid (vitamin C), and $\beta$-carotene (vitamin A). $\alpha$-Tocopherol and $\beta$-carotene scavenge free radicals to prevent lipid peroxidation [12]; of note, SCD patients have approximately a 40% reduction in plasma carotene levels and a 30% reduction in vitamin E levels [25,55]. Additionally, there is an inverse correlation between vitamin E levels and the percentage of irreversibly sickled RBCs [56].
Although it appears that vitamin E depletion may play a role in the development of vaso-occlusion, the effect of vitamin E supplementation is unclear. In two small prospective studies that evaluated the effect of vitamin E supplementation in SCD patients [57,58], there was a decrease in the number of irreversibly sickled cells, but this did not correlate with a reduction in the number of occurrences or severity of VOC. Measurement of ascorbic acid levels in SCD has produced conflicting results; several studies [54,59–62] have demonstrated reduced levels in the plasma and WBCs of SCD patients, but others have found no significant difference. This disparity may reflect differences in the populations studied, but makes it less likely that vitamin C represents an important antioxidant in this population. To date, no clinical trials have been conducted to evaluate the efficacy of either vitamin A or C supplementation in the SCD population.

Homocysteine
Homocysteine, a sulfur-containing amino acid that is produced during methionine metabolism, can produce reactive oxygen species via auto-oxidation [12]. Although no clear link with ACS has been demonstrated, hyperhomocysteinemia is associated with increased risk for cerebrovascular disease in SCD patients [63], suggesting an increased propensity toward vascular disease. Additionally, serum levels of key cofactors in homocysteine metabolism (ie folate, and vitamins B$_{12}$ and B$_{9}$) are depressed in SCD patients, suggesting that they are more prone to hyperhomocysteinemia. Further work is needed to elucidate the role of homocysteine in vaso-occlusion.

Conclusion
Reactive oxygen species may play an important role in the vascular dysfunction that is observed during ACS and VOC of SCD. Currently, however, there is little direct information available to confirm this hypothesis. In addition to being directly toxic to the endothelium via peroxidation of the lipid membrane, reactive oxygen species can upregulate expression of molecules such as VCAM-1, ICAM-1, and endothelin-1. The adhesion molecules VCAM-1 and ICAM-1 facilitate interaction between sickle RBCs and WBCs and the endothelium, thereby promoting vaso-occlusion. Endothelin-1 is a potent vasoconstrictor and an important mediator of vascular tone. By upregulating endothelin-1 expression and deactivating the vasodilator NO, oxygen radicals modulate vascular tone, and thereby could increase vaso-occlusion.

Although the genetic hemoglobinopathy of SCD is responsible for a percentage of the oxygen radicals that are produced, deficiencies in antioxidant defense mechanisms and the presence of other sites of oxidant production suggest that other genetic polymorphisms exist within the SCD population. It is possible that both genetic and dietary heterogeneity exist among SCD patients, and that this is in part responsible for the clinical variability in disease course. Further work is necessary to define more clearly the oxidant/antioxidant profile of SCD patients at baseline and during VOCs, including ACS, and to examine the clinical effect of pharmacologic intervention to reduce oxidant production in SCD. This will hopefully lead to a better understanding of the role of reactive oxygen species in the pathogenesis of vaso-occlusion.

Acknowledgements
This work was funded by an American Lung Association Research Training Fellowship (RT-030-N; ESK) and by an American Heart Association Grant-In-Aid (0150155N; HWF).

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