Published in final edited form as:

Int J Biochem Cell Biol. 2006 ; 38(8): 1237–1243.

Sickle cell disease and nitric oxide: A paradigm shift?

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Abstract

Traditionally the pathophysiology of sickle cell disease is thought to result from the polymerization of hemoglobin S in red cells, under hypoxic conditions, resulting in the occlusion of blood vessels. Adhesion of cells to the venular endothelium also appears to play a role. Recent studies have also suggested that in addition to the polymerization of hemoglobin S in the red blood cell, a deficiency of the endogenous vasodilator, nitric oxide may be involved. Hemoglobin released as a result of hemolysis rapidly consumes nitric oxide resulting in a whole program of events that inhibit blood flow. Therapies directed at decreasing the destruction of nitric oxide, increasing the production of nitric oxide, or amplifying the nitric oxide response may prove beneficial.

Keywords

Sickle cell; Nitric oxide; Blood flow

1. Introduction

Sickle cell disease is an autosomal recessive disorder and the most common genetic disease affecting African-Americans. Approximately, 0.15% of African-Americans are homozygous for sickle cell disease, and 8% have sickle cell trait. The single amino acid change in the beta subunit causes sickle hemoglobin to polymerize, especially under low oxygen tension. Patients with sickle cell disease suffer from acute and chronic vascular occlusion due to polymerization of sickle hemoglobin in red cells, inducing red cell rigidity that is often accompanied by a change in morphology to a sickle or crescent shape. Furthermore, hemoglobin S polymerization can lead to hemolysis, inflammation, cell adhesion and end-organ ischemia-reperfusion injury and infarction. Patients with sickle cell disease often have characteristic vaso-occlusive crises, which are intermittent painful episodes due to acute vascular obstruction. Recent studies indicate that up to 50% of patients with sickle cell disease have endothelial dysfunction due to impaired bioavailability of endogenous nitric oxide (NO), due in large part to scavenging of NO by cell-free plasma hemoglobin. These observations suggest that therapies directed at restoring NO bioavailability might prove beneficial.

1.1. Nitric oxide and vascular function

NO is a soluble gas with a half-life of seconds, continuously synthesized in endothelial cells from the amino acid L-arginine by isoforms of the NO synthase enzyme. NO released from

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the endothelium activates soluble guanylyl cyclase in smooth muscle after binding to its heme group, resulting in increased intracellular cyclic GMP (Ignarro, Byrns, Buga, & Wood, 1987). Cyclic GMP activates cGMP-dependent kinases that decrease intracellular calcium concentration in smooth muscle, producing relaxation, vasodilation, and increased regional blood flow (Furchgott & Zawadzki, 1980). In addition, NO induces a coordinated program of cellular events that promote blood flow, primarily by suppressing platelet aggregation, expression of cell adhesion molecules on endothelial cells, and secretion of procoagulant proteins (Hebbel, 1985; Reiter et al., 2002; Rother, Bell, Hillmen, & Gladwin, 2005; Voetsch, Jin, & Loscalzo, 2004).

2. Pathogenesis

2.1. Reduced nitric oxide bioavailability in sickle cell disease

Recent studies suggest that patients with sickle cell disease suffer from decreased NO reserves. Blood plasma levels of L-arginine (the precursor to NO) are depressed in patients with sickle cell disease, particularly during vaso-occlusive crisis and the acute chest syndrome, and these levels vary inversely with pain symptoms (Enwonwu, Xu, & Turner, 1990; Morris, Kuypers, Larkin, Vichinsky, & Styles, 2000). Furthermore, NO-dependent blood flow is impaired in patients with sickle cell disease (Belhassen et al., 2001; Eberhardt et al., 2003; Gladwin et al., 2003). In comparison to healthy controls, inhibition of NOS with L-NMMA (a NOS-dependent inhibitor) produces little change in blood flow in half of patients with SCD, indicating that little of their baseline blood flow is provided by NO (Eberhardt et al., 2003; Gladwin et al., 2003). These data suggest that dysfunctional vascular endothelium may contribute to the clinical events suffered by patients with sickle cell disease. Although reports have varied, some investigators have found low levels of NO_x (nitrate and nitrite) levels in patients with sickle cell disease and these reduced levels are consistent with impaired endothelial generation of NO, and its subsequent reactions with hemoglobin and oxygen. The three major mechanisms of impaired NO bioavailability appear to be due to decreased plasma L-arginine and consumption of NO by cell-free plasma hemoglobin and by reactive oxygen species.

2.2. L-Arginine

L-Arginine is an important amino acid in the endogenous production of NO. Arginine is taken up into cells via a cationic amino acid transporter and is converted to NO via the enzyme NO synthase. Arginine alternatively can be broken down into ornithine via the enzyme arginase, and ultimately converted into proline and polyamines. Pathological release of arginase into blood plasma appears to contribute to decreased NO bioavailability. Patients with sickle cell disease have low plasma arginine levels (Morris et al., 2003). We have found in these patients high plasma arginase levels, correlating with low ratios of plasma arginine to ornithine (Morris et al., 2005). Plasma arginase levels are highest in those patients with markers of accelerated hemolytic rate. Arginase is particularly abundant in young erythrocytes, which predominate in these patients because of rapid erythrocyte turnover. The enzyme is released into blood plasma as those cells lyse, depleting the plasma pool of arginine. Patients with the lowest ratio of arginine to ornithine are highly prone to develop pulmonary hypertension and early death (Morris et al., 2005). Thus, intravascular hemolysis results in pulmonary vascular disease related to impaired production of NO due in part to deficiency of L-arginine. Although low arginine levels are thought to be related to the development of pulmonary hypertension independent of sickle cell disease, patients with sickle cell disease have ongoing hemolysis and release of arginase, which continually depletes NO synthesis and is associated with risk of mortality from pulmonary hypertension.

2.3. Consumption of NO by cell-free hemoglobin

Laminar flowing blood and compartmentalization of hemoglobin inside the red cell produce naturally occurring barriers to the consumption of NO by intraerythrocytic hemoglobin (Azarov et al., 2005; Minneci et al., 2005). In contrast, cell-free plasma hemoglobin resulting from intravascular hemolysis consumes NO 1000-fold more rapidly (Liu et al., 1998), dramatically limiting NO bioavailability in patients with sickle cell disease (Reiter et al., 2002). Thus, intravascular hemolysis produces a state of resistance to NO-dependent vasodilation. Consistent with this thesis, in patients with the highest plasma heme levels (≥ 6 μM), the blood flow responses to the NO donor sodium nitroprusside were abolished (Gladwin et al., 2003). During vaso-occlusive pain crisis and the acute chest syndrome, hemolysis intensifies with increases of plasma hemoglobin by up to fourfold suggesting that NO scavenging or inactivation may play a major role in vascular instability during crisis (Reiter et al., 2002). This in vitro association of cell-free hemoglobin with NO consumption has been confirmed in a canine model, in which in vivo hemolysis produces pulmonary hypertension reversed by exogenously administered NO (Minneci et al., 2005).

Hemoglobin released during intravascular hemolysis is scavenged by haptoglobin, the complex then binding to CD-163 (hemoglobin scavenger receptor) on macrophages and undergoing phagocytosis and degradation, depleting plasma haptoglobin. Cell-free plasma hemoglobin will accumulate only when the hemolytic rate exceeds the scavenging capacity of plasma haptoglobin. This accumulated hemoglobin reacts stoichiometrically with NO, inactivating it in a nearly diffusion limited reaction.

2.4. Reactive oxygen species

Reactive oxygen species scavenge NO thus providing an additional mechanism by which NO bioavailability is decreased. The production of reactive oxygen species in patients with sickle cell disease is multi-factorial. Tissue ischemia, occurring during vaso-occlusion may initiate a program of ischemia-reperfusion pathophysiology that may play an important role. When the occlusion is relieved there is an increase in blood levels of oxygen and oxygen free radicals, which may cause oxidative damage. Free radical damage can result in membrane lipid peroxidation and cell destruction and NF- κ B activation. NF- κ B induces increased endothelial cell adhesion molecule expression, which can bind with integrins on leukocytes and young erythrocytes, resulting in cell adherence which can cause more vaso-occlusion, further exacerbating the cycle of ischemia and reperfusion (Aslan, Thornley-Brown, & Freeman, 2000).

In addition to these general mechanisms, additional mechanisms appear to produce high levels of reactive oxygen species specifically in patients with sickle cell disease. Patients with sickle cell disease produce more oxidative species like hydrogen peroxide and superoxide and have impaired free radical defense mechanisms (Aslan & Freeman, 2002). There are several pathways that potentially may produce clinically significant amounts of superoxide in patients with sickle cell disease. First, xanthine oxidase activity, present in large amounts on endothelial cells in patients with SCD, produces superoxide as a side product of uric acid production (Aslan et al., 2001). Second, marrow transplantation experiments in a murine model of sickle cell disease strongly implicate NADPH oxidase production of superoxide in cerebral blood vessels with consequent vascular dysfunction (Wood, Hebbel, & Granger, 2005). Third, once oxidative damage occurs in the red cell, hemoglobin denatures and Fe^{3+} interacts with the red cell membrane causing lipid peroxidation, membrane damage, inactivation of membrane enzymes and DNA damage. Sickled red blood cells have increased oxidized lipids and a greater tendency for lipid peroxidation in comparison to normal red blood cells (Aslan et al., 2000). These cells may then hemolyze, release free hemoglobin, which consumes NO. Finally, the reduced availability of L-arginine as a substrate for NOS not only may reduce NO synthesis,

but also cause uncoupling of NOS activity that produces reactive oxygen species (Morris et al., 2005). Each of these mechanisms may cooperate in increasing superoxide production in sickle cell disease, causing NO consumption and vascular dysfunction.

2.5. Hemolysis-endothelial dysfunction syndrome

As a consequence of impaired NO bioavailability, patients with significant intravascular hemolysis are at risk for a recently recognized constellation of medical complications, which we have called the hemolysis-endothelial dysfunction syndrome. In addition to pulmonary hypertension, the patients with the most severe hemolysis tend to frequently develop leg ulcers and priapism (painful persistent penile erection) (Nolan, Wyszynski, Farrer, & Steinberg, 2005; Kato et al., 2006). It is counterintuitive that the latter complication would result from impaired NO bioavailability, since NO production is believed to induce normal penile erection. However, consistent with the human data, the mouse doubly deficient in both the endothelial and inducible forms of NO synthase also demonstrates priapism, as does the sickle cell transgenic mouse. It has been proposed that NO deficiency down regulates expression of the counterbalancing phosphodiesterase 5 (PDE5), resulting in impaired ability to dampen surges in cyclic GMP level in the penile blood vessels (Champion, Bivalacqua, Takimoto, Kass, & Burnett, 2005). Other symptoms potentially related to impaired NO bioavailability can include esophageal dysmotility, abdominal pain, renal dysfunction, high blood pressure and ischemic stroke. Each of these is regulated in part by the NO pathway and it is attractive to speculate that impaired NO bioavailability plays a role in their development (Rother et al., 2005) (see Table 1).

Pulmonary hypertension is a particularly ominous complication of hemolysis-endothelial dysfunction syndrome. Patients with sickle cell disease and pulmonary hypertension have a mortality rate of approximately 1% per month, far higher than those without pulmonary hypertension (Gladwin et al., 2004). Pulmonary hypertension is a stronger predictor of early death than any other chronic complication of sickle cell disease in adults. This provides a rationale for clinical trials of pulmonary hypertension treatment in adults with sickle cell disease, although it remains to be proven whether this will reduce mortality. There are a number of therapeutic strategies that might restore NO-dependent blood flow in patients with sickle cell disease and potentially reduce the morbidities of the hemolysis-endothelial dysfunction syndrome, such as administration of NO or NO donors, or drugs that either reduce oxidant stress or increase responsiveness to NO.

2.6. Reaction of nitrite with hemoglobin

Recent data suggest a capability of red cell hemoglobin to reduce nitrite to NO, inducing vasorelaxation in human volunteers (Cosby et al., 2003). Red cell hemoglobin, at 50% oxygen saturation (i.e., tissue levels) provides optimal reaction velocity for this nitrite reductase activity, providing a potential for NO delivery by red cells to hypoxic tissue, improving its regional blood flow (Crawford et al., 2006; Huang et al., 2005a,b) (Scott Isbell et al., 2005). Other heme proteins also may catalyze the reaction of nitrite to NO and it is indeed possible that myoglobin or others may also be physiologically important.

3. Therapy

3.1. Increase NO production

3.1.1. Inhaled NO, inhaled nitrite, intravascular nitrite—One therapeutic approach to conditions of decreased NO bioavailability is to directly deliver NO and NO donor compounds. Inhaled NO, already FDA approved for newborns with pulmonary hypertension, in pilot clinical trials has reduced the severity and duration of vaso-occlusive pain crisis in children with sickle cell disease (Weiner et al., 2003). A larger scale clinical trial is currently in progress.

Inhaled NO produces a rapid decrease in increased pulmonary pressures and improves ventilation-to-perfusion matching and oxygenation. However, inhaled NO is expensive and requires specialized handling and administration. Hunter and colleagues have demonstrated in an animal model that inhaled nebulized sodium nitrite, a novel NO donor, is an effective pulmonary vasodilator; notably effective under pathophysiologic conditions including hypoxia and acidosis. Furthermore, inhaled nitrite produced an effective and longer lasting reduction in pulmonary pressures than inhaled NO (Hunter et al., 2004). Detailed translational and toxicity studies in humans and multiple other species are ongoing. One mechanism by which NO or nitrite improves vasodilatory function in hemolytic diseases is by oxidizing cell-free plasma hemoglobin (Minneci et al., 2005). This will reduce cell-free hemoglobin-mediated scavenging of NO in the peripheral circulation. Our group is investigating intravascular nitrite in healthy volunteers and in patients with sickle cell disease, providing encouraging preliminary results, with improvement in regional blood flow. Sodium nitrite is a promising NO pharmaceutical, already FDA approved as an intravenous antidote for cyanide poisoning in humans, that has a putative unique property of NO formation and delivery selectively to hypoxic tissue as discussed above.

3.1.2. Hydroxyurea—Hydroxyurea induces expression of fetal hemoglobin, which reduces hemoglobin S polymerization in patients with sickle cell disease reducing mortality and vaso-occlusive crises (Steinberg et al., 2003). In vitro and animal studies have demonstrated that hydroxyurea may play an additional role as an NO donor (King, 2004). Activation of fetal hemoglobin expression by hydroxyurea appears to occur through this NO pathway (Cokic et al., 2003). Hydroxyurea is a proven treatment for sickle cell disease that may exert part of its therapeutic effect through the NO pathway.

3.1.3. Arginine—Correction of plasma L-arginine deficiency may be possible with oral L-arginine administration, hypothetically improving NO bioavailability. Although pilot studies have shown that arginine alone does not increase NO species in patients with sickle cell disease in steady state, there is evidence that it may do so when given in conjunction with hydroxyurea (Morris et al., 2003). Further investigation is warranted with such therapies in patients with sickle cell disease.

3.2. Decrease NO destruction

3.2.1. Allopurinol—Allopurinol is a xanthine oxidase inhibitor in widespread clinical use to prevent gout due to excessive uric acid. Since allopurinol therapy should prevent xanthine oxidase from producing both uric acid and reactive oxygen species, this familiar drug merits investigation in sickle cell disease. Reducing generation of reactive oxygen species by xanthine oxidase might reduce its consumption of NO.

3.3. Amplify NO response

3.3.1. Statins—At least two drugs in clinical use appear capable of increasing sensitivity to NO. Statins, inhibitors of HMG-CoA reductase conceived originally to reduce serum cholesterol, also improve NO-dependent blood flow, even in healthy volunteers with normal baseline cholesterol levels. Statins suppress the expression of a variety of cell adhesion molecules and helps to protect against increased tissue factor expression (Solovey et al., 2004). Use of statins therefore could have the beneficial effect of altering the vascular pathobiology of sickle cell disease.

3.3.2. Sildenafil—Cyclic GMP (cGMP) production leads to a cascade of intracellular signaling events that results in smooth muscle relaxation. Sildenafil inhibits hydrolysis of cGMP by phosphodiesterase 5 (PDE5), prolonging cGMP effect. This has the ultimate effect of amplifying NO responsiveness in the vascular beds expressing PDE5, primarily penile and

pulmonary blood vessels. Although use of this drug is familiar for penile erectile dysfunction, it has also found a role in pulmonary hypertension, with preliminary indications of similar effectiveness in pulmonary hypertension due to sickle cell disease, and more clinical trials anticipated (Machado et al., 2005). There is considerable concern that sildenafil may increase the rate of priapism, persistent painful penile erections, in males with sickle cell disease. Our pilot experience suggests that these concerns may be exaggerated, but further study is required before sildenafil can be recommended for males with sickle cell disease and pulmonary hypertension. Thus far there is no published evidence that other PDE5 inhibitors are as effective as sildenafil for pulmonary hypertension (Fig. 1).

4. Conclusions

Sickle cell disease is the first disease in which the specific genetic defect has been identified. However, there is a large spectrum of phenotypic expression. Although much of its pathophysiology relates to polymerization of sickle hemoglobin inside the red cell, there is a paradigm shift in which hemolysis, NO deficiency, ischemia-reperfusion injury, cell adhesion and inflammation are thought to also play a role. These alternative mechanisms are providing new targets for therapeutic intervention. NO, and drugs that alter NO bioavailability, will be important therapeutic agents for clinical investigation.

Acknowledgements

This research was supported in part by the Intramural Research Program of the United States National Institutes of Health, National Heart, Lung and Blood Institute and the Clinical Center Critical Care Medicine Department.

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Fig. 1.

New investigational therapeutic targets for patients with sickle cell disease at the level of the pre-capillary arteriole, capillary and post-capillary venule. These therapeutic targets include (a) potential NO donors, (b) drugs that decrease oxidative stress and (c) drugs that increase the response to NO. Hemolysis from sickle cell disease results in release of hemoglobin and arginase from the red cell. Cell free hemoglobin rapidly consumes nitric oxide, an important mediator of blood flow. Arginase breaks down arginine, the substrate for nitric oxide production. Under hypoxic conditions, polymerization of hemoglobin S results in a rigid, sickled shape cell that can occlude blood flow. As a result of hemolysis, young red blood cells enter the circulation and can stick to the endothelium via $\alpha_4\beta_1$ integrins binding to VCAM-1. Due to occlusion of flow, ischemia-reperfusion pathophysiology may play a role in sickle cell disease with oxidative damage resulting in cell destruction. Xanthine oxidase and NADPH oxidase may increase oxidative damage in patients with sickle cell disease. (a) Hydroxyurea, inhaled nitric oxide, inhaled/intravascular nitrite may act as NO donors and decrease the expression of cell adhesion molecules which may prevent red cell adhesion to the endothelium and result in improved blood flow. L-Arginine supplementation may replenish arginine under conditions of hemolysis. (b) Allopurinol inhibits xanthine oxidase mediated superoxide production which may lead to decreased cellular damage. (c) Statin therapy may decrease the expression of a variety of adhesion molecules. Sildenafil is a phosphodiesterase 5 inhibitor which inhibits the breakdown of cGMP and results in improved NO responsiveness.

Table 1

Hemolysis endothelial dysfunction syndrome

Organ system	Manifestation
Lungs	Pulmonary hypertension
Genito-urinary	Priapism
Skin	Leg ulcers
Brain	Stroke
Renal	Renal insufficiency
Gastrointestinal	Esophageal dysmotility abdominal pain