Anti-Sickling Property of Fetal Hemoglobin Enhances Nitric Oxide Bioavailability and Ameliorates Organ Oxidative Stress in Transgenic-Knockout Sickle Mice

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ABSTRACT

In sickle cell disease (SCD), the events originating from hemoglobin S polymerization and intravascular sickling lead to reperfusion injury, hemolysis, decreased nitric oxide (NO) bioavailability and oxidative stress. Oxidative stress is implicated as a contributing factor to multiple organ damage in SCD. We hypothesize that inhibition of sickling by genetic manipulation to enhance anti-sickling fetal hemoglobin (HbF) expression will have an ameliorating effect on oxidative stress by decreasing intravascular sickling and hemolysis and enhancing nitric oxide (NO) bioavailability. We tested this hypothesis in BERK mice expressing exclusively human α- and βS-globins and varying levels of HbF, i.e., BERK (<1% HbF), BERKγM (20% HbF) and BERKγH (40% HbF). Intravascular sickling showed a distinct decrease with increased expression of HbF, which was accompanied by decreased hemolysis and increased NO metabolites (NOx) levels. Consistent with decreased intravascular sickling and increased NO bioavailability, BERKγM and BERKγH mice showed markedly decreased lipid peroxidation accompanied by increased activity/levels of antioxidants (superoxide dismutase [SOD], catalase, glutathione peroxidase [GPx] and reduced glutathione [GSH]) in the muscle, kidney and liver as compared with BERK mice (P<0.05-0.0001). NOx levels showed a strong inverse correlation with hemolytic rate and oxidative stress. Decreased oxidative stress in the presence of elevated HbF levels led to an anti-inflammatory effect as evidenced by decreased peripheral leukocyte counts. These results show that the protective effect of HbF is mediated primarily by decreasing intravascular sickling resulting in decreased oxidative stress and increased NO bioavailability.
Key words: sickle cell disease, fetal hemoglobin, hemolysis, nitric oxide, oxidative stress, inflammation.
INTRODUCTION

Central to the vaso-occlusive pathophysiology of sickle cell disease (SCD) is polymerization of hemoglobin S (HbS) and intravascular sickling under deoxygenated conditions, the consequences of which can lead to vaso-occlusion, reperfusion injury, hemolysis, decreased nitric oxide (NO) bioavailability and oxidative stress (15; 39; 46; 48). Oxidative stress in SCD results from hypoxia-reperfusion (vaso-occlusive events) and inactivation of anti-inflammatory nitric oxide (NO) by oxidants and plasma heme (hemolysis). Chronic oxidative stress is implicated as a critical factor in endothelial activation, inflammatory effects (e.g., higher peripheral leukocyte counts) and multiple organ damage in SCD (22).

Since intravascular sickling is considered etiologic to the pathophysiology of SCD, attempts have been made to employ anti-sickling strategies to alleviate pathophysiology of this disease. In view of the anti-sickling properties of fetal hemoglobin (HbF), recent therapies have been designed to elevate HbF levels in sickle patients. For example, hydroxyurea has been commonly used to induce higher levels of HbF (7; 53). However, the mechanism of hydroxyurea action is somewhat controversial since hydroxyurea also results in increased NO production and thereby in decreased inflammation (i.e., diminished leukocyte counts) prior to the elevation of HbF (10). Recent reports suggest that hydroxyurea enhances HbF levels in sickle patients by an NO-derived mechanism (9; 10). However, it is relevant to address the issue whether genetic manipulation to increase antisickling HbF (i.e., without any pharmacological intervention) will decrease intravascular sickling, hemolysis, vaso-occlusive events
(reperfusion injury) and consequently decrease oxidative stress and improve NO bioavailability.

We have previously shown that introduction of anti-sickling HbF in transgenic-knockout (BERK) mice (expressing exclusively human α- and βS-globins) results in improved hematologic and microvascular flow parameters (27). Both human SCD and transgenic sickle mouse models are characterized by decreased NO bioavailability (4; 29; 49). The decreased NO bioavailability in BERK mice is associated with compensatory up-regulation of cyclooxygenase-2 (COX-2), a vasodilatory species, and impaired vascular reactivity to NO-mediated vasoactive stimuli (27). In contrast, BERK mice expressing 20% HbF show markedly improved microvascular reactivity to NO-mediated vasodilators and decreased COX-2 expression (27). These effects were attributed to anti-sickling effect of HbF.

Intravascular sickling and associated transient vaso-occlusive events (reperfusion injury) contribute to chronic oxidative stress in SCD, resulting in multiple organ damage. Among the sources of excessive superoxide (O$_2^-\$) generation in human SCD and transgenic sickle mice are plasma membrane NADPH oxidase (21; 57), endothelial NO synthase decoupling (2; 23), and increased plasma xanthine oxidase (4). Our recent studies have shown that arginine supplementation of BERK mice significantly decreases lipid peroxidation and increases the activity/level of endogenous anti-oxidants secondary to enhanced NO production (13). The availability of BERK mice expressing varying levels of HbF provides a unique opportunity to address the relationship between intravascular sickling, hemolysis, NO bioavailability and organ oxidative stress.
In the present studies, we have used BERK mice expressing >1%, 20% and 40% HbF levels (BERK, BERKγM and BERKγH mice, respectively) to resolve the following issues: 1. What is the effect of elevated HbF levels on intravascular sickling, hemolysis and NO bioavailability?; 2. Is increased NO bioavailability in HbF expressing sickle mice associated with decreased organ oxidative stress? 3. What is the relationship between intravascular hemolysis, NO bioavailability and organ oxidative stress?

In our studies, we have evaluated the effect of HbF on lipid peroxidation and selected anti-oxidants. Lipid peroxidation is implicated in cellular injury and organ damage. The first line of cellular defense against oxidative insults consists of the antioxidant superoxide dismutase (SOD) that converts $O_2^-$ into $H_2O_2$, whereas the antioxidants catalase and glutathione peroxidase (GPx) convert $H_2O_2$ into water. Reduced glutathione (GSH) is the most abundant non-enzymatic antioxidant in the cell and its protective action is based on the thiol group of its cysteine, with the formation of oxidized glutathione (GSSG) (5; 14; 38).

The current study reveals that the introduction of anti-sickling HbF in BERK mice significantly alleviates oxidative stress, and this effect is mediated by anti-sickling property of HbF, which leads to increased NO bioavailability and decreased organ oxidative stress.
MATERIALS AND METHODS

Chemicals

Butylated hydroxytoluene (BHT), deferoxamine (DFO), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), glutathione (GSH), oxidized glutathione (GSSG), pyrogallol, triton X-100, ethylenediamine tetracetic acid (EDTA), bovine serum albumin (BSA), thiobarbituric acid (TBA), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma/Aldrich (St. Louis, MO).

Transgenic mice

Berkeley (BERK) mice expressing cointegrated 6.4-kb miniLCR, a 1.5-kb PstI fragment of human α1 gene, and a 39-kb KpnI fragment containing human GγAγδ and βS globin genes were generated as described (44). These mice are homozygous for the mouse α-knockout (45) and homozygous for mouse β-knockout (52), and express exclusively human hemoglobin via the hemizygous copy of the BERK transgene (44). Two different γ-constructs generated by Gilman et al. (20) (γM and γH) as previously described were used for generation of HbF expressing BERKγM and BERKγH mice. The γM (G203) construct was identical to the γH construct except that it included a PCR generated 4.3 kb fragment (HumHBB 912 to 5200) containing HS 3 and 4 of the LCR instead of HS4. Injection of these constructs into fertilized mouse oocytes resulted in production of several founder mice. To breed mice on a day-to-day basis the following schemes are used: 1) The BERKγH mice (γH construct) breed successfully with each other. 2) For BERK γM (γM or G203 construct) mice, fully knocked out healthy females expressing neutral hemoglobins such as HbA (miniLCRaβA Hba0/Hba0 Hbb0/Hbb0) or
HbC and a BERK γM male in which the γM is homozygous were used. This leads to a projected breeding efficiency similar to that for the BERK mice. The globin composition in adult transgenic-knockout mice was determined by HPLC as described (17). These mice were maintained in a specific pathogen-free facility at Albert Einstein College of Medicine. These knockout mice were extensively backcrossed (an average of 8 generations) onto C57BL/6J background. We used BERK mice expressing >1.0%, ~20% and ~40% HbF (termed BERK, BERKγM and BERKγH mice, respectively). Hematological characteristics of these mice have been described (17; 27).

Control C57BL mice were maintained on a standard diet and water \textit{ad libitum}. Sickle mice were maintained on “sickle chow” developed by Paszty et al. (44) without added arginine and obtained from Purina (Purina Mills, St. Louis, MO) as diet no. 5740C and had access to Nestlets (Anacare, Bellmore, NY) nesting material. All experimental protocols were approved by the institutional animal studies committee.

\textbf{Measurement of globin chains}

The globin composition was determined by denaturing HPLC as previously described (16). The percent HbF was calculated as the percent of all beta-like globins.

\textbf{Intravascular sickling}

To determine intravascular sickling, blood samples were drawn from the tail vein into airtight syringes containing 2.5 % glutaraldehyde solution in 0.1 mol/L cacodylate buffer, pH 7.4 as described (28).

\textbf{Measurement of hemolysis}

For plasma free hemoglobin, blood samples were drawn from the abdominal aorta, and determinations made using a tetramethyl-benzidine-based assay kit (Catachem
Inc., Bridgeport, CT) that measures plasma free hemoglobin and other heme-containing proteins present in plasma.

**NO metabolites (NOx) determination**

For the determination of NO metabolites, NOx, the blood was drawn from bifurcating abdominal aorta using EDTA as an anticoagulant. Total NOx concentration in plasma was determined by nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) using manufacturer’s instructions (13; 29).

**Preparation of homogenates, cytosol and microsome fractions**

Mice were sacrificed by cervical dislocation and the entire liver was then perfused immediately with cold 0.9% NaCl and thereafter carefully removed, trimmed free of extraneous tissue and rinsed in chilled 0.15M Tris-KCl buffer (0.15M KCl + 10mM Tris-HCL, pH 7.4). The liver was then blotted dry, weighed quickly and homogenized in ice cold 0.15 M Tris-KCl buffer (pH 7.4) to yield 10% (w/v) homogenate. An aliquot of this homogenate (0.5 ml) was used for assaying reduced glutathione levels while the remainder was centrifuged at 10,000 rpm for 30 min. The resultant supernatant was transferred into pre-cooled ultracentrifugation tubes and centrifuged at 105, 000 x g for 60 min in a Beckman ultracentrifuge (Model - L870M). The supernatant (cytosol fraction), after discarding any floating lipid layer and appropriate dilution, was used for the assay of antioxidant enzymes, whereas the pellet representing microsomes was suspended in homogenizing buffer and used for assaying lipid peroxidation. The kidney and the muscle from the legs were carefully removed, along with the liver, trimmed free of extraneous tissue and rinsed in chilled 0.15 M Tris-KCl (pH 7.4). The organs were then blotted dry, weighed quickly and homogenized in ice cold 0.15 M Tris-KCl buffer.
(pH 7.4) to yield a 10% (w/v) homogenate. A 0.5ml aliquot of this homogenate was used
for assaying reduced glutathione. The rest of the homogenate was processed as described
for the liver.

Assay methods

Lipid peroxidation (LPO). LPO in the microsomes was estimated spectrophotometrically
by thiobarbituric acid reactive substances (TBARS) method, as described by Ohkawa et al.
(42) and is expressed in terms of malondialdehyde (MDA) formed per mg protein. LPO
was measured using an iron chelator (DFO) and an antioxidant butylated hydroxytoluene
(BHT) in the microsomal preparation as described (13). DFO prevents further iron-
catalyzed TBARS formation (6), and BHT is used to prevent further lipid peroxidation
(19).

Glutathione (GSH). GSH was estimated as total non-protein sulphydryl group by the
method as described by Moron et al. (37).

Superoxide dismutase (SOD). Total SOD was assayed utilizing the technique of
Marklund and Marklund (35), which involves inhibition of pyrogallol autoxidation at pH
8.0. A single unit of enzyme was defined as the quantity of superoxide dismutase required to
produce 50% inhibition of autoxidation.

Catalase. Catalase was estimated at 240 nm by monitoring the disappearance of H₂O₂ as
described by Aebi (1). The reaction mixture (1 ml, vol) contained 0.02 ml of suitably diluted
cytosol in phosphate buffer (50 mM, pH 7.0) and 0.1ml of 30 mM H₂O₂ in phosphate buffer.
Catalase enzyme activity has been expressed as moles of H₂O₂ reduced / min / mg protein.

Glutathione peroxidase (GPx). GPx activity was measured by the coupled assay
method as described by Paglia and Valentine (43). One unit of enzyme activity have been
defined as nmoles of NADPH consumed /min /mg protein based on an extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$.

**Peripheral leukocyte counts:** Peripheral leukocyte counts on blood samples obtained from mice were measured using the Bayer Advia 120 (Tarrytown, NY).

**Statistical analysis.** Statistical analysis of hematological parameters was performed using one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls multiple comparisons. Comparisons between groups were made using student’s t-test. $P<0.05$ was considered significant. Regression analysis was done using linear model $Y=a+bX$. Statistical analysis was performed using Statgraphics plus 5.0 program for Windows (Manugistics, Inc. Rockville, MD). Plasma NOx calculation was performed using the analysis spread sheet provided by Cayman chemical at http://www.caymanchem.com/app/template/Home.vm.
RESULTS

Hematological parameters, intravascular sickling and hemolysis. As depicted in Table 1, BERK mice (HbF <1.0) show low hematocrit level (20.0% ± 2.0%), high reticulocyte counts (39.5% ± 1.5%), and maximal intravascular (venous) sickling (19.9% ± 1.9%) and hemolysis (7.8 ± 0.4 μmol measured as plasma heme); these associations reflect increased red cell destruction in BERK mice. In contrast, with an increase in HbF expression in BERK mice, percent sickled red cells in venous samples showed a distinct decrease as follows: BERKγM (~20% HbF) - 13.3% ± 0.9%; BERKγH (~40% HbF) - 6.0% ± 1.0% (P<0.05 vs. BERK mice, multiple comparisons by ANOVA). The decreased sickling in BERKγM and BERKγH mice resulted in increased hematocrit levels and decreased reticulocyte counts, as well as in decreased hemolysis compared with BERK mice (P<0.05, multiple comparisons by ANOVA) (Table 1). BERKγH mice (40% HbF) showed maximal reduction in anemia as evidenced by higher hematocrit (40.8% ± 1.1%) and a concomitant decrease in percent reticulocytes compared with C57BL, BERK and BERKγM mice (P<0.05 vs. BERK, multiple comparisons). However, in BERKγH mice, plasma hemoglobin levels (5.3 ± 0.2 μmol), although lower than in BERK mice, remained higher compared with C57BL mice (3.0 ± 0.2 μmol, P<0.01), indicating persistence of hemolysis.

Plasma NO metabolites (NOx) levels. Since NO is effectively inactivated by plasma hemoglobin, we measured NO metabolites (NOx) as a measure of NO. Consistent with maximal hemolysis (Table 1), BERK mice showed marked 78% decrease in NOx levels compared with C57BL mice (each n=4; P<0.001) (Figure1). In contrast, BERKγM and BERKγH mice expressing 20% and 40% γ-globin levels respectively (each n=4), showed
marked increases in NOx levels as compared to BERK mice. While BERKγM mice showed 2.6-fold in NOx values compared to BERK mice ($P<0.001$), maximal NOx levels were observed in BERKγH mice that were 1.3-fold greater than in BERKγM mice ($P<0.001$) and 3.42-fold greater than in BERK mice ($P<0.001$). The resulting NOx levels in BERKγH mice were almost 75% of the control C57BL values.

**Lipid peroxidation (LPO).** TBARS assay was performed in the presence of deferoxamine (DFO) and BHT to prevent further lipid peroxidation during the procedure as described in the Methods. Figure 2 depicts the protective effect of HbF on LPO in various tissues (muscle, liver and kidney) of BERK mice. Evidently, in any given tissue, BERK mice (n=4) showed marked increase in LPO compared with C57BL mice (i.e., 5.4 to 6.9-fold increase vs. C57BL [n=6], $P<0.01$). In contrast, BERKγM (n=6) and BERKγH (n=4) showed marked 73.2% to 81% decreases in LPO respectively in muscle and kidney tissues ($P<0.001$), but a smaller (24.8% and 37.2%) decrease in the liver ($P<0.001$) when compared with BERK mice. LPO values in BERKγH mice showed further but modest 17% to 27% decreases in these tissues when compared with BERKγM mice ($P<0.05$-$0.001$). The resulting LPO values of BERKγH mice were not significantly different from control C57BL baseline levels in muscle and kidney, but remained significantly (2.4-fold) higher in liver.

**Antioxidants:**

**Glutathione (GSH).** As shown in Figure 3, in each group of mice (n = 4-6), GSH levels were maximal in the liver followed by kidney and the muscle tissues in that order ($P<0.05$, multiple comparisons by ANOVA). Furthermore, for any given tissue, GSH levels were lowest in BERK mice and ~43%-65% lower than those in control C57BL
mice (n=6) (P<0.001). In BERK mice expressing HbF, GSH levels increased significantly in any given tissue compared with BERK mice (BERKγM, % increase: muscle ~17%, kidney 23% and liver 38%, P<0.05-0.01 vs. BERK; BERKγH, % increase: 28%, 37.2% and 92% in that order, P<0.01-0.001 vs. BERK) (Figure 3). In BERKγH mice, the greater increase in GSH levels was clearly associated with higher expression of HbF, and the values approached almost 63% to 74% of the C57BL baseline values in the tissues examined.

**Superoxide dismutase (SOD).** As shown in Figure 4, BERK mice showed lowest total SOD activity in any given tissue compared with other groups. Compared with C57BL controls, BERK mice tissues showed a marked ~48%-63.3% decrease in SOD activity (P<0.001). Compared with BERK mice, SOD activity was significantly augmented in BERKγM and BERKγH mice. In BERKγM mice, SOD activity increased by ~25%, 11% and 27% in the muscle, kidney and the liver, respectively, (P<0.05-<0.001 vs. BERK), while in BERKγH mice, the increase was more pronounced, i.e., 48%, 30% and 59% in the corresponding tissues (P<0.001 vs. BERK). Compared with BERKγM mice, BERKγH mice exhibited significant 18.7% and 25% increases in SOD activity in the muscle and liver, respectively (P<0.05 and P<0.001), although the resulting values remained lower than in control C57BL mice.

**Catalase.** In the tissues examined, BERK mice exhibited marked decreased in catalase activity (Figure 5). The muscle and kidney catalase showed maximal decrease (68.4% and 68.7%, decrease, respectively, each P<0.001 vs. C57BL mice) followed by 32% decrease in the liver (P<0.001 vs. C57BL). In contrast, BERKγM expressing 20% HbF levels showed significant 28.2% and 50.5% increases in catalase activity in the muscle
and kidney, respectively ($P<0.05$ and $P<0.01$ vs. BERK), but no significant change in the liver. On the other hand, marked increase in catalase activity was observed in any given tissue of BERK$\gamma$H mice (muscle, 70.0%; kidney, 74.3%; liver 22.4%; $P<0.01$, $P<0.001$ and $P<0.01$, respectively, vs. BERK).

**Glutathione peroxidase (GPx).** In the tissues examined, GPx activity showed marked 45.5%-59.3% decrease ($P<0.01$) in BERK mice as compared with C57BL controls (Figure 6). In contrast, introduction of HbF resulted in 31.1%-40.0% increases in GPx activity in BERK$\gamma$M mice ($P<0.001$ vs. BERK), while maximal increase (43.8%-53.5%) was observed for BERK$\gamma$H mice ($P<0.001$ vs. BERK). Although BERK$\gamma$H mice showed marked increases in GPx activity over BERK mice, the resulting values did not reach the level of C57BL baseline values.

**Correlates of oxidative stress**

Next, we analyzed our data to ascertain whether the decreased organ oxidative stress in HbF expressing BERK mice was a consequence of decreased hemolysis and increased NO bioavailability. As shown in Table 1, the decrease in intravascular sickling paralleled percent HbF expression in BERK$\gamma$M and BERK$\gamma$H mice, and it was associated with increased hematocrit and decreased reticulocyte counts. Importantly, linear regression analysis of the data showed a strong relationship between plasma hemoglobin and plasma NOx levels as NO bioavailability increased commensurate with the decrease in plasma hemoglobin (Figure 7A). Similarly, a strong inverse relationship was noted when plasma NOx levels were plotted against the lipid peroxidation (LPO) values in the liver tissue (Figure 7B). Thus, in BERK mice (HbF, <1.0%), lowest plasma NOx values were associated with maximal lipid peroxidation; a similar relationship between NOx
levels and lipid peroxidation was observed in the muscle and kidney (data not shown). Conversely, with the introduction of anti-sickling HbF, the marked increase in NOx levels was associated with decreased lipid peroxidation.

**HbF decreases peripheral leukocyte counts**

To ascertain if the decrease in oxidative stress observed in the presence of HbF was associated with attenuated inflammation, we measured peripheral leukocyte counts in C57BL, BERK and BERK\(^\gamma\)H mice. Compared with C57BL mice (n=20), BERK mice (n=7) showed 3.2-fold greater peripheral leukocyte counts (10,915 ± 1190 vs. 35,064 ± 3,255 counts/μl, \(P<0.001\)). In contrast, compared with BERK mice, BERK\(^\gamma\)H mice (n=14; HbF ~40%) showed marked 3-fold decrease in peripheral leukocyte counts (11,542 ± 1,085 counts/μl, each \(P<0.001\) vs. BERK), and the resulting values were not significantly different from the control C57BL values.
DISCUSSION

The multiple (pleiotropic) effects of the $\beta^S$ gene include intravascular sickling, dense red cell generation, reticulocytosis, abnormal cation transport, hemolysis, reperfusion injury and multiple organ damage. On the other hand, the presence of modifier (epistatic) genetic factors such as HbF, a product of $\gamma$-globin genes ($^G\gamma$ and $^A\gamma$ genes), significantly alter disease severity and progression by inhibiting HbS polymerization and intravascular sickling, which is the primary defect in SCD. In the present study, we show that the presence of HbF in transgenic-knockout sickle (BERK) mice significantly ameliorates several expressed abnormalities. In this mouse model of SCD, increasing HbF levels results in commensurate decrease in intravascular sickling decreased hemolysis, enhanced NO production and, importantly, decreased organ oxidative stress. Finally, the results show a strong relationship between NO bioavailability and hemolysis on one hand, and between NO bioavailability and organ oxidative stress on the other, clearly indicating that events originating from the primary defect (i.e., HbS polymerization and sickling) lead to increased organ oxidative stress.

Oxidative damage in SCD and mouse models of SCD has been attributed to oxygen free radicals resulting from reperfusion injury and heme-mediated peroxidase chemistry (22; 26; 32; 54). BERK mice, expressing exclusively human $\alpha$- and $\beta^S$-globins, show maximal intravascular sickling that can intermittently disrupt blood flow to the tissues and result in reperfusion injury and oxidant generation. BERK mice also show maximal hemolysis and minimal NO metabolites (NOx) compared to other groups of mice examined. We believe that contribution of oxidative stress to NO consumption in BERK mice will be significant and even independent of hemolysis particularly during
episodes of transient vaso-occlusive events and reperfusion injury. NO inactivation by plasma ferrous hemoglobin, as shown by Gladwin, Schechter and coworkers (49), is a major contributor to oxidative stress. NOS itself can be decoupled by oxidation of the essential cofactor tetrahydrobiopterin (BH4) (2; 11), and decoupled NOS produces superoxide in place of nitric oxide. Previous studies indicate that oxidative stress is modulated by NO bioavailability and vice versa (18; 25; 55).

In BERK mice, decreased NO bioavailability will contribute to oxidative stress in a manner similar to that observed after inhibiting NO synthase activity in transgenic (NY1DD) sickle mice that have mild pathology (13). In fact, the severe pathology of BERK mice is associated with intense organ oxidative stress as evidenced by marked increase in lipid peroxidation, a marker of peroxidation of membrane lipids by peroxides, and diminished activity of antioxidants, which is in accord with our previous studies (13). BERK mice show 5.4 to 6.9-fold increases in lipid peroxidation in the tissues examined. Lipid peroxidation causes damage to the cell membrane and mitochondria, and is a likely contributor to multiple organ damage in SCD. In BERK mice, depleted levels of tissue antioxidants such as GSH, total SOD, catalase and GPx will result in diminished capacity to quench oxygen radicals and thereby enhance lipid peroxidation (8; 12). Moreover, during reperfusion (i.e., transient vaso-occlusive events), overproduction of oxygen radicals is implicated in degradation and consumption of antioxidants (40). Hence, failure to replenish antioxidants will exacerbate oxidative stress, and contribute to inflammation and multiple organ damage in SCD.

Our studies show that introduction of HbF in BERK mice resulted in marked decrease in intravascular sickling leading to recovery of hematological parameters and
decreased hemolysis in BERKγM and BERKγH mice, respectively expressing 20% and 40% HbF (balance HbS). Compared with BERK mice, BERKγH mice showed >100% increase in Hct and reduced erythropoietic stress as evidenced by ~85% decrease in peripheral reticulocytes counts (see Table 1). Decreased sickling is likely to diminish transient vaso-occlusive events and oxidant generation. Decreased hemolysis was evident by 32% decrease in cell-free plasma hemoglobin, which will also result in decreased release of arginase that consumes arginine, the substrate of NOS (23). Importantly, BERKγM and BERKγH mice showed a significant increase in NO metabolite (NOx) levels, which is indicative of decreased NO consumption/inactivation secondary to diminished plasma heme levels and global oxidative stress in these mice. Although NOx values in BERKγH mice did not reach the C57BL baseline levels, the increase was 3.42-fold over the BERK values and within 75% of the C57BL levels.

In conformity with increased NO bioavailability, BERKγM and BERKγH mice showed marked, 54% to 81%, decrease in lipid peroxidation in muscle, kidney and liver tissues (see Figure 2). In contrast to 5.4 to 6.9-fold increase in lipid peroxidation in the tissues of BERK mice over the control C57BL values, lipid peroxidation levels in BERKγH mice showed almost complete recovery to the control levels in muscle and kidney, and nearly 40% decrease in liver compared with BERK mice, demonstrating a marked ameliorating effect of anti-sickling HbF in these mice. These observations are consistent with previous reports that termination of lipid peroxidation, involving the reaction of NO with lipid-oxy and –peroxy radicals, is one of the most important antioxidant properties of NO (41; 55; 56).
Consistent with attenuated lipid peroxidation, BERK mice expressing HbF, show distinct increase in the level/activity of antioxidants (both non-enzymatic and enzymatic). Because the decrease in antioxidants in BERK mice suggests consumption/inactivation of antioxidants by excessive generation of reactive oxygen species, the decreased oxidative stress in BERKγM and BERKγH mice, as exemplified by diminished lipid peroxidation, is likely to result in the observed recovery of antioxidants. Non-enzymatic GSH, which was depleted by ~43%-65% in the tissues of BERK mice compared with C57BL mice, showed a distinct rebound in BERKγH mice with the values approaching 63.5% to 73.4% of the C57BL baseline levels (see Figure 3). GSH makes major contributions to the recycling of other antioxidants that become depleted due to excessive oxidative stress (3; 36). GSH effectively scavenges reactive oxygen species directly and indirectly by enzymatic reactions (58). Moreover, GPx catalyzes GSH-dependent reduction of H$_2$O$_2$ and other peroxides (34). Increased oxidant generation and depleted GSH and glutamine levels have been associated with compromised red cell integrity, hemolysis and decreased NO bioavailability, all factors that might as well contribute to the pathogenesis of this disease (23; 38). Oxidant generation is also associated with damage to protein 4.1 binding ability (51) and damage to red cell flipase (33), which will contribute to phosphatidylserine exposure and rapid removal from the circulation.

Increased O$_2^-$ generation not only results in endothelial dysfunction and inflammation in BERK mice, but also in excessive nitrotyrosine formation that is the result of protein nitration by peroxynitrite (ONOO$^-$) (27; 29), a molecule that is implicated in lipid peroxidation (47; 50) and impaired vascular responses (27; 30; 31). ONOO$^-$ formation and nitration of tyrosine residues of proteins (i.e., formation of
nitrotyrosine) may also be driven by plasma heme-mediated peroxidase chemistry (32; 54). Importantly, decreased sickling in the BERK mice expressing 20% HbF is associated with diminished nitrotyrosine expression (27), and NO replenishment by arginine in this mouse model increases NO production, decreases lipid peroxidation and increases endogenous antioxidants (13).

The antioxidant effect of HbF is further evident by significant increases in antioxidant enzymes SOD, catalase and GPx. With an increase in HbF levels, BERKγM and BERKγH mice show a corresponding increases in these antioxidants in all the tissues examined, although the resulting values remained lower than in control C57BL mice (see Figures 4 -6). This incomplete recovery is likely, in part, due to persistent albeit reduced hemolysis in HbF expressing BERK mice, and may reflect heme-mediated oxidative stress and an incomplete recovery of NOx levels (i.e., 75% of C57BL baseline) as observed in BERKγH mice expressing 40% HbF. The persistent hemolysis in BERK mice expressing HbF is a condition comparable to that observed in sickle patients on hydroxyurea therapy (24). Nevertheless, the observed antisickling effect of HbF in BERK mice is likely to decrease sickling-induced vaso-occlusive events and result in reduced hemolysis, increased plasma NOx production and reduced oxidative stress.

In the present studies, we show that reduction of intravascular sickling by introduction of HbF has wide-ranging effects leading to an increase in NO bioavailability and decreased organ oxidative stress. A strong relationship between plasma NOx levels and lipid peroxidation (see Figure 7) further supports a role of NO in decreased organ oxidative stress. Decreased oxidative stress is accompanied by a significant decrease in peripheral leukocyte counts, demonstrating the anti-inflammatory effect of HbF. Thus,
replenishment of NO by genetic manipulation to elevate HbF levels would result in decreased disease severity by attenuating oxidative stress and inflammation. We have previously shown that NO replenishment by arginine significantly elevates NO metabolites in transgenic-knockout sickle mice, accompanied by attenuated lipid peroxidation and enhanced activity of antioxidants (13), suggesting a role for NO in modulating oxidative stress in SCD. Future studies will be needed to examine if combining HbF elevation with arginine therapy will have a greater therapeutic effect in this disease. Nevertheless, the results of this study show that the protective effects of antisickling HbF are derived from its ability to decrease oxidative stress and enhance NO bioavailability.

**Perspectives and Significance**

The events originating from the primary defect of SCD (i.e., hemoglobin S polymerization and intravascular sickling) lead to multiple pathologies that include red cell abnormalities, vaso-occlusive events (reperfusion injury), and hemolysis, which contribute to increased oxidative stress and decreased NO bioavailability. Increased oxidative stress and NO deficiency in this disease are a major source of endothelial dysfunction, inflammation, vascular pathobiology and multiple organ damage. The interaction between NO and oxidative stress includes but is not limited to peroxynitrite (OONO⁻) formation; oxidative decoupling of endothelial nitric oxide synthase (eNOS); red cell hemolysis due to oxidative stress leading to release of arginase and the consumption of arginine, the substrate of NOS, and release of cell-free hemoglobin. Thus, the pathophysiology of sickle cell disease is driven by both increased oxidative stress and reduced NO bioavailability.
The introduction of anti-sickling HbF into BERK mice significantly alleviates oxidative stress and increases NO production, primarily by decreasing intravascular sickling and its consequences. We have previously demonstrated that arginine supplementation increases NO production, decreases oxidative stress and improves microvascular flow in transgenic sickle mice (13; 29). Taken together these results support the contention that the interaction of oxidative stress and NO deficiency impact multiple aspects of the pathophysiology of SCD. Depleted levels of antioxidants likely result in increased susceptibility to lipid peroxidation. Lipid peroxidation causes damage to the cell and mitochondrial membranes, and will contribute to multiple organ damage in this disease. Degradation and consumption of antioxidants by the release of oxidants during reperfusion has been recognized (40). Our observations support the concept that multiple organ damage in SCD is associated with increased oxidative stress, and elevated levels of anti-sickling HbF lead to an ameliorating effect.
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GRANTS

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DISCLOSURES

None.
REFERENCES


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FIGURE LEGENDS

**Figure 1.** Plasma NOx levels in C57BL, BERK, BERKγM and BERKγH mice: the effect of fetal hemoglobin (HbF). BERK mice showed minimal NOx levels compared with other groups, while BERKγH mice had 3.42-fold greater NOx levels compared with BERK mice. The NOx values in BERKγH mice were almost 75% of the control C57BL values. *P<0.001 vs. C57BL controls; †P<0.001 vs. BERK mice; ‡P<0.001 vs. BERKγM mice.

**Figure 2.** The effect of fetal hemoglobin (HbF) on lipid peroxidation (LPO) in muscle, kidney and liver of C57BL, BERK, BERKγM and BERKγH mice. TBARS assay was performed in the presence of deferoxamine (DFO) and BHT to prevent further lipid peroxidation during the procedure as described in Methods. Lipid peroxidation levels in BERKγH mice showed almost complete recovery to the control levels in the muscle and kidney tissues, and nearly 40% reduction in the liver compared with BERK mice *P<0.01-0.001 vs. C57BL controls; †P<0.001 vs. BERK mice; ‡P<0.05-0.001 vs. BERKγM mice.

**Figure 3.** Glutathione (GSH) in C57BL, BERK, BERKγM and BERKγH mice: the effect of fetal hemoglobin (HbF). In BERKγH mice, GSH values approached almost 63% to 74% of the C57BL baseline levels in these tissues. *P<0.001 vs. C57BL controls; †P<0.05-0.001 vs BERK mice; ‡P<0.01 vs. BERKγM mice.

**Figure 4.** Superoxide dismutase (SOD) activity in C57BL, BERK, BERKγM and BERKγH mice: the effect of fetal hemoglobin (HbF). *P<0.01-0.001 vs. C57BL controls,
+P<0.05-0.001 vs. BERK mice, ‡P<0.05-0.001 vs. BERKγM mice in the muscle and kidney.

**Figure 5.** Catalase activity in C57BL, BERK, BERKγM and BERKγH mice: the effect of fetal hemoglobin (HbF). *P<0.05-0.001 vs. C57BL controls, †P<0.05-0.001 vs. BERK mice.

**Figure 6.** Glutathione peroxidase (GPX) activity in C57BL, BERK, BERKγM and BERKγH mice: the effect of fetal hemoglobin (HbF). *P<0.01-0.001 vs. C57BL controls, †P<0.001 vs. BERK mice, ‡P<0.01 vs. BERKγM mice in the liver.

**Figure 7.** A: relationship between plasma heme and NOx values. Linear regression revealed a strong correlation between plasma heme and NOx levels, indicating that a decrease in hemolysis was associated with increased NO bioavailability. B: relationship between liver lipid peroxidation (LPO) and plasma NOx values showed a strong correlation between the two parameters. With an increase in plasma NOx, the LPO values showed a distinct decrease in BERKγM, BERKγH and control C57BL mice. These results indicate that a reduction in hemolysis results in increased NO bioavailability that in turn has an antioxidant effect.
Table 1

The effect of HbF on hematological parameters, intravascular sickling and hemolysis (plasma hemoglobin) in knockout sickle (BERK) mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>HbF (%)</th>
<th>Hematocrit (%)</th>
<th>Retics (%)</th>
<th>Intravascular sickling (%)</th>
<th>Plasma hemoglobin (μmol heme)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>0</td>
<td>46.0 ± 1.4 (9)*</td>
<td>1.6 ± 0.3 (6)*</td>
<td>0</td>
<td>3.0 ± 0.2 (5)*</td>
</tr>
<tr>
<td>BERK</td>
<td>&lt;1.0</td>
<td>20.0 ± 2.0 (9)*</td>
<td>39.5 ± 1.9 (5)*</td>
<td>19.9 ± 1.9 (4)*</td>
<td>7.8 ± 0.4 (8)*</td>
</tr>
<tr>
<td>BERKγM</td>
<td>21.0</td>
<td>36.3 ± 0.9 (11)*</td>
<td>31.9 ± 1.9 (5)*</td>
<td>13.3 ± 0.9 (4)*</td>
<td>5.5 ± 0.4 (6)*</td>
</tr>
<tr>
<td>BERKγH</td>
<td>40.0</td>
<td>40.8 ± 1.1 (6)§</td>
<td>9.1 ± 1.6 (4)*</td>
<td>6.0 ± 1.0 (4)*</td>
<td>5.3 ± 0.2 (5)§</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; the numbers in parentheses represent the number of mice used in these measurements. Retics = reticulocytes. *P<0.05 among the groups indicated; §P<0.05 vs. C57BL and BERK mice (multiple comparisons by ANOVA).
Plasma NOx (µmol)

- C57BL
- BERK
- BERKγM
- BERKγH

* +,‡
Lipid peroxidation (LPO)

A

Muscle

LPO (nmol/mg protein)

DFO + BHT

B

Kidney

LPO (nmol/mg protein)

DFO + BHT

C

Liver

LPO (nmol/mg protein)

DFO + BHT

C57BL BERK BERK\gamma M BERK\gamma H
Figure 3

**Glutathione (GSH)**

**A** muscle tissue

**B** kidney tissue

**C** liver tissue

Muscle GSH (nmol/g tissue)

- 0
- 20
- 40
- 60
- 80
- 100

Kidney GSH (nmol/g tissue)

- 0
- 20
- 40
- 60
- 80
- 100
- 120
- 140
- 160

Liver GSH (nmol/g tissue)

- 0
- 100
- 200
- 300
- 400

* C57BL BERK BERKγM BERKγH
Superoxide dismutase (SOD)

A. Muscle

B. Kidney

C. Liver

*+, ‡
Figure 5

Catalase

**A**

Muscle

Catalase (μmol/min/mg protein)

**B**

Kidney

Catalase (μmol/min/mg protein)

**C**

Liver

Catalase (μmol/min/mg protein)

- C57BL
- BERK
- BERK$_{M}$
- BERK$_{H}$
Kidney

Muscle

Liver

A

B

C

Glutathione peroxidase (GPx)

GPx activity (nmol NADPH/min/mg protein)

0
20
40
60
80

C57BL BERK BERK\(^{\gamma}M\) BERK\(^{\gamma}H\)

* + *,+

* *

* * *
Plasma hemoglobin (μMol heme)

$\text{Plasma NOx (μMol)}$

$\text{C57BL}$

$\text{BERK}$

$\text{BERK}_7^M$

$\text{BERK}_7^H$

Figure 7

$R^2 = 0.96$

$P < 0.023$

$R^2 = 0.77$

$P < 0.05$