Anti-inflammatory therapy ameliorates leukocyte adhesion and microvascular flow abnormalities in transgenic sickle mice

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Anti-inflammatory therapy ameliorates leukocyte adhesion and microvascular flow abnormalities in transgenic sickle mice. Am J Physiol Heart Circ Physiol 287: H293–H301, 2004. First published March 4, 2004; 10.1152/ajpheart.01150.2003.—In sickle cell disease, inflammatory activation of vascular endothelium and increased leukocyte-endothelium interaction may play an important role in the occurrence of vasoocclusion. In sickle mouse models, inflammatory stimuli (e.g., hypoxia-reoxygenation and cytokines) result in increased leukocyte recruitment and can initiate vasoocclusion, suggesting that anti-inflammatory therapy could be beneficial in management of this disease.

We have tested the hypothesis that inhibition of endothelial activation in a transgenic mouse model by anti-inflammatory agents would lead to reduced leukocyte recruitment and improved microvascular blood flow in vivo. In transgenic sickle mice, hypoxia-reoxygenation resulted in greater endothelial oxidant production than in control mice. This exaggerated inflammatory response in transgenic mice, characterized by increased leukocyte recruitment and microvascular flow abnormalities, was significantly attenuated by antioxidants (allopurinol, SOD, and catalase). In contrast, control mice exhibited a muted response to antioxidant treatment. In addition, hypoxia-reoxygenation induced activation of NF-κB in transgenic sickle mice but not in control mice. In transgenic sickle mice, sulfasalazine, an inhibitor of NF-κB activation and endothelial activation, attenuated endothelial oxidant generation, as well as NF-κB activation, accompanied by a marked decrease in leukocyte adhesion and improved microvascular blood flow. Thus targeting oxidant generation and/or NF-κB activation may constitute promising therapeutic approaches in sickle cell disease.

endothelium; antioxidants; sulfasalazine; sickle cell anemia

SICKLE CELL DISEASE IS CHARACTERIZED by recurring episodes of painful vasoocclusive crisis. The pathophysiology of this disease is due to a single amino acid replacement that results in polymerization of hemoglobin S and sickling of red blood cells under deoxygenated conditions. Although sickling and increased red cell rigidity are quintessential to this disease, the emerging evidence suggests that vascular endothelial abnormalities and increased blood cell-endothelium interactions would play a significant role in the onset of a vasoocclusive episode. Vascular endothelial abnormalities in sickle cell disease may result from intravascular sickling and red blood cell-endothelium interactions, as well as from inflammatory processes involving transient vasoocclusive events (21, 29).

Thus the rheological insult by sickle red blood cells and reperfusion injury may contribute to endothelial damage (22) and endothelial cell detachment (36, 38), as reported in other ischemic diseases (10). Circulating endothelial cells in patients with sickle cell disease have an abnormally activated phenotype, as evidenced by increased expression of adhesion molecules such as vascular cell adhesion molecule, intercellular adhesion molecule-1 (ICAM-1), P-selectin, and E-selectin, as well as tissue factor (34–36). Similarly, transgenic sickle mice have an active inflammatory response, as manifested by up-regulation of endothelial adhesion molecules and activation of nuclear factor-κB (NF-κB), a transcription factor critical for inflammatory response (6, 29).

Intravital microscopic studies in transgenic sickle mice have shown that inflammatory activation of endothelium and the associated leukocyte-endothelium interactions can induce venular flow abnormalities and initiate vasoocclusion. In transgenic sickle (βS) mice, expressing 75% of all β-globins as βS, hypoxia-reoxygenation results in a marked inflammatory response characterized by endothelial oxidant generation, increased leukocyte-endothelium interactions, and significant flow abnormalities (21). In C57BL mice transplanted with bone marrow from the homozygous sickle mice (Berkeley model), infusion of the inflammatory cytokine tumor necrosis factor frequently resulted in venular occlusion, primarily initiated by leukocyte adhesion to endothelium and accompanied by trapping and binding of sickled red blood cells to adherent leukocytes (39). These intravital findings buttress the paradigm that inflammatory activation of endothelium in human sickle cell disease would have direct consequences on the occurrence of vasoocclusive episodes. Sickle cell patients show higher-than-normal leukocyte counts, and infections are often followed by a vasoocclusive crisis (5, 25, 30).

Inflammatory response following reoxygenation (reperfusion) entails generation of endothelial oxidants (e.g., H2O2), which are implicated in the activation of NF-κB transcription factor (15). NF-κB can rapidly activate transcription of endothelial adhesion molecules involved in leukocyte-endothelium interactions (9). The consequences of NF-κB inhibition on inflammatory response (i.e., leukocyte recruitment) have not been ascertained under in vivo microcirculatory flow conditions. Development of transgenic sickle mouse models presents an opportunity to test the hypothesis that inhibition of endothelial activation would lead to reduced leukocyte recruitment...
and improved microvascular flow in vivo. To this end, we have used the β² mouse model to compare the relative efficacies of selected antioxidants, as well as to examine the efficacy of sulfasalazine. Sulfasalazine has long been used as an anti-inflammatory agent in humans with inflammatory bowel disease, and its anti-inflammatory activity is attributed to its ability to inhibit NF-κB activation (41, 43). Also, recent studies have shown that sulfasalazine has an inhibitory effect on endothelial adhesion molecule expression in sickle cell patients and β⁸ mice (37), but its effect on leukocyte recruitment in vivo has not been explored.

The β⁸ mice show clear evidence of organ damage, accompanied by higher mean corpuscular hemoglobin concentration and increased reticulocyte counts compared with control mice, as well as the presence of irreversibly sickled cells (13). Additionally, these transgenic mice exhibit an exaggerated response to hypoxia-reoxygenation (21) and, therefore, are a suitable model to test the therapeutic modulations of endothelial activation and its consequences on hemodynamic parameters and leukocyte recruitment in the microcirculation.

MATERIALS AND METHODS

Transgenic Mice

Transgenic sickle mice expressing human α (α⁸) and β⁸-globins on a homozygous mouse β⁸ME mouse background (β⁸ME) were raised in a specific pathogen-free facility at the University of Minnesota Medical School. In these mice, β⁸-globin forms symmetrical tetramers with human α-globin (42%) and with mouse α-globin (~30%). The total β⁸-globin levels are ~75% of all β-globin, as determined by denaturing HPLC (13). We refer to these as "β² mice." The transgenic mice carrying the mutant gene were backcrossed for a total of eight generations with C57BL/6J mice.

 Intravital Microscopy

Transgenic β² mice and matching C57BL controls were shipped to Albert Einstein College of Medicine, where intravitral studies were performed with the approval of the Animal Care and Use Committee. Male C57BL/6J control (n = 33) and transgenic β⁸ (n = 53) mice weighing ~25–30 g (4–6 mo old) were used. The mice were maintained on a standard diet and water ad libitum. Mice were anesthetized intraperitoneally with 10% urethane and 2% chloralose in saline (5 ml/kg). The animals were tracheostomized. The right jugular vein was cannulated for infusion of antioxidants. In vivo microcirculatory observations were made in the open cremaster muscle preparation, prepared according to the method of Baev (3). Sulfonamide and maintenance of the mouse cremaster preparation were done as described elsewhere (20). Briefly, the preparation was suffused with a Ringer bicarbonate solution (in mM: 135.0 NaCl, 5.0 KCl, 27.0 NaHCO₃, 0.64 MgCl₂, and 11.6 glucose), with pH adjusted to 7.35–7.4 by continuous bubbling with 94.6% N₂-5.6% CO₂. The osmolarity of the solution, as measured by a Microosmometer (Precision Systems, Sudbury, MA), was adjusted to 330 mosM, as described for mouse plasma (7). The temperature of the suffusion solution (flow rate 5–6 ml/min) was maintained at 34.5–35°C and monitored by a telemetherometer (Yellow Springs Instruments, Yellow Springs, OH). Microscopic observations were carried out using a Nikon microscope (model E400, Morrell Instrument, Melville, NY) equipped with a charge-coupled device (CCD) television camera (model CCD-300T-RC, Dage-MTI, Michigan City, IN) and a U-matic video recorder (model VO5800, Sony, Teaneck, NJ).

Intravitral measurements were initiated within 15 min of surgical exteriorization of the tissue and completed within the next 30 min. Red cell velocity (V_RBC) and leukocyte adhesive behavior were determined in randomly chosen postcapillary venules (~23–38 μm diameter). Vessel luminal diameter (D) was measured online using an image-shearing device (model 907, Instruments for Physiology and Medicine, San Diego, CA). V_RBC was measured along the vessel centerline using the "dual-slit" photodiode and a velocity cross correlator (33, 42) (model 102 BF, Instruments for Physiology and Medicine). The centerline V_RBC was converted to the mean V_RBC across the vessel diameter using a conversion factor of 1.6 (V_RBC / mean = 1.6, where V_mean is mean velocity), originally described by Wayland and Wayland (4) and later validated by Seki and Lipowsky (32). Volumetric flow rates (Q) were determined from V_RBC and the vessel vessel cross-sectional area (πD²/4), as described elsewhere (4, 26). Shear rates along the wall of a microvessel of a given luminal diameter were calculated as follows: 8 V_mean/D (32).

Rolling leukocytes (repeated transient contacts) were defined as those that distinctly roll along the endothelial surface at a velocity lower than that of leukocytes and red blood cells in the flow (40). Rolling leukocyte flux (cells/min) was determined as the number of leukocytes rolling through a given point in a vessel. The rolling is followed by firm adhesion of leukocytes. A leukocyte was considered adherent if it remained stationary for >30 s. Adherent leukocytes were counted along the length of a given venule and expressed as the average number of cells per 100-μm length of the vessel. Peripheral leukocyte counts on blood samples obtained from mice were determined using a Neubauer chamber. Neutrophil counts were made on blood smears stained with May-Grunwald-Giemsa stain (11).

Antioxidants

Wild-type controls and β² mice were subjected to 3 h of hypoxia in 8% O₂-0.5% CO₂-balance N₂ and then returned to room air (reoxygenation). In control and β² mice, hypoxia induced a decrease in arterial hemoglobin oxygen saturation (monitored using an oximeter and oxypoxy probe; model 3770, Ohmeda, Madison, WI) from baseline values of ~90% and 85%, respectively, to <70% (64–67%) in each case. Reoxygenation resulted in complete recovery of arterial percent hemoglobin oxygen saturation. The protocol consisted of the following experimental groups. Group 1 (n = 5 control and 6 β² mice) served as normoxic controls (room air). Group 2 (n = 6 each) was subjected to 3 h of hypoxia followed by 1.5 h of reoxygenation. Group 3 (n = 5 each) was treated with allopurinol (Sigma, St. Louis, MO; 50 mg/kg ip) 1 day before the surgery; an additional dose of allopurinol (50 mg/kg) was administered before hypoxia (19). Group 4 (n = 4 control and 6 β² mice) was infused with an intravenous (via tail vein) bolus (750 U) of SOD (Sigma) at the onset of reoxygenation followed by a continuous intravenous (via jugular vein) infusion of SOD (2,000 U·kg⁻¹·min⁻¹) for 15 min. Group 5 (n = 4 control and 6 β² mice) was infused with an intravenous bolus (5,000 U) of catalase (Sigma) followed by a continuous intravenous infusion of catalase (5,000 U·kg⁻¹·min⁻¹) for 15 min. The dose of SOD and catalase used here is comparable to that employed previously (1, 28).

In the above experiments, hemodynamic parameters and leukocyte-endothelium interactions were recorded after 1.5 h of reoxygenation.

We determined endothelial oxidant generation in control and β² mice (n = 2 each) from each of the above experimental groups by adding the oxidant-sensitive fluorochrome probe dihydrorhodamine 123 (DHR; Molecular Probes, Eugene, OR) to the suffusing bath the cremaster preparation. Stock solution of DHR in DMSO was stored at −20°C. Working solution of DHR (10 μmol/l) was made in Ringer bicarbonate solution (continuously bubbled with 94.6% N₂-5.6% CO₂). After the exteriorization procedure (~15 min) under ambient conditions, the cremaster preparation was suffused with DHR for 15 min (total reoxygenation period ~30 min). DHR has been previously used to detect intracellular generation of DHR in a variety of cell types, including vascular endothelium (8, 21, 23). In the presence of oxidants, nonfluorescent DHR is oxidized to fluorescent rhodamine 123, which is localized in mitochondria. Fluorescent im-

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ages were videotaped using a Nikon microscope equipped with epifluorescence (model E400, Nikon, Melville, NY) and a low-light-sensitive cooled television camera (model CCD-300, Dage-MTI) in fixed-gain mode. Images were digitized into a Macintosh Quadra 840AV computer with a QuicKapture card with auto gain off. NIH Image was used to quantify fluorescence intensities, as described previously (21). The difference between the background and DHR fluorescence (∆Intensity) was used to estimate the relative levels of oxidized DHR, indicative of H₂O₂ formation. Fluorescence intensities were measured in 14–33 venular segments (total = 202 venular segments) under a given experimental condition. The use of rhodamine 123 (10 μmol/l) as control revealed no significant differences in fluorescence intensity after hypoxia-reoxygenation compared with the normoxic condition.

Sulfasalazine

In a series of experiments, we subjected the β8 mice to sulfasalazine (Sigma) therapy. Sulfasalazine was dissolved in normal mouse saline (1.024% NaCl adjusted to pH 8.0). Sulfasalazine was administered twice daily at 0.057 and 0.114 g·kg⁻¹·day⁻¹ ip for 3 days, with controls receiving the vehicle alone. Sulfasalazine at the lower of the two concentrations (i.e., 0.057 g·kg⁻¹·day⁻¹, or 4 g/d for a 70-kg human) has been found to be effective in inhibiting endothelial adhesion molecule expression in human sickle cell patients and transgenic mice (37). After the treatment period, the mice (n = 17) were examined, under normoxic conditions or after 3 h of hypoxia and 1.5 h of reoxygenation, for hemodynamic parameters and leukocyte recruitment in the cremaster muscle venules. In separate experiments, we examined endothelial oxidant generation in cremaster venules of β8 mice treated with vehicle or sulfasalazine (0.114 g·kg⁻¹·day⁻¹) under normoxia and after hypoxia-reoxygenation (n = 8). DHR intensities were determined as described above in 17–21 venular segments in each group (total venular segments = 74).

EMSA for NF-κB Expression in Kidneys

NF-κB expression was determined in control and β8 mice (untreated and after sulfasalazine treatment at 0.114 g·kg⁻¹·day⁻¹, n = 4 in each group) under normoxic conditions and after hypoxia-reoxygenation. Lipopolysaccharide (LPS, 10 μg; Escherichia coli; Sigma)-treated control mice served as positive controls for NF-κB activation. Mice were asphyxiated with CO₂, the left kidney was removed, external fat and connective tissue were excised, and the kidney was frozen in liquid N₂. Kidney homogenates were prepared as previously described (12). Whole kidney NF-κB was extracted and measured as described for mouse lungs (6). Briefly, kidney nuclear proteins were extracted from kidney homogenates and incubated with end-labeled 32P-double-stranded DNA containing a murine NF-κB DNA binding sequence (underlined base pairs): 5’-AGTTGAGGG-GACTTCCCAGGC-3’ (Santa Cruz Biotechnology). Each DNA-protein binding reaction contained nuclear protein extracts equivalent to 260 ng of kidney DNA. Reactions were carried out for 30 min at room temperature and then separated by electrophoresis. Radioactive bands corresponding to NF-κB were quantified by phosphorescence densitometry and ImageQuant 5.0 software (Molecular Dynamics), as previously described by us (6).

Statistical Analysis

A total of 444 venules (in addition to fluorescence studies) were analyzed for various microcirculatory flow parameters. Statistical analysis of the data on venular segments of each treatment group was performed using one-way ANOVA followed by Newman-Keuls multiple comparisons. Comparisons between groups (control vs. transgenic) were made using Student’s t-test. Where tests for normality failed or Bartlett’s test for homogeneity of variance showed significant difference in the standard deviations, nonparametric tests, such as Kruskal-Wallis test for ANOVA or the Wilcoxon two-sample test, were used. P < 0.05 was considered significant. Statistical analysis was performed using Statgraphics plus 3.0 program for Windows (Manugistics, Rockville, MD).

RESULTS

Hematocrit and Peripheral Leukocyte Counts

Hematocrit levels in C57BL controls (n = 5) and β8 mice (n = 5) were 46.2 ± 0.3% and 48.3 ± 1.1% (P = 0.11), respectively. During steady-state conditions, compared with control mice (n = 10), the β8 mice (n = 9) showed higher peripheral leukocyte counts (8,915 ± 800 vs. 11,536 ± 773 counts/µl, P < 0.025) accompanied by an ∼2.8-fold increase in neutrophil counts (1,384 ± 206 vs. 3,873 ± 428 counts/µl, P < 0.0001). The differences in the leukocyte and neutrophil counts are consistent with those reported in our previous studies (21).

Endothelial Oxidant Generation

Endothelial oxidant production in cremaster venules was monitored by suffusing the preparation with DHR and quantifying ∆I between the background and the venular endothelium. Figure 1 presents pseudo-colored images to illustrate DHR fluorescence in the venular endothelium of the β8 mice during normoxia and at 3 h of hypoxia and 30 min of reoxygenation (ambient air) with or without treatment with a given antioxidant. The β8 mice showed ∼60–85% greater ∆I than control

![Figure 1](http://ajpheart.physiology.org/)
mice under normoxia and after reoxygenation ($P < 0.01$ and $P < 0.001$, respectively; Fig. 2). Treatment with allopurinol resulted in a significantly decreased DHR fluorescence in $\beta^S$ mice ($P < 0.00001$) but not in normal mice ($P = 0.058$). In both groups, significant decreases in DHR oxidation were seen after SOD and catalase ($P < 0.0001–0.00001$), with catalase resulting in maximal antioxidative effect.

**Hemodynamic Parameters**

Table 1 shows the effect of antioxidants on hemodynamic parameters induced by hypoxia-reoxygenation in transgenic $\beta^S$ and control wild-type mice. Under normoxic conditions, the $\beta^S$ mice showed $\sim$60% lower $V_{\text{RBC}}$ and wall shear rates than control mice (each $P < 0.0001$), accompanied by a $>60\%$ decrease ($P < 0.0001$) in Q, which is consistent with our previous observations (21). Hypoxia for 3 h followed by 1.5 h of reoxygenation caused significant decreases in $V_{\text{RBC}}$, wall shear rates, and Q in normal and $\beta^S$ mice ($P < 0.05–0.0001$), but the $\beta^S$ mice showed significantly lower values for any given parameter (Table 1). All the hemodynamic parameters showed greater improvement in the $\beta^S$ mice treated with allopurinol, SOD, and catalase than in the treated control mice (Table 1). After infusion of catalase in $\beta^S$ mice, $V_{\text{RBC}}$ and wall shear rates showed a $>165\%$ increase ($P < 0.00001$) compared with the untreated group, and the resulting values were not significantly different from the normoxic values in control mice (Table 1). In control mice, catalase treatment restored all the hemodynamic parameters to the normoxic level.

The next effective antioxidant was SOD, which caused a $>100\%$ increase in $V_{\text{RBC}}$ and wall shear rates in the $\beta^S$ mice compared with the untreated group ($P < 0.04–0.0001$). In contrast, control mice receiving SOD showed only a $\sim40\%$ increase in wall shear rates ($P < 0.05$). Allopurinol caused a $>50\%$ increases in $V_{\text{RBC}}$ and wall shear rates in the $\beta^S$ mice ($P < 0.01–0.001$) but had no significant effect in control mice.

**Leukocyte Rolling Fluxes and Adhesion**

To determine whether the improvement in hemodynamic parameters after antioxidant therapy was due to an ameliorating effect on leukocyte-endothelium interactions, we examined leukocyte rolling fluxes and adhesion in the same individual venules of $\beta^S$ and control mice.

Figure 3 shows the effect of antioxidants on leukocyte rolling flux and adhesion induced by hypoxia-reoxygenation in transgenic and control mice. During normoxia, the $\beta^S$ mice showed a greater than twofold increase in rolling leukocyte fluxes and a more than threefold increase in leukocyte adhesion ($P < 0.001–0.00001$) compared with control mice (Fig. 3). In the $\beta^S$ mice, hypoxia-reoxygenation resulted in a markedly exaggerated response, as was evident by an almost twofold greater leukocyte rolling flux ($P < 0.000001$) and a nearly fivefold increase in adherent leukocytes ($P < 0.0000001$) compared with controls (Fig. 3). In the $\beta^S$ mice, SOD and catalase caused a significant reduction in the reoxygenation-induced increase in leukocyte rolling flux ($P < 0.026$ and $P < 0.000001$, respectively), whereas the decrease in rolling flux after allopurinol treatment was not significant. However, with any antioxidant, the effect was more pronounced on leukocyte adhesion than on rolling flux. The reoxygenation-induced increase in leukocyte adhesion in the $\beta^S$ mice was attenuated by allopurinol (60% reduction), SOD (74% reduction), or catalase.

### Table 1. Effect of antioxidants on hemodynamic parameters in microcirculation of control and $\beta^S$ mice subjected to hypoxia-reoxygenation

<table>
<thead>
<tr>
<th>Hypoxia-Reoxygenation</th>
<th>Untreated</th>
<th>Allopurinol</th>
<th>SOD</th>
<th>Catalase</th>
</tr>
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<tbody>
<tr>
<td><strong>Venular diameter, $\mu$m</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wild type</td>
<td>28.9 ± 1.1 (28)</td>
<td>27.1 ± 0.9 (32)</td>
<td>29.0 ± 0.8 (28)</td>
<td>26.1 ± 1.0 (24)</td>
</tr>
<tr>
<td>Transgenic</td>
<td>28.8 ± 1.1 (28)</td>
<td>28.0 ± 0.7 (36)</td>
<td>27.1 ± 1.0 (25)</td>
<td>27.7 ± 0.7 (50)</td>
</tr>
<tr>
<td><strong>Red cell velocity, mm/s</strong></td>
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</tr>
<tr>
<td>Wild type</td>
<td>5.9 ± 0.50</td>
<td>3.3 ± 0.27*</td>
<td>3.8 ± 0.40*</td>
<td>4.3 ± 0.66*</td>
</tr>
<tr>
<td>Transgenic</td>
<td>2.4 ± 0.17†</td>
<td>1.8 ± 0.13‡</td>
<td>3.0 ± 0.26†</td>
<td>4.3 ± 0.40‡</td>
</tr>
<tr>
<td><strong>Wall shear rate, s⁻¹</strong></td>
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<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1.016 ± 74</td>
<td>613 ± 43*</td>
<td>672 ± 82*</td>
<td>852 ± 129†</td>
</tr>
<tr>
<td>Transgenic</td>
<td>435 ± 31‡</td>
<td>335 ± 25*‡</td>
<td>542 ± 41*†</td>
<td>796 ± 77*†</td>
</tr>
<tr>
<td><strong>Venular blood flow, n/s</strong></td>
<td></td>
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<tr>
<td>Wild type</td>
<td>2.75 ± 0.39</td>
<td>1.31 ± 0.18*</td>
<td>1.61 ± 0.20*</td>
<td>1.23 ± 0.19*</td>
</tr>
<tr>
<td>Transgenic</td>
<td>1.04 ± 0.11‡</td>
<td>0.73 ± 0.07‡</td>
<td>1.16 ± 0.16‡</td>
<td>1.67 ± 0.18‡</td>
</tr>
</tbody>
</table>

Values are mean ± SE number of venules shown in parentheses. *$P < 0.05–0.0001$ vs. respective normoxic values. †$P < 0.05–0.0001$ vs. respective untreated hypoxia-reoxygenation controls. ‡$P < 0.03–0.00001$ vs. corresponding wild-type controls.
and after hypoxia-reoxygenation.

In contrast, antioxidants had no significant effect in control mice (Fig. 3).

Leukocytes rolling and adherent leukocytes in βS mice after catalase treatment, the number of rolling and adherent leukocytes in βS mice was more markedly inhibited than leukocyte rolling. In contrast to control mice, allopurinol and SOD infusion in βS mice resulted in significant decreases in leukocyte adhesion. *P < 0.05–0.0001 vs. corresponding wild-type controls; +P < 0.002–0.000001 vs. respective normoxic values; #P < 0.005–0.00001 vs. respective untreated hypoxia-reoxygenation group (Kruskal-Wallis test for ANOVA).

(95% reduction; each P < 0.000001 vs. untreated group). With catalase treatment, the number of rolling and adherent leukocytes in βS mice after reoxygenation was not significantly different from that of normoxic values in control mice (Fig. 3). In contrast, antioxidants had no significant effect in control mice, except for a reduction in adherent leukocytes (P < 0.04) after catalase treatment.

Effect of Sulfasalazine in βS Mice

As shown above, antioxidants resulted in significant protection against hypoxia-reoxygenation-induced leukocyte recruitment in the βS mice. Because oxidants can activate NF-κB, a transcription factor that promotes expression of adhesion molecules involved in the inflammatory response, we tested the efficacy of sulfasalazine, an inhibitor of NF-κB and endothelial activation, on microcirculatory parameters, endothelial oxidant generation, and NF-κB expression in βS mice. The βS mice received sulfasalazine twice daily at 0.057 and 0.114 g·kg⁻¹·day⁻¹ for 10 days, with control βS mice receiving vehicle alone (see MATERIALS AND METHODS).

After treatment, the mice were examined under normoxic conditions and at 3 h of hypoxia + 1.5 h of reoxygenation.

Hemodynamic parameters. Table 2 depicts the effect of sulfasalazine on the hemodynamic parameters in βS mice during normoxia and after hypoxia-reoxygenation. Consistent with the adverse effect of reoxygenation, as noted above, V_RBC, wall shear rates, and Q showed a significant decline after 1.5 h of reoxygenation compared with normoxic values in transgenic mice receiving vehicle only (P < 0.05–0.00001). In contrast, sulfasalazine treatment at either concentration caused a marked improvement in V_RBC and wall shear rates under normoxic conditions and after reoxygenation compared with the respective baseline values in control βS mice: 33–66% increase (P < 0.05–0.001) in normoxia and 170–190% increase (P < 0.00001) in hypoxia-reoxygenation. Although V_RBC and wall shear rates showed an upward trend with reoxygenation in the treated groups, as well as with the higher concentration of sulfasalazine, the differences were not statistically significant.

The changes in Q followed a pattern that, overall, was similar to that for V_RBC and wall shear rates.

Leukocyte rolling fluxes and adhesion. In control βS mice receiving vehicle only, hypoxia-reoxygenation caused a ~30% greater leukocyte rolling flux (P < 0.02; Fig. 4A) accompanied by more than twofold increase (P < 0.0001) in the number of adherent leukocytes (Fig. 4B) compared with the normoxic values. Under normoxic conditions, sulfasalazine caused a dose-dependent decrease (29% and >50% decrease at 0.57 and 1.14 g·kg⁻¹·day⁻¹, respectively) in rolling flux compared with the baseline normoxic values (P < 0.016 and P < 0.0001; Fig. 4A). With hypoxia-reoxygenation, leukocyte rolling showed an increase in each group, but the overall rolling flux was significantly lower than the control values (P < 0.001). The observed decreases in the rolling fluxes in the treated mice, under normoxic conditions and after reoxygenation, were accompanied by a marked decline in leukocyte adhesion (P < 0.0005–0.00001) compared with the respective baseline values (Fig. 4B), suggesting an anti-inflammatory effect of sulfasalazine. The observed decrease in leukocyte rolling fluxes and adhesion with sulfasalazine treatment is consistent with the above-noted increase in V_RBC and wall shear rates.

Table 2. Effect of sulfasalazine on hemodynamic parameters in microcirculation of βS mice during normoxia and after hypoxia-reoxygenation

<table>
<thead>
<tr>
<th></th>
<th>Vehicle Only (Controls)</th>
<th>Sulfasalazine</th>
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<tbody>
<tr>
<td></td>
<td>Normoxia (n = 22)</td>
<td>H/R (n = 24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normoxia (n = 20) H/R (n = 24) Normoxia (n = 23) H/R (n = 24)</td>
</tr>
<tr>
<td>Venular diameter, μm</td>
<td>27.5 ± 0.9</td>
<td>29.8 ± 0.8</td>
</tr>
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</table>
| Red cell velocity, mm/s | 2.7 ± 0.22             | 1.7 ± 0.10*  | 3.6 ± 0.36* | 4.7 ± 0.33* | 4.4 ± 0.35* | 4.6 ± 0.33*
| Wall shear rate, s⁻¹  | 491 ± 37                | 287 ± 18*    | 678 ± 64* | 808 ± 54* | 813 ± 74* | 833 ± 63.3*
| Venular flow, nl/s   | 1.04 ± 0.13             | 0.76 ± 0.07* | 1.3 ± 0.18 | 2.1 ± 0.26* | 1.8 ± 0.22* | 1.9 ± 2.5*

Values are means ± SE; n, number of venules. H/R, hypoxic-reoxygenation. *P < 0.03–0.001 vs. normoxic controls. †P < 0.003–0.00001 vs. controls exposed to hypoxia-reoxygenation.
NF-κB expression in control mice. Treatment with sulfasalazine clearly blunted the effect of hypoxia-reoxygenation on NF-κB expression in βS mice, and the level of NF-κB expression was not significantly different from that in normal mice or normoxic βS mice receiving sulfasalazine treatment.

**Endothelial oxidant generation.** The βS mice treated with the higher concentration of sulfasalazine (0.114 g·kg⁻¹·day⁻¹) were examined for endothelial oxidant generation in venular endothelium under normoxic conditions and after hypoxia-reoxygenation. Under normoxic conditions, sulfasalazine-treated βS mice showed an ~25% decrease in DHR fluorescence intensities compared with control βS mice (vehicle only, Fig. 5; P < 0.0005). In contrast, after hypoxia-reoxygenation, sulfasalazine-treated βS mice showed a marked 72% decrease in DHR fluorescence compared with controls receiving vehicle only. **P < 0.00001 vs. normoxic βS mice (vehicle only); *P < 0.00001 vs. normoxic βS control; #P < 0.000001 vs. βS control (vehicle only) after hypoxia-reoxygenation.**

**Effect on NF-κB expression.** To further ascertain the anti-inflammatory effect of sulfasalazine in βS mice, we measured NF-κB expression in the kidney of normal and βS mice treated with the higher concentration of sulfasalazine (1.114 g·kg⁻¹·day⁻¹). NF-κB expression was increased ~1.7-fold in the kidneys of βS mice after exposure to hypoxia-reoxygenation (P < 0.001) and >2-fold in LPS-treated normal mice (P < 0.05; Fig. 6). Hypoxia-reoxygenation had no effect on the effect of sulfasalazine on leukocyte adhesion. Sulfasalazine caused a marked decrease in leukocyte rolling. With hypoxia-reoxygenation, rolling flux in treated groups was significantly lower than in controls (vehicle). B: effect of sulfasalazine on leukocyte adhesion. Sulfasalazine caused a marked decrease in leukocyte adhesion in normoxic conditions, as well as after hypoxia-reoxygenation, indicating anti-inflammatory efficacy of sulfasalazine. *P < 0.02–0.0001 vs. normoxic βS controls; +P < 0.016–0.00001 vs. normoxic βS controls; +++P < 0.0001 compared with sulfasalazine-treated (0.057 g·kg⁻¹·day⁻¹) normoxic βS mice; #P < 0.0012–0.000001 vs. untreated βS mice subjected to hypoxia-reoxygenation.

- **A:** effect of sulfasalazine on leukocyte rolling flux and adhesion in βS mice. Treated βS mice were examined under normoxic conditions and after hypoxia-reoxygenation (H/R). A: effect of sulfasalazine on leukocyte rolling flux. Under normoxic conditions, sulfasalazine caused a dose-dependent decrease in leukocyte rolling. With hypoxia-reoxygenation, rolling flux in treated groups was significantly lower than in controls (vehicle).

**Fig. 4.** Effect of sulfasalazine treatment on leukocyte rolling flux and adhesion in βS mice. Treated βS mice were examined under normoxic conditions and after hypoxia-reoxygenation (H/R). A: effect of sulfasalazine on leukocyte rolling flux. Under normoxic conditions, sulfasalazine caused a dose-dependent decrease in leukocyte rolling. With hypoxia-reoxygenation, rolling flux in treated groups was significantly lower than in controls (vehicle). B: effect of sulfasalazine on leukocyte adhesion. Sulfasalazine caused a marked decrease in leukocyte adhesion in normoxic conditions, as well as after hypoxia-reoxygenation, indicating anti-inflammatory efficacy of sulfasalazine. *P < 0.02–0.0001 vs. normoxic βS controls; +P < 0.016–0.00001 vs. normoxic βS controls; +++P < 0.0001 compared with sulfasalazine-treated (0.057 g·kg⁻¹·day⁻¹) normoxic βS mice; #P < 0.0012–0.000001 vs. untreated βS mice subjected to hypoxia-reoxygenation.

**Fig. 5.** Effect of sulfasalazine on endothelial oxidant generation. Sulfasalazine-treated transgenic mice were examined under normoxic conditions and after hypoxia-reoxygenation. Under normoxic conditions, there was ~25% increase in DHR fluorescence intensities in sulfasalazine-treated βS mice compared with βS controls (vehicle only). After hypoxia-reoxygenation, sulfasalazine-treated mice showed a marked 72% decrease in DHR fluorescence compared with controls receiving vehicle only. *P < 0.0005 vs. normoxic βS mice (vehicle only); **P < 0.000001 vs. normoxic βS control; #P < 0.000001 vs. βS control (vehicle only) after hypoxia-reoxygenation.

**Fig. 6.** EMSA demonstrates that NF-κB is upregulated in kidneys of transgenic βS mice after exposure to hypoxia-reoxygenation and LPS-treated normal mice (18 h after LPS injection) compared with normal mice. Sulfasalazine treatment blunted effects of hypoxia-reoxygenation on NF-κB. Bands from kidneys of 1 LPS-treated normal mouse, 4 transgenic βS mice, and 4 normal control mice in each group are shown. Kidney NF-κB expression is plotted for each mouse group. *P < 0.05 vs. normal normoxic mice receiving vehicle; +P < 0.001 compared with βS mice receiving vehicle after hypoxia-reoxygenation.
ANTI-INFLAMMATORY THERAPY IN TRANSGENIC SICKLE MICE

DISCUSSION

The concept that sickle cell disease is a state of inflammation is supported by a number of recent studies showing abnormally activated endothelium in human sickle cell patients and transgenic sickle mouse models (6, 34, 36). In sickle mouse models, inflammatory stimuli (e.g., hypoxia-reoxygenation and cytokines) result in increased leukocyte recruitment and can initiate vasoostriction, suggesting that anti-inflammatory therapy could be beneficial in ameliorating leukocyte-induced vasoostriction in this disease.

In the present studies, we show that hypoxia-reoxygenation induces an exaggerated inflammatory response in transgenic sickle mice that is characterized by a greater endothelial oxidant generation than in control mice. This exaggerated inflammatory response in βS mice is markedly attenuated by antioxidant therapy. In contrast, control mice show a muted effect of antioxidant therapy on leukocyte-endothelium interactions. In addition, we show that hypoxia-reoxygenation induces activation of NF-κB in transgenic mice but not in control mice. Importantly, in βS mice, treatment with sulfasalazine, an inhibitor of NF-κB transcription factor as well as endothelial activation, results in an attenuation of leukocyte adhesion and improved microvascular flow in vivo.

We evaluated the anti-inflammatory effects of antioxidants 1) by monitoring generation of oxidants in cremaster venules using DHR, an H2O2-sensitive fluorochrome; and 2) by measuring hemodynamic parameters, leukocyte rolling flux, and adhesion in postcapillary venules. Previous studies have shown that reperfusion injury is associated with enhanced production of oxygen-derived free radicals, which play an important role in the mediation of neutrophil adhesion to venular endothelium (14). Studies using DHR probe have indicated that endothelial oxidant generation occurs as early as 5 min after reperfusion (8). In the present studies, we chose the shortest-possible time interval after reoxygenation to determine the effect of antioxidants on DHR oxidation. This brief reoxygenation period (~30 min) is attributable to cremaster tissue exteriorization (~15 min) under ambient conditions plus the application of DHR sulfusate (15 min) after the hypoxic period (i.e., exposure to 8% oxygen in the breathing mixture). As demonstrated earlier, increasing the reoxygenation period results in a distinct decrease in DHR oxidation in the βS mice (21).

Our results show ~60–80% greater DHR oxidation in the βS mice under ambient conditions and after hypoxia-reoxygenation, indicating a greater endothelial activation than in control mice. The exaggerated inflammatory response to hypoxia-reoxygenation in the βS mice is characterized by greater leukocyte-endothelium interactions and reductions in Vrbc, wall shear rates, and Q than in control mice. As noted in our previous study (21), the exaggerated response to reoxygenation in the βS mice is probably due to relative ischemia, resulting from intravascular sickling during the hypoxic period.

The present study shows that, in the βS mice, allopurinol, SOD, and catalase resulted not only in significant decreases in the DHR oxidation in vascular endothelium after hypoxia-reoxygenation but also in subsequent decreases in leukocyte rolling and adhesion at 1.5 h of reoxygenation. Importantly, the anti-inflammatory effect of antioxidants was accompanied by a marked improvement in the microvascular flow, as evidenced by significant increases in Vrbc, wall shear rates, and Q. In contrast, in control mice, although SOD and catalase, but not allopurinol, resulted in decreased DHR oxidation, these antioxidants had no effect on leukocyte rolling, and only catalase resulted in decreased leukocyte adhesion (Fig. 3). These results imply that the overall modest inflammatory response to hypoxia-reoxygenation in control mice may not reach the threshold of endothelial activation (as well as leukocyte activation) for antioxidant to have a marked effect on leukocyte recruitment.

In the βS mice, the anti-inflammatory effect of allopurinol, an inhibitor of xanthine oxidase (XO) activity, is consistent with the involvement of XO-derived oxidants. In the vasculature, the endothelium is generally considered the principal source of XO, which catalyzes oxygen radical production upon reoxygenation (15). Pretreatment with XO inhibitors (e.g., allopurinol and oxypurinol) is known to inhibit ischemia-reperfusion-induced injury (24, 27, 31). Reperfusion allows XO to act on its purine substrate hypoxanthine, with the resulting generation of superoxide. Spontaneous dismutation of superoxide by the enzyme SOD results in the formation of H2O2. The increased oxidant production and subsequent leukocyte accumulation after ischemia (induced by vessel occlusion)-reperfusion or cytokine application are inhibited by pretreatment with SOD and catalase (8, 17, 23, 28, 31). Our results show that, in the βS mice, exogenous SOD and catalase, given at the onset of reoxygenation, were even more effective than allopurinol in inhibiting leukocyte-endothelium interactions and restoring the microvascular flow parameters almost to the control level. In addition to the endothelium-generated oxidants, another major source of oxidants is circulating XO in the plasma, which can bind vascular endothelium at sites remote from its source (i.e., splanchic system) (18). Recent studies have demonstrated higher circulating levels of XO in sickle cell patients, as well as in sickle mice under steady state (2). Exposure of sickle mice to hypoxia-reoxygenation may increase the levels of circulating plasma XO and its binding to vascular endothelium. The endothelial cell surface-bound XO may then participate in the generation of oxidants. The efficacy of SOD and catalase in the βS mice may be in part due to their ability to scavenge oxidant products of endothelium-bound XO. In addition, under ischemic conditions, (i.e., 3 h of hypoxia), xanthine oxidoreductase may be released (from sources other than vascular endothelium) into the circulation, where it can undergo rapid proteolytic conversion to XO (18). Thus the possibility cannot be excluded that the apparent greater effect of SOD and catalase is due to their ability to scavenge oxygen radicals and H2O2 derived from XO generated during hypoxia. However, SOD and catalase, because of their large molecular weights, are more likely to scavenge oxidants on or near the endothelial surface.

Because oxidant radicals are implicated in the activation of NF-κB, the transcription factor that promotes expression of several genes involved in inflammatory response (9, 15), we examined the efficacy of sulfasalazine, an inhibitor of NF-κB (41, 43) and endothelial adhesion molecule expression (37). Hypoxia-reoxygenation resulted in a significant increase in endothelial oxidant generation in the cremaster venules, as well as in the activation of NF-κB in the transgenic mouse kidneys, but not in control mice, confirming an exaggerated response to hypoxia-reoxygenation in these mice. Furthermore, sulfasalazine treatment markedly attenuated not only endothelial oxidant generation in cremaster venules, but also NF-κB activa-
though the mechanism(s) involved in the divergent effects on (i.e., P-selectin, E-selectin, and ICAM), but it may not be NF-κB activation may attenuate the expression of endothelial by 56% (Fig. 4). The marked inhibitory effect on leukocyte recruitment was accompanied by marked increases in V_{HBC} and wall shear rates. Thus the present studies provide the first in vivo demonstration that sulfasalazine not only attenuates endothelial oxidant generation and inhibits NF-κB activation but also attenuates leukocyte recruitment and improves microvascular flow. Previous studies have shown that sulfasalazine results in significant decreases in the expression of endothelial adhesion molecules such as ICAM-1, E-selectin, and vascular cell adhesion molecule-1 in sickle cell anemia patients and β5 mice (37), probably as a result of the inhibition of NF-κB by this agent. Our results show that, in the β5 mice, sulfasalazine had a more pronounced inhibitory effect on leukocyte adhesion than on leukocyte rolling (Fig. 4). The marked inhibition of leukocyte adhesion indicates an effective inhibition of ICAM expression, which mediates firm leukocyte adhesion to the endothelium. We found that sulfasalazine reduced ICAM expression in sickle mouse lungs after hypoxia-reoxygenation by 56% (P < 0.05; unpublished studies). The inhibition of NF-κB activation may attenuate the expression of endothelial adhesion molecules involved in leukocyte rolling and adhesion (i.e., P-selectin, E-selectin, and ICAM), but it may not be effective against constitutively expressed L-selectin on leukocytes. Also, treatment with antioxidants was more effective in inhibiting firm leukocyte adhesion than leukocyte rolling. Although the mechanism(s) involved in the divergent effects on leukocyte rolling and adhesion remains to be elucidated, our studies demonstrate that preventing firm leukocyte adhesion by antioxidants and sulfasalazine would be an effective therapeutic approach to ameliorate reoxygenation-induced microvascular flow abnormalities, vessel blockage, and tissue injury.

In conclusion, our studies show the efficacy of anti-inflammatory therapy in ameliorating hypoxia-reoxygenation-induced leukocyte recruitment and associated microvascular flow abnormalities in transgenic sickle mice. The exaggerated inflammatory response in β5 mice is significantly alleviated by pretreatment with antioxidants, as well as by sulfasalazine (an inhibitor of NF-κB and endothelial adhesion molecule expression), resulting in a marked improvement of microvascular flow. Because oxidants activate NF-κB, treatments directed against oxidant generation and/or NF-κB activation may form promising therapeutic avenues in sickle cell disease.

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GRANTS

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