Inhibition of sickle red cell adhesion and vasoocclusion in the microcirculation by antioxidants

Dhananjay K. Kaul, Xiao-du Liu, Xiaoqin Zhang, Li Ma, Carleton J. C. Hsia, and Ronald L. Nagel

Department of Medicine, Albert Einstein College of Medicine, Bronx, New York; and SynZyme Technologies, LLC, Irvine, California

Submitted 17 October 2005; accepted in final form 24 January 2006

SICKLE CELL ANEMIA (SCA) is characterized by recurring episodes of painful vasoocclusive crisis and multiple organ damage. Although hemoglobin S polymerization and red cell sickling under deoxygenated conditions are central to the pathophysiology of this disease (23, 43), growing evidence indicates that sickle cell disease is a state of inflammation characterized by vascular endothelial activation and increased blood cell-endothelium interactions (13, 18, 20). Abnormal interaction of sickle (SS) red blood cells with vascular endothelium is considered a key contributor to the initiation of vasoocclusion in this disease. Adhesion of SS red blood cells involves contribution from SS red cell abnormalities (induced by repeated sickling, expression of adhesion molecules, and dense red cell formation) and upregulation of endothelial adhesion molecules (17, 20). Ischemia and reperfusion in the microcirculation may lead to endothelial oxidant generation, endothelial activation, and upregulation of adhesion molecules (2, 25). In addition to leukocyte recruitment, inflammatory activation of the endothelium may have an indispensable role in enhanced SS red blood cell-endothelium interactions. SS red cell adhesion in postcapillary venules can increase microvascular transit times and initiate vasoocclusion (21, 22).

Several studies have shown involvement of an array of adhesion molecules expressed on SS red blood cells [CD36, αvβ3-integrin, intercellular adhesion molecule-4 (ICAM-4), and basal cell adhesion molecule] and activated endothelium [P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and αvβ3-integrin] and an important role of plasma factors and adhesive proteins [thrombospondin, von Willebrand factor (vWF), and laminin] in this interaction (18, 20). For example, the induction of VCAM-1 and P-selectin on activated endothelium is known to enhance SS red cell interactions (36, 45). In addition, αvβ3-integrin is upregulated in activated endothelium in sickle cell patients (42). αvβ3-Integrin binds to several adhesive proteins (thrombospondin, vWF, red cell ICAM-4, and, possibly, soluble laminin) involved in SS red cell adhesion (7, 16, 28, 31, 49), and antibodies to this integrin dramatically inhibit SS red cell adhesion (28). In addition, under inflammatory conditions, increased leukocyte recruitment, in combination with adhesion of SS red blood cells, may further contribute to stasis. We and others have shown that adhesion of SS red blood cells and/or leukocytes in postcapillary venules leads to vasoocclusion by secondary trapping of SS and dense SS red blood cells (21, 22, 46).

We hypothesize that endothelial activation will have an indispensable role in the SS red cell adhesion and that antioxidants will inhibit this interaction. To test our hypothesis, we used the ex vivo mesocecum vasculature, a well-tested model that allows measurement of peripheral resistance and microvascular oxidant generation, endothelial activation, and microvascular

Address for reprint requests and other correspondence: D. K. Kaul, Dept. of Medicine, Rm. U-917, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461 (e-mail: kaul@aecom.yu.edu).

http://www.ajpheart.org 0363-6135/06 $8.00 Copyright © 2006 the American Physiological Society

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
injury (34, 44), all of which characterize sickle cell disease, have been well documented in several studies. PAF is elevated twofold in sickle cell patients (39), suggesting that PAF has a role in chronic endothelial activation and inflammation in this disease. In addition to its ability to upregulate adhesion molecules involved in leukocyte-endothelium interactions (10, 33, 34), PAF, as well as other inflammatory stimuli (e.g., thrombin), may also result in induction of P-selectin and increased expression of endothelial vWF (28, 37), both of which are colocalized in Weibel-Palade bodies (38) and are implicated in SS red cell adhesion (27, 37).

In the present invratual studies, we examined the efficacy of selected antioxidants, i.e., superoxide dismutase (SOD), catalase, and polynitroxyl albumin (PNA), to inhibit SS red cell adhesion in the ex vivo preparation pretreated with PAF. Although the antioxidant enzymes SOD and catalase remove superoxide and H$_2$O$_2$, respectively, nitroxide molecules covalently attached to albumin in PNA act as an intravascular SOD mimetic and also facilitate heme-mediated catalytic removal of H$_2$O$_2$ (6, 32). PNA has two important activities in vivo: 1) it has been found to be effective in ameliorating leukocyte-endothelium interactions caused by ischemia-reperfusion (6, 41), and 2) its action is prolonged compared with that of unbound nitroxide in vivo. This is achieved by linking nitroxide molecules to biological macromolecules (e.g., polynitroxylation of albumin) prepared by covalently linking albumin with a high molar ratio of nitroxide (6).

Our results show that antioxidant enzymes (SOD and catalase) and PNA have pronounced inhibitory effects on SS red cell adhesion in a PAF-activated ex vivo preparation. Furthermore, PNA, with a maximal inhibitory effect in PAF-treated preparations, also abolished SS red cell adhesion in exteriorized ex vivo preparations that were not activated by PAF. These findings demonstrate that inflammatory activation of vascular endothelium as a consequence of increased oxidant generation is crucial for increased SS red cell adhesion, and antioxidants such as PNA are potential candidates for use in antiadhesive therapy in this disease.

**MATERIALS AND METHODS**

**Preparation of Cells**

Heparinized blood was obtained with informed consent from adult patients with SCA (i.e., homozygous for sickle hemoglobin) who were not in crisis and had not received a blood transfusion in the preceding 4 mo (n = 9). The buffy coat was removed, and the blood was washed three times in normal saline and once in bicarbonate Ringer-albumin solution (118 mM NaCl, 5 mM KCl, 2.5 mM CaCl$_2$, 0.64 mM MgCl$_2$, 27 mM NaHCO$_3$, and 0.5% bovine albumin, equilibrated with 95% O$_2$-5% O$_2$, pH 7.4, 295 mosmol/kgH$_2$O) and resuspended in Ringer-albumin solution. In each case, hematocrit (Hct) was adjusted to 30% for perfusion studies. Blood samples obtained from SS patients were analyzed for mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), percent reticulocytes, and Hct using a Sysmex KX-21N analyzer (Sysmex America, Mundelein, IL). Fetal hemoglobin was estimated using HPLC (Bio-Rad, Hercules, CA).

**Preparation and Perfusion of Rat Mesocecum Vasculature**

Perfusion studies were performed in the isolated, acutely denervated, and artificially perfused rat mesocecum vasculature (n = 40) according to the method of Baez et al. (3) as modified by Kaul et al. (24) for the infusion of erythrocytes. Details of the procedure have been described previously (24). Briefly, perfusion pressure in the mesocecum was maintained at 60 mmHg, and venous outflow pressure was kept at 3.8 mmHg. During perfusion with Ringer-albumin solution containing 3% bovine albumin, a 0.2-ml bolus of SS red cell suspension (Hct 30%) was infused via the arterial injection port over ~5 s. Peripheral resistance units (PRU, mmHg·ml$^{-1}$·min$^{-1}$·g$^{-1}$) were determined as described previously (14): PRU = ΔP/Q, where ΔP is arteriovenous pressure difference and Q is rate of venous outflow (ml·min$^{-1}$·g tissue wt$^{-1}$). Pressure-flow recovery time ($t_{pf}$), defined as the time (s) required for arterial pressure and venous outflow to return to baseline levels, was determined after red cell infusion.

**Intravital Microscopic Observation and Adhesion Quantification**

Direct intravital microscopic observations and simultaneous video recording of the microcirculatory events were carried out using a microscope (model E400, Nikon, Melville, NY) equipped with a television camera (model CCD-300T-RC, Dage-MIT, Michigan City, IN) and a U-Matic video recorder (model V05800, Sony, Teaneck, NJ). The number of adherent SS cells per 100 µm$^2$ was calculated from the counts of individual adherent cells and the surface area (µm$^2$) of the inner wall of the vessel segment as described previously (22). Adhesion data for each experimental group were pooled for statistical comparisons.

**Protocols of Experiments With Antioxidants**

To demonstrate the involvement of reactive oxygen species, the ex vivo mesocecum preparation was pretreated with PAF. PAF supplied in chloroform solution (Sigma Aldrich, St. Louis, MO) was first diluted in DMSO (Sigma Aldrich) and then serially diluted in Ringer-albumin solution. The ex vivo preparation was perfused with 40 ml of Ringer-albumin solution containing PAF (200 pg/ml) for 10 min and then infused with SOD (1,500 U in 5 ml of Ringer-albumin solution) or catalase (10,000 U in 5 ml of Ringer-albumin solution; Sigma Aldrich). In each case, after 15 min of incubation at room temperature, the preparation was perfused briefly (~5–7 min) with Ringer-albumin solution at 37°C, and a bolus of SS red cells was infused during constant perfusion with Ringer-albumin solution. In control preparations, treatment with PAF was followed by incubation with Ringer-albumin solution for 15 min. In experiments designed to evaluate the effect of PNA, the preparations were treated with PNA (SynZyme Technologies) or control human serum albumin (HSA; SynZyme Technologies; each 33 mg/ml diluted in Ringer-albumin solution). The ex vivo mesocecum preparation of the rat was first infused with PAF as described above. Then PNA or HSA solution (40 ml) was infused over a 10-min period, and the vasculature was incubated for an additional 20 min (total incubation 30 min). In addition, in separate experiments, we examined the adhesion of SS red cells in ex vivo preparations incubated with HSA (control) and PNA as described above, but without PAF pretreatment.

In separate experiments, endothelial oxidant generation in control (untreated) and PAF-treated preparations (n = 2 each) was determined. In each case, during perfusion with Ringer-albumin solution, the mesocecum was superfused by addition of the oxidant-sensitive fluorochrome probe dihydrodorhamine 123 (DHR; Molecular Probes, Eugene, OR) to the suffusate bathing the preparation as described previously (25). The preparation was superfused with DHR (10 µmol/l in bicarbonate Ringer solution bubbled with 94.6% N$_2$-5.6% CO$_2$) for 15 min. DHR has been previously used to detect intracellular generation of H$_2$O$_2$ in a variety of cell types, including vascular endothelium (8, 25, 34). In the presence of oxidants, nonfluorescent DHR is oxidized to fluorescent rhodamine 123. Fluorescent images were videotaped using a microscope equipped with epifluorescence (model E400, Nikon) and a low-light-sensitive cooled television camera (model CCD-300, Dage-MTI) in a fixed-gain mode. Images were digitized, and fluorescence intensities were quantified using Meta-
Morph Imaging Software (Universal Imaging, Downingtown, PA). The difference between background and DHR fluorescence [Δintensity (ΔI)] was used to estimate the relative levels of oxidized DHR. Fluorescence intensities were measured in five to six venular segments in each preparation (total 23 venular segments). The profile of fluorescence intensity across vessel segments was examined as described elsewhere (25, 44).

**Statistical Analysis**

Paired or Student’s t-test was applied to analyze hemodynamic data and fluorescence intensity in venules. Regression line analysis of the number of adherent red blood cells per 100 μm² (y) vs. the venular diameter (x) was performed using the following equation for the best fit: y = ax⁻ᵇ. For comparison of the regression lines, both variables were logarithmically transformed. Linear regression analysis of the transformed variables allowed comparison of the intercept and slopes of the regression lines between experimental groups by multiple linear regression analysis (30). Homogeneity of variance between groups for y values in relation to x was confirmed using Bartlett’s test. The statistical tests or tests for hypotheses were performed using a type I error and were two-tailed. Statistical analysis was performed using STATGRAPHICS Plus 5.0 for Windows (Manugistics, Rockville, MD).

**RESULTS**

**Hematologic Parameters**

Table 1 depicts hematologic values (Hct, MCV, MCH, MCHC, reticulocytes, and fetal hemoglobin) from blood samples of SS patients used in ex vivo experiments to investigate the effect of antioxidants on hemodynamic behavior and adhesion of SS red cells. Evidently, the hematologic parameters were not significantly different among the two groups of patients used for SOD/catalase or PNA experiments.

**PAF-Induced Endothelial Oxidant Generation**

Endothelial oxidant generation in the ex vivo vasculature was monitored by suffusion of the preparation with DHR and quantification of ΔI between the background and the venular endothelium, the site of SS red cell adhesion (22). Images of Ringer-albumin-perfused single venules and their corresponding pseudocolored DHR fluorescence images and intensity profiles in control (untreated) and PAF-treated preparations are shown in Fig. 1. In contrast to the low level of DHR oxidation in control vessels, venular endothelium in PAF-treated preparations revealed a pronounced increase in DHR fluorescence intensity: ΔI was fivefold greater in PAF-treated than in control preparations (Fig. 2).

**Effect of SOD and Catalase on PAF-Treated Ex Vivo Vasculature**

**Hemodynamic behavior of SS red cells.** In these experiments, we evaluated the effect of the antioxidants SOD and catalase on the hemodynamic behavior of SS red blood cells in PAF-treated ex vivo mesocoeum preparations. We tested the effect of each antioxidant in five preparations (4 patients). In each case, ΔPRU was calculated from the percent increase in the baseline PRU (Ringer-albumin solution) after SS red cell bolus infusion (Table 1). PRU was significantly increased in PAF-treated preparations compared with preparations perfused with Ringer-albumin solution alone before infusion of SS red blood cells: 4.0 ± 0.8 vs. 7.8 ± 1.9 PRU (Table 2).

Inflow of SS red cells into PAF-treated preparations resulted in an ~70% increase in ΔPRU compared with untreated preparations (Table 2). The PAF-induced increase in ΔPRU for SS red blood cells is consistent with our previous observations (28). When PAF treatment was followed by incubation with SOD, infusion of SS red blood cells resulted in a significant (40%) decrease in ΔPRU compared with the group treated with PAF alone. Incubation with catalase resulted in a 46.9% decrease in ΔPRU. In each case, the resulting ΔPRU values were not significantly different from those of the untreated preparations.

The tₙₚ results were consistent with the PRU data (Table 2), in that infusion of SS red blood cells into PAF-treated preparations induced a maximal increase in tₙₚ compared with the

---

Table 1. *Hematologic parameters in ScA patients*

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Hct, %</th>
<th>MCV, μm²</th>
<th>MCH, pg/cell</th>
<th>MCHC, g/dl</th>
<th>Retics, %</th>
<th>HbF, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SOD/catalase experiments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>174</td>
<td>21.6</td>
<td>82.1</td>
<td>28.1</td>
<td>34.3</td>
<td>7.5</td>
<td>5.8</td>
</tr>
<tr>
<td>216</td>
<td>23.9</td>
<td>72.0</td>
<td>29.4</td>
<td>33.9</td>
<td>3.6</td>
<td>ND</td>
</tr>
<tr>
<td>666</td>
<td>24.5</td>
<td>86.9</td>
<td>33.1</td>
<td>35.9</td>
<td>7.5</td>
<td>8.9</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>24.3 ± 2.3</td>
<td>83.3 ± 8.6</td>
<td>28.5 ± 4.1</td>
<td>34.1 ± 1.5</td>
<td>5.9 ± 2.0</td>
<td>5.9 ± 3.0</td>
</tr>
<tr>
<td><strong>PNA/HSA experiments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>086</td>
<td>27.9</td>
<td>86.1</td>
<td>27.5</td>
<td>34.9</td>
<td>6.2</td>
<td>ND</td>
</tr>
<tr>
<td>153</td>
<td>22.0</td>
<td>79.1</td>
<td>27.7</td>
<td>35.0</td>
<td>13.2</td>
<td>1.2</td>
</tr>
<tr>
<td>174</td>
<td>21.6</td>
<td>82.1</td>
<td>28.1</td>
<td>34.3</td>
<td>7.5</td>
<td>5.8</td>
</tr>
<tr>
<td>216</td>
<td>23.9</td>
<td>72.0</td>
<td>23.2</td>
<td>32.2</td>
<td>4.9</td>
<td>3.0</td>
</tr>
<tr>
<td>266</td>
<td>23.8</td>
<td>86.3</td>
<td>36.6</td>
<td>34.5</td>
<td>7.1</td>
<td>14.6</td>
</tr>
<tr>
<td>283</td>
<td>24.1</td>
<td>80.1</td>
<td>26.6</td>
<td>33.2</td>
<td>9.8</td>
<td>2.5</td>
</tr>
<tr>
<td>623</td>
<td>17.5</td>
<td>96.7</td>
<td>32.0</td>
<td>33.1</td>
<td>15.3</td>
<td>8.4</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>23.0 ± 3.2</td>
<td>83.2 ± 7.7</td>
<td>28.8 ± 4.3</td>
<td>33.9 ± 1.1</td>
<td>9.1 ± 3.8</td>
<td>5.9 ± 2.0</td>
</tr>
</tbody>
</table>

In polynitroxyl albumin (PNA)/human serum albumin (HSA) experiments, of 7 patients with sickle cell anemia (SCA), blood from 5 patients (patient nos. 086, 153, 216, 283, and 623) was used in platelet-activating factor (PAF)-treated ex vivo preparations and 4 patients (patient nos. 174, 216, 266, and 283) in non-PAF-activated preparations. Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; HbF, hemoglobin F; ND, not determined.
untreated preparations infused with SS red blood cells. The $t_{pf}$ declined 24% in PAF-treated preparations incubated with SOD, whereas catalase caused a maximal 55% decrease in $t_{pf}$.

**Effect on SS red cell adhesion.** Infusion of SS red blood cells into untreated and PAF-treated preparations resulted in an adhesion of these cells that was inversely correlated (using the equation $y = ax^{-b}$) with venular diameter (Fig. 3). Untransformed data showed that PAF clearly caused a greater adhesion of SS red blood cells in venules (Fig. 3B), with frequent blockage of small-diameter venules, as described elsewhere (28). Comparison of regression lines after logarithmic transformation of the data confirmed significantly greater adhesion of SS red blood cells in the PAF-treated than in the untreated group, as revealed by differences in $y$-intercepts ($P < 0.0001$; Fig. 3B, inset).

In contrast, when PAF was followed by SOD or catalase, the adhesion of SS red blood cells was noticeably inhibited in venules of all diameters (Fig. 4A). In either case, a plot of untransformed data revealed a lack of correlation with venular diameter. A comparison of regression lines of the transformed data revealed significant differences in $y$-intercepts (each $P < 0.00001$) compared with PAF-treated preparations (Fig. 4, insets). The slopes were also markedly different (each $P < 0.00001$), because each antioxidant inhibited adhesion in venules of all diameters. Nevertheless, catalase resulted in greater inhibition of adhesion, especially in small-diameter venules (Fig. 4B), the sites of frequent blockage in PAF-treated preparations. No postcapillary blockage was evident in preparations treated with SOD or catalase.

**Effect of PNA in PAF-Treated Ex Vivo Vasculature**

**Effect on hemodynamic behavior of SS red blood cells.** In a separate series of experiments, we tested the effect of control HSA and PNA in six preparations (5 patients). In control preparations treated with PAF and HSA, infusion of SS red blood cells caused an increase in ΔPRU and $t_{pf}$ (Table 3) similar in magnitude to that in the preparations treated with PAF alone (Table 2). In contrast, pretreatment with PAF and PNA resulted in a $>50\%$ decrease in ΔPRU and a 33% decrease in $t_{pf}$ compared with control HSA-treated preparations (Table 3).

**Effect on SS red cell adhesion.** Intravital microscopy and video analysis revealed that, in the PAF- and HSA-treated control preparations, infusion of SS red blood cells resulted in widespread adhesion of these cells in the postcapillary venules (Fig. 5, A and B), often resulting in complete
blockage of small-diameter venules, as was noted for the PAF-treated preparations. The adhesion showed a strong inverse correlation with the vessel diameter ($r = 0.84$, $P < 0.00001$). In marked contrast, in preparations treated with PAF and PNA, there was little or no adhesion of SS red blood cells in venules of all diameters (Fig. 5, D and E), resulting in a lack of correlation with the vessel diameter ($r = 0.16$, $P > 0.28$). The marked inhibition of adhesion by PNA in the PAF-treated preparation resulted in no postcapillary blockage. Quantitative analysis of the untransformed data (i.e., number of adherent SS red blood cells as a function of venular diameter) revealed almost complete inhibition of SS red cell adhesion by PNA (Fig. 6, A and B).

### Table 2. Effect of SOD and catalase on hemodynamic behavior of SS red blood cells in PAF-activated ex vivo mesocecum preparations

<table>
<thead>
<tr>
<th></th>
<th>PRU</th>
<th>Ringer mmHg/ml $\cdot$ min$^{-1}$</th>
<th>Cells, mmHg/ml $\cdot$ min$^{-1}$</th>
<th>$\Delta$, %</th>
<th>tpf, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS RBC</td>
<td>4.0±0.8</td>
<td>4.9±0.9</td>
<td>21.2±1.3</td>
<td>37.0±2.7</td>
<td></td>
</tr>
<tr>
<td>PAF-SS RBC</td>
<td>7.8±1.9</td>
<td>10.6±2.7</td>
<td>36.0±1.5*</td>
<td>69.0±7.4†</td>
<td></td>
</tr>
<tr>
<td>PAF-SOD-SS RBC</td>
<td>7.1±1.9</td>
<td>8.5±2.0</td>
<td>21.6±4.8‡</td>
<td>52.0±5.7§</td>
<td></td>
</tr>
<tr>
<td>PAF-catalase-SS RBC</td>
<td>6.2±1.1</td>
<td>7.4±1.5</td>
<td>19.1±4.5‡</td>
<td>38.0±5.7</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD ($n = 5$). SS red blood cells (RBC) were infused into untreated preparations (SS RBC), preparations treated with PAF only (200 pg/ml, PAF-SS RBC), and preparations treated with PAF and then incubated with SOD (1,500 U in 5 ml, PAF-SOD-SS RBC) or catalase (10,000 U in 5 ml, PAF-catalase-SS RBC). PRU, peripheral resistance units; $t_{pf}$, pressure-flow recovery time to baseline. *$P < 0.01$ and †$P < 0.002$ vs. respective value for SS RBC; ‡$P < 0.001$ and §$P < 0.005$ vs. respective values for PAF-SS RBC (paired $t$-test).

![Fig. 3.](image_url) **Fig. 3.** Regression line plots of number of adherent sickle (SS) red blood cells (RBC) per 100 $\mu$m$^2$ relative to venular diameter in ex vivo mesocecum microvasculature. A: untransformed data showing moderately strong SS red cell adhesion in untreated (Ringer/SS) group. B: data from PAF-treated (PAF/SS) group showing adhesion of SS red blood cells strongly correlated with vessel diameter. Inset: linear regression after logarithmic transformation of data allowing comparisons of intercepts and slopes between experimental groups. Because logarithm of zero is undefined, values of $y$ (number of adherent cells) were coded by addition of 1. Comparison of regression lines of transformed data confirmed significantly higher $y$-intercept for PAF/SS than for Ringer/SS.

![Fig. 4.](image_url) **Fig. 4.** Effect of SOD and catalase on SS red cell adhesion in PAF-treated ex vivo mesocecum vasculature. A: regression plot of number of adherent SS red blood cells per 100 $\mu$m$^2$ relative to venular diameter in PAF-activated preparation incubated with SOD (PAF/SOD). Note distinct decrease in venules of all diameters, resulting in a lack of correlation with vessel diameter. Inset: linear regression after logarithmic transformation of data showing significant differences in $y$-intercepts and slopes. B: regression plot for number of adherent SS red blood cells per 100 $\mu$m$^2$ relative to venular diameter in PAF-treated preparation incubated with catalase (PAF/catalase). Note even more marked decrease in adherent SS red blood cells, particularly in small-diameter venules, resulting in no correlation with vessel diameter. Inset: comparison of regression lines of transformed data revealing markedly lower $y$-intercept in PAF/catalase than in PAF. Marked differences in slopes indicate that catalase inhibited adhesion in venules of all diameters.
Table 3. Effect of PNA on hemodynamic behavior of SS red blood cells in PAF-activated ex vivo mesocecum preparations

<table>
<thead>
<tr>
<th>PRU</th>
<th>Ringer, mmHg/ml/min/g</th>
<th>Cells, mmHg/ml/min/g</th>
<th>Δ, %</th>
<th>tpf, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAF-HSA-SS RBC</td>
<td>6.2±0.6</td>
<td>8.6±1.1</td>
<td>38.3±10.0</td>
<td>70.8±8.0</td>
</tr>
<tr>
<td>PAF-PNA-SS RBC</td>
<td>5.5±0.7</td>
<td>6.7±0.9</td>
<td>20.9±2.2*</td>
<td>47.5±2.7†</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 6). Isolated mesocecum preparation was perfused with PAF (200 pg/ml, 40 ml over 10 min) and then infused with 40 ml of human serum albumin (HSA) or polynitroxyl albumin (PNA), 10 min and vasculature was incubated for 20 min. *P < 0.012 and †P < 0.002 vs. respective values for PAF-HSA-SS RBC (paired t-test).

Effect PNA in Ex Vivo Vasculature Not Activated by PAF

To ascertain whether the low level of SS red cell adhesion observed in non-PAF-treated preparations is due to endothelial activation secondary to the effect of tissue exteriorization, we examined the effect of PNA, the most effective antioxidant in the above experiments, in non-PAF-activated preparations.

Infusion of SS red blood cells into ex vivo preparations treated with PNA alone resulted in markedly lower ΔPRU (40%) and tpf (30.1%) than in preparations treated with control HSA (Table 4). These results show a distinct improvement in hemodynamic behavior with PNA treatment in untreated preparations. Intravital microscopy showed a marked inhibition of adhesion in the PNA- compared with the HSA-treated preparation. Moreover, the inhibition of adhesion by PNA resulted in a markedly lower y-intercept (P < 0.0001) and significant differences in slopes of the regression lines (P < 0.00001) after transformation of the data (Fig. 7), indicating an ameliorating effect of PNA.

DISCUSSION

Our present studies show that endothelial activation caused by PAF, which induces endothelial oxidant generation, results in enhanced SS red blood cell-endothelium interactions in the...
ex vivo mesocecum preparation. Importantly, we show that treatment with antioxidants significantly decreases peripheral resistance in the ex vivo vasculature and inhibits SS red cell adhesion in postcapillary venules, the sites of adhesion and vasoocclusion in PAF-treated preparations. These results also suggest that upregulation of endothelial adhesion molecules involved in SS red cell adhesion is a consequence of endothelial activation, a condition that characterizes SCA.

Our intravital observations demonstrate that PAF treatment results in a pronounced increase in endothelial oxidant generation in the ex vivo preparation (Figs. 1 and 2) compared with untreated preparations. SCA is characterized by increased oxidative stress (18), and increased endothelial oxidant generation is followed by endothelial activation (22, 26). Sickle cell patients and transgenic-knockout sickle mice show enhanced vascular superoxide production and elevated plasma xanthine oxidase activity (1, 40). In SCA, chronic effects of intravascular sickling, recurring vasoocclusive events (reperfusion injury) (25), and elevated plasma PAF levels (39) may contribute to increased oxidant generation and endothelial activation.

Direct microscopic observations and video analysis revealed that, in preparations treated with PAF alone, adhesion showed a strong inverse correlation with venular diameter. Maximal adhesion in small-diameter venules often resulted in their frequent blockage, which is consistent with our previous observations (28). Importantly, we show that antioxidants, by scavenging endothelial oxidants, result in striking inhibition of human SS red cell adhesion in the ex vivo preparation. Thus, when PAF was followed by SOD or catalase, the adhesion was markedly decreased in venules of all diameters, with catalase resulting in greater inhibition of adhesion in small-diameter venules. Maximal inhibition of SS red blood cell adhesion was observed after treatment with PNA, which is an SOD mimetic but can also scavenge H2O2 (6, 32). Importantly, no postcapillary blockage was evident after treatment with any given antioxidant, which is in marked contrast to the frequent blockage observed after infusion of SS red blood cells into preparations treated with PAF or HSA (control for PNA).

We previously showed that, in contrast to SS red blood cells, normal (AA) red blood cells do not adhere in PAF-treated or untreated ex vivo preparations (28, 29), suggesting that only SS red blood cells express the adhesion molecules that are required for interaction with vascular endothelium.

Table 4. Effect of PNA on hemodynamic behavior of SS red blood cells in non-PAF-treated ex vivo mesocecum preparations

<table>
<thead>
<tr>
<th>PRU</th>
<th>Ringer, mmHg·ml−1·min−1·g−1</th>
<th>Cells, mmHg·ml−1·min−1·g−1</th>
<th>Δ, %</th>
<th>Lt, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ringer-HSA-SS RBC</td>
<td>4.8±0.5</td>
<td>5.7±0.4</td>
<td>23.1±4.7</td>
<td>43.8±7.5</td>
</tr>
<tr>
<td>Ringer-PNA-SS RBC</td>
<td>4.1±0.6</td>
<td>4.6±0.6</td>
<td>12.9±2.7*</td>
<td>30.6±6.6†</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 4). Isolated mesocecum preparation was perfused with Ringer-albumin solution and then infused with 40 ml of HSA or PNA solution over 10 min, and vasculature incubated for 20 min. *P < 0.05 and †P < 0.002 vs. respective value for Ringer-HSA-SS RBC (paired t-test).
Our results show that endothelial oxidant generation by PAF in the ex vivo preparation, as also demonstrated in previous in vivo studies (34, 44), is likely involved in the upregulation of adhesion molecules. Other inflammatory substances such as thrombin and tumor necrosis factor-α enhance adhesion of not only leukocytes (34), but also SS red blood cells (37, 49). The enhanced adhesion of SS red blood cells in the presence of PAF and its marked inhibition by antioxidants demonstrate that oxidant generation and inflammatory activation of the endothelium have an important role in SS red cell adhesion and vasoocclusion. Similarly, the inhibitory effect of antioxidants on leukocyte adhesion has been reported by us and others (25, 35). Oxidant radicals are implicated in the activation of nuclear factor-κB, the transcription factor that promotes expression of several genes involved in the inflammatory response and upregulation of adhesion molecules (9, 15), and PNA is reported to inhibit activation of nuclear factor-κB in transgenic sickle mice (35).

In human SCA and transgenic sickle mice, inflammatory activation of the endothelium may result in the observed upregulation of an array of endothelial adhesion molecules involved in leukocyte and red cell adhesion (i.e., VCAM-1, ICAM-1, E-selectin, P-selectin, and α3β1-integrin) (5, 18, 42, 47). However, the mechanistic aspects of red cell adhesion in the current transgenic sickle mouse lines have not been characterized, and the factors that stimulate or inhibit red cell adhesion in transgenic mice have not been defined. In contrast, the ex vivo model used in the present studies, similar to cultured human endothelial cells, allows testing of known stimulating and inhibitory agents on adhesion of human SS red blood cells (4, 27, 28). Importantly, the ex vivo model enables distinction of microvascular sites of adhesion and vasoocclusion. Thus this ex vivo preparation has allowed us to test the inflammatory effect of PAF, as well as the ameliorating action of antioxidants on human SS red blood cell adhesion and vasoocclusion. In the present studies, we have tested PAF at a dose approximating that reported in sickle cell patients (24).

Further support for the role of oxidative stress in SS red cell adhesion is provided by our experiments with non-PAF-activated ex vivo preparations. Previous studies showed that tissue exteriorization itself results in inflammatory effects (12, 46) that cause transient induction of P-selectin (19). Thus the low level of SS red blood cell adhesion observed invariably in untreated (Ringer-perfused) preparations may be due to the inflammatory effects (i.e., endothelial activation) of the exteriorization procedure. This interpretation is supported by J1 low levels of endothelial oxidant generation in untreated preparations (Figs. 1 and 2) and 2 the experiments where treatment with PNA improved hemodynamic behavior and completely inhibited adhesion compared with preparations treated with control HSA (Fig. 5). Future studies are required to reaffirm the efficacy of PNA and other antioxidants in relevant transgenic models and to establish the contribution of other blood cells to red cell adhesion-mediated vasoocclusion.

Growing evidence shows that the SS red blood cell-endothelium interaction involves multiple adhesion molecules on red blood cells and endothelium (20, 43). The results of the present studies strongly support the view that antioxidant therapy with a stable and long-acting molecule, such as PNA, may constitute a useful therapeutic approach to inhibit receptor-ligand interactions involved in SS red blood cell adhesion and related vasoocclusion.

GRANTS
This work was supported by National Institutes of Health Grants U54 38655, RO1 HL-070047, and HL-55552.

DISCLOSURES
PNA and HSA were provided, free of charge, by SynZyme Technologies. L. Ma and C. J. C. Hsia (SynZyme Technologies) provided advice about the dose of PNA and HSA and contributed comments to this paper. L. Ma and C. J. C. Hsia are officers of SynZyme Technologies, which holds patents for PNA and HSA.

REFERENCES