

Divergent Nitric Oxide Bioavailability in Men and Women With Sick Cell Disease

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Background—Although reduced endothelial nitric oxide (NO) bioavailability has been demonstrated in arteriosclerotic vascular disease, the integrity of this system in sickle cell disease remains uncertain.

Methods and Results—We measured forearm blood flow in 21 patients with sickle cell disease (hemoglobin SS genotype) and 18 black control subjects before and after intra-arterial infusions of acetylcholine, nitroprusside, and the NO synthase inhibitor NG-monomethyl-L-arginine (L-NMMA). Endothelium-dependent vasodilation, measured by the percent increase in flow induced by acetylcholine infusion, was significantly greater than in controls ($252 \pm 37\%$ for patients versus $134 \pm 24\%$ for controls; $P < 0.0001$). However, there was a large sex difference in blood flow responses between female and male patients ($340 \pm 46\%$ versus $173 \pm 41\%$; $P = 0.035$). Similarly, basal NO bioactivity, as measured by the percent decrease in flow induced by L-NMMA, was depressed in male compared with female patients ($-17 \pm 5\%$ versus $-34 \pm 4\%$; $P = 0.01$), as was the response to nitroprusside ($86 \pm 21\%$ versus $171 \pm 22\%$; $P = 0.008$). L-NMMA reduced the blood flow response to acetylcholine in women, but not in men. Sex differences in vascular cell adhesion molecule-1 were appreciated, with significant correlations between levels of soluble vascular cell adhesion molecule-1 and blood flow responses to L-NMMA and nitroprusside ($r = 0.53$, $P = 0.004$ and $r = -0.66$, $P < 0.001$, respectively).

Conclusions—NO bioavailability and NO responsiveness are greater in women than in men with sickle cell disease and determines adhesion molecule expression. Endothelium-dependent blood flows are largely non-NO mediated in male patients. These results provide a possible mechanism for reported sex differences in sickle cell disease morbidity and mortality and provide a basis for novel pharmacological interventions. (*Circulation*. 2003;107:271-278.)

Key Words: nitric oxide ■ endothelium ■ anemia, sickle cell ■ acetylcholine ■ cell adhesion molecules

Similar to atherosclerotic vascular disease, sickle cell disease is characterized by chronic inflammation¹ and ischemia-reperfusion injury,^{2,3} presumably as a consequence of the continuous occlusion of the microvasculature by erythrocytes made rigid by intracellular polymerization of deoxyhemoglobin S. Although reduced endothelial nitric oxide (NO) production has been demonstrated in coronary artery disease and its risk factors, hypertension, diabetes, and hypercholesterolemia, the integrity of this system in sickle cell disease remains uncertain and controversial. Clinical investigations have mostly focused on plasma NO metabolite levels, nitrite and nitrate (NOx), and plasma arginine levels. Although some studies have suggested that these levels are high in patients with sickle cell disease,⁴ others find that NOx levels and L-arginine are depressed, particularly during vaso-occlusive crisis and the acute chest syndrome, and that these

levels vary inversely with pain symptomatology.⁵⁻⁸ Such studies are limited by confounding effects of illness, diet, and renal function. Endothelial function studies from transgenic sickle cell mice and humans provide similarly conflicting data. Although 3 animal studies demonstrated increases in tissue NO synthase levels and basal activity (determined by reduction in blood flow during NO synthase inhibition) and decreased vasodilatory responses to acetylcholine or calcium ionophore,⁹⁻¹¹ endothelial function studies performed in 7 patients with sickle cell disease showed a marked increase in forearm blood flow during the intra-arterial infusion of acetylcholine and normal constrictor responses to NO synthase inhibition, consistent with preserved NO bioactivity.¹²

To better understand the role of endothelial NO bioavailability and endothelial function in humans with sickle cell disease, we measured forearm blood flow in 21 adult sickle

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Patient Characteristics Distinguished by Sex

	Men (n=11)	Women (n=10)	P
Mean age, y	31 (31, 21, 47)	33 (36, 21, 42)	0.61
Hydroxyurea therapy, n	4	4	...
Days with mild to severe pain (per year)	40 (24, 3, 188)	55 (12, 0, 360)	0.98
Number of emergency department visits (per year)	4.8 (4, 0, 12)	4.4 (3, 0, 12)	0.68
No. of patients taking oral narcotic medications (codeine, OxyContin, hydrocodone, hydromorphone) with acetaminophen	8	7	...
No. of patients with a history of leg ulcers	3	5	...
Arterial blood pressure, mm Hg	86 (82, 77, 107)	77 (78, 62, 94)	0.08
Hemoglobin SS genotype, n (%)	11 (100)	10 (100)	...
Fetal hemoglobin, %	11.9 (12.4, 0.6, 24.0)	12.1 (7.3, 2.2, 35.0)	0.95
Hemoglobin A ₂ , %	4.1 (4.2, 3.5, 4.9)	4.3 (4.1, 3.5, 4.8)	0.58
Hemoglobin, g/dL	8.9 (9.0, 6.3, 12.0)	9.5 (9.0, 7.5, 12.6)	0.41
Hematocrit, %	25.7 (26.4, 17.1, 33.5)	27.6 (26.7, 20.4, 36.3)	0.35
Reticulocytes, 1000 cells/ μm^3	269 (244, 123, 406)	279 (214, 73.5, 702)	0.88
Mean corpuscular volume, μm^3	94 (99, 57, 119)	95 (94, 78, 119)	0.85
White blood cell count, 1000 cells/ μm^3	10.1 (7.4, 4.4, 19.2)	9.5 (9.0, 4.6, 19.4)	0.76
Platelet count, 1000 cells/ μm^3	365 (359, 200, 694)	431 (417, 251, 788)	0.34
LDL, mg/dL	82 (78, 52, 132)	72 (70, 52, 116)	0.37
HDL, mg/dL	33 (33, 25, 45)	38 (38, 26, 55)	0.19
CRP, mg/dL	0.69 (0.63, 0.03, 2.48)	0.45 (0.33, 0.01, 1.97)	0.39
Ferritin, $\mu\text{g/L}$	1152 (257, 67, 5656)	601 (235, 39, 3040)	0.44
Total bilirubin, mg/dL	3.2 (3.2, 0.8, 4.9)	2.7 (2.8, 1.0, 4.5)	0.42
Creatinine, mg/dL	0.7 (0.6, 0.5, 1.2)	0.5 (0.5, 0.3, 0.7)	0.13
Arginine, $\mu\text{mol/L}$	34.9 (38, 16, 61)	43 (45, 16, 74)	0.50
Ornithine, $\mu\text{mol/L}$	57 (53, 23, 108)	43 (46, 15, 63)	0.33

Values are mean (median, minimum, and maximum) or number.

cell patients with hemoglobin SS genotype and 18 black control subjects, using venous-occlusion strain-gauge plethysmography before and after intra-arterial infusions of acetylcholine to test endothelium-dependent vasodilation, sodium nitroprusside to test vascular responsiveness to exogenous NO, and the NO synthase inhibitor *N*^G-monomethyl-L-arginine (L-NMMA) to measure basal NO production. Relationships between endothelial NO bioavailability and clinical characteristics, including markers of NO metabolism and systemic inflammation, were evaluated.

Methods

Subjects

The National Heart, Lung, and Blood Institute's Institutional Review Board approved all protocols. All subjects provided written informed consent. Twenty-one patients with sickle cell disease and hemoglobin S-only phenotype were selected for study (Table). Inclusion criteria for the sickle cell individuals were age 18 to 55 years, electrophoretic and chromatographic diagnosis of sickle cell disease (hemoglobin S-only), and a hematocrit >17%. Potential patients with sickle cell disease were excluded if they were smokers, had been in vaso-occlusive crisis in the last 2 weeks, had received blood transfusions within the preceding 3 months (or had hemoglobin A >10%), or had risk factors for endothelial dysfunction (fasting blood sugar >120 mg/dL, LDL cholesterol >130 mg/dL, or blood pressure

>140/80 mm Hg). No subjects evaluated had high glucose, high LDL cholesterol levels, or blood pressures >140/80 mm Hg requiring exclusion, but 1 male patient was excluded before study because of a history of cigarette use and 1 female patient for a history of recent blood transfusion. Study subjects were asked not to take nonsteroidal anti-inflammatory medications for at least 1 week, and the only additional medications used by our patients, other than hydroxyurea, were oral narcotic preparations with or without acetaminophen. Demographic and forearm hemodynamic data for the 18 black control subjects with hemoglobin A-only and no risk factors for endothelial dysfunction have been published previously (sex-specific blood flow data from these subjects have not been reported previously).¹³ None of the control subjects had risk factors for endothelial dysfunction, and their mean age was 43 ± 1.7 years. Female control subjects and patients were all premenopausal.

Forearm Blood Flow Measurements

Brachial artery and antecubital vein catheters were placed in the arm, with the intra-arterial catheter connected to a pressure transducer for blood pressure measurements and an infusion pump that delivered 5% dextrose-in-water at 0.5 mL/min. After 20 minutes of rest, baseline arterial and venous blood samples were obtained, and forearm blood flow measurements were made by strain gauge venous-occlusion plethysmography, as reported previously.^{14–16}

Patients were randomized to receive either acetylcholine or sodium nitroprusside as the first infusion, before receiving the alternate infusion, after a 25-minute rest with intra-arterial infusion of 5% dextrose-in-water and repeat baseline measurement. Acetyl-

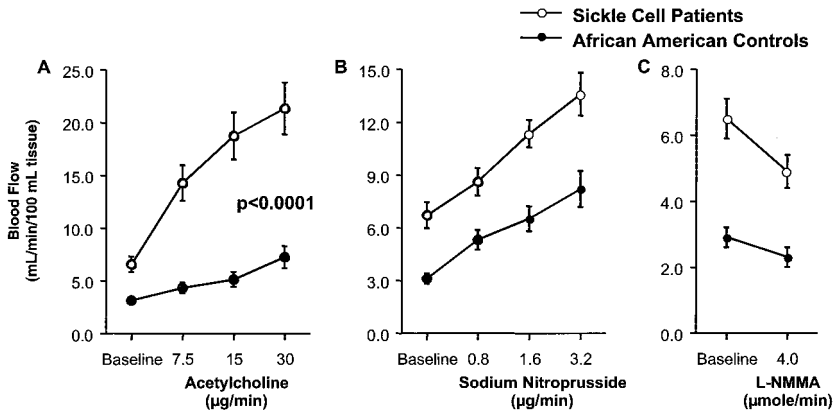


Figure 1. Forearm blood flow during intra-arterial infusions of acetylcholine (A), sodium nitroprusside (B), and L-NMMA (C) in 21 patients with sickle cell disease compared with 18 black control subjects. Data are mean \pm SEM.

choline was infused at 7.5, 15, and 30 μ g/min, and sodium nitroprusside was infused at 0.8, 1.6, and 3.2 μ g/min, each for 5 minutes. After 3 minutes of each infusion dose, forearm blood flows were measured. After a completion of testing with these agonists and a 25-minute rest period, repeat baseline blood flow measurements were obtained, and L-NMMA was infused at 4 μ mol/min. After 5 minutes of L-NMMA infusion, forearm blood flow was measured. In 6 of the patients (3 men and 3 women), acetylcholine was infused at 30 μ g/min for 5 minutes during continuation of L-NMMA infusion to test the contribution of non-NO endothelium-derived relaxing factors to acetylcholine-mediated vasodilation.

Laboratory Evaluations

All sickle cell disease patients had complete blood cell counts and standard laboratory chemistries, hemoglobin electrophoresis and high-performance liquid chromatography, lipid profiles, high-sensitivity C-reactive protein (CRP) assay, plasma amino acids, soluble vascular cell adhesion molecule-1 (sVCAM-1) levels, iron-binding studies, and red cell G6PD activity measurements. CRP was measured by a high-sensitivity (0.01 mg/dL) chemiluminescent immunometric assay (Immulite 2000; Diagnostic Products Corp). sVCAM-1 in plasma was measured by ELISA (R&D Systems) according to the manufacturer's instructions.

Statistical Analysis

Two-sided probability values were calculated by unpaired *t* test for the comparisons between men and women with sickle cell disease and between controls and patients with sickle cell disease for baseline blood flow and changes in flow during acetylcholine, nitroprusside, and L-NMMA infusions. Repeated-measures ANOVA was performed to study the effects of sex on changes in blood flow over all doses of drugs. Linear regression was performed to evaluate the effect of subject characteristics (sex, age, fetal hemoglobin levels, total hemoglobin levels, white blood cell count, creatinine, and hydroxyurea therapy) on forearm blood flow responses to infused medications and to investigate the effects of these variables on the sex-blood flow response relationship. Measurements shown are mean \pm SEM. Analysis was performed with Stata 7.0 (Stata Corporation) and SAS, version 8.0 (SAS Institute Inc) software.

Results

Patient characteristics by sex are shown in the Table. Male patients tended to have higher mean blood pressures and higher creatinine levels. Ornithine, which is metabolized from arginine by arginase, tended to be higher in the men. A history of leg ulcers (3 men and 5 women) and central nervous system events (1 man with a stroke and 1 woman with a seizure) constituted the only major vascular complications reported by these patients. In all patients, markers of inflammation and hemolysis were increased compared with

reference values from our laboratory. LDL and plasma arginine levels were low-normal and G6PD activity was normal in all patients.

Forearm Hemodynamics at Baseline and During Acetylcholine Infusion

Basal blood flow was higher in patients than in control subjects (6.3 ± 0.7 versus 3.1 ± 0.3 mL/min per 100 mL of forearm tissue; $P < 0.01$; Figure 1). The increased basal blood flow was inversely correlated with hematocrit ($r = -0.38$; $P = 0.05$). Basal blood flows tended to be higher in men with sickle cell disease than in women (7.4 ± 1.0 versus 5.0 ± 0.6 mL/min per 100 mL of forearm tissue; $P = 0.11$) and was significantly higher in male control subjects than in women (3.7 ± 0.3 versus 2.1 ± 0.2 mL/min per 100 mL of forearm tissue; $P = 0.01$). Intra-arterial infusions of acetylcholine produced significantly greater increases in forearm blood flow in the 21 patients with sickle cell disease than in 18 black control subjects ($P < 0.0001$; Figure 1). Whereas blood flow increased $134 \pm 24\%$ in black control subjects, it increased $252 \pm 37\%$ in patients with sickle cell disease. The acetylcholine effect was similar in male and female black control subjects but differed substantially between men and women with sickle cell disease (Figures 2A and 2B). In black control subjects, acetylcholine increased forearm blood flow $148 \pm 24\%$ in men and $116 \pm 24\%$ in women ($P = 0.51$), whereas in patients with sickle cell disease, acetylcholine increased forearm blood flow $173 \pm 45\%$ in men and $340 \pm 49\%$ in women ($P = 0.035$). Female blood flow responses to acetylcholine in patients were significantly different from female controls ($P = 0.003$), whereas responses in male patients were not significantly different from male controls. Similar sex differences were observed for forearm vascular resistance (data not shown).

Forearm Hemodynamics During Sodium Nitroprusside Infusion

Intra-arterial infusions of nitroprusside produced similar relative increases in forearm blood flow in the 21 patients with sickle cell disease and 18 black control subjects (Figure 1). Whereas blood flow increased to a similar extent in black control men and control women, a sex-based difference was observed in patients with sickle cell disease, which suggests differential sensitivity to exogenous NO (Figures 2C and 2D).

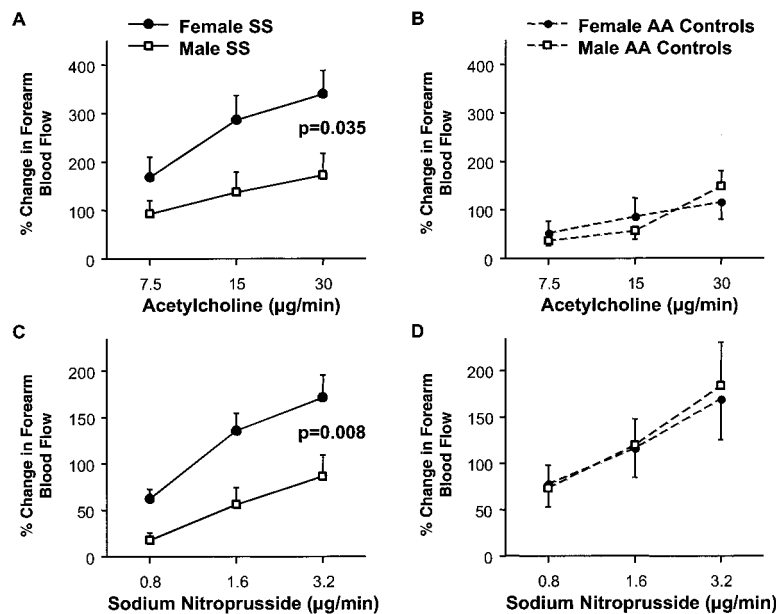


Figure 2. Forearm blood flow, expressed as percentage change from baseline during intra-arterial infusion of acetylcholine (A and B) and sodium nitroprusside (C and D), in 21 patients with sickle cell disease (A and C) and 18 black control subjects (B and D). *P* values refer to comparison of blood flow responses at 3 doses of acetylcholine and sodium nitroprusside between the 2 curves. Data are mean \pm SEM.

In patients with sickle cell disease, nitroprusside increased forearm blood flow $86 \pm 23\%$ in men and $171 \pm 24\%$ in women ($P=0.008$). Similar results were observed for forearm vascular resistance (data not shown). Male sickle cell disease patients also tended to have lower blood flow responses to nitroprusside than male controls ($P=0.07$).

Forearm Hemodynamics During L-NMMA Infusion

Intra-arterial infusions of L-NMMA produced similar relative decreases in forearm blood flow from baseline values in the 21 patients with sickle cell disease as a group compared with the 18 black control subjects ($-25 \pm 4\%$ versus $-24 \pm 5\%$, respectively; $P=\text{NS}$; Figure 1). Although the decrease in blood flow in black men and women in the control group was similar, there was a markedly greater decrease in women with sickle cell disease than in men with sickle cell disease ($-34 \pm 4\%$ versus $-17 \pm 5\%$, respectively; $P=0.01$; Figure 3). Male sickle cell disease patients tended to have reduced responsiveness to L-NMMA compared with male controls ($-17 \pm 5\%$ versus $-27 \pm 5\%$; Figure 3A; $P=0.25$). Similar results were observed for forearm vascular resistance (data not shown).

Forearm Hemodynamics During Coinfusion of L-NMMA and Acetylcholine

In the final 6 patients studied (3 men and 3 women), during continued infusion of L-NMMA, acetylcholine at 30 $\mu\text{g}/\text{min}$ was reinfused for 5 minutes. This allowed a comparison of the blood flow responses to acetylcholine alone (performed earlier) with blood flow responses to acetylcholine during NO synthase inhibition (Figure 4). In the women, L-NMMA infusion blunted the increase in blood flow induced by acetylcholine from $448 \pm 89\%$ to $212 \pm 121\%$ ($P=0.02$). In contrast, L-NMMA infusion had no effect on blood flow during acetylcholine infusion in men ($221 \pm 89\%$ to $237 \pm 56\%$; $P=\text{NS}$), consistent with blood flow responses not being mediated by NO in men with sickle cell disease.

Although we did not perform this measurement in the black control subjects in the present study, we have previously published such blood flow responses in normal volunteers (mostly white) and found that blood flow responses to acetylcholine were 43% reduced with coinfusion of L-NMMA.¹⁴ This is similar to the response observed in female sickle cell patients in the present study but is very different from the lack of effect of L-NMMA seen in our male sickle cell patients.

Analysis of the Relationship Between Clinical Variables and Blood Flow Responses to Acetylcholine and L-NMMA

We found no significant correlations between markers of inflammation (white blood cell count, erythrocyte sedimentation rate, and CRP levels), fetal hemoglobin levels, genotype, plasma L-arginine levels, the number of emergency room visits for pain, age, or hydroxyurea therapy and blood flow responses to acetylcholine or L-NMMA. Plasma CRP levels were highly correlated with other markers of inflammation, such as ferritin ($r=0.75$; $P<0.0001$) and white blood cell count ($r=0.57$; $P=0.002$). To determine whether clinical variables, such as hydroxyurea therapy, creatinine, fetal hemoglobin, and other variables shown in the Table, confounded the effect of sex on blood flow responses, multiple linear regression analyses were performed. None of the potentially confounding variables changed the differences in percentage change in blood flow with acetylcholine or L-NMMA between men and women by 10% or more.

Effect of Endothelial NO Bioavailability on sVCAM-1 Levels

sVCAM-1 levels were significantly elevated in all patients with sickle cell disease (479 ± 46 ng/mL) compared with a group of 15 healthy black subjects (184 ± 28 ng/mL; $P<0.001$ for the comparison). Consistent with our earlier work, sVCAM-1 levels were inversely correlated with fetal hemoglobin ($r=-0.58$; $P=0.001$) and directly correlated with

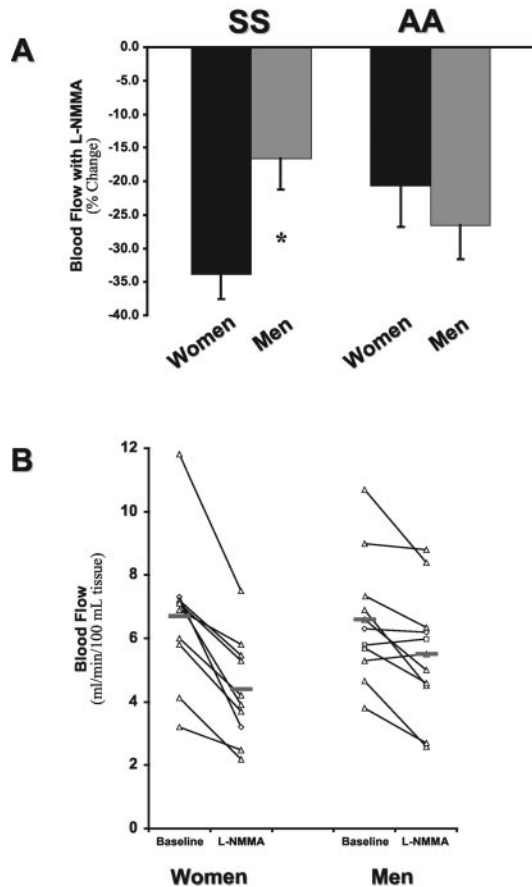


Figure 3. A, Forearm blood flow expressed as percentage change from baseline during intra-arterial infusion of 4 mmol/min L-NMMA in 21 patients with sickle cell disease (SS genotype) compared with 18 black control subjects (AA). * $P=0.01$ by unpaired t test for male vs female patients with sickle cell disease. B, Absolute blood flow responses before and during L-NMMA infusion in all male and female patients with sickle cell disease.

white blood cell counts ($r=0.55$; $P=0.002$).¹⁷ Additionally, sVCAM-1 levels were significantly correlated with measures of endogenous NO production and bioavailability (Figure 5). Thus, levels of sVCAM-1 correlated directly with blood flow responses to L-NMMA ($r=0.53$; $P=0.004$; Figure 5A) such that patients with the lowest basal NO production (limited blood flow decrease during L-NMMA infusion) had the highest sVCAM-1 levels. Similarly, levels of sVCAM-1 correlated inversely with blood flow responses to sodium nitroprusside ($r=-0.66$; $P<0.0001$; Figure 5B) and acetylcholine ($r=-0.41$; $P=0.03$). Consistent with a sex difference in NO bioavailability, sVCAM-1 levels were significantly higher in men than in women with sickle cell disease (572 ± 58 and 378 ± 50 ng/mL, respectively; $P=0.03$). No significant sex differences in adhesion molecule expression were observed in black control subjects.

Effects of Hydroxyurea Therapy on Endothelial Function and Plasma sVCAM-1

Analysis of the 8 patients undergoing chronic hydroxyurea therapy compared with the 13 patients not undergoing therapy revealed expected differences in fetal hemoglobin ($21\pm3\%$ versus $6\pm1\%$; $P<0.0001$), hemoglobin (10.6 ± 0.5

versus 8.3 ± 0.3 g/dL; $P=0.0005$), white blood cell count (6.6 ± 0.7 versus 11.8 ± 1.2 1000 cells/ μm^3 ; $P=0.005$), and plasma levels of sVCAM-1 (345 ± 75 versus 562 ± 48 ng/mL; $P=0.02$). The differences in blood flow responses to acetylcholine in patients taking hydroxyurea versus nontreated patients ($209\pm50\%$ versus $280\pm52\%$; $P=0.37$), and to L-NMMA ($-30\pm6\%$ versus $-22\pm5\%$; $P=0.31$) did not achieve statistical significance. However, forearm blood flow responses to sodium nitroprusside were significantly improved ($175\pm36\%$ versus $97\pm18\%$ change in forearm blood flow; $P=0.04$).

Discussion

In this study of 21 subjects with sickle cell disease, we found that although NO pathways play a dominant role in maintaining basal dilator tone and blood flow responsiveness in women with sickle cell disease, this system is impaired in male patients. Belhassen and colleagues¹² tested endothelial responsiveness to L-NMMA and acetylcholine in 7 patients with sickle cell disease and found that responses to L-NMMA were normal but that acetylcholine responsiveness was increased. An analysis of all of the patients in the present study as a group is consistent with the results of Belhassen et al¹² with normal blood flow responses to L-NMMA and augmented blood flow responses to acetylcholine (Figure 1). However, the greater number of patients evaluated in the present study revealed that both of these effects were limited to women with sickle cell disease but that in men, NO bioavailability and responses to exogenous NO were reduced. Our results also suggest that there is a significant upregulation of non-NO vasodilators (which may include prostacyclin and endothelium-derived hyperpolarizing factor) in males and females with sickle cell disease. Although the number of patients treated with hydroxyurea was small in this study, their flow responses suggest a normalization of vascular

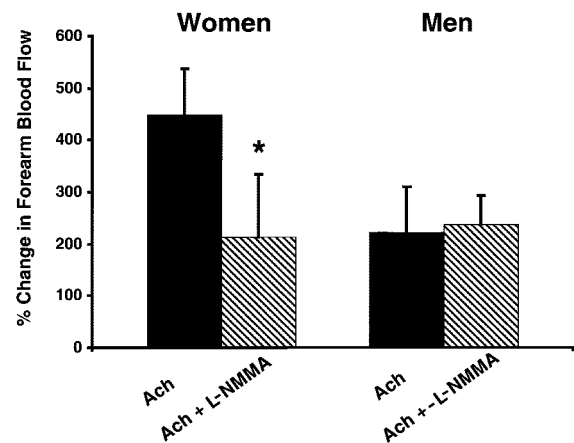


Figure 4. Forearm blood flow, expressed as percentage change from baseline during coinfusion of L-NMMA and acetylcholine (Ach) in men and women with sickle cell disease. L-NMMA infusion significantly reduced change in blood flow with acetylcholine alone in women (* $P=0.02$). Consistent with blood flow responses not being mediated by NO in men with sickle cell disease, L-NMMA infusion had no effect on male blood flow during acetylcholine infusion ($P=NS$).

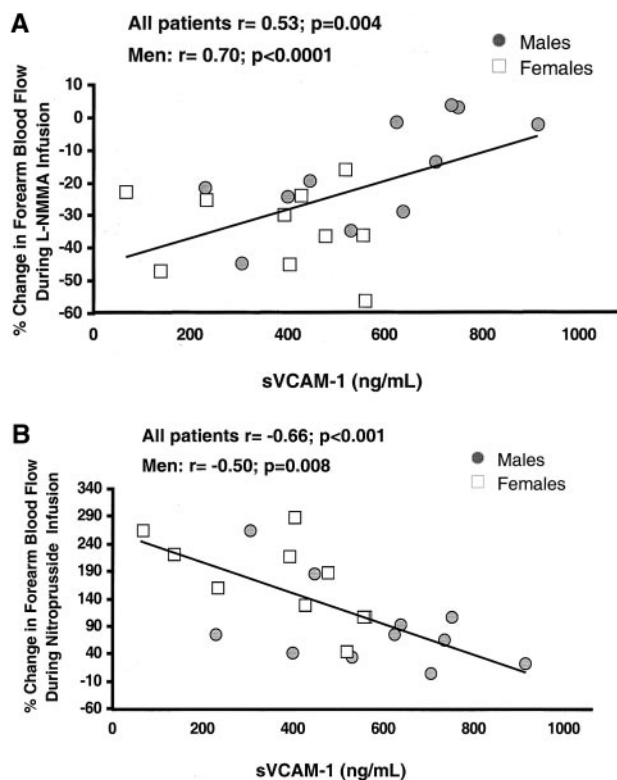


Figure 5. Relationship between basal endothelial NO bioavailability, reflected by decrease in forearm blood flow during infusion of NO synthase inhibitor L-NMMA, and sVCAM-1 levels in plasma (A). Women (□) had greater basal NO bioavailability (ie, significant blood flow decreases during NO synthase inhibition) and lower sVCAM-1 levels, whereas men (●) had reduced NO bioavailability (ie, limited blood flow decreases during NO synthase inhibition) and increased sVCAM-1 levels. Similar results were observed for infusions of sodium nitroprusside, with higher levels of sVCAM-1 observed in patients with lower blood flow responses to nitroprusside (B).

responsiveness to the agonists infused and levels of circulating markers of inflammation.

NO is the major endothelium-derived relaxing factor in normal physiology and plays a central role in vascular homeostasis by maintaining basal and stimulated vasomotor tone, limiting platelet aggregation and ischemia-reperfusion injury, and modulating endothelial proliferation. In recent years, there has been increasing evidence for depressed bioavailability of NO in syndromes associated with atherosclerosis and its risk factors, with defects in both basal and pharmacologically stimulated endothelial NO production reported.^{14,18,19} Like atherosclerotic vascular disease, sickle cell disease is characterized by chronic inflammation¹ and ischemia-reperfusion injury,^{2,3} and similar mediators and markers of inflammation, most notably plasma CRP, oxidized lipids, and sVCAM-1, are elevated in plasma of patients with both diseases. VCAM-1 is produced by endothelial cells, particularly during cytokine stimulation (interleukin [IL]-1 α , IL-1 β , and IL-4, and tumor necrosis factor- α), and facilitates recruitment of monocytes and lymphocytes to sites of vascular inflammation.^{20–23} sVCAM-1 is elevated in plasma from patients with sickle cell disease, and its expression on endothelial cells promotes adherence of reticulocytes via its interactions with integrin- $\alpha_4\beta_1$.^{24–29}

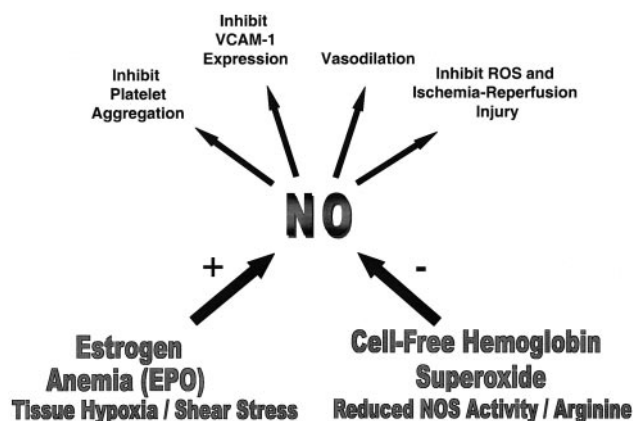


Figure 6. NO bioavailability in patients with sickle cell disease. NO likely plays a critical compensatory role in sickle cell disease by maintaining vasomotor tone, limiting platelet aggregation, inhibiting ischemia-reperfusion injury, and modulating endothelial adhesion molecule expression. Increased shear-stress, anemia, and erythropoietin production,⁴⁹ as well as compensatory responses to chronic vascular injury, drive increases in endothelial NO production,^{9,10} which is facilitated by estrogens. NO is simultaneously scavenged by superoxide, which is produced by xanthine oxidase,¹¹ and by cell free hemoglobin,⁴¹ which is liberated by steady-state hemolysis. Reduced NO production secondary to NO synthase inhibition or arginine deficiency may also contribute.⁶ Results of this study show that in women, compensatory increases in NO production and bioactivity are maintained and even augmented, whereas in men, this system fails. ROS indicates reactive oxygen species; EPO, erythropoietin; and NOS, NO synthase.

NO pharmacologically reduces VCAM-1 gene transcription in endothelial cells by inhibiting nuclear factor- κ B, particularly during cytokine stimulation.³⁰ Furthermore, the blockade of endogenous NO by L-NMMA induces VCAM-1 in cultured endothelial cells.³⁰ The present results are consistent with such a model in sickle cell disease. Chronic cycles of tissue ischemia-reperfusion injury secondary to intraerythrocytic hemoglobin S polymerization and microvascular obstructive events create an inflammatory state driven by monocyte-derived cytokines (tumor necrosis factor- α and IL-1 β) and leukocyte- and xanthine oxidase-derived oxygen radicals (superoxide). Subsequent nuclear factor- κ B gene activation stimulates increases in VCAM-1 gene transcription. Consistent with this model, we have observed increases in plasma sVCAM-1 levels that correlate with markers of inflammation. In keeping with previous *in vitro* studies that show that NO tonically downregulates VCAM-1 gene transcription, the present study demonstrates that diminished endothelial NO bioavailability and increased NO destruction, as suggested by the limited blood flow responses to L-NMMA and nitroprusside infusions, respectively, may increase plasma sVCAM-1 levels. These data are consistent with recent studies demonstrating that patients with the acute chest syndrome have increases in VCAM-1 levels that are inversely correlated with plasma NO metabolite levels.^{7,27} Thus, NO appears to play a critical compensatory role in maintaining endothelial homeostasis during the steady-state ischemia and oxidant- and cytokine-driven stress characteristic of sickle cell disease. Our current understanding of the

major factors that affect NO bioavailability in sickle cell disease is depicted in Figure 6.

We also observed an apparent protective effect of ovarian estrogens on endothelial function in sickle cell disease. Estrogens increase endothelial NO synthase expression and basal endothelial NO production and appear to prevent endothelial dysfunction in the setting of classic risk factors for atherosclerosis.^{31–37} For example, women with hypercholesterolemia have less impairment in endothelial function than men with hypercholesterolemia.³⁸ In addition to increasing NO synthase expression, estrogen infusions into the brachial or coronary artery acutely enhance NO-dependent vasodilation, consistent with enhanced NO synthase activity or antioxidant protection from NO destruction.^{33,39,40} It is therefore not surprising that there would be sex-based differences in the responses to chronic vascular injury between men and women of reproductive age with sickle cell disease. Indeed, the present data suggest that men have reduced endothelial NO production, on the basis of reduced blood flow responses to L-NMMA and acetylcholine, as well as increased NO inactivation, on the basis of reduced blood flow responses to the exogenous NO donor sodium nitroprusside. The latter observation is consistent with an increase in NO consumption by superoxide¹¹ or cell free hemoglobin⁴¹ in patients with sickle cell disease that is limited by estrogen-induced NO production in women.

Considering the effects of estrogen on NO bioavailability, we hypothesize that NO production can account for sex differences in morbidity and mortality observed in sickle cell disease. The Cooperative Study of Sickle Cell Disease reported a median age of death of 42 years for men and 48 years for women.⁴² Although similar sex differences in mortality are observed in black control subjects, it is notable that mortality differences between male and female patients with sickle cell disease become evident only in adulthood after 30 years of age, and the magnitude of this difference is greater in sickle cell patients.⁴² Further supporting a sex difference in clinical outcomes, Baum and colleagues⁴³ observed a striking increase in vaso-occlusive crisis in men with sickle cell disease after age 15 years, resulting in a greater rate of pain attacks in men than in women. Others have described a similar increase in pain rates in men; however, this was only observed between the ages of 20 and 29 years.⁴⁴ Female patients have slightly greater fetal hemoglobin and F cell levels, and this has been proffered as a mechanistic explanation for the observed sex differences in morbidity and mortality.^{45–47} Indeed, NO has now been linked to fetal hemoglobin transcriptional control, which suggests that NO bioavailability may possibly contribute to sex differences in fetal hemoglobin expression.⁴⁸ Further studies that carefully measure adult sickle cell complications, especially large-vessel central nervous system disease, may provide greater insight into the role of sex as a disease severity modifier.

In conclusion, both men and women display enhanced non-NO-driven vasodilation, likely prostacyclin and/or endothelium-derived hyperpolarizing factor, which raises important questions about nonsteroidal anti-inflammatory drug use for analgesia in this population. Women with sickle cell disease upregulate basal and acetylcholine-dependent NO

production, whereas men with sickle cell disease have depressed NO bioavailability and responsiveness to exogenous NO. Furthermore, this *in vivo* reduction in NO bioavailability is correlated with enhanced endothelial adhesion molecule expression. These observations provide a possible mechanism for reported sex differences in sickle cell disease morbidity and mortality and may contribute to the great phenotypic heterogeneity that characterizes this disease. Our data suggest that therapies that restore NO bioactivity by supplying exogenous NO, reducing NO scavenging by superoxide or cell free hemoglobin, or inducing NO synthase expression or activity may prove beneficial for patients with sickle cell disease.

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