Imaging hemoglobin oxygen saturation in sickle cell disease patients using noninvasive visible reflectance hyperspectral techniques: effects of nitric oxide

Karel J. Zuzak,1 Mark T. Gladwin,2 Richard O. Cannon III,3 and Ira W. Levin1

1Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, 2Critical Care Medicine Department, Warren G. Magnuson Clinical Center, and 3Cardiovascular Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892-0510

Submitted 18 March 2003; accepted in final form 29 May 2003

Zuzak, Karel J., Mark T. Gladwin, Richard O. Cannon III, and Ira W. Levin. Imaging hemoglobin oxygen saturation in sickle cell disease patients using noninvasive visible reflectance hyperspectral techniques: effects of nitric oxide. Am J Physiol Heart Circ Physiol 285: H1183–H1189, 2003. First published June 5, 2003; 10.1152/ajpheart.00243.2003.—Sickle cell disease is characterized by microvascular occlusion and hemolytic anemia, factors that impair tissue oxygen delivery. We use visible reflectance hyperspectral imaging to quantitate skin tissue hemoglobin oxygen saturation (HbO2) and to determine whether changes in blood flow during nitric oxide (NO) stimulation or gas administration (therapies proposed for this disease) improve skin tissue oxygen saturation in five patients with sickle cell disease. Compared with six healthy African-American subjects, sickle cell patients exhibited higher forearm blood flows (7.4 ± 1.8 vs. 3.2 ± 0.4 ml·min⁻¹·100 ml tissue⁻¹, P = 0.037) but significantly reduced percentages of skin HbO2 (61.0 ± 0.2 vs. 77.5 ± 0.2%, P < 0.001). Administration of acetylcholine to patients increased blood flow by 15.1 ± 3.8 ml·min⁻¹·100 ml tissue⁻¹ and the percentage of skin HbO2 by 4.1 ± 0.3% (P = 0.02, P < 0.001, respectively, from baseline values). Sodium nitroprusside, a direct NO donor, increased blood flow by 3.9 ± 1.1 ml/min and the percentage of skin HbO2 by 2.9 ± 0.3% (P = 0.02, P < 0.001, respectively). NO inhalation had no effect on forearm blood flow, yet increased the percentage of skin HbO2 by 2.5 ± 0.3% (P < 0.001). Percentages of skin HbO2 were exponentially related to blood flow (R = 0.97, P < 0.001), indicating a limit to skin tissue oxygen saturation at high blood flows. Thus, for acetylcholine infusion leading to blood flows sevenfold greater than those of healthy resting African-American subjects, patients still exhibited lower percentages of skin HbO2 (65.2 ± 0.2 vs. 77.5 ± 0.2%, P < 0.001). Visible reflectance hyperspectral imaging demonstrates that either the stimulation or the administration of NO pharmacologically or by gas inhalation improves, but does not normalize, skin tissue oxygen saturation in patients with sickle cell disease.

Peripheral vascular disease; cardiovascular pharmacology; blood flow

SICKLE CELL DISEASE is an autosomal recessive genetic disorder caused by a variant of the β-globin gene that affects ∼50,000 Americans and results in high mortality rates, events largely attributable to acute and chronic vascular occlusion of vital organs (2, 22). For the disease to be expressed, either two copies of sickle hemoglobin (HbS) or one copy of HbS associated with another β-globin variant (e.g., HbC) is required. These genetic alterations result in the intracellular polymerization of HbS (4, 16, 29). The extent of the polymerization depends on the percentages of oxygenated hemoglobin and the total intracellular hemoglobin concentrations and composition (4). Because HbS polymerization leads to increased erythrocyte rigidity, red blood cell passage through the microvasculature and arteriolar sphincters of various organs is impaired and results in ischemia and infarction (7). Although the rheological mechanisms responsible for initiating vascular occlusion leading to tissue pathology are poorly understood for this disease, acute and chronic inflammation with increased adhesion molecule expression likely contribute (12, 20, 21, 27).

A suggested therapy for alleviating vasoocclusive crisis is administration of nitric oxide (NO) gas directly or by a stimulated endothelial NO release in an effort to augment blood flow to critical ischemic regions (7). NO, produced endogenously by the endothelium, is a potent inorganic vasodilator, an inhibitor of platelet activation and adhesion, and an inhibitor of both endothelial adhesion molecule expression and leukocyte adhesion (6, 13, 17). NO gas administered to humans selectively improves blood flow and skin tissue oxygen saturation in the forearm in which regional NO synthesis is inhibited pharmacologically, simulating endothelial dysfunction (5, 30).

In the present study, we consider the adequacy of blood flow measurements alone to reflect skin tissue oxygen saturation in patients with sickle cell disease. Specifically, we measure forearm blood flows and, separately, the saturation of hemoglobin with oxygen perfusing skin tissue by using a novel noninvasive imaging device (31). Measures were made before and after brachial artery infusions of acetylcholine, an agonist...
that stimulates the release of NO from the endothelium, sodium nitroprusside, a direct NO donor and endothelium-independent vasodilator, and \(N^G\)-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthesis. We then administered NO gas at a dose of 80 parts/million, a procedure that is currently under evaluation in clinical trials.

**MATERIALS AND METHODS**

**Study population.** The study population consisted of non-smoking African-American volunteer subjects between the ages of 18 and 59 yr: six healthy subjects with the hemoglobin AA genotype (4 men, 2 women) and five patients with sickle cell disease with HbS (4 men, 1 woman), as determined by hemoglobin high-performance liquid chromatography. No patient presented a hematocrit of ≤18%, received a blood transfusion within the preceding 4 wk, or displayed hemoglobin A of >5%. Neither the healthy subjects nor patients possessed risk factors for endothelial dysfunction (that is, smoking within 2 yr, values of fasting blood sugar of >120 mg/dl, low-density lipoprotein cholesterol of >130 mg/dl, high-density lipoprotein cholesterol of <30 mg/dl, diastolic blood pressure of >90 mmHg, and creatinine of >1.0 mg/dl). This study was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute, and participants provided informed consent for all procedures that were followed in accordance with institutional guidelines.

**Spectroscopic imaging of percentage of skin oxyhemoglobin.** Visible reflectance hyperspectral imaging provides a general, noninvasive tool for in vivo quantitation of blood constituents during tissue perfusion (30, 31). Each pixel within the acquired image provides a value for an average hemoglobin oxygen saturation (HbO2) from a volume of skin tissue. Because hemoglobin provides the spectroscopic image contrast, an infusion of invasive contrast agents is unnecessary. Briefly, after illumination of the skin by light, a visible reflectance spectrum is obtained for the 645- to 520-nm wavelength region. Light within this spectral range penetrates the skin up to 2 mm (11) with, however, most of the signal penetrating to depths between 0.23 and 0.65 mm (1). The reflected radiation is detected by a charge-coupled device (CCD). The resulting spectral information is converted to an image in which skin tissue oxygen saturation is presented in terms of percentages of HbO2 and deoxyhemoglobin.

The visible reflectance hyperspectral imaging system, which has been described in detail (31), consists of a liquid crystal tunable filter (Cambridge Research & Instrumentation), a CCD (Roper Scientific), optics (Nikon), and a stable quartz-tungsten-halogen source with illuminating optics (Oriel Instruments) mounted on a standard movable surgical tripod (QuickSet International). All instrument functions are computer controlled (Dell). A desktop computer was also used for spectral deconvolutions and image analyses by using Matlab programs (MathWorks).

**Clinical protocol.** Study participants fasted overnight and refrained from drinking alcoholic and caffeinated beverages for at least 12 h before the study. Studies were initiated in the morning in a quiet room with a temperature of ~22°C. An intra-arterial catheter was placed into the brachial artery with an infusion of 5% dextrose in water. Blood flow was determined by venous occlusion plethysmography, as previously described (18).

Imaging commences by illuminating the area of interest on the subject, the palm of the hand, in this study, with a stable quartz-tungsten-halogen source. Because ultraviolet and infrared wavelengths were filtered, the tissue, if necessary, could be illuminated by the visible radiation at relatively high intensities for extended periods of time without damage. The subject remains immobile for 1–2 min during data acquisition. The data consist of a series of digital images at multiple, contiguous wavelengths that span the visible spectral range from 645 to 520 nm in 1-nm increments; the liquid crystal tunable filter spectral bandwidth is ~0.5 nm. The array size of the detector, a CCD device, consists of 768 × 512 pixels. With the subject positioned 5.5 ft from the detector and for a spatial resolution of 0.45 mm, a 17-cm-diameter field of view is delineated. The spectra acquired at each detector pixel are deconvolved by a multivariate least-squares fit based on linear combinations of reference spectra of HbO2 and deoxyhemoglobin. The acquired data define the grayscale images. Thus each pixel within an image represents a quantitative value for the percentage of skin HbO2 at a spatially independent point. In our experience, skin tissue of the hypoparenchymal region within the palm of the hand provides a suitable surface for reproducible hyperspectral visible reflectance measurements. Accordingly, a predetermined 36-mm2 rectangular area in the hypoparenchymal region, representing 100 pixels, was sampled systematically on all subjects.

After baseline measurements of the percentages of skin HbO2 in all study participants, infusions of acetylcholine at 30 \(\mu\)g/min, sodium nitroprusside at 3.2 \(\mu\)g/min, and L-NMMA at 4 \(\mu\)mol/min were administered sequentially to patients with sickle cell disease, separated by 20-min stabilization periods after each infusion, while 5% dextrose in water was continuously administered. The stabilization periods allowed blood flow to return to basal values. After 3 min of continuously infusion of either acetylcholine or sodium nitroprusside, hyperspectral images of the hand were recorded, followed by blood flow measurements. During a 5-min interval while L-NMMA was administered, three sequential hyperspectral measurements, followed by a blood flow measurement, were acquired. After this series of infusions and measurements, NO was inhaled by all patients at 80 parts/million via an anesthesia facemask with a reservoir bag (inspired oxygen fraction = 0.21) using the INOvent delivery system (INO Therapeutics, Clinton, NJ). After 90 min of NO inhalation, percentages of skin HbO2 and forearm blood flows were recorded, and L-NMMA was reintiated.

**Statistical analysis.** To describe the independently measured values from the subjects, data are presented as means ± SE. A paired \(t\)-test was used to determine whether the mean values for the sickle cell disease patients were significantly different before and after pharmacological intervention. An unpaired \(t\)-test was used to determine the significance of differences between sickle cell disease patients and healthy African-American subjects. The level of significance, the probability of a difference due to random chance, was set to \(P < 0.05\), and critical values for these tests were determined from a nondirectional, two-tail distribution (28).

**RESULTS**

Correlation between oxygen saturation in skin and in underlying tissue. Figure 1 displays for a population of sickle cell disease patients a linear relationship between skin tissue HbO2 determined by the visible reflectance imaging measurements and oxygen saturation of venous blood sampled from a 20-gauge catheter placed into the antecubital vein. This correlation demonstrates that HbO2 in the microcirculation of the perfused areas of the skin is linearly related to the
Effects of NO stimulation on blood flow and skin tissue oxygen saturation. Infusion of acetylcholine into the brachial artery of patients increased forearm blood flow from 7.4 ± 1.8 to 22.5 ± 5.1 ml·min⁻¹·100 ml tissue⁻¹ (P = 0.02). Hyperspectral imaging demonstrated that acetylcholine infusion increased the percentage of skin HbO₂ from 61.0 ± 0.2% for baseline values to 65.2 ± 0.2% (P < 0.001) (Fig. 3A, 1 and 2). After the acetylcholine infusion was terminated and the blood flow was allowed to return to basal levels, infusion of sodium nitroprusside increased forearm blood flow to 11.3 ± 2.2 ml·min⁻¹·100 ml tissue⁻¹ (P = 0.02 vs. baseline values) and increased the percentage of skin HbO₂ to 63.9 ± 0.3% (P < 0.001 vs. baseline values; Fig. 3, B and C).

Effects of NO inhibition and inhalation on blood flow and skin tissue oxygen saturation. Infusion of the NO synthase inhibitor l-NMMA into the brachial artery of patients for 5 min decreased both forearm blood flow from 7.4 ± 1.8 to 6.3 ± 1.1 ml·min⁻¹·100 ml tissue⁻¹ and the percentage of skin HbO₂ from 61.0 ± 0.2 to 57.1 ± 0.2% (P < 0.001). Time-resolved hyperspectral measurements during l-NMMA infusion (Fig. 4) showed a stepwise decrease in the percentage of skin HbO₂.

After the termination of l-NMMA infusion, inhalation of NO for 90 min increased the percentage of skin HbO₂ from the room air value of 61.0 ± 0.2 to 63.3 ± oxygen saturation values of venous blood within the underlying tissue.

Blood flow and skin tissue oxygen saturation of hemoglobin. Forearm blood flow in healthy, resting African-American subjects was 3.2 ± 0.4 ml·min⁻¹·100 ml tissue⁻¹. This value is similar to that of healthy, resting Caucasian subjects studied previously by our laboratory (2.7 ± 1.0 ml·min⁻¹·100 ml tissue⁻¹) (30). Forearm blood flow in patients was significantly greater at 7.4 ± 1.8 ml·min⁻¹·100 ml tissue⁻¹ (P = 0.037) compared with African-American controls.

The visible reflectance hyperspectral method for determining the percentage of HbO₂ distributed throughout the skin tissue within the palm of a hand is shown in Fig. 2. The white light images present general morphological features and illustrate the relative melanin content of an African-American control subject and a patient with sickle cell disease, respectively (Fig. 2A, 1 and 2). The associated hyperspectral images display quantitatively the percentages of HbO₂ distributed within the skin tissue as a function of grayscale, where increasing, brighter pixel intensity indicates an increasing percentage of skin HbO₂ (Fig. 2B, 1 and 2). Sampling from the hypothenar region of African-American controls (Fig. 2B, 1), the area within the square on the image, we determined the percentage of skin HbO₂ to be 77.5 ± 0.2%, which is similar to the skin HbO₂ percentage in healthy Caucasian subjects of 78.2 ± 0.2% (30). In contrast, the percentage of skin HbO₂ in patients was significantly less at 61.0 ± 0.2% (P < 0.001).

Fig. 1. Linear relationship between skin tissue hemoglobin oxygen saturation (HbO₂) and underlying tissue oxygen saturation for sickle cell disease patients. The correlation represents averaged data for patients breathing room air (1), during acetylcholine (ACh) infusion (2), during N⁵-monomethyl-L-arginine (l-NMMA) infusion (3), inhaling nitric oxide (NO) (4), and during l-NMMA infusion while continuing to inhale NO (5). The skin tissue oxygen saturation of hemoglobin was determined to be linearly related to the oxygen saturation of venous blood sampled from the underlying tissue by standard techniques, where y = 0.23x + 44.89 (R = 0.94, P = 0.007). This correlation was determined by using the mean values for the patients during each experimental treatment. The circles delineate the error in the venous blood oxygen saturation; the height of the measurement point reflects the error in skin tissue HbO₂.
0.2% \((P < 0.001)\), despite no increase in forearm blood flow \((6.9 \pm 1.2 \text{ ml/min}^{-1} \cdot 100 \text{ ml tissue}^{-1})\). Continued inhalation of NO while L-NMMA was infused for an additional 5 min reduced blood flow to \(4.9 \pm 1.0 \text{ ml/min}^{-1} \cdot 100 \text{ ml tissue}^{-1}\) \((P = 0.003)\), as well as reduced the percentage of skin \(\text{HbO}_2\) from 63.3 \(\pm 0.2\) to 59.2 \(\pm 0.3\% \((P < 0.001)\). However, the reduction in percentage of skin \(\text{HbO}_2\) as a result of L-NMMA infusion was significantly less during NO breathing than observed during L-NMMA infusion with room air breathing (Fig. 4).

Relation between blood flow and skin tissue oxygen saturation. Forearm blood flow and skin \(\text{HbO}_2\) percentages for patients obtained at rest and during infusions of acetylcholine, sodium nitroprusside, and L-NMMA are plotted in Fig. 5. The relationship between blood flow and skin \(\text{HbO}_2\) was best fit by an exponential function. Of note, the highest skin \(\text{HbO}_2\) values measured in sickle cell disease patients during vasodilation either by acetylcholine \((65.2 \pm 0.2\%\) or by sodium nitroprusside \((63.9 \pm 0.3\%\) were less than those values in healthy African-American subjects at rest \((77.5 \pm 0.2\%, P < 0.001\) vs. patient values). This apparent below-normal limit in skin tissue oxygen saturation even at seven times the normal blood flow was also indicated by the gentle slope of the linear relationship for oxygen saturation values for skin and for underlying tissue, as shown in Fig. 1.

Hyperspectral imaging of small vessels. An area in the thenar region of the palm with visible blood vessels, represented by the white dotted square on the hyperspectral image in Fig. 6A, was examined while patients were at rest and during infusion of acetylcholine (Fig. 6B, i–ii). Line profiles were determined by averaging the percentages of skin \(\text{HbO}_2\) within the image and plotting these values as a function of pixel position (Fig. 6C). These images display the percentages of skin \(\text{HbO}_2\) within two distinct vessels (Fig. 6C,

**Fig. 3.** Visible reflectance hyperspectral images and quantitative assessment of sickle cell disease patients. A: hyperspectral imaging for patient 5. Ach, acetylcholine; SNP, sodium nitroprusside. B: comparisons of blood flow. C: skin \(\text{HbO}_2\) percentage measurements for Ach and SNP infusions.

**Fig. 4.** Time-resolved visible-reflectance hyperspectral image analysis. The percentage of skin \(\text{HbO}_2\) measured during NO inhibition (continuous L-NMMA infusion) while sickle cell disease patients either breathed ambient air or inhaled NO is shown. *Time at which the values while inhaling NO gas became distinguished from breathing ambient air \((P < 0.001)\).

**Fig. 5.** Empirical relationship between the percentage of skin \(\text{HbO}_2\) and forearm blood flow for sickle cell disease patients. Data were averaged while subjects breathed room air (1), were infused with Ach (2), SNP (3), or L-NMMA (4), inhaled NO (6), or were infused with L-NMMA while continuing to inhale NO (6). Blood flow was found to be an exponential function of percentage of skin \(\text{HbO}_2\), where \(y = \ln[1.21 \times 10^2x - 7 \times 10^2]\) \((R = 0.97, P < 0.001)\).
subject levels, despite their forearm blood vessel (Fig. 6 features were veins, possibly bifurcating from a single surrounding skin tissue, suggesting that these structures and lower than the microvasculature of the skin microcirculatory HbO2 values well below healthy patients at rest and breathing ambient air exhibited relationship of skin tissue oxygen saturation to forearm blood flow.

**DISCUSSION**

We demonstrate the use of noninvasive, visible reflectance hyperspectral imaging to determine both the spatial distribution of skin tissue oxygen saturation as a measure of the adequacy of tissue perfusion and the relationship of skin tissue oxygen saturation to forearm blood flow in patients with sickle cell disease. Patients at rest and breathing ambient air exhibited skin microcirculatory HbO2 values well below healthy subject levels, despite their forearm blood flows being twice those of healthy African-American subjects. These data are consistent with the observation that tissues and organ systems in sickle cell disease are subject to hypoxia despite high overall blood flow because of diminished oxygen transport, which is due to reduced hemoglobin, and vascular shunting arising from chronic microvascular occlusions (9, 25). Although the percentages of HbO2 of the skin and the deeper tissue (Fig. 1) showed linearity, the dependence between skin blood flow and total blood flow may not be linear as a consequence of shunts resulting from HbS polymerization leading to microvascular occlusions.

The infusion of acetycholine, an endothelium-dependent vasodilator, stimulates the release of relaxant factors, including NO (15). Furthermore, recent work (8) suggests that acetycholine increases endothelium-derived relaxing factors such as NO, which accounts for 40% of the induced blood flow and for the upregulation of non-NO vasodilators such as prostacyclin and endothelium-derived hyperpolarizing factor. By contrast, sodium nitroprusside, an endothelium-independent vasodilator, is a direct NO donor. Both acetycholine and sodium nitroprusside increased forearm blood flows and skin tissue oxygenation in patients with sickle cell disease, indicating a preserved capacity of the endothelium to release NO and of the vasculature to respond to NO. Despite the two- to threefold increases in blood flow with NO stimulation or delivery, the associated increases in the skin microcirculatory hemoglobin oxygen saturation were far below the levels determined in healthy African-American subjects at rest. NO gas inhalation by patients increased skin tissue oxygenation even when local synthesis of NO was pharmacologically inhibited. This represents the first observation of a direct effect of NO gas inhalation on skin tissue oxygenation in sickle cell patients. Our laboratory reported previously (30) that, for a normal healthy subject population, NO inhalation had no significant effect on either blood flow or skin tissue hemoglobin oxygen saturation. When the endogenously produced NO was blocked with l-NMMA, however, NO inhalation returned skin tissue HbO2 saturation toward basal levels, as noted in this study with sickle cell disease patients. For the sickle cell patients, however, the HbO2 saturation values remained well below those of healthy subjects.

Of potential mechanisms for explaining the effect of NO gas on tissue perfusion and skin tissue oxygen saturation, one may be that NO gas is peripherally delivered as nitrite, iron-nitrosyl-hemoglobin, or as a plasma or red blood cell S-nitrosothiol (5). Because NO gas can survive in the vasculature in erythrocyte-free zones for seconds, direct action of the NO radical may be involved (23, 26). Although no net effect of inhaled NO on blood flow was determined in patients, improved tissue oxygenation was observed by reflectance spectroscopy imaging. This may indicate that sickle cell disease patients inhaling NO have an increased NO bioavailability at the tissue level. A possible contributing factor to the increased oxygen saturation, in the blood, may be due to a reduction in tissue oxygen consumption through a NO-dependent mechanism in vitro, which has been reported previously (3, 19). Another contributing factor might be the dilation of constricted microvessels, as shown in Fig. 6, with diminished shunting and improved perfusion of skin. Such changes in skin blood flow may have been too subtle for detection by venous occlusion plethysmography. In
healthy resting subjects, blood flowing through the skin, which is primarily controlled by the sympathetic nervous system, has been found to be 8.5% of the total blood flow, whereas in diseased states 10–20% of the cardiac output may be diverted to the skin (10). Furthermore, patients with sickle cell disease have been recently observed to have high circulating plasma levels of cell-free hemoglobin released during chronic hemolysis. Because hemoglobin scavenges NO at near diffusion-limited reaction rates, NO-dependent vasodilation is impaired (24). Inhaled NO gas will react in the lung vasculature with plasma hemoglobin to oxidize it to methemoglobin, thus eliminating the ability of hemoglobin to inactivate NO or to carry oxygen in the peripheral circulation (8, 24).

We have shown that visible reflectance spectroscopic imaging allows the selection of an area of vascular interest for determining, in real time, skin tissue oxygen saturation values, which in sickle cell disease may be nonlinearly dependent on blood flow. Studies have shown empirical evidence to suggest the adhesion of HbS red blood cells to the vascular endothelium correlates with the severity and pain of sickle cell disease (4). Digital enlargement of hyperspectral images shows the microvasculature at the basal condition to be lower in percentage of HbO2 than the surrounding tissue, which may be a result of HbS red blood cells adhering with the endothelium of the vasculature. Furthermore, the dynamic nature of the disease is demonstrated by which may be a result of HbS red blood cells adhering to the vascular endothelium correlates with the severity and pain of sickle cell disease (4). Digital enlargement of hyperspectral images shows the microvasculature at the basal condition to be lower in percentage of HbO2 than the surrounding tissue, whereas other vessels become equivalent. The visible reflectance hyperspectral imaging method clearly has potential for determining oxygen saturation of hemoglobin percentages either within specific vessels or averaged over tissue regions of interest.

In summary, patients with sickle cell disease at rest exhibit impaired skin tissue oxygen saturation despite having resting blood flow values that are twofold higher than those of healthy African-American subjects. Furthermore, when blood flow is pharmacologically increased by sevenfold, skin tissue oxygen saturation was improved, although it still remained well below healthy subject values. NO stimulation or administration improved, but did not normalize, skin tissue oxygen saturation. Finally, because visible reflectance hyperspectral imaging can determine skin tissue oxygen saturation independent of blood flow in patients with sickle cell disease, the technique may provide a novel approach for assessing disease severity and disease progression.

REFERENCES