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

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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.—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 ± 2.9 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 2.3 ± 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 ARTERIOsplenic VARIOUS ORGANS IS IMPAIRED AND RESULTS IN ischemia and infarction (7, 14). ALTHOUGH THE ROLEMOLOGICAL 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 vasocclusive 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.

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that stimulates the release of NO from the endothe-
lum, sodium nitroprusside, a direct NO donor and
endothelium-independent vasodilator, and \( \text{NG-mono-
methyl-L-arginine (L-NMMA), an inhibitor of NO syn-
}

thase. We then administered NO gas at a dose of 80
parts/million, a procedure that is currently under eval-
uation 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 hemoglo-
in 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 hemo-
globin 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 par-
ticipants 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 ac-
quired image provides a value for an average hemoglobin
oxygen saturation (HbO\(_2\)) from a volume of skin tissue.
Because hemoglobin provides the spectroscopic image con-
trast, 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 pene-
trates 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 HbO\(_2\) and deoxyhemoglobin.

The visible reflectance hyperspectral imaging system,
which has been described in detail (31), consists of a liquid
crystal tunable filter (Cambridge Research & Instrumenta-
tion), 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 \(\sim 22^\circ\)C
in a 645- to 520-nm wavelength region. After baseline mea-
surements of the percentages of skin HbO\(_2\) in all study partici-
pants, infusions of acetycholine 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 contin-
uously administered. The stabilization periods allowed blood
doses to return to basal values. After 3 min of continuously
infusion of either acetycholine or sodium nitroprusside, hy-
perspectral 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 HbO\(_2\) and forearm blood flows were
recorded, and L-NMMA was reintroduced.

**Statistical analysis.** To describe the independently mea-
sured values from the subjects, data are presented as
means \(\pm\) SE. A paired \(t\)-test determined 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 non-directional, 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 be-
tween skin tissue HbO\(_2\) determined by the visible re-
fectance imaging measurements and oxygen satura-
tion of venous blood sampled from a 20-gauge catheter
placed into the antecubital vein. This correlation dem-
strates that HbO\(_2\) in the microcirculation of the
perfused areas of the skin is linearly related to the

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Effects of NO stimulation on blood flow and skin tissue oxygen saturation. Infusion of acetycholine 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 acetycholine 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 acetycholine 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 ±

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).
0.2% (P = 0.001), despite no increase in forearm blood flow (6.9 ± 1.2 ml·min⁻¹·100 ml tissue⁻¹). Continued inhalation of NO while L-NMMA was infused for an additional 5 min reduced blood flow to 4.9 ± 1.0 ml·min⁻¹·100 ml tissue⁻¹ (P = 0.003), as well as reduced the percentage of skin HbO₂ from 63.3 ± 0.2 to 59.2 ± 0.3% (P < 0.001). However, the reduction in percentage of skin HbO₂ 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 HbO₂ percentages for patients obtained at rest and during infusions of acetycholine, sodium nitroprusside, and L-NMMA are plotted in Fig. 5. The relationship between blood flow and skin HbO₂ was best fit by an exponential function. Of note, the highest skin HbO₂ values measured in sickle cell disease patients during vasodilation either by acetycholine (65.2 ± 0.2%) or by sodium nitroprusside (63.9 ± 0.3%) were less than those values in healthy African-American subjects at rest (77.5 ± 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 acetycholine (Fig. 6B, i–ii). Line profiles were determined by averaging the percentages of skin HbO₂ within the image and plotting these values as a function of pixel position (Fig. 6C). These images display the percentages of skin HbO₂ within two distinct vessels (Fig. 6C).
subject levels, despite their forearm blood flow, 
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 arm blood flow in patients with sickle cell disease.

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 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

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) show 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.

Fig. 6. Digitally enlarged area from the thenar region of the visible reflectance hyperspectral images. An area of vascular interest in a patient is chosen from a hyperspectral image (A), as depicted by the white dotted square within the thenar region of palm at rest (B, i) and during ACh infusion (B, ii). This image reveals a small bifurcating vein (1 and 2) with surrounding skin tissue. C: line profiles show the percentage of skin HbO2 as a function of pixel position across the region of interest.

J and 2) of ~1 mm in width. During basal conditions, the percentage of skin HbO2 was similar for the two vessels and lower than the microvasculature of the surrounding skin tissue, suggesting that these structures were veins, possibly bifurcating from a single vessel (Fig. 6C, i). During the acetycholine infusion (Fig. 6C, ii), skin HbO2 increased within the region of interest as a whole and in both vessels, thus imaging the dynamic vasococlusive nature of sickle cell disease.

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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 vascucature. Furthermore, the dynamic nature of the disease is demonstrated by the infusion of acetylcholine as some of the microvasculature perfusing the skin tissue continues 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.

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