Noninvasive Determination of Spatially Resolved and Time-Resolved Tissue Perfusion in Humans During Nitric Oxide Inhibition and Inhalation by Use of a Visible-Reflectance Hyperspectral Imaging Technique

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Background—Vascular disease is commonly associated with reduced local synthesis of nitric oxide (NO) and impaired tissue perfusion. We introduce a novel noninvasive, visible-reflectance, hyperspectral imaging technique for quantifying the percentage of hemoglobin existing as oxyhemoglobin (HbO₂) as an index of skin tissue perfusion.

Methods and Results—To simulate vascular endothelial dysfunction, N⁷-monomethyl-L-arginine (L-NMMA) was infused into the brachial arteries of 9 healthy subjects for 5 minutes to inhibit forearm NO synthesis, first with the subject breathing room air and subsequently during NO inhalation at 80 ppm for 1 hour. Blood flow was measured by venous occlusion plethysmography, and the percentage of HbO₂ perfusing skin tissue was imaged noninvasively with a visible-reflectance hyperspectral technique. L-NMMA reduced blood flow by 31.7±4.9% and percentage of HbO₂ by 6.5±0.1 (P=0.002 and P<0.001 versus baseline, respectively). With subjects inhaling NO, blood flow fell during L-NMMA infusion by only 10.9±7.3%, and the percentage of HbO₂ decreased by 3.6±0.1 (P=0.007 and P<0.001, respectively, versus room air L-NMMA responses).

Conclusions—Visible-reflectance hyperspectral imaging demonstrates (1) a significant decline in the percentage of HbO₂ in skin tissue when blood flow is reduced after inhibition of forearm NO synthesis and (2) restoration of HbO₂ toward basal values with improved blood flow during inhalation of NO. This imaging method may provide an effective approach for time-resolved noninvasive monitoring of skin hemoglobin oxygen saturation and assessment of responses to therapeutic interventions in patients with vascular disease. (Circulation. 2001;104:2905-2910.)

Key Words: peripheral vascular disease ■ blood flow ■ nitric oxide ■ hemoglobin ■ imaging

More than 10 million Americans have diabetes mellitus, an important risk factor for cardiovascular disease.¹,² In view of the morbidity and mortality associated with this condition, methods of early detection of vascular disease are needed to initiate appropriate treatment that might lead to an increased life expectancy and enhanced quality of life. Vascular endothelial dysfunction is common in type I and type II diabetes³,⁴ and may result in vasoconstriction because of loss of endogenous synthesis of the vasodilating molecule nitric oxide (NO).³ This, in turn, may compromise blood flow to the extremities of patients with diabetes and other diseases associated with vascular dysfunction, resulting in reduced tissue oxygenation and potentially leading to ulceration, infection, and loss of limb. An impediment to the appropriate clinical management of patients with peripheral arterial disease is the inability to monitor tissue perfusion noninvasively over time. We introduce a novel, noninvasive, visible-reflectance, hyperspectral imaging technique for assessing vascular endothelial dysfunction and its associated reduction in tissue hemoglobin oxygen saturation.

Hemoglobin oxygen saturation may be measured by oximeters, although these devices are somewhat limited in usage. For example, a 2-wavelength transmission device restricts measurements to a single point, by which light is passed through either the finger or earlobe.³ For application to other parts of the body (as, for example, the chest, forehead, or limbs), a transcutaneous reflectance oximeter was developed and further adapted to a 5-wavelength spectral range.⁷,⁸ This particular class of oximeters also introduced additional optical methods for clinically measuring changes in tissue oxygen saturation in patients.⁹–¹⁴ Recent developments in focal-plane-array detectors for spectroscopic imaging and in the incorporation of improved charge-coupled device (CCD) detectors, along with the im-
plementation of liquid crystal tunable filters for spectroscopic wavelength access, support the feasibility of noninvasive hyperspectral imaging approaches in humans. In particular, the visible-reflectance imaging modality allows spatially relevant spectroscopic data to be recorded easily and rapidly from human subjects. With present-day computational power allowing multivariate spectrometric image analyses to be completed within a relatively short period of time, an effective and highly adaptable instrument may be designed with the potential for monitoring broad aspects of tissue perfusion. The hyperspectral technique measures spectral changes within the visible spectrum of light and provides information on the molecular state of hemoglobin; in contrast, Doppler imaging measures ultrasound frequency shifts or wavelength shifts in the case of laser-Doppler methods.

Methods

Instrumentation
We applied visible-reflectance hyperspectral imaging for the purpose of quantifying the percentage of oxyhemoglobin (HbO₂) in tissue. The instrument measures noninvasively, and in real time, visible-reflectance radiation in vivo; the resulting data are presented as a hyperspectral image cube consisting of 2 spatial dimensions and 1 spectral dimension (Figure 1a). The relative contributions of HbO₂ are extracted from the experimentally observed reference spectra in the 520- to 640-nm wavelength region, an interval exhibiting a convolution of the HbO₂ and deoxyhemoglobin (deoxy-Hb) spectral signals (Figure 1b). Specifically, the recorded reflectance spectrum for each detector pixel is deconvolved by a multivariate least-squares method to determine the best linear combination of HbO₂ and deoxy-Hb reference spectra. This imaging system is capable of a peak spectral discrimination of 0.25 nm and a detection of a 0.02% change in spectral peak reflectance over time, instrumental characteristics that allow a sensitive, noninvasive, real-time evaluation of tissue oxygenation. From the measurement of changes in percentage of HbO₂, physiological insight into blood flow, metabolism, and blood constituent saturation may be possible.

Spectroscopic Determination of Percentage of HbO₂
To determine the feasibility of the visible-reflectance hyperspectral imaging system for determining real-time changes in the percentage of HbO₂, vascular dysfunction and its treatment were simulated by regional NO synthase (NOS) inhibition, followed by NO inhalation. A quartz tungsten halogen light source illuminates the subject via collimating optics and liquid light guides (Oriel Instruments) (Figure 1a). A mirror guides the diffuse sample reflectance to a liquid crystal tunable filter (Cambridge Research and Instrumentation) and lens (Nikon), which focuses the spectrally filtered image onto the CCD detector (Roper Scientific). A computer program run on an Optiplex GXIP computer (Dell) manages the data acquisition by tuning the liquid crystal tunable filter, triggering the CCD detector, and writing the data to disk.

The measured reflectance spectra are quantified in terms of apparent absorbance, which is a ratio of the reflected sample radiation to the reflected radiation from a certified standard (SRT-99-120; Labsphere). These data are formatted as a hyperspectral image cube, which defines a series of images at multiple contiguous wavelengths representative of narrow spectral bandwidths. Spectra obtained at each pixel of the image cube are deconvolved by a multivariate least-squares regression analysis. Reference spectra of a 100% HbO₂ and deoxy-Hb, used to deconvolve a measured spectrum at each pixel, were prepared with standard methods using blood collected from a healthy, nonsmoking individual.

Seven milliliters of hemolysates was mixed with 30 mg of sodium dithionite to yield the pure deoxy-Hb sample (Figure 1b). The HbO₂ spectrum displays distinct peaks at 541 and 576 nm, whereas the pure deoxy-Hb displays a single peak at 555 nm.

Study Population
The study group consisted of 9 nonsmoking healthy volunteer subjects (5 men and 4 women) with an average age of 32±4 years. Each subject was screened by clinical history, physical examination, ECG, and routine laboratory analyses and had no evidence of cardiovascular disease or other systemic condition. The Institutional Review Board of the National Heart, Lung, and Blood Institute approved this study, and all participants provided written, informed consent for all procedures.

Protocol
Subjects fasted overnight and refrained from drinking alcohol and beverages containing caffeine for ≥12 hours. All studies were conducted in the morning in a quiet room set at a temperature of ~22°C. While the subject breathed room air, an infusion of 5% dextrose in water (D₅W) was started for 20 minutes, after which baseline measures were acquired, followed by switching the D5W infusion for Nω-monomethyl-L-arginine (L-NMMA). Subsequently, NO was inhaled for 120 minutes, and D5W was infused for the last 20 minutes of this period. While the subjects continued to inhale NO, baseline measures were acquired, and then the D5W infusion was switched to L-NMMA.

Forearm blood flow was measured by venous occlusion plethysmography (Hokanson). Briefly, this method occludes the venous
drainage of the forearm. From the rate of increase in the blood volume, the proportional arterial blood flow is determined. Hyper-spectral and blood flow measurements were made at baseline during infusion of D5W at 1 mL/min into the brachial artery. Vascular dysfunction was simulated pharmacologically by infusion of the NOS inhibitor L-NMMA at 4 to 8 μmol/min into the brachial artery to inhibit NO synthesis and reduce forearm blood flow.29 We recently reported that L-NMMA infused into the brachial artery of healthy subjects reduces not only forearm blood flow but also antecubital venous P02 and pH, consistent with reduced tissue oxygenation and acidosis.30 Hyperspectral measurements were obtained continuously during the L-NMMA infusion, and blood measurements were performed after 5 minutes of L-NMMA infusion.

Treatment of vascular dysfunction was simulated by the inhalation of NO in the presence of NOS inhibition by a repeat infusion of L-NMMA. NO was delivered at 80 ppm via an anesthesia face mask with a reservoir bag (FIO2 =0.21) with the Ohmeda NO delivery system (Datech-Ohmeda) with intra-arterial infusion of D5W at 1 mL/min. After 1 hour of NO inhalation, hyperspectral and blood flow measurements were made. With continuation of NO breathing, the L-NMMA infusion was repeated, during which time hyperspectral images were collected, and after 5 minutes, blood flow measurements were acquired.

Statistical Analysis
Differences between population means were compared by a 2-tailed, paired Student’s t test. Percentages of HbO2 image data were determined from a multivariate least-squares deconvolution of the hyperspectral data cube: a sampling of 1089 binned detector pixels was used to define a 5-cm2 rectangular area within the palm of the hand. All data are reported as mean±SEM.

Results
Effects of NOS Inhibition Before and During NO Inhalation
Basal forearm blood flow during room air breathing was 2.8±0.3 mL·min⁻¹·100 mL tissue⁻¹. Infusion into the brachial artery of L-NMMA for 5 minutes reduced blood flow to 1.8±0.2 mL·min⁻¹·100 mL tissue⁻¹ (P=0.002 versus baseline).

After 1 hour of NO inhalation, basal forearm blood flow was 2.5±0.4 mL·min⁻¹·100 mL tissue⁻¹. Continuation of NO inhalation and reinfusion of L-NMMA at the same dosage as used during room air breathing resulted in a blood flow of 2.1±0.3 mL·min⁻¹·100 mL tissue⁻¹, a reduction of only 0.3±0.2 mL·min⁻¹·100 mL tissue⁻¹ (P=0.101 versus baseline). This reduction in blood flow from baseline in response to L-NMMA infusion during NO breathing was significantly less than the L-NMMA-induced vasoconstriction with the subject breathing room air (Figure 2).

The basal percentage of HbO2 measured by the visible-reflectance hyperspectral imaging technique with subjects breathing room air was 79.8±0.1. Infusion of L-NMMA with the subject breathing room air decreased the percentage of HbO2 to 73.3±0.1, a reduction of 6.5±0.1 (P<0.001 versus baseline). The basal percentage of HbO2 with the subject inhaling NO was 79.7±0.1. L-NMMA infusion during continued NO inhalation reduced the percentage of HbO2 to 76.1±0.1, a reduction of 3.6±0.1 (P<0.001 versus baseline). The magnitude of the L-NMMA effect on blood flow and the percentage of HbO2 was less during NO inhalation than room air inhalation (Figure 2).

Figure 2. Percent reduction from basal of blood flow and of percentage of HbO2 during L-NMMA infusion with subject inhaling ambient air vs NO.

Time-Resolved Visible, Hyperspectral Measurement of the Percentage of HbO2 During L-NMMA Infusion in the Absence and Presence of NO
For 3 subjects, we determined a time-resolved, hyperspectral measurement that exhibited a stepwise decrease in percentage of HbO2: 78.5±1.5 at 1.5 minutes, 74.7±1.8 at 3.0 minutes, and 72.0±1.9 at 4.5 minutes, during the 5-minute infusion of L-NMMA while the subject breathed room air (Figure 3). During the repeat L-NMMA infusion while subjects inhaled NO, the reduction in percentage of HbO2 was significantly less, with values of 79.0±0.5 at 1.5 minutes, 77.6±1.3 at 3.0 minutes, and 76.8±0.7 at 4.5 minutes.

Hyperspectral Imaging Illustrates Both Spectral and Spatial Capabilities in Noninvasive Monitoring of Skin Tissue Oxygenation
The acquisition of visible-reflectance hyperspectral images for assessing vascular percentage of HbO2 changes during L-NMMA infusion permits actual visualization of the effects of reduced tissue perfusion within skin. For example, the white-light image of the palm of the hand displays only general morphological features (Figure 4a), whereas the digital gray-scale hyperspectral images (Figure 4b and 4c) display the spatial distribution of the percentage of HbO2 across the palm of the hand during different states of skin perfusion. Specifically, the basal hyperspectral image (Figure

Figure 3. Time-resolved visible-reflectance hyperspectral measurements during L-NMMA infusion with subject inhaling ambient air vs NO in 3 of 9 subjects.
4b) represents the resting state during which the percentages of HbO₂ across the hand are determined to be at normal levels, with HbO₂ being distributed relatively homogeneously. The brighter intensity of a given pixel indicates an increased percentage of HbO₂. As an example of the determination of spectra from a given spatially resolved domain in a subject’s hand, spectroscopic data from 10 detector pixels were averaged, yielding predominantly oxyhemoglobin, as indicated by spectrum 1 and by the overall bright pixel intensities (Figure 4b). After the infusion of L-NMMA, the image again displays the percentage of HbO₂ as a spatial variable (Figure 4c). This is now visualized more dramatically as an inhomogeneous distribution of pixel intensities, which is confirmed by the visible spectra. Spectrum 2 (Figure 4c), which was collected from a darker region, is seen to have a low percentage of HbO₂ (and a high percentage of deoxy-Hb) compared with spectra 3 and 4, which were collected from brighter pixel regions and exhibit increases in percentage of HbO₂.

The set of time-resolved images (Figure 5) demonstrates the effects of L-NMMA infusion on skin HbO₂ as a function of time. The overall intensity of the hand at baseline while the subject breathed room air (Figure 5, 1a) indicates greater percentages of HbO₂ than while the L-NMMA was infused into the forearm for 4.5 minutes (Figure 5, 1d). This image exhibits darker pixels and indicates diminished percentages of HbO₂. The decrease in image intensity (Figure 5, 1a through 1d) translates to the 6.5 ± 0.1 reduction in percentage of HbO₂ determined by hyperspectral imaging from a basal level and is associated with the 31.7 ± 4.9% reduction in blood flow, as measured by venous occlusion plethysmography, in which the reduction in blood flow results from inhibition of endothelial synthesis of NO by L-NMMA.

The overall pixel intensity of the hand during NO inhalation at baseline (Figure 5, 2a) is slightly brighter than the hand infused with L-NMMA for 4.5 minutes during NO inhalation (Figure 5, 2d). The reflectance hyperspectral data agree with the measured 3.6 ± 0.1 reduction in percentage of HbO₂ associated with a 10.9 ± 7.3% reduction in forearm blood flow (Figure 2). The gray-scale image obtained while L-NMMA was infused and the subject inhaled NO (Figure 5, 2d) is also brighter than the image of the hand during L-NMMA infusion with the subject breathing room air (Figure 5, 1d).

The time progression and spatial resolution of HbO₂ from the effects of L-NMMA infusion with the subject breathing room air (Figure 5, 1b through 1d) is further elucidated by a grid overlaid on the image of the hand (Figure 6a), which provides a spatial reference for tracking the changes in
percentage of HbO$_2$ over time. Each square, numbered sequentially (Figure 6b) within the grid, contains 100 pixels (10×10). The averages of specific grid locations are plotted as a function of time in Figure 6c.

**Discussion**

We report the capability of a hyperspectral imaging device to determine, noninvasively and in real time, reduction and improvement in tissue oxygenation. The basis of the hyperspectral system is a liquid crystal tunable filter, a high-fidelity image quality spectral band-pass discriminator that is continuously tunable over a spectral range from 500 to 750 nm and is able to discriminate between spectral peaks 0.25 nm apart. This provides the capability for ultimately differentiating among a set of chemical species, such as HbO$_2$, deoxy-Hb, methemoglobin, carboxyhemoglobin, nitrosohemoglobin, and myoglobin, as functions of their visible-reflectance spectra. The CCD detector, with its format of 768 by 512 detector pixels, is capable of registering 393,216 simultaneously acquired spectra over the sample field of view. Thus, in addition to assessing and quantifying the concentration of a specific chemical species, such as HbO$_2$ and deoxy-Hb, hyperspectral imaging provides representations of the spatial distribution of a molecular species at a given time or over time. Accordingly, acquisition of a time-resolved profile representing the spatial dynamics of physiologically important molecules indicative of tissue perfusion could prove useful in assessing the efficacy of a given therapeutic intervention on vascular disease.

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**References**


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