Human respiratory muscle blood flow measured by near-infrared spectroscopy and indocyanine green

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Human respiratory muscle blood flow (RMBF) is an indicator of respiratory muscles' metabolic and oxygen demands, which are closely related to the work of breathing. Previous studies have used invasive methods to measure RMBF in anesthetized subjects, but these methods are not feasible in awake humans. Near-infrared spectroscopy (NIRS) has been used to measure RMBF in awake humans, but its accuracy has been questioned.

In this study, the authors report the development of a noninvasive method to measure RMBF in awake humans, which involves the use of indocyanine green (ICG) dye and near-infrared spectroscopy (NIRS). The method was validated against previously established methods and showed high correlation with RMBF values obtained in awake humans.

The results of this study provide a noninvasive method for measuring RMBF in awake humans, which can be used to better understand the metabolic and oxygen demands of respiratory muscles during different levels of ventilation. This method has potential applications in clinical settings, such as in the assessment of respiratory muscle function in patients with respiratory illness or during exercise in healthy individuals.
Recent investigations have used NIRS on specific respiratory muscles to determine oxygenation status during exercise (24, 25, 31, 43). However, to our knowledge, there are no published studies that have used NIRS in combination with ICG to estimate RMBF in humans. The ability to measure RMBF in humans has implications for understanding blood flow distribution patterns and circulatory regulation in health and disease. For example, the ability to measure RMBF may provide insight into the “steal” phenomenon, where it is thought that blood is redirected from locomotor muscles to the respiratory muscles during fatiguing diaphragmatic contractions (11, 42). This technique may also help elucidate some of the mechanisms involved in exertional dyspnea and exercise intolerance in patients with heart failure and chronic lung disease.

The purpose of this study was to determine if the NIRS-ICG technique could be applied to quantify regional RMBF in humans as it has previously been used for locomotor muscles during exercise (5, 7). In this study, RMBF of the internal and external intercostals was measured at the seventh intercostal space at the apposition of the costal diaphragm. The intercostal muscles were used because they are easily accessible, they are active across a wide range of ventilations, and because previous studies have shown a linear relationship between intercostal muscle electromyography (EMG) activity and the work of breathing (45, 46). Since limb skeletal muscle blood flow increases in close relation to increasing workload, we hypothesized that intercostal RMBF would also progressively increase with increases in minute ventilation (VE) and would correlate closely with changes in the work of breathing (WOB), transdiaphragmatic pressure (Pdi), and simultaneously measured cardiac output.

METHODS

Experimental design. Five competitive male cyclists performed an incremental cycle test to exhaustion to determine maximal aerobic capacity (VO2max). On a separate testing day, subjects performed three 5-min bouts of isocapnic hyperpnea at ventilations corresponding to approximately 30, 55, and 75% of their previously determined maximum VE. Respiratory and quadriceps muscle blood flow and cardiac output were measured during the final minute of each 5-min hyperpnea bout. All experimental procedures and protocols received institutional approval and conformed to the Declaration of Helsinki. Each subject provided written informed consent before participating in this study. All subjects were healthy and had no history of cardiopulmonary disease or allergies.

VO2max and pulmonary function (day 1). Basic pulmonary function data were obtained in all subjects while seated, utilizing standard protocols as outlined in the American Thoracic Society Guidelines (1). Pulmonary function variables included forced vital capacity (FVC), forced expired volume in 1 s (FEV1.0), and FEV1.0/FVC and were measured using an automated ventilatory analysis system (Vmax 229; Sensor Medics, Anaheim, CA). Subjects then performed an incremental exercise test on an electromagnetically braked cycle ergometer (Ergoline 800; Sensor Medics) starting at 30 W and increasing by 30 W every minute until volitional fatigue. All gas exchange and ventilatory data were obtained on a breath-by-breath basis using the same metabolic cart (Vmax 229; Sensor Medics). Specific variables measured included oxygen consumption (VO2), carbon dioxide production (VCO2), respiratory exchange ratio (RER), VE, tidal volume (VT), and breathing frequency (f). This allowed maximal exercise VE to be measured for each subject.

Isoacapnic hyperpnea (day 2). Subjects were asked to maintain a target VE, and experimenters provided verbal guidance to adjust the rate and depth of their breathing such that the target VE was obtained and held constant to within 5%. Isoacapnia was maintained by having subjects inspire from a Douglas bag (5% CO2, 21% O2, balance N2) that was connected to a two-way nonbreathing valve (model 2700, Hans Rudolph) by a piece of tubing. The flow into the Douglas bag was constant, and subjects breathed the gas mixture at the rate that they demanded.

Subject preparation. Subjects were instrumented with gastric and esophageal balloon catheters (no. 47-9005, Ackrad Laboratory, Cranford, NJ) to measure gastric pressure (PG) and esophageal pressure (Pes) such that Pdi and the WOB could be calculated. Viscous lidocaine hydrochloride (2%) was applied to the nasal and pharyngeal passages to minimize discomfort, and the two balloon tips were positioned in the middle third of the stomach and esophagus, respectively. Balloon catheters were connected to differential pressure transducers (MP-45, ±250 cmH2O; Validyne, Northridge, CA) and calibrated using a custom-made water manometer.

Under local anesthesia (2% lidocaine), two 20-cm-long, 16-gauge catheters (CV-04301, Arrow International, Reading, PA) were inserted percutaneously into the right femoral arterial and vein ~2 cm below the inguinal ligament according to the Seldinger technique. Catheters were positioned in the proximal direction and secured by a 3-0 skin suture and adhesive tape. The femoral catheters were used for arterial and venous blood sampling and for cardiac output and muscle blood flow measurements (see below).

Two sets of NIRS optodes were placed, one on the vastus lateralis of the right leg and the other over the left seventh intercostal space, to measure quadriceps and RMBF, respectively. The NIRS optodes on the seventh intercostal space were positioned by an experienced respiratory physician and secured using double-sided adhesive tape. For safety purposes, subjects were connected to an ECG (Marquette Max, Marquette Hellige) and a digital pulse oximeter (Nonin 8600, Nonin Medical). Data from these two monitors were recorded continuously.

Respiratory variables. The mechanical WOB was determined by ensemble averaging several breaths using a customized software program (LabVIEW software V6.1, National Instruments) to integrate the averaged esophageal pressure-tidal volume loop (32). The WOB was then multiplied by the breathing frequency to obtain the total amount of work done per minute by the respiratory system. The Pdi was determined by taking the difference between gastric and esophageal pressure. The WOB and Pdi were each determined during the final minute at each level of ventilation, at which time cardiac output and respiratory and quadriceps muscle blood flow were also measured.

Cardiac output. Cardiac output was determined by the dye dilution method (12), using known volumes of ICG (range: 0.8–1.2 ml at 5 mg/ml) injected into the right femoral vein followed by a 10-ml flush of isotonic saline. Blood was withdrawn from the femoral artery using an automated pump (Harvard Apparatus) at 20 ml/min through a linear photodensitometer (Pulsion ICG, ViCare Medical) connected to a cardiac output computer (Waters CO-10, Rochester, MN) through a closed-loop, sterile-tubing system. The blood was reinfused into the femoral vein immediately on completion of the measurements. The cardiac output computer was connected to a data-acquisition system (DI-720, Dataq). Data were sampled at 100 Hz and stored on a computer for subsequent analysis. To remove the influence of dye recirculation, the downslopes of the dye concentration curves were linearly extrapolated using a semilogarithmic scale in the conventional manner (12). Cardiac output was calculated as the ratio of ICG mass injected to the mean arterial ICG concentration over the time interval of the curve and expressed as liters per minute. ICG calibration curves were obtained following each experiment by measuring the raw voltage deflection from three 20-ml blood samples containing.
various concentrations of ICG. Calibrations at each concentration were performed two to three times to ensure linearity and consistency.

**NIRS-ICG blood flow.** Dual-channel laser diodes with emitting and receiving optodes were carefully placed over the vastus lateralis of the right leg and on the skin over the left seventh intercostal space. The optode separation distance for both muscles was 4 cm, corresponding to a penetration depth of ~2 cm. The left intercostal space was used to avoid potential blood flow contributions from the liver on the right side of the body. Muscles within view of the intercostal space included primarily the internal and external intercostals. Optodes were connected to a NIRO 300 spectrophotometer (Hamamatsu Photonics KK, Hamamatsu, Japan), which was used to measure ICG concentration following a 4- to 5-mg bolus injection of ICG in the right femoral vein (the very same bolus as used above for cardiac output determination). As previously described (7, 20), the ICG bolus circulates to the right heart and lungs and enters the arterial circulation where arterial blood is then withdrawn by a pump. The arterial ICG concentration is measured by photodensitometry, while in the tissue microcirculation, ICG is detected transcutaneously by measuring light attenuation with NIRS at 775-, 813-, 850-, and 913-nm wavelengths and analyzed using an algorithm incorporating the modified Beer-Lambert law (7, 13, 44). Since the measured light attenuation in the tissue is influenced by ICG and oxy- and deoxyhemoglobin, the independent contribution of ICG to the light-absorption signal was isolated using a matrix operation (MATLAB). The matrix operation incorporates pathlength-specific extinction coefficients for each of the light-absorbing chromophores (hemoglobin + myoglobin and ICG) at each wavelength employed by the NIRS machine (Hamamatsu Photonics KK).

Blood flow was calculated from the rate of tissue ICG accumulation over time measured by NIRS according to the Sapirstein principle (41). Accordingly, for any time interval less than the time to reach peak tissue accumulation of tracer, the tissue receives the same fraction of the ICG bolus as quantified in arterial blood (input function). Two separate time points within the time range of the curve were used to calculate flow, and the average value was taken to represent the tissue ICG accumulation. Therefore total blood flow was calculated using the following equation:

$$\text{blood flow (ml·100 ml}^{-1} \text{· min}^{-1}) = k \cdot \frac{\int_{0}^{t} [ICG]_{\text{dr}}}{[ICG]_{\text{in}} \cdot t}$$

where $k$ is the molecular weight of ICG for the conversion of ICG in moles to grams per liter; $[ICG]_{\text{in}}$ is the accumulation of ICG in tissue over time $t$ expressed in micromoles; and $\int_{0}^{t} [ICG]_{\text{dr}}$ is the time integral of the arterial ICG concentration expressed in milligrams per liter (7). The ICG calibration procedure as described for cardiac output was also used to quantify the input function for calculation of the regional tissue blood flow with NIRS.

**Blood gas analysis.** Femoral arterial blood samples were analyzed using a calibrated ABL 520 system (Radiometer, Copenhagen, Denmark) operating at 37.0°C. This system provided direct measures of P_{O2}, P_{CO2}, saturation, hemoglobin concentration, P_{O2}/P_{CO2}, pH, and whole blood lactate concentration. Samples of 2 ml were collected anaerobically in heparinized syringes, and any bubbles were immediately expressed. Every arterial sample was analyzed within 10 s of collection.

**Statistical analysis.** Linear regression analysis was used to determine if RMBF increased with increasing levels of Ve. To do this, linear regressions relating RMBF and Ve were performed for each of the five subjects. The individual slopes were then compared with zero using paired $t$-tests. The same procedure was done to determine if leg blood flow increased with an increase in Ve. Linear regression analysis was also performed to determine closeness of correlation between selected dependent variables (cardiac output, RMBF, Ve, WOB, and Pdi). The level of significance was set at $P < 0.05$ for all statistical comparisons. All data are presented as means ± SE. We chose SE rather than SD because we are most concerned with precision in identifying mean values. With five subjects, SD, which reflects variance in individual measurements, can be calculated as 2.236 × SE for each variable.

**RESULTS**

**Descriptive characteristics.** Table 1 shows basic physical characteristics and pulmonary function data. Table 2 shows power output, oxyhemoglobin saturation, heart rate, and ventilatory and gas exchange variables from the maximal incremental cycle test performed on day 1. All subjects had normal pulmonary function.

**Hyperpnea.** Baseline Ve (20.2 ± 4.3 l/min) was above normal resting levels because there was a single outlier during resting breathing due to an experimental error. This subject was mistakenly given the CO_{2} mixture to breathe during rest, which elevated his Ve and RMBF. This subject’s resting data were therefore excluded from the analysis because of the confounding influence of hypercapnia on Ve and blood flow. Ve without the outlier was 16.0 ± 1.4 l/min. Resting breathing was 9.9 ± 1.5% of maximal Ve, and the three isocapnic hyperpnea bouts corresponded to 27.1 ± 3.2, 56.0 ± 6.1, and 75.9 ± 5.7% of their maximal Ve as determined on day 1. Absolute Ve at these percentages corresponded to 44.0 ± 2.4, 90.0 ± 3.9, and 123.1 ± 3.6 l/min, respectively. There were no significant differences between the arterial partial pressure of CO_{2} (39.8 ± 2.2, 41.5 ± 1.0, 40.4 ± 0.6, 41.1 ± 0.6, respectively) or pH (7.374 ± 0.009, 7.366 ± 0.005, 7.380 ± 0.006, 7.378 ± 0.004, respectively) during rest and the three hyperpnea bouts.

**Cardiac output and muscle blood flow.** Figure 1 shows cardiac output plotted against Ve at rest and during the three progressively increasing levels of hyperpnea. A strong, positive linear relationship was observed between Ve and cardiac output ($r = 0.993$; cardiac output = 0.0345·Ve + 5.84). Figure 2 shows the respiratory muscle ICG response measured by NIRS in a representative subject. There is a progressive increase in dispersion and peak dye concentration, indicating a faster rate and magnitude of ICG accumulation to the respiratory muscles, reflecting increasing blood flow with higher levels of Ve. Figure 3 shows mean RMBF and also that of the inactive control muscle (vastus lateralis) during rest and isocapnic hyperpnea. The mean slope relating RMBF and Ve was 0.37 ± 0.12, which was significantly different from zero ($P =$
0.02) (see METHODS). This is in contrast to the slope relating leg blood flow and V˙E (0.036 /min), which was not different from zero (P = 0.19), indicating that leg blood flow remained constant throughout the hyperpnea protocol while RMBF increased. Figure 4 shows a strong relationship between RMBF and cardiac output across the four ventilatory conditions (r = 0.994; RMBF /min = 10.804 cardiac output /min 62.35). The WOB plotted against V˙E is shown in Fig. 5, while Fig. 6 demonstrates the nearly perfect linear relationship between RMBF and the WOB (r = 0.998; RMBF = 0.834-Pdi + 2.68). Table 3 shows individual r² and r values relating RMBF with cardiac output, the WOB, and Pdi. Table 3 demonstrates the consistently strong linear relationships and low variability observed between subjects.

DISCUSSION

The present investigation is the first to quantify absolute changes in respiratory muscle perfusion in conscious humans over a wide range of ventilations, from resting to ~120 l/min. Blood flow was quantified using an NIRS-ICG technique that has previously been validated in locomotor muscle during exercise in humans (7). We have demonstrated that RMBF measured by NIRS and the tracer ICG in the region of the seventh intercostal space increases progressively with increased V˙E, while blood flow to an inactive control muscle remains constant. We also found a strong and linear correlation between RMBF and cardiac output (r = 0.994), the WOB (r = 0.995), and Pdi (r = 0.998). These results indicate that the NIRS-ICG technique has potential to advance our understanding of human respiratory muscle circulation, particularly during exercise.

Alternative approaches to measuring respiratory muscle blood flow. Traditional techniques for assessing human RMBF are capable of measuring both instantaneous and average blood flow. Instantaneous diaphragm blood flow can be assessed by recording blood-drop count rates from the cannulated left inferior phrenic vein (3) or by using electromagnetic and ultrasonic Doppler flowmeters to measure blood flow in arteries supplying the diaphragm (17, 30). Alternatively, average

![Fig. 1. Relationship between cardiac output and minute ventilation (V˙E). Values are means ± SE.](image)

![Fig. 2. Respiratory muscle indocyanine green dye (ICG) response measured by near-infrared spectroscopy (NIRS) at 4 different ventilatory loads in a single representative subject. There is a progressive increase in slope and peak ICG concentration with increasing levels of V˙E, indicating a faster rate of dye accumulation and therefore a higher blood flow response. Actual blood flow values in this subject at 20, 42, 85, and 121 l/min were 10.2, 23.5, 32.9, and 76.0 ml·100 ml⁻¹·min⁻¹, respectively.](image)

![Fig. 3. Group mean blood flow responses at the 7th intercostal space and vastus lateralis. Respiratory muscle blood flow (RMBF) significantly increases from rest while leg blood flow remains constant during isocapnic hyperpnea. Values are means ± SE.](image)

Table 2. Maximal incremental exercise data on day 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaO2, %</td>
<td>89±3</td>
</tr>
<tr>
<td>V˙E, l/min</td>
<td>165.6±12.3</td>
</tr>
<tr>
<td>VT, liters</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>fb, breaths/min</td>
<td>54.6±5.0</td>
</tr>
<tr>
<td>V˙O2, l/min</td>
<td>4.6±0.3</td>
</tr>
<tr>
<td>V˙O2, ml·kg⁻¹·min⁻¹</td>
<td>62.8±5.4</td>
</tr>
<tr>
<td>V˙CO2, l/min</td>
<td>5.6±0.2</td>
</tr>
<tr>
<td>RER</td>
<td>1.20±0.03</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>184±4</td>
</tr>
<tr>
<td>Power, W</td>
<td>372±9</td>
</tr>
</tbody>
</table>

Values are means ± SE. SaO2, arterial oxyhemoglobin saturation; V˙E, minute ventilation; VT, tidal volume; fb, breathing frequency; V˙O2, oxygen consumption; V˙CO2, carbon dioxide production; RER, respiratory exchange ratio; HR, heart rate.
blood flow can be measured using the inert radioactive-labeled tracer gas method (37) or by trapping radioactive-labeled microspheres in the microcirculation (36). Because of the invasive nature of these methods, the vast majority of studies examining RMBF have been conducted in animals.

**Validation of the NIRS-ICG technique.** As previously described, the NIRS-ICG technique has been validated for cerebral blood flow (9, 22, 33, 35) and skeletal muscle blood flow (7) measurements. On the basis of the Fick principle, Roberts et al. (35) used the NIRS-ICG technique to validate cerebral blood flow against the microsphere method in piglets undergoing cardiopulmonary bypass. Cerebral blood flow values from the NIRS-ICG technique were strongly correlated with the microsphere method. Within each animal, the linear least-squares method gave values of \( r^2 \) in the range of 0.91–0.99, but the slopes of these fits were variable and ranged from 0.5 to 1.8. These findings suggest that the NIRS-ICG technique provides a good measure of relative changes in cerebral blood flow, but individual variability exists compared with the microsphere method where blood flow is quantified by cumulative deposition of microspheres in tissue over time.

For validation of skeletal muscle blood flow, Boushel et al. (7) compared the NIRS-ICG technique against the conventional “gold standard” of limb dye dilution with arterial blood sampling to quantify ICG by photodensitometry. Normalization of unit 100 ml tissue flow was then done by precise measures of muscle mass by MRI scans. A Bland-Altman plot showed a mean difference between methods of only 5 ml·100 ml·min\(^{-1}\) with a 95% confidence interval for the bias of −10 to +20 ml·100 ml·min\(^{-1}\) for flow rates ranging from resting to peak plantar flexion exercise up to 75 ml·100 ml·min\(^{-1}\). Thus the NIRS-ICG technique is in good agreement with the Fick method; however, variability does exist, which results from several factors such as regional differences in blood flow within muscle (the Fick method assumes even distribution of flow for all muscle regions, whereas the NIRS-ICG technique measures only superficial muscle regions). For blood flow validation for tendon regions, they used the standard \(^{133}\)Xe washout technique. The mean difference between the \(^{133}\)Xe washout and the NIRS-ICG technique compared across all subjects and exercise loads was 0.41 ml·100 ml·min\(^{-1}\), and the 95% confidence interval for the bias was −5.1 to +5.4 ml·100 ml·min\(^{-1}\). Taken together, the method has been well validated and is based on sound tracer theory. However, directly validating this technique for human...
It is important to recognize that we were only accessing a portion of the respiratory muscles that were involved in generating the required ventilation. Despite the linearity of RMBF and cardiac output (Fig. 4), the magnitude of intercostal blood flow cannot account for the full magnitude of the increase in cardiac output. Clearly, the numerous other respiratory muscles (i.e., diaphragm, parasternals, sternocleidomastoid, scalenus, abdominal and triangularis sternalis) were also active to varying degrees during the hyperpnea tasks. These muscles will certainly have different perfusion levels, depending on the mass activated, force of contraction, and flow capacity defined by muscle characteristics such as fiber type and capillary density. For example, Poole et al. (34) has shown that the rat intercostal muscles receive a relatively lower portion of the cardiac output compared with other respiratory muscles during maximal treadmill running. Specifically, these authors demonstrated that intercostal blood flow (68 ± 6 ml·min⁻¹·100 g⁻¹) was considerably lower than that of the diaphragm (360 ± 26 ml·min⁻¹·100 g⁻¹), scalenus (152 ± 36 ml·min⁻¹·100 g⁻¹), and abdominal muscles (148 ± 21 ml·min⁻¹·100 g⁻¹). Given this extensive respiratory muscle blood flow heterogeneity that exists, it remains difficult to estimate the mass or volume of the intercostals in the present study because we did not measure blood flow to all of the additional respiratory muscles and because it is not currently possible to measure blood flow to deeper respiratory muscles such as the diaphragm. It would be of interest for future studies to develop methods to quantify

**Table 3. Individual $r^2$ and $r$ values relating RMBF to cardiac output, the work of breathing, and transdiaphragmatic pressure**

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>RMBF vs. CO</th>
<th>RMBF vs. WOB</th>
<th>RMBF vs. Pdi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>$r$</td>
<td>$r^2$</td>
</tr>
<tr>
<td>1</td>
<td>0.884</td>
<td>0.940</td>
<td>0.970</td>
</tr>
<tr>
<td>2</td>
<td>0.795</td>
<td>0.892</td>
<td>0.785</td>
</tr>
<tr>
<td>3</td>
<td>0.829</td>
<td>0.910</td>
<td>0.972</td>
</tr>
<tr>
<td>4</td>
<td>0.983</td>
<td>0.992</td>
<td>0.965</td>
</tr>
<tr>
<td>5</td>
<td>0.860</td>
<td>0.927</td>
<td>0.963</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>0.870±0.032</td>
<td>0.932±0.17</td>
<td>0.931±0.037</td>
</tr>
</tbody>
</table>

Values are means ± SE. RMBF, respiratory muscle blood flow; CO, cardiac output; WOB, work of breathing; Pdi, transdiaphragmatic pressure.
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some of these muscles (particularly the diaphragm) during ventilatory maneuvers and during exercise in humans.

In this study, subjects were seated and performed voluntary hyperpnea up to 76% of maximal VE. On the basis of the VE obtained in these subjects during maximal exercise (Table 2), we extrapolated the mean data from Fig. 3 (RMBF vs. VE) using a quadratic curve-fitting function [RMBF = 9.53 – 0.0219(VE) + 0.0028(VE)^2; r^2 = 0.9988] and determined that RMBF would be ∼83 ml·100 ml^-1·min^-1 at maximal ventilations (i.e., 165.6 l/min). This value is slightly higher than that found for intercostal blood flow in rats (68 ± 6 ml·min^-1·100 g^-1) (34) but lower than intercostal blood flow in ponies (132.1 ± 10.0 ml·min^-1·100 g^-1) (26) during maximal treadmill exercise. Nevertheless, our values are well within normal blood flow ranges based on these animal studies using radionuclide-labeled microspheres, and any discrepancy is likely due to species differences.

**Unique advantages and applications of the NIRS-ICG technique.** The NIRS-ICG technique has the unique ability (with alternative placement of other chest wall optodes) to simultaneously measure blood flow to other superficial muscles such as the scalenus, sternocleidomastoids, parasternals, and abdominals from a single ICG injection. This has the potential to provide insight into RMBF heterogeneity in humans. Furthermore, the NIRS-ICG technique has the ability to measure blood flow and indexes of tissue oxygenation in very close time sequence (within 5–10 s). These factors, coupled with the fact that whole body VO2 and cardiac output can also be measured simultaneously, may provide novel information into the mechanisms regulating respiratory muscle oxygen delivery and utilization under various physiological and pathophysiological conditions. Additional practical advantages of the NIRS-ICG technique are that it is affordable and nontoxic (9).

**Assumptions in using indicator dilution to measure flow.** There are five important assumptions that must be fulfilled when determining the accuracy of NIRS-ICG blood flow values. First, there must be sufficient mixing of the ICG bolus with the blood. This was achieved by injecting the ICG into the peripheral venous circulation to allow mixing during passage through the heart before the blood entered the arterial system. Second, recirculation of ICG affects the dye-concentration curve, which will influence the calculated blood flow values. To account for this, we measured ICG accumulation only during the first half of the tracer transit time (i.e., as the signal was rising). Third, it is important that blood flow remains constant during the measurement period. Steady-state conditions were achieved by having subjects maintain constant levels of ventilation for 3–4 min before the ICG injection. Heart rates were not different across time, and there was never a difference of more than 4 beats/min in a given bout of hyperpnea. This provides additional assurances that steady-state conditions were achieved. Fourth, the tracer dose response must be linear to ensure accurate blood flow calculations. Linearity of the photodensitometry-derived ICG response was established by performing a three-point calibration using known concentrations of ICG thoroughly mixed in measured volumes of each subject’s own blood. Linearity of the NIRS light absorption signal using various concentrations of ICG has previously been established (7). Finally, the ICG should not be retained in the tissue nor metabolized during the measurement (23). As previously described (7), ICG binds to plasma proteins and does not access extravascular tissue spaces in the first pass. Furthermore, there is no measurable hepatic clearance of ICG during the first pass of the bolus. Hepatic parenchymal cells clear ICG at ∼0.8 mg/min in healthy adults once the ICG enters the hepatic circulation (39).

**Limitations of the NIRS-ICG technique.** There are some limitations remaining in the present investigation. For example, blood flow was measured in milliliters of blood per 100 ml of tissue per minute. We have no estimate of respiratory muscle volume, as this would have required magnetic resonance imaging. Future studies characterizing RMBF would be able to determine total blood flow to a particular respiratory muscle if its volume is accurately determined. However, one would have to assume uniform perfusion to a given respiratory muscle. Another consideration is the potential contribution of skin and subcutaneous tissue to the light-absorption signal. Nonmuscular tissue would be expected to result in an underestimation of absolute blood flow values, but this would not influence the relative changes in blood flow observed in the present study. It is also important to consider that all subjects were lean competitive endurance athletes, and therefore nonmuscular contributions would only account for a small portion of the signal. This factor could become more significant in individuals with high subcutaneous adipose deposition and/or when exercise is performed in hot environmental conditions where cutaneous blood flow is elevated.

In the present study, we measured blood flow to the left seventh intercostal space. Previous studies examining respiratory muscle oxygenation with NIRS have used the parasternal muscle (24, 25). We chose to use the seventh intercostal space on the left side of the body because it is located at the apposition of the costal diaphragm such that we would obtain a robust blood flow response. We have used the general term “respiratory muscle blood flow” throughout the manuscript because we are unable to determine “precisely” the relative blood flow contributions of the various muscles located within the field of view of the optodes over the seventh intercostal space, but with a high degree of certainty, the blood flow values presented here reflect predominantly the intercostal muscles.

Another factor that may impose minor imprecision in the absolute values presented here is that the differential pathlength factor (DPF) for the intercostal muscles is not known. Light pathlength is needed to fulfill the components of the Beer-Lambert equation for quantification of the concentration of light-absorbing compounds in the field of view in the selected tissue. Pathlength can only be determined by direct time-of-flight (TOF) measurements or phase-resolved spectroscopy, which was not determined in the present study. We used a DPF of 4.5, which is in close range (4–5) of directly measured pathlengths from other skeletal muscles (10, 13). We are unable to determine the exact impact that the DPF would have on our absolute blood flow values relative to a directly measured pathlength for the intercostal muscles. This potential limitation in the absolute blood flow values would not influence the relative changes in flow or the strong linear relationships observed in the present study.

**Summary.** We have shown that the NIRS-ICG technique is a sensitive tool to assess regional blood flow to the respiratory muscles in humans. RMBF significantly increased with an increase in VE while blood flow to an inactive control muscle remained constant. RMBF was strongly correlated with cardiac output, the WOB, and Pdi. This novel approach to studying...
respiratory muscle circulation has future potential for understanding many factors in cardiorespiratory physiology in both health and disease at rest and during exercise.

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