Changes in whole tissue heme concentration dissociates muscle deoxygenation from muscle oxygen extraction during passive head-up tilt

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Adami A, Koga S, Kondo N, Cannon DT, Kowalchuk JM, Amano T, Rossiter HB. Changes in whole tissue heme concentration dissociates muscle deoxygenation from muscle oxygen extraction during passive head-up tilt. J Appl Physiol 118: 1091–1099, 2015. First published February 12, 2015; doi:10.1152/japplphysiol.00918.2014.—Skeletal muscle deoxygenated hemoglobin and myoglobin concentration ([HHb]), assessed by near-infrared spectroscopy (NIRS), is commonly used as a surrogate of regional O2 extraction (reflecting the O2 delivery-to-consumption ratio, Q˙/V˙O2). However, [HHb] change ([Δ[HHb]]) is also influenced by capillary-venous heme concentration, and/or small blood vessel volume (reflected in total heme; [THb]). We tested the hypotheses that [Δ[HHb]] is associated with O2 extraction, and insensitive to [THb], over a wide range of Q˙/V˙O2 elicited by passive head-up tilt (HUT; 10-min, 15° increments, between −10° and 75°). Steady-state common femoral artery blood flow (FBF) was measured by echo-Doppler, and time-resolved NIRS measured [Hb], and [THb] of vastus lateralis (VL) and gastrocnemius (GS) in 13 men. EMG confirmed muscles were inactive. During HUT in VL [HHb] increased linearly (57 ± 10 to 101 ± 16 μM; P < 0.05 above 15°) and was associated (r² = 0.80) with the reduction in FBF (618 ± 75 ml/min at 0° to 268 ± 52 ml/min at 75°; P < 0.05 above 30°) and the increase in [THb] (228 ± 30 vs. 252 ± 32 μM; P < 0.05 above 15°). GS response was qualitatively similar to VL. However, there was wide variation within and among individuals, such that the overall limits of agreement between [Δ[HHb]] and ΔFBF ranged from −35 to +19% across both muscles. Neither knowledge of tissue O2 saturation nor vascular compliance could appropriately account for the [Δ[HHb]]-ΔFBF dissociation. Thus, under passive tilt, [HHb] is influenced by Q˙/V˙O2, as well as microvascular hematocrit and/or tissue blood vessel volume, complicating its use as a noninvasive surrogate for muscle microvascular O2 extraction.

time-resolved near-infrared spectroscopy; echo-Doppler; blood flow; tissue saturation; vascular compliance

NEAR-INFRARED SPECTROSCOPY (NIRS) provides a noninvasive window on the microvascular and intramuscular oxygenation status of skeletal muscle under a wide range of conditions, at rest or in exercise, in health, and in clinical populations. NIRS measures absorbance of light in the near-infrared range (∼750-950 nm) by tissues ∼1.5–2.0 cm beneath the skin, allowing the relative concentrations of oxygenated (HbO2) and deoxygenated (HHb) heme chromophores to be calculated from the absorbance spectrum. Thus total heme concentration (THb; the sum of HHb and HbO2) and tissue O2 saturation (StO2; the ratio of HbO2 to THb) can be estimated. While NIRS does not discriminate between absorbance by hemoglobin (Hb) or myoglobin (Mb) (7, 32, 45, 46), absorbance in large vessels is high due to high regional tissue [Hb]; this means that NIRS provides information on the oxygenation status within small blood vessels (arterioles, capillaries, and venules), and myocytes, i.e., the site of oxygen exchange. NIRS-derived estimates of muscle deoxygenation are proposed to reflect the oxygen delivery-to-consumption ratio (Q˙/V˙O2) within the NIRS field of view (43).

Often, in skeletal muscle studies HHb, rather than StO2, has been the variable of interest because 1) unlike HbO2, it is relatively insensitive to changes in skin blood volume or flow (12, 17, 23, 26, 35; c.f. 31), and therefore better reflects the deoxygenation status of the muscle under the probe; and 2) changes in HHb are assumed to be localized within the capillary-venous compartment, at least under conditions where change in arterial saturation or Hb concentration is unlikely. Thus, by conceptually replacing venous oxygen concentration in the Fick equation, NIRS-derived ΔHHb measurement is commonly used as a noninvasive surrogate for microvascular O2 extraction (e.g., 6, 8, 9, 13, 17, 28, 34). However, ΔHHb depends on 1) extraction-related mechanisms, or Q˙/V˙O2; and 2) microvascular hematocrit and/or tissue blood-vessel-volume-related mechanisms that influence heme concentration within the interrogated tissue. This latter reflects the fact that heme concentration within the tissue may change with limb blood volume (47), independently of the microvascular heme concentration or hematocrit. Thus ΔHHb is influenced by changes in hematocrit, capillary-venous heme concentration, and/or volume of small blood vessels. Each of these qualifying conditions includes the assumption that [Mb] and arterial oxygen concentration are constant. Thus the valid use of ΔHHb as an index of O2 extraction depends on the assumption that tissue heme concentration is constant (41–44), or that any change in tissue heme concentration is isolated to change in [HbO2].

To challenge these assumptions we used passive incremental head-up tilt (HUT) to redistribute blood volume in the dependent leg muscles (21), and to incrementally reduce femoral artery blood flow (FBF). We combined this with time-resolved (TRS) NIRS to assess muscle heme concentration change ([ΔTHb]). TRS NIRS is different from conventional NIRS instruments in that it uses diffusion theory to measure mean photon time-of-flight and photon loss in skeletal muscle, allowing the optical pathlength, scattering, and absorption coef-
ponents to be quantified, and calibrated absolute concentrations of heme chromophores to be calculated (10, 25). Thus, by confirming leg muscle quiescence with electromyography (EMG) at the NIRS site (to validate the assumption of constant muscle VO$_2$), we inferred muscle ΔQ/V˙O$_2$ in the steady-state from Doppler ultrasound assessment of ΔFBF during HUT.

We used this model to examine the assumptions that 1) Δ[HHb] is dependent on O$_2$ extraction (e.g., as argued in Ref. 23); and 2) Δ[HHb] is essentially unaffected by changes in total heme concentration (THb) (e.g., see methods in Ref. 28). Thus we tested the hypothesis that Δ[HHb] is closely associated with O$_2$ extraction over a wide range of Q˙/V˙O$_2$. Accepting this hypothesis would imply that Δ[HHb] is relatively insensitive to changes in hematocrit, capillary-venous heme concentration, and/or the volume of small blood vessels, compared with the magnitude of the change in O$_2$ extraction.

MATERIALS AND METHODS

Participants. Sixteen young, healthy, moderately active, nonsmoking men volunteered to participate in this study. Three participants experienced syncope during the HUT protocol (above 45°) and were unable to complete the study. Therefore, responses from the 13 volunteers who completed the HUT protocol were included in the analysis (mean ± SD: age, 20 ± 2 yr; height, 171 ± 4 cm; body mass, 61 ± 4 kg). All participants were informed about the procedures and risks associated with the approved experimental protocol and provided written informed consent approved by the Human Subjects Committee of Kobe Design University in accordance with the Declaration of Helsinki. Participants visited the laboratory twice within 5 days to perform passive HUT at visit 1, and adipose tissue thickness was measured at visit 2. Participants were instructed 1) to refrain from strenuous activity the day before each visit; 2) to sleep at least 8 h the night before the tests; and 3) to have a light meal no less than 2 h before reporting to the laboratory.

Tilt protocol. The HUT protocol was a modification of Ferrari et al. (11) and Binzoni et al. (2). Participants lay supine on a padded tilt-table and were secured with a harness system over the shoulders, chest, and waist (in a similar arrangement to a backpack harness) to minimize movement and prevent falling. Padding was removed from the table in the region of the calf muscles to avoid contact with NIRS and electromyography probes. The participant was asked to lie still and quiet, and to relax the lower limb muscles during ~2 h protocol. The protocol started with 10 min basal measurement at 0°, during which the common femoral artery was localized (see Femoral artery blood flow by Doppler ultrasound for details). The table was tilted manually over 10 s and remained at each of the following angles for 10 min: −10°, 0°, 15°, 30°, 45°, 60°, 75°, −10°, and 75°. At the end of the incremental tilt phase, repeated tilts to −10° and to 75° (each for 10 min) were completed as an internal control, which together with the repeated measurements at 0° were used to assess reproducibility. The ambient temperature was maintained at ~20°C by air conditioning.

TRS NIRS. [HHb] and [HbO$_2$] were measured at the right vastus lateralis (VL) and gastrocnemius (GS) muscles using TRS NIRS (TRS-20, Hamamatsu Photonics KK, Hamamatsu, Japan) to assess oxygenation distribution among different tissues during HUT. The principles and algorithms of TRS NIRS have been presented elsewhere (38, 39). Briefly, heme chromophore concentrations (in μM) are measured by emitting a picosecond light pulse at three different wavelengths (760, 795, and 830 nm). Light is emitted at a frequency of 5 MHz and a full width at half maximum of 100 ps. The optical pathlength, reduced scattering, and absorption coefficients are estimated by a function, based on diffusion theory, fitted to the intensity-time profiles of the reflected light (3, 5, 24, 25, 38). Calibration of the instrument was performed before each test by measuring the response when the emitting and receiving optodes face each other through a neutral-density filter in a black tube.

Each NIRS probe was housed in a black plastic holder to maintain optode spacing and prevent extraneous light entering the field. The emitter-receiver optode spacing was 3 cm. The skin at the site of the NIRS probe placement was cleaned with alcohol (and shaved where needed). Probes were fixed to the skin with double-sided adhesive tape and secured medical tape. The probe positions were marked with ink to facilitate localization of the adipose tissue thickness measurement in visit 2. NIRS variables were recorded at 0.5 Hz throughout the tilt protocol. Steady-state [HHb] and [HbO$_2$] were measured over the final minute at each tilt angle (minutes 9–10). [THb] was calculated from measurement of absolute [HHb] + [HbO$_2$], and [O$_2$] was calculated from [HbO$_2$]/[THb].

Femoral artery blood flow by Doppler ultrasound. A Doppler ultrasound (Logiq400, GE-Yokogawa Medical Systems, Tokyo, Japan) with flat linear array probe operating at a frequency of 11 MHz was used to measure blood velocity in the right common femoral artery. During baseline (0°) the vessel was localized approximately 2–3 cm above the common femoral artery bifurcation, and perpendicular to the image of the artery measured during B-mode, after having frozen the optimized image, and was remeasured in the steady state at each tilt angle.

Blood velocity was measured for 1 min between minutes 9 and 10 of each tilt angle with a probe insonation angle of ~60° and a sample volume maximized according to the vessel size and was centered within the vessel. The vessel diameter was measured perpendicular to the central axis of the artery by caliper in B-mode, after having frozen the optimized image, and was remeasured in the steady state at each tilt angle.

Surface electromyography (EMG). To assess lower limb muscle activity, and confirm passive tilt, EMG was recorded from the VL and GS. The skin was shaved and cleaned with alcohol, and bipolar electrodes were placed 0.5 cm below the NIRS probes. To improve signal-to-noise, electrode cables were fixed to the skin with adhesive tape. EMG signals were acquired at 2 kHz throughout the HUT protocol and were digitally converted (MP 150, Biopac System, Goleta, CA) band-pass filtered (Butterworth, 5th order, 5–450 Hz), full-wave rectified, and smoothed with a high-pass filter (Butterworth, 5th order, 15 Hz). Root mean square (RMS) EMG activity for VL and GS were calculated during the last minute of each tilt angle and compared with the baseline value. EMG analysis was performed using Matlab 7.14 (Mathworks, Natick, MA).

Central hemodynamics by Finometer. Mean arterial pressure (MAP, mmHg) and heart rate (HR, min$^{-1}$) were derived from the arterial pulse pressure profile, continuously recorded at the fingertip using noninvasive photoplethysmography (Finometer, FMS, The Netherlands). Throughout the experiment, the participant’s arm was kept in a comfortable position at the side with the fingers extended. Pressure was corrected in real-time for the vertical distance between the heart and the finger cuff. At the beginning of the protocol (at 0°) the Finometer finger pressure was automatically calibrated to the pressure at the brachialis artery using a cuff on the upper arm, according to the manufacturer’s instructions. Stroke volume (SV, ml) was calculated beat-by-beat using the Modelflow method (49) applied to the pulse pressure profile. Beat-by-beat cardiac output (Q, l/min) was calculated off-line as the product of SV and HR.

Estimation of vascular compliance. Vascular compliance (VC) within the NIRS field of view was estimated (in units of μM/mmHg) in the VL and GS from the ratio of Δ[THb]/ΔP, where P is intravascular pressure above MAP (which remained constant throughout the experiment; see RESULTS) (2; also see 47). Briefly, P was calculated, by 1) the vertical distance (l) at each tilt angle (α) between the heart and...
Adipose tissue thickness measurement. NIRS variables were corrected within each individual for variation in adipose tissue thickness (ATT) at the VL and GS (25, 37). At visit 2 participants lay supine for 15 min while the ATT was determined by Doppler ultrasound. [THb] was measured by TRS NIRS at rest for 2 min at the two muscle sites (previously marked by ink). The [THb]-ATT linear regression was used to normalize TRS NIRS variables to a common ATT of 0 mm, as previously described by Bowen et al. (3).

Statistics and analyses. All data are means ± SD, unless otherwise specified. Data were analyzed by one-way ANOVA for repeated measures. When a significant difference was detected, a Dunnett’s multiple comparison test was used to determine the location of significant differences compared with baseline (0°) (Prism v6.0b, GraphPad Software, San Diego, CA). Regression analysis was used to estimate the association between ΔFBF and Δ[HHb] during the HUT protocol. Individual and group variability in the association between normalized ΔFBF and Δ[HHb] was assessed using the intraclass correlation coefficient (ICC) and a difference plot. Significant differences were accepted at P ≤ 0.05.

RESULTS

Adipose tissue thickness. ATT at VL was 4.9 ± 1.5 mm, and at GS was 4.0 ± 1.3 mm. ATT was inversely linearly correlated with [THb] (y = −18.8x + 218; r² = 0.64), similar to Bowen et al. (3).

Cardiovascular and electromyographic responses to HUT. Femoral artery diameter was 9 ± 0.8 mm at baseline (0°) and did not differ throughout the HUT protocol (P = 0.79). VL and GS RMS EMG also was unchanged compared with baseline throughout HUT. Relative to baseline, the mean change during HUT of RMS EMG in the VL was −0.6 ± 2.2% (n = 9, P = 0.15) and was −0.3 ± 12.1% (n = 7, P = 0.57) in the GS.

Figure 1 shows the central cardiovascular responses to HUT. SV was 94 ± 10 ml at baseline. As expected, SV decreased with increased tilt angle (P < 0.05 from 45° vs. baseline; Fig. 1A), reaching 57 ± 13 ml at 75°. HR (baseline HR = 63 ± 12 min⁻¹) increased during HUT (P < 0.05 from 45°; Fig. 1B), reaching 94 ± 16 min⁻¹ at 75°. Consequently cardiac output (baseline cardiac output = 5.9 ± 1.3 l/min) was unchanged during HUT (P > 0.05; Fig. 1C). MAP, which was 80 ± 9 mmHg at baseline, also was unchanged (P > 0.05) up to 75°. Despite the length of the 2-h protocol, the internal control measurements (repeated tilts to −10° and 75°) suggested little variance in central hemodynamics: there was a small difference in SV at 75° between repeated measurements (57 ± 13 vs. 61 ± 10 ml; P = 0.02), but HR and cardiac output did not differ between repeated measurements at either angle.

The tilt protocol was successful in redistributing cardiac output away from the dependent limbs. FBF was 618 ± 75 ml/min at baseline and decreased progressively during HUT (P < 0.05 from 30°) to 268 ± 52 ml/min at 75°, a 56 ± 11% reduction (Fig. 2C). TRS NIRS responses to HUT. In the VL (Fig. 2A), [HHb] was 57.2 ± 10.5 µM at baseline and increased progressively during HUT (P < 0.05 from 15°) to 100.6 ± 15.5 µM at 75°, a 78 ± 20% increase. In concert, [HbO2] decreased from 170.4 ± 21.9 µM at baseline to 151.8 ± 25.6 µM (P < 0.05 from 30°), an 11 ± 7% decrease. On average [THb] increased by 11 ± 5% in the VL during tilt (P < 0.05 from 15°). The responses in the GS were qualitatively similar to the VL, but with greater magnitude (Fig. 2B), due to the greater tilt-induced change in intravascular pressure. [HHb] increased from 84.4 ± 16.7 µM to 155.7 ± 36.7 µM between 0° and 75° (P < 0.05 from 15°); [HbO2] decreased from 152.5 ± 14.8 µM to 127.3 ± 15.6 µM between 0° and 75° (P < 0.05 from 30°); and [THb] increased by 19 ± 5% (P < 0.05 from 15°). Overall the average increase in [HHb] in both muscles (approximately +57 µM) was greater than the average decrease in [HbO2] (approximately −22 µM), meaning that the larger portion (~60%) of the Δ[HHb] response to tilt was a result of Δ[HHb] increase.

Relationships among TRS NIRS variables and FBF during HUT. To facilitate comparisons among individuals, Δ[HHb] and ΔFBF were normalized between the minimum (0%) and
shown for each individual in Fig. 3, the ICC was less than 0.70 in 9 (VL) and 10 (GS) of the 13 participants (Fig. 3, E and F) ranged from −35 to +19%. These limits exceeded the test-retest variability of the individual [HHb] and FBF measurements. Repeated measures at −10°, 0°, and 75° showed low individual coefficients of variation among subjects for both TRS NIRS (VL: 5, 5, 4%; GS: 3, 5, 4%, respectively) and for FBF (9, 12, 11%, respectively).

Between 0° and 75°, the predominant contributor to ∆[THb] was [HHb], which increased more than [HbO2] decreased in both muscles (VL, ∆[HHb] = 43 ± 10 μM, ∆[HbO2] = −18 ± 11 μM; GS, ∆[HHb] = 72 ± 24 μM, ∆[HbO2] = −25 ± 13 μM). This meant that across muscles, ~60% of the ∆[THb] response to tilt was a result of ∆[HHb] increase. Similarly, ∆[HHb] was the predominant determining variable for ∆StO2. Therefore, we next sought to establish whether vascular compliance could explain dissociation between ∆[HHb] and ∆FBF where it occurred, greater compliance presumably predisposing toward a greater dissociation. To estimate VC we used the linear relationship between ∆[THb] and ∆P (r² = 0.87 ± 0.12; Fig. 4). However, while estimated VC ranged widely (from 0.14 to 0.62 μM/mmHg), there was no relationship between VC and variance in the [HHb] to FBF relationship (VL, r² = 0.001; GS, r² = 0.03).

**DISCUSSION**

Using TRS NIRS, this study examined two common assumptions in the application of NIRS to skeletal muscle. First, we used passive HUT and noninvasive assessment of bulk FBF to generate a broad range of muscle Q˙/V˙O2 values, to determine the agreement between regional [HHb] and the change in limb O2 extraction. On average, over the entire passive HUT protocol, we found good agreement between ∆FBF and ∆[HHb] in both VL and GS (ICC ranged from 0.70 to 0.96). However, the variability in this association across Q˙/V˙O2 values was wide both within and among individuals (confidence intervals were −35% to +19%; Fig. 3, E and F). Second, we assessed whether ∆[HHb] was essentially unaffected by changes in [THb], but found that the increase in [HHb] was the predominant contributor to ∆[THb], and that the decrease in [HbO2] provided only a small influence on [THb].

The overall good agreement between ∆[HHb] and ∆FBF suggests that muscle deoxygenation is a good index of limb O2 extraction across a wide range of Q˙/V˙O2. However, the wide limits of agreement of the individual measurements, and strong relationship with [THb], emphasizes that [HHb] is not necessarily “insensitive” to changes in tissue heme concentration, i.e., changes in hematocrit, capillary-venous heme concentration, and/or volume of small blood vessels, which can each contribute to dissociating ∆[HHb] from O2 extraction. In an attempt to correct NIRS variables for ∆[THb], we found that neither StO2 nor vascular compliance could correct the dissociation between ∆FBF and ∆[HHb]. Thus, under the passive conditions of this study (i.e., no measureable muscle activity), the sensitivity of [HHb] to both Q˙/V˙O2 and tissue heme concentration may complicate its use as a noninvasive surrogate for muscle microvascular O2 extraction.
[HHb] as a surrogate for microvascular O₂ extraction. The notion that skeletal muscle [HHb] provides an index of microvascular O₂ extraction is intuitively attractive. NIRS light absorption changes in muscle reflect changes in oxygenation in small blood vessels (small arterioles, capillaries, venules) and intracellular sites of O₂ transport (myoglobin) and consumption (cytochrome c). Following this line of thought, NIRS provides a noninvasive method for interrogating the site of O₂ exchange at the capillary-myocyte interface that is otherwise inaccessible in humans. However, there is controversy in this interpretation (e.g., 23, 40, 41, 43), because [HHb] may be influenced by both Q˙/V˙O₂ and tissue heme concentration.

Various interventions have been used to investigate the response of NIRS variables to alterations in muscle O₂ extraction, such as exercise, tilt, or lower body negative/positive pressure. Each of these approaches is likely to have a different influence on the extraction-related (from Q˙/V˙O₂) and volume-related (from microvascular hematocrit, capillary-venous heme concentration, and/or tissue vascular volume) changes in [HHb]. For example, active compression of vessels during muscle contractions may minimize gross vascular distension elicited in tilt or lower-body negative pressure interventions. Consequently, it has been argued that (16, 23) during exercise, the close similarity among the dynamics of [HHb], femoral venous O₂ concentration (both in humans; 17, 18, 29, 48), microvascular PO₂ (1), and intramyocyte PO₂ (19) (both in animals) implies that any change in heme concentration has a negligible influence on [HHb], at least in contracting muscle. Recent findings from Vogiatzis et al. (48) showed that (with the exception of one outlier) the relationship between NIRS-derived SO₂ and femoral venous O₂ saturation was approximately linear, and the coefficient of determination (r²) ranged between 0.60 and 0.82 in rest and exercise in humans, similar to our data. This further supports the notion that the [HHb] shows overall good agreement with limb O₂ extraction.

Koga et al. (24) used a visual light spectroscopic method (C9183, Hamamatsu Photonics K.K; 20) to study the kinetics of muscle deoxygenation in relation to microvascular deoxygenation measured by phosphorescence quenching in surface vessels of the rat gastrocnemius muscle. They showed that
muscle deoxygenation kinetics by the two methods were similar, supporting the view that [HHb] is closely associated with microvascular deoxygenation and O2 extraction during exercise (24).

Wüst et al. (51) compared NIRS-derived HHb and femoral venous saturation (SvO2) kinetics during contractions in canine hindlimb. In this preparation, NIRS was measured directly on the muscle surface (i.e., without overlying skin and adipose tissue) and SvO2 was measured in a peripheral vein immediately downstream from the site of O2 exchange. Similar to Vogiatzis et al. in humans (48), Wüst et al. (51) found a strong association ($r^2 = 0.69$) between $\Delta$[HHb] and measured O2 extraction in canine muscle; but only when the kinetics of [THb] were similar to those of [HHb]. In the first ~20 s of exercise, where [THb] was changing (either more rapidly or more slowly than [HHb]), $\Delta$[HHb] was dissociated from $\Delta$SvO2. Interestingly, when the muscle was vasodilated by adenosine infusion and blood flow was experimentally controlled at a high rate, the association between $\Delta$[HHb] and $\Delta$SvO2 was stronger ($r^2 = 0.93$). These data highlight that although $\Delta$[HHb] and O2 extraction can be dissociated from each other, the degree of dissociation depends on an interaction between extraction-related and volume-related changes in [HHb].

In our study we determined average [HHb] over 1 min following 9 min of steady-state rest. Under these very controlled conditions, we found that [HHb] was influenced by both extraction-related and volume-related mechanisms. The question arises, to what degree can volume-related change in [HHb] influence the utility of NIRS measurements as a surrogate for microvascular O2 extraction? The variability that we observed between $\Delta$FFB and $\Delta$[HHb] (~15–30%) suggests that this influence is important during passive HUT where muscle activity is minimal, and that [HHb] may not consistently provide an accurate surrogate for O2 extraction (Fig. 3). The relative dispersion between $\Delta$FFB and $\Delta$[HHb] we found was similar to the dispersion between $\Delta$STO2 and femoral venous O2 saturation in Vogiatzis et al. (48). Interestingly, using NIRS-derived measurements from six sites of the superficial quadriceps, Vogiatzis et al. (48) also showed that the $V_\text{O2}/Q$ ratio was low (~0.1) and varied very little among sites or among experimental conditions (rest, exercise, and hypoxia). The relatively low dispersion in regional $V_\text{O2}/Q$ (~12–15%) compared with the dispersion between $\Delta$FFB and $\Delta$[HHb] from our study (~15–30%) highlights that volume-related mechanisms likely contribute to dissociating $\Delta$[HHb] from regional O2 extraction, at least during passive HUT.

The most common NIRS application in muscle is during exercise. While our study likely elicited a similar magnitude of O2 extraction as expected during high-intensity exercise (limb blood flow was halved from ~620 ml/min at 0°, to ~270 ml/min at 75°; thus estimated arteriovenous oxygen concentration difference [Ca-\text{O2}/O2] across the leg was also halved from ~0.08 ml O2/ml blood at baseline to ~0.16 ml/ml at 75°), it is difficult to extend our results to exercise conditions. This is because passive HUT likely maximizes the volume-related influence on [HHb], whereas muscle contractions in exercise are expected to counter the vascular distension and microvascular hemococoncentration (28) that we observed. Exercise NIRS studies typically observe that either 1) [THb] does not change during exercise (6, 17; although see 3, 4, 7 for contrasting evidence); and/or 2) [HHb] does not follow the dynamics of [THb], but more closely resembles changes in [HbO2] (e.g., 3, 9, 11, 15, 24, 25, 28, 29). However, our data emphasize that neither of these observations can be taken as evidence that [HHb] is insensitive to volume-related mechanisms during exercise. This is because in our study we saw a volume-related increase in [HHb] that was partly countered by a decrease in [HbO2], meaning that constancy in [THb] does not necessarily mean that [HHb] is isolated from volume-related change. This influence on [HHb] may be particularly important when considering deoxygenation kinetics, where mechanisms that cause variability in the association between [HHb] and O2 extraction could have a larger influence on values for kinetic parameters than during the steady-state (50).

Thus, until these concerns are addressed during exercise, the results of our study urge caution about the accuracy for using $\Delta$[HHb] as a quantitative surrogate for O2 extraction, e.g., in mathematical calculations to estimate the adequacy of microvascular O2 delivery in relation to muscle O2 consumption (14; also see 36 for review).

**Approaches to account for changes in tissue heme concentration.** [HHb] is dependent on heme concentration within the NIRS field of view (hemoglobin, myoglobin, and potentially cytochrome c concentration; 31). The sensitivity of [HHb] to volume-related change is therefore dependent on the magnitude of heme capacitance compared with the magnitude of O2 extraction. We found ~10–20% increase in [THb] during HUT, similar to Davis and Barstow (7), who found ~1–30% increase in [THb] during cycling exercise eliciting a similar magnitude of estimated O2 extraction. In an attempt to account for this, Quaresima and colleagues have suggested to use STO2 to correct for changes in [THb] (41–43): this suggestion is particularly relevant because STO2 is often measured in studies using conventional, continuous-wave NIRS, but $\Delta$[HHb] is used preferentially in many reports. STO2 should be less sensitive to volume-related HHb change and more sensitive to extraction-related HHb change. To our surprise, however, our data using TRS NIRS did not support this view: during HUT, $\Delta$[HHb] and $\Delta$STO2 were strongly correlated ($r^2 = 0.99$). The dependence of $\Delta$STO2 on $\Delta$HHb during passive HUT also implies that volume-correction algorithms (e.g., developed for use during vascular occlusion; 44) would not be expected to increase the precision of $\Delta$[HHb] as an O2 extraction index during free flow conditions. Similarly, we found that individual differences in estimated vascular compliance (calculated by [THb]; 2) did not relate to the variability observed in the $\Delta$FFB and $\Delta$[HHb] relationship (Figs. 3 and 4).

Thus, in this study, common volume-correction methods were not successful to account for the influence of volume-related heme shifts on $\Delta$[HHb].

The finding that the volume-related component of $\Delta$[HHb] is large during HUT is consistent with previous studies. Ferrari et al. (11) used HUT and exercise (separately) to alter O2 extraction and measured HHb and HbO2 with continuous-wave NIRS. During intermittent exercise, after an initial volume expansion phase during the first three 5-min exercise bouts, they found an ~5 µM increase in [HHb] that appeared to be independent of [THb]. These data are frequently used to suggest that [HHb] is insensitive to volume-related heme shifts, and therefore closely reflects O2 extraction. However, Q/V$\text{O2}$ was unknown in Ferrari et al. (11), and therefore the
degree to which [HHb] was influenced by changes in heme concentration and/or \( Q/V_O_2 \) during exercise also was unknown.

Consistent with our findings, Ferrari et al. (11), Binzoni et al. (2), and Truijen et al. (47) each found a large increase in \( \Delta[Hb] \) during HUT, which was strongly related to \( \Delta[THb] \) (a \( \sim 20-40 \mu M \) increase). These changes are greater than that commonly seen during exercise (a \( \sim 5-20 \mu M \)) (11, 28, cf. 7). Truijen et al. (47) found that vascular volume and \( [THb] \) were closely associated (\( r^2 = 0.95 \)) and continued to change during sustained HUT, approaching a steady state by 10 min (the duration of each tilt increment used in our study). This was explained largely by a biexponential prolonged filling of small lower-limb vessels that occurred concurrently with distension of the large veins. The duration to reach a steady state in \([THb]\) is relatively long compared with many exercise studies, and therefore, any exercise-related change in microvascular volume may continue throughout in studies focusing on less prolonged exercise responses, complicating \( \Delta[HHb] \) interpretation.

Together our data demonstrate that during passive HUT: \( \Delta[HHb] \) is sensitive to increases tissue heme concentration; the magnitude of volume-related change in the \( [HHb] \) NIRS signal significantly dissociated the \( [HHb] \) measurement from the extraction-related mechanism of increase; and common methods to correct \( [HHb] \) for heme concentration changes may not be effective.

Limitations. This study made some assumptions that are important to highlight. We assumed leg muscle \( V_O_2 \) to be constant, based on little to no change in EMG activity; but regional \( V_O_2 \) within the TRS NIRS field of view is unknown. More importantly we assumed that tilt-induced \( \Delta FBF \) represented change in microvascular blood flow within the small vessels interrogated by TRS NIRS. The TRS NIRS signals are derived from surface tissues (including skin, adipose, and muscle), and thus this sample will not be representative of the entire vastus lateralis or gastrocnemius. While these muscles have a heterogeneous expression of fiber type and capillary density, surface muscle tends to have a greater expression of type II fibers with lower rates of perfusion (22, 33). Additionally, blood flow control may differ between conduit vessels perfusing regions of muscle expressing fast- or slow-twitch muscle fibers (27). Therefore, the degree with which \( \Delta FBF \) reflected regional, microvascular \( \Delta Q/V_O_2 \) in this study is dependent on these assumptions. It may be that the deviations from identity between \( [HHb] \) and \( \Delta FBF \) (Fig. 3) reflect the magnitude of the variance in microvascular \( \Delta Q/V_O_2 \) around the mean measured across the whole muscle (i.e., approximately \( \pm 15-30\% \); 27, 48). In an attempt to reduce the influence of these variables, we made our measurements in the steady state, 9 min after each tilt increment. Also, the normalization process (i.e., to the maximum and minimum \([HHb]\) at each muscle site) should account for differences in absolute \( Q/V_O_2 \) between muscles (i.e., were the relationship between blood flow and tilt angle to differ between vastus lateralis and gastrocnemius), and focuses the analysis on the degree to which change in \([HHb]\) is reflected change in \( Q/V_O_2 \). Thus, our interpretation relies on the assumption that \( [HHb] \) is a linear function of \( \Delta FBF \) (under the passive conditions of this experiment), and is independent of the absolute FBF change, should it vary between muscles.

Estimating intravascular pressure in the arterioles, capillaries, and venules is complex, i.e., the vessels from which the TRS NIRS signal derives. The VC calculation reflects pressure change across tilt angles within large-conduit arteries outside of the muscle, and to a lesser extent, resistance arterioles within the muscle. The intravascular pressures calculated using Eq. 2 may therefore not well represent the vascular pressures within the vessels measured by TRS NIRS. As the main purpose of the experiment was to determine the effect of changes FBF using HUT (measured by Doppler ultrasound) on \([HHb]\), the uncertainly associated with VC estimation in the microvascular does not influence the main study conclusion.

Conclusions. This study examined two common assumptions in the application of NIRS to skeletal muscle, using passive head-up tilt and Doppler measurement of femoral artery blood flow: that \( [HHb] \) is dependent on \( O_2 \) extraction, and that \( [HHb] \) is essentially unaffected by changes in \( [THb] \). Over a wide range of muscle \( O_2 \) extraction we found a good agreement between \( \Delta FBF \) and \( [HHb] \) (ICC > 0.70) in the lower limb muscles during HUT. However, using quantitative TRS NIRS we also found that \( [THb] \) was mostly a result of an increase in \( [HHb] \) with a small contribution from reduced \([HbO_2]\). Together these data suggest that over a wide range of \( O_2 \) extraction in passive HUT, \( [HHb] \) is mainly dependent on an extraction-related mechanism (\( Q/V_O_2 \)), but volume-related mechanisms (microvascular hematocrit, capillary-venous heme concentration, and/or tissue vascular volume) may contribute \( \sim 15-30\% \) of the response. Attempts to account for the volume-related influence (e.g., by normalizing for \([THb]\), or estimating vascular compliance) were not successful in adjusting the \([HHb]\) signal to better reflect \( O_2 \) extraction. Thus, under the passive conditions of this study where muscle activity is minimal, it appears that the sensitivity of \( [HHb] \) to both \( Q/V_O_2 \) and heme capacitance may complicate its use as a noninvasive surrogate for muscle microvascular \( O_2 \) extraction. While this concern may be mitigated during exercise by the effects of muscle contractions, our data emphasize the complexity in interpreting NIRS deoxygenation (and other NIRS-derived) signals.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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