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ORIGINAL RESEARCH

Intracellular oxygen tension limits muscle contraction-induced change in muscle oxygen consumption under hypoxic conditions during Hb-free perfusion

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Abstract

Under acute hypoxic conditions, the muscle oxygen uptake ($m\dot{V}O_2$) during exercise is reduced by the restriction in oxygen-supplied volume to the mitochondria within the peripheral tissue. This suggests the existence of a factor restricting the $m\dot{V}O_2$ under hypoxic conditions at the peripheral tissue level. Therefore, this study set out to test the hypothesis that the restriction in $m\dot{V}O_2$ is regulated by the net decrease in intracellular oxygen tension equilibrated with myoglobin oxygen saturation ($\Delta P_{mb}O_2$) during muscle contraction under hypoxic conditions. The hindlimb of male Wistar rats (8 weeks old, $n = 5$) was perfused with hemoglobin-free Krebs–Henseleit buffer equilibrated with three different fractions of O_2 gas: 95.0%O_2, 71.3%O_2, and 47.5% O_2. The deoxygenated myoglobin (Mb) kinetics during muscle contraction were measured under each oxygen condition with a near-infrared spectroscopy. The Δ[deoxy-Mb] kinetics were converted to oxygen saturation of myoglobin ($S_{mb}O_2$), and the $P_{mb}O_2$ was then calculated based on the $S_{mb}O_2$ and the O_2 dissociation curve of the Mb. The $S_{mb}O_2$ and $P_{mb}O_2$ at rest decreased with the decrease in O_2 supply, and the muscle contraction caused a further decrease in $S_{mb}O_2$ and $P_{mb}O_2$ under all O_2 conditions. The net increase in $m\dot{V}O_2$ from the muscle contraction ($\Delta m\dot{V}O_2$) gradually decreased as the $\Delta P_{mb}O_2$ decreased during muscle contraction. The results of this study suggest that $\Delta P_{mb}O_2$ is a key determinant of the $\Delta m\dot{V}O_2$.

Introduction

Under acute hypoxic conditions, the decrease in O_2 supply reduces muscle oxygen consumption ($m\dot{V}O_2$) during exercise (Calbet et al. 2003; Lundby et al. 2004; Calbet et al. 2009). However, a series of O_2 transport steps (ventilation, diffusion from the lung to the blood, bulk delivery by the cardiovascular system, and the transfer of O_2 from the blood to the skeletal muscle) control the $m\dot{V}O_2$ and oxidative ATP generation (Bassett and Howley 2000; Howlett et al. 2009). The way the various steps involved in the determination of the $\dot{V}O_2$ contribute to the respiration during

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exercise under hypoxia conditions remains unclear. Recent research has reported that in hypoxia $\dot{V}O_2$ might be limited by factors aside from arterial oxygen content (Lundby et al. 2004). Taken together, these facts suggest the existence of factors regulating the m$\dot{V}O_2$ at the peripheral tissue level.

As the value of the m$\dot{V}O_2$ depends on the O_2 flux – that is, the O_2 diffusion conductance (DO_2) and the O_2 gradient between the microvasculature and the myocytes – the decrease in m$\dot{V}O_2$ under hypoxic conditions may be caused by decreases in both the DO_2 and O_2 gradients across the plasma membrane (Richardson et al. 1995; Takakura et al. 2010). However, previous studies have not considered the contribution of the O_2 gradient as based on the intracellular oxygen tension equilibrated with myoglobin oxygen saturation ($P_{mb}O_2$) (Gonzalez et al. 2006). Currently, only the $P_{cap}O_2$ is regarded as an adequate measure of the O_2 diffusion between the plasma membrane. The study assumed that the $P_{mb}O_2$ at rest was close to zero and that it remained unchanged during moderate- to high-intensity exercise (Richardson et al. 1995). However, under hypoxic condition, Richardson et al. (2006) could not detect any deoxy myoglobin (Mb) signal at rest due to presumably a low signal to noise ratio. Therefore, it remains unclear whether muscle contraction under hypoxic conditions causes a further decrease in $P_{mb}O_2$. Other groups have reported that the $P_{mb}O_2$ decreases with the increase in exercise intensity under normoxic conditions, and that the O_2 gradient from the vasculature to the cell could play a key role in the control of the $\dot{V}O_2$ (Molé et al. 1999; Chung et al. 2005). These conflicting results necessitate an examination of the relationship between the m$\dot{V}O_2$ and the intracellular O_2 environment during muscle contraction under hypoxic conditions.

We recently demonstrated that the intracellular Mb dynamics during muscle contraction involved in enhancing the O_2 flux to meet the increased muscle O_2 demand (Masuda et al. 2010; Takakura et al. 2010). Indeed, the $P_{mb}O_2$ decreased with increasing exercise intensity under normoxic conditions (Molé et al. 1999; Takakura et al. 2010). This change in the O_2 gradient as reflected by the decrease in the $P_{mb}O_2$ can contribute to the muscle O_2 uptake. Therefore, this study considers the effect of hypoxia on the intracellular O_2 environment and assesses whether the vasculature-to-cell O_2 gradient contributes in regulating the m$\dot{V}O_2$ during muscle contraction.

Materials and Methods

Experimental animals and preparation of hindlimb perfusion

Male Wistar rats were employed as subjects. All were housed in a temperature-controlled room at 23 ± 2°C with a 12-h light–dark cycle and maintained on a commercial diet with water ad libitum. The procedures conformed to the "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions" (published by the Ministry of Education, Culture, Sports, Science and Technology, Japan) and was approved by the Ethics Committee for Animal Experimentation of Kanazawa University (Protocol AP-101821).

The hindlimb perfusion was performed to the rats at 8 week of age (body weight at experiment; 256.0 ± 8.7 g). Preparation of isolated rat hindlimb and the perfusion apparatus are described in previous reports (Masuda et al. 2010; Takakura et al. 2010, 2015). All surgical procedures were performed under pentobarbital sodium anesthesia (64.8 mg/kg intraperitoneal). After finishing a surgical procedure, the rats were killed by injecting 1 mol/L KCl solution directly into the heart and a hemoglobin-free Krebs–Henseleit buffer (NaCl, 118 mmol/L; KCl, 5.9 mmol/L; KH_2PO_4, 1.2 mmol/L; $MgSO_4$, 1.2 mmol/L; $CaCl_2$, 1.8 mmol/L; $NaHCO_3$, 20 mmol/L; Glucose, 15 mmol/L) equilibrated with 95% O_2 + 5% CO_2 at 37°C was perfused into the abdominal aorta in flow through mode, at a constant flow rate. In order to adjust the perfusion pressure to approximately 80.0 mmHg, the flow rate was set to 22.2 ± 0.5 mL min^{-1} throughout the perfusion period. In this condition, the average perfusion pressures were 77.5 ± 3.1 mmHg, and the perfusion resistance was unchanged throughout the perfusion period. In addition, no sign of edema in the hindlimb was seen at the given flow rate. The effluent was collected from the inferior vena cava in order to measure m$\dot{V}O_2$ and the lactate and pyruvate concentrations.

During the perfusion period, the oxygen supply was modulated by adjusting the O_2 fraction of the equilibration gas using nitrogen gas, while 5% CO_2 concentration was maintain to keep pH in perfusate. This study set three levels of O_2 concentration: a 95.0% O_2 fraction, a 71.3% O_2 fraction, and a 47.5% O_2 fraction. After establishing a sufficient equilibrium in each oxygen condition, muscle contraction was performed on a rat at the maximal twitch tension in all oxygen conditions. The order of the three oxygen conditions was randomized.

Measurement parameters

The twitch contraction protocol and measurement of Mb oxygenation and m$\dot{V}O_2$ followed the previous methods (Masuda et al. 2010; Takakura et al. 2010). The sciatic nerve of the left hindlimb was then exposed and connected to two parallel stainless steel wire electrodes (Unique Medical, Tokyo, Japan) and the Achilles' tendon

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was connected to a sensitive strain gauge with a string (MLT500/D, AD Instrument, Castle Hill, NSW, Australia). The stimulation pulse via the sciatic nerve derived by an electrostimulator system (Model RU-72, Nihon Koden, Tokyo, Japan) was 1 Hz in frequency (delay, 10 μsec; duration, 1 msec) for 120 sec (120 twitch contractions). Target tension was controlled by changing the voltage of stimuli to obtain 100% of peak tension under buffer-perfused conditions (3–8 volts). Twitch tension was calculated as the average of a series of contractions. The muscle also showed no sign of fatigue at the set stimulation intensity.

An NIRS instrument (NIRO-300 + Detection Fibre Adapter Kit, Hamamatsu Photonics, Shizuoka, Japan) was employed to measure oxygenation of Mb at rest and during muscle contraction. The distance between the photodiode and the LED was fixed at 10 mm. The toe of the foot was secured by a clamp with the rat laid on its back. After that, the NIRS probes were firmly attached to the skin of the gastrocnemius muscle and were fixed by clamps on both sides of the muscle. During the initial period, for at least 30 sec before the start of contraction, the average fluctuation in the NIRS signals was adjusted to a reference value of zero. After the exercise protocol, the anoxic buffer (equilibrated with 95% N_2 + 5% CO_2 gas) was perfused for 30 min to obtain maximal Mb desaturation. The muscle then received electrical stimulation to contract for 2 min. No further increase in change in NIRS signal associated with concentration of deoxygenated Mb (Δ[deoxy-Mb]) signal was evident. The final Δ[deoxy-Mb] signal intensity served as the normalization constant for 100% Mb deoxygenation.

The value of m$\dot{V}O_2$ was calculated from the arteriovenous oxygen content difference multiplied by the flow rate, using the equation:

$$\mathrm{m}\dot{V}\mathrm{O}_2(\mu\mathrm{mol\ g}^{-1}\mathrm{min}^{-1}) = \text{flow rate} \times [(\mathrm{PO}_{2_{\mathrm{in}}} - \mathrm{PO}_{2_{\mathrm{out}}}) \times \mathrm{O}_2 \text{ solubility}]/\text{muscle weight}$$

where flow rate is the flow in milliliters per minute, and PO_{2in} and PO_{2out} are the arterial and venous oxygen tensions after adjusting for the vapor pressure of water. Inflow PO_2 and outflow PO_2 were measured continuously using two O_2 electrodes (5300A, YSI, Yellow Springs, OH) along tubing before and after perfusion of the hindlimb. The vapor pressure at 37°C was 47.03 mmHg. The solubility of oxygen in the buffer was 0.00135 μmol mL^{-1} mmHg^{-1} at 37°C (Philip and Dorothy 1971). The m$\dot{V}O_2$ at rest and during muscle contraction was calculated by using the values of $PO_{2in}-PO_{2out}$ averaged over 15 sec in the steady-state conditions before and during muscle contraction.

The sampling rate for the NIRS data was 1 Hz. The other parameters (tension, perfusion pressure, O_2 content at the inflow and outflow) were collected using a data acquisition system (PowerLab 8SP, AD Instruments, Australia) at a sampling rate of 1 kHz. All the data were transferred to a personal computer with acquisition software (Chart ver. 5.5.6. AD Instruments).

Data analysis

The data analysis followed our previous methods (Takakura et al. 2010, 2015). A simple moving average smoothed the Δ[deoxy-Mb] NIRS signals using a rolling average of 5 points, which corresponds to a 5 sec timeframe(Box et al. 1978). The Δ[deoxy-Mb] signals were calibrated against two different NIRS signal values: one at rest as 10% Mb deoxygenation and the other during steady state with anoxic buffer perfusion as 100% Mb deoxygenation. While the $S_{mb}O_2$ at rest could not be determined by NIRS, the value was assumed to be 90% based on previous studies reporting that the $S_{mb}O_2$ at rest was greater than 90% (Chung et al. 2005). The %Δ[deoxy-Mb] plots were converted to $S_{mb}O_2$ (%) plots using the following equation:

$$\mathrm{S_{mb}O_2} = 100 - \%\Delta[\mathrm{deoxy-Mb}]$$

$S_{mb}O_2$ plots were fitted by the following single-exponential equation to calculate kinetics parameters using an iterative least-squares technique by means of a commercial graphing/analysis package (KaleidaGraph 3.6.1, Synergy Software, Reading, PA):

$$\mathrm{S_{mb}O_2} = \mathrm{BL} + \mathrm{AP} \times [1 - \exp^{-(t-\mathrm{TD})/\tau}]$$

where BL is the baseline value, AP the amplitude between BL and the steady-state value during the exponential component, TD the time delay between onset of contraction and appearance of $S_{mb}O_2$ signals, and τ the time constant of $S_{mb}O_2$ signal kinetics. MRT calculated by TD + τ was used as an effective parameter of the response time for Mb deoxygenation at onset of muscle contraction. Dividing 63% of AP by MRT yields a value for the time-dependent change in Mb deoxygenation. The $P_{mb}O_2$ value (mmHg) at rest and steady state during muscle contraction was converted from the $S_{mb}O_2$ value using the following equation:

$$\mathrm{P_{mb}O_2} = \frac{\mathrm{S_{mb}O_2} \cdot \mathrm{P_{50}}}{(1 - \mathrm{S_{mb}O_2})}$$

where P_{50} is the partial oxygen pressure required to half-saturate Mb. A P_{50} of 2.4 mmHg was used for this equation, assuming a muscle temperature of 37°C (Schenkman et al. 1997). The calculated $P_{mb}O_2$ plots were evaluated to obtain an MRT of its kinetics using the same single exponential equation as for $P_{mb}O_2$. The $_{0.63}$AP/MRT for

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$P_{mb}O_2$ indicates a rate of decrease in $P_{mb}O_2$ at muscle contraction onset. $P_{mb}O_2$ at steady state was calculated by using the $S_{mb}O_2$ value at steady state. Since O_2 partial pressure corresponds to a specific amount of dissolved O_2, intracellular $[O_2]$ (μM) was calculated from the $P_{mb}O_2$ value at rest and at each O_2 condition using the following equation:

$$\text{Intracellular } [O]_2 = P_{mb}O_2 \times O_2 \text{ solubility}$$

with $P_{mb}O_2$ is in mmHg, and O_2 solubility in buffer is 0.00135 μmol mL^{-1} mmHg^{-1} at 37°C (Philip and Dorothy 1971).

The relationship of m$\dot{V}O_2$ to conductance and O_2 gradient used the following equation:

$$m\dot{V}O_2 = kDO_2 \times (P_{cap}O_2 - P_{mb}O_2)$$

where m$\dot{V}O_2$ is muscle oxygen consumption, k is constant, D is conductance, $P_{cap}O_2$ is microvascular oxygen tension, $P_{mb}O_2$ is intracellular oxygen tension equilibrated with myoglobin oxygen saturation as determined from the Mb signal. Because study used a constant flow perfused hindquarter model, the analysis has assumed a proportional relationship between the outflow and capillary PO_2 and has set 30 mmHg as the reference the normoxic $P_{cap}O_2$ value (Behnke et al. 2001; McCullough et al. 2011; Kano et al. 2014; Ferguson et al. 2015).

Statistical analyses

All data are expressed as mean ± SD. Statistical differences were examined using one-way paired measures analysis of variance (ANOVA) (factor: O_2 fraction). A Turkey–Kramer post hoc test was applied if the ANOVA indicated a significant difference. The level of significance was set at $P < 0.05$.

Results

In this study, the oxygen supply to the hindlimb muscle decreased from 17.1 ± 1.3 μmol min^{-1} at 95% O_2 saturation to 12.0 ± 1.2 and 8.8 ± 0.9 μmol min^{-1} in the 71.3% and 47.5% O_2 saturation, respectively. The correspondence between the O_2 saturation and the measured O_2 concentration in the perfusate confirmed the appropriateness of the experimental conditions. As the O_2 supply volume decreased, the m$\dot{V}O_2$ in resting muscle decreased gradually (Table 1). Even though $S_{mb}O_2$, and $P_{mb}O_2$ also decrease, the measurements could not detect a significant change in the estimated O_2 gradient as reflected in ($P_{cap}O_2$-$P_{mb}O_2$), and did not reveal any significant alteration. Under all hypoxia conditions, the lactate to pyruvate ratio (L/P) showed no increase (Table 1).

Relative to the resting m$\dot{V}O_2$, muscle contracting at a similar tension (77-83 g) increased m$\dot{V}O_2$ under all O_2

Table 1. Muscle oxygen consumption and intracellular O_2 parameter at rest during hindlimb perfusion with different O_2 fraction.

		O_2 fraction in pefusate		
Parameter	Unit	47.5%	71.3%	95.0%
Inflow PO_2	mmHg	291.1 ± 37.4[12]	399.1 ± 48.0[1]	566.9 ± 53.7
$[O_2]$ in perfusate	μmol/L	18.0 ± 2.1[12]	24.5 ± 2.7[1]	35.1 ± 3.0
Outflow PO_2	mmHg	112.3 ± 20.7[12]	170.5 ± 31.8[1]	291.1 ± 37.4
Relative outflow PO_2		0.41 ± 0.08[12]	0.63 ± 0.12[1]	1.00 ± 0.15
Estimated $P_{cap}O_2$	mmHg	12.4 ± 2.3[12]	18.8 ± 3.5[1]	30.0 ± 4.6
m$\dot{V}O_2$	μmol g^{-1} min^{-1}	0.34 ± 0.05[12]	0.43 ± 0.07[1]	0.55 ± 0.12
$S_{mb}O_2$	%	55.0 ± 7.9[12]	74.1 ± 11.7[1]	90.0 ± 0.1
$P_{mb}O_2$	mmHg	3.1 ± 0.9[12]	8.5 ± 4.6[1]	21.7 ± 0.2
Intracellular $[O_2]$	μmol/L	4.1 ± 1.2[12]	11.5 ± 6.2[1]	29.3 ± 0.3
$P_{cap}O_2$-$P_{mb}O_2$	mmHg	9.3 ± 3.0	10.3 ± 5.2	8.3 ± 4.5
L/P		19.6 ± 3.0	17.8 ± 2.3	18.3 ± 2.0

Values are mean ± SD (n = 5 in each condition). Inflow PO_2, oxygen tension before perfusion of the hindlimb; $[O_2]$ in perfusate, O_2 concentration in perfusate; Outflow PO_2, oxygen tension after perfusion of the hindlimb; Relative outflow PO_2, relative value of outflow PO_2 based on the outflow PO_2 value at 95.0%O_2 fraction; Estimated $P_{cap}O_2$, estimated microvasucular oxygen tension based on 30 mmHg of $P_{cap}O_2$ at 95.0%O_2 fraction. m$\dot{V}O_2$, muscle oxygen consumption; $S_{mb}O_2$, intracellular O_2 myoglobin saturation; $P_{mb}O_2$: intracellular O_2 tension equilibrated with myoglobin O_2 saturation; Intracellular $[O_2]$, intracellular O_2 concentration; $P_{cap}O_2$-$P_{mb}O_2$, the difference in oxygen tension between $P_{cap}O_2$-$P_{mb}O_2$; L/P, lactate to pyruvate ratio measured in effluent perfusate.

[1]$P < 0.05$ versus 95.0% condition

[2]$P < 0.05$ versus 71.3% condition.

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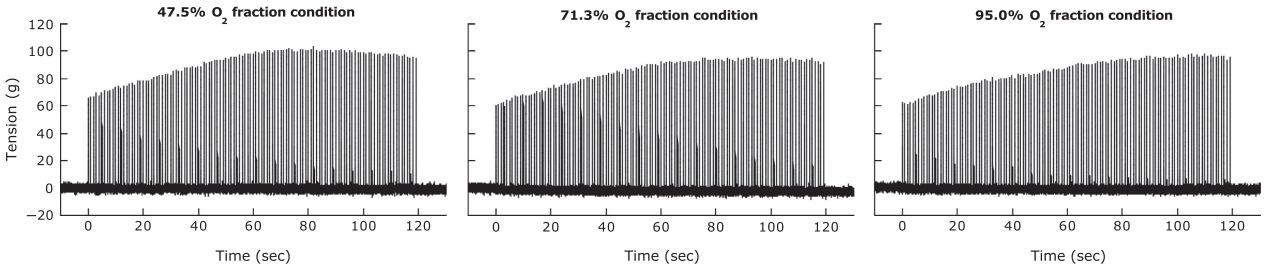

Figure 1. Representative muscle tension generation during muscle contraction for 120 sec under each O_2 fraction conditions. Maximal twitch muscle contractions were elicited every 1 sec (1 Hz) by stimulating sciatic nerve. The time courses of the change in twitch tension at each O_2 fraction condition are shown. No sign of fatigue was observed during muscle contraction regardless of the O_2 fraction conditions.

supply conditions. The representative time course of the change in twitch tension at each O_2 fraction condition was shown in Figure 1. However, the largest increase occurred at 95% O_2, where the $\Delta m\dot{V}O_2$ rose by 0.28 μmol g^{-1} min^{-1}. At 47.5% and 71.3% O_2 saturation, $\Delta m\dot{V}O_2$ rose only by 0.11 and 0.16 μmol g^{-1} min^{-1}, respectively. Even though $m\dot{V}O_2$ increased under hypoxia conditions, it increased much less than muscle under normoxic condition (Table 2).

In contrast, L/P increased with declining O_2 supply. L/P increased significantly higher at 47.5% O_2 (3.6 ± 3.4) than at 95.0% O_2 (1.8 ± 1.0). At 95.0% O_2 the muscle

Table 2. Muscle oxygen consumption and intracellular O_2 parameter during muscle contraction during hindlimb perfusion with different O_2 fraction.

		O_2 fraction in perfusate		
Parameter	Unit	47.5%	71.3%	95.0%
Muscle tension	g	76.7 ± 15.2	81.5 ± 8.9	82.8 ± 12.9
$m\dot{V}O_2$	μmol g^{-1} min^{-1}	0.45 ± 0.08[1†]	0.60 ± 0.07[1]	0.87 ± 0.15
$\Delta m\dot{V}O_2$	μmol g^{-1} min^{-1}	0.11 ± 0.04[12]	0.16 ± 0.07[1]	0.28 ± 0.05
Outflow PO_2	mmHg	104.9 ± 20.6[1†]	158.7 ± 30.1[1]	252.2 ± 41.1
Relative outflow PO_2		0.42 ± 0.08[12]	0.63 ± 0.12[1]	1.00 ± 0.15
Estimated $P_{cap}O_2$	mmHg	12.5 ± 2.4[12]	18.9 ± 3.6[1]	30.0 ± 4.9
$S_{mb}O_2$ kinetics				
Steady-state value	%	12.3 ± 8.0[12]	42.0 ± 16.6[1]	68.7 ± 3.0
AP	%	−42.7 ± 7.5[1]	−32.1 ± 8.8	−21.4 ± 3.0
MRT	s	39.4 ± 7.8	37.3 ± 8.0	42.4 ± 11.8
$_{0.63}$AP/MRT	% s^{-1}	−0.69 ± 0.10[1]	−0.57 ± 0.23[1]	−0.34 ± 0.11
$P_{mb}O_2$ kinetics				
Steady-state Value	mmHg	0.4 ± 0.3[12]	2.1 ± 1.2[1]	5.5 ± 0.9
AP	mmHg	−2.7 ± 0.8[1]	−6.5 ± 3.8[1]	−16.2 ± 0.9
MRT	sec	30.6 ± 5.4	35.3 ± 8.2	33.4 ± 11.7
$_{0.63}$AP/MRT	mmHg sec^{-1}	−0.03 ± 0.07[1]	−0.13 ± 0.18[1]	−0.41 ± 0.14
Intracellular [O_2]	μmol/L	0.5 ± 0.4[12]	2.8 ± 1.7[1]	7.4 ± 1.2
$P_{cap}O_2$-$P_{mb}O_2$	mmHg	12.1 ± 2.5[12]	16.8 ± 3.8[1]	24.5 ± 2.5
ΔL/P		3.6 ± 3.4[1]	2.5 ± 1.7	1.8 ± 1.0

Values are mean ± SD (n = 5 in each condition). $m\dot{V}O_2$, muscle oxygen consumption; $\Delta m\dot{V}O_2$, the net increase in $m\dot{V}O_2$ due to muscle contraction; Outflow PO_2: oxygen tension after perfusion of the hindlimb, Relative outflow PO_2, relative value of outflow PO_2 based on the outflow PO_2 value at 95.0 %O_2 fraction; Estimated $P_{cap}O_2$, estimated microvasucular oxygen tension based on 30 mmHg of $P_{cap}O_2$ at 95.0% O_2 fraction; $S_{mb}O_2$, intracellular O_2 myoglobin saturation; AP is the amplitude between BL (baseline) and the steady-state value during the exponential component; MRT is the time required to reach 63% of AP from the onset of muscle contraction. $_{0.63}$AP/MRT is calculated by dividing $_{0.63}$AP by MRT; $P_{mb}O_2$, intracelluar O_2 tension equilibrated with myoglobin O_2 saturation; Intracellular [O_2], intracellular O_2 concentration; $P_{cap}O_2$-$P_{mb}O_2$, the difference in oxygen tension between $P_{cap}O_2$-$P_{mb}O_2$; ΔL/P, the net increase in lactate to pyruvate ratio measured in effluent perfusate.

[1] $P < 0.05$ versus 95.0% condition.

[2] $P < 0.05$ versus 71.3% condition.

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contraction causes no significant change in the L/P ratio, consistent with previous observations (Takakura et al. 2010).

During muscle contraction, the steady $S_{mb}O_2$ and $P_{mb}O_2$ still decreased with decreasing oxygen supply. However, the $S_{mb}O_2$ and $P_{mb}O_2$ kinetics did not show a significant difference (Table 2). Figure 2 shows representative Δ[deoxy-Mb] kinetics during different levels of O_2 delivery conditions and during anoxic perfusion, as assessed by NIRS. The roman numerals I, II and III represent the NIRS signal response to 1 Hz maximal twitch contractions at 95.0%, 71.3% and 47.5% O_2 fraction condition, respectively. In protocol IV, the noncontracting muscle received a perfusate equilibrated with 95% N_2 + 5% CO_2.

Figure 3 shows the net increase in m$\dot{V}O_2$ (Δm$\dot{V}O_2$) relative to its resting value during muscle contraction in each O_2 fraction condition. The Δm$\dot{V}O_2$ due to muscle contraction (0.11 ± 0.04, 0.16 ± 0.07, and 0.28 ± 0.05 μmol g^{-1} min^{-1} at 47.5%, 71.3%, and 95% O_2 fraction) decreased progressively with hypoxia. Under resting conditions, the measurements indicate $P_{mb}O_2$ values of 3.1, 8.5, and 21.7 mmHg at 47.5, 71.3, and 95% O_2. With muscle contraction, the $P_{mb}O_2$ values decrease correspondingly to 0.4, 2.1, and 5.5 mmHg. Figure 4 shows then net decrease in $P_{mb}O_2$ ($\Delta P_{mb}O_2$) due to muscle contraction (2.50 ± 0.81, 6.12 ± 3.52, and 16.10 ± 0.87 mmHg in 47.5, 71.3, and 95% O_2 fraction conditions, respectively). Even though $P_{mb}O_2$ decreases under all

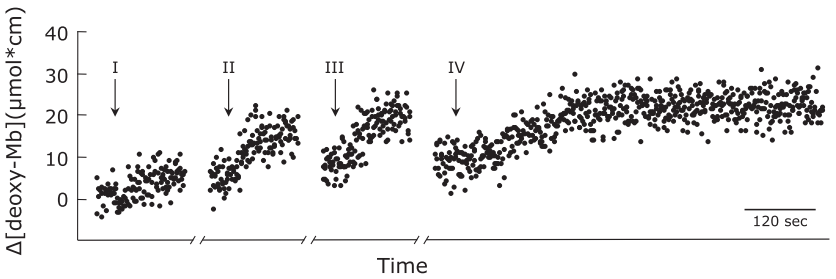

Figure 2. Representative time courses of the Δ[deoxy-Mb] NIRS signals during muscle contraction under different O_2 fraction conditions (I–III) and during anoxia perfusion (IV). The arrows indicate the onset of muscle contraction (I–III) and anoxic perfusion (95%N_2 + 5%CO_2; IV). Protocols I, II and III show the Δ[deoxy-Mb] signals during maximal twitch contraction under 95.0%, 71.3% and 47.5% O_2 fraction conditions in the Hb-free perfusion model. The Δ[deoxy-Mb] signals changed immediately at the onset of contraction and reached the steady state. As protocol IV was conducted after finishing protocol III, desaturated Mb already existed at a certain level at rest before perfusing anoxia buffer.

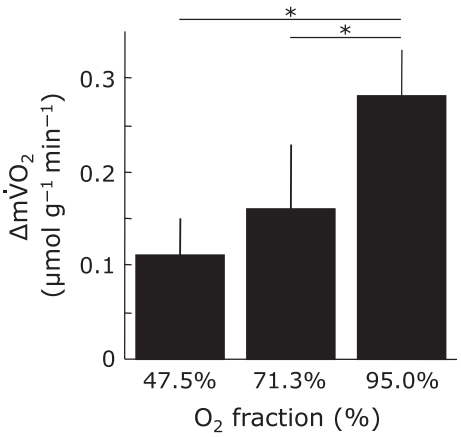

Figure 3. Change in net increase in muscle O_2 consumption (Δm$\dot{V}O_2$) due to muscle contraction for each O_2 fraction. The Δm$\dot{V}O_2$ due to muscle contraction decreased with the decrease in the O_2 supply volume. Δm$\dot{V}O_2$: net increase in muscle oxygen consumption due to contraction. Values are expressed as means ± SD (n = 5). *P < 0.05 vs. 95.0% O_2 fraction.

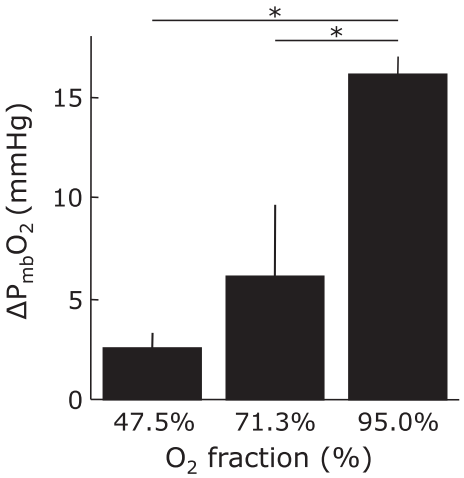

Figure 4. Net decrease in intracellular oxygen tension ($\Delta P_{mb}O_2$) due to muscle contraction for each O_2 fraction. The Δm$\dot{V}O_2$ due to muscle contraction decreased with the decrease in the O_2 supply volume. $P_{mb}O_2$ during contraction, hatched bars: net decrease in $P_{mb}O_2$. $\Delta P_{mb}O_2$: net decrease in intracellular oxygen tension equilibrated with O_2 saturation myoglobin. Values are expressed as means ± SD (n = 5). *P < 0.05 vs. 95.0% O_2 fraction.

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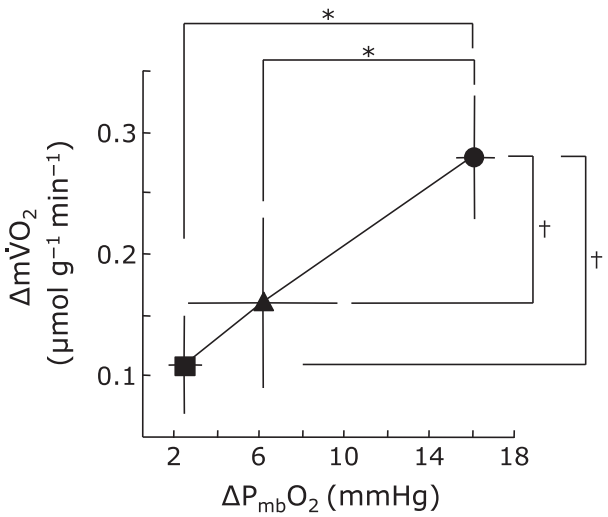

Figure 5. Relationship between net decrease in intracellular oxygen tension ($\Delta P_{mb}O_2$) and net increase in muscle oxygen consumption ($\Delta m\dot{V}O_2$) during muscle contraction. The $\Delta P_{mb}O_2$ and $\Delta m\dot{V}O_2$ gradually decreased as the O_2 supply volume decreased. The relationship between the $\Delta P_{mb}O_2$ and the $\Delta m\dot{V}O_2$ was represented with a line graph. $\Delta m\dot{V}O_2$: net increase in muscle oxygen consumption due to contraction. $\Delta P_{mb}O_2$: net decrease in intracellular oxygen tension equilibrated with O_2 saturation myoglobin. Each data point represents a mean ± SD. $n = 5 \times 3$ points. $^*P < 0.05$ for $\Delta m\dot{V}O_2$ parameter. $^\dagger P < 0.05$ for $\Delta P_{mb}O_2$ parameter.

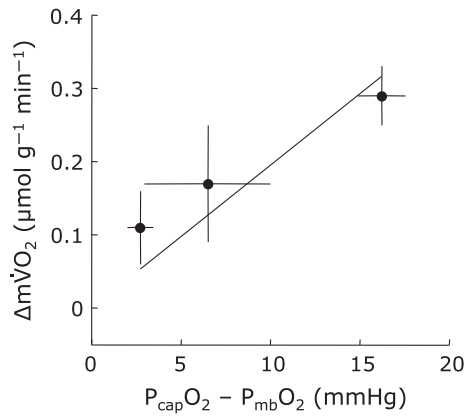

Figure 6. Relationship between delta change in muscle oxygen consumption ($\Delta m\dot{V}O_2$) and estimated O_2 gradient between microvascular oxygen tension ($P_{cap}O_2$) and intracellular oxygen tension ($P_{mb}O_2$) under exercising condition. Delta change in $m\dot{V}O_2$ due to muscle contraction increased linearly as a function of estimated O_2 gradient ($P_{cap}O_2$-$P_{mb}O_2$). Regression line is based on mean values ($m\dot{V}O_2 = 0.020 \times (P_{cap}O_2\text{-}P_{mb}O_2)$, $R^2 = 0.99$, $n = 5$ in each point). Each data point represents a mean ± SD.

exercise conditions, the value changes the most at 95% O_2. Figure 5 shows the relationship between the $\Delta m\dot{V}O_2$ and the $\Delta P_{mb}O_2$ in each O_2 fraction condition. During muscle contraction, the decrease in oxygen supply caused a decrease in the $\Delta P_{mb}O_2$, which led to a decrease in the $\Delta m\dot{V}O_2$.

The linear relationship between $\Delta m\dot{V}O_2$ and $\Delta P_{mb}O_2$ suggests that the O_2 gradient as reflected in ($P_{cap}O_2$-$P_{mb}O_2$) has changed. Using an estimate $P_{Cap}O_2$, the analysis shows that indeed $\Delta m\dot{V}O_2$ changes linearly with ($P_{cap}O_2$-$P_{mb}O_2$) (Fig. 6).

Discussion

Intracellular O_2 environment under hypoxic conditions

As oxygen delivery decreases, the $P_{mb}O_2$ at rest decreased correspondingly from 21.7 to 8.5 to 3.1 mmHg. The $m\dot{V}O_2$ decreases correspondingly from 0.55 to 0.43 to 0.34 μmol g^{-1} min^{-1}. It might appear that the decreasing O_2 supply leads consequently and directly to the decreasing $m\dot{V}O_2$. Yet in normoxic muscle the decreasing intracellular O_2 leads to an increasing $m\dot{V}O_2$, which implies a critical role for the O_2 gradient instead of the O_2 supply alone. An analysis of the O_2 gradient as reflected in ($P_{cap}O_2$-$P_{mb}O_2$) shows that despite the decreasing intracellular O_2, the O_2 gradient appears to have increased during hypoxia. The low $m\dot{V}O_2$ in resting muscle most likely creates a shallow gradient, which obscures measurement accuracy. Nevertheless, the constant L/P ratio would argue that the O_2 supply and $m\dot{V}O_2$ have not compromised oxidative metabolism, which would require glycolysis to compensate for any energy deficit. Consistent with a previous findings, the decreasing O_2 supply did not affect the low $m\dot{V}O_2$ at rest (Shiota and Sugano 1986).

However, when muscle begins to contract, $m\dot{V}O_2$ rose and the $P_{mb}O_2$ decreased further from the resting levels. At 47.5% O_2, the $P_{mb}O_2$ drops below the critical PO_2 level that limits oxidative phosphorylation in mitochondria (1.5 mmHg) (Kreutzer et al. 1992). L/P rises more at 47.5% O_2 than at 95.0% O_2. However, the drop in $P_{mb}O_2$ also expands the O_2 gradient under all O_2 saturation conditions during muscle contraction. But the widening O_2 gradient does not sufficiently enhance the O_2 flux to meet the cell's oxidative needs. Anaerobic glycolysis commences to supplement the energy demand, especially as the buffer PO_2 drops to 47%.

These findings indicate that even under hypoxia $m\dot{V}O_2$ rises during contraction. Given the same O_2 saturation conditions, the rise in $m\dot{V}O_2$ required an increase in DO_2 or ($P_{cap}O_2$-$P_{mb}O_2$) as shown in equation (Richardson et al. 1995; Takakura et al. 2010). Even though the $P_{mb}O_2$ decreases, the O_2 gradient has widened to increase the O_2 flux to balance out the O_2 supply and demand. A decreasing $P_{mb}O_2$ with a rising $m\dot{V}O_2$ agree with the

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previously reported observations (Molé et al. 1999; Takakura et al. 2010). In contrast, Richardson et al. (1995) reported that under normoxic or hypoxic condition, the $P_{mb}O_2$ did not decrease proportionally to muscle contraction, once the contraction has exceeded about 40% of maximum work exercise workload. Even under hypoxic condition, the Mb desaturation level does increase as the leg $\dot{V}O_2$ rose to its maximum level (Richardson et al. 1995). In addition, the $P_{mb}O_2$ reaches a low level (<5 mmHg) in the skeletal muscle, even when moderate levels of work were performed (Molé et al. 1999; Chung et al. 2005; Takakura et al. 2010). These findings have led researchers to ignore the $P_{mb}O_2$ variable from the formula for the calculation of the amount of O_2 consumption in the muscle tissue in hypoxic environments (Gonzalez et al. 2006).

However, probably due to a signal-to-noise ratio limitation, Richardson et al. (2006) could not detect the $P_{mb}O_2$ at rest in hypoxic muscle. As a result, the study could not determine the true magnitude of the change in the intracellular oxygen environment during exercise. Our study's unique experimental model, with the subjects placed under Hb-free perfusion, suggests that changes in the intracellular oxygen environment and the O_2 gradient can contribute to the $\Delta m\dot{V}O_2$ during hypoxia. In addition, the $P_{mb}O_2$ at the resting muscle did not reach a nadir as a result of the hypoxia. Instead, the $P_{mb}O_2$ can decreased further during the muscle contraction even in the hypoxic conditions.

Change in $P_{mb}O_2$ from muscle contraction modulating change in m$\dot{V}O_2$ under Hb-Free perfusion

The study investigated whether the m$\dot{V}O_2$ during muscle contraction in various oxygen concentration conditions depend on the changes in the $P_{mb}O_2$ or the O_2 gradient. Because the oxygen delivery remained constant at any muscle contraction condition in the perfusion model at a constant flow, the decrease in $P_{mb}O_2$ due to muscle contraction widen the O_2 gradient between the microvasculature and myocyte as reported in the our previous study (Takakura et al. 2010). This change contributes the increase in m$\dot{V}O_2$ due to muscle contraction. This interpretation is proven by the following equation;

$$m\dot{V}O_2 = kDO_2 \times (P_{cap}O_2 - P_{mb}O_2)$$

Because kDO_2 does not significantly contribute to the increase in m$\dot{V}O_2$ due to muscle contraction in the constant-flow hindlimb perfusion model, the m$\dot{V}O_2$ cannot increase unless the O_2 gradient expands by decrease in $P_{mb}O_2$ in our previous studies (Takakura et al. 2010, 2015). In the present study, the results have confirmed the hypothesis that the delta change in $P_{mb}O_2$ and in the O_2 gradient contributes to delta increase in m$\dot{V}O_2$ during muscle contraction. Our results show that under all oxygenation conditions, m$\dot{V}O_2$ rises but $P_{mb}O_2$ continues to decline by muscle contraction between 77% and 88% of maximal voluntary contraction (MVC). However, rise in the m$\dot{V}O_2$ and O_2 gradient expansion under hypoxia conditions are much less than the corresponding changes under normoxic conditions. The observation then agree with the studies by Molé et al. (1999) and Takakura et al. (2010), which indicate the progressive expansion of the O_2 gradient between the $P_{cap}O_2$ and the $P_{mb}O_2$ during muscle contraction contribute to the O_2 flux supporting the rising m$\dot{V}O_2$. No $P_{mb}O_2$ plateau even under hypoxia condition seems apparent at MVC well above 40%.

Moreover, a changing m$\dot{V}O_2$ in constant flow model also supports a $P_{mb}O_2$ contribution. In constant flow perfusion, a constant flow-induced vasodilatation occurs at a given flow rate. As a consequence, the model maintains a constant diffusion conductance throughout the perfusion (Hepple et al. 2003). Consequently, any change in $\Delta m\dot{V}O_2$ must have a contribution from $P_{mb}O_2$. Therefore, the mechanism behind the m$\dot{V}O_2$ in hypoxic environments, includes then a contribution $P_{mb}O_2$ (Molé et al. 1999; Takakura et al. 2010).

The mechanism of the oxygen transport to the mitochondria in the myocytes is dependent on the Mb-mediated oxygen transport or the dissolved O_2 flux. The contribution from the Mb-mediated oxygen transport increases with decreasing $P_{mb}O_2$. Below the equipoise diffusion PO_2 value (1.77 mmHg), 50% of the total oxygen transport to the mitochondria stems from the Mb-mediated oxygen transport (Lin et al. 2007). The $P_{mb}O_2$ during muscle contraction in the 47.5% O_2 fraction condition was 0.4 ± 0.3 mmHg. As this was below the equipoise diffusion PO_2 value, the Mb-mediated oxygen transport mechanism will dominate. In addition, any increase in Mb concentration will raise the equipoise PO_2 (Lin et al. 2007; Takakura et al. 2015). Because people acclimated to high altitudes often have a high concentration in Mb, muscle contraction under hypoxic conditions in these people may also benefit from Mb-mediated transport (Reynafarje 1962; Reynafarje and Morrison 1962).

Van der Laarse et al. (2005) have examined about the value of the diffusion coefficient for oxygen from extracellular region in muscle. The diffusion coefficient is important because it is a determinant of the extracellular oxygen tension at which the core of muscle fibers becomes anoxic (PO_{2crit}). According to Hill (1965), PO_{2crit} at the maximum rate of oxygen consumption ($\dot{V}O_{2max}$; in nmol mm^{-3} sec^{-1}) is given by

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$$PO_2crit = \dot{V}O_2max \times CSA/4\pi D\alpha$$

where CSA is the cross-sectional area of the cell (mm^2), D is the diffusion coefficient for oxygen in the muscle cell (mm^2/sec), and α is the solubility of oxygen in muscle cell (mmol/L per mmHg). Dα is known as Krogh's diffusion coefficient. Dα seems to depend on fat content, extracellular space, and unknown factors (Jones and Kennedy 1986; Dutta and Popel 1995; Baranov et al. 2000). Indeed, Dα increased with enlargement of extracellular space in the preparation (van der Laarse et al. 2005). However, the results of the previous study indicated that Hill's model for oxygen diffusion in valid for single muscle fibers without myoglobin and a fairly homogeneous mitochondrial distribution consuming oxygen at the maximum rate. Because above Hill's equation is based on the following assumption: (1) the cross section of the fiber is a circle, and oxygen diffuses in the radial direction only; (2) $\dot{V}O_2$ is distributed homogeneously in the cells; (3) $\dot{V}O_2$ by mitochondria is independent of local intracellular PO_2; and (4) myoglobin-facilitated oxygen diffusion is negligible, the role of Mb was not taken account of above Dα. We have conducted the maximal twitch contraction under three O_2 fraction conditions to the same individual in the present perfusion model. Moreover, as the order of the three O_2 condition was randomized and no sign of edema in the hindlimb was seen at the given flow rate, change in extracellular space would be almost none. Thus, Dα in the above equation would not been affected throughout the perfusion experiment in this study. On the other hand, others and we reported the effect of Mb on the intracellular O_2 diffusion to the mitochondria (Lin et al. 2007; Takakura et al. 2010, 2015). As mentioned above, Lin et al. (2007) reported that 50% of the total oxygen transport to the mitochondria stems from the Mb-mediated oxygen transport below the equipoise diffusion PO_2 value (1.77 mmHg). Also, we reported that decrease in $P_{mb}O_2$ directly contribute the expansion of the O_2 gradient to lead the increased m$\dot{V}O_2$ (Takakura et al. 2010, 2015). As for a key factor regulating O_2 diffusion to intracellular mitochondria, the change in $P_{mb}O_2$ might be one of candidates, because the change in $P_{mb}O_2$ affects O_2 gradient, Mb-mediated O_2 transport and mitochondria respiratory.

Conclusion

This study investigated the effects of reduced oxygen supply on the $P_{mb}O_2$ at rest and during muscle contraction, as well as the effect of the $\Delta P_{mb}O_2$ on the $\Delta m\dot{V}O_2$ during muscle contraction. Our findings showed that the resting $P_{mb}O_2$ decreases with the decrease in oxygen supply. However, the decrease in $P_{mb}O_2$ expands the O_2 gradient, which then supports the rising $\Delta m\dot{V}O_2$ during muscle contraction even under hypoxic conditions. The results suggest that the $\Delta P_{mb}O_2$ is a key determinant factor of the $\Delta m\dot{V}O_2$.

Conflict of interest

There is no conflict of interest for this study.

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