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At onset of muscle contraction, myoglobin (Mb) immediately releases its bound O₂ to the mitochondria. Accordingly, intracellular O₂ tension (P_{mb}O₂) markedly declines in order to increase muscle O₂ uptake (m \dot{V} O₂). However, whether the change in P_{mb}O₂ during muscle contraction modulates m \dot{V} O₂ and whether the O₂ release rate from Mb increases in endurance-trained muscles remain unclear. The purpose of this study was, therefore, to determine the effect of endurance training on O₂ saturation of Mb (S_{mb}O₂) and P_{mb}O₂ kinetics during muscle contraction. Male Wistar rats were subjected to a 4-week swimming training (Tr group; 6 days per week, 30 min \times 4 sets per day) with a weight load of 2% body mass. After the training period, deoxygenated Mb kinetics during muscle contraction were measured using near-infrared spectroscopy under hemoglobin-free medium perfusion. In the Tr group, the m \dot{V} O₂ peak significantly increased by 32%. Although the P_{mb}O₂ during muscle contraction did not affect the increased m \dot{V} O₂ in endurance-trained muscle, the O₂ release rate from Mb increased because of the increased Mb concentration and faster decremental rate in S_{mb}O₂ at the maximal twitch tension. These results suggest that the Mb dynamics during muscle contraction are contributing factors to faster \dot{V} O₂ kinetics in endurance-trained muscle.

Relative to control muscle, endurance-trained muscle increases O₂ consumption at the same level of maximal voluntary contraction (MVC) and increases maximal O₂ consumption, which is considered an indicator of improved aerobic exercise capacity. The increased O₂ consumption in the trained skeletal muscle depends on both O₂ utilization and vascular O₂ supply. Muscle O₂ utilization capacity is mainly determined by mitochondrial function and the quantity of mitochondria, whereas O₂ supply capacity to the mitochondria is determined by capillarization. Many studies have reported that endurance training upregulates mitochondrial function, and mitochondria number and volume^{1–3}. It also increases capillarization^{1–3}. However, the contribution of O₂ diffusion from the capillary to the mitochondria is still unknown, especially with respect to the intracellular factors involved in O₂ transport from the sarcolemma to the mitochondria.

Recent studies have shown that the O₂ gradient can contribute to the enhanced O₂ flux to meet the increased muscle O₂ demand during contraction⁴. The O₂ saturation of Mb (S_{mb}O₂), which reflects the intracellular O₂ tension (P_{mb}O₂), decreases as work intensity and muscle oxygen consumption (m \dot{V} O₂) increase. The decreasing P_{mb}O₂ expands the O₂ gradient from the capillary to the muscle cell to increase the O₂ flux from the vasculature to the mitochondria. Whether the O₂ gradient contributes to the increased O₂ uptake in endurance-trained muscle remains uncertain. With the experimental model to investigate the intracellular O₂ dynamics⁴, we have hypothesized that the increase in O₂ consumption in endurance-trained skeletal muscle is accompanied with increased expansion of the O₂ gradient across the plasma membrane⁴, because studies have already shown that the change in P_{mb}O₂ during exercise can play a key role in \dot{V} O₂ regulation^{5,6}.

Because training also induces an acceleration of \dot{V} O₂ kinetics at the onset of muscle contraction, which pulmonary \dot{V} O₂ measurements have detected and have attributed to adjustments of oxidative metabolism at the skeletal muscle level^{7–9}, a faster \dot{V} O₂ on-kinetics could be an important adaptation, as it would potentially incur a smaller O₂ deficit. Previous studies have already shown Mb contribution to the intracellular O₂ dynamics,



which affects the $m\dot{V}O_2$ response at the onset of muscle contraction^{4,5,10,11}. Nuclear magnetic resonance and near-infrared spectroscopic (NIRS) experiments clearly show that Mb immediately releases O_2 at the onset of muscle contraction and provides the initial O_2 supply to support the rapid increase in $m\dot{V}O_2$ ^{4,5,10,11}. The sudden increase in $m\dot{V}O_2$ does not appear to depend on muscle adenosine diphosphate concentration and thereby implicates a direct and immediate role for Mb-mediated O_2 delivery⁵.

By using a hemoglobin (Hb)-free rat hind limb perfusion model, the present study shows that at all relative levels of MVC, the $m\dot{V}O_2$ of endurance-trained muscle exceeds that of the control muscle. Trained muscle also reaches a higher peak $m\dot{V}O_2$. Even though the $P_{mb}O_2$ of both the control and endurance-trained muscle decreases with increasing exercise intensity, the O_2 gradient from capillary to the mitochondria does not change significantly to accommodate the differences in the observed $m\dot{V}O_2$. At any given MVC, the endurance-trained muscle exhibits a smaller O_2 gradient. However, the endurance-trained muscles exhibit a faster release of O_2 from Mb at the initiation of contraction, consistent with the enhanced $m\dot{V}O_2$ and with the Mb-mediated O_2 supply. Indeed the kinetics of O_2 release from Mb can serve as an index of the change in intracellular $m\dot{V}O_2$ as muscle undergoes training⁵.

Results

Descriptive data for muscle weight are presented in Table 1. Although endurance training caused a significant reduction in body and muscle mass, the ratio of muscle mass to body mass differed slightly between the groups (with a difference in mean value of 0.1%).

Table 2 shows the contractile and metabolic properties of the control and trained hind limb muscles. Although both groups showed no significant difference in $m\dot{V}O_2$ at rest, at maximal tension, the values of the peak $m\dot{V}O_2$ per gram per minute in the swimming training (Tr) group were significantly higher than those in the control (Con) group. [Mb] and citrate synthase (CS) activities in the deep portion of gastrocnemius muscle significantly increased after endurance training, whereas the lactate-to-pyruvate ratio (L/P) decreased at peak maximal twitch tension. Table 3 summarizes muscle tension, the net increase in $m\dot{V}O_2$ due to muscle contraction ($\Delta m\dot{V}O_2$), O_2 cost, and kinetics parameters for $S_{mb}O_2$ and $P_{mb}O_2$ at each tension level for both groups. Muscle tension, $\Delta m\dot{V}O_2$, and O_2 cost increased as work intensity increased.

Figure 1 shows the representative kinetics of $S_{mb}O_2$ in each group. As for the $S_{mb}O_2$ kinetic parameters, the steady-state value, amplitude (AP), and mean rate of change to 63% of the AP value ($_{0.63}AP$ /mean response time [MRT]) increased as work intensity increased in both groups, whereas MRT tended to accelerate in both groups. At maximal tension, the steady-state value and AP of $S_{mb}O_2$ kinetic parameters in the Tr group were unchanged, but $_{0.63}AP$ /MRT of the kinetic parameters for $S_{mb}O_2$ increased. The MRT also tended to be faster in the Tr group. At submaximal tension levels, the steady-state value, AP, and $_{0.63}AP$ /MRT in the Tr group were also unchanged, but the MRT of the kinetic parameters for $S_{mb}O_2$ tended to be faster. The kinetic parameters for $P_{mb}O_2$ showed that the steady-state value, AP, and $_{0.63}AP$ /MRT increased, and the MRT

became faster in both groups as work intensity increased. When the kinetic parameters for $P_{mb}O_2$ were compared at the same relative tension level between both groups, the relative temporal parameters for $P_{mb}O_2$ kinetics in the trained muscle showed a tendency to accelerate to a higher level. In the present study, while the MRT was used to describe the overall dynamics of $S_{mb}O_2$ and $P_{mb}O_2$ fall following the onset of muscle contraction, $_{0.63}AP$ /MRT is the effective temporal parameter to show deoxygenation rate of Mb- O_2 per unit time in the initial phase of muscle contraction. The $_{0.63}AP$ /MRT would reflect the steep change in mitochondrial oxygen demand, because we previously reported that $_{0.63}AP$ /MRT increased in response to change in mitochondrial oxygen demand due to muscle contraction. Kindig et al.¹² also used AP/time constant in intracellular PO_2 kinetics during muscle contraction as an index of initial metabolic response. As for $_{0.63}AP$ /MRT parameter in the present study, while its value showed significant difference at 100% of the maximal twitch tension by endurance training, it did not differ at 50% and 75% of the maximal twitch tension between Con and Tr group. This result at submaximal tension level might be caused by non-significant difference in O_2 demand level during muscle contraction at the relative same intensity between groups. $\Delta m\dot{V}O_2$ did not actually show the significant difference at the 50% and 75% of the maximal twitch tension between groups. On the other hand, at the maximal twitch tension level, $_{0.63}AP$ /MRT parameter and $\Delta m\dot{V}O_2$ in the trained muscle showed significant difference compared with that in the control muscle.

Figure 2 shows the relationship between muscle tension and the net increase in $m\dot{V}O_2$ during muscle contraction for both groups. While muscle tension and $\Delta m\dot{V}O_2$ were significantly correlated in both groups, the mean individual slope in the Tr group ($0.36 \pm 0.11 \times 10^{-2} \mu\text{mol}/[\text{g}^2 \cdot \text{min}]$) tended to be higher than that in the Con group ($0.27 \pm 0.06 \times 10^{-2} \mu\text{mol}/[\text{g}^2 \cdot \text{min}]$; $p = 0.058$).

Figure 3 shows the relationship between intracellular $[O_2]$ and $\Delta m\dot{V}O_2$ during muscle contraction. Intracellular $[O_2]$ was based on the $S_{mb}O_2$ - $P_{mb}O_2$ equilibrium. In the present study, the $S_{mb}O_2$ at rest was assumed to be 90%. Intracellular $[O_2]$ decreased markedly from 29.2 μM at rest to 9.2 ± 3.0 , 5.1 ± 2.1 , and $3.3 \pm 1.0 \mu\text{M}$ at 50%, 75%, and 100% of maximal contraction in the Con group, respectively; and from 29.2 μM at rest to 12.1 ± 4.7 , 5.8 ± 1.9 , and $3.2 \pm 0.7 \mu\text{M}$ at 50%, 75%, and 100% of maximal contraction in the Tr group, respectively. Although intracellular $[O_2]$ decreased markedly with the $\Delta m\dot{V}O_2$ in both groups, the Tr group curve showed a smaller $[O_2]$ decline. At the same level of intracellular $[O_2]$ in the muscle cell, $\Delta m\dot{V}O_2$ in the trained muscle was higher than in the control muscle, suggesting that the trained muscle had more oxidative potential capacity compared with the control muscle.

Figure 4 shows the O_2 release rate from Mb at same percent of MVC in the Tr and Con groups. The O_2 release rate from Mb increased progressively with the twitch tension level as follows: 1.1 ± 0.3 , 2.3 ± 0.4 , and $3.7 \pm 0.8 \times 10^{-2} \mu\text{mol}/(\text{g} \cdot \text{min})$ at 50%, 75%, and 100% of maximal contraction in the Con group, respectively; and 1.1 ± 0.5 , 2.6 ± 0.7 , and $4.6 \pm 0.5 \times 10^{-2} \mu\text{mol}/(\text{g} \cdot \text{min})$ at 50%, 75%, and 100% of maximal contraction in the Tr group, respectively. At maximal tension, the O_2 release rate from Mb showed a significant increase in the Tr group, suggesting more O_2 supply from Mb to the mitochondria at the onset of muscle contraction.

Table 1 | Descriptive data for the muscle weight

Parameter	Unit	Con	Tr
Body Mass	g	269.0 ± 11.6	255.4 ± 22.7
Muscle Mass			
m. Gastrocnemius	mg	1711.1 ± 73.8	1541.0 ± 136.7
m. Plantaris	mg	346.9 ± 15.0	289.3 ± 25.7 *
m. Soleus	mg	126.4 ± 5.5	98.2 ± 8.7 *
GPS	mg	2141.6 ± 92.4	1928.4 ± 171.0 *

Values are mean ± SD (n = 9 in each group). Con: control group. Tr: training group. GPS: gastrocnemius-plantaris-soleus. A superscript (*) indicates a significant difference ($p < 0.05$ vs. Con).



Table 2 | Contractile and metabolic properties of hindlimb muscles

Parameter	Unit	Con	Tr
Maximal Tension	g	73.7 ± 10.6	92.0 ± 21.9 *
m $\dot{V}O_2$ at rest	$\mu\text{mol g}^{-1} \text{min}^{-1}$	0.48 ± 0.09	0.58 ± 0.13
m $\dot{V}O_2$ peak	$\mu\text{mol g}^{-1} \text{min}^{-1}$	0.70 ± 0.10	0.93 ± 0.16 *
[Mb]	$\mu\text{mol g}^{-1}$	0.10 ± 0.01	0.12 ± 0.01 *
CS activity	$\mu\text{mol g}^{-1} \text{min}^{-1}$	28.4 ± 1.1	40.8 ± 6.7 *
L/P ratio		19.1 ± 2.22	12.5 ± 5.2 *

Values are mean ± SD (n = 9 in each group for physiological parameters, n = 6 in each group for biochemical parameters). Con: control group. Tr: training group. [Mb]: Mb concentration. CS activity: citrate synthase activity. L/P ratio: Lactate to pyruvate ratio measured in effluent perfusate at the maximal twitch tension. As for [Mb] and CS activity, the deep portion of gastrocnemius muscle was used for the measurement as a representative muscle. A superscript (*) indicates a significant difference (p < 0.05 vs. Con).

Discussion

Effect of endurance training on muscle oxidative capacity. In the present study, 4 weeks of swimming endurance training resulted in an increase in m $\dot{V}O_2$ peak, even when O₂ delivery to the endurance-trained hind limb was not greater than that supplied to sedentary muscles. This increase in m $\dot{V}O_2$ peak value at constant flow was consistent with previous studies^{2,15}. This increase in m $\dot{V}O_2$ peak value without increase in O₂ delivery to the hind limb muscle would be caused by increased of both O₂ supply capacity to the mitochondria and O₂ utilization capacity such as capillary density, mitochondrial respiration capacity, and Mb function in the active muscle. At the equivalent muscle tension, the Tr group showed a slightly higher $\Delta m\dot{V}O_2$ than the Con group (Fig. 2). As reflected by a higher CS activity, the endurance-trained muscle had higher muscle oxidative potential. The increase in O₂ consumption and O₂ cost at a given work rate would imply a shift to more aerobic metabolism during muscle contraction.

The decrease in L/P at the maximal twitch tension also suggested a greater capacity to oxidize carbohydrate and a tightening in the

coupling between ATP supply and demand^{13,14}. This tight integration of ATP supply and demand is associated with less stimulation of glycolysis, resulting in a decrease in lactate production and a lower cytosolic redox state, and thus an improved coupling between pyruvate oxidation and glycolytic flux^{13,15}. Collectively, the swimming endurance training in the present study enhanced muscle oxidative capacity, in agreement with evidence previous studies^{1,16,17}.

Relationship between P_{mb}O₂ kinetics and muscle oxygen consumption. Endurance training increases both m $\dot{V}O_2$ peak and $\Delta m\dot{V}O_2$ at the same percentage of MVC. However, both control and trained muscle show a steady decline in P_{mb}O₂ with increasing MVC. The declining P_{mb}O₂ and the increasing O₂ consumption indicates an expansion of the O₂ gradient across the plasma membrane. Both the O₂ diffusion conductance (DO₂) and O₂ gradient between P_{cap}O₂ and P_{mb}O₂ can influence the O₂ flux into the cell, which then supports the m $\dot{V}O_2$. In our perfusion model, the O₂ diffusion conductance would show little change even at the onset of contraction. Fick's first law of diffusion relates explicitly the

Table 3 | Muscle tension, muscle oxygen consumption, S_{mb}O₂ and P_{mb}O₂ kinetics parameters during muscle contraction at each tension level

Parameter	Unit	Group	Tension Level (% of maximal twitch tension)		
			50%	75%	100%
Muscle Tension	g	Con	36.4 ± 6.4 ^{abc}	54.2 ± 9.6 ^a	73.7 ± 10.6
		Tr	53.5 ± 15.0 ^a	70.9 ± 18.3 ^a	92.0 ± 21.9
$\Delta m\dot{V}O_2$	$\mu\text{mol g}^{-1} \text{min}^{-1}$	Con	0.08 ± 0.04 ^{abc}	0.13 ± 0.05 ^{ab}	0.22 ± 0.06 ^a
		Tr	0.13 ± 0.06 ^{ab}	0.25 ± 0.11 ^a	0.36 ± 0.11
O ₂ cost	10 ⁻² $\mu\text{mol g}^{-2} \text{min}^{-1}$	Con	0.22 ± 0.08 ^a	0.25 ± 0.10 ^a	0.30 ± 0.08
		Tr	0.25 ± 0.08 ^a	0.35 ± 0.13	0.40 ± 0.12
S _{mb} O ₂ kinetics					
Steady-State Value	%	Con	72.7 ± 6.5 ^{acd}	58.7 ± 10.9 ^c	49.0 ± 8.1 ^b
		Tr	76.8 ± 7.9 ^{abd}	62.7 ± 7.9 ^a	49.5 ± 5.3
AP	%	Con	-19.2 ± 7.0 ^{acd}	-31.2 ± 11.0 ^c	-41.0 ± 8.1 ^b
		Tr	-13.2 ± 7.9 ^{abd}	-27.3 ± 7.9 ^a	-40.5 ± 5.3
MRT	s	Con	63.0 ± 18.2 ^{ac}	52.2 ± 14.0	43.7 ± 6.6
		Tr	52.6 ± 12.4	48.3 ± 10.2	39.3 ± 5.9
0.63AP/MRT	% s ⁻¹	Con	-0.18 ± 0.06 ^{abcd}	-0.37 ± 0.07 ^{ac}	-0.58 ± 0.10 ^a
		Tr	-0.15 ± 0.07 ^{abcd}	-0.36 ± 0.10 ^a	-0.65 ± 0.08
P _{mb} O ₂ kinetics					
Steady-State Value	mmHg	Con	6.9 ± 2.2 ^{ac}	3.8 ± 1.5	2.4 ± 0.8
		Tr	8.9 ± 3.4 ^{abcd}	4.3 ± 1.4	2.4 ± 0.5
AP	mmHg	Con	-15.0 ± 2.3 ^{ac}	-17.6 ± 1.8	-19.1 ± 0.7
		Tr	-12.6 ± 3.4 ^{abcd}	-17.3 ± 1.4	-19.2 ± 0.5
MRT	s	Con	43.8 ± 10.3 ^{abc}	34.6 ± 5.6	29.5 ± 5.7
		Tr	43.2 ± 9.4 ^{ac}	33.7 ± 4.6	25.9 ± 5.2
0.63AP/MRT	mmHg s ⁻¹	Con	-0.23 ± 0.06 ^{abcd}	-0.33 ± 0.06 ^a	-0.42 ± 0.07
		Tr	-0.19 ± 0.06 ^{abcd}	-0.23 ± 0.06 ^{ac}	-0.47 ± 0.09

Values are mean ± SD (n = 9 in each group). Con: control group. Tr: training group. $\Delta m\dot{V}O_2$ is the net increase in m $\dot{V}O_2$. 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. 0.63AP/MRT is calculated by dividing 0.63AP by MRT. Superscripts indicate a significant difference (a: p < 0.05 vs. Tr × 100%; b: p < 0.05 vs. Tr × 75%; c: p < 0.05 vs. Con × 100%; d: p < 0.05 vs. Con × 75%).

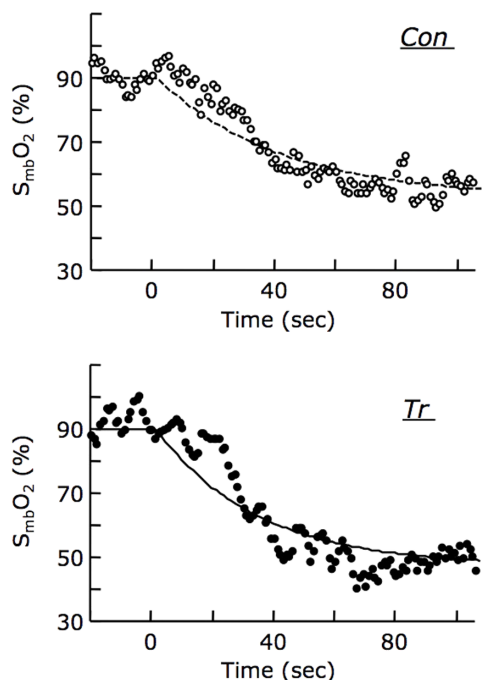


Figure 1 | Representative kinetics of myoglobin (Mb) saturation (S_{mbO_2}) during maximal twitch contraction (1 Hz) in the training (Tr) and control (Con) groups. The plots of S_{mbO_2} show representative data at the maximal twitch tension from the single experiment in each group. While the S_{mbO_2} kinetics (dotted line) in the representative control rat declined with a mean response time (MRT) of 39.5 sec (upper panel), the S_{mbO_2} kinetics (solid line) in the representative trained rat declined with a MRT of 33.0 sec (lower panel). The MRT in the S_{mbO_2} kinetics was shortened by 5 sec on average due to endurance training. By contrast, the S_{mbO_2} value at steady state did not show any significant difference between the two groups.

change in substance concentration over time depends upon the gradient of concentration over space. In a one-dimension case for O_2 diffusion in the x-direction, the equation clearly states that:

$$J = -D \frac{\partial C}{\partial x} \quad (1)$$

J = diffusion flux (amount of O_2 crossing a unit area per unit time), D = diffusion coefficient (length of unit area squared \times time $^{-1}$), $\frac{\partial C}{\partial x}$ = the change in O_2 concentration along dimension x or the O_2 gradient along the x -direction. 1H -NMR experiments show Mb desaturating and the cellular PO_2 decreasing rapidly upon the initiation of muscle contraction 5,6 . The debate remains whether with increasing exercise intensity and associated increasing respiration, does the gradient expand or does it reach a plateau. Our experiment data show the gradient expanding. Conductance may still contribute to O_2 transport into the cell. However, our experiments provide no supporting or contradictory data. They only show that blood flow has not changed. Indeed, we used the constant flow mode and an Hb-free Krebs-Henseleit buffer as perfusate in this perfusion experiment. By using the constant flow mode, the perfusion pressure remained constant throughout the perfusion period, which suggested the convective O_2 delivery did not change both at rest and during muscle contraction. Additionally, based on the fact that a 30-min equilibrium period elicited a flow-induced vasodilatation corresponding to the given flow rate 18 , we assumed that convective O_2 delivery was maintained during the perfusion period. Also, as the perfusate did not contain an Hb in this study, muscle contraction would have little

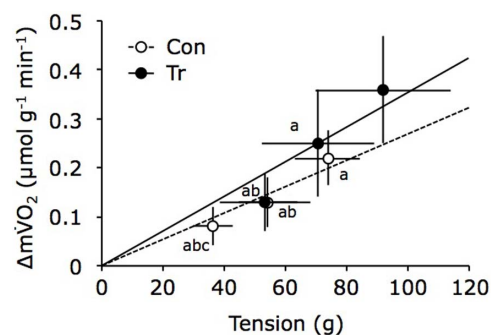


Figure 2 | Relationship between muscle tension and $\Delta m\dot{V}O_2$ during twitch contraction in the training (Tr) and control (Con) groups. Changes in muscle O_2 uptake ($\Delta m\dot{V}O_2$) due to muscle contraction increased linearly as a function of muscle tension in both groups. Regression lines are based on mean values ($n = 9$ in each group; Con: $\Delta m\dot{V}O_2 = 0.003 \times$ Tension, $R^2 = 0.98$, $p < 0.05$; Tr: $\Delta m\dot{V}O_2 = 0.004 \times$ Tension, $R^2 = 0.99$, $p < 0.05$). The data represent the mean \pm standard deviation values. The superscript indicates a significant difference (a: vs. Tr \times 100%, $p < 0.05$; b: vs. Tr \times 75%, $p < 0.05$; c: vs. Con \times 100%, $p < 0.05$).

affect to skeletal muscle capillary hemodynamic in contrast to the previous study of Kindig et al. 19 that muscle contraction induced rapid increase of RBC flux and velocity, and capillary hematocrit at the microcirculatory level. Thus, the supposition of a requisite and predominant conductance role in diffusion cannot undermine the observation of an expanding O_2 gradient that coincides with an increased $m\dot{V}O_2$.

Because the steady-state $P_{cap}O_2$ level doesn't change 20 and the $P_{mb}O_2$ decreases with increasing exercise intensity, the increase in $\Delta m\dot{V}O_2$ and $m\dot{V}O_2$ peak in the Tr group must also have a contribution from an increased capillarization and/or a greater DO_2 1 . In the extracellular segment, the angiogenesis of the muscle capillary in the hind limb muscles during training would enhance the supply of O_2 . An increase in capillarity would improve blood flow-to- $\dot{V}O_2$ relationships within the muscle, allowing for a greater O_2 extraction 1 . The change in capillarity can also affect DO_2 . A greater DO_2 after exercise training implies a decreased mean O_2 diffusion distance from the tissue capillaries to the muscle mitochondria 21,22 . In the intracellular segment, O_2 transport to the mitochondria depends upon Mb-mediated O_2 flux and free O_2 . The Mb-mediated O_2 flux in the trained muscle would show a greater value at a relatively higher

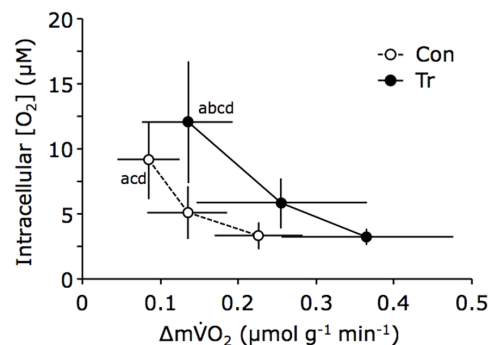


Figure 3 | Relationship between intracellular $[O_2]$ and $\Delta m\dot{V}O_2$ during twitch contraction in the training (Tr) and control (Con) groups. Intracellular $[O_2]$ (in μM) decreased gradually with the increase in changes in muscle O_2 uptake ($\Delta m\dot{V}O_2$) in both groups. The relationship between intracellular $[O_2]$ and $\Delta m\dot{V}O_2$ was shown as a line graph. Each data point represents the mean \pm standard deviation. The superscript letters indicate significant differences (a: vs. Tr \times 100%, $p < 0.05$; b: vs. Tr \times 75%, $p < 0.05$; c: vs. Con \times 100%, $p < 0.05$; and d: vs. Con \times 75%).

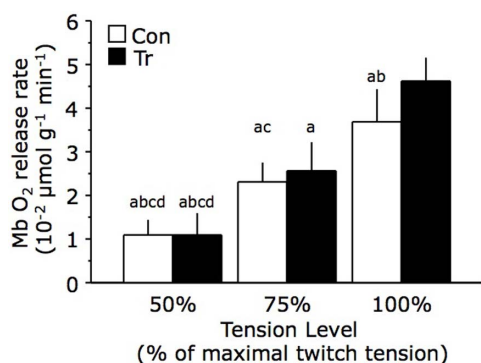


Figure 4 | The O₂ release rate from myoglobin (Mb) at the each tension level in the training (Tr) and control (Con) groups. At the onset of muscle contraction, Mb released its bound O₂ to the mitochondria with the twitch tension level. The data show the mean ± standard deviation values. The superscript letters indicate significant differences (^a: vs. Tr × 100%, $p < 0.05$; ^b: vs. Tr × 75%, $p < 0.05$; ^c: vs. Con × 100%, $p < 0.05$; and ^d: vs. Tr × 50%, $p < 0.05$).

tension level where intracellular O₂ tension (equivalent with $P_{mb}O_2$) became lower, because an adaptive increase in Mb concentration led to the increase in Mb-O₂ flux, as also shown by a previous study²³. Therefore, increased augmentation of capillarity and Mb-mediated O₂ flux during muscle contraction can enhance the capacity supplying O₂ to the mitochondria to support the increased $\Delta m\dot{V}O_2$ and $m\dot{V}O_2$ peak after endurance training. The experiment results show that the hypothesized expansion of O₂ gradient due to further decrease in $P_{mb}O_2$ does not explain the overall increase in $\Delta m\dot{V}O_2$ and $m\dot{V}O_2$ peak in endurance-trained muscle.

O₂ release rate from Mb at the onset of muscle contraction.

Endurance training usually results in faster $\dot{V}O_2$ kinetics²⁴, which will presumably experience result in a smaller decrease in muscle phosphocreatine concentration, a smaller increase in lactate and proton (H⁺) production, and a reduced degradation of muscle glycogen, compared with an individual with slow $\dot{V}O_2$ on-kinetics^{25–27}. Improvement in mitochondrial respiration capacity itself would largely contribute to this adaptation as a result of endurance training. However, no study has investigated the O₂ supply to the mitochondria at the intracellular level.

We previously found that Mb supplied O₂ immediately at the onset of muscle contraction and that the O₂ release rate from Mb increased linearly as the O₂ demand increased^{4,28}. These facts suggest that Mb provides an immediate O₂ source for the sudden increase in $m\dot{V}O_2$ at the onset of muscle contraction. The present study reveals an increase in the O₂ release rate from Mb at the onset of muscle contraction at the maximal twitch tension after endurance training. Myocyte experiments have also suggested that a direct Mb-mediated oxygen delivery might contribute to mitochondrial respiration²⁹. The blockade of Mb oxygen-binding capacity suppressed approximately 70% of mitochondrial respiration, even under the condition of sufficiently available O₂²⁹. Indeed, the fact that the O₂ release rate from Mb at the onset of muscle contraction increases progressively as O₂ demand increases might indicate that Mb-supplied O₂ may directly influence $m\dot{V}O_2$ kinetics⁴. Taking these findings together, both mitochondrial respiration capacity and O₂ release rate from Mb might be important factors that regulate $m\dot{V}O_2$ kinetics at the onset of muscle contraction.

The binding of O₂ to Mb and Hb certainly proceeds much faster than transport^{30,31}, even though the rate-determining step depends on a much slower off rate. But, dismissing any contribution of Mb in regulating respiration in the cell (an inhomogeneous and compartmentalized system) based on just the steady-state rate determining step argument seems tenuous. If the blood delivers a sufficient O₂

supply, the cell would not need to withdraw O₂ from its Mb reservoir at the start of muscle contraction. But the cell does withdraw O₂ from Mb, as our data and all ¹H-NMR data show, and takes a finite amount of time to reach a new steady state^{5,6}. Thermodynamics requires a demand to elicit the loss of O₂ from Mb, and both ATP utilization and respiration surge once contraction starts. The kinetics coincides with O₂ release from Mb. Thus, the postulated regulatory relationship between the O₂ release rate from Mb and $m\dot{V}O_2$ seems quite reasonable and consistent with the postulated role of Mb. Once Mb desaturation has reached a new steady state, vascular O₂ supply must begin to contribute significantly to sustain the rising $m\dot{V}O_2$. To avoid the missteps in the rate limiting step approach, metabolic control theory vantage advocates examining the relative contribution of MbO₂ and O₂ to the regulation of $m\dot{V}O_2$. Note that Mb never resaturates to its control level as long as the muscle contraction is sustained. The cellular PO₂ drops during contraction, consistent with an enhanced O₂ gradient.

The O₂ release rate from Mb reflects the intracellular $m\dot{V}O_2$ ^{5,6}. Consequently, the enhanced intracellular $m\dot{V}O_2$ observed after endurance training could be induced by a 30% increase in [Mb] concentration and a 12% acceleration in the Mb deoxygenation rate at the maximal twitch tension. Tables 2 and 3 show that an increase in [Mb] predominantly contributes to an increase in O₂ release rate from Mb in the trained skeletal muscles. Although several studies have reported that endurance training produces an increased [Mb] in rat limb muscle^{16,17,32,33}, the physiological significance of increased [Mb] has not been demonstrated *in vivo*. The present study also demonstrated that the deoxygenation rate of Mb became faster at the onset of muscle contraction after endurance training, suggesting a more efficient O₂ transport from Mb to the mitochondria at the transient phase.

In addition, the $P_{mb}O_2$ response at the onset of muscle contraction showed the tendency to be faster after endurance training in the present study. Hirai et al.²⁰ reported that endurance training led to slower $P_{cap}O_2$ kinetics during 1-Hz twitch contraction, indicating a relatively greater increase in muscle blood flow at the microvascular level than in O₂ diffusivity. This adaptation would be mainly caused by an increase in capillary density. Meanwhile, the intracellular O₂ environment was reported to adjust more effectively to the abrupt increase in oxygen demand at the onset of muscle contraction, before the microcirculatory O₂ environment has adapted⁴. In fact, the MRT of $P_{mb}O_2$ kinetics became approximately 5 seconds faster on average by endurance training. The acceleration of $P_{mb}O_2$ kinetics with slower $P_{cap}O_2$ kinetics would imply a sharper expansion of O₂ gradient at the onset of muscle contraction, resulting in a more efficient O₂ transport from Mb to the mitochondria at the transient phase.

In the present study, we have performed additional statistical analyses on kinetics parameters to check the existence of type II error. As for MRT in $P_{mb}O_2$ kinetics, the effect size was 0.052, and the statistical power was 0.109. This level of statistical power implies the existence of a type II error. However, based on our experimental content, increasing the sample size would not necessarily improve the accuracy or precision of our results. This might be one of limitations in this type of experiment. Actually, although significant difference was not recognized for kinetics parameters such as MRT between groups at the relative same tension level, the shorting of MRT by 5 sec on average at the maximal tension level in both of $S_{mb}O_2$ and $P_{mb}O_2$ kinetics suggests the possibility that swimming endurance training accelerates both kinetics during muscle contraction.

In summary, the results presented herein suggest that Mb plays an important role in the faster $m\dot{V}O_2$ response and increased $m\dot{V}O_2$ in trained skeletal muscles. However, how Mb-bound O₂ is supplied to the mitochondria at the onset of muscle contraction remains unclear. Recently, Yamada et al.³⁴ suggested the possibility that the presence of Mb in mitochondrial fractions indicates involvement in



the immediate O_2 release from Mb at the onset of muscle contraction. If so, endurance training might impact Mb localization in the muscle cell. Further research is required to elucidate the mechanism of O_2 transport to the mitochondria within muscle cells and the causal relationship between cellular factors altering the O_2 off rate in Mb and mitochondrial respiration activity.

Methods

Experimental Animals and Preparation of Hindlimb Perfusion. Male Wistar rats were employed as subjects. All were housed in a temperature-controlled room at $23 \pm 2^\circ\text{C}$ 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-101636).

Five-week-old Wistar rats were randomly divided into the Con and 4 weeks Tr groups ($n = 9$ in each group). The training protocol for the swimming group was as follows: On the first and second days, the rats swam for 1 h in two 30-min bouts separated by 5 min of rest. On the third and fourth days, the rats swam for 1.5 hours in three 30-min bouts separated by 5 min of rest. On and after the fifth day, the rats swam for 2 hours in four 30-min bouts separated by 5 min of rest. Except for the first bout of swimming training until the sixth day, a weight equal to 2% of the rats’ body weight was tied to the bodies of the rats. The rats performed the above swimming protocol six days per week. During swimming exercise, the water temperature was kept at around 35°C . The tank’s shape was square and its characteristics were 48 cm depth, 80 cm longitudinal and 60 cm width. All rats swam in that tank and an average surface area of at least $600\text{ cm}^2/\text{rat}$. Also, we kept monitoring to prevent the climbing, diving and bobbing of rat during swimming training. In cases where these behaviors were observed, they were dealt with immediately.

After 4 weeks (at 9-week of age), hindlimb perfusion was performed in each group (Con group: initial body weight (BW) at 5 weeks old; 143–168 g, final BW at 9 weeks old; 257–295 g, Tr group: initial BW; 140–176 g, final BW; 226–250 g). Preparation of isolated rat hindlimb and the perfusion apparatus are described in previous reports^{2,28}. All surgical procedures were performed under pentobarbital sodium anesthesia (45 mg kg^{-1} intraperitoneal). The rats were killed by injecting 1 M KCl solution directly into the heart, followed by a surgical procedure, and an Hb-free Krebs-Henseleit buffer (NaCl, 118 mM; KCl, 5.9 mM; KH_2PO_4 , 1.2 mM; MgSO_4 , 1.2 mM; CaCl_2 , 1.8 mM; NaHCO_3 , 20 mM; Glucose, 15 mM) 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 $22.0 \pm 0.0\text{ ml min}^{-1}$ in the Con group and $22.1 \pm 0.9\text{ ml min}^{-1}$ in the Tr group. In this condition, the average perfusion pressures were $78.6 \pm 7.7\text{ mmHg}$ in the Con group and $74.2 \pm 5.2\text{ mmHg}$ in the Tr group. Therefore, the perfusion resistance was unchanged throughout the perfusion period. In addition, no sign of oedema 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.

Measurement Parameters. The twitch contraction protocol and measurement of Mb oxygenation and $m\dot{V}O_2$ followed the previous methods^{4,28}. 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 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 50%, 75% and 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, even at the highest 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 ($\Delta[\text{deoxy-Mb}]$) signal was evident. The final $\Delta[\text{deoxy-Mb}]$ signal intensity served as the normalization constant for 100% Mb deoxygenation.

$m\dot{V}O_2$ ($\mu\text{mol g}^{-1}\text{ min}^{-1}$) was calculated from the arteriovenous O_2 content differential multiplied by flow rate, using two O_2 electrodes (5300A, YSI, Yellow Springs, Ohio, USA). Two O_2 electrodes were adjusted for the vapour pressure of water before hindlimb perfusion experiment. The oxygen cost was calculated by dividing change in

$m\dot{V}O_2$ due to muscle contraction by muscle tension. The L/P remains constant and shows no significant increase from the resting muscle value. Lactate levels can increase for a number of reasons, including lack of adequate O_2 delivery. Therefore, limited oxygen availability should lead to an increase in lactate level as anaerobic glycolysis starts. Indeed, the L/P increases during perfusion with anoxia buffer (95% N_2) increases. O_2 availability and delivery can sustain maximal contraction with no sign of fatigue.

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 methods⁴. A simple moving average smoothed the $\Delta[\text{deoxy-Mb}]$ and $\Delta[\text{oxy-Mb}]$ NIRS signals using a rolling average of 5 points, which corresponds to a 5-sec timeframe³⁵. The $\Delta[\text{deoxy-Mb}]$ 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 $S_{\text{mb}O_2}$ at rest could not be determined by NIRS, the value was assumed to be 90% based on previous studies reporting that the $S_{\text{mb}O_2}$ at rest was greater than 90%^{5,36}.

The $\% \Delta[\text{deoxy-Mb}]$ plots were converted to $S_{\text{mb}O_2}$ (%) plots using the following equation:

$$S_{\text{mb}O_2} = 100 - \% \Delta[\text{deoxy-Mb}] \quad (2)$$

$S_{\text{mb}O_2}$ 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, USA):

$$S_{\text{mb}O_2} = \text{BL} + \text{AP} \times [1 - \exp^{-(t-\text{TD})/\tau}] \quad (3)$$

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 $S_{\text{mb}O_2}$ signals, and τ the time constant of $S_{\text{mb}O_2}$ signal kinetics. MRT calculated by $\text{TD} + \tau$ 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. These parameter, $0.63\text{AP}/\text{MRT}$, for $S_{\text{mb}O_2}$ shows the O_2 release rate from Mb, which indicates the amount of O_2 released from Mb per unit time at onset of exercise. The O_2 release rate from Mb was calculated using the following equation:

$$O_2 \text{ release rate from Mb} = \frac{0.63\text{AP} \cdot [\text{Mb}]}{\text{MRT}} \quad (4)$$

where $0.63\text{AP}/\text{MRT}$ for $S_{\text{mb}O_2}$ was the Mb deoxygenation rate in %/sec. Inserting this value for Mb into the equation led to determination of the O_2 release rate from Mb in micromoles per gram per minute.

We reconstructed $P_{\text{mb}O_2}$ kinetics based on the resulting $S_{\text{mb}O_2}$ kinetics parameters. The model $S_{\text{mb}O_2}$ kinetics was converted to $P_{\text{mb}O_2}$ (mmHg) using the following equation:

$$P_{\text{mb}O_2} = \frac{S_{\text{mb}O_2} \cdot P_{50}}{(1 - S_{\text{mb}O_2})} \quad (5)$$

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 ³⁷. The calculated $P_{\text{mb}O_2}$ plots were evaluated to obtain an MRT of its kinetics using the same single exponential equation as for $P_{\text{mb}O_2}$. The $0.63\text{AP}/\text{MRT}$ for $P_{\text{mb}O_2}$ indicates a rate of decrease in $P_{\text{mb}O_2}$ at muscle contraction onset. $P_{\text{mb}O_2}$ at steady state was calculated by using the $S_{\text{mb}O_2}$ 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 $P_{\text{mb}O_2}$ value at rest and at each exercise intensity using the following equation:

$$\text{Intracellular } [O_2] = P_{\text{mb}O_2} \times O_2 \text{ solubility} \quad (6)$$

with $P_{\text{mb}O_2}$ is in mmHg, and O_2 solubility in buffer is $0.00135\text{ }\mu\text{mol ml}^{-1}\text{ mmHg}^{-1}$ at 37°C ³⁸.

Mb Concentration and CS Activity in Buffer-Perfused Muscle Tissue. After buffer perfusion experiment, Mb concentration in muscle tissue was measured by a modified Reynafarje method³⁹. CS activity, a mitochondrial enzyme and marker of muscle oxidative potential, was measured in whole muscle homogenates by using the spectrophotometric method of Srere⁴⁰.

Statistical Analyses. All data are expressed as mean \pm SD. Statistical differences were examined using two-way unpaired measures analysis of variance (ANOVA) (tension level \times training). A Turkey-Kramer post-hoc test was applied if the ANOVA indicated a significant difference. An unpaired *t*-test was used in comparing biochemical and physiological parameters between groups. Pearson’s correlation



coefficient was calculated when the relationship between two variables was evaluated. The level of significance was set at $p < 0.05$.

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Author contributions

H.T., K.M. and T.J. designed the research, H.T., Y.F., T.Y. and M.O. conducted the experiment and analysed the data, H.T. wrote the manuscript, K.M. and T.J. edited paper and T.H., S.I., T.H. and T.I. helped experiments.

Additional information

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