Endurance training facilitates myoglobin desaturation during muscle contraction in rat skeletal muscle

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At onset of muscle contraction, myoglobin (Mb) immediately releases its bound O2 to the mitochondria. Accordingly, intracellular O2 tension (PmbO2) markedly declines in order to increase muscle O2 uptake (mV. O2). However, whether the change in PmbO2 during muscle contraction modulates mV. 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 (SmbO2) and PmbO2 kinetics during muscle contraction. Male Wistar rats were subjected to a 4-week swimming training (Tr group; 6 days per week, 30 min × 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 mV. O2peak significantly increased by 32%. Although the PmbO2 during muscle contraction did not affect the increased mV. O2 in endurance-trained muscle, the O2 release rate from Mb increased because of the increased Mb concentration and faster decremental rate in SmbO2 at the maximal twitch tension. These results suggest that the Mb dynamics during muscle contraction are contributing factors to faster VO2 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 volume1–3. It also increases capillarization1–3. 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 (SmbO2), which reflects the intracellular O2 tension (PmbO2), decreases as work intensity and muscle oxygen consumption (mVO2) increase. The decreasing PmbO2 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 PmbO2 during exercise can play a key role in VO2 regulation4.

Because training also induces an acceleration of VO2 kinetics at the onset of muscle contraction, which pulmonary VO2 measurements have detected and have attributed to adjustments of oxidative metabolism at the skeletal muscle level5–7, a faster VO2 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 mVO₂ response at the onset of muscle contraction⁴,⁵,¹⁰,¹¹. Nuclear magnetic resonance and near-infrared spectroscopic (NIRS) experiments clearly show that Mb immediately releases O₂ at the onset of muscle contraction and provides the initial 0.63AP/MRT parameter. This effect is consistent with the enhanced mVO₂ at the initiation of contraction, consistent with the enhanced mVO₂ and with the Mb-mediated O₂ supply. Indeed the kinetics of O₂ release from Mb can serve as an index of the change in intracellular mVO₂ as muscle undergoes training⁷.

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 mVO₂ at rest, at maximal tension, the values of the peak mVO₂ 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 mVO₂ due to muscle contraction (ΔmVO₂), O₂ cost, and kinetics parameters for SmbO₂ and PmbO₂ at each tension level for both groups. Muscle tension, ΔmVO₂, and O₂ cost increased as work intensity increased.

Figure 1 shows the representative kinetics of SmbO₂ in each group. As for the SmbO₂ kinetic parameters, the steady-state value, amplitude (AP), and mean rate of change to 63% of the AP value (0.63AP/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 SmbO₂ kinetic parameters in the Tr group were unchanged, but 0.63AP/MRT of the kinetic parameters for SmbO₂ increased. The MRT also tended to be faster in the Tr group. At submaximal tension levels, the steady-state value, AP, and 0.63AP/MRT increased, and the MRT became faster in both groups as work intensity increased. When the kinetic parameters for PmbO₂ were compared at the same relative tension level between both groups, the relative temporal parameters for PmbO₂ 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 SmbO₂ and PmbO₂ fall following the onset of muscle contraction, 0.63AP/MRT is the effective temporal parameter to show deoxygenation rate of Mb-O₂ per unit time in the initial phase of muscle contraction. The 0.63AP/MRT would reflect the steep change in mitochondrial oxygen demand, because we previously reported that 0.63AP/MRT increased in response to change in mitochondrial oxygen demand due to muscle contraction. Kindig et al. also used AP/time constant in intracellular PO₂ kinetics during muscle contraction as an index of initial metabolic response. As for 0.63AP/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 O₂ demand level during muscle contraction at the relative same intensity between groups. ΔmVO₂ 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, 0.63AP/MRT parameter and ΔmVO₂ 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 mVO₂ during muscle contraction for both groups. While muscle tension and ΔmVO₂ were significantly correlated in both groups, the mean individual slope in the Tr group (0.36 ± 0.11 × 10⁻² μmol/(g²-min)) tended to be higher than that in the Con group (0.27 ± 0.06 × 10⁻² μmol/(g²-min); p = 0.058).

Figure 3 shows the relationship between intracellular [O₂] and ΔmVO₂ during muscle contraction. Intracellular [O₂] was based on the SmbO₂–PmbO₂ equilibrium. In the present study, the SmbO₂ at rest was assumed to be 90%. Intracellular [O₂] decreased markedly from 29.2 μM at rest to 9.2 ± 3.0, 5.1 ± 2.1, and 3.3 ± 1.0 μM at 50%, 75%, and 100% of maximal contraction in the Tr group, respectively; and from 29.2 μM at rest to 12.1 ± 4.7, 5.8 ± 1.9, and 3.2 ± 0.7 μM at 50%, 75%, and 100% of maximal contraction in the Tr group, respectively. All intracellular [O₂] decreased markedly with the ΔmVO₂ in both groups, the Tr group curve showed a smaller [O₂] decline. At the same level of intracellular [O₂] in the muscle cell, ΔmVO₂ 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 O₂ release rate from Mb at same percent of MVC in the Tr and Con groups. The O₂ release rate from Mb increased progressively with the twitch tension level as follows: 1.1 ± 0.3, 2.3 ± 0.4, and 3.7 ± 0.8 × 10⁻² μmol/(g·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 ± 0.5 × 10⁻² μmol/(g·min) at 50%, 75%, and 100% of maximal contraction in the Tr group, respectively. At maximal tension, the O₂ release rate from Mb showed a significant increase in the Tr group, suggesting more O₂ 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        | mg   | 1711.1 ± 73.8 | 1541.0 ± 136.7 |
| m. Gastrocnemius   | mg   | 346.9 ± 15.0 | 289.3 ± 25.7 * |
| m. Plantaris       | mg   | 126.4 ± 5.5  | 98.2 ± 8.7  * |
| m. Soleus          | 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).
Discussion

Effect of endurance training on muscle oxidative capacity. In the present study, 4 weeks of swimming endurance training resulted in an increase in mVO₂ peak, even when O₂ delivery to the endurance-trained hind limb was not greater than that supplied to sedentary muscles. This increase in mVO₂ peak value at constant flow was consistent with previous studies. This increase in mVO₂ peak value without increase in O₂ delivery to the hind limb muscle would be caused by increased 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 ΔmVO₂ 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. 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. Collectively, the swimming endurance training in the present study enhanced muscle oxidative capacity, in agreement with evidence from previous studies.

Relationship between Pₘ₉O₂ kinetics and muscle oxygen consumption. Endurance training increases both mVO₂ peak and ΔmVO₂ at the same percentage of MVC. However, both control and trained muscle show a steady decline in Pₘ₉O₂ with increasing MVC. The declining Pₘ₉O₂ 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 Pₘ₉O₂ and Pₘ₉O₂ can influence the O₂ flux into the cell, which then supports the mVO₂. 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 2 | Contractile and metabolic properties of hindlimb muscles

| Parameter             | Unit          | Con     | Tr     |
|-----------------------|---------------|---------|--------|
| Maximal Tension       | g             | 73.7 ± 10.6 | 92.0 ± 21.9 |
| mVO₂ at rest          | μmol g⁻¹ min⁻¹ | 0.48 ± 0.09 | 0.58 ± 0.13 |
| mVO₂ peak             | μmol g⁻¹ min⁻¹ | 0.70 ± 0.10 | 0.93 ± 0.16 |
| [Mb]                  | μmol g⁻¹      | 0.10 ± 0.01 | 0.12 ± 0.01 |
| CS activity           | μmol g⁻¹ 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. 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)."

Table 3 | Muscle tension, muscle oxygen consumption, Sₘ₉O₂ and Pₘ₉O₂ kinetics parameters during muscle contraction at each tension level

| Parameter        | Unit       | Group | 50%       | 75%       | 100%      |
|------------------|------------|-------|-----------|-----------|-----------|
| Muscle Tension   | g          | Con   | 36.4 ± 6.4 | 54.2 ± 9.6 | 73.7 ± 10.6 |
|                  |            | Tr    | 53.5 ± 15.0 | 70.9 ± 18.3 | 92.0 ± 21.9 |
| ΔmVO₂            | μmol g⁻¹ min⁻¹ | Con   | 0.08 ± 0.04 | 0.13 ± 0.05 | 0.22 ± 0.06 |
|                  |            | Tr    | 0.13 ± 0.06 | 0.25 ± 0.11 | 0.36 ± 0.11 |
| O₂cost           | 10⁻² μmol g⁻¹ min⁻¹ | Con   | 0.22 ± 0.08 | 0.25 ± 0.10 | 0.30 ± 0.08 |
|                  |            | Tr    | 0.25 ± 0.08 | 0.35 ± 0.13 | 0.40 ± 0.12 |
| Sₘ₉O₂ kinetics   |            |       |           |           |           |
| Steady-State Value | %          | Con   | 72.7 ± 6.5 | 58.7 ± 10.9 | 49.0 ± 8.1 |
|                  |            | Tr    | 76.8 ± 7.9 | 62.7 ± 7.9 | 49.5 ± 5.3 |
| AP               |            |       |           |           |           |
|                  | %          | Con   | -19.2 ± 7.0 | -31.2 ± 11.0 | -41.0 ± 8.1 |
|                  |            | Tr    | -13.2 ± 7.9 | -27.3 ± 7.9 | -40.5 ± 5.3 |
| MRT              | s          | Con   | 63.0 ± 18.2 | 52.2 ± 14.0 | 43.7 ± 6.6 |
|                  |            | Tr    | 52.6 ± 12.4 | 48.3 ± 10.2 | 39.3 ± 5.9 |
| MRT/ΔAP/MRT      | % s⁻¹      |       | -0.18 ± 0.06 | -0.37 ± 0.07 | -0.58 ± 0.10 |
|                  |            |       | -0.15 ± 0.07 | -0.36 ± 0.10 | -0.65 ± 0.08 |
| Pₘ₉O₂ kinetics   |            |       |           |           |           |
| Steady-State Value | mmHg      | Con   | 6.9 ± 2.2 | 3.8 ± 1.5 | 2.4 ± 0.8 |
|                  |            | Tr    | 8.9 ± 3.4 | 4.3 ± 1.4 | 2.4 ± 0.5 |
| AP               | mmHg       |       |           |           |           |
|                  |            | Con   | -15.0 ± 2.3 | -17.6 ± 1.8 | -19.1 ± 0.7 |
|                  |            | Tr    | -12.6 ± 3.4 | -17.3 ± 1.4 | -19.2 ± 0.5 |
| MRT              | s          |       |           |           |           |
|                  |            | Con   | 43.8 ± 10.3 | 34.6 ± 5.6 | 29.5 ± 5.7 |
|                  |            | Tr    | 43.2 ± 9.4 | 33.7 ± 4.6 | 25.9 ± 5.2 |

*Values are mean ± SD (n = 9 in each group. Con: control group. Tr: training group. ΔmVO₂ is the net increase in mVO₂. 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. ΔAP/MRT is calculated by dividing ΔAP by MRT. Superscripts indicate a significant difference (p < 0.05 vs. Tr x 100%; b: p < 0.05 vs. Tr x 75%; c: p < 0.05 vs. Con x 100%; d: p < 0.05 vs. Con x 75%)."
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:

$$J = -D \frac{\partial C}{\partial x}$$

where $J$ is the diffusion flux (amount of $O_2$ crossing a unit area per unit time), $D$ is the diffusion coefficient (length of unit area squared x time $^{-1}$), $\frac{\partial C}{\partial x}$ is the change in $O_2$ concentration along dimension x or the $O_2$ gradient along the x-direction. $^1$H-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 $O_2$ diffusion flux (amount of $O_2$ crossing a unit area per unit time) in the trained muscle would show a greater value at a relatively higher exercise intensity and associated increasing respiration.

Intracellular $[O_2]$ (in $\mu$M) decreased gradually with the increase in changes in muscle $O_2$ uptake ($\Delta mVO_2$) in both groups. The relationship between intracellular $[O_2]$ and $\Delta mVO_2$ was shown as a line graph. Each data point represents the mean ± 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. $Con \times 100\%$, $p < 0.05$; $^d$: vs. $Con \times 75\%$).

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**Figure 1** | Representative kinetics of myoglobin (Mb) saturation ($S_{mbo2}$) during maximal twitch contraction (1 Hz) in the training (Tr) and control (Con) groups. The plots of $S_{mbo2}$ show representative data at the maximal twitch tension from the single experiment in each group. While the $S_{mbo2}$ kinetics (dotted line) in the representative control rat declined with a mean response time (MRT) of 39.5 sec (upper panel), the $S_{mbo2}$ kinetics (solid line) in the representative trained rat declined with an MRT of 33.0 sec (lower panel). The MRT in the $S_{mbo2}$ kinetics was shortened by 5 sec on average due to endurance training. By contrast, the $S_{mbo2}$ value at steady state did not show any significant difference between the two groups.

**Figure 2** | Relationship between muscle tension and $\Delta mVO_2$ during twitch contraction in the training (Tr) and control (Con) groups. Changes in muscle $O_2$ uptake ($\Delta mVO_2$) 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: $\Delta mVO_2 = 0.003 \times Tension$, $R^2 = 0.98$, $p < 0.05$; Tr: $\Delta mVO_2 = 0.004 \times Tension$, $R^2 = 0.99$, $p < 0.05$). The data represent the mean ± 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. $Con \times 100\%$, $p < 0.05$).

**Figure 3** | Relationship between intracellular $[O_2]$ and $\Delta mVO_2$ during twitch contraction in the training (Tr) and control (Con) groups. Intracellular $[O_2]$ (in $\mu$M) decreased gradually with the increase in changes in muscle $O_2$ uptake ($\Delta mVO_2$) in both groups. The relationship between intracellular $[O_2]$ and $\Delta mVO_2$ was shown as a line graph. Each data point represents the mean ± 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. $Con \times 100\%$, $p < 0.05$; $^d$: vs. $Con \times 75\%$).
Superscript letters indicate significant differences (a: vs. Tr
Endurance training usually results in faster V. O2 kinetics24, which
itself would largely contribute to this adaptation as a result of
that the hypothesized expansion of O2 gradient due to further
demand increases might indicate that Mb-supplied O2 may directly
supply to the mitochondria at the intracellular level.

O2 release rate from Mb at the onset of muscle contraction.
Endurance training usually results in faster VO2 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 VO2 on-
kinetics25–27. 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 increased24,28. These facts suggest
that Mb provides an immediate O2 source for the sudden increase in
VO2 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 suffi-
ciently 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 mVO2 kinetics3. Taking these findings together, both mito-
chondrial respiration capacity and O2 release rate from Mb might be
important factors that regulate mVO2 kinetics at the onset of muscle
contraction.

The binding of O2 to Mb and Hb certainly proceeds much faster
than transport30,31, 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 compart-
mentalized 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 state34. 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 relation-
ship between the O2 release rate from Mb and mVO2 seems quite
reasonable and consistent with the postulated role of Mb. Once Mb
desaturates has reached a new steady state, vascular O2 supply must
begin to contribute significantly to sustain the rising mVO2. 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 mVO2. Note that Mb never resa-
turates 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 mVO25,6.
Consequently, the enhanced intracellular mVO2 observed after
endurance training could be induced by a 30% increase in [Mb]
centration 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 muscle16,17,32,33, 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 PmbO2 response at the onset of muscle contraction
showed the tendency to be faster after endurance training in the
present study. Hirai et al.34 reported that endurance training led to
slower PmbO2 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 had adapted3. In fact, the MRT
of PmbO2 kinetics became approximately 5 seconds faster on average
by endurance training. The acceleration of PmbO2 kinetics with
slower PmbO2 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 ana-
lyses on kinetics parameters to check the existence of type II error. As
for MRT in PmbO2 kinetics, the effect size was 0.052, and the statisti-
cal power was 0.109. This level of statistical power implies the exist-
ence of a type II error. However, based on our experimental content,
increasing the sample size would not necessarily improve the accu-
racy 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 SmbO2 and PmbO2
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 mVO2 response and increased mVO2 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 pre-
sence of Mb in mitochondrial fractions indicates involvement in
the immediate O2 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 O2 transport to the mitochondria within muscle cells and the causal relationship between cellular factors altering the O2 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 ± 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-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 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 cm²/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. The surgery 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; KH₂PO₄, 1.2 mM; MgSO₄, 1.2 mM; CaCl₂, 1.8 mM; NaHCO₃, 20 mM; Glucose, 15 mM) equilibrated with 95% O₂ and 5% CO₂ gas) was perfused for 30 min to each rat. The perfusion pressure, O2 content at the inflow and outflow) were collected using a data acquisition system (PowerLab-BSP, 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. A single moving averaged the S[deoxy-Mb] and A[oxy-Mb] NIRS signals using a rolling average of 5 points, which corresponds to a 5-sec timeframe. The S[deoxy-Mb] signals were calibrated against two different NIRS signal values: one at rest as 10% Mb oxygenation and the other during steady state with anoxic buffer perfusion as 100% Mb deoxygenation. While the S[oxy-Mb] at rest could not be determined by NIRS, the value was assumed to be 90% based on previous studies reporting that the S[oxy-Mb] at rest was greater than 90%.

The %A[deoxy-Mb] plots were converted to S[deoxy-Mb] (%) using the following equation:

\[ S_{deoxy-Mb} = 100 - \%A[deoxy-Mb] \]

(2)

S[deoxy-Mb] 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):

\[ S_{deoxy-Mb} = BL + AP \times \left(1 - \exp\left(-\frac{t - TD}{MRT}\right)\right) \]

(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[deoxy-Mb] signals, and MRT the time constant of S[deoxy-Mb] signal kinetics. MRT calculated by TD + t was used as an effective parameter of the response time of 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 parameters, e.g., AP/MRT, for S[deoxy-Mb] shows the O2 release rate from Mb, which indicates the amount of O2 released from Mb per unit time at onset of exercise. The O2 release rate from Mb was calculated using the following equation:

\[ O_2 \text{ release rate from } Mb = \frac{e^{0.63AP} \times [Mb]}{MRT} \]

(4)

where e0.63AP/MRT for S[deoxy-Mb] was the Mb deoxygenation rate in %/sec. Inserting this value for Mb into the equation led to determination of the O2 release rate from Mb in micromoles per gram per minute.

We reconstructed PmbO2 kinetics based on the resulting S[deoxy-Mb] kinetics. The model S[deoxy-Mb] kinetics was converted to PmbO2 (mmHg) using the following equation:

\[ P_{mbO_2} = \frac{S_{deoxy-Mb} \times P_{mbO_2}}{1 - S_{deoxy-Mb}} \]

(5)

where PmbO2 is the partial oxygen pressure required to half-saturate Mb. A PmbO2 of 2.4 mmHg was used for this equation, assuming a muscle temperature of 37°C. The calculated PmbO2 plots were evaluated to obtain an MRT of its kinetics using the same single exponential equation as for PmbO2. The e0.63AP/MRT for PmbO2 indicates a rate of decrease in PmbO2 at muscle contraction onset. PmbO2 at steady state was calculated by using the S[deoxy-Mb] value at steady state. Since O2 partial pressure corresponds to a specific amount of dissolved O2, intracellular [O2] (μM) was calculated from the E/PmbO2 value at rest and at each exercise intensity using the following equation:

Intracellular [O2] = PmbO2 \times O2 solubility

(6)

with PmbO2 in mmHg, and O2 solubility in buffer is 0.00135 μmol ml⁻¹ mmHg⁻¹ at 37°C.

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

Statistical Analyses. All data are expressed as mean ± SD. Statistical differences were examined using two-way unpaired measures analysis of variance (ANOVA) (tension level × 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
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 experiments. H.T., Y.F., T.Y. and M.O. helped experiments.

**Additional information**

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