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NIRS Measurement of O_2 Dynamics in Contracting Blood and Buffer Perfused Hindlimb Muscle

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Abstract In order to obtain evidence that Mb releases O_2 during muscle contraction, we have set up a buffer-perfused hindlimb rat model and applied NIRS to detect the dynamics of tissue deoxygenation during contraction. The NIRS signal was monitored on hindlimb muscle during twitch contractions at 1 Hz, evoked via electrostimulator at different submaximal levels. The hindlimb perfusion was carried out by perfusion of Krebs Bicarbonate buffer. The NIRS still detected a strong signal even under Hb-free contractions. The deoxygenation signal (Δ[deoxy]) was progressively increased at onset of the contraction and reached the plateau under both blood- and buffer-perfused conditions. However, the amplitude of Δ[deoxy] during steady state continued to significantly increase as tension increased. The tension-matched comparison of the Δ[deoxy] level under buffer-perfused and blood perfused conditions indicate that Mb can contribute approximately 50% to the NIRS signal. These results clarify the Mb contribution to the NIRS signal and show a falling intracellular PO_2 as workload increases.

1 Introduction

The transport of O_2 into myocytes and the regulation of respiration depend upon an extensive series of mechanisms and influences [1]. To clarify these mechanisms *in vivo*, researchers have developed non-invasive magnetic resonance spectroscopy (^{1}H-MRS) and near infrared spectroscopy (NIRS) techniques [2-4]. Indeed, these non-invasive methods have shed unique insights but have also raised controversial questions: Does the intracellular oxygen continue to decline or plateau as workload increases? Does the NIRS signal detect Hb or Mb desaturation? The near infrared spectroscopy (NIRS) is a powerful tool for detecting O_2 dynamics in contracting muscle *in vivo*, because it can detect O_2 changes with high sensitivity and time resolution. However, NIRS cannot distinguish the contribution from Mb and Hb. Currently, most researchers believe that the NIRS signal derives mainly from blood (Hb) [5-6]. In contrast, recent NMR experiments have suggested that Mb contributes significantly to NIRS signal [7].

To address these questions, we have conducted an NIRS study with buffer perfused hindlimb. Buffer-perfused muscle avoids any Hb interference. The NIRS signals reflect only the intracellular O_2 dynamics during muscle contraction. The results indicate that NIRS detects a significant contribution from Mb and that Mb desaturation does not reach a plateau with increased workload.

2 Materials and Methods

2.1 Experimental Animals and Protocol

Male Wistar rats (9 weeks old, 242 - 280 g, n=3) were used for the present experiment. All procedures performed in the present study were approved by the ethics committee on Animal Experimentation of Kanazawa University.

The present experiment was consisted of two conditions: 1) under evoked twitch contractions without buffer perfusion (normal blood-perfused condition), and 2) under evoked twitch contractions with buffer-perfusion

(without Hb interference). First of all, the rats were subjected to twitch contractions *in vivo*. The evoked tension via the sciatic nerve was set at several levels of the maximal tension (25~100%), and a constant tension was maintained during a 2-min contraction. A 10-minute recovery period between series of 2-min contractions to eliminated muscle fatigue. The NIRS signal returned to its control level, before the next set of stimulation began.

After the *in vivo* stimulations, the rats were prepared for hindlimb perfusion. Under buffer-perfused condition, the hindlimb muscle was stimulated via the sciatic nerve as same as the condition of *in vivo* stimulation.

2.2 Preparation of Electrostimulation and Hindlimb Perfusion, and Measurement of Muscle Oxygenation

Under anesthesia with pentobarbital sodium (45 mg/kg i.p.), the sciatic nerve of the left hindlimb was connected to two parallel stainless steel wire electrodes. The Achilles' tendon was connected to a strain gauge with a string (MLT500/D, ADInstrument, Australia). At the muscle's optimum length, where the muscle generated peak tension, the electric stimuli on the sciatic nerve elicited a series of isometric twitch contractions. The stimulation consisted of a single square wave (delay 10 μsec, interval: 2 msec, duration: 1 msec) was controlled by the electrostimulator system (Model RU-72, Nihon Koden, Japan). The stimulation was 1 Hz of frequency for 120 sec. The target tension was controlled by changing stimuli voltage to obtain 25~75% of peak tension (4-8 volts). The twitch tension was recorded via data acquisition system (PowerLab 8SP, ADInstrument, Australia).

Preparation of the isolated rat hindlimb and the perfusion apparatus were described in the previous reports [8-10]. Fig. 1 shows the system. Surgery was modified from those in previous reports [11-13]. The inflow catheter was placed in the descending aorta with its tip 4-5 mm proximal to the aorta bifurcation. Initially the hypogastric trunk was occluded to evaluate whether blood flow to the trunk region could be eliminated. Subsequently ligature was placed around the contralateral (right hindlimb) common iliac artery. Blood flow to the tail was eliminated by ligation at its base. Venous effluents were collected separately from the vena cava.

Regular Krebs-Henseleit buffer contained 15 mM glucose was equilibrated with 95% O_2 + 5% CO_2. A circulating water bath and temperature jacketed reservoir and tubings maintained at 37°C. A peristaltic pump maintained a constant, non-recirculating perfusate flow. After the cannulation of the abdominal aorta, the Krebs-Henseleit buffer containing heparin (2000 U/l) was perfused into hindlimb for 30-min to prevent clotting blood and to wash out blood from hindlimb. Buffer was continued to perfuse the hindlimb until the end of experiment.

The NIRS instrument (NIRO-300 + Detection Fiber Adapter Kit, Hamamatsu Photonics, Japan) provided separate measurements of changes in deoxygenated Hb and Mb concentrations (Δ[deoxy]), changes in oxygenated Hb and Mb concentrations (Δ[oxy]), and changes in the sum of these two variables as total Hb and Mb concentrations (Δ[total]). Since previous study suggested that the Δ[oxy] was affected by the variation of blood flow compared with Δ[deoxy] signal [14], the Δ[deoxy] signal was used as the muscle oxygenation index in the present study.

The distance between the photodiode and the LED was fixed at 10 mm on the skin of m. gastrocnemius. The kinetics of Δ[deoxy] during evoked contraction was mathematically evaluated by fitting the data to the single exponential function to determine amplitude (the amplitude between baseline and the steady-state value during the exponential component, μMcm).

2.3 Statistical Analysis

All data are expressed as means ± SD. To compare measurements between tension levels or between conditions, repeated measurement of one-way analysis of variance (ANOVA) was used. Scheffé's post-hoc test was conducted if ANOVA indicated a significant difference. The level of significance was set at $p < 0.05$.

3. Results

Buffer-perfused muscle contained no Hb. Yet, NIRS still detected a very strong signal. The evoked twitch tension under buffer-perfusion remained constant during stimulation but reached half the maximal tension observed under blood-perfusion. Estimated contributions of Mb and Hb on the NIRS Δ[deoxy] amplitude during contraction is shown in Table 1. At 50% of maximal twitch tension (71.05 ± 28.47 g), the amplitude of the Δ[deoxy] signal contains about 50% contribution from Mb (Table 1). The Mb deoxygenation (at steady state) at 50%, 75% and 100% of maximal twitch tension obtained under buffer-perfusion was 6.10 ± 3.57, 10.35 ± 6.90, 15.57 ± 6.60 (μMcm) respectively, and progressively amplified as tension level increased ($p < 0.01 \sim 0.05$). Preliminary calibration experiments with anoxic buffer have yielded an estimate of total Mb desaturation at maximal exercise under buffer perfused condition to be about 50% ($S_{mb}O_2$). Since blood perfused muscle reaches a higher tension and shows almost a 50% increased in amplitude, Mb should desaturate even lower than 50%.

4. Discussion

According to the traditional assumption, Mb serves merely as an O_2 store in myocytes [15]. Mb releases O_2 only under extremely hypoxic condition. Based on this assumption, many researchers have attributed the NIRS signal from muscle to Hb rather than intracellular Mb. However, NMR experiments have raised questions about the predominant Hb contribution in the NIRS experiments. ^{1}H-NMR can detect the signals from the His F8 NH of deoxy Mb and Hb in human skeletal muscle and can monitor directly Mb and Hb desaturation during contraction [16-17]. Based on a comparative measurement of NMR and NIRS, Mb contributes significantly to the NIRS signal [7].

Mb deoxygenation has an influence on the optical measurement such as NIRS. Surprisingly, the Mb contributes nearly 50% to the NIRS deoxy signal (Table 1). Researchers usually ignore the Mb contribution in human muscle experiments [14, 18]. Previous rat hindlimb experiments that applied chemical infusion to modify the muscle O_2 uptake have suggested that 90% of the NIRS signal comes from Hb [9]. In contrast, the present study shows a parallel change in Mb and Hb desaturation and suggests that Mb contributes equally to the Δ[deoxy] signal [7].

The present study also provides a preliminary answer to the controversial question if Mb plays a role as an O_2 store and if the O_2 gradient continues to widen as energy demand increases. Mb surely releases O_2 at the onset of the contraction, and the Mb release of O_2 depends upon the O_2 demand of mitochondria. The present result shows a progressive increase in the amplitude of the Δ[deoxy] signal for both blood- and buffer-perfused conditions. The trend does not appear to reach any plateau, and Mb desaturation increases as O_2 demand rises with muscle contraction. In essence, the O_2 gradient continues to widen with increased work. The present result supports the observation that Mb desaturation continues to deoxygenate with increasing muscle activity and does not reach any plateau [17, 19].

Physiological significance of Mb desaturation at the onset of contraction remains unclear. However, it is obvious that the gradient from extracellular layer to intracellular layer must expand once Mb desaturates. The gradient would improve the efficiency of diffusion conductance (DO_2) in the muscle tissue [20]. Even though further experiments are still required to clarify these changes in cellular O_2 during muscle contraction, our results indicate that Mb continues to deoxygenate as work increases and contributes significantly to the NIRS signal in exercising skeletal muscle.

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Figure legends:

Fig. 1 The illustration of buffer perfused hindquarter system.

Table 1. Estimated Mb and Hb Contribution on the NIRS Δ[deoxy] Amplitude during Contraction at a Given Tension Level.

	Hb+Mb	Mb	Hb
Amplitude	26 ± 11	13 ± 6*	12 ± 5*

Unit: μMcm, means ± SD, n=3, *: $p < 0.05$ vs. Hb+Mb. The "Hb+Mb" denotes the amplitude under blood-perfused condition. "Mb" corresponds to the amplitude under buffer perfusion. The "Hb" was obtained by subtracting the amplitude of "Mb" from "Hb+Mb".