Microvascular and interstitial Po2 measurements in rat skeletal muscle by phosphorescence quenching

MASAHIRO SHIBATA, SHIGERU ICHIOKA, JOJI ANDO, AND AKIRA KAMIYA
Department of Biomedical Engineering, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan

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Shibata, Masahiro, Shigeru Ichioka, Joji Ando, and Akira Kamiya. Microvascular and interstitial Po2 measurements in rat skeletal muscle by phosphorescence quenching. J Appl Physiol 91: 321–327, 2001.—To clarify the transport of O2 across the microvessels in skeletal muscle, we designed an intravital laser microscope that utilizes a phosphorescence quenching technique to determine both the microvascular and tissue Po2. After we injected the phosphorescent probe into systemic blood, phosphorescence excited by a N2 dye pulse laser was detected with a photomultiplier over a 10 μm in diameter area. In vitro and in vivo calibrations confirmed that the present method is accurate for Po2 measurements in the range of 7–90 Torr (r = 0.958) and has a rapid response time. This method was then used to measure the Po2 of microvessels with different diameters (40–130 μm) and of interstitial spaces in rat cremaster muscle. These measurements showed a significant drop in Po2 in the arterioles after branching (from 74.6 to 46.6 Torr) and the presence of a large Po2 gradient at the blood-tissue interface of arterioles (15–20 Torr). These findings suggest that capillaries are not the sole source of oxygen supply to surrounding tissue.

oxygen transport; intravital microscope; microcirculation; palladium-porphyrin; cremaster muscle

ALTHOUGH RESEARCHERS HAVE KNOWN for over 100 years that one of the principal functions of the microcirculation is O2 delivery to tissues (19, 20, 28), our understanding of the processes involved in the transport of O2 from blood to tissue is incomplete (6, 9, 14). A major problem is that we do not know how Po2 levels in microvessels and tissues vary, particularly in the vicinity of blood vessels and around individual cells. Several methods have been utilized in efforts to measure these parameters. Recent progress in O2 microelectrodes (17, 30), which are currently the most widely used and successful method, has produced many advantages in numerous physiological studies because of the rapid time response and wide range of Po2 measurements these microelectrodes make possible (1, 40, 41). However, the microelectrodes must be inserted into the tissue, disturbing the local environment. Optical methods using spectrophotometry and O2 indicators, such as hemoglobin or myoglobin, have also been utilized (3, 4, 7, 24, 37). These methods allow noninvasive and reliable measurements to be performed, but only when the signals of interest have a homogeneous distribution. In addition, these methods cannot simultaneously measure the Po2 of blood and tissue, and these parameters are probably necessary for complete analysis of O2 delivery to tissues surrounding the microvessels.

In the last decade, methods involving the O2-dependent quenching of fluorescence or phosphorescence (2, 23, 27, 38, 46) have undergone considerable improvement, and several successful measurements of microvascular and interstitial Po2 values in normal tissues (15, 16, 32, 44) and tumors (34, 43) have been made. In addition, this technique has been used to measure microvascular and interstitial Po2 gradients in rat mesentery (35, 45) and hamster skin (33) to clarify the distribution of O2 on the order of a micrometer. However, these measurements have only been performed in thin tissues, and only a few reports have been made of microvascular and interstitial Po2 measurements in thick tissues like skeletal muscle (46). The reason for this is that, with incident light using a conventional excitation like a xenon flash lamp, it is difficult to excite the phosphorescent probe in a limited volume. Thus the light cannot penetrate deeper tissues because it is absorbed and scattered.

In a previous study (29), we reported on the development of a fluorescence intravital microscope equipped with a laser illuminator. The use of the laser illuminator allows deeper tissues to be penetrated, enabling tomographic observations of the microcirculation in thicker tissues. Herein, we report the development of a new phosphorescence quenching microscope based on the laser illuminator technique that allows noninvasive and regional determination of intravascular and interstitial Po2 values in skeletal muscle.

MATERIALS AND METHODS

Animal preparation. Adult male Wistar rats weighing between 150 and 180 g were anesthetized with urethane intr-
arterioles were designated as second order (A2, 50–80 μm) and branches of first-order arterioles (A1) were classified according to their branching order in the microscopic field. Large arterioles with a diameter of 60–100 μm were designated as first order (A1). Branches of first-order arterioles were designated as second order (A2, 50–80 μm in diameter). Third-order arterioles (A3) had a diameter of <60 μm and branched from second-order arterioles. Venules were also classified by their branching order. The large venules entering the central cremasteric artery were designated as first order (V1), branches from V1 as second order (V2), and branches from V2 as third order (V3). The diameter of each vessel is indicated in parentheses (in μm).

PO2 measurements were performed at several locations in the rat cremaster muscle (Fig. 1). Arterioles and venules were classified according to their branching order in the microscopic field. Large arterioles with a diameter of ~80–120 μm branching from the central cremasteric artery were designated as first order (A1). Branches of first-order arterioles were designated as second order (A2, 50–80 μm in diameter). Third-order arterioles (A3) had a diameter of <60 μm and branched from second-order arterioles. Venules were also classified by their branching order. The large venules entering the central cremasteric vein were designated as first order (V1), branches from V1 as second order (V2), and branches from V2 as third order (V3). The diameter of each vessel is indicated in parentheses (in μm).

Fig. 1. Classification of vessel order in rat cremaster muscle. Branches from the central cremasteric artery were designated as first-order (A1), branches from A1 as second-order (A2), and branches from A2 as third-order (A3) arterioles. The large venules entering the central cremasteric vein were designated as first order (V1), branches from V1 as second order (V2), and branches from V2 as third order (V3). The diameter of each vessel is indicated in parentheses (in μm).
camera (DXC-107A, Sony, Tokyo) connected to a videotimer (VTG-33, For-A, Tokyo, Japan) and videocassette recorder (SLV-RS1, Sony), and the image was displayed on a 14-in. high-resolution television monitor (PVM-1442Q, Sony) at a final magnification of approximately \( \frac{3}{800} \). The Pd-porphyrin phosphorescent probe was excited by epi-illumination using a \( \text{N}_2 \)-dye pulse laser (LN120C, Laser Photonics) with a 535-nm line at 20 Hz via the objective lens. The average optical power and pulsewidth of the laser were 1.2 mW and 300 ps, respectively. The area of the epi-illuminated tissue was 10 \( \mu \text{m} \) in diameter on the surface. The phosphorescent emissions from the tissue were captured by a photomultiplier (C6700, Hamamatsu Photonics, Hamamatsu, Japan) through a long-pass filter at 610 nm. Signals from the photomultiplier were converted to 10-bit digital signals at intervals of 3 \( \mu \text{s} \). The averaged 20–40 data were calculated by mathematically fitting the decay of the phosphorescence to the rectangular model equation (11) using online computer analysis. Intra-vascular \( \text{PO}_2 \) measurements were carried out immediately, whereas interstitial \( \text{PO}_2 \) measurements were started 30 min after injection of the Pd-porphyrin solution (~25 mg/kg body wt) into the cannulated jugular vein. In the measurements of interstitial \( \text{PO}_2 \), Pd-porphyrin solution injection was adjusted to the magnitude of the photomultiplier signal and equivalent in changing the sensitivity of the photomultiplier at three to four times.

**In vitro and in vivo calibration.** The \( \tau \) values of the phosphorescent probe at various \( \text{PO}_2 \) levels were measured in vitro to obtain \( \tau_0 \) and \( K_q \) for the present system because these values depend on pH and temperature. In vitro calibration was carried out to keep the temperature of the phosphorescent probe at 37°C and the pH at 7.3–7.4, at which the probe solution was stationary during measurement. The in vitro \( \tau \) value of the phosphorescent probe as a function of \( \text{PO}_2 \) is shown in Fig. 3. The values of \( \tau_0 \) and \( K_q \) calculated from this result were 545 \( \mu \text{s} \) and 362 Torr\( ^{-1} \)s\(^{-1} \), respectively. These values were used for all \( \text{PO}_2 \) calculations in this study.

We also examined the accuracy of the tissue \( \text{PO}_2 \) measurements calculated with our system by simultaneous in vivo measurements with the \( \text{O}_2 \) microelectrodes (13) in rat mesentery (Fig. 4). The measurement area of the electrodes was \( \sim 20 \mu \text{m} \) in diameter, and the \( \text{PO}_2 \) level in the mesentery varied between 7 and 90 Torr with the inhalation of \( \text{O}_2 \) or \( \text{N}_2 \). An adequate correlation between the two methods was found \((r = 0.958)\), and the maximum divergence was <12% in this \( \text{PO}_2 \) range.

**Statistics.** All data are means ± SD. Order-related differences were analyzed using a one-way ANOVA and multiple comparisons test. For all statistical tests, a \( P \) value of <0.05 was considered significant.

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**Fig. 2.** Arrangement of the intravital laser microscope using \( \text{O}_2 \)-dependent phosphorescence quenching. A general observation of the microcirculation was performed using a modified Nikon microscope with a charge-coupled device camera. Pd-porphyrin was excited by epi-illumination with a \( \text{N}_2 \)-dye pulse laser with 535-nm line at 20 Hz via the objective. The epi-illuminated tissue area was 10 \( \mu \text{m} \) in diameter. Phosphorescence emission was captured by a photomultiplier through a long-pass filter at 610 nm, and signals from the photomultiplier were converted to 10-bit digital signals at intervals of 3 \( \mu \text{s} \). ADC, analog-to-digital signal converter; PMT, photomultiplier tube.

**Fig. 3.** In vitro calibration of \( \text{PO}_2 \) measurements. Pd-meso-tetra(4-carboxyphenyl)porphyrin bounded to bovine serum albumin was dissolved in Krebs solution to achieve a concentration of \( \sim 500 \mu \text{mol/l} \) and adjusted to a pH of 7.3–7.4 and a temperature of 37°C. \( \tau_0 \), Phosphorescence lifetime in the absense of \( \text{O}_2 \); \( K_q \), quenching constant.
RESULTS

Systemic arterial PO2, PCO2, and pH were measured using a blood analysis system (Series 2000, Diametrics Medical, St. Paul, MN) in samples from the carotid arteries of four rats. Arterial PO2 averaged 98.1 ± 12.9 Torr, whereas arterial PCO2 and pH averaged 48.7 ± 9.2 Torr and 7.32 ± 0.05, respectively.

A typical example of a digitized phosphorescence decay curve measured in the interstitial space of the rat cremaster muscle is shown in Fig. 5. The symbols indicate the original data, whereas the solid line represents the theoretical curve fitted to the original data. This curve was obtained from the average of 20 curves. These results show clearly that the phosphorescence decay curve is virtually equal to the theoretical curve. In addition, the application of the pulse laser illuminator enables the phosphorescence quenching curve to be quickly detected in the initial segment by excluding the afterglow produced by incidental light. In this example, the calculated values of τ and PO2 were 87 µs and 26.7 Torr, respectively.

Table 1 summarizes the results of the statistical analysis on data from four animal experiments. The mean arteriolar PO2 values of the different arteriole orders decreased significantly after branching, but the venular PO2 showed no dependency on vessel order. Mean PO2 values in the interstitial spaces adjacent to the arterioles of different orders were significantly lower than intravascular PO2 values. In addition, PO2 values measured in the interstitial spaces at a distance of more than 100 µm from the arterioles showed no significant differences with respect to vessel order, although their values were significantly lower than the PO2 values at locations adjacent to the arterioles. Figure 6 shows the PO2 profile for the data in Table 1. The

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Arterioles</th>
<th>Venules</th>
<th>Interstitial Spaces</th>
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<tbody>
<tr>
<td></td>
<td></td>
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<td>d &lt; 20 µm</td>
</tr>
<tr>
<td>1st order</td>
<td>74.6 ± 7.7</td>
<td>29.7 ± 3.5</td>
<td>50.7 ± 4.2</td>
</tr>
<tr>
<td>2nd order</td>
<td>54.4 ± 6.0</td>
<td>30.6 ± 6.1</td>
<td>39.4 ± 10.1</td>
</tr>
<tr>
<td>3rd order</td>
<td>46.6 ± 7.3</td>
<td>33.6 ± 9.9</td>
<td>28.8 ± 6.1</td>
</tr>
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</table>

Values are means ± SD in Torr of the average of 4 animal experiments. Distance (d) < 20 µm signifies interstitial spaces within 20 µm of the arterioles; d > 100 µm signifies interstitial spaces farther than 100 µm from the arterioles.
systemic arterial P O2 value, which is significantly higher than that of the A1 arteriole, is also indicated in Fig. 6. A large drop in P O2 across the blood-tissue interface followed by a gentle decrease is clearly demonstrated for every order of arteriole.

**DISCUSSION**

O2-dependent quenching of phosphorescence has proven to be a powerful method for measuring the P O2 of tissues in living animals because it allows rapid and noninvasive measurements to be performed. However, to determine P O2 values using in vivo measurements obtained by the phosphorescence quenching technique, the heterogeneous distribution of P O2 in the measuring area always becomes a large problem (8, 21). In an effort to resolve this problem, many researchers have proposed an effective algorithm for analyzing a heterogeneous system (39, 46). These investigations generally encourage the use of multiexponential analysis because the phosphorescence decay curve in a heterogeneous system consists of several exponents. On the other hand, Golub et al. (11) proposed the continuous distribution model, in which the fitting procedure based on this model can result in more accurate values than other methods, for determining P O2 values in a heterogeneous system. However, both investigations suggested that measurement accuracy depends on the volume of the detection area. In this study, a 535-nm N2-dye pulse laser was used to excite the phosphorescent probe through a long-working-distance objective lens. This optical arrangement enabled the phosphor in a limited volume to be excited for short time (1–2 s).

Buerk et al. (2) reported direct comparison of tissue P O2 measurement in hamster skinfold preparations using the phosphorescence quenching technique with microelectrodes. The major difference in the optical arrangement between their system and ours is the lighting source. They used a xenon strobe lamp with an optical window for detecting the phosphorescence emission in a limited area (10 × 10 μm), whereas our system was equipped with a N2-dye pulse laser. They found that there was no significant difference between the optical and microelectrode tissue P O2 measurements and that there was no significant depletion of O2 during excitation of the phosphor (3 s). Consequently, our measurements, made under similar conditions, suggest that, even though the fluid movement in the interstitial space was slow, the photoactivated consumption of O2 might have had a minimal effect on our data.

In the last decade, the phosphorescence quenching technique has been substantially improved and applied to in vivo measurements by several investigators (38, 44). Torres Filho et al. (33) reported on the intravascular P O2 gradients in arterioles and venules of different orders in a hamster skin preparation. In addition, Tsai et al. (35) reported on the distribution of P O2 in the microvessels and interstitial spaces of a rat mesentery preparation. However, both of these investigations used thin preparations. Only a few reports have been made of intravascular and interstitial P O2 measurements in skeletal muscle using the phosphorescence technique (46) because skeletal muscle is generally too thick for the phosphorescent probe to be excited by the xenon flash lamp that is usually used in this method. In this study, a 535-nm N2-dye pulse laser was used to excite the phosphorescent probe through a long-working-distance objective lens. This optical arrangement might anticipate the application of the P O2 measurement in the deeper tissue.

In a previous study from our laboratory (29), we evaluated the divergence of the incident light in tissue as a result of light scattering and absorption when a laser illuminator was used to excite a fluorescent probe in rabbit tenuissimus muscle. Our results indicated that, at tissue depths of 100 and 200 μm below the surface, the intensity of the incident light was at least 50 and 30%, respectively. Moreover, each augmentation was no more than 1.7–2.5 times wider. Pulse laser can also be used to excite the phosphorescent probe in regions where the P O2 is higher than 100 Torr and the lifetime of Pd-porphyrin is shorter than 30 μs. With a xenon flash lamp, the decay in the light from the xenon flash remains for the first 30 μs of the phosphorescence decay curve (32). Thus high P O2 values cannot be measured.

A significant drop in the P O2 levels of different order arterioles was found (see Table 1). Similar levels of decrease in P O2, including the longitudinal P O2 gradient in arterioles, were also found by many investigators (6, 9, 21, 31). For instance, in hamster skin arterioles, a similar tendency was reported by Torres Filho et al. (33), although the arterioles in their study were smaller than those in the present study. The P O2 values of A1, A2, and A3 in our results were 74.6, 54.4, and 46.6 Torr, respectively. These values are higher than the data for skin (A1, 51.8; A2, 44.1; A3, 39.9 Torr). However, the systemic arterial P O2 value in our preparation (98.1 Torr) was also much higher than that of the skin preparation (71.1 Torr). These differences may be due to the poorer respiratory gas exchange of hamsters relative to that of rats and/or the organ specificity. When arteriolar P O2 values for vessels with a similar diameter were compared, our A2 and A3 P O2 values were consistent with the A1 and A2 P O2 values in the skin. In addition, the P O2 value of the venules (31.3 Torr) was consistent with the venular P O2 value in the skin (30.8 Torr).

In this study, a significant reduction in the P O2 level was found at the interface between the blood vessels and the tissue as shown in Fig. 6. A similar P O2 gradient has been reported for rat mesenteric arterioles (35). The interstitial P O2 values adjacent to the A1, A2, and A3 arterioles were 50.7, 39.4, and 28.8 Torr, respectively. These values are 15–20 Torr lower than the P O2 values in each arteriole. These findings, and a significant drop in the P O2 levels of different order arterioles as was stated previously, strongly support the view that capillaries are not the sole source of O2 supply to surrounding tissue. These phenomena are with the hypothesis that the intravascular O2 flux is facilitated.
by a high diffusion constant (26) and/or they are due to high O₂ solubility in tissue (25). In contrast, a possibility remains that there is a high rate of O₂ consumption in the vessel walls (14) because O₂ consumption decreased by 34% when the endothelium was removed from the hindlimb of a dog (5). More recently, Vadapalli et al. (36) predicted that some other extramitochondrial pathways are associated with O₂ utilization. Further study is necessary to clarify these phenomena.

P₀₂ levels in the interstitial spaces at points near and far from the arterioles were significantly different (see Table 1). The interstitial P₀₂ values adjacent to the A1, A2, and A3 arterioles were significantly higher than the interstitial P₀₂ values located farther away (10–13 Torr). It is difficult to compare these values with those of other reports using different preparations and measurement techniques because the area of measurement depends on the microvascular arrangement. For instance, in the hamster skin preparation in which the photoquenching technique was used to obtain measurements, interstitial P₀₂ values were between 11 and 36 Torr (33). However, in the hamster cremaster muscle measurement using microelectrodes, the tissue P₀₂ values were between 14 and 17 Torr (12). In addition, using the same preparation and the same technique, the mean tissue P₀₂ value was lowered still further (11–15 Torr) under superfusion with a low P₀₂ solution (18). In our experiment, the muscle surface was suffused with 0% O₂ solution before P₀₂ measurement, and then the suffusion was stopped and a coverslip was placed on the muscle surface during P₀₂ measurement. Consequently, no O₂ supply from room air through the coverslip may be one of the causes of low interstitial P₀₂ values. In addition, the relatively high temperature of sulfamate (37°C) in our experiment may have induced high metabolic activity in the tissue. Otherwise, the differences between our values and those reported before are likely due to the differences in tissue metabolism between rats and hamsters.

This study has shown that the phosphorescence quenching laser microscope can be used to clarify the transport of O₂ across microvessels and the distribution of O₂ in tissues, although more regional data on P₀₂ values in tissues are required at the present. The use of lasers to excite phosphorescent probes enables a smaller area of tissue to be illuminated (<10 μm) if the potential for tissue damage is clarified in detail. In addition, intracellular measurements of P₀₂ will require the phosphorescent probe to penetrate the cell membrane.

In conclusion, a new intravital microscope has been designed that utilizes O₂-dependent phosphorescence quenching. This microscope may feasibly be applied to studies for O₂ transport across microvessels and the distribution of O₂ in skeletal muscle. In addition, we found a large reduction in P₀₂ levels of different order arterioles as well as a large P₀₂ gradient at the interface between blood and tissue. These findings suggest that capillaries are not the sole source of oxygen supply to surrounding tissue.

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