Oxygen pressures in the interstitial space and their relationship to those in the blood plasma in resting skeletal muscle

David F. Wilson,1 William M. F. Lee,2 Sosina Makonnen,2 Olga Finikova,1 Sofia Apreleva,1 and Sergei A. Vinogradov

Departments of 1Biochemistry and Biophysics and 2Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Submitted 3 April 2006; accepted in final form 24 July 2006

Wilson, David F., William M. F. Lee, Sosina Makonnen, Olga Finikova, Sofia Apreleva, and Sergei A. Vinogradov. Oxygen pressures in the interstitial space and their relationship to those in the blood plasma in resting skeletal muscle. J Appl Physiol 101: 1648–1656, 2006.—This study compared oxygen pressures (PO2), measured by oxygen-dependent quenching of phosphorescence, in the intravascular (blood plasma) space in the muscle with those in the interstitial (pericellular) space. Our hypothesis was that the capillary wall would not significantly impede oxygen diffusion from the blood plasma to the pericellular space. A new near-infrared oxygen sensitive probe, Oxyphor G3, was used to obtain oxygen distributions in the interstitial space. Oxyphor G3 is a Pd-tetrabenzoporphyrin encapsulated inside generation 2 poly-arylglycine (AG) dendrimer. The periphery of the dendrimer is modified with oligoethylene glycol residues (average molecular weight 350) to make the probe water soluble and biologically inert. Oxyphor G3 was injected into thigh muscle using a 30-gauge needle. Histograms of the PO2 in the interstitial space were measured in awake and anesthetized animals and compared with those for Oxyphor G2 in the intravascular (blood plasma) space. For awake mice, the lowest 10% of PO2 values for the interstitial and intravascular spaces (believed to represent capillary bed) were not significantly different [23.8 (SD 4.5) and 25 Torr (SD 4.3), respectively], whereas, in isoflurane-anesthetized mice, there was a small but significant (P = 0.01) difference [20.4 (SD 6.3) and 27.9 Torr (SD 3.5), respectively]. The peak values for the histograms for the interstitial space in awake and isoflurane-anesthetized mice were 40.8 (SD 7.5) and 36.9 Torr (SD 8.3), respectively, whereas those for the intravascular space were 52.2 (SD 4.9) and 55.9 Torr (SD 8.4), respectively, showing no significant difference due to isoflurane anesthesia. The histograms for the intravascular space were significantly wider, with more contribution at higher PO2 values. A different anesthetic, ketamine plus xylazine injected intraperitoneally, caused a marked decrease in the tissue PO2 values in both spaces, with the time course and extent of the decrease dependent on the time after injection and variable among mice. It was, therefore, not further used.

OXYGEN IS TRANSPORTED FROM THE LUNG TO OTHER TISSUES WHILE BOUND TO HEMOGLOBIN, BUT, UPON REACHING THOSE TISSUES, IT MOVES FROM THE BLOOD PLASMA TO THE MITOCHONDRIA BY DIFFUSION. THIS MEANS THE OXYGEN FIRST DIFFUSES FROM THE BLOOD PLASMA THROUGH THE WALLS OF THE MICROVESSELS INTO THE INTERSTITIAL (PERICELLULAR) SPACE. FROM THE INTERSTITIAL SPACE, IT PASSES INTO THE CELLS AND THEN TO THE MITOCHONDRIA. IN DIFFUSING FROM A SOURCE (BLOOD PLASMA) TO A SINK (MITOCHONDRIA), AN OXYGEN PRESSURE (PO2) GRADIENT IS FORMED, WITH PRESSURE LOWER AT THE SINK THAN AT THE SOURCE. THE DIFFERENCE IN PO2, AND THEREFORE THE GRADIENT, INCREASES WITH INCREASE IN THE RATE OF OXYGEN CONSUMPTION BY THE MITOCHONDRIA. THE DISTANCE OVER WHICH OXYGEN CAN BE SUPPLIED TO THE MITOCHONDRIA IS DETERMINED BY 1) THE RATE OF OXYGEN CONSUMPTION BY THE MITOCHONDRIA; 2) THE DISTANCE FROM THE BLOOD VESSEL (THE OXYGEN SOURCE) TO THE MITOCHONDRIA; AND 3) THE PO2 IN THE BLOOD PLASMA. QUANTITATIVE MEASUREMENTS OF PO2 VALUES IN DIFFERENT COMPARTMENTS OF TISSUE ARE, THEREFORE, NECESSARY TO UNDERSTAND MUSCLE BIOCHEMISTRY AND PHYSIOLOGY.

OXYGEN-DEPENDENT QUENCHING OF PHOSPHORESCENCE PROVIDES A NONINVASIVE OPTICAL METHOD FOR DETERMINING PO2 VALUES IN BIOLOGICAL AND OTHER SAMPLES (26,35). THIS METHOD HAS BEEN SHOWN TO BE EFFECTIVE FOR MEASUREMENTS IN MANY TYPES OF BIOLOGICAL MEDIA, INCLUDING BIOLOGICAL FLUIDS AND THE MICROVASCULARITY OF TISSUE IN VIVO (2–7, 12, 14, 18–22, 29, 36, 37). THE AVAILABLE OXYGEN-SENSITIVE PHOSPHORS, SUCH AS OXYPHORS R0, R2, AND G2 (OXYGEN ENTERPRISES, PHILADELPHIA, PA), CONTAIN PD-PORPHYRIN CORES THAT ARE AT LEAST PARTIALLY EXPOSED TO THE MEDIUM. AS A RESULT, THEY BIND TO BIOLOGICAL MACROMOLECULES SUCH AS ALBUMIN. OXYGEN SENSITIVITY OF PHOSPHORESCENCE IS DEPENDENT ON THE MICROENVIRONMENT OF THE Porphyrin AND, THEREFORE, STRONGLY DEPENDENT ON THE IDENTITY OF THE MACROMOLECULES IN THE ENVIRONMENT AND THE EXTENT OF THE PHOSPHOR BINDING TO THOSE MACROMOLECULES. IN BLOOD PLASMA, OXYPHORS R0, R2, AND G2 ARE ESSENTIALLY QUANTITATIVELY BOUND TO ALBUMIN. ALBUMIN PLAYS AN IMPORTANT ROLE, HELPING BOTH TO LIMIT ACCESS OF OXYGEN TO THE PORPHYRIN CORE, FACILITATING OXYGEN MEASUREMENTS IN THE PHYSIOLOGICAL RANGE (0–120 TORR), AND PROVIDING A RELATIVELY HOMOGENEOUS PORPHYRIN MICROENVIRONMENT.

IF OXYPHORS R0, R2, OR G2 LEAK OUT OF THE INTRAVASCULAR COMPARTMENT, THERE MAY BE MUCH LESS ALBUMIN IN THE ENVIRONMENT. AS A RESULT, THEY MAY NOT BE ENTIRELY BOUND TO ALBUMIN AND MAY INSTEAD BIND TO BIOMOLECULES OTHER THAN ALBUMIN. EACH DIFFERENT BINDING SITE PROVIDES A DIFFERENT MICROENVIRONMENT AND THUS A DIFFERENT OXYGEN SENSITIVITY OF THE OXYPHOR. SUCH HETEROGENEITY IN THE OXYPHOR PROPERTIES LEADS TO UNCERTAINTY IN OXYGEN MEASUREMENTS. TO MINIMIZE THIS UNCERTAINTY IN THE METHOD, WE HAVE DESIGNED A NEW FAMILY OF DENDRITIC PHOSPHORS, WHOSE PERIPHERY IS MODIFIED WITH OLIGOETHYLENE GLYCOL FRAGMENTS (15–17). OXYPHOR G3 IS ONE SUCH OXYGEN SENSOR, AND ITS OXYGEN-QUenchING PROPERTIES ARE NOT AFFECTED BY BIOLOGICAL MACROMOLECULES SUCH AS ALBUMIN, WHILE ITS PHOSPHORESCENT PARAMETERS ARE WELL SUITED FOR MEASURING OXYGEN IN VIVO AND IN VITRO.

The purpose of this communication is twofold: first, to demonstrate the utility of Oxyphor G3 for oxygen measure-
ments in the interstitial space of tissue; and second, to test the hypothesis that the walls of microvessels, particularly capillaries, provide a very minimal barrier to oxygen delivery to the cells. To test this hypothesis, we have determined the difference in PO₂ between the interstitial space and the blood plasma. The vascular wall is the only physical barrier between these two compartments, and oxygen consumption within the interstitial space is very small. As a result, differences in PO₂ between the compartments are a measure of the barrier to oxygen diffusion/oxygen consumption by the walls of the vessels and of the distance from the vessel lumen. The oxygen measurements have been made in both awake and anesthetized mice to control for the possibility of stress-induced changes in blood pressure and vascular resistance in awake mice. By measuring in both awake and anesthetized mice, it is possible to quantify the effect of anesthesia on tissue oxygenation. This is important because anesthetics can have marked effects not only on cardiopulmonary function but also on vascular regulation.

MATERIALS AND METHODS

Oxygen Histograms Obtained by Oxygen-dependent Quenching of Phosphorescence

With the use of oxygen-dependent quenching of phosphorescence, PO₂ histograms can be obtained from a single-point measurement. A cartoon illustrating the distribution measurement experiment is shown in Fig. 1.

For an excitation source and a phosphorescence detector (light guide tips), positioned on the surface of tissue and separated by distance d, the sampled volume will be a banana-like shaped region, formed by overlapping three-dimensional “globes” of diffuse light centered in front of the tip of the light guides (see Ref. 10 for published measurements of the photon density distributions). Different parts of the volume may contribute unequally to the signal due to the differences in the absorption and scattering coefficients for the excitation light and phosphorescence. For Oxyphors G2 and G3, the excitation light (635 nm) is more absorbed and scattered than is the excitation light and phosphorescence. For Oxyphors G2 and G3, the differences in the absorption and scattering coefficients for the different parts of the volume may contribute unequally to the signal due to the differences in the absorption and scattering coefficients for the excitation light and phosphorescence. For Oxyphors G2 and G3, the excitation light (635 nm) is more absorbed and scattered than is the phosphorescence (800 nm). As a result, volumes closer to the excitation source contribute more to the detected signal than those further from the source. Most of the detected signal comes from the banana-shaped volume (10), shown in Fig. 1.

When sampling normal tissue, the measured distribution of PO₂ does not depend on the positions of the excitation and emission light guides, because the measured volume is much larger than the individual vascular beds in the tissue. The 6-mm center to center of the positioning of light guides in the present studies was used in part to be sure that sampled volume was representative of the tissue as a whole.

Measurement of PO₂ Histograms

Phosphorescence lifetime measurements were performed using a PMOD-5000 phosphorometer (Oxygen Enterprises, Philadelphia, PA) (28). The PMOD-5000 is a frequency domain instrument operating in the frequency range of 100–100,000 Hz. The measured phosphorescence lifetimes are independent of local phosphor concentration and are insensitive to the presence of endogenous tissue fluorophores and chromophores. The excitation light was carried to the animal through one glass fiber bundle and the emission collected by another 3-mm-diameter glass fiber bundle (center-to-center distance of 6 mm). The emission was filtered through a 695-nm long-pass glass filter (Schott glass) and detected by an avalanche photodiode (Hamamatsu). The resulting photocurrent was converted into voltage, amplified, digitized, and transferred to the computer for analysis.

In the present study, PMOD-5000 was used in multifrequency mode (28) to determine distributions of phosphorescence lifetimes. The lifetime distributions were used to calculate distributions of PO₂ values, i.e., oxygen histograms (31, 32). The excitation light (maximum wavelength = 635 nm) was modulated by a waveform consisting of 37 sinusoids with equal amplitudes and frequencies ranging from 100 Hz to 38 kHz. The tips of the light guides were brought into contact with the skin, but care was taken not to apply pressure that might restrict flow in the surface blood vessels. The obtained signal was used to calculate the dependence of the phosphorescence amplitude and phase on the modulation frequency. The resulting phase/ amplitude dependence was analyzed using the Maximal Entropy Method (32) to yield the distribution of phosphorescence lifetimes. This distribution was converted into the distribution of PO₂ in the sample, as described previously (28, 32). The basis for the conversion is the Stern-Volmer relationship:

$$I'/I = T'/T = 1 + k_qI^*T^*PO_2$$  \( (1) \)

where I' and T', are the phosphorescence intensities and lifetimes in the absence of oxygen, and I and T are the phosphorescence intensities and lifetimes at PO₂, respectively. The quenching constant, k_q, is a second-order rate constant, describing the quenching of the excited state of the phosphor by oxygen. The values of T' and k_q have been determined for each phosphor for the experimental conditions (temperature, etc., as appropriate).

According to Eq. 1, intensities (amplitudes) of phosphorescent signals decrease with increasing PO₂ values. Thus, for two equal volumes of tissue, containing equal amounts of the phosphorescent probe and excited by equal numbers of photons, the accuracy in signal is higher for lower PO₂ values. The decrease in signal with increasing PO₂ [decrease in signal-to-noise ratio (S/N)] results in asymmetric broadening of oxygen histograms, as seen in the “tail” effect on the high-oxygen end of the histogram. This asymmetric broadening is intrinsic to the Maximal Entropy Method analysis, reflecting the fact that uncertainty in determination of phosphorescence lifetimes increases with decreasing S/N. Thus, although the histograms are very reliable at lower PO₂ values where there is little broadening, for PO₂ values above ~80 Torr, the histograms are sufficiently broadened; they should be used only for qualitatively comparisons. The presented histograms were arbitrarily truncated at 140 Torr.
Phosphorescent Probes Oxyphor G2 and Oxyphor G3

Two phosphorescent probes were used in our experiments, both based on Pd-tetrabenzo-porphyrin cores (30), Oxyphor G2 (15) (Fig. 2A), and Oxyphor G3 (Fig. 2B). The synthesis will be published elsewhere, but synthesis of similar dendritic porphyrins has been reported (27).

Pd-tetrabenzo-porphyrin dendrimers G2 and G3 differ by the dendrimer composition (G2, polyglutamate; G3, polyarylglycine) and surface coatings [G2, none; G3, polyethylene glycol, average molecular weight (MW) 350]. G2 (MW 2,642) is designed to be used in combination with albumin, which provides a uniform microenvironment for the phosphor. In contrast, G3 (MW 16,100) is designed not to interact with albumin and other biomolecules by adding a surface layer of polyethylene glycols. The dendrimer in G3 folds tightly around the core in aqueous media and controls accessibility of oxygen to the porphyrin. The absorption and the phosphorescence spectra of G2 and G3 are nearly identical (Fig. 2). Both phosphors have quantum yields of ~10% and lifetimes of ~270 μs in deoxygenated aqueous solutions.

The oxygen $k_Q$ of G2 and G3 in aqueous buffered solutions at pH 7.2 at 38°C are 2,800 and 180 Torr$^{-1}$·s$^{-1}$, respectively. Thus unbound Oxyphor G3, but not G2, can be used to measure oxygen in physiological range. In the blood, however, Oxyphor G2 binds tightly to albumin, and this lowers the oxygen $k_Q$. The $k_Q$ for the G2-albumin complex at 38°C is 280 Torr$^{-1}$·s$^{-1}$. This value is well suited for oxygen measurements in vivo. Oxyphors G2 and G3 have lifetimes at zero oxygen of 250 and 270 μs, respectively.

The phosphorescence of Oxyphor G3 is completely insensitive to the presence of albumin (at 1–5% by weight range). It is also insensitive to changes in pH and ionic strength throughout the physiological range. Stern-Volmer plots of both Oxyphor G3 and Oxyphor G2-albumin complex exhibit slight nonlinearity, due to the multiple conformations of their large polymeric structures.

Measurements of Oxygen in the Blood Plasma and Interstitial Space of Muscle

Mouse preparation. The fur on the right and left rear quarters was removed by first using electrical clippers and then depilated. Care was taken not to cause any abrasions to the skin. The oxygen measurements were then made noninvasively through the skin. The fur was removed because, in dark-colored mice, the fur absorbs both the excitation light and the emitted phosphorescence, greatly attenuating the phosphorescence signal.

Measuring Oxygen Histograms in the Blood Plasma (Oxyphor G2)

Isoflurane anesthesia. Anesthesia was induced with 1.5% isoflurane in air, and 0.1 ml of a solution of Oxyphor G2 (3.2 mg/ml) in physiological saline was injected into the tail vein. As soon as anesthesia was induced, isoflurane was decreased to 1.2%, and the oxygen histograms were measured ~10 min after induction of anesthesia. It has been previously noted (37), and confirmed in the present study, that induction of anesthesia with isoflurane causes a transient decrease in tissue PO2 values that recovers within 10 min of continuing anesthesia. After the oxygen histograms (anesthetized) were measured, the nose cone supplying the isoflurane was removed, and the mice were replaced in their cage. Mice recover quickly from isoflurane anesthesia, typically recovering full activity within 5 min. After ~40 min without inhaled anesthetic, the oxygen histograms were again measured (awake).

Ketamine + xylazine anesthesia. A mixture of ketamine and xylazine (ketamine 85 mg/kg + xylazine 6.5 mg/kg) was given as an intraperitoneal injection. Oxygen histograms were measured periodically until the anesthetic wore off and they began to move about.

Throughout the periods of anesthesia, body temperature was maintained by laying the mice on a 38° isothermal pad covered with a terrycloth towel to be sure they did not overheat.

Measuring Oxygen Histograms in the Interstitial Space (Oxyphor G3)

The mice were shaved and depilated as described above. They were anesthetized with isoflurane (nose cone, 1.5% mixed with air) and given injections of Oxyphor G3 solution (80 μM in physiological saline) along three different 1-cm tracks (20 μl containing 1.6 nmol of Oxyphor per track) in the thigh muscle using a 30-gauge needle. The nose cone was removed, and the mice were returned to their cage. They were allowed to wake up and run about in the cage for 70–90 min to help distribute the phosphor within the interstitial space of the muscle, and then the oxygen histograms were measured in the awake mouse. Each mouse was then anesthetized with either isoflurane or ketamine plus xylazine, and the oxygen histograms measured are described above. The amount of Oxyphor G3 injected into the muscle was ~2% of that required to give a similar phosphorescence intensity (a concentration similar to that used for Oxyphor G2) when injected into the blood. Thus the measured phosphorescence comes from the interstitial space, since any Oxyphor G3 entering the blood would be distributed throughout the body, attenuating the signal to insignificance.

Fig. 2. Phosphorescent oxygen probes Oxyphor G2 (A), Oxyphor G3 (B), and their absorption and emission spectra (C).
Table 1. \( \text{PO}_2 \) at the peak of the oxygen histograms as measured using Oxyphor G3 and Oxyphor G22

<table>
<thead>
<tr>
<th>Phosphor</th>
<th>( \text{PO}_2 ) Peak Awake</th>
<th>( \text{PO}_2 ) Peak Isoflurane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxyphor G3 (n = 11): interstitial space</td>
<td>40.8 (7.5)</td>
<td>36.9 (8.3)</td>
</tr>
<tr>
<td>Oxyphor G2 (n = 10): intravascular space</td>
<td>52.2 (4.9)</td>
<td>55.9 (8.4)</td>
</tr>
<tr>
<td>Oxyphor G3: awake vs. isoflurane</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Oxyphor G2: awake vs. isoflurane</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Oxyphor G3 vs. Oxyphor G2</td>
<td>( P &lt; 0.001 )</td>
<td>( P &lt; 0.0001 )</td>
</tr>
</tbody>
</table>

Values are means (SD) in rows 1 and 2; \( n \), no. of animals. The calculated \( P \) values for the differences in peak position are given in rows 3–5. NS, not significant.

The experiments were carried out by investigators trained to handle mice. All of the experimental procedures were reviewed and approved by the local Institutional Animal Care and Use Committee. At the end of the experiment, the mice were euthanized according to guidelines established by the American Veterinary Medical Association Panel on Euthanasia.

Statistical Analysis

The presented results are the means (SD). Statistical analysis was comparison of two independent populations. Differences were considered significant if \( P < 0.05 \).

RESULTS

\( \text{PO}_2 \) Distributions in the Interstitial Space of Awake and Anesthetized Mice: Isoflurane Anesthesia

After injection of Oxyphor G3 into the muscle, there was a strong phosphorescence, which was not present before the phosphor was injected. Good S/N (>50) was required, typical values were from 65 to 120) was obtained using data collection periods of 200–600 ms. This allowed the measurements to be made quickly, minimizing sensitivity to movement of the mouse. The mice were accustomed to being handled, and care was taken that the foot of the measured leg was not in contact with a surface. This prevented the leg muscle from developing tension by pushing against the surface. The light guides were brought gently in contact with the skin and did not cause a reaction by the mouse. In a total of 11 independent experiments, the peak position was 40.8 (SD 7.5) (Table 1), with very little of the muscle volume having \( \text{PO}_2 \) values <10 Torr. The individual histograms were analyzed to determine the \( \text{PO}_2 \) values for which the cumulative volume fractions were 5, 10, or 40% of the signal (tissue volume). These were 15.9 (SD 6.1), 23.8 (SD 4.5), and 46.2 Torr (SD 4.5), respectively (see Table 2). The distributions are asymmetric with a tail on the higher oxygen side. The asymmetry is always present but variable in magnitude (see MATERIALS AND METHODS).

When measurements were made in animals anesthetized with isoflurane, the histograms were very similar to those in awake mice. In 11 experiments, the histograms had peak values of 36.9 Torr (SD 8.3) (Table 1), again with little or none of the signal <10 Torr. The \( \text{PO}_2 \) values were determined for which the cumulative sum of the volume fractions were 5, 10, or 40% of the signal (tissue volume). These were 13.8 (SD 5.9), 20.4 (SD 6.3), and 42.6 Torr (SD 7.6), respectively (see Table 2). Although the values for awake mice were slightly higher than for anesthetized mice, the differences were not statistically significant. This indicates isoflurane anesthesia had little effect on the \( \text{PO}_2 \) distribution in the interstitial space. Figure 3A shows histograms formed by separately averaging the measurements for awake and anesthetized mice.

\( \text{PO}_2 \) Distribution in the Blood Plasma of Awake and Anesthetized Mice: Isoflurane Anesthesia

To measure oxygen distributions in the blood plasma of the microcirculation, Oxyphor G2 was injected into the tail vein of the mice. Since the optical properties of Oxyphors G2 and G3 are very similar (Fig. 2C), they cannot be used to make simultaneous measurements in the two compartments. We first did an experiment in which mice were given injections of Oxyphor G2 in the tail vein while awake, and the oxygen distributions were measured in the awake animals. The resulting oxygen histograms were highly variable, and many had very broad histograms with peaks at \( \text{PO}_2 \) values of 60 Torr and above. These broad distributions with increased high \( \text{PO}_2 \) values correlated with the visual evidence of agitation, indicating that stress related to the tail vein injection was causing increased blood flow in the muscle. The evidence of stress-induced increase in flow was variable and persisted for periods of many minutes. To avoid the stress associated with tail vein injections in awake mice, it was decided to do the tail vein injections under isoflurane anesthesia. After the intravenous injection, the mice were kept under anesthesia, and the oxygen histograms were measured 10–15 min later. The isoflurane was withdrawn, and the mice were returned to their cage. The histograms for the awake mice were measured 40–60 min after recovery from anesthesia.

Table 2. Comparison of the tissue oxygen histograms obtained with Oxyphor G3 (interstitial space) and Oxyphor G2 (intravascular space) for normal mouse muscle

<table>
<thead>
<tr>
<th>Phosphor</th>
<th>5% of Volume</th>
<th>10% of Volume</th>
<th>40% of Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awake</td>
<td>Isoflurane</td>
<td>Awake</td>
<td>Isoflurane</td>
</tr>
<tr>
<td>Oxyphor G3 (n = 11)</td>
<td>15.9 (6.1)</td>
<td>23.8 (4.5)</td>
<td>46.2 (6.9)</td>
</tr>
<tr>
<td>Oxyphor G2 (n = 10)</td>
<td>17.4 (4.0)</td>
<td>25 (4.3)</td>
<td>55.2 (5.1)</td>
</tr>
<tr>
<td>G3: awake vs. isoflurane</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>G2: awake vs. isoflurane</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>G2 vs. G3</td>
<td>( P &lt; 0.05 )</td>
<td>( P &lt; 0.01 )</td>
<td>( P &lt; 0.01 )</td>
</tr>
</tbody>
</table>

The oxygen pressures for which 5, 10, and 40% of the signal (sampled volume) has oxygen pressures less than that value are given with SDs in rows 1 and 2; \( n \), no. of animals. Statistical analysis was then used to determine whether there were significant differences in the values when comparing Oxyphor G3 (row 3) and Oxyphor G2 (row 4) in awake and isoflurane-anesthetized mice, and when comparing Oxyphor G3 with Oxyphor G2 in awake or isoflurane-anesthetized mice (row 5).
Comparison of the Po2 Distributions in the Interstitial and Intravascular Spaces of Skeletal Muscle of Awake and Anesthetized Mice

Ketamine + xylazine anesthesia. One of the most widely used methods for anesthetizing rodents is intraperitoneal injection of a combination of ketamine and xylazine. This is a convenient and effective method. It does not require an expensive anesthesia machine and can be used to maintain anesthesia for substantial periods of time. In the present studies, we minimized stress for the mice by first anesthetizing them with isoflurane and injecting Oxyphor G3 into the muscle while they were anesthetized. The isoflurane was removed, and the mice were allowed to recover from the anesthetic. A 90-min awake period was allowed for complete removal of the isoflurane from the mouse and for Oxyphor G3 to distribute through the interstitial space of the muscle. The Po2 histograms were measured (awake), and an intraperitoneal injection of ketamine plus xylazine was given. Beginning ~5 min after injection of anesthetic, oxygen histograms were measured at intervals until the anesthesia had decreased to where the mouse was again attempting to walk. A representative set of histograms is shown in Fig. 4A. The 0-min histogram was taken just before injection of the anesthetic. The histograms show a progressive decrease in tissue Po2 values during the first 15 min, with the peak value falling from 40 Torr to ~18 Torr. The trend then reversed, and the Po2 values increased again to near the preanesthetic values in 45 min. This pattern is typical, but the extent of the decrease in oxygen and the time before the mouse recovered from anesthesia varied from mouse to mouse. In all cases, the lowest Po2 values in the histogram fell to near zero, indicating that regions of near anoxia were present in the interstitial space. The effect of the anesthetic was quite long lasting, and some decrease in oxygen persisted for 45 min or 1 h, a time point at which the mice were moving about with poor coordination.

A similar pattern was observed when Oxyphor G2 was used (Fig. 4B). The oxygen histograms shift strongly toward lower oxygen levels with minimal values occurring in ~10–30 min, followed by recovery toward the preanesthetic values. For both Oxyphor G3 and Oxyphor G2, the changes in Po2 induced by ketamine + xylazine anesthesia were large enough to mask any differences in Po2 values between the intravascular and interstitial spaces in the tissue.

Relationship of the Po2 Values in the Intravascular and Interstitial Spaces

In awake mice. The relationship of the oxygen distributions in the interstitial space with those of the intravascular (blood plasma) space was examined by comparing the relevant histograms. In awake animals, the peaks of the histograms were at 40.8 (SD 7.5) and 52.2 Torr (SD 4.9), for Oxyphor G3 and G2, respectively, and this difference was statistically significant (P < 0.001) (Table 1). The cumulative sums of the volume fractions of 5, 10, or 40% of 5.1, respectively (see Table 2).

Although the values for awake mice were slightly lower than for anesthetized mice, the differences were not statistically significant. Figure 3B shows the very similar Po2 histograms obtained using for Oxyphor G2 in both awake and anesthetized mice.
broadened compared with those for the interstitial space, and
there is a significantly higher fraction of the volume at higher
PO2 values.

In isoflurane-anesthetized mice. In mice under isoflurane
anesthesia, the peak values for the histograms were 36.9 Torr
(SD 7.5) for Oxyphor G3 and 55.9 Torr (SD 8.4) for Oxyphor
G2 (Table 1). The former is significantly less than the latter
\((P < 0.001)\). The histograms were analyzed for the PO2 values
for which 5, 10, or 40% of the signal (tissue volume) had lower
PO2 values. These were 13.8 (SD 5.9), 20.4 (SD 6.3), and 42.6
Torr (SD 7.6), respectively, for Oxyphor G3 and 19.9 (SD 3.7),
27.9 (SD 3.5), and 59.2 (SD 5.9), respectively, for Oxyphor G2
(see Table 2). The values for the vascular compartment were
significantly higher than for the interstitial space \((P \text{ values }<
0.05, 0.01, \text{ and } 0.001\), respectively). The average of histograms
for awake mice are shown in Fig. 5A, whereas those for
isoflurane-anesthetized mice are in shown in Fig. 5B.

DISCUSSION

Oxyphor G3 as a Sensor for Oxygen in the Interstitial Space

The termini of the dendrimers in Oxyphor G3 are esterified
with oligoethylene glycol residues, which make the probe
water soluble and at the same time uncharged. Addition of
albumin, a biological macromolecule with a wide variety of
binding functions, does not affect the oxygen-dependent
quenching of phosphorescence of Oxyphor G3, and there is no
evidence that any other biological agents affect its oxygen
sensitivity. When Oxyphor G3 is injected into the interstitial
space of muscle, the phosphorescence could be measured for at
least 3 h, with little attenuation of the signal. Measurements
made 2 days after injection, however, did not detect significant
remaining phosphorescence, consistent with the Oxyphor be-
ing removed through the lymphatic system.

There are no other methods for selectively measuring inter-
stitial PO2 values. The values can, however, be compared with

Fig. 4. Oxygen histograms measured over time after subcutaneous injection of
ketamine plus xylazine to induce anesthesia. A: Oxyphor G3 (interstitial
space); B: Oxyphor G2 (intravascular space). The 0-min curve was measured
just before injection of the anesthetic.

Fig. 5. Comparison of the oxygen histograms measured for the interstitial
space (Oxyphor G3) and intravascular space (Oxyphor G2) of skeletal muscle
in awake (A) and isoflurane-anesthetized (B) mice. The histograms are the
means for 11 (Oxyphor G3) or 10 (Oxyphor G2) independent experiments,
with error bars indicating the SE of the mean (±SE) for the measurements.
those obtained using microoxygen electrodes and solid electron paramagnetic resonance probes (22), which measure a mixture of the interstitial space and capillary oxygenation, and to those obtained using nitroimidazole binding, which measures intracellular oxygenation. Most microoxygen electrode measurements have been made in softer tissue, such as the kidney, liver, and brain. Baumgärtl and coworkers (1) published detailed histograms of the oxygen distribution in dog kidney with sufficient numbers of measurements (630–1,105) to yield good histograms. The kidney histograms were reported for six experiments and the mean PO2 values were 36.8 Torr (SD 6.0). These histograms were also asymmetric, with very few values below 10 Torr, but including values up to 80–90 Torr. The anesthetic used was not reported, preventing more detailed comparison. There have been oxygen measurements in rodent muscles using oxygen electrodes and phosphorescence quenching. Whalen and coworkers (33, 34) used electrodes with very small tips to measure PO2 values within the cells in living tissue in animals anesthetized with urethane and barbital, and the muscles were surgically exposed. They reported that 75% of the values were between 0 and 5 Torr in guinea pig gracilis and cat heart muscles, whereas those in the cat soleus muscle were higher, having a mean value of 18.9 Torr (SD 1.8). At that time, measurements could only be made in anesthetized animals, so the influence of the anesthetic on PO2 in the tissue was not appreciated. As a result, these and other early oxygen electrode measurements gave rise to a widely held, but erroneous, view that the PO2 values in tissue were very low and there were small regions of anoxia within the tissue. Other reported mean values for muscle tissue include 19 Torr (12) and 26.8 Torr (7) for the rat cremaster muscle, and 31.4 Torr (2) for the rat spinotrapezius microvasculature. These are lower than the 46.2 Torr (awake) or 36.9 Torr (isoflurane anesthesia) values reported in the present communication for the interstitial space but are more comparable to those for ketamine plus xylazine anesthesia. The measurements in the present study were of the bulk muscle, with no differentiation among the muscle groups of the leg. It remains to be established whether different muscles and muscle types have different PO2 values, although in resting muscle such differences would likely be small.

Tissue oxygen measurements using electron paramagnetic resonance active particles injected into the tissue (see Refs. 23, 24) were reported to give PO2 values in the rat brain of 39.3 Torr (SD 4.1) in isoflurane-anesthetized rats (11).

Nitroimidazole binding has been used to measure intracellular oxygenation (for review, see Ref. 8), primarily of tumor tissue and other tissues having lower PO2 values than normal tissue. This is because nitroimidazole binding increases with decreasing PO2 values, making it very effective for detecting tissue volumes with below-normal oxygenation. Although the decreased sensitivity at higher PO2 values has thus far precluded generation of oxygen histograms, this method can determine the lower limit of the tissue PO2 values very effectively. In awake and isoflurane-anesthetized animals, normal muscle and other tissues show little 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide binding, indicating there are very few cells with intracellular PO2 values less than ~15 Torr. This is in agreement with the current observations for the interstitial space.

Oxygen measurements using a variety of different methods are in agreement that only a very small fraction of the tissue vascular volumes has PO2 values less than 15 Torr and the maximal volume fractions are near 35–40 Torr. In the present study, the PO2 values in awake and isoflurane-anesthetized mice were not significantly different as long as care was taken to keep the animals from becoming stressed by the experimental procedure and the foot was not in contact with a surface.

Comparison of PO2 Distributions in the Interstitial Space (Oxyphor G3) With Those in the Blood Plasma (Oxyphor G2)

The oxygen distributions measured in the interstitial (pericellular) space of awake mice were slightly lower than those in the intravascular space. In the lower oxygen portion of the histograms, which are attributed to the capillary bed, there was no significant difference between the pericellular and intravascular PO2 values, showing the PO2 difference from the capillary blood plasma to the interstitial space is <1.5 Torr. Oxygen delivery (flux) by diffusion is proportional to the PO2 difference. Thus, if a pressure difference of 2 Torr occurred for resting muscle, a normalized consumption rate of 1.0, this pressure difference would increase to an impossibly high value of 100 Torr for a flux of 50 (approaching maximal oxygen consumption rates by the muscle). Thus a measured difference of <1.5 Torr is consistent with the fact that the maximal oxygen consumption rates by skeletal muscle are 50- to 100-fold that in resting muscle. As hypothesized, the capillary walls do not present a significant barrier for oxygen diffusion, nor do they consume enough oxygen to generate a pressure difference large enough to be measured in these experiments between the blood plasma in the capillary lumen and the interstitial space.

The intravascular space has a greater fraction of its volume at higher PO2 values than the interstitial space. This suggests the presence of a population of larger/high-flow vessels for which there are substantial PO2 differences from the plasma in the lumen to the surrounding interstitial space. These larger caliber and higher flow vessels “shunt” the oxygen histograms for the microvasculature toward higher PO2 values than those for the interstitial space. With further technical development, it should become possible to quantify the volume mismatch between the blood plasma in the capillary lumen and the interstitial space. This information would aid understanding of the distribution of blood flow in the microvessels and the role of “shunting” in tissue physiology.

It has been suggested by Tsai and coworkers (25) that the vascular walls are responsible for a large fraction of the oxygen consumed by resting muscle and that there is a substantial (18 Torr) PO2 difference across the vascular wall. Their measurements and conclusions are very different from those in the present study. Our data show the pressure difference in the low-oxygen portion of the histograms for the vascular and interstitial space is small, less than ~1.5 Torr. The low-oxygen portion of the oxygen histograms likely applies to the capillary bed, however, whereas the measurements of Tsai and coworkers were for small arterioles. The internal volume of the small arterioles contributes only a small fraction of the total vascular volume of the tissue, and the PO2 values in the arterioles are generally higher than in the capillaries. The arterioles, with their higher flow velocities and heavier walls, may have plasma...
PO\textsubscript{2} values well above those in the surrounding interstitial space. It is also true that, in the experiments of Tsai and coworkers, the phosphor in the interstitial space had leaked from the vessels and may have bound to biomolecules other than albumin. This would introduce uncertainty into calibration of the phosphor and therefore into the calculated PO\textsubscript{2} values in the interstitial space.

Comparing mice under isoflurane anesthesia with awake mice, there is a slight, not significant, decrease in the PO\textsubscript{2} values in the interstitial space but a slight increase in the intravascular space. As a result, under isoflurane anesthesia, the interstitial space has significantly lower oxygenation than the intravascular space at all PO\textsubscript{2} values, although the difference is larger at higher PO\textsubscript{2} values than at low-PO\textsubscript{2} values. This suggests isoflurane anesthesia causes a decrease in vascular tone, resulting in blood flow becoming more heterogeneous and significantly shifting flow to the larger caliber/higher flow vessels. The high-flow vessels have less efficient transfer of the oxygen from the plasma to the interstitial space, increasing the difference in PO\textsubscript{2} between the compartments. At the present time, it is not clear whether this effect is due to isoflurane anesthesia per se or to physiological differences between being awake and asleep, although we suggest the former is more likely.

**Anesthesia Using Ketamine and Xylazine and Its Effect on Tissue Oxygenation**

After bolus injection of this anesthetic, the PO\textsubscript{2} in the tissue, both in the vasculature and the interstitial space, decreased dramatically and then slowly recovered. Although this anesthetic protocol can be very useful in many applications, it should be used only when the tissue oxygen levels are not an important experimental variable. Not only are the PO\textsubscript{2} values decreased, the oxygen distributions show increased heterogeneity (broadening of the histogram), and the PO\textsubscript{2} values are not very stable over time or reproducible from animal to animal. Similar effects of injected anesthetics have been reported by others (see, for example,Refs. 9, 24).

In summary, a new oxygen sensor, Oxyphor G3, has been microinjected into the interstitial (pericellular) space of normal skeletal muscle in mice and used to selectively measure the oxygen distribution in this compartment. The oxygen distributions in the interstitial space are compared with those in the intravascular space using Oxyphor G2 dissolved in the blood plasma. In awake mice, the differences in PO\textsubscript{2} values between the intravascular and interstitial spaces were not significant for the lower oxygen region histograms (<10% of the tissue volume) but become significant for higher PO\textsubscript{2} values. Isoflurane anesthesia increased the difference to where it is significant at all PO\textsubscript{2} values, consistent with the anesthetic causing a decrease in vascular tone. The PO\textsubscript{2} difference across the capillary walls is <1.5 Torr, supporting the hypothesis that the vessel walls do not provide a significant barrier to oxygen delivery and that oxygen consumption by the endothelial cells is also low to induce an oxygen difference across the wall. The data do show significant PO\textsubscript{2} differences across the walls of a subset of vessels with higher PO\textsubscript{2} values, vessels tentatively identified as larger caliber vessels such as arterioles and veins.

Ketamine plus xylazine anesthesia given intraperitoneally resulted in substantial suppression of the tissue PO\textsubscript{2} values, an effect that lasted through most of the period of anesthesia. Both the time course and extent of the PO\textsubscript{2} decrease differed from mouse to mouse, and it was concluded that this anesthetic should not be used when evaluating oxygen delivery to tissue.

**ACKNOWLEDGMENTS**

The authors are indebted to Dr. Cameron Koch for critiquing this manuscript and for many valuable discussions on tissue oxygenation and its measurement.

**GRANTS**

The work was supported in part by National Institutes of Health Grants U54 CA105008–01 (W. M. F. Lee and D. F. Wilson), NS-31465 (D. F. Wilson), HL-081273 (D. F. Wilson and S. A. Vinogradov), and EB003663 (S. A. Vinogradov).

**REFERENCES**


18. Rumsey WL, Pawlowski M, Lejarrardi N, and Wilson DF. Oxygen pressure distribution in the heart in vivo and evaluation of the ischemic...


