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OXYGEN PRESSURES IN THE INTERSTITIAL SPACE OF SKELETAL MUSCLE AND TUMORS IN VIVO

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Abstract

A new Oxyphor (Oxyphor G3) has been used to selectively determine the oxygen pressure in interstitial (pericellular) spaces. Oxyphor G3 is a Pd-tetrabenzoporphyrin, encapsulated inside generation 2 poly-arylglycine (AG) dendrimer, and therefore is a true near infrared oxygen sensor, having a strong absorption band at 636nm and emission near 800nm. The periphery of the dendrimer is modified with oligoethylene glycol residues (Av. MW 350) to make the probe water soluble and biologically inert. Oxyphor G3 was injected along “tracks” in the tissue using a small needle (30gauge or less) and remained in the pericellular space, allowing oxygen measurements for several hours with a single injection. The oxygen pressure distributions (histograms) were compared with those for Oxyphor G2 in the intravascular (blood plasma) space. In normal muscle, in the lower oxygen pressure region of the histograms (capillary bed) the oxygen pressure difference was small. At higher oxygen pressures in the histograms there were differences consistent with the presence of high flow vessels with oxygen pressures substantially above those of the surrounding interstitial space. In tumors, the oxygen pressures in the two spaces were similar but with large differences among tumors.

In mice, anesthesia with ketamine plus xylazine markedly decreased oxygen pressures in the interstitial and intravascular spaces compared to awake or isoflurane anesthetized mice.

1. INTRODUCTION

Oxygen transported to tissue, after reaching the tissue microcirculation, diffuses from the blood plasma through the walls of the micro-vessels into the interstitial (pericellular) space and then from interstitial space into the cells and finally to the mitochondria. As it diffuses, from the source (blood plasma) to a sink (mitochondria), an oxygen pressure gradient is formed in which the pressure is lower at the sink than at the source. The difference in oxygen pressure between the blood plasma and the mitochondria increases with increase in the rate of oxygen consumption by the mitochondria and the distance from the vessel to the mitochondria. The distance over which oxygen can be supplied to the mitochondria is, therefore, determined by a) the rate of oxygen consumption by the mitochondria, b) the distance from the blood plasma (the oxygen source) to the mitochondria and c) the oxygen pressure in the blood plasma.

Oxygen dependent quenching of phosphorescence is a minimally non-invasive optical method that can quantitate oxygen pressures in biological and other samples.^{1–4} Although it has been widely used^{1–17} for measurements *in vivo*, focus has been on the intravascular space. The available oxygen sensitive phosphors, such as Oxyphors R0, R2 and G2 (Oxygen Enterprises, Ltd, Philadelphia, PA), contained Pd-porphyrin cores that are at least partially exposed to the medium. As a result, the oxygen sensitivity is dependent on the microenvironment of the porphyrin and therefore on the macromolecule to which it is bound, and on the fraction of the Oxyphor bound to that macromolecule. 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 to provide a relatively homogeneous microenvironment for the phosphor.

A new family of Oxyphors has been synthesized that can be used in a much wider range of media, particularly in highly heterogeneous environments such as the interstitial space. The porphyrin core is first coated with dendrons and then the external surface of the dendrimer modified with oligoethylene glycol fragments^{18–20}. Oxyphor G3 is a member of this oxygen sensor family. Not only are its oxygen quenching properties unaffected by biological macromolecules such as albumin, but also its oxygen quenching constant and phosphorescent lifetimes are well suited for measuring oxygen *in vivo* and *in vitro*.

2. MATERIALS AND METHODS

2.1. Measurement of oxygen pressure histograms

Phosphorescence lifetime measurements were performed using a PMOD-5000 phosphorometer (Oxygen Enterprises, Ltd., Philadelphia, PA, USA)⁴, a frequency domain instrument with a range of 100–100,000 Hz. Phosphorescence lifetimes are independent of local phosphor concentration and insensitive to endogenous tissue fluorophores and chromophores. The PMOD-5000 was used in multifrequency mode⁴ in order to determine distributions of phosphorescence lifetimes. The lifetime distributions were used to calculate distributions of oxygen pressures, i.e. oxygen histograms^{22, 23}. The excitation light (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^{22, 23} to yield the distribution of phosphorescence lifetimes. This distribution was converted into the distribution of oxygen pressure in the sample as described previously^{22, 23}. The basis for the conversion is the Stern-Volmer relationship:

$$I^0/I = T^0/T = 1 + k_Q * T^0 * pO_2, \quad (1)$$

where, I^0 , T^0 and I , T are the phosphorescence intensities and lifetimes in the absence of oxygen and at oxygen pressure pO_2 , 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^0 and k_Q have been determined for each phosphor for the experimental conditions⁴ (temperature etc. as appropriate).

According to (1), intensities (amplitudes) of phosphorescent signals decrease with increasing oxygen pressures. Thus, for equal volumes of tissue, containing equal amounts of the phosphorescent probe and excited by equal numbers of photons, the accuracy in determination of lifetimes and/or amplitudes will be higher for volumes with lower oxygen pressures. The decrease in accuracy (decrease in signal level) causes asymmetric broadening of oxygen histograms. This broadening increases with increasing oxygen pressure (decreasing signal) and this is responsible for the “tail” effect on the high oxygen end of the histogram. This broadening is intrinsic to the MEM analysis, reflecting the fact that uncertainty in determination of phosphorescence lifetimes increases as the signal-to-noise ratio (S/N) decreases. At lower oxygen pressures there is little broadening, less than 3 Torr for pressures below 20 Torr, but for oxygen pressures above about 80 Torr the histograms are substantially broadened and are only qualitative. The presented histograms were arbitrarily truncated at 140 Torr.

2.2. Phosphorescent probes Oxyphor G2 and Oxyphor G3

Both Oxyphors G2²³ and G3 are based on Pd-tetrabenzoporphyrin cores²⁰. The structure of G3 is published in Wilson et al²⁴ and synthesis of similar dendritic porphyrins has been reported²⁵. Pd tetrabenzoporphyrin (PdTBP) dendrimers G2 and G3 differ by the dendrimer composition (G2 - polyglutamate; G3 - polyarylglycine) and surface coatings (G2 - none; G3 - PEG, Av. 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 not affected by albumin and other biomolecules due to the surface layer of polyethyleneglycols (PEG's). The absorption and the phosphorescence spectra of G2 and G3 are nearly identical. Both phosphors have quantum yields of about 10% and lifetimes of about 270 μ s in deoxygenated aqueous solutions. Oxygen quenching constants (k_Q) of G2 and G3 in aqueous buffered solutions at pH 7.2 at 38°C are 2,800 Torr⁻¹s⁻¹ and 180 Torr⁻¹s⁻¹ respectively. Unbound Oxyphor G2 cannot be used to measure oxygen in physiological range. In the blood, however, it binds tightly to albumin, and the oxygen quenching constant (k_Q) of the G2-albumin complex at 38°C is 280 Torr⁻¹sec⁻¹. Phosphorescence lifetime and oxygen quenching constant of Oxyphor G3 are insensitive to the presence of albumin (at 1–5 percent by weight) as well as changes in pH and ionic strength throughout the physiological range.

2.3. 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 made non-invasively through the undisturbed 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)—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 about 10 min after injection of the Oxyphor. It has been previously noted^{17, 24} that induction of anesthesia with isoflurane causes a transient decrease in tissue oxygen pressures that recovers within 10 min of continuing anesthesia. After measuring the oxygen histograms (anesthetized), the nose cone supplying the isoflurane was removed and the mice replaced in their cage. After about 40 min without inhaled anesthetic, the oxygen histograms were again measured (awake).

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

Measuring oxygen 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 micromolar in physiological saline) along 3 different 1 cm tracks (20 μ L containing 1.6 nmoles of Oxyphor per track) in the thigh muscle using a 30 gage needle. The nose cone was removed and the mice 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 measured in the awake mouse. Each mouse was then anesthetized with either isoflurane or ketamine xylazine and the oxygen histograms measured described above. The amount of Oxyphor G3 injected into the muscle was about 4% of that required to give the concentration of Oxyphor G2 injected into the blood. Thus, the measured phosphorescence comes from the interstitial space.

The experiments were carried out by investigators trained to handle mice. All of the experimental procedures were reviewed and approved by the local IACUC committee. At the end of the experiment the mice were euthanized according to guidelines established by the AVMA Panel on Euthanasia.

3. RESULTS

Preliminary measurements have been made in subcutaneous tumors grown on the hind quarter of mice. These tumors grow under the skin and the measurements can be made that are selective for tumor tissue since the tumor tissue is readily separated from the underlying muscle tissue. Illustrative measurements of the oxygen histograms for the interstitial space of muscle and tumors are shown in Figure 1A and B. For Figure 1A measurements were made for Oxyphor G3 in the interstitial space of a RENCA tumor and muscle measured on the same mouse. In this case the mouse was awake, illustrating that the measurements can be made in awake animals. It is important, however, that the animals be preconditioned to not become anxious when being handled. Although their becoming agitated does not affect the tumor measurements very much, if the leg muscles are being used to try to escape, or if the mice are stressed, this alters vascular regulation, blood pressure, and local blood flow. As a result, the tissue oxygen pressures are altered.

In normal muscle essentially all of the interstitial space of normal muscle has oxygen pressures greater than 10 Torr and there is a very small fraction with oxygen pressures less than 15 Torr. This is consistent with the results published earlier as part of a comparison of the oxygen pressures in the interstitial space and the vascular space in resting muscle²⁴. In both the RENCA and Lewis Lung tumors the interstitial space oxygenation is heterogeneous and generally lower than those in normal tissue. Particularly evident, for these two tumor types, is that a substantial part of both the interstitial and the vascular spaces have oxygen pressures less than 15 Torr. The tumor oxygen pressure distributions are, however, sufficiently different among tumors of the same type that much more detailed studies will be required to determine if further generalizations can be made. In addition, our preliminary measurements indicate that the anesthetic may also affect tissue oxygen pressures in the tumors more than in the muscle, and this needs to be studied in more detail.

4. DISCUSSION

Oxygen pressures in the interstitial space can not be measured by other methods, making it impossible to compare the measured values with values from the literature. Micro-oxygen electrodes and solid EPR probes²⁶ measure a mixture of the interstitial space and capillary oxygenation, whereas nitroimidazole binding measures intracellular oxygenation. Most micro oxygen electrode measurements for normal tissue have been made in softer tissue, such as the kidney, liver and brain. Baumgärtl and coworkers²⁷ published histograms of the oxygen distribution in dog kidney with mean PO_2 values of 36.8 ± 6.0 ($\pm$ SD) Torr, but did not indicate the anesthetic that was used. Oxygen measurements have been made in rodent muscles using oxygen electrodes and phosphorescence quenching. The electrode measurements were, however, typically made in urethane and/or barbiturate anesthetized animals and the muscle tissues were surgically exposed. Whalen and coworkers^{28, 29} used electrodes with very small tips to measure oxygen pressures within the cells in living tissue in animals anesthetized with urethane and barbiturate. They reported 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 ± 1.8 Torr. The influence of the anesthetic on oxygen pressure in the tissue was not appreciated, and, partly for this reason early oxygen electrode measurements gave rise to the erroneous view that the oxygen pressures in normal tissue are very low and there were significant volumes with effectively zero oxygen pressures. Later measurements

have given higher values, and mean values reported for muscle tissue include 19³⁰ and 26.8³¹ for the rat cremaster muscle, and 31.4¹⁴ Torr for the rat spinotrapezius microvasculature. These are still much lower than the 46.2 Torr (awake) or 36.9 Torr (isoflurane anesthesia) values obtained with phosphorescence quenching for the interstitial space²⁴, but are more consistent with those for ketamine plus xylazine anesthesia.

Tissue oxygen measurements using EPR active particles injected into the tissue²⁶ are reported to give oxygen pressures in the rat brain of 39.3 ± 4.1 Torr in isoflurane anesthetized rats³³.

Nitroimidazole binding has been used to measure intracellular oxygenation (for review see³⁴). Binding is small in normoxic tissue but increases strongly with decreasing oxygen pressures. Normal muscle and other tissues show little binding of the nitroimidazole, EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide] in awake and isoflurane anesthetized animals, indicating there are few cells with intracellular oxygen pressures less than about 15 Torr.

We conclude that the currently available data are consistent with mean oxygen pressures in normal skeletal muscle interstitium of 35 to 45 Torr and with there being negligible volumes with oxygen pressures less than 15 Torr. Further, direct measurements of oxygen pressures in the intravascular and interstitial spaces (see Wilson et al.²⁴) shows that the difference in oxygen pressure across the capillary walls under resting conditions is very small, less than 1.5 Torr. Thus, the capillary walls consume insignificant oxygen and provide very little resistance for oxygen movement from the blood plasma to the pericellular space. This contrasts with the suggestion by Tsai et al.³⁵ that the walls of small arterioles consume a substantial fraction of the available oxygen, resulting in a difference in oxygen pressure across the wall of tens of Torr.

Tumors, in contrast to normal tissue, are now well recognized as having substantial heterogeneity within individual tumors and among different tumor types. Preliminary measurements have shown that the oxygen pressures measured in the intravascular space and the interstitial spaces are very similar, although this is expected to depend on the tumor being measured. Particularly important will be the extent of tumor necrosis, as necrotic volumes will contribute to the interstitial space, but not the vascular space, oxygen measurements. In tumors, there seems no alternative to making the oxygen measurements in the tumor at the time of treatment if this important parameter is to be useful for developing therapeutic protocols. It is clear that conclusions concerning the efficacy of therapeutic protocols based on experiments in which the tumor tissue oxygenation was not measured must be interpreted with great caution.

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Figure 1.

A. Oxygen pressure histograms for the interstitial space in RENCA tumors and normal muscle tissue. The Oxyphor G3 was microinjected and the oxygen histograms measured as described in Methods. The measurements were made while the mouse was awake and held in the hand.

B. Oxygen pressure histograms for the interstitial space in a Lewis Lung carcinoma and in normal muscle. The mouse was anesthetized with 1.2% isoflurane while the measurements were made. The histograms have been normalized to the same area under the curve for both tumor and muscle in order to eliminate differences in the total Oxyphor and illumination intensities.

Figure 2.

2A. Oxygen pressure in the intravascular space in RENCA tumors and normal muscle. Oxyphor G2 was injected i.v. in the tail vein and then the oxygen pressure histograms measured in an awake mouse. Three histograms are presented, each measured for a different region of the tumor to emphasize the heterogeneity of this tumor¹⁷. **2B.** Oxygen histograms from a Lewis Lung carcinoma and normal muscle in an isoflurane anesthetized mouse.