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Application of In Vivo EPR for Tissue pO₂ and Redox Measurements

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Summary

The technique of electron paramagnetic resonance (EPR) spectroscopy is more than 50 years old, but only recently it has been used for in vivo studies. Its limited application in the past was due to the problem of high nonresonant dielectric loss of the exciting frequency because of high water content in biological samples. However, with the development of spectrometers working at lower frequencies (1,200 MHz and below) during the last 15 years, it is now possible to conduct in vivo measurements on a variety of animals and isolated organs. This is further facilitated by the development of new resonators with high sensitivity and appropriate stability for in vivo applications. It now has become feasible to obtain new insights into the complex aspects of physiology and pathophysiology using in vivo EPR. Among several important applications of this technique, the in vivo tissue pO₂ (partial pressure of oxygen) and redox measurements seem to be the most extensive use of this technique. In this chapter, we describe the procedure for in vivo pO₂ and redox measurements in animal models.

Keywords

EPR; ESR; Oximetry; pO₂; Redox; Nitroxide; Heart; In vivo

1. Introduction

The amount of oxygen in tissue plays an important role in many physiological and pathological processes, especially those associated with reactive intermediates (1–3). Over the last several years, considerable effort has been invested to develop techniques that can provide noninvasive and reliable measure of tissue oxygen (reported as the concentration [O₂] or the partial pressure pO₂). The methods, such as the polarographic electrode, fluorescence quenching, O₂ binding to myoglobin, chemiluminescence, phosphorescence quenching, and spin label oximetry are useful but have significant limitations, especially when used in vivo. Methods which assess perfusion, such as magnetic resonance imaging, do not provide a direct measure of tissue pO₂. These methods have been reviewed recently (4–7).

EPR oximetry is a new emerging technique which has the capability to provide repeated, direct, and accurate measurement of tissue oxygen and the redox status of the tissue. The other important applications include the measurement of biophysical parameters such as macromolecular motion, membrane fluidity, viscosity, membrane potential, paramagnetic metal ions, pH, thiols, and detection and identification of free radicals by using the method of spin trapping.

1.1. In vivo EPR Oximetry for pO₂ Measurement

The basis of EPR oximetry is the paramagnetic nature of molecular oxygen, which therefore affects the EPR spectra of other paramagnetic species in its vicinity by altering their relaxation rates. The magnitude of this effect is directly related to the amount of oxygen that is present in the environment of the paramagnetic materials. Therefore, the line width of the EPR spectra of the paramagnetic materials, when injected into tissue, provides direct measurement of tissue pO₂. The placement of the paramagnetic material in the tissue of interest is minimally invasive (it usually requires an insertion via a 25 or 23 gauge needle, or direct implantation during a surgical procedure required for other reasons), but all subsequent measurements are entirely noninvasive (8,9). Two types of oxygen sensitive paramagnetic materials are used: particulates (such as lithium phthalocyanine (LiPc) crystals) and soluble probes (such as nitroxides and trityl radicals). The unpaired electrons in particulates are distributed over complex arrays of atoms and crystalline materials, such as in LiPc (10,11). LiPc is so far the most commonly used probe for pO₂ measurements. These are metabolically inert, and their EPR spectra have good response to the presence of oxygen. Once introduced into the tissue of interest, they allow repeated measurement of pO₂ at the same site for up to years after implantation (12,13). The region that is measured directly is that immediately surrounding the paramagnetic material. If the paramagnetic material is a macroscopic particle (such as LiPc), then it reflects the pO₂ in the tissue that is in contact with the surface of the particle. If the paramagnetic material is a slurry of small particles (such as ink), then it reports the average pO₂ of the sum of the surfaces that are in contact with the individual components of the slurry. Under appropriate conditions, EPR oximetry using particulates can measure pO₂ levels in tissues over a wide range from the extremes of very low (<0.1%) to very high (up to 100%) oxygen levels. Extensive reviews of EPR oximetry with more information on the principles, methodology, and technical aspects are available in recent reports (14,15). An important recent development in EPR oximetry is the capability to measure pO₂ simultaneously from several sites (16–18). The High Spatial Resolution Multi-Site (HSR-MS) EPR oximetry technique can be used to provide pO₂ estimates from multiple LiPc implants with greatly increased spatial resolution (16). This technique has been recently refined to achieve improved oximetric sensitivity by the application of overmodulation during EPR measurements (18).

EPR oximetry with particulates has features that often compliment those available with other methods. These include the capability to make repeated noninvasive measurements with high sensitivity and accuracy, and the measurements can be made quickly (in as little as a few minutes) with a high degree of stability and inertness. The existing EPR oximetry techniques already are very useful for small animal studies where the depth of measurements is not an overriding issue. In large animals and potentially in human subjects, this technique seems to be immediately applicable for up to a distance of 10 mm from the surface. For depths greater than 10 mm, approaches such as implantable resonators can be successfully used for tissue pO₂ measurements; however, this involves some degree of invasiveness. The clinical use of EPR oximetry seems especially promising and is likely to be used for long-term monitoring of the status and response to treatments of peripheral vascular diseases and also to optimize radiotherapy by enabling it to be modified on the basis of the pO₂ measured in the tumor.

EPR oximetry has been used to study the tissue pO₂ in a wide range of experimental systems, including heart (19,20), muscle (21), brain (22,23), kidney (24,25), liver (26), skin (27), and tumors (28,29). In order to measure pO₂ using EPR oximetry, a detector (also referred to as a resonator) is placed on the surface of the skin above the tissue injected with the paramagnetic material. Through the application of an appropriate combination of an electromagnetic field (excitation frequency near 1,200 MHz) and a magnetic field (around 400 G), the scanning of the magnetic field produces a characteristic resonance signal (14,15). By using an appropriate

calibration curve, the line width of the EPR signal provides a sensitive measurement of tissue oxygen (19–29).

1.2. Measurement of Redox State of the Isolated Perfused Heart

Nitroxides, molecules with a stabilized unpaired electron on N–O bond, have been widely utilized in biophysical studies. The discovery that the reduction of nitroxides is dependent on the oxygen concentration has raised additional possibilities for measuring oxygen concentrations and related redox metabolism. Recently, EPR has been widely used for noninvasive studies of the pharmacokinetics of nitroxides. This provides an effective approach to understand the fundamental aspects of the metabolism and distribution of nitroxides in vivo.

Nitroxides can act as electron acceptors, forming hydroxylamines and, as electron donors, giving oxoammonium cations. Nitroxides may also react with other free radicals to give radical adducts, or disproportionate under strong acidic conditions to the hydroxylamine and oxoammonium salt. Oxygen-dependent changes in the rate of reduction of nitroxides occur when the oxygen concentration is relatively low (the apparent K_m for oxygen is approximately 1 μ M (30)). The potential applications of EPR using nitroxides include biophysical and biochemical studies, such as oximetry, analysis of membrane fluidity, and polarity; detection of free radicals; and measurement of redox interactions with antioxidants and oxidants. Some of the potential biological uses of nitroxides have been summarized by Kocherginsky and Swartz (31).

2. Materials

2.1. In vivo pO₂ Measurements in Heart

1. Paramagnetic oximetry probe (such as LiPc).
2. 23 gauge needle/plunger.
3. Suitable anesthetic and inhaled oxygen (we use 1.5–1.8% isoflurane with 30% FiO₂ in our experiments).
4. 1.2 GHz (L-band) EPR spectrometer.

2.2. Redox Status Measurement in Isolated Perfused Heart

1. Nitroxides such as TEMPO (2,2,5,5-tetramethyl-4-piperidine-1-oxyl; Sigma-Aldrich) or PCA (2,2,5,5-tetramethyl-3-carboxylpyrrolidine-*N*-oxyl; Sigma-Aldrich) could be used for this purpose.
2. 1.2 GHz (L-band) EPR spectrometer.
3. Sodium pentobarbital 80 mg/kg, i.p. (or other appropriate anesthetic).
4. Anticoagulant (heparin sodium, 500 IU/kg, i.v.).
5. Langendorff isolated heart perfusion apparatus.
6. Modified Krebs–Henseleit bicarbonate buffer (KHB): 118 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl₂, 25 mM Na₂HPO₄, 0.36 mM KH₂PO₄, 1.2 mM MgSO₄ and 10 mM glucose.

3. Methods

3.1. In vivo pO₂ Measurements in Heart

1. Paramagnetic oximetry probe (such as LiPc) should be sterilized (autoclaved) prior to implantation in the tissue.

2. A response of the EPR signals of the LiPc crystals to different concentrations of perfused oxygen should be measured, either using a 1.2 GHz (or a ~9 GHz) EPR spectrometer to obtain the calibration plot. The dependence of the line width of EPR spectrum on pO_2 is determined by measuring the line width (LW) as a function of pO_2 in the perfused gas. The LW is defined as the difference in the magnetic field between the maximum and the minimum of the first-derivative EPR signal. The resulting calibration is fitted to a first-order regression equation, which then is used to convert the values of LW measured in the heart into appropriate values of pO_2 .
3. LiPc crystals (40–60 μg) should be injected into the myocardial tissue of interest using a 23 gauge needle/plunger. This will involve minor surgery to open the chest and access the heart for LiPc implantation. This procedure also can be done during other surgeries, for example during the implantation of a pneumatic occluder for ischemia-reperfusion studies. The animals should be allowed to recover for 5–7 days. For studies in isolated perfused heart, the LiPc crystals can be injected after the heart is excised; the experiment set up is described by Grinberg et al. (19,20).
4. For pO_2 measurements, the animals should be anesthetized using a suitable anesthetic and inhaled oxygen and gently placed in between the EPR magnets (we use 1.5–1.8% isoflurane with 30% FiO_2 in our experiments). This anesthetic maintains reasonably good SpO_2 , heart beat, and blood pressure. The body temperature of the animals should be maintained at 37°C using a warm water pad and warm air blower.
5. The EPR resonator should be positioned on the skin above the myocardial tissue with the LiPc implant and the spectrometer tuned. The spectrometer parameters should be optimized and EPR data collected as desired.
6. The EPR spectra should be averaged to obtain better signal to noise ratio which will provide accurate estimates of tissue pO_2 .
7. The fit of the EPR spectrum of the implanted LiPc crystals will provide line width, which is then converted to pO_2 using the calibration plot as described above (*see* Note 1).

This procedure will allow continuous noninvasive measurement of tissue pO_2 , and these measurements can be repeated over days or weeks as desired. If the study is acute, a small incision in the chest could be used to gently insert the resonator for its placement directly on the myocardial tissue to achieve better signal to noise ratio of the EPR spectra. Currently, implantable resonators are being developed, which can provide much better signal to noise ratio, and myocardial tissue pO_2 can be measured repetitively for several weeks. The initial implantation procedure will be invasive, but the rest of the oximetry measurements will be entirely noninvasive.

¹For tissue pO_2 measurements, the EPR spectrometer parameters especially the microwave power, modulation amplitude, and time constant should be carefully selected to avoid any EPR signal distortion. The power saturation should be checked by recording the EPR spectra at different microwave powers and then by plotting the EPR signal intensity vs. the square root of power. The EPR signal intensity should grow as the square root of the microwave power; however, at saturation the EPR signal intensity will become weaker (saturate). One can measure either the signal intensity or the line width to check power saturation (at saturation, the line width will broaden) and then select the microwave power which does not lead to saturation. To avoid line broadening due to field modulation, the “rule of thumb” is to keep the modulation amplitude at least half of the EPR line width. The time constant filters out the noise but, on the other hand, a high time constant will distort the signal. Generally, it is best to keep the time constant nearly ten times less than the scan time used to acquire the EPR signal. The oximetry probe can be requested from Professor Harold M. Swartz, Director, EPR Center (<http://www.dartmouth.edu/~eprctr/>), Dartmouth Medical School, Hanover, NH 03755, USA. The readers are encouraged to contact the authors for any query or for a potential collaboration to make these measurements.

3.2. Redox Status Measurement in Isolated Perfused Heart

A 1.2-GHz EPR spectrometer is used to determine the redox status of the isolated perfused heart by evaluating the reduction of a nitroxide during the ischemic challenge. The experiment setup is similar to that reported by Grinberg et al. (19,20), Khan et al. (32), and references cited therein.

1. An appropriate nitroxide should be selected and its concentration should be determined for use at 1.2-GHz EPR spectrometer. TEMPO (2,2,5,5-tetramethyl-4-piperidine-1-oxyl) or PCA (2,2,5,5-tetramethyl-3-carboxylpyrrolidine-N-oxyl) are the most commonly used nitroxide for this purpose. A concentration of 0.2 mM or less is suggested for isolated perfused heart experiments. For direct in vivo measurements, 150 mg/kg of the nitroxide could be used.
2. The rat should be anesthetized (sodium pentobarbital 80 mg/kg, i.p. or other appropriate anesthetic), and an anticoagulant injected (heparin sodium, 500 IU/kg, i.v.).
3. After ensuring sufficient depth of anesthesia, a thoracotomy is performed, and the heart is perfused in the retrograde Langendorff mode at 37°C at a constant perfusion pressure of 100 cm of water (10 kPa) for a 5-min washout period. The perfusion buffer slightly varies from lab to lab. We use a modified Krebs–Henseleit bicarbonate buffer (KHB) as described in **Subheading 2**.
4. The heart is perfused with the KHB buffer containing the nitroxide for 15 min, and global ischemia is performed for 30 min. The reduction of the nitroxide by the myocardial tissue during ischemia will result in a decrease in EPR signal intensity with time.
5. The change in signal intensity with time should be plotted, and the data should be fitted depending on the nature of the reduction (first or second order exponential decay) to determine the reduction rate of the nitroxide. This provides the redox status of the heart which can be compared with other experimental conditions such as treatment of the heart with other drugs, etc. Details of the procedure can be obtained from a recent publication by Das et al. (33) (*see Note 1*).

The oxygen-dependent metabolism of nitroxides can be used with NMR to provide images that reflect these processes (34). In vivo EPR spectroscopy of nitroxides also provides a noninvasive method to measure the presence of reactive free radicals by their effects on the concentration of the nitroxides (35–37).

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