Measuring Brain Tissue Oxygenation under Oxidative Stress by ESR/MR Dual Imaging System

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The in vivo measurement of oxygen in tissues is of great interest because of oxygen’s fundamental role in life. Many methods have been developed for such measurement, but all have been limited, especially with regard to repeated measurement, degree of invasiveness, and sensitivity. We describe electron spin resonance (ESR) oximetry with paramagnetic oxygen-sensing probe for in vivo measurement of oxygen in brain tissues by homemade ESR/MR dual imaging spectroscopy. Lithium 5, 9, 14, 18, 23, 27, 32, 36-octan-butoxy-2,3-naphthlocyanine (LiNc-BuO) radical was employed as the solid oxygen-sensing probe, and we confirmed its ability to report partial pressure of oxygen (pO2) in brain tissues of live animals under normal and pathological conditions for more than a month. pO2 measurements could also be made repeatedly on the same animal and at the same location. The implantation site of LiNc-BuO in examined rats was verified by 0.5T magnetic resonance (MR) imaging. Septic-shock rats were used to monitor tissue oxygenation during pathological state. A decline in pO2 levels from severe hypotension during sepsis was detected, and generation of nitric oxide (NO) in brain tissues was confirmed by NO spin trapping. ESR oximetry using oxygen-sensing probe and NO spin-trapping can be used to monitor pO2 change and NO production simultaneously and repeatedly at the same site in examined animals.

Keywords: ESR, MRI, oximetry, sepsis, brain

Introduction

Oxygen is crucial in metabolism in the tissue of mammals and considered a very important biological substance.1 Under normal physiological conditions, the delivery of oxygen to the tissues by blood and tissue oxygenation are securely regulated to maintain a balance, which seems to change during many pathological states. Therefore, an accurate and reliable method to determine oxygen concentration in selected tissue locations within the body is particularly anticipated.2 The brain is the highest oxygen-consuming organ in the body, and its functions are extremely affected by oxygen depletion. Although adequate cerebral oxygen is critical to neuron survival, monitoring oxygen levels in the brain in vivo is very technically challenging because of the difficulty in making repeated measurements in the same location of the brain. There are a number of useful methods for measuring oxygen in the brain in vivo, including Clark-type electrodes,3 fluorescence quenching,4 NMR (19F, 17O, or blood oxygen level-dependent (BOLD)),5–7 and ESR.2,8-11 All of these techniques have limitations, especially with regard to the ability to make repeated measurements, the degree of invasiveness, and sensitivity.

Among these methods, ESR oximetry is adequate to measure repeatedly the oxygen in the rodent brain and with minimum invasiveness, and the method permits quantitation of absolute
oxygen levels, rather than relative changes in the oxygen level in vivo.

ESR oximetry requires the incorporation of a paramagnetic oxygen-sensing probe in the region of interest. Two types commonly used are a soluble probe that reports the concentration of dissolved oxygen and an insoluble solid probe that measures partial oxygen pressure (pO2) in examined regions.\(^2,8,12,13\) The soluble probes are limited in that allowing long-term monitoring of pO2 in animals. In contrast, solid microcrystalline paramagnetic probes are reported to be characterized by higher sensitivity for oximetry, tissue stability, and minimal toxicity, thus allowing long-term monitoring of pO2 in animals in vivo by implantation in tissues.

We monitored the changes in pO2 in the brains of animals under pathological conditions by ESR oximetry with insoluble paramagnetic oxygen-sensing probe, lithium 5, 9, 14, 18, 23, 27, 32, 36-octa-n-butoxy-2,3-naphthlocyanine (LiNc-BuO) radical,\(^13\) using an ESR/MR dual imaging system.\(^14\) As the pathological condition, animal models of sepsis were used. During sepsis, overproduction of nitric oxide (NO) catalyzed by induced NO synthase causes hypotension. For practical applications of the ESR oximetry, we monitored the pO2 levels in the rat brains during sepsis. We also simultaneously measured pO2 and NO generated in the brains of rats under sepsis with the aid of spin-trapping reagents for NO produced.

**Materials and Methods**

**Animals**

The animal protocols were institutionally approved according to the National Institute of Health Animal Care and Use protocol. Male Wister rats (150–200 g, 8–10 weeks old) were maintained on laboratory chow and water ad libitum on a 12-hour light/dark cycle. Rats were anesthetized with urethane(SIGMA-ALDRICH JAPAN K.K. Tokyo, Japan) at a dosage of 1.2 g/kg, intraperitoneally (i.p.) for ESR/MR imaging measurements.

**Materials**

Oxygen-sensing probe, lithium 5, 9, 14, 18, 23, 27, 32, 36-octa-n-butoxy-2,3-naphthlocyanine (LiNc-BuO) radical was prepared according to the method previously reported.\(^13\) Diethyldithiocarbamate (DETC), Escherichia coli (E. coli) lipopolysaccharide 026:B6 (LPS), urethane, and N\(^\text{O}^-\)monomethyl-L-arginine (L-NMMA) were purchased from SIGMA-ALDRICH JAPAN K.K. (Tokyo, Japan), and gadopentetate dimeglumine (Gd-DTPA) from Nihon Schering K.K. (Osaka, Japan). Other chemical reagents were of analytical grade.

**Implantation of LiNc-BuO**

An oxygen-sensing probe, LiNc-BuO, with a diameter less than 50 \(\mu\)m was suspended in phosphate-buffered saline (PBS), and about 20 \(\mu\)g of LiNc-BuO suspension (10 \(\mu\)L volume) was used for implantation in the animals. The rats were anesthetized with ketamine/xylazine at dosages of 50 and 20 mg/kg, and a pin hole measuring 1 mm across was made in the parietal skull. About 10 \(\mu\)L of LiNc-BuO suspension in PBS was implanted stereotactically into the brain 1 mm posterior and 3 mm to the left of the bregma using a 24-gauge needle to a depth of 3 mm from the dura. After a minimum of 24 h to allow for healing, pO2 levels were measured.

**NO spin trapping**

Male Wister rats (150–200 g) were used for NO trapping experiments according to the same procedures published previously.\(^15\) Briefly, rats were injected with LPS (50 mg/kg in saline, i.p.), and 5.5 hours later, DETC (100–500 mg/kg, prepared in PBS) was injected intraperitoneally followed by subcutaneous injection of ferrous sulfate (100 mg/kg in PBS). Thirty minutes after these injections, rats were anesthetized with urethane (1.2 g/kg), and in vivo ESR spectra of septic-shock rats were recorded on an L-band in vivo ESR spectrometer.

**In vivo ESR measurements**

L-band in vivo ESR experiments were performed on a custom-built ESR/MR imager previously reported.\(^14\) This system works as an independent in vivo ESR spectrometer with an imaging capability that consists of a surface-coil resonator (10-mm diameter) and an L-band microwave bridge assembly.\(^16,17\) Typical spectroscopic conditions were: frequency = 1.2 GHz; applied magnetic field = 42 mT; incident microwave power = 25 mW; 10 KHz modulation amplitude = 0.02 to 0.2 mT; sweep rate = 5 mT/min; time constant = 0.1 to 0.3 s.

**MR imager**

MR imaging was conducted with a home-built ESR/MR dual imaging system based on 0.5T MRmini (MR Technology, Tsukuba, Japan). The detail in this imager was shown in the previous work.\(^14\) Conventional spin-echo sequence, 3D T1-weighted sequence, and 3D Flash sequence were employed.
Fig. 1. Effect of molecular oxygen on ESR spectra of LiNc-BuO at various partial pressures. (A) L-band ESR spectra of microcrystalline LiNc-BuO (diameter less than 50 μm) suspended in PBS was recorded under the following conditions: frequency, 1.2 GHz; incident microwave power, 0.5 mW; modulation frequency, 10 KHz; modulation amplitude, 0.02 mT. (B) Relationship between pO2 and the peak-to-peak ESR line width of LiNc-BuO. Various pO2 values were obtained by mixing pure oxygen and nitrogen gases. Pure argon and air were used for 0% and 21% oxygen, individually. ESR spectrometer conditions were identical to those in (A), except modulation amplitude: 0.02–0.04 mT depending on the obtained line-width.

Results and Discussion

Stability of LiNc-BuO to chemicals and its response to oxygen

The paramagnetism of LiNc-BuO was tested in a variety of biological conditions. LiNc-BuO was stable, and its ESR spectral line-shape and its intensity were not changed when exposed to superoxide (generated by 0.2 mM xanthine + 0.01 U/mL xanthine oxidase), hydroxyl (generated by 0.1 mM Fe2+ + 1 mM H2O2), hydrogen peroxide (1 mM), nitric oxide (generated by 1 mM SNAP), and ascorbate (5 mM) for 30 min.13 The presence of molecular oxygen broadens the ESR spectrum of LiNc-BuO. ESR spectra of LiNc-BuO in the presence of 0 and 10% oxygen are depicted in Fig. 1A. The broadening of the ESR spectrum in the presence of molecular oxygen is generally attributed to the Heisenberg spin exchange between the LiNc-BuO probe and molecular oxygen, and subsequent shortening of the spin-spin relaxation time.18,19 The variation of the peak-to-peak width with pO2 observed for LiNc-BuO is shown in Fig. 1B. The line width increased linearly with pO2, and the curve was quite linear for LiNc-BuO, in the 0-to-400 mmHg range examined in this study.

When used in vivo, the oxygen-sensing probe requires long-term stability in tissues; inflammatory response to the implanted probe must be avoided. The microcrystalline particles of LiNc-BuO (diameter: less than 50 μm) were suspended in PBS, and an aliquot of LiNc-BuO suspension was implanted in muscle and brain tissues. No apparent inflammation was observed where the probes were implanted, and none of the animals died from the implan-
Fig. 3. Magnetic resonance (MR) images of rat brain implanted with LiNc-BuO microcrystalline particles taken by 0.5T MR imaging. Axial MR images of rat brain implanted with LiNc-BuO were taken separately from 2 individual rats. Ten μL of PBS suspension of LiNc-BuO particles (about 20 μg) were implanted, as shown in the text. Gd-DTPA (final 0.01 mM) was used only for the higher contrast MR images, as added in PBS suspension of LiNc-BuO. The implantation site of LiNc-BuO is indicated by the arrow. Axial Flash images were taken under these conditions: repetition time (TR) = 35 ms; echo time (TE) = 7 ms; field of vision = 38 mm × 38 mm; image matrix 256 × 256; number of excitations (NEX) = 12.

The obtained ESR spectrum of LiNc-BuO implanted in the cortex is shown in Fig. 2, in which the line width of LiNc-BuO reveals pO₂ in the cortex to be 38.2 ± 2.8 mmHg (n = 6). The location of the implanted LiNc-BuO was examined by MR imaging. MR images of the rat brain implanted with suspensions of LiNc-BuO crystals (about 20 μg) are shown in Fig. 3, in which each MR image (Fig. 3A or B) was taken separately from 2 different rats. Because so little of the oxygen probe (LiNc-BuO) was implanted for MR imaging sensitivity, the probe itself was not visible on the 0.5T MR imager. However, the implantation site could be identified within the cortex on MR images by the line trace made by the needle when the probe was implanted. To enhance the contrast in MR images (Fig. 3), Gd-DTPA was added with the suspension of LiNc-BuO. MR images taken from 2 different rats clearly show the implantation site of LiNc-BuO (shown by the arrow in Fig. 3). In every animal, the site of implantation of the probe was examined after all experiments, and the implanted site identified by MR imaging was in accord with that obtained from the brain tissues of the sacrificed rats. Therefore, our 0.5T MR imager on the ESR/MRI system was able to confirm the exact insertion sites of the oxygen probes non-invasively, conveniently, and rapidly. An MR imager with a higher magnetic field might allow the insertion site to be found in more detail.

The pO₂ levels in the cortex were followed in the same rat up to 14 days after implantation. Monitoring was started 24 hours after implantation of LiNc-BuO into the rat brain. The initial pO₂ value in the cortex was 38.5 ± 3.6 mmHg. Figure 2B shows the ESR spectrum recorded on the 15th day after monitoring pO₂ in the brain. After 14 days, the signal intensity of LiNc-BuO implanted in the brain tissues decreased from the initial value, so that the signal-to-noise ratio seemed decreased. The decrease in signal intensity (about 38%) shown in Fig. 2B might be attributable to the loss of paramagnetism of some LiNc-BuO particles implanted in the brain tissues. However, 14 days from implantation, the line width did not change, and the oxygen level calculated from the obtained spectrum was 36.9 ± 3.2 mmHg, which implies that pO₂ remained constant over this period. The LiNc-BuO implanted in the brain tissue (cortex) was recovered from examined rat brains, and the oxygen sensitivity of recovered LiNc-BuO was examined. The implanted LiNc-BuO continued to respond to change in oxygen concentration and did not lose oxygen sensitivity.

We used animal models of sepsis to demonstrate the monitoring of tissue oxygenation during a pathological state. The relatively high dose of 50 mg/kg LPS was injected into rats intraperitoneally, and severe lethal hypotension resulted within 6 to 8 hours of injection. The overproduction of NO catalyzed by NOS induced with LPS has been implicat-
Fig. 4. Changes in pO2 levels in the brains of septic-shock rats. LiNc-BuO particles were implanted in the cortex of rat brains. Twenty-four hours after implantation of LiNc-BuO, sepsis was induced by administering LPS (50 mg/kg). L-band ESR spectra were recorded by placing the surface-coil resonator above the rat brain implanted with LiNc-BuO, and pO2 values were assessed from the obtained ESR spectra. Each point indicates the mean pO2 values (n = 4). ESR spectrometry conditions were identical to those in Fig. 1.

Fig. 5. Simultaneous measurement of nitric oxide (NO) generation and pO2 in the rat brain during sepsis. Top spectrum shows in vivo ESR spectrum obtained by placing the surface-coil resonator above the rat brain implanted with LiNc-BuO during sepsis. Bottom spectrum is similar to top spectrum, except that the surface-coil resonator is placed above the brain without LiNc-BuO in the same rat. In vivo ESR spectrometry conditions were identical to those in Fig. 1.
in intact animals.\textsuperscript{22–24} In most studies, cell labeling is accomplished by endocytosis of iron oxide particles. Because paramagnetic LiNc-BuO microcrystals are very inert in biological systems and can be made in sub-micron-sized particles or smaller, internalization of LiNc-BuO particles into a wide variety of cells is feasible.\textsuperscript{13} Cells labeled with LiNc-BuO can be tracked indirectly by high-field MR imaging based on the effect of relaxation time-dependent contrast enhancement or directly by ESR imaging. At least one advantage of LiNc-BuO over iron oxide is that we can monitor the absolute oxygen concentration in the examined cells or tissues rather than just tracking the labeled site of the target object.

**Conclusion**

We monitored the change in pO\textsubscript{2} levels in vivo for relatively long terms using an ESR/MR dual imaging system with the oxygen-sensing probe, LiNc-BuO. The technique allowed us to measure changes in the pO\textsubscript{2} level in the brains of septic-shock rats. The implantation site of the oxygen-sensing probe was examined non-invasively, conveniently, and quickly by the newly developed 0.5T MR imaging system.

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**References**

19. Pandian RP, Kim Y, Woodward PM, Zweier JL,


