In Vivo Electron Paramagnetic Resonance Imaging of Tumor Heterogeneity and Oxygenation in a Murine Model

Periannan Kuppusamy, Mobae Afeworki, Ravi A. Shankar, Deborah Coffin, Murali C. Krishna, Stephen M. Hahn, James B. Mitchell, and Jay L. Zweier

Molecular and Cellular Biophysics Laboratories, Department of Medicine [P. K., R. A. S., J. L. Z.] and the Electron Paramagnetic Resonance Center [P. K., R. A. S., J. L. Z.], Johns Hopkins University, School of Medicine, Baltimore, Maryland 21224; Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892 [M. A., D. C., M. C. K., J. B. M.]; and Department of Radiation Oncology, The Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania 19104 [S. M. H.]

ABSTRACT

Nitroxides are redox-sensitive probes, which are useful in noninvasively delineating tissue heterogeneity especially with respect to metabolic activity and tissue oxygenation. Recent studies have shown that nitroxides are in vitro and in vivo radioprotectors and selectively protect normal tissue compared to tumor tissue. It has been postulated that the basis for selective radioprotection of normal tissues is greater bioreduction of nitroxides in tumor tissue compared to normal tissue. The aim of the present study was to investigate the distribution and lifetime of nitroxides in tumor and normal tissues. Mice were implanted with tumor cells (RIF-1) in the thigh, and the tumor was allowed to grow to about 10–15 mm in diameter. After i.v. infusion of nitroxides, in vitro electron paramagnetic resonance spectroscopy and imaging of the tumor were performed using a specially built bridged-loop surface resonator. The pharmacokinetic and spatial distribution of the nitroxides in tumor tissue were followed and compared with those in normal tissue. Three-dimensional spatial images showed significant heterogeneity in the nitroxide distribution as well as reduction rates. The nitroxide reduction rates were significantly higher in tumors than in the normal tissue. Measurements using spin label oximetry showed a substantial difference in the level of oxygenation between normal tissue (muscle) and tumor tissue. Average pO2 levels in tumor tissue were found to be 3-fold lower than in a corresponding volume of normal tissue. The lower pO2 levels in tumor compared to normal tissue may explain the more rapid reduction of nitroxides in these tissues. This study demonstrates that electron paramagnetic resonance imaging can perform noninvasive anatomical as well as functional imaging and provide in vivo physiological information regarding cellular metabolism in tumor and normal tissues.

INTRODUCTION

Therapeutic regimens exploiting the physiological differences between normal and malignant tissue would provide options for the efficacious treatment of tumor while protecting the normal tissue (1, 2). Some of the differences between normal tissue and tumors include oxygen status, redox status, and intracellular pH. These differences, at least in part, could be attributed to the physical architecture of the tumors with compromised vascularity (3). Methods of detecting these subtle differences would greatly aid in appropriate treatment strategies. Redox status and tissue pO2 are two factors that are hypothesized to be different in the two tissue types (4–6). The noninvasive measure of these two parameters therefore would be valuable. EPR3 of in vivo objects is a recently available technique, which can be adapted to measure tissue pO2 and redox status using appropriate spin probes (7–15). In many EPR studies in cellular models as well as in vivo objects, stable nitroxides have been used as biophysical probe molecules (16, 17). The nitroxides exist in at least two different oxidation states in biological systems, namely nitroxide free radical form and the diamagnetic hydroxylamine, which is the one-electron reduction product of the nitroxide free radical (18, 19). Prior studies indicate that the nitroxides are reduced to the corresponding hydroxylamines in cellular incubations as well as in vivo (20–24). The rate of reduction has been found to be significantly enhanced under hypoxic conditions, presumably by intercepting cellular reducing equivalents (25). Hence, the pharmacology of nitroxides in an EPR experiment should reflect some of these differences, which might exist in tissues having different oxygen status. The exchange interaction between molecular oxygen and nitroxides has been shown to modulate the EPR spectral characteristics of the nitroxide from which tissue oxygen content can be obtained (14, 26–28).

Nitroxides, in addition to being used as biophysical probes, have recently been found to be effective antioxidants (29–34). The antioxidant effects of nitroxides against ionizing radiation have been observed in many cellular and in vivo experiments (35–38). The protective effects of nitroxides have been attributed to several processes, including (a) SOD-mimicking activity; (b) inducing catalase-like activity in hemeproteins; and (c) radical scavenging. In a systematic study of the cellular radiomodifying effects of nitroxides and its precursors, it was noted that the nitroxide free radical provides radioprotection, whereas the hydroxylamine does not have any protective effects (35). Because nitroxides are rapidly converted to the hydroxylamines in hypoxic tissue and there might be regions of hypoxia in tumors, a recent study evaluated the effects of nitroxides on the tumor growth delay after radiotherapy (38). It was found that administration of nitroxides had no effect on tumor growth delay (38). The levels of the oxidized form of the nitroxide, were also estimated from tumor homogenates and were found to be significantly lower in tumor compared to bone marrow (38). Although the systemic pharmacology of nitroxides has been studied using isolated processed samples, it is important to determine the reduction profiles in tumor and normal tissue in vivo. This might provide important physiological information in terms of tissue redox status and oxygen status noninvasively. We have used the recently developed in vivo EPR instrumentation (11–13) to measure tissue nitroxide levels, their distribution, and differential reduction rates in tumor versus normal tissues. The imaging techniques were found to enable noninvasive measurement of physiological information in intact biological objects.

MATERIALS AND METHODS

Chemicals. TPL and 3-CP were purchased from Aldrich Corp. (Milwaukee, WI). The 15N analogue of TPL (15N-TPL) and PDT were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). All solutions of nitroxide labels were freshly prepared at a stock concentration of 20 mM in saline. Mice. Female C3H mice were supplied through the National Cancer Institute Animal Production Area (Frederick, MD). The animals were received at 6
The animals were observed closely, and the tumors became palpable approximately 5 days after injection. Tumors were allowed to grow to a size of about 10–15 mm in the greatest dimension. A minimum of five animals were used for each study described below.

**EPRI Instrumentation.** Imaging measurements were performed using the EPRI imaging instrumentation built at the Johns Hopkins University EPRI Center, consisting of an L-band EPRI spectrometer, three sets of water-cooled gradient coils, and a personal computer-based data acquisition system (11–13, 39). EPRI spectra were recorded using a custom-built surface resonator (40). The resonator consisted of two concentric quartz tubes, 10 and 12 mm in diameter, with the outer surfaces covered with silver foil strips forming a metallic tubular shield with one open end, 22 mm in diameter, functioning as the active surface. The resonator was capable of sampling a cylindrical volume measuring 10 mm in diameter and 5 mm deep. The open structure of this resonator was ideal for localized measurements on large objects and thus was not limited by the size of the object.

**Animal Preparation.** Mice were anesthetized with a mixture of 80 mg/kg of ketamine and 15 mg/kg of lignocaine, given i.m. The tail vein was cannulated with heparin-filled 30-gauge catheter for infusion of drugs. Either the tumor in the right leg or the normal tissue (muscle) on the left leg was prepared for measurements. The hair on the observation spot of the skin was shaved, and the animal was placed on a bedplate with a circular slot in such a way that the observation spot was centered at the slot (see Fig. 1). The animal was secured to the plate with adhesive tape and placed on top of the resonator such that the tumor or the normal muscle was in direct contact with the active surface of the resonator. The animal was allowed to breathe normally. An IR lamp was used to maintain normal body temperature.

**Projection Acquisition and Image Reconstruction.** Projection data were acquired using the angular sampling method. All of the projections were acquired as single scans (1024 points per projection) using a constant sweep time. The measured projections were corrected for removal of any hyperfine-based artifacts and deconvoluted with the corresponding zero-gradient projection (41). The deconvoluted projections were then convoluted with a second difference (three-point) filter function (42) and subsampled to 64 points for backprojection. A two-stage, filtered backprojection reconstruction algorithm was used to recover the image (43). Projection data acquisition and subsequent image reconstruction were performed using an Intel Pentium 200 MHz personal computer equipped with an IEEE-488 GPIB board (Capital Equipment Corp., Burlington, MA).

**Oximetry Calibration.** Calibration measurements of EPR line widths of PDT against oxygen (pO2) were performed at L-band using the surface bridged-loop resonator. A 0.25 mm solution of PDT in approximately 2-fold concentrated fetal bovine serum (Life Technologies, Inc.) was placed in a gas-permeable Tetlon tube (Zeus Industries, Raritan, NJ), and equilibrated with known concentrations of oxygen and nitrogen gases for at least 20 min. The EPR spectra were recorded for oxygen partial pressures ranging from 0 to 190 mm Hg (0–25% oxygen) using the following instrument settings: modulation amplitude, 50 mG; microwave power, 5 mW; scan range 2 G; scan time, 15 s. A linear response of peak-to-peak derivative line width to pO2 was observed in the range of oxygen concentration studied.

**RESULTS**

The present study used the following three nitroxides: TPL, 3-CP, and PDT, all of which have been shown to be in vitro radioprotectors (36). Recently, TPL has been reported to be an in vivo radioprotector (38). The 3-CP has greater biostability in tissues than TPL and hence was chosen for in vivo EPRI studies (21, 44, 45). The nitroxide PDT was used as an oximetry probe for oxygenation measurements (28, 45). All of the experiments were performed in mice with tumor sizes in the range of 10–15 mm in the greatest dimension. A minimum of five animals were used for each study described below.

**Pharmacokinetics of Nitroxides.** Nitroxides are paramagnetic compounds, whereas the corresponding reduction products (notably hydroxylamines) are not. This enables selective real-time monitoring...
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Fig. 2. EPR spectra of nitroxides in tumor. Mice were infused via the tail vein with a mixture of 100 mg/kg 3-CP and 100 mg/kg 15N-TPL, and the pharmacokinetics of the nitroxides in the leg tumor were measured in vivo using u-band EPR spectrometer. The triplet signal due to 3-CP peaked around 4 min and decayed gradually, whereas the doublet signal (*) due to 15N-TPL peaked at 1 min and decayed rapidly. Measurement parameters: microwave frequency, 1.25 GHz; microwave power, 10 mW; modulation amplitude, 0.5 G; modulation frequency, 100 kHz; scan time, 10 s.

of tissue nitroxide levels, in vivo. Mice were anesthetized and infused with a mixture of 15N-TPL (100 mg/kg) and 3-CP (100 mg/kg) via tail vein catheter. The pharmacokinetics of the nitroxides from the tumor-bearing right leg or from the normal muscle on the left leg was followed continuously in real time using EPR spectroscopy. A few typical spectra obtained during the infusion and clearance of nitroxides from the tumor-bearing right leg or from the normal muscle on the left leg are shown in Fig. 2. Because the spectrum of 15N-TPL has a doublet with a hyperfine coupling constant, \( [a(15N) = 22.80 \text{ G}] \) and that of 3-CP was a triplet [mean \( a(14N) = 16.80 \text{ G} \)], the presence and metabolism of both of the nitroxides could be simultaneously measured. The normalized plot of nitroxide concentration as a function of postinfusion time is shown in Fig. 3. The data from normal tissue showed that the 3-CP concentration peaked (100%) at about 4 min after infusion and thereafter slowly decayed. The TPL levels peaked at about 1 min and quickly decayed to baseline in about 10 min. Further, the maximum concentration of TPL that was observed in the normal tissue was only about 60% of the 3-CP level. These pharmacological data from the tumor indicated that the peak nitroxide level was significantly lower in the tumor compared to that in the normal tissue. In addition, the concentration of the nitroxides was observed to decay faster in the tumor.

In the above experiment, the observation was performed in a limited region of the tissue (tumor). The systemic nitroxide is expected to function as a reservoir with continuous infusion of the compound into the observation volume of the tumor. Thus, at any time, there will be two opposing processes: infusion of nitroxide from the systemic vasculature into the tumor and local decay due to bioreduction. Assuming that the tissue nitroxide is an intermediate in the infusion-decay process, we modeled the pharmacokinetic data to determine the rates of infusion and decay (Fig. 3). The half-life of TPL was 0.5 ± 0.1 min in tumor compared to 0.9 ± 0.2 min in normal tissue. The half-life of 3-CP was 9.8 ± 0.5 min in tumor compared to 14.6 ± 0.5 min in normal tissue. No significant differences were observed in the infusion rates of nitroxides in tumor versus normal tissue. Taken together, the pharmacokinetic data suggest that nitroxides are rapidly reduced in the tumor and that tumors contain significantly lower amounts of nitroxides compared to the systemic levels.

Imaging of Nitroxide Clearance in the Tumor. EPR spectroscopic measurements give the total amount of nitroxide in the EPR detectable volume of the tissue. We performed EPRI to determine spatially defined alterations in the distribution and decay of nitroxides. Anesthetized mice were infused (via the tail vein) with 160 mg/kg of 3-CP, and a series of two-dimensional spatial images were acquired at 1.5-min intervals. The images are shown in Fig. 4, along with similar time-matched images obtained from normal tissue. It was seen that although the nitroxide in normal tissue persisted for longer than 16 min, in tumor it was cleared within 10 min of infusion. The observed nitroxide clearance also correlates well with the spectroscopic measurements on the tissue volume shown in Fig. 3.

Tumor Heterogeneity. To investigate the tumor heterogeneity in terms of vascularization, oxygen content, and redox status, we performed three-dimensional imaging of the nitroxide distribution in tumor and normal tissues. Anesthetized tumor-bearing mice were given 160 mg/kg 3-CP i.v. After allowing 4 min for the nitroxide to reach maximum tissue levels, three-dimensional spatial EPR images...
were acquired. Fig. 5 shows some representative slices obtained from the three-dimensional images. The slices from the normal tissue show uniform distribution of nitroxide. However, the tumor slices show significant heterogeneity. A lower 3-CP content was observed in the middle of the tumor, which is consistent with an area of compromised blood flow. 3-CP has been shown previously to penetrate the cell membrane and accumulate intracellularly (36). Hence, this nitroxide would normally be expected to be present both intracellularly and extracellularly, producing a homogenous distribution in the image. In normal tissue, a homogenous distribution of the 3-CP was observed. In tumor tissue, however, there was heterogeneity that might be due to several factors, including reduced vascularity, hypoxia, and cellular necrosis.

**Oximetry in Tumor.** The RIF-1 model with a tumor size of 10–15 mm that was used in this study is known to have a radiobiological hypoxic fraction of 5–10% (4). These percentages were determined indirectly by comparing in vitro radiation dose-response curves of cells taken from tumors irradiated under control conditions versus conditions where the blood flow to the tumor was restricted by clamping several minutes prior to irradiation (4). Although this invasive technique is useful in establishing the fraction of radiobiological hypoxic cells in an experimental tumor, it does not give an assessment of intermediate oxygen levels. Because the bioreduction of nitrooxides in tissues is significantly affected by the tissue oxygenation and might be the cause of the observed heterogeneity, as shown in Fig. 5, further noninvasive oxygen measurements were performed. Molecular oxygen is paramagnetic, and thus, it causes exchange broadening of the nitroxide EPR signal. Because the magnitude of this broadening is directly correlated to the concentration of oxygen in solutions, EPR spectroscopy offers a unique method of noninvasive tissue oxygen measurement (26). When combined with the spectral-spatial imaging technique, this offers an even greater potential for measuring site-specific oxygen concentrations in biological tissues and small animals (10, 11, 14, 15, 46).

Because the nitroxides show different degrees of oxygen sensitivity in their EPR line width, we used PDT, which has been shown to have better oxygen sensitivity (28, 45). Mice were given 100 mg/kg PDT i.v. After a 1-min period to allow the PDT concentration to peak, the tumor or the normal leg was clamped to restrict tissue blood flow. EPR spectra were continuously acquired during the ischemic period for 10 min, and the line shapes were analyzed and compared with a calibration curve to determine oxygen concentration. It should be noted that the nitroxide spectral width response to oxygen in tissues is proportional to both dissolved oxygen concentration and the diffusion constant of oxygen and nitroxide in the medium. Halpern et al. (14) have used concentrated fetal bovine serum as a medium of rough equivalence to calibrations and tissue measurements. The solubility of oxygen in tissue is another important factor that needs to be considered in using calibration data obtained in aqueous medium. Oxygen has a higher solubility in lipids, and hence, nitroxides (such as PDT), which are lipophilic, would report higher concentrations of oxygen in lipid compartments compared to aqueous compartment, under similar pO2. In the present study, we approximated the solubility of oxygen in lipid to that in olive oil, and calculated the equivalent oxygen pressure in tissues. Fig. 6 shows the initial and change in EPR line width and the corresponding tissue pO2 during tissue ischemia. Whereas the normal tissue had an oxygen partial pressure of 40 ± 4 mm Hg before the onset of ischemia, the tumor showed a value of only 10 ± 2 mm Hg. Both tissues showed a drop in oxygen concentration to about 8 ± 2 mm Hg in 10 min. The reason that the tissue levels did not reach 0 mm Hg mostly likely resides in the inability to completely restrict blood flow of internal blood vessels by the clamping technique used. The significant hypoxia may explain the reduced nitroxide content as well as greater bioreduction of nitroxides in the tumor.

**DISCUSSION**

The present data, obtained using in vivo EPR techniques and nitroxide spin probes to study the RIF-1 tumor implanted in a C3H mouse model, show the feasibility of obtaining information on the differential physiology between normal and tumor tissue. After i.v. infusion, the amounts of the nitroxides TPL and 3-CP accumulating in
Normal Tissue

![Images of normal tissue slices](image1.jpg)

RIF-1 Tumor

![Images of tumor tissue slices](image2.jpg)

Fig. 5. Visualization of tumor heterogeneity monitored by nitroxide uptake. Following a tail vein infusion of 160 mg/kg of 3-CP, three-dimensional EPR images of the nitroxide in normal muscle and tumor were measured. A few selected adjacent slices of 0.3-mm thickness obtained from the three-dimensional image of normal (a–d) and tumor (e–h) tissue are shown. The images show some differences between the two tissues in terms of anatomy and physical architecture. The tumor tissue shows significant heterogeneity of nitroxide uptake compared to the normal tissue. Image acquisition parameters: number of projections, 100; gradient, 15 G/cm; acquisition time, 10 min.

The tumor have been found to be markedly lower than that in normal tissue. Furthermore, the reduction of these nitroxides has been found to be enhanced in tumors compared to the normal tissue. Some differences in the pharmacokinetics between the nitroxides have been found. The five-membered ring nitroxide 3-CP has been found to be relatively more stable in vivo, in tumor as well as normal tissue, compared to the six-membered nitroxide, TPL. These differences have been explained in terms of the redox behavior of the spin probes themselves. The in vivo stability of nitroxides depends primarily on the ease of the oxidation of the nitroxide to the oxoammonium cation (47) or the reduction to the hydroxylamine (25, 44). Consistent with this hypothesis are observations that the in vivo stability of the six-membered nitroxides is inversely related to the ease of reduction of the nitroxide (24). Likewise, the five-membered nitroxides are resistant to reduction and also have prolonged in vivo lifetime (21, 24, 48). Further, in vivo lifetimes of nitroxides are dependent on the oxygen content, as well as on levels of endogenous reducing agents (25, 49). The enhanced reduction of the nitroxides in tumor compared to normal tissue is consistent with the corresponding oxygen status of the two tissues. Furthermore, these results are in agreement with the lack of protective effects of TPL spin probe on tumor growth delay and the diminished nitroxide levels in tumor homogenates (38). Hence, using the differential reduction rates of nitroxides, in vivo EPR spectroscopy can be used to distinguish regions in vivo with differing redox states. Nitroxides, in addition to being probes, are also effective radioprotectors, and therefore, their pharmacokinetics can be studied.

In addition to pharmacokinetic information, a series of cross-sectional images of the spin probe distribution, obtained from tumor and normal muscle of mice administered with 3-CP spin probe, show some differences between the two tissues in terms of anatomy and physical architecture. Whereas the normal tissue exhibited no significant heterogeneity across the direction of the various slices, the tumor tissue was found to show significant heterogeneity. This is consistent with the compromised vasculature in tumors compared to normal tissue (3, 50, 51). To further explore the possible nature of the differential nitroxide accumulation and bioreduction in tumor versus normal tissues, EPR oximetry was performed. The level of oxygen in
normal muscle as determined by EPR oximetry of 40 ± 4 mm Hg is consistent with reported values using oxygen electrodes (52), whereas the average oxygen level in the tumor volume sampled was 10 ± 2 mm Hg. This 3-fold difference in \( pO_2 \) values in normal \textit{versus} tumor tissue may be one reason why TPL and 3-CP were more rapidly reduced in tumor tissue (Fig. 3). When blood flow was restricted to normal tissue, a steady decrease in nitroxide line width was observed, consistent with the onset of hypoxia. A decrease in oxygen levels was also noted in tumor tissue upon restriction of blood flow; however, the extent of decrease was much less. Although the present results report average \( pO_2 \) values over the volume sampled, spectral-spatial EPR with long-lived spin probes will be required to provide a detailed map of \( pO_2 \) distributions in tissue. With newly available spin probes, which have a long circulation half-life (53), such experiments should be feasible.

We have shown that \textit{in vivo} EPR spectroscopy/imaging is a potentially valuable tool for correlation of anatomical information with relevant physiological and metabolic parameters of tissues. The approach uses nontoxic redox-active probes in a noninvasive fashion and enables real-time measurements and correlation of tissue nitroxide levels and oxygenation. These qualities afford EPR the opportunity to perform anatomical as well as functional imaging that may prove useful in the selection of optimal nitroxide radioprotective agents for clinical use. Likewise, the ability to perform “metabolic profiling” based on the redox status of different tissues should provide new information that heretofore has not been available.

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