Oxymetry deep in tissues with low-frequency electron paramagnetic resonance

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ABSTRACT We have measured the oxygen concentration in the body water of murine FSa and NFSa fibrosarcomas using a new method for quantitative oxygen concentration determination deep in the tissues of a living animal. The measurement uses unusually low-frequency electron paramagnetic spectroscopy sensitive to substrate 7 cm deep in tissue, partially deuterated spin probes (spin labels of molecular mass 195, approximating that of glucose) whose distribution compartment can be targeted with facile adduct substitution, and novel analytic techniques. We show that the water-compartment oxygen concentration of the tumors decreases as the tumor size increases and also shows a trend to decrease as radiobiologic hypoxia increases. An oxymetric spectral image of the tumor is presented. The technique will improve with larger human tissue samples. It provides the potential to quantitatively assess tissue hypoxia in ischemic or preischemic states in stroke and myocardial infarction. It will allow direct assessment of tumor hypoxia to determine the usefulness of radiation and chemotherapy adjuvants directed to hypoxic cell compartments.

The measurement of molecular oxygen concentration deep in living tissues remains difficult despite its enormous biologic importance. The effectiveness of cancer therapy is affected by the oxygenation status of normal and tumor tissues (1). The oxygenation status of ischemic tissue in stroke and myocardial infarction is measured only with difficulty and invasiveness that frustrates clinical application. Current techniques use electrodes, which are invasive and have little control of the pharmacologic compartment sensitivity (2–4), or the quenching of luminescence by oxygen with sensitivity in tissues limited only to very superficial structures (5). Other techniques, hemoglobin dissociation, cytochrome oxidase status, metabolic and biologic assays, are indirect measures that are nonlinear in their response, making interpretation of heterogeneous samples difficult.

EPR (electron paramagnetic resonance) oxymetry has been used previously to monitor oxygen consumption in cellular and enzymatic systems. The homogeneous components of EPR spectral linewidths are linearly related to the rate with which these molecules encounter molecular oxygen (6, 7). This linewidth component is therefore proportional to local oxygen concentrations in the solution sampled by the spin label. The spectrum from a spin probe dissolved in the medium is often obtained under spectroscopic conditions where individual (homogeneous) hydrogen hyperfine lines are resolved, their widths giving the oxygen concentration. A major confounding variable is the concentration of the spin probe which, as an environmental paramagnet, broadens spectral lines. Most of these spin label measurements rely on an easily measured distribution volume to determine the spin probe concentration. Full correction for the line broadening

due to spin label concentration can be made, yielding a quantitative oxymetry.

In living tissue, however, the volume of distribution is difficult if not impossible to measure. Differential bioreduction (in various pharmacologic compartments) of the spin probe further diminishes measurability of even relative concentrations (8, 9). Prior work with spin label EPR oxymetry in tissue has been less quantitative, ignoring this effect (10). The information potentially available in the EPR spectrum is not fully exploited to extract quantitative oxygen concentrations by correcting for other environmental influences on the oxygen signature or to extract other important aspects of the spin probe environment. Others have used implantable ampoules with confined spin label that equilibrates with the implanted environment (11). Insertion of high-spin-density carbon particulate gives high oxygen sensitivity (12). But both techniques make definition of the sampled compartment problematic and are invasive.

We present here an EPR oxymetry at very low frequency that obviates these problems (13). Using innovative spectroscopic and analytic techniques, we have measured the EPR spectrum from small (molecular mass 195, approximating that of glucose) spin probe molecules infused directly into living tissue. Conventional EPR, performed at 10 GHz, suffers from the superficial sensitivity of other oximetric techniques. However, at frequencies more characteristic of nuclear magnetic resonance—250 MHz—the penetration depth of the radiofrequency used in the spectroscopy increases about 2 orders of magnitude from 0.1 cm to 7 cm in living tissues, sufficient to gain access to deep human tissues (13, 14). The reduction in frequency involves sacrifice in sensitivity. This has been thought to obviate the possibility of an EPR oxymetry deep in tissues. However, spectral sensitivity is increased both with the use of partially deuterated spin probes (15) and spectral fitting techniques (16) to reflect the full information in the EPR spectrum. This allows direct measurement, deep in tissue, of the many aspects of solution environment to which EPR spectra are sensitive, including molecular oxygen. This magnetic resonance technique allows imaging, not only of the quantity of spin probe but of its spectrum, allowing an oxygen image.

MATERIALS AND METHODS

Tissue Penetration with Very-Low-Frequency EPR Spectroscopy. EPR spectroscopy is conventionally done at frequencies (≈ 10 GHz) (17) so high that the electromagnetic fields that stimulate resonant absorption of energy cannot penetrate the conductive aqueous material of living tissue to

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Abbreviation: mHCTPO, 4-protio-3-carbamoyl-2,2,5,5-tetraproteromethyl-3-pyrrolinyl-1-¹⁵N-oxy.

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a depth of more than 1 mm. Operation at a frequency of 250 MHz increases skin depth from 1 mm to 7 cm (14), at which deep human tissues become accessible to EPR measurement. An EPR spectrometer was constructed (13) to operate at this frequency by using a highly stable, uniform magnetic field of 90 gauss (G; 1 G = 0.1 mT) produced by a Helmholtz coil. The relationship between the reduction in operating frequency and diminished EPR sensitivity (signal-to-noise ratio) in living tissue samples is approximately linear (18). A precise *in vivo* oxymetry requires operating conditions that further compromise the signal-to-noise ratio to avoid spectral distortion (15) such as the following conditions: 0.1-s time constant = data-point acquisition time; each scan of 246 points per spectral window (2.5-G intervals), 5 scans per spectrum; modulation field of 0.1 G; modulation frequency of 5.12 kHz; and a radiofrequency B_1 of 0.03 G, where B_1 is the radiofrequency root-mean-square magnetic field. An acrylic immobilization jig mechanically isolates the mouse from a resonator designed to accommodate the tumor-bearing limb.

Sensitivity Enhancement with a Modified Oximetric Spin Probe. A modified form of the spin label CTPO (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy) was used (15). Methyl deuteration collapses the 13 methyl hydrogen hyperfine line splittings. The ring hydrogen splits each nitrogen manifold by ≈ 500 mG. This allows a modulation field increase by a factor of 2 to 5 (to 100 mG), focusing spectral attention on two spectral lines of each nitrogen manifold as shown in Fig. 1. The resulting infusible nitroxide spin probe is 4-protio-3-carbamoyl-2,2,5,5-tetraprodeuteromethyl-3-pyrrolinyl-1- ^{15}N -oxy (mHCTPO) whose spectrum is shown in Fig. 1. The oxymetric sensitivity relative to CTPO is increased by a factor of 20 (15).

The three-carbon substituent largely determines the solubility of the spin probe without substantially changing its spectral characteristics. By modifying this group, the solubility characteristics of the spin label may be altered. Thus, it can target pharmacologic compartments. With the polar carboxamide substituent, this spin probe distributes in the body water compartment (19); it is nontoxic to mice with

repeated infusions. It was administered i.p. at 30 mM concentration as a 0.7-ml bolus.

Sensitivity Enhancement with Spectral Fitting. A second, well-known strategy for applying the information in the entire spectrum to its characteristic parameters is spectral fitting to an adequate analytic hypothesis (16). This can be thought of as a noise-reduction strategy. Each spectral line can be characterized under the specified spectral acquisition conditions as a weighted sum of Lorentzian and Gaussian forms. As an approximation to the more commonly used (Voigt) convolution of the two forms, the sum-function form is fitted by using a standard Levenberg-Marquardt nonlinear least-squares fitting algorithm to the spectral data. The fits are shown as the solid lines in Fig. 1. Parameters extracted from these fits are 1 order of magnitude more accurate than those depending on a few spectral points. The fitting algorithm also gives criterion-derived estimates of the uncertainties of the spectral parameters from an individual spectrum.

Spectral Analysis. Parameters extracted from the spectral-fit function are (i) R , the ratio of flanking peak-trough to central trough-peak heights, which is linear in molecular oxygen concentration (15, 16) and more robust in the face of noise than ΔB (the peak-to-peak linewidth) (Fig. 1); (ii) A_H , the ring hydrogen splitting extracted from the fits, which is linearly responsive to local spin label concentration and independent of oxygen concentration (15, 16); (iii) $\Delta R = R_1 - R_2$ or $\Delta \Delta B = \Delta B_1 - \Delta B_2$ where R_1 , R_2 , ΔB_1 , and ΔB_2 are defined in Fig. 1, which gives a parameter proportional to the rotational hinderance of the pharmacologic compartment (solvent) in which the spin probe distributes, in the limit of small correlation time (20, 21); and (iv) A_N , the nitrogen splitting, which is responsive to solvent (environment) polarity (22). The first three (R , A_H , and ΔR) parameters allow measurement of the effect on linewidth of the medium in which the spin probe distributes. Thus, the calibration medium can be adjusted to give the same ΔR as tumor or tissue measurements, on average. Deviations of individual tumor or tissue ΔR measurements from the average can be used to correct the individual tumor measurements.

Calibration. A calibration grid was used to evaluate the relationship between spin label concentration and oxygen concentration and the spectral parameters R and A_H . Spectral width response to oxygen depends on the interaction rate of the spin probe with molecular oxygen. This is, by the Smoluchowsky equation (6), proportional to both the oxygen concentration and the solvent diffusion constant for molecular oxygen (which we assume is proportional to that for the spin probe). Tissue oxygenation is quoted in terms of partial pressure because calibration involved equilibration of spin probe of known concentration with gases whose oxygen partial pressures are known. We assure the rough equivalence of the calibration and tissue measurements by calibrating in a medium consisting of fetal calf serum prepared as follows. The volume of fetal calf serum was reduced by slow desiccation to five-ninths of its original volume so that its rotational hinderance approximated that of the large and medium tumors ($\Delta \Delta B = 0.012$ G, $\Delta R = 0.010$), and it was heated to $37 \pm 0.1^\circ\text{C}$. To samples of fetal calf serum prepared in this manner, amounts of mHCTPO were added to give specific concentrations of spin label. These samples were equilibrated with N_2/O_2 gas mixtures (Linco, Hillside, IL), each with individual elemental analyses. Spectra from samples were obtained and fit parameters were extracted. The calibration grid was similar to water grid measurements previously published (15, 16). Regression analyses of the grid gave the following relations between spin label concentration ([SL]), oxygen equivalent partial pressure ([O_2]), R value, and ring hydrogen splitting (A_H) for the lowfield ^{15}N manifold.

FIG. 1. EPR spectrum (individual data points are dots) and spectral fit (solid line) from the oxymetrically optimized spin probe mHCTPO measured in an FSa leg tumor of a C3H/SeD mouse. R_1 for the low-field line and R_2 for the high-field line are defined in the figure and are derived from the fit. ΔB_1 and ΔB_2 are respective peak-to-peak linewidths; and A_N and A_H are nitrogen splittings.

$$[\text{SL}] = (0.5103 - A_H) \cdot 2.5732 \cdot 10^4 \mu\text{M}, \quad [1]$$

$$[O_2] = (0.628 - \delta R_{\text{Rotational hindrance}} - R - 5.11 \cdot 10^{-4} [\text{SL}]) \cdot 442 \text{ torr. [2]}$$

A temperature correction of ≈ 2.5 torr (t ; 1 torr = 133 Pa)/ $^{\circ}\text{C}$ was used. Contributions of normal tissue to oxygen estimates were corrected by a volume-weighted subtraction and renormalization based on normal tissue volume and oxygenation measurements.

Tumor Model. The animals used were 12- to 20-week-old C3H/SeD mice (Edwin Steele Laboratories, Massachusetts General Hospital, Boston). Tumors were grown from chunks of F5a and NFSa transplantable fibrosarcomas (the latter generously provided by L. Milas and K. Mason, M. D. Anderson Hospital, Houston) implanted subcutaneously in the legs of the mice. The tumors were allowed to grow to varied sizes as measured by necropsy after oxygen measurement.

Animal Measurement Conditions. The animals were anesthetized with ketamine (45 mg/kg) and diazepam (20 mg/kg) to reduce animal motion. Animals were further restrained with elastic adhesive bandage (Elastoplast, Beiersdorf, Norwalk, CT) and with gentle tension to extend the tumor-bearing limb through the resonator. Heating with goose neck lamps maintained core and surface temperatures to $37.0 \pm 0.5^{\circ}\text{C}$. Rectal and subcutaneous temperatures were measured with a Physitemp Th-6D thermocouple thermometer (Physitemp Instruments, Clifton, NJ).

Imaging. Spectral-spatial imaging was accomplished by imposing a highly uniform gradient in the direction of the leg tibia. This yielded one-spatial dimensional images distinguishing tumor-leg slices along the tibia. Spectral-spatial images were derived from 32 projections uniformly covering the accessible pseudo-object space [in the terminology of Lauterbur *et al.* (23) and Maltempo (24)]. A maximum gradient of 3 G/cm and an object length of 3.5 cm were used. The reconstruction algorithm of Maltempo, Eaton, and Eaton (25) was used to extract the two-dimensional spectral-spatial image by using the code given to us by S. Eaton and then modified for our use.

RESULTS

The basic signature of the presence of tissue oxygen is a linear increase in the homogeneous component of the linewidth; it is also related to the R parameter (15, 16), as defined in Fig. 1, extracted from the fitted function. Both as the spectral lines broaden and as the splitting A_H decreases, the R parameter diminishes. The calibration in tumor-equivalent fetal calf serum was similar to that seen in water (15, 16), demonstrating a linear relation between the R parameter and oxygen concentration at physiologically relevant oxygen and spin probe concentrations. The R parameter also diminishes with increasing spin probe concentration. The resolution of this confounding effect is found in the measurement of the ring hydrogen splitting, A_H , as noted (15, 16). A_H diminishes linearly with increasing spin probe concentration via spin exchange (26) shown over the entire range of physiologic concentrations. Oxygen does not induce a spin exchange shift in the position of the spectral lines, likely because of the enormous width of the oxygen EPR line. Thus, the spin probe concentration can be measured independently (Eq. 1) and used to evaluate the correction factor of Eq. 2, giving a quantitative oxymetry. Various other EPR spectral parameters report a number of environmental conditions, including the local polarity (22), ordering (27), and diffusion constants (related to local viscosity) (20). These parameters are useful to ensure that the medium in which the calibration measurements are made fully reflects tissue conditions. The small effects of these parameters on the oxymetric signature are

included in the final oxygen concentration evaluation as corrections.

This technique was used to measure oxygen within the body water in mouse leg tumors. Spin probe was injected i.p. in the mice 10 min after administration of anesthesia. Measurements began 2–4 min after spin probe administration. Spectra were obtained in 4 min. A maximum in signal intensity typically appeared 5 min after measurements began and diminished by a factor of 5–10 because of bioreduction of the nitroxide to hydroxylamine to a much more slowly decreasing plateau phase ≈ 40 min later. Thus, the major oxymetric information is obtained in the first 20 min after the injection.

Spectra were fit to the hypothetical shape of Fig. 1 to extract spectral parameters. The calibration grid was generated in a medium with the viscosity that reflects that of the tumor water compartment, measured to be 1.6 ± 0.05 cP (1 poise = 0.1 Pa-sec) from the difference in the R and linewidth (ΔB) values between the two spectral lines as shown in Fig. 1. The nitrogen splitting, A_N , of 22.855 ± 0.010 G measured in the tumor is consistent with water compartment distribution expected from the polar mHCTPO (22, 28). Equivalent oxygen partial pressure was extracted from spectral parameters according to the algorithms of Eqs. 1 and 2.

Fig. 2 *Upper* shows the spin label concentration estimates from a single tumor derived from the ring hydrogen splitting of

FIG. 2. (*Upper*) [SL] from Eq. 1 in a single medium-size tumor versus time. EPR maximum signal appeared in the tumors beginning 5 min after the i.p. injection. (*Lower*) $[O_2]$ from Eq. 2 versus time.

FIG. 3. Sensitivity of the spectrum of the tumor to the inhaled O_2 fraction. ○, Tumor spectrum from the anesthetized animal breathing air; ●, tumor spectrum from the anesthetized animal breathing nearly 100% O_2 with a 2-min equilibration between spectra. The R value for the 100% O_2 -inspired gas is $0.29 \pm .02$, while that for air breathing is 0.37 ± 0.02 (2-min measurements), corresponding to pO_2 of 42 ± 8 t and 7 ± 8 t. Despite the small apparent changes in the spectra, the large number of contributing spectral points give significant pO_2 differences.

the low-field nitroxide manifold plotted vs. time. This is to be distinguished from the spectral signal intensity mentioned earlier, which cannot measure concentration in the absence of a measurement of distribution volume. After a maximum at 5 min, the concentration diminishes because of bioreduction, with a stabilization at ≈ 40 min. Fig. 2 *Lower* also shows the spectral estimates of the tumor tissue oxygen concentrations measured as a function of time after spin probe injection. Scatter in the values indicate a present uncertainty of 7 t. The large error bar in the fourth datum is due to uncaptured animal motion and is an important indicator of a contaminated datum.

Fig. 3 shows spectra from a single nitrogen manifold obtained from a large FSa tumor under two conditions: the mouse was first bathed for 4 min in a gas atmosphere of oxygen; next it was bathed in air for 4 min. As can be seen, despite compensatory vasoconstriction thought to prevent

changes in oxygenation with the administered oxygen atmosphere, there are clear changes in the tumor oxygenation, from 42 ± 8 t with oxygen breathing to 7 ± 8 t in air. These differences diminished in three cycles to undetectable, presumably representing an adaptive response. This demonstrates the sensitivity of the technique to difficult tumor measurements. The adaptation noted, if true in humans, argues that the common practice of "equilibrating" the patient with O_2 or 95% O_2 /5% CO_2 breathing for 0.5–1 hr prior to radiation treatment may have been self-defeating. Studies were carried out applying and releasing a tourniquet to a small well-oxygenated tumor. These showed dramatic changes with pO_2 of 3 ± 8 t with tourniquet applied and $pO_2 = 48 \pm 8$ t with it released.

Fig. 4 shows the equivalent pO_2 measured in the body water of tumors of varied sizes. There is a dependence of the equivalent pO_2 on tumor size. There are lower oxygen concentrations in the body water of larger tumors. This is consistent with prior electrode measurements (29) made without pharmacologic compartment distinction. The spin probe equivalent pO_2 measurements are similar in value to measurements with a polarographic electrode (29). The radiobiologic hypoxic fractions of the fibrosarcomas are 3% for the NFSa and 27% for the FSa histologies (30). In Fig. 4 there is a suggestion that in the body water of NFSa tumors—the histologic type with the lower radiobiologic hypoxic fraction—the equivalent pO_2 appears to remain above zero for larger tumors than in the more hypoxic FSa tumors, but the difference is not significant. The scatter of the pO_2 values substantially exceeds the measurement errors within each category of tumor size and histology. Given the consistency of the multiple measurements taken with the same tumor (e.g., Fig. 2 *Lower*), these appear to be real variations.

The imposition of magnetic-field gradients of the sample allows localization of the signal within the sample—imaging. The integral representation of the spectrum is shown along the horizontal axis in Fig. 5, while the spatial distribution along the mouse thigh is seen projected into the page. A deeper dip between the spectral lines indicates the lines to be narrower. With other conditions equal, narrower lines imply a lower oxygen concentration. The one-dimensional distribution of spin label along the axis of the mouse leg is shown in Fig. 5. This is, then, a one-dimensional oxygen image of the tumor.

DISCUSSION AND CONCLUSIONS

Using both an unusually low-frequency EPR spectroscopy to secure tissue penetration and a novel spin probe, mHCTPO,

FIG. 4. Equivalent pO_2 vs. tumor size (determined by necropsy) for tumors of different histologic types. (Left) FSa tumors. (Right) NFSa tumors.

FIG. 5. A two-dimensional (1 spatial/1 spectral) spectral-spatial image with the spatial dimension along the mouse thigh, parallel to the femur. The spectral display (along the bottom of the page) is the integral of the spectra shown in Fig. 1; this corresponds directly to paramagnetic absorption. The requirement of positive definite image values for the back projection reconstruction algorithm forces this display. The spatial distribution (into the page) is consistent with a uniform distribution of spin label in the leg tumor. This image is oxygen sensitive in that the depth of the valley between the spectral peaks indicates the extent of tumor hypoxia.

we have quantitatively measured, with minimal tissue invasion, oxygen concentration in the body water compartment of a murine fibrosarcoma implanted in the mouse leg. By modifying the spin probe at the three-carbon, the pharmacologic distribution can be altered to sample hydrophobic, intracellular (alone), intravascular, and other pharmacologic compartments. Recent electrode measurements in human tumors have shown dramatic differences in response to therapy for localized head and neck (31) and cervical cancer (1): This technique yields values that are consistent with recent microelectrode measurements in murine tissues (4, 29, 32) without the corresponding invasiveness and microenvironment perturbation and with full knowledge of the compartment being sampled. It provides measures of other characteristics of the fluid compartments of living tissue heretofore unavailable, including their polarity and viscosity. At the very low frequencies used, it is capable of measurements deep in human tissues. Human measurements with larger samples containing more substrate should yield even higher resolution than seen here (33). The technique is capable of providing an oxygen image even for the small samples provided by this mouse leg tumor. It promises a quantitative imagable oxymetry deep in living tissues that may allow direct measurement of molecular oxygen concentrations in various pathologic states, including those of stroke, myocardial infarction, and cancer, both to predict and to evaluate the efficacy of therapeutic intervention.

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