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EPR IMAGING OF TISSUE REDOX STATUS

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The purpose of the study was to obtain spatially resolved redox information (redox mapping) from biological tissues under noninvasive conditions using electron paramagnetic resonance imaging (EPRI) method. The method is based on the ability of the EPRI to measure spatially resolved pharmacokinetics data using a redox sensitive nitroxide probe. The principle of the redox mapping is described and validated using a biological sample. Redox mapping experiments were performed in RIF-1 murine tumor using low-frequency *in vivo* EPR imaging techniques with 3-CP nitroxide redox probe. The data show the existence of significant heterogeneity of redox status in the tumor. Also mice pretreated with BSO, a glutathione depleting agent, showed a decrease in the magnitude and distribution of the reducing equivalents in the tumor. Thus it is demonstrated that the redox mapping method by EPRI provides a noninvasive means of obtaining spatial and time-resolved pharmacokinetics information, which may be important in the understanding tumor physiology and therapy.

INTRODUCTION

The redox status of cells and tissue are governed by several independent factors including perfusion, oxygenation, levels of antioxidants, and a network of enzymatic systems (Schafer & Buettner 2001). A variety of intracellular molecules contribute to the overall redox status in tissues including glutathione (GSH), thioredoxins, NADPH, flavins, ascorbate, and others (Schafer & Buettner 2001). Oxidative or reductive stress and alterations in the endogenous/exogenous antioxidant levels cause an imbalance in the redox homeostasis of the cells. The cells then revert to the normal redox status by mounting a stress response using various signaling pathways and enzymatic systems.

Tumor cells are known to have significant alterations in the redox status. The tumor redox status is an important determinant in the response of the tumor to certain chemotherapeutic agents (Russo, Carmichael et al. 1986), radiation (Mitchell & Russo 1987), and bioreductive hypoxic cell cytotoxins (Yu & Brown 1984; Brown 1993; Stratford, Adams et al. 1994). Therefore, a noninvasive assessment of such information will be useful for tumor treatment planning. Magnetic resonance spectroscopy and positron emission tomography (PET) are some radiologic methods which are capable of providing such information using endogenous probes. More recently, electron paramagnetic resonance (EPR) imaging has been developed to provide such information in small animals and potentially in humans (Kuppusamy, Afeworki et al. 1998). The EPR methods, however, require the use of exogenously infused redox sensitive spin probes such as nitroxides. Two critical properties of nitroxides provide such capability:

(i) nitroxide radicals participate in redox-reactions (Figure 1) where the nitroxide (paramagnetic, EPR detectable) are reduced to the corresponding hydroxylamine (diamagnetic, EPR silent) and the hydroxylamine can undergo reoxidation back to the nitroxide (Krishna, Grahame et al. 1992)

(ii) in cells nitroxides undergo reduction to the corresponding hydroxylamines more efficiently under hypoxic conditions than under aerobic conditions via intracellular enzymatic processes (Swartz 1990).

When nitroxides are administered *in vivo*, a rapid equilibrium is established between the levels of nitroxide and hydroxylamine. The level of the nitroxide detectable *in vivo* is independent of whether the nitroxide or the hydroxylamine is administered. Based on the above properties, several studies were carried out using stable nitroxides as probes and in vivo EPR spectroscopy and imaging experiments to non-invasively obtain tissue/tumor redox status. Recent studies of the levels of nitroxide in normal tissue and tumors, which contain hypoxic regions, suggest that the nitroxides are metabolized more efficiently in tumors than in normal tissue (Kuppusamy, Afe-

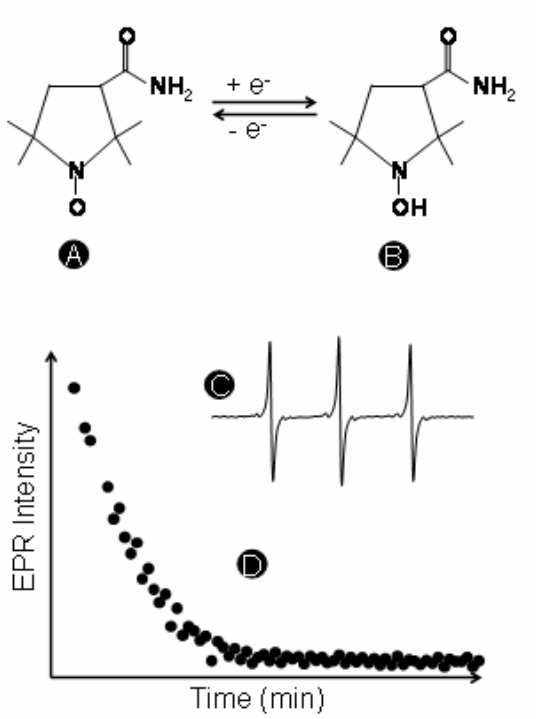

Fig. 1. Structure of 3-CP and its tissue metabolite. The 'EPR active' 3-CP (3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl, or 3-carbamoyl-proxyl) nitroxide (**A**) probe undergoes one-electron reduction in tissues to 'EPR-silent' hydroxylamine (**B**). A typical L-band (1.3 GHz) EPR spectrum of 3-CP in tissues is shown (**C**). The triplet, arising due to hyperfine splitting from the ^{14}N nucleus, is characterized with coupling constants 15.78 G and 16.30 G and peak-to-peak width 1.50 G. The conversion of the nitroxide to hydroxylamine in tissues is conveniently measured by following the signal intensity of the nitroxide as a function of time (**D**). The decay profile is a measure of nitroxide clearance from the tissue by processes including metabolic reduction and elimination.

worki et al. 1998). This observation was supported by an *in vivo* EPR imaging study examining the nitroxide clearance in normal tissue and tumors. Such studies suggest that the levels of nitroxides and the rate at which they are reduced may be dependent on the tissue redox status.

The purpose of this study was to develop a non-invasive method based on EPR imaging to measure and image tissue redox status. Experiments were performed using low-frequency *in vivo* EPR imaging techniques with a nitroxide redox probe. In this manuscript, we report the application of the EPR imaging method to obtain spatially resolved redox status information (redox mapping) in a RIF-1 (radiation induced fibrosarcoma) murine tumor. The results demonstrate that EPRI is capable of providing useful redox information from experimental murine tumors.

MATERIALS AND METHODS

Chemicals

The nitroxide probe 3-CP (3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-n-oxyl, or 3-carbamoyl-proxyl) was purchased from Aldrich (Milwaukee, WI). Solutions of the nitroxide were freshly prepared at a stock concentration of 300 mM in saline. L-buthionine-S,R-sulfoximine was purchased from Sigma.

EPR Imaging Instrumentation

Imaging measurements were performed using the EPR imaging instrumentation consisting of an L-band EPR spectrometer, three sets of water-cooled gradient coils and a personal computer-based data acquisition system (Kuppusamy, Chzhan et al. 1994; Kuppusamy, Chzhan et al. 1995; Kuppusamy, Afeworki et al. 1998). EPR spectra were recorded using a custom-built surface resonator.

RIF-1 Tumor Growth and Animal Preparation

Female C3H mice were used. The animals were on average 50 days old at the time of experimentation and weighed 25±3 g. RIF-1 tumor cells were grown in monolayered culture until injection in mice. The mice were injected subcutaneously in their right hind leg with a single cell suspension of tumor cells (10^6 cells in 0.1 ml) in PBS. 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 8-10 mm in the greatest dimension.

Mice were anesthetized by breathing air containing 1% isoflurane delivered through a nose cone. The tail vein was cannulated with a heparin-filled 30-gauge catheter for infusion of the nitroxide probes. The animal was placed on a bedplate with a circular slot (20 mm diameter) in such a way that the tumor was centered at the slot. The animal was secured to the plate with an adhesive tape and placed on top of the resonator such that the tumor was in direct contact with the active surface of the resonator. An infrared lamp was used to maintain normal body temperature, which was measured using a rectal thermistor probe.

Projection Acquisition and Image Reconstruction

Projection data were acquired using angular sampling method. The projections were acquired as single scans (1024 points/projection) using

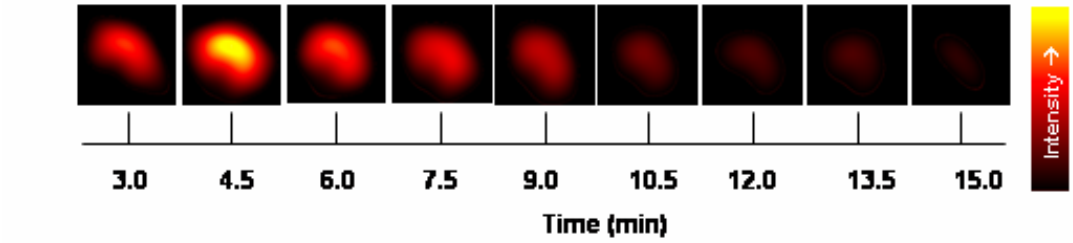

Fig. 2. 2D images nitroxide pharmacokinetics in a RIF-1 tumor. Following tail vein infusion of 3-CP (180 mg/kg), a series of two-dimensional images of the nitroxide from a RIF-1 tumor was obtained using L-band EPR imaging method. A few selected images and the corresponding time after infusion of the probe are shown. The images represent the mean nitroxide concentration in a 2-D projection of the tissue volume (10x10 mm^2; depth 5 mm) averaged over 1.5 min. The image data were acquired using a magnetic field gradient of 15 G/cm at 16 orientations in the 2-D plane.

constant sweep time. The measured projections were corrected for removal of hyperfine-based artifacts and deconvoluted with the corresponding zero-gradient projection (Kuppusamy & Zweier 1996). The deconvoluted projections were then convoluted with a Shepp-Logan filter and subsampled to 128 points for backprojection. A single-stage, filtered backprojection reconstruction algorithm was used to recover the image. Projection data acquisition and subsequent image reconstruction were performed using an Intel Pentium 600 MHz Personal Computer equipped with an IEEE-488 GPIB board (Capital Equipment Corporation, Burlington, MA).

RESULTS

Imaging of Nitroxide Metabolism in Tumor

Nitroxides, such as 3-CP, exist in a biological tissue as a redox pair (Figure 1), namely the nitroxide free radical form and the diamagnetic hydroxylamine, which is the one-electron reduction product of the nitroxide free radical. The 3-CP shows a triplet EPR spectrum, whose intensity is a measure of its concentration in the tissue. Thus the nitroxide probe, when injected into a tissue will undergo reduction to reach the equilibrium status with its hydroxylamine form. The rate of reduction will depend on the cellular reducing equivalents, oxygen concentration, pH, or collectively the tissue redox status. Hence it is hypothesized that the experimentally measured rate (or rate constant) of the nitroxide signal loss is a measure of the tissue redox status. Even if an absolute quantification of the tissue redox status is not obtained from this method, it should still provide information regarding the differences that exist in tissues having different redox status.

Figure 2 shows a representative measurement of

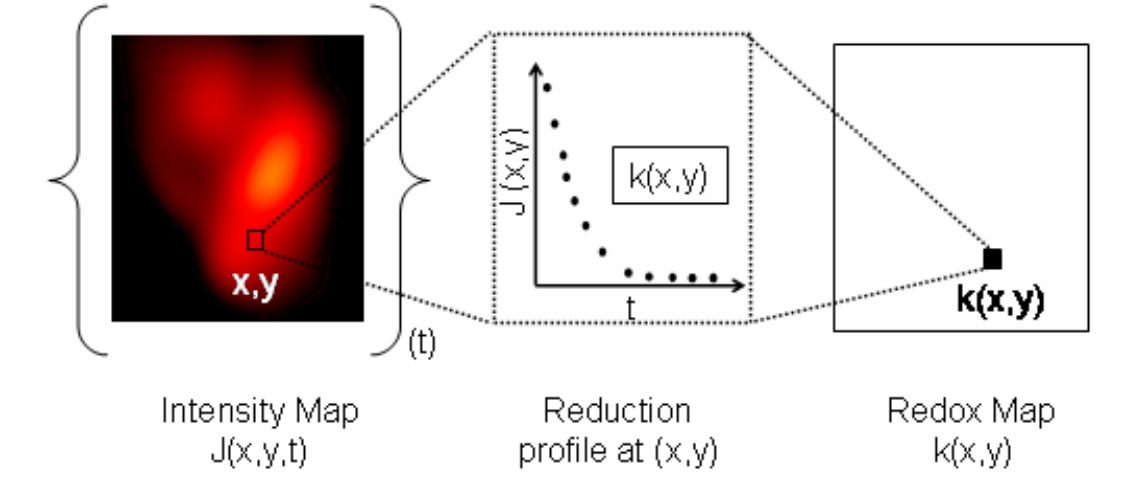

Fig. 3. Reconstruction of redox image. The procedure of obtaining redox image from a 2D pharmacokinetic image data is illustrated. Nitroxide intensity, J(x,y,t) from a given voxel within the image is followed as a function of time to obtain the reduction profile J(x,y) versus t. The profile is modeled with an appropriate kinetic expression, usually a pseudo first order decay process, to obtain rate constant, k(x,y). The computation is repeated over all the voxels to reconstruct the redox image.

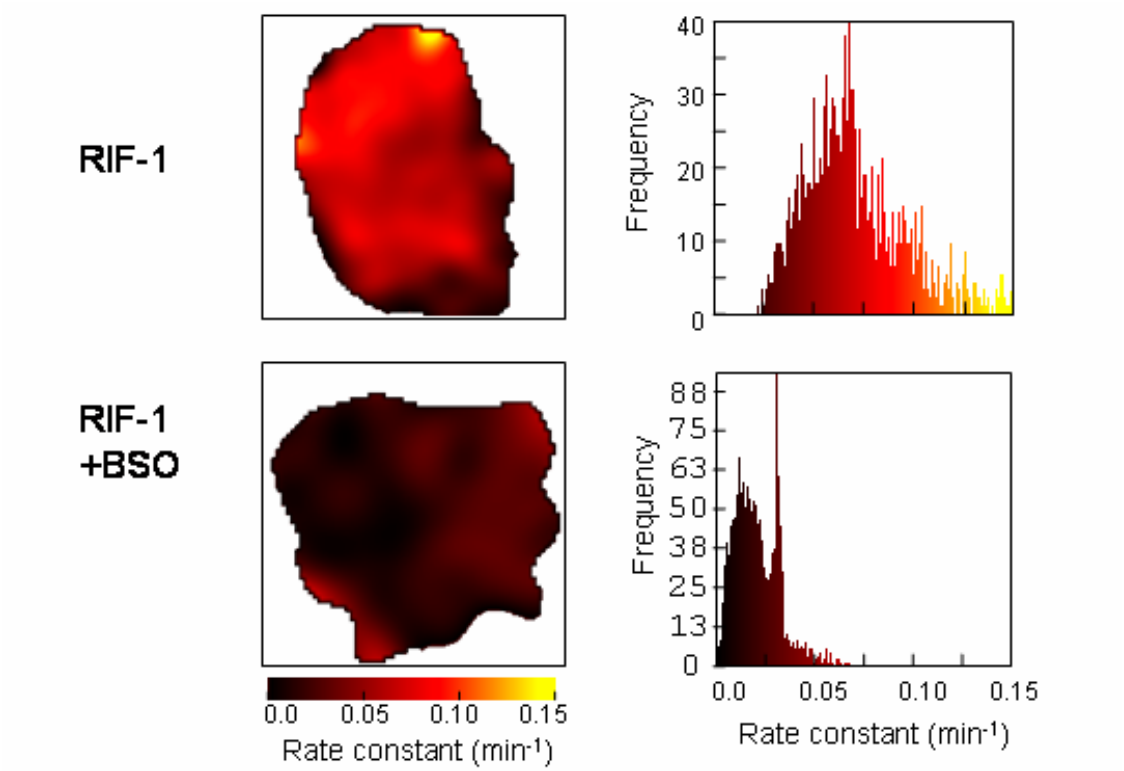

Fig. 4. Redox mapping of tumor. 2-D spatial mapping of pseudo-first order rate constants (left panels) and frequency plot (right panels) of the nitroxide reduction were obtained in RIF-1 tumors of untreated and BSO-treated.

the pharmacokinetics of nitroxide (3-CP) uptake and clearance from a RIF-1 tumor implanted in the upper leg of mouse and measured using *in vivo* L-band (1.3 GHz) EPR imaging. The mouse was infused, as a bolus dose *via* tail vein catheter, with a saline solution of 3-CP (180 mg/kg). The EPR images were acquired continuously during the clearance of the probe. It is observed that the nitroxide concentration in the tumor peaked at about 4.5 min and then continued to decrease thereafter. The images also show the existence of spatially resolved signal loss.

Redox Mapping by EPR

The principle of redox mapping by EPR imaging is schematically described in Figure 3. The pharmacokinetics images in Figure 2 are 64×64 pixel 2D images cropped from 128×128 pixels of original data obtained from a field of view of 20×20 mm^2. Thus, each pixel in the image represents a theoretical volume of 0.2×0.2×5.0 mm^3. The time-series image data can be used to follow time-dependent changes of nitroxide concentration in each voxel to obtain spatially resolved decay curves and hence rate constants as illustrated in Figure 3. If the decay is assumed as a pseudo-first order kinetic process, which will be case in most of the cases, the fitting may require only about 8 – 10 points along the curve to obtain a reasonable decay constant. The rate constants for the clearance of the probe in each pixel are computed and displayed in the form a color-coded image or frequency (histogram) plot. An appropriate threshold of intensity level needs to be defined to exclude those regions where the nitroxide concentration is too small to make calculations. This means that the redox data are applicable to regions with nitroxide intensity above the threshold level.

Redox Imaging of Tumor

Figure 2 shows images from a series of 2-D spatial maps of nitroxide content in the tumor tissue obtained as a function of time after 3-CP infusion. The rate constants for the clearance of the probe in each pixel were computed and displayed in the form a color-coded image in Figure 4. A frequency plot of the rate constants is also shown in Figure 4. The rate constant map and the frequency plot show the presence of a range of rate constants, which is a measure of heterogeneity in the redox status within the tissue. To determine the effect of alterations in the tumor redox environment on the redox map, similar experiments were performed in tumors of mice treated with 2.25 mmol/kg of BSO, a GSH depleting agent, for 6 hours. The redox data show that the BSO treatment significantly decreased the rate constant of nitroxide reduction in the tumor tissue (Figure 4).

The effect of BSO treatment on the redox constants obtained in the present experiments thus can be attributed to alterations in the tissue glutathione levels.

DISCUSSION

Non-invasive assessment of tissue redox status in intact biological organs will be valuable. In the pharmacokinetics experiments we have followed changes in the nitroxide signal intensity in the tumor, *in vivo*, as a function of post-infusion period. The measured rate of decrease of nitroxide signal intensity at the tumor site may depend on the following dynamic processes: (i) infusion of the probe into the tumor, (ii) changes in the probe concentration due to bioreduction in the tumor tissue, (iii) elimination of the probe out of the tumor volume by blood flow.

Thus, the decrease in the EPR signal intensity of the nitroxide cannot be attributed to bioreduction processes alone. However, it has been reported by Gallez, et al. (Gallez, Bacic et al. 1996) that bioreduction at the local site is the major factor that contributes to the overall decrease. This is also evident from the earlier observations different rate constants of clearance are observed between the tumor tissue and muscle on the nontumor-bearing leg (Kuppusamy, Afeworki et al. 1998). If perfusion is the major contributor then any local measurement should reflect on the systemic levels of the probe, and thus the rates constants would have been identical between the two tissues. Thus, the clearance rate constant observed by this localized measurement can be used as a parameter of tumor tissue redox status, which is correlated to the bioreduction of the probe.

Several studies have shown that tissue oxygenation is an important factor that will affect the tissue redox status and hence the reduction rate of the nitroxide. Particularly, tissue hypoxia, which is known to be present in tumors, has been shown to enhance the bioreduction of nitroxides (Swartz 1990). While this is evident from the enhanced rate of reduction from the tumor as compared to muscle tissue, which is relatively well oxygenated, the involvement of other factors such as altered redox state, and pH may also be important.

Thiols such as intracellular glutathione and protein thiols are important molecules that maintain the redox status of tissues. Chemically, nitroxides do not react with thiols. However, in microsomal preparations, thiol-containing biomolecules have been shown to play a significant role in the bioreduction of nitroxides (Tomasi, Albano et al. 1988). Since thiol/disulfide status generally reflects the redox state of the tissue, altering the concentration of the thiol or changing the ratio of redox pairs should have an impact on the clearance of the nitroxide and thus provide a means to study the importance of this class of compounds as a function of bioreduction. For example, depleting the GSH levels by using specific inhibitors of its synthesis such as L-buthionine-S,R-sulfoximine (BSO) and monitoring the pharmacokinetics of redox sensitive nitroxide probes in both normal as well as tumor tissue should delineate the role of thiols in modulating tissue redox state.

GSH is present in significantly large quantities inside cells and it is a versatile protector. Some of the protective roles of the glutathione include radical scavenging, restoration of damaged molecules by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the reduced state. Of these roles, hydrogen atom donation to DNA radicals is probably the most important (Biaglow, Varnes et al. 1983). Since competing reactions are very rapid, particularly with oxygen in well-oxygenated normal tissues, high concentrations of GSH are required for protection. Thus, moderate depletion of GSH in normal cells may have no effect on the radiosensitivity while under hypoxic conditions, such as that occurs in tumor tissues, GSH may play a dominant protective role. Conversely, the depletion of GSH in the hypoxic tumor tissue may have beneficial effects on the tumor treatment.

SUMMARY AND CONCLUSIONS

An EPR imaging method for mapping tissue redox status is described. Noninvasive measurements and mapping of tumor redox status in RIF-1 tumor implanted in mice were performed. The nitroxide pharmacokinetics images showed significant heterogeneity of reducing equivalents in the tumor. Mice treated with BSO, a glutathione depleting agent, showed an overall decrease in the magnitude as well as distribution of reducing equivalents in the tumor.

Acknowledgement

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