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Comparative Studies with EPR and MRI on the *In Vivo* Tissue Redox Status Estimation using Redox Sensitive Nitroxyl Probes: Influence of the Choice of the Region of Interest

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Abstract

In vivo decay rates of a nitroxyl contrast agent were estimated by a MR redox imaging (MRRI) technique and compared with the decay rates obtained by the electron paramagnetic resonance spectroscopy (EPRS) and imaging (EPRI). MRRI is a dynamic imaging technique employing T₁-weighted pulse sequence, which can visualize a nitroxyl-induced enhancement of signal intensity by T₁-weighted contrast. EPR techniques can directly measure the paramagnetic nitroxyl radical. Both the SCC tumor-bearing and normal legs of a female C3H mouse were scanned by T₁-weighted SPGR sequence at 4.7 T with the nitroxyl radical, carbamoyl-PROXYL (CmP), as the contrast agent. Similarly, the time course of CmP in normal muscle and tumor tissues was obtained using an 700 MHz EPR spectrometer with a surface coil. The time course imaging of CmP was also performed by 300 MHz CW EPR imager. EPRS and EPRI gave slower decay rates of CmP compared to the MRRI. Relatively slow decay rate at peripheral region of the tumor tissues, which was found in the image obtained by MRRI, may contribute to the slower decay rates observed by EPRS and/or the EPRI measurements. To reliably determine the tissue redox status from the reduction rates of nitroxyls such as CmP, heterogenic structure in the tumor tissue must be considered. The high spatial and temporal resolution of T₁-weighted MRI and the T₁-enhancing capabilities of nitroxyls support the use of this method to map tissue redox status which can be a useful biomarker to guide appropriate treatments based on the tumor microenvironment.

Keywords

redox mapping; redox sensitive contrast agent; nitroxide radical; electron paramagnetic resonance; magnetic resonance functional imaging; tumor physiology

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Introduction

Nitroxyl free radicals were initially evaluated as T_1 -enhancing MRI contrast agents [1–3]. The T_1 -weighted MR contrast observed by the nitroxyl contrast agent decayed in proportion to the *in vivo* reduction of the paramagnetic nitroxyl radical to the corresponding diamagnetic hydroxylamine [4–6]. The *in vivo* instability, i.e. fast reduction, was a considered to be an undesirable feature in the early days of development of MRI contrast agents. On the other hand, nitroxyl radicals are widely used as redox sensitive contrast agents in fields of *in vivo* electron paramagnetic resonance spectroscopy (EPRS) and imaging (EPRI) [7–12]. When a nitroxyl contrast agent was administered to a living animal, the chemical and/or enzymatic reduction of the nitroxyl contrast agent was estimated and used as an index of *in vivo* redox status. Using a low frequency EPR spectrometer and/or imager, a time course of EPR signal decay of the nitroxyl contrast agent can be analyzed.

The *in vivo* decay constant of an EPR signal of the nitroxyl contrast agent measured in a particular region of an animal can reflect the redox status of tissues/organs. *In vivo* EPR measurement using localized detector, such as a surface coil resonator (Fig. 1A), can give redox information specific to the sampled region [13, 14]. Using a CW EPRI technique, 2D or 3D mapping of distribution of a nitroxyl contrast agent in a sample is possible. However, low temporal resolution of the conventional CW modality does not permit 3D EPRI to follow *in vivo* EPR signal decay rates under most conditions. Therefore, in practice, *in vivo* time-course experiment using a nitroxyl contrast agent typically employs 2D EPRI (Fig. 1B) [15–17]. In addition, the broad EPR linewidth of nitroxyl contrast agent resulted in images with sub-optimal spatial resolution for useful extraction of redox status information in the region examined. Unlike with MRI, the lack of anatomical information on the EPRI makes identification of a specific tissue/organ of interest difficult.

MRI instruments utilize efficient pulse sequences and can obtain small T_1 changes sensitively with high spatial and temporal resolution [18]. The relatively low relaxivity of the nitroxyl contrast agents ($0.17 \text{ mM}^{-1}\text{s}^{-1}$ at 4.7 T) exhibits a linear relation between the concentration of the contrast agent and the enhancement of T_1 -weighted image intensity at low concentrations ($\sim 2 \text{ mM}$), a dose which is usually used in *in vivo* experiments [19]. Therefore, an *in vivo* decay rate of the nitroxyl T_1 -contrast based on the spoiled gradient recalled acquisition in steady state (SPGR) can be a useful method to assess tissue redox status [20]. The T_1 -weighted SPGR MR sequence can obtain *in vivo* redox status based on reduction of a nitroxyl contrast agent simultaneously with a high spatial and temporal resolution (Fig. 1C).

The MR-based *in vivo* nitroxyl decay rates obtained in normal and tumor regions from mouse reported usually higher values than the results obtained by *in vivo* EPRS and EPRI [19]. However a simulation study [20] and phantom studies [19, 21, 22] showed good correspondence between the MR-based and the EPR-based decay rates of a nitroxyl contrast agent. It is possible that *in vivo* EPR-based experiment may have underestimated the actual *in vivo* decay rates of nitroxyl contrast agent due to the unsuitable and/or insufficient volume of the measurement due to its intrinsic sub-optimal resolution.

In this paper, *in vivo* decay rates of a nitroxyl contrast agent estimated by T₁-weighted SPGR MRI scanning with several ROI selections were compared with the decay rates obtained by the EPRS and EPRI. The importance of the choice of measuring volume and suitability of T₁-weighted SPGR MRI scanning for redox status estimation in a heterogenic tumor tissue are discussed.

Materials and Methods

Chemical

Carbamoyl-PROXYL (3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl: CmP) was purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO). CmP was prepared as 300 mM (isotonic) solution in deionized water.

Animal

Female C3H mice were supplied by the Frederick Cancer Research Center, Animal Production (Frederick, MD). Animals, received at six weeks of age, were housed five per cage in climate controlled, circadian rhythm-adjusted rooms and were allowed food and water ad libitum. Experiments are carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the National Cancer Institute Animal Care and Use Committee. Experiments were performed within 4 weeks of their arrival at the facility. Their body weight was measured before the experiments and ranged from 22–27 g. A squamous cell carcinoma (SCC) was implanted and grown on the right hind leg for 9 days.

MRI experiment

Mice were anesthetized and secured on a special mouse holder by adhesive skin tape, stomach side down. The tail vein was cannulated for the injection of contrast agent. The mouse was then placed in the MR resonator, which was previously warmed up by a hot water-cycling pad covering the resonator. The resonator unit including the mouse was placed in the magnet. The MR measurements were started after the mouse's body temperature came up to 37°C. The mouse body temperature was kept at 37 ± 1°C during the MRI measurement with the warm water-cycling system. MRI measurements were performed at 4.7 T controlled with ParaVision[®] 3.0.1 (Bruker BioSpin MRI GmbH, Rheinstetten, Germany). Prior to the experiments, MSME based T₂ mapping was observed. SPGR (also referred as gradient echo fast imaging, GEFI) (TR = 75 ms, TE = 3 ms, FA = 45°, NEX = 3) was employed to observe T₁ effect. Axial slices were obtained with 128 × 128 image resolution, 3.2 × 3.2 cm FOV, 2 mm slice thickness. The scan time for one SPGR data set (which included 4 slices) was 30 s. Fifty data sets were repeatedly scanned (total scan time was 25 min). The 1.5 μmol/g b.w. 3CP was injected from tail vein cannulation 5 min after starting scan.

In vivo EPR spectroscopic measurement of the nitroxyl probe

Mice were anesthetized and placed on special mouse holder and fixed with adhesive tapes. The single loop surface coil (7.3 mm i.d.) was placed on the normal or the tumor-bearing leg. EPR signal was measured by a surface coil type resonator worked at 700 MHz CW EPR

[23]. The EPR measurement was started after the mouse body temperature reached to 36.5°C. The mouse body temperature was kept at $37 \pm 1^\circ\text{C}$ during the EPR measurement using an air heater. EPR conditions were as follows: microwave frequency = 700 MHz, microwave power = 10 mW, field modulation frequency = 13.5 kHz, field modulation amplitude = 0.3 Gauss, time constant = 0.03 s, sweep width = 60 Gauss, scan time = 16 s. The center line of the triplet was repeatedly monitored every 20 s for 20 min. Decay rates were obtained from the linear part from 7.5 min to end of measurement (20 min) by minimum square method. Tumor and normal legs were measured separately using individual mouse, where the “normal leg” means the normal leg of a tumor bearing mouse.

EPR imaging

Mice were anesthetized by isoflurane (1.5 %) in medical air (700 mL/min). The mouse was fixed on special mouse holder with adhesive tape and put in a Litz coil resonator (30 mm diameter and 50 mm length) [24]. The tail vein was cannulated for the injection of nitroxyl contrast agent. The mouse body temperature was kept at $37 \pm 1^\circ\text{C}$ during the EPR measurement using an air heater. Data acquisition was started immediately after the injection of the nitroxyl contrast agent (1.5 $\mu\text{mol/g}$ b.w.). EPRI data acquisition was carried out using home build 300 MHz CW EPR imager [25]. Coronal 2D images were obtained with 6×6 cm FOV. Twelve projections for each image were obtained every 1.9 min. Other EPR conditions were as follows; microwave frequency = 300 MHz, microwave power = 5 mW, field modulation frequency = 13.5 kHz, field modulation amplitude = 1.5 Gauss, time constant = 0.1 s, sweep width = 10 Gauss, scan time = 8 s, and the magnitude of the field gradient was 1.7 Gauss/cm. EPR image was reconstructed on 128×128 matrix by filtered back-projection (FBP) with Shepp-Logan filter.

Image handling and ROI selection

The MRI and EPRI data were analyzed using the ImageJ software package (a public domain Java image processing program inspired by NIH Image that can be extended by plug-ins, <http://rsb.info.nih.gov/ij/>). T_2 mapping was calculated using a plug-in (MRI analysis calculator, Karl Schmidt, HypX Laboratory, Brigham and Women's Hospital) available in ImageJ. ROI-TC, ROI-TP, ROI-TW, ROI-NC, ROI-NP, and ROI-NW were selected based on T_2 mapping for center of tumor, center of normal leg, periphery of tumor leg, and periphery of normal leg, respectively (Fig 2 lower panel). The peripheral ROIs were selected on the outer back side, because the EPR surface-coil was put on this side when the EPRS measurement was carried out. The peripheral ROI on the normal leg was put on the axisymmetric outer back side of the control leg. For the T_1 -weighted SPGR MRI data, percent-enhancement of the image intensity after administration of the nitroxyl contrast agent was calculated pixel-wise describe as previous paper [19]. Since the EPR image intensity was directly related to the levels of CmP, ROIs for tumor and normal legs were directly selected from the EPR image. Logarithmic values of averages in each selected ROI were plotted with time.

Results and Discussion

Figure 2 upper panel shows an MSME based anatomic image and a T_2 map calculated from the MSME echo train. The T_2 map (Fig. 2 lower panel) showed a clear difference between the tumor and normal tissues. The normal tissue showed shorter T_2 (0.041 ± 0.001 ms, $n = 4$), which was colored violet. The 9-day SCC tumor showed longer T_2 (0.074 ± 0.004 ms, $n = 4$), which was colored pink. Normal tissues were included in inner side of the tumor bearing leg. Therefore, the EPR surface-coil was put on the outer back side of the tumor bearing leg, when the EPRS measurement was carried out. The tumor region was almost flat at this size of tumor (1.3 cm), and the internal structures were not distinct from the T_2 map. Several ROIs were selected using the T_2 map as a guide as described in the methods section.

The nitroxyl contrast agent used in this study, i.e. CmP, is basically hydrophilic molecule with slight lipophilicity. The CmP molecule can not freely go through blood-brain barrier [26], while can be reduced enzymatically in the microsomal fraction [27]. Therefore, the signal from the lipophilic phase should decay faster due to enzymatic reduction. In addition, signal enhancement can be lower when the nitroxyl contrast agent distributes in the lipid phase [28]. For these reasons, most of MR signal may originate from water phase. If so, EPR signal, which can reflect a higher level of CmP from lipophilic phase, can decay faster. However the result showed slower CmP decay in EPR experiment, which may suggest a minimal level of CmP in lipophilic phase.

Comparison of T_1 -weighted SPGR images obtained before and after the administration of the CmP showed apparently recognizable signal increasing after the administration of the CmP (Fig. 3A). The %-signal increase after the CmP administration is proportional to the concentration of CmP (Fig. 3B). The CmP distributed in both legs immediately after the *i.v.* injection, followed by a gradual decrease. Although the distribution of CmP in the tumor region showed heterogeneity in this time period, varieties of distribution patterns in the tumor region were observed depending on the mouse. Relatively larger tumors often showed some lack of distribution as shown in Fig. 3B. On the other hand, relatively smaller tumors showed uniform distribution (data not shown). Decay rate mapping (Fig. 3C) was obtained from a pixel-wise time course of the %-signal increase. Faster decay rate in the tumor leg compared with the normal leg can be apparently obtained, although the distribution of decay rates in the tumor leg is heterogeneous. Figure 4 shows MRI based decay profiles of CmP obtained for several different ROIs. In all cases, profiles in the ROI on the tumor or tumor bearing tissue showed faster decay compared to the corresponding ROI in normal muscle.

The decay rates resulting from the reduction of CmP to its corresponding hydroxylamine were determined using EPR spectroscopy using a surface coil and EPR imaging with a volume coil. With EPRS using a surface coil, the sampled volume is restricted to the peripheral region where the coil is placed, whereas with EPRI using a volume coil, the whole tumor is probed. Typical decay profiles of CmP in the mouse tumor and normal muscle tissues showed a single phase decay after the intensity once reached maximum level. Figure 4 shows an example of EPRS based decay profiles of CmP obtained in normal muscle and in tumor tissues. A typical EPRI based decay profiles of CmP obtained in normal muscle and in tumor tissues in Figure 4. MRI based decay profiles also showed

similar patterns (Fig. 4). Decay constants were obtained from the linear decay part after the peak using by least square line fitting.

Table 1 summarizes the decay constants obtained by several ROI selections of MRI, EPRS, and EPRI. Decay constant in the tumor leg was faster than the normal leg in all experiments. The values from the peripheral-ROI and the central-ROI showed significant difference between the tumor and normal legs. The result of EPRS also showed significance. In the MRI experiment, center-ROI showed largest values, and peripheral-ROI showed smallest values. Decay constants from EPRS were slightly lower than the results from EPRI. Decay constants obtained from EPRS were at a similar level of that from peripheral-ROI of MRI. The values obtained by EPRI and values from whole-ROI of MRI experiments also showed similar trends.

The T_2 map can show a clear difference between tumor and the normal tissues, while the internal structure of the tumor tissue could not be discerned. Distribution pattern of CmP obtained from a %-change of SPGR image showed the internal structure in the tumor tissue clearly, while the normal and tumor tissues can not be distinguished only from this image. The region showing a lack of distribution of the CmP may have no blood supply, suggesting the tissue to be necrotic. A fusion of the %-change image and the T_2 map may help to delineate active tumor tissue.

The results from this study clearly showed the influence of ROI selection on the apparent decay constant in the target tissue. The relatively slow decay rate obtained by EPRS and EPRI studies can be attributed to the effect of slow decay rate from the peripheral region. The center part of tumor tissue showed significantly faster decay rate than the normal tissue. This result suggests that the center part of the tissue is apparently significantly hypoxic compared to the peripheral regions. However, the distribution of hypoxic area in the tumor may depend on the heterogeneous internal structure of the tumor tissue. Necrotic regions distributed in the tumor did not show any reduction rate due to low levels of the nitroxyl contrast agent, which may underestimate the decay rates in a ROI. Therefore, the decay rate mapping will be more convenient to compare individual tumor and can be an index to find active regions in the tumor tissue.

The decay profiles of MRI, EPRI, and/or EPRS signals observed in this study were reflecting redox response of CmP. CmP is basically hydrophilic with slight lipophilicity. As described by Matsumoto et al. [19], the concentration of total CmP molecule, i.e. oxidized and reduced form of CmP, was stable during 20 min experimental period in both tumor and normal muscle tissues. However, the hydrophilic nitroxyl contrast agent, such as carboxy-PROXYL, which is membrane-impermeable, showed washout pattern, i.e. clearance-depending decay profile [21]. Therefore, effect of site specific clearance on the differences of decay profiles of CmP may be minimal in this study.

Surface coil resonator is good for a localized detection, while the application is limited for the surface of an intact animal and/or a surgically exposed region. Since traditional EPRI can not achieve slice selection, all information distributing perpendicular to the slice were projected onto one slice and the decay rate information mixed up. Although the T_1 -weighted

signal change caused by a nitroxyl contrast agent is an indirect way to detect the nitroxyl radical, the slice selection technique and high image resolution achieved by MRI can make it possible to compare the *in vivo* decay rates of nitroxyl contrast agent among specific tissues/organs.

Conclusions

MRI based *in vivo* nitroxyl decay rates of a nitroxyl contrast agent CmP observed from a center ROI of the tissues were much faster than that from the EPRS and/or the EPRI. The under-estimation of the overall decay rate constants of CmP can be attributed to the relatively slow decay rate at peripheral region of the tissues also included with the tumor regions in with the surface coil in EPRS and the poor resolution in CW EPRI using projection reconstruction approaches. The results of this study strongly support the suitability of MR redox imaging with its superior spatial and temporal resolution, for reliable estimation of mapping reduction rates in the tissue.

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Figure 1.

Volume selection by several measurement techniques. (A) Surface coil resonator can detect EPR signals from limited surface region. (B) 2D EPRI technique can map a distribution of free radicals, while the 2D EPR image is equal to a transmission image, such as an X-ray radiograph. (C) MRI can detect NMR signals from a particular thin slice, and map a distribution of protons and/or accompanied relaxation information.

Figure 2.

ROI selection on the MRI. The upper panel is an MSME axial slice of mouse thigh measured with following conditions; the TR was 4000 ms, the TE was 15 ms, the FOV was 3.2 mm, the slice thickness was 2 mm. The lower panel is the T₂ map calculated from a MSME echo train, which took 16 TEs every 15 ms. The T₂ calculation used 8 data points from 45 ms to 150 ms, which region showed highest linearity. Based on the T₂ mapping as a scout image, six ROIs, i.e. ROI-TC, ROI-TP, ROI-TW, ROI-NC, ROI-NP, and ROI-NW, were selected for center of the tumor, periphery of the tumor, whole region of the tumor leg,

center of the normal leg, periphery of the normal leg, and whole region of the normal leg, respectively.

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Figure 3.

MRI based redox estimation using a nitroxyl contrast agent, CmP. (A) Raw SPGR images obtained before and 1.3 min after the injection of CmP (1.5 $\mu\text{mol/g}$ b.w., i.v.). A sequence of T_1 -weighted SPGR images was obtained every 30 s with following conditions; the TR was 75 ms, the TE was 3 ms, the FA was 45° , the FOV was 3.2 mm, the slice thickness was 2 mm. (B) Time course of %-signal changes before and after the administration of CmP were calculated from the sequence of T_1 -weighted SPGR images. (C) A decay rate mapping was calculated from a sequence of %-signal change images.

Figure 4.

Decay profiles of CmP estimated from MRI based redox imaging. Averaged decay profiles estimated for (A) center, (B) peripheral, and (C) whole ROIs. Black and gray circles indicate the time courses of MR signal intensities in normal and tumor tissues, respectively. Marks and error bars indicate average $\pm$ SD of 5 experiments. Least linear approximations were calculated using region indicating with closed marks.

Figure 5.

An example of EPRS based tissue redox estimation. Decay profiles of CmP in normal and tumor tissues were measured with a surface coil type resonator. Black and gray circles indicate the time courses of EPR intensities in normal and tumor tissues, respectively.

Figure 6. An example of EPR image of CmP distributed in mouse thighs and the decay profiles. (A) EPR image obtained 2 min after CmP injection. (B) EPRI based decay profiles of CmP in normal (black circles) and tumor (gray circles) tissues.

Table 1.

In vivo decay rates of a nitroxyl radical, carbamoyl-PROXYL, measured by EPRS, EPRI, or MRI.

	<i>n</i>	Normal leg Decay rate (min ⁻¹)	Tumor leg Decay rate (min ⁻¹)
MRI (center)	7	0.083 ± 0.021	0.105 ± 0.024 *
MRI (periphery)	7	0.059 ± 0.016	0.082 ± 0.008 **
MRI (whole)	7	0.077 ± 0.024	0.091 ± 0.008
EPRS	5 §	0.058 ± 0.005	0.077 ± 0.009 **
EPRI	3	0.075 ± 0.027	0.084 ± 0.026

Values are indicated as mean ± SD. *n* indicates number of animal.

§ Same number of animals were used for individual measurement of each normal and tumor leg.

* and ** indicate significances between the normal leg and the tumor leg by $p < 0.05$ and $p < 0.01$ respectively.