Probing the Intracellular Redox Status of Tumors with Magnetic Resonance Imaging and Redox-Sensitive Contrast Agents

Fuminori Hyodo, Ken-ichiro Matsumoto, Atsuko Matsumoto, James B. Mitchell, and Murali C. Krishna

Radiation Biology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland

Abstract

Nitroxide radicals are paramagnetic contrast agents, used in magnetic resonance imaging (MRI), that also exert antioxidant effects. Participating in cellular redox reactions, they lose their ability to provide contrast as a function of time after administration. In this study, the rate of contrast loss was correlated to the reducing power of the tissue or the “redox status.” The preferential reduction of nitroxides in tumors compared with normal tissue was observed by MRI. The influence of the structure of the nitroxide on the reduction rate was investigated by MRI using two cell-permeable nitroxides, 4-hydroxy-2,2,6,6,-tetramethyl-1-piperidynyloxyl (Tempol) and 3-carbamoyl-2,2,5,5-tetramethylpyrroolidine-1-oxyl (3CP), and one cell-impermeable nitroxide, 3-carboxy-2,2,5,5-tetramethylpyrroolidine-1-oxyl (3CxP). Pharmacokinetic images of these nitroxides in normal tissue, tumor, kidney, and artery regions in mice were simultaneously obtained using MRI. The decay of Tempol and 3CP in tumor tissue was significantly faster than in normal tissue. No significant change in the total nitroxide (oxidized + reduced forms) was noted from tissue extracts, suggesting that the loss in contrast as a function of time is a result of intracellular bioreduction. However, in the case of 3CxP (membrane impermeable), there was no difference in the reduction rates between normal and tumor tissue. The time course of T1 enhancement by 3CxP and the total amount of 3CxP (oxidized + reduced) in the femoral region showed similar pharmacokinetics. These results show that the differential bioreduction of cell-permeable nitroxides in tumor and normal tissue is supported by intracellular processes and the reduction rates are a means by which the intracellular redox status can be assessed noninvasively. (Cancer Res 2006; 66(20): 9921-8)

Introduction

Differences in the biochemical profile between benign and neoplastic tissue present important opportunities in diagnosis and prognostic evaluation (1, 2). Malignant cells exhibit several differences in the levels of endogenous molecules which reflect the differences in the metabolism and the energetics of a transformed cell (3, 4). Imaging techniques that can noninvasively distinguish these differences provide significant advantage for diagnosis, choice of treatment, and monitoring the efficacy of treatment (5, 6).

Cellular energy is derived from the transport of electrons through a series of oxidation reactions to inspired molecular oxygen, resulting in an overall reducing environment. Several redox couples exist in cells contributing to the overall redox status (7). GSH, NADH, and NADPH are a few examples of intracellular molecules, which, in coordination with the corresponding enzymatic cycles, collectively determine the global redox status of tissue (7). These redox couples are responsive to changes in oxidizing-reducing conditions which can result from changes in blood flow, oxygenation, and other variables (8, 9). Many of the biochemical mechanisms that underlie the observed metabolic differences between malignant and normal tissue are well understood, making it possible to profile tissues based on the relative levels of key metabolites (4).

Noninvasive imaging modalities capable of detecting the levels of individual molecules related to cellular metabolism can potentially provide diagnostic and prognostic information. Localized phosphorus-31 magnetic resonance spectroscopy (31P-MRS) has been successfully used to distinguish malignant tissue from benign tissue by monitoring levels of endogenous organic and inorganic phosphates indicative of the energetic status (3, 10). MRS imaging (MRSI) techniques, detecting 1H chemical shifts of endogenous metabolites being monitored may impose a limitation on the temporal and spatial resolutions.

In addition to biochemical differences from normal tissue, several, but not all, solid tumors exhibit hypoxic regions resulting from inadequate vasculature and/or compromised blood flow (11). These tumors, although exhibiting radiation resistance because of hypoxia, might be selectively sensitized by hypoxic cell cytotoxins (12). Thus, techniques that noninvasively distinguish biochemical/physiologic differences can provide a means to characterize malignant tissue with specificity and sensitivity. Radioactive tracers are also useful in providing functional/biochemical information in imaging modalities. The uptake and retention of the radiotracer 18-fluorodeoxyglucose in tumors is monitored by positron emission tomography imaging to differentiate benign lesions from malignant lesions (13, 14).

More recently, dynamic contrast–enhanced magnetic resonance imaging (MRI) is being successfully applied to identify tumors using T1 contrast agents such as Gd3+ complexes, which provide enhancement to MR images (15–17). This technique uses perfusion differences between benign and malignant tissue and therefore examines extracellular differences in tumor vasculature based on the characteristic time-intensity profiles (18). Because the contrast agent enhances the water/proton–based image intensity, temporal and spatial resolutions tend to be much better compared to MRSI. The image intensity monitored as a function of time with dynamic
contrast–enhanced MRI after injecting the contrast agent provides information of the perfusion and clearance of the contrast agent. However, Gd\(^{3+}\) is biologically "redox-inert" and any time-dependent loss in contrast is solely by clearance, which is an advantage in perfusion studies. Because of their size, the Gd\(^{3+}\) complexes are restricted to extracellular regions and do not provide contrast from intracellular regions.

Nitroxides are stable organic free radicals having a single unpaired electron and are therefore capable of providing image contrast via shortening T\(_1\) similar to Gd\(^{3+}\) complexes (19, 20). Although nitroxides compare unfavorably to conventional T\(_1\) contrast agents such as Gd\(^{3+}\) complexes in terms of relaxivity, being cell permeable, their volume distribution is significantly greater (21, 22). This partially compensates for their lower relaxivity compared with the cell-impermeable gadolinium complexes and provides useful T\(_1\) contrast enhancement per unit volume similar to unchelated Mn\(^{2+}\) (23).

Nitroxides were initially evaluated as potential contrast agents in MRI and were found suboptimal in the 1980s because of their lower molar relaxivity compared with Gd\(^{3+}\) (19). In addition, the rapid bioreduction of the paramagnetic nitroxide to the corresponding diamagnetic hydroxylamine compromised their utility as contrast agents (24). However, their propensity to undergo bioreduction might provide useful information pertaining to tissue redox status, provided that sufficient contrast enhancement is available initially (25).

Nitroxides are redox-active species which can be oxidized or reduced by the corresponding reactants in cells (26). Nitroxides were shown to undergo oxidation to the corresponding oxammonium by various oxidants such as hypervalent heme, H\(_2\)O\(_2\) or NO\(_2\) radicals (27–30). Thus, in tumors, low levels of oxidative stress may generate these species, which can decrease the nitroxide levels faster than in normal tissue. Oxammonium cations can be reduced to the nitroxide level by superoxide at diffusion-limited rates. Nitroxides can also be reduced to the corresponding hydroxylamines by reductants such as ascorbate, semiquinone radical, and also by intercepting reducing equivalents from the electron transport chain. The hydroxylamines, on the other hand, can be oxidized to the nitroxides in the presence of hydrogen peroxide and other oxidants such as transition metal complexes (31).

In cell culture experiments, nitroxides were found to undergo accelerated conversion to hydroxylamines under hypoxic conditions compared with normoxia (32). Furthermore, in normoxic cells, the conversion was significantly retarded when the thiol levels were depleted or in cells deficient in the enzyme thiol-6-phosphate dehydrogenase (9, 33). In in vivo studies, irrespective of whether nitroxide or hydroxylamine is administered, the ratio of the nitroxide/hydroxylamine is constant and depends on the redox status of the tissue (9, 34, 35). These observations suggest that nitroxides participate in cellular redox reactions and that their levels may be indicative of the global cellular redox status. Nitroxides also offer structural flexibility in that there are numerous derivatives available that can be directed to localize in specific subcellular compartments or be restricted to extracellular spaces (22, 36).

Earlier studies have shown that a cell-permeable nitroxide was reduced faster in tumor than in normal tissue (8, 9, 37). Therefore, monitoring the rate of transformation of suitable nitroxides to the corresponding diamagnetic species using MRI can provide in vivo assessment of redox status in a noninvasive manner. In this study, the structural determinants on the nitroxide ring influencing the reduction rates and their differences between tumor and normal tissue were investigated.

### Materials and Methods

#### Chemical

Carbamoyl-proxyl (3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl; C\(_3\)P); carboxy-proxyl (3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl; C\(_3\)CP); Tempol (4-hydroxy-2,2,6,6-tetramethyl-1-piperidinylxoyl) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Deionized water (deionization by the Milli-Q system) was used for all experiments. Other materials used were of analytic grade.

#### Animal Studies

Female C3H mice were supplied by the Frederick Cancer Research Center, Animal Production (Frederick, MD). The mice were 6 to 8 weeks of age at the time of experimentation, weighing 20 to 30 g, and were housed, five per cage, in climate-controlled, circadian rhythm–adjusted rooms, and were allowed food and water ad libitum. All in vivo experiments were 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. Tumor cells were injected s.c. as a single cell suspension of 10\(^6\) squamous cell carcinoma (SCCVII) cells in the right hind leg. The tumors grew to a diameter of 1.5 cm (1,800 mm\(^3\)) in 10 days, at which time, all imaging experiments were carried out.

#### MRI and Pulse Sequence

MRI measurements were done with a 4.7 T scanner controlled with ParaVision 3.0.1 (Bruker BioSpin MRI GmbH, Rheinstetten, Germany). For T\(_1\) mapping, spin echo images were obtained using a multi-slice multi-echo (MSME) sequence with a 10-echo train and an echo time of 15 ms. The scan time for a T\(_1\) mapping image set (\(N_{EX} = 1\)) by the MSME sequence was 10 minutes. Spooled gradient echo (SPGR) also referred to as gradient echo fast imaging: repetition time (TR), 75 ms; echo time (TE), 3 ms; flip angle (FA), 45 degrees; \(N_{EX}, 2\) was employed to observe the T\(_1\) effect. The scan time for six slices with the SPGR sequence was 20 seconds. Other common image variables are as follows: image resolution was 256 \times 256, field of view (FOV) was 7.0 \times 7.0 cm, slice thickness was 2.0 mm.

"Redox phantom". Five phantom tubes were prepared as shown in Fig. 1B. Tube (i) contained PBS in the absence of Tempol. Tubes (ii), (iii), (iv), and (v) contained 2 mmol/L of Tempol in the presence of 0, 0.5, 1, and 2 mmol/L of ascorbic acid (AsA), respectively, and were horizontally set in the resonator. After the addition of the AsA/PBS (pH 7.4) solution using a SP-10 catheter from a syringe, the MRI measurement with SPGR was immediately started and continuously measured for up to 10 minutes. The experiments were repeated four times with freshly prepared solutions. Semilogarithmic plots of the time course of MRI signal change in the region of interest (ROI, 30 \times 30 pixels) were used for decay rate calculation. The decay rates of tubes (iii), (iv), and (v) were calculated by using filled symbols (Fig. 1C). The MRI conditions used were as follows: TR, 75 ms; TE, 3 ms; FA, 45 degrees; \(N_{EX}, 2\); scan time, 20 seconds. Image resolution, 256 \times 256; FOV was 3.2 \times 3.2 cm; slice thickness was 2.0 mm.

To compare the decay rate between EPR and MRI, the phantoms including 3CP and 5 or 10 mmol/L of the AsA solution were prepared (\(n = 3\)). After adding the AsA solution, the scan was immediately started and continuously measured using both modalities. After data acquisition, decay rates were obtained. The MRI conditions used were identical to the above conditions. EPR data acquisition was carried out using a home-built 300 MHz CW EPR imager (38). A set of 12 projections was obtained every 1.9 minutes. Other EPR conditions were as follows: microwave frequency, 300 MHz; microwave power, 10 mW; field modulation frequency, 13.5 kHz; field modulation amplitude, 0.8; time constant, 0.01 seconds; sweep width, 10 G; scan time, 2 seconds; field gradient, 2.5 G/cm; and number of averages, 1. EPR images were reconstructed on a 128 \times 128 matrix by filtered back-projection with a Shepp-Logan filter. FOV was 4.0 \times 4.0 cm.
In vivo MRI Experiments

Mice were anesthetized by isoflurane (1.5%) in medical air (700 mL/min) and secured on a special holder by adhesive skin tape, stomach-side down. A breathing sensor (SA Instruments, Inc., Stony Brook, NY) was placed on the back of the mice. A nonmagnetic temperature probe (FISO, Quebec, Canada) was used to monitor the rectal temperature of the mice. The tail vein was cannulated for the injection of contrast agent. The mouse was then placed in the resonator, which was previously warmed up using a hot water cycling pad. The theresonator, which was previouslywarmed up using a hot water cyclingpad. The resonator unit, including the mouse, was placed in the magnet bore.

The MR measurements were started after the mouse’s core body temperature stabilized to 37°C. The mouse body temperature was kept at 37 ± 1°C during the experiment. Before taking the measurements with the nitroxide contrast agents, native images of the mouse were taken with the variables mentioned above. The middle two slices including normal tissue, tumor tissue, kidney, and artery area, were selected as reference for calculating signal enhancement in the presence of the nitroxide agents. T1-weighted images were acquired continuously for 20 minutes, using SPGR. A solution of nitroxide contrast agent in PBS (pH 7.4) was injected into the tail vein of C3H mice bearing SCCVII tumors at a dose of 1.5 μmol/g body weight. The concentration in the bolus was 150 μmol/L for 3CxP and 300 μmol/L for Tempol and 3CP (three animals each). The blood and organs weight. The concentration in the bolus was 150 μmol/L for 3CxP and 300 μmol/L for Tempol and 3CP (three animals each). The blood and organs were homogenized with 4-fold volumes of PBS. Ferricyanide (200 μmol/L solution) was added to each sample solution (800 μL). Ferricyanide quantitatively converts the hydroxylamine produced as a result of continuous irreversible one-electron reduction-oxidation showing the interconversion between the oxidized nitroxide (EPR active) and the corresponding reduced hydroxylamine (EPR inactive). B, continuous T1-weighted MR images of the Tempol phantom by reaction of AsA. Tube i, PBS; tube ii, 2 mmol/L of Tempol; tube iii, 2 mmol/L of Tempol and 0.5 mmol/L of AsA; tube iv, 2 mmol/L of Tempol and 1 mmol/L of AsA; tube v, 2 mmol/L of Tempol and 2 mmol/L of AsA. After the addition of the AsA/PBS solution using SP-10 tubes, the MRI measurement was started immediately and continuously measured for up to 10 minutes. C, the decay curves of Tempol with AsA as a function of concentration (0.5, 1, and 2 mmol/L). The experiments were repeated four times using freshly prepared solutions. Semilogarithmic plots of the time course of MRI signal change in the ROI (30 × 30 pixels) were used for decay rate calculation using ImageJ software. Semilogarithmic plots for time course of average MRI signal change in the ROI. Decay rate constants were obtained from the slope of linear portion of the decay curves (n = 4).

Figure 1. A, reversible one-electron reduction-oxidation showing the interconversion between the oxidized nitroxide (EPR active) and the corresponding reduced hydroxylamine (EPR inactive). B, continuous T1-weighted MR images of the Tempol phantom by reaction of AsA. Tube i, PBS; tube ii, 2 mmol/L of Tempol; tube iii, 2 mmol/L of Tempol and 0.5 mmol/L of AsA; tube iv, 2 mmol/L of Tempol and 1 mmol/L of AsA; tube v, 2 mmol/L of Tempol and 2 mmol/L of AsA. After the addition of the AsA/PBS solution using SP-10 tubes, the MRI measurement was started immediately and continuously measured for up to 10 minutes. C, the decay curves of Tempol with AsA as a function of concentration (0.5, 1, and 2 mmol/L). The experiments were repeated four times using freshly prepared solutions. Semilogarithmic plots of the time course of MRI signal change in the ROI (30 × 30 pixels) were used for decay rate calculation using ImageJ software. Semilogarithmic plots for time course of average MRI signal change in the ROI. Decay rate constants were obtained from the slope of linear portion of the decay curves (n = 4).

Table 1. Decay constant of 3CP with AsA by means of EPR spectroscopy, EPRI, and MRI

<table>
<thead>
<tr>
<th></th>
<th>AsA</th>
<th>EPRI (n = 3)</th>
<th>MRI (n = 3)</th>
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<tbody>
<tr>
<td>3CP</td>
<td>5 mmol/L</td>
<td>0.066 ± 0.004</td>
<td>0.072 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>10 mmol/L</td>
<td>0.136 ± 0.009</td>
<td>0.130 ± 0.006</td>
</tr>
<tr>
<td>Normal leg</td>
<td>0.08 ± 0.02</td>
<td>0.067 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>Tumor leg</td>
<td>0.09 ± 0.02*</td>
<td>0.097 ± 0.009*</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: The significant difference between EPRI and MRI was not observed by Student’s t test. In vivo data was referenced by Matsumoto et al. (25). Values are indicated as mean ± SD.

*Indicates significant differences between muscle and tumor (P < 0.05), when values were analyzed by paired t tests with two-tail comparison. The conditions of EPRI and MRI were follows: EPRI, microwave frequency, 300 MHz; microwave power, 10 mW; field modulation frequency, 13.5 kHz; field modulation amplitude, 0.8 G; time constant, 0.01 seconds; sweep width, 10 G; scan time, 2 seconds; field gradient, 2.5 G/cm; and number of averages, 1. EPRI images were reconstructed on a 128 × 128 matrix by filtered back-projection with a Shepp-Logan filter. FOV was 4.0 × 4.0 cm. MRI: TR, 75 ms; TE, 3 ms; FA, 45 degrees; NEX, 2; scan time, 20 seconds. Image resolution, 256 × 256; FOV was 3.2 × 3.2 cm, slice thickness was 2.0 mm (two slices).

The MR measurements were started after the mouse’s core body temperature stabilized to 37°C. The mouse body temperature was kept at 37 ± 1°C during the experiment. Before taking the measurements with the nitroxide contrast agents, native images of the mouse were taken with the variables mentioned above. The middle two slices including normal tissue, tumor tissue, kidney, and artery area, were selected as reference for calculating signal enhancement in the presence of the nitroxide agents. T1-weighted images were acquired continuously for 20 minutes, using SPGR. A solution of nitroxide contrast agent in PBS (1.5 μmol/g body weight) was injected via tail vein cannulation, 2 minutes after starting the scan.

Image Analysis

The MRI data were analyzed using the ImageJ software package. T2 maps were calculated using a plug-in (MRI analysis calculator, Karl Schmidt, HypX Laboratory, Brigham and Women’s Hospital) available in ImageJ.

Total Tissue Nitroxide Concentration Assessment

Nitroxide contrast agents dissolved in PBS (pH 7.4) were injected into the tail vein of C3H mice bearing SCCVII tumors at a dose of 1.5 μmol/g body weight. The concentration in the bolus was 150 mmol/L for 3CP and 300 mmol/L for Tempol and 3CP (three animals each). The blood and organs were collected and wet weight was determined at different times (2.5, 5, 7.5, 10, 15, and 20 minutes). Blood samples were diluted and tissue samples were homogenized with 4-fold volumes of PBS. Ferricyanide (200 μL of 10 mmol/L solution) was added to each sample solution (800 μL) to obtain the final concentration of 2 mmol/L. The ferricyanide quantitatively converts the hydroxylamine produced as a result of in vivo reduction back to the oxidized form (27–29). The signal intensities of the 100 μL samples were measured using a Varian E-9 X-band EPR spectrometer. The EPR spectrometer operating conditions were: modulation frequency, 100 kHz; microwave power, 1 mW. Because there is linearity up to 2 mmol/L between the concentration and EPR signal magnitude of nitroxide contrast agents (data not shown), EPR signal magnitudes of homogenate mixtures were converted to the concentration using previously obtained standard curves.

1 https://rsb.info.nih.gov/ij/.

www.aacrjournals.org 9923 Cancer Res 2006; 66: (20). October 15, 2006
(100 μmol/L-2 mmol/L). Finally, nitroxide concentrations in the tissues were calculated based on a dilution factor of 4.

Statistical Analyses
The statistical differences were estimated with the TTEST function in Microsoft Excel XP. The suitable "type" and "tail" for the test was selected according to the correspondence and variance of the data.SIGNIFICANCES were estimated at \( P < 0.05 \).

Results
The two cell-permeable nitroxides, Tempol and 3CP, exhibit amphipathic behavior, whereas the cell-impermeable nitroxide, 3CxP, exhibits minimal lipophilicity. The relaxivity differences were minimal between the three nitroxides and were \( \sim 0.2 \) mmol/L \( \cdot \) s \( \cdot \) M \( ^{-1} \).

Phantom experiments were carried out to examine the capability of Tempol to participate in redox reactions and elicit time-dependent changes in image contrast using AsA as a model reductant, which can convert the paramagnetic nitroxide molecule to the corresponding diamagnetic hydroxylamine (Fig. 1A). Figure 1B (left) shows the schematics of the five-tube phantom used. Tube (i) is a PBS solution with no additives. Tubes (ii), (iii), (iv), and (v) contained 2 mmol/L of Tempol in the presence of 0, 0.5, 1, and 2 mmol/L AsA, respectively. \( T_1 \)-weighted MRI images were collected using the SPGR sequence at different times after the reaction was initiated. As expected, no enhancement in intensity was observed in tube (i), in which Tempol was not included. In tube (ii), which contained 2 mmol/L of Tempol, a significant enhancement in image intensity was noted, which did not change with time, consistent with the notion that no reduction reaction takes place in the absence of AsA. However, in tubes (iii), (iv), and (v), which contained AsA and Tempol, a time-dependent loss in image intensity was noticed. The rate at which the intensity decreased in these tubes was dependent on AsA concentration. Tube (v), which contained Tempol in the presence of 2 mmol/L of AsA, exhibited a loss in intensity characterized by a rate constant of \( k = 0.33 \pm 0.03 \) min \( ^{-1} \), whereas tubes (iii) and (iv), which had 0.5 or 1 mmol/L of AsA, exhibited a reduction constant rate of \( k = 0.07 \pm 0.02 \) min \( ^{-1} \) and \( k = 0.18 \pm 0.03 \) min \( ^{-1} \), respectively, suggesting that the reduction rate was directly dependent on the concentration of the reductant (Fig. 1C). To compare the decay rate between EPRI and MRL, 3CP and AsA (5 and 10 mmol/L) phantoms were prepared. The decay rate constants, obtained using both modalities, were similar (5 mmol/L of AsA: EPRI,

![Figure 2. Comparison of pharmacokinetic/redox images (slice 1) of three nitroxide contrast agents by SPGR MRI. Sixty serial images of Tempol (A), 3CP (B), and 3CxP (C) were obtained during 20 minutes of continuous imaging. Slice 1, normal and tumor tissue area. Enhanced image intensity (%) against preinjection image by \( T_1 \)-weighted MRI (green). D, for \( T_2 \) mapping, spin echo images were obtained using a MSME sequence with eight echo trains and 15-ms echo times.](cancerres.aacrjournals.org)
The pharmacokinetic MR images of the three nitroxides were simultaneously examined in normal tissue, tumor tissue, kidney, and artery area utilizing SPGR to acquire six slices every 20 seconds. Two middle slices were considered because they could be used to visualize the target organs (normal tissue, tumor tissue, kidney, and blood). Figure 2 shows slice 1 containing normal tissue and tumor tissue at several time points after the injection of three different nitroxide analogues. The two cell-permeable nitroxides, Tempol and 3CP, exhibited distinct differences in the decay rates of image intensity between normal and tumor tissue. In the case of Tempol, the decay rate was approximately 3.5 times that observed in the normal tissue. For 3CP, the decay rate in the tumor was approximately 2 times higher than in the normal tissue. However, in the case of the cell-impermeable nitroxide, 3CxP, no significant differences in signal decay rates between tumor and muscle tissue were detected. When the signal decay rates of the three nitroxides in the normal leg were compared, they ranked, from fastest to slowest (Tempol > 3CP > 3CxP). The levels of nitrooxide + hydroxylamine as a function of time were as follows: TR, 75 ms; TE, 3 ms; FA, 45 degrees; cannulation, 2 minutes after starting the scan. The MRI conditions during a scan time of 20 minutes, using SPGR. A solution of nitroxide contrast agents in PBS (1.5 mol/L of AsA: EPRI, 0.136 ± 0.009 min⁻¹; MRI, 0.130 ± 0.006 min⁻¹) and changed as a function of AsA concentration (Table 1). These results show that MRI decay rates can validly extract the reduction rates of 3CP to hydroxylamine as well as EPRI. The in vivo data with 3CP using MRI also showed that MR signal change between normal and tumor tissues was similar to EPRI (Table 1; ref. 25).

The pharmacokinetic MR images of the three nitroxides were simultaneously examined in normal tissue, tumor tissue, kidney, and artery area using SPGR to acquire six slices every 20 seconds. Two middle slices were considered because they could be used to visualize the target organs (normal tissue, tumor tissue, kidney, and blood). Figure 2 shows slice 1 containing normal tissue and tumor tissue at several time points after the injection of three different nitroxide radicals. As can be seen, a time-dependent increase in image intensity, indicating the accumulation of these agents, was observed. However, each individual nitroxide exhibited different times to reach a peak value in intensity enhancement, as well as temporally different patterns of tumor enhancement. In the case of Tempol, a modest enhancement in intensity in all the tissues was observed as early as 30 seconds after injection, followed by a rapid loss in enhancement, and after 2.8 minutes, those changes were no longer observed (Figs. 2A and 3A). Interestingly, the area of signal increase in tumor tissue displayed an inhomogeneous pattern. However, in the case of 3CP, the contrast in tumor tissue increased until 2.8 minutes after injection, and its distribution was homogeneous. A decrease in image intensity was observed after 7.8 minutes, in contrast to Tempol, in which the intensity decrease was noticed at earlier times. The images in the kidney and artery area also showed enhancement, which decreased as a function of time. On the other hand, when the cell-impermeable 3CxP was injected, the accumulation in tissues was very slow and the enhancement was high compared with other nitroxides. The maximum intensity in tumor area was observed at 9.3 minutes after injecting 3CxP. Figure 3 shows slice 2, containing kidney and artery areas at time points corresponding to those in Fig. 2. The kidney area showed continuous enhancement up to 17.8 minutes.

Figure 2D shows an example of a T2 map generated from slice 1, containing muscle and tumor tissue. To monitor the reduction rates of the three nitroxides, a single ROI was selected on the T2 areas at time points corresponding to those in Fig. 2. The kidney area also showed continuous enhancement up to 17.8 minutes.

**Table 2. Decay rate of nitroxide contrast agents in various tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Decay rate (min⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>Tempol</td>
</tr>
<tr>
<td>Normal leg</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>Tumor leg</td>
<td>1.1 ± 0.2*</td>
</tr>
<tr>
<td>Blood</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Left kidney</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Right kidney</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>

NOTE: Values are indicated as means ± SD. After each nitroxide injection, three mice (total, nine mice) were measured and six slices were obtained from each mouse for serial images (total, 360 images) during a scan time of 20 minutes. T1-weighted images (six slices and 60 continuous imaging; total, 360 images) were acquired during the scan time of 20 minutes, using SPGR. A solution of nitroxide contrast agents in PBS (1.5 μmol/g body weight) was injected via tail vein cannulation, 2 minutes after starting the scan. The MRI conditions were as follows: TR, 75 ms; TE, 3 ms; FA, 45 degrees; NEX, 2; scan time, 20 seconds. Image resolution, 256 × 256; FOV was 32 × 32 cm, slice thickness was 2 mm (two slices).

*P < 0.01, significant difference between the muscle and tumor.

1P < 0.05, significant difference between the muscle and tumor.
after i.v. administration of the three nitroxides were examined by analyzing the tissue extracts after oxidation with ferricyanide using EPR spectroscopy. The results of EPR analysis are summarized in Fig. 4. Although Figs. 2 and 3 show that MR image intensity decays in both tumor and normal muscle, the total (nitroxide + hydroxylamine) does not change significantly during the imaging time in either tumor or muscle. Furthermore, similar patterns were observed in blood and kidney levels in which the MR intensity exhibited a rapid decay as a function of time, whereas the total levels remained unchanged. It was suggested that the decrease in image intensity observed in T1-weighted imaging is associated with the bioreduction of nitroxides predominantly in the imaging time windows. Furthermore, the contributions of clearance of nitroxide to the observed intensity decay rates were minimal. Intracellular reducing equivalents and electrons intercepted from the mitochondrial electron transport chain were proposed as the mechanisms which underlie the reduction of nitroxides (32, 39).

Discussion

Tumors and normal tissue may differ in several physiologic properties including oxygen status, reducing capability, pH, interstitial pressure, etc. (11). These differences can, at least in part, be attributed to the compromised blood flow and leaky vasculature in tumors (12). Furthermore, differences between intracellular and extracellular pH may have a profound influence on the uptake of exogenous molecules including chemotherapy drugs and intracellular contrast agents, such as the nitroxide radicals, by providing conditions favoring the uptake of weakly acidic drugs (40). Treatment strategies that exploit such differences, such as hypoxic cell cytotoxins, are being explored (41). Imaging modalities that can detect such subtle differences will aid in the development of appropriate treatment strategies (5).

MRI-based techniques such as MRS and MRSI can detect levels of endogenous metabolites that reflect the tissue redox status (1). These techniques, which have been shown to be of significant diagnostic value, were used to successfully monitor treatment progress (5, 42). However, the low levels of the metabolites impose limitations on the resolution (temporal and spatial) achievable with MRS and MRSI. Paramagnetic contrast agents in MRI provide information on tumor perfusion (16). However, clinically used contrast agents are extracellular, do not participate in redox reactions, and are therefore insensitive to tissue redox status.

Nitroxides have a combination of unique properties that make them promising candidates as redox status–sensitive MRI contrast agents. The paramagnetic nitroxide radicals can undergo reduction to the corresponding diamagnetic hydroxylamine, which can revert to the nitroxide in the presence of oxidants or if the cellular oxygen status permits (27, 31). The nitroxide reduction is supported by intracellular enzymatic processes, and is more efficient in hypoxic cells.
than in aerobic cells (43, 44). They are also effective antioxidants, which offer protection to cells in culture and were used successfully in animals exposed to diverse types of oxidative insult (26). Earlier studies established the feasibility of using MRI to monitor the spatial distribution of nitroxides and their redox transformations in vivo with high spatial and temporal resolution (25).

Several studies showed that thiol-dependent processes contribute, at least in part, to \textit{in vivo} nitroxide reduction (9, 45). Earlier studies using EPR spectroscopy in cellular incubations of nitroxides, the cell-permeable nitroxides, Tempol and 3CP, were found to accumulate intracellularly, whereas 3CxP was not detectable from intracellular regions even after prolonged incubations. However, anionic transport pathways for 3CxP cannot be ruled out. Additionally, the accumulation of nitroxides in extracellular spaces as a result of leaky vasculature may make the clearance pathways of nitroxides less efficient. In this study, an established EPR method of invasively detecting total nitroxide/hydroxylamine levels was used to show that SPGR MRI signal intensity loss, after nitroxide injection, is not due to washout. The combination of the nitroxide contrast agent and SPGR MRI studies provided us with pharmacokinetic information for various tissues. The availability of anatomic information, as well as the superior spatial and temporal resolution, make MRI more efficient at monitoring the pharmacokinetic properties of nitroxides compared with EPR imaging methods.

The MRI studies clearly show that among the nitroxides tested, the six-membered piperidine nitroxide, Tempol, was reduced rapidly compared with the two five-membered pyrrolidine nitroxides, 3CP and 3CxP. Tempol and 3CP (both cell permeable) were reduced in tumors more rapidly than in normal muscle. However, the cell-permeable nitroxide, 3CxP, exhibited no difference between tumor and normal tissue. To determine the contribution of clearance processes to the time-dependent signal intensity loss in MRI experiments, \textit{ex vivo} EPR analyses of tissues from an independent set of animals were done. EPR studies show that during the scan time of 20 minutes, the total (nitroxide + hydroxylamine) levels remained unchanged, suggesting that clearance is not a major contributor to the observed time-dependent intensity change. Importantly, the data further indicates that the observed time-dependent disappearance of MR image intensity enhancement due to Tempol and 3CP (both cell permeable) in various tissues occurred as a result of intracellular reduction.

**Conclusion**

In this study, we have shown that the pharmacokinetics of nitroxide contrast agents and high-quality anatomic structure using SPGR MRI could be simultaneously acquired. Such information could be extremely useful to noninvasively monitor the intracellular redox status in various tissues, and to outline the redox differences between normal and tumor tissue. Nitroxide contrast agents such as Tempol and 3CP show significant differences in decay rates between normal and tumor tissues depending on the ability of nitroxides to cross the cellular membrane. The decay rate of Tempol in tumor tissue was fastest among the three nitroxide contrast agents compared with normal tissue. However, there was no difference between normal and tumor tissues in the case of 3CxP. The data from the current study fully supports the use of cell-permeable nitroxides coupled with MRI to noninvasively examine differences in the redox status of tissues. Although, in the present study, particular emphasis was placed on differences in the redox status in tumors compared with selected normal tissues, the technique possesses the potential to have broad applications to the study of other disease states, inflammatory processes, and other circumstances in which oxidative stress is implicated.

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**References**


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Fuminori Hyodo, Ken-ichiro Matsumoto, Atsuko Matsumoto, et al.


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