

NMR AND MRI SPIN TRAPPING: USING NMR TO LEARN ABOUT FREE RADICAL REACTIONS

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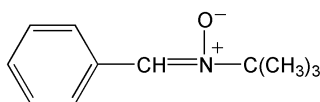
The paper supports the idea that NMR spectroscopy and imaging can enhance the classical EPR spin trapping techniques. NMR was used to follow and identify the diamagnetic products of DEPMPO/R• spin adduct breakdown. Oxyradical adducts of DEPMPO led to recycling of the spin trap, however, without sufficient long-term accumulation that would enhance EPR detection *in vivo*. Fluorinated derivative of the same spin trap, FDMPO, although not specific for •OH or •O₂⁻, was giving much more stable EPR signal. High sensitivity of H-MRI, allowing to distinguish tissue boundaries very clearly, could be coupled with proton relaxation enhancing capabilities of paramagnetic spin traps to better visualize free radicals *in vivo*. This approach can be exemplified by the image of NO generation in the liver of a living rat. In general, NMR-, MRI-, and EPR spin trapping appear excellent complementary methods to follow localization of short-living radicals *in vivo*, under variable physiological conditions causing biodestruction of trapped radicals.

INTRODUCTION

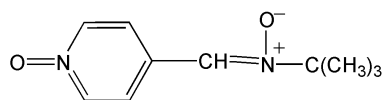
Spin traps were developed in order to accumulate (“trap”) highly reactive, primary radicals which cannot otherwise be observed directly. The ultimate goal in biomedical research is to detect critical bioradicals *in vivo*. Two key oxyradicals, •OH and •O₂⁻ are very short lived and may only be directly observed by fast freeze quenching at liquid nitrogen or lower temperatures. Certainly the feasibility of quickly immersing a mouse or a rat into a cryogen compromises a truly *in vivo* experiment! Many of the applications, successes and pitfalls of *in vivo* spin trapping have been recently reviewed (Mason & Kadiiska, 2001; Timmins & Liu, 2001).

The most popular, commercially available spin traps are nitrones, several of which are enumerated in Figure 1. The most recent class, typified by DEPMPO (diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) exhibit unique specificity in the spin trap adduct EPR spectra with substantial improvement in stability (Frejaville, Karoui, Tuccio, Le Moigne, Culcasi, Pietri, Lauricella & Tordo, 1995; Roubaud, Sankarapandi, Kappusamy, Tordo & Zweier, 1997). Despite these improvements, the *in vivo* “stability” of paramagnetic spin trap adducts is sharply reduced, probably due to biological reduction of the resulting nitroxide to the hy-

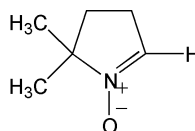
phenyl-tert-butyl nitron (PBN)



α-4-pyridyl-1-oxide N-tert-butyl nitron (4-POBN)



5,5-dimethylpyrroline N-oxide (DMPO)



5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO)

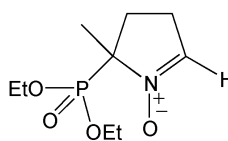
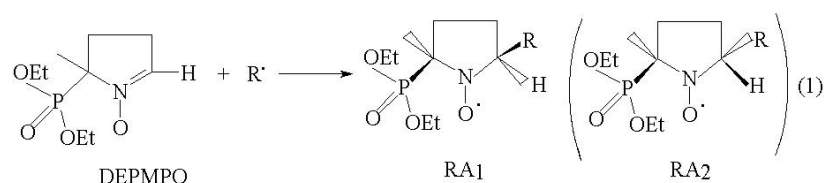
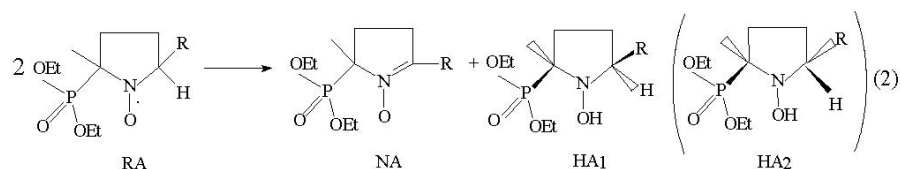


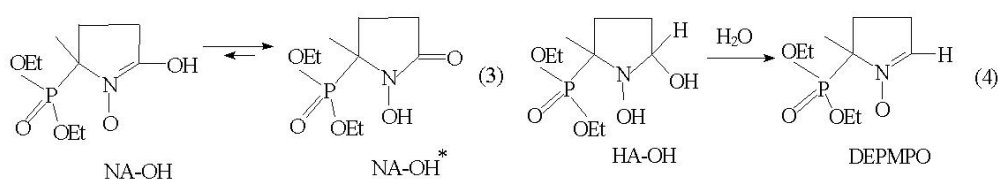
Fig. 1. Structures of nitron spin traps.



Scheme A



Scheme B



Scheme C

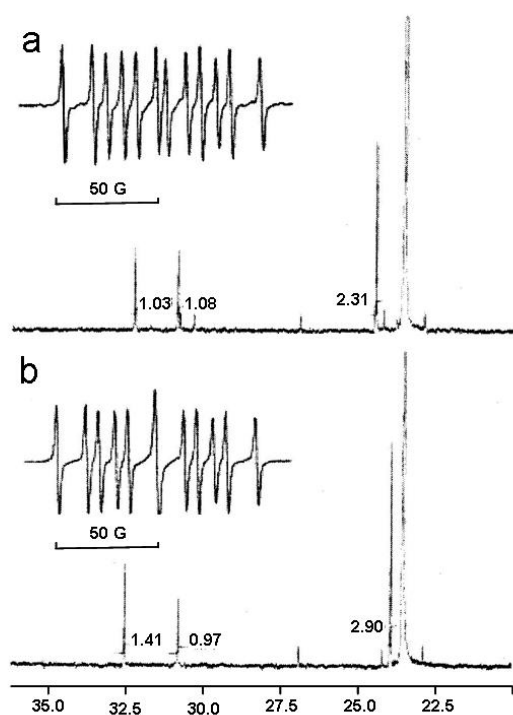


Fig. 2. ^{31}P -NMR spectra of 0.1 M DEPMPPO in 0.1 M cacodylate buffer, 20 mM DTPA, pH 7.0, 10% DMSO, detected 2 h after addition of 5 mM FeSO_4 and 5 mM H_2O_2 at $T=300$ K (a); the same but in the presence of 10 % MeOH instead of DMSO (b). The spectrometer settings were: frequency 121.5 MHz (^{31}P); number of scans 512; acquisition time 1.69 s. Inset: EPR spectra observed 10 min after addition of 5 mM FeSO_4 and 5 mM H_2O_2 in the presence of DMSO (Fig. 2a) or MeOH (Fig. 2b). Spectrometer settings were as follows: microwave power 20 mW, modulation amplitude 0.8 G. Experimental spectra are found in a good agreement with calculated ones with parameters $a_{\text{N}} = 14.56$ G, $a_{\text{H}} = 21.8$ G, $a_{\text{P}} = 46.95$ G for DEPMPPO/ $\cdot\text{CH}_3$ and $a_{\text{N}} = 14.5$ G, $a_{\text{H}} = 20.7$ G, $a_{\text{P}} = 49.95$ for DEPMPPO/ $\cdot\text{CH}_2\text{OH}$ adducts, which are in agreement with literature data (Frejaville *et al.* 1995). From Khramtsov *et al.* (1999) with permission.

droxylamine, but also by other destructive chemical processes.

NMR SPIN TRAPPING

Several years ago Selinsky, Levy, Motten and London (1989) suggested the use of ^{19}F NMR to

study free radical reactions with fluorinated spin traps. They were able to monitor organic free radical reactions in the test tube by both EPR and NMR. The extrapolation of this approach, however, to *in vivo* biological systems was complicated by both the absolute free radical levels *in vivo* as well as other limitations noted above. Nonetheless, recent reports have appeared with

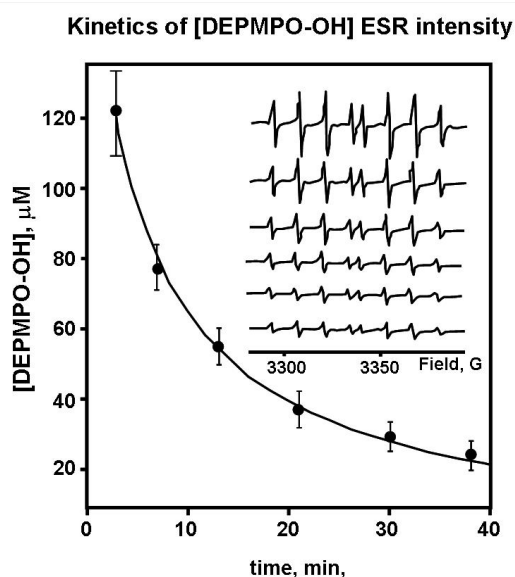


Fig. 3 Decay of DEPMPO/ \cdot OH spin adduct formed in the presence of 0.1 M DEPMPO in a Fenton system (0.2 mM FeSO_4 and 0.2 mM H_2O_2 , see text for details). Inset: Time-evolution of X-band EPR spectrum. The spectrometer settings were: microwave power 20 mW; modulation amplitude 0.8 G; sweep time 1 min. The solid line is a non-linear least-squares fit of the data to the equation of a bimolecular decay of the spin adduct, SA ($[\text{SA}] = [\text{SA}]_0 / (k_d t [\text{SA}]_0 + 1)$) giving $k_d = (15.8 \pm 1.5) \text{ M}^{-1} \text{ s}^{-1}$; initial concentration of spin adduct $[\text{SA}]_0 = 0.173 \text{ } \mu\text{M}$.). From Khramtsov *et al.* (1999) with permission.

some biological spin traps using 31-P NMR and 19-F detection (Berliner, Khramtsov, Fujii, & Clanton, 2001a; Khramtsov, Berliner & Clanton, 1999; Khramtsov, Reznikov, Berliner, Litkin, Grigor'ev & Clanton, 2001b).

The modus operandi of this approach is based on the following questions:

- i. Do the diamagnetic (decomposition or transformed) products of a spin trap adduct accumulate to sufficient levels for detection by NMR?
- ii. Does the resultant NMR spectrum allow extrapolation back to a specific radical adduct?

DEPMPO

The generic reaction of DEPMPO with a radical, $\text{R}\cdot$ is shown in Scheme A, elucidating the fact that two stereoisomers are obtained as the result of addition of a radical moiety to an asymmetric center, which should be reflected in two pairs of EPR spectra (shown in Figure 2) such as the EPR spectra of the methyl (Figure 2a) and hydroxymethyl (Figure 2b) radical adducts of DEPMPO, respectively. Within two hours the radical signal

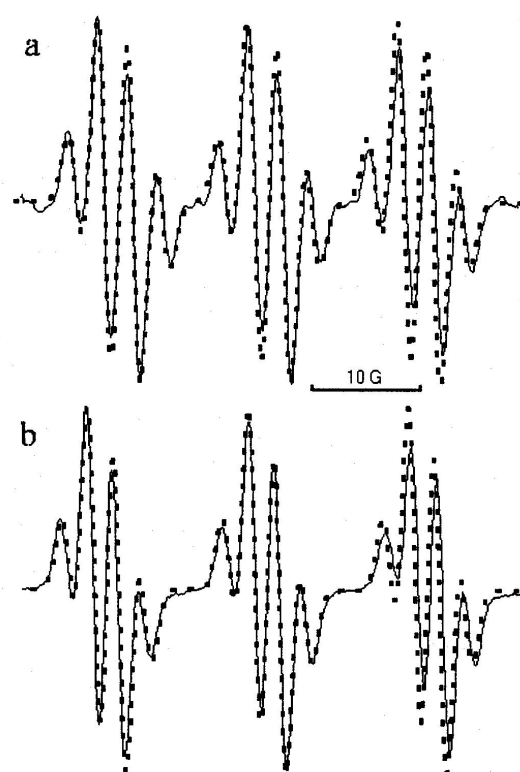


Fig. 4 X-band ESR spectra of FDMPO spin adducts with \cdot OH (a) and \cdot CH₂OH (b). Spectra were obtained in 0.1 M K-phosphate buffer, pH 7.0, 2 mM DTPA, 0.1 mM H_2O_2 , 50 mM FDMPO, after addition of 0.1 mM FeSO_4 solution, and in 10% (v/v) methanol (b). Spectrometer settings were: microwave power 20 mW; modulation amplitude, 0.63 G; sweep time, 1 min. The dotted lines are calculated spectra. From Khramtsov *et al.* (2001b) with permission.

decays away, leaving the 31-P NMR spectrum depicted in the insets in Figure 2, which gave evidence of e.g., several new, unique peaks at 24.54 ppm with additional peaks at 30.83 and 32.52 ppm. A very small DEPMPO/ \cdot OH product resonance at 27.05 ppm also occurred since the radicals were produced by a Fenton \cdot OH generating system (Khramtsov, Berliner & Clanton, 1999).

When considering the origin of these new peaks one must also include the bimolecular disproportionation chemistry outlined in reaction in (2) Scheme B. Note that both products of this disproportionation reaction were diamagnetic (ie., the new nitrene, NA) and the hydroxylamine adducts, HA1 and HA2. Consequently, one should obtain three peaks, one of which is 50% total intensity and two of which are 25% each, depending upon the stereospecificity of radical addition. While one would not expect an appreciable contribution of disproportionation *in vivo*, direct reduction of the

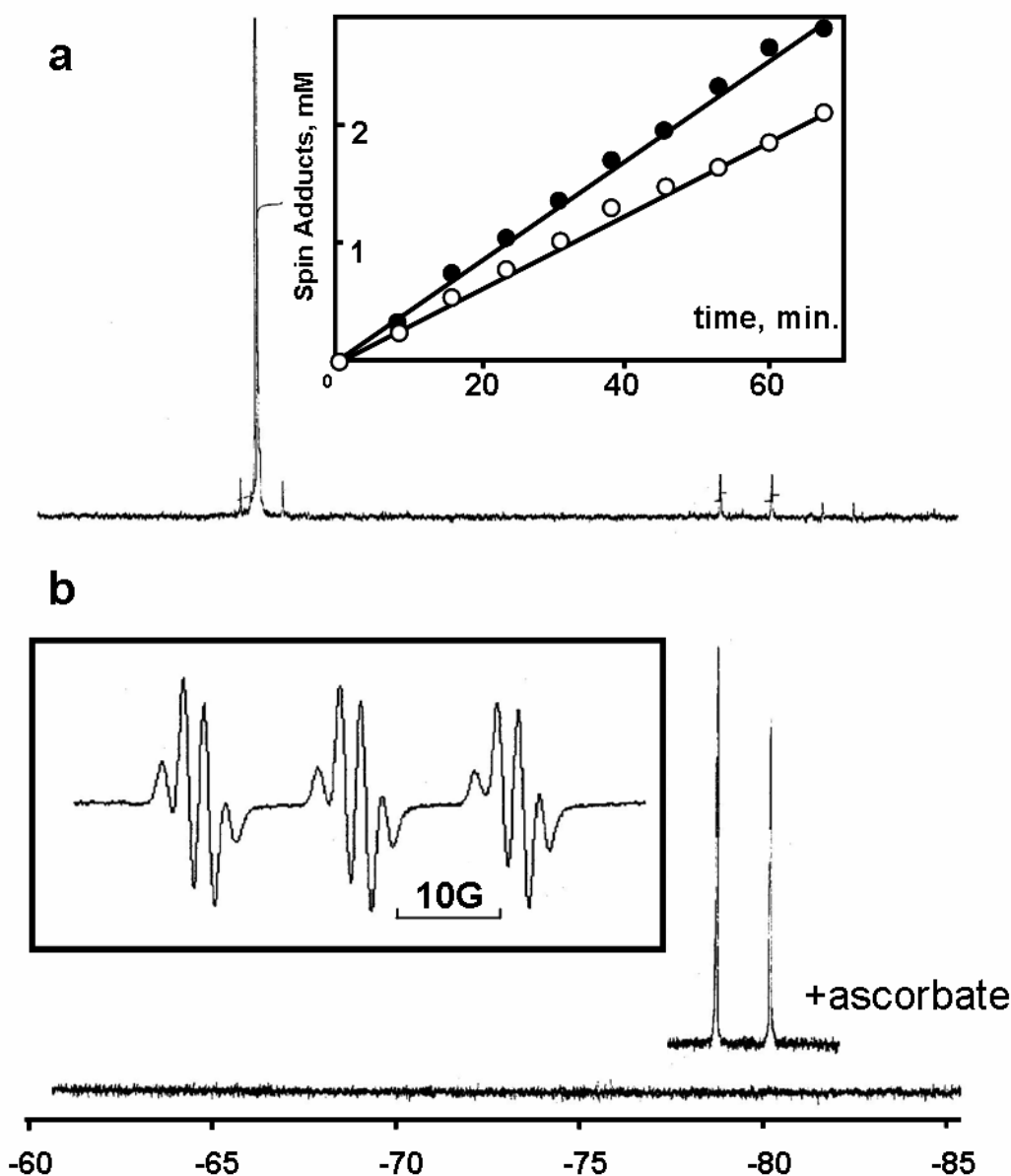


Fig. 5 ^{19}F -NMR spectra of FDPMPPO/ $\bullet\text{CH}_3$ adduct products in a Fenton "iron recycling" system in 0.1 M K-phosphate buffer, pH 7.0, 2 mM DTPA, 20 mM H_2O_2 , 50 mM FDPMPPO, 10% v/v of DMSO, 20 mM of ascorbate, 30 min after addition of 0.5 mM FeSO_4 solution; number of scans 208; Inset: kinetics of product accumulation. The symbols (●) and (○) denote integral intensities of the ^{19}P -NMR peaks of reduced FDPMPPO/ $\bullet\text{CH}_3$ products at -78.6 ppm and -80.0 ppm, respectively; (b) ^{19}F -NMR spectra of 3 mM of FDPMPPO/ $\bullet\text{CH}_3$, synthesized in Scheme 2, in 0.1 M phosphate buffer, pH 7.0, before and after addition of 10 mM ascorbate; number of scans 120; Inset: EPR spectrum of 0.1 mM FDPMPPO/ $\bullet\text{CH}_3$ adduct; spectrometer settings were as in Figure 4. From Khramtsov *et al.* (2001b) with permission.

DEPMPPO adduct to the hydroxylamines HA1 and HA2 is highly facile, and hence, likely. In fact, when ascorbic acid was included in the radical generation system only direct conversion was observed to the hydroxylamine adducts, HA1 and HA2, as shown in Scheme B. Consequently the two new 31-P lines at 32.31 and 30.83 ppm are a direct "history" of spin trapping and subsequent

formation and disproportionation of the methyl radical adduct of DEPMPPO.

Oxyradical adducts are much less stable

Figure 3 depicts the intensity of the DEPMPPO/ $\bullet\text{OH}$ EPR adduct spectrum with time. This bimolecular decay reaction is relatively rapid and the adduct remains for a few minutes unless drastically high steady state levels of radical pro-

duction occurs simultaneously (Liu, Miyake, Panz & Swartz, 1999a; Liu, Bechara, Kotake & Swartz, 1999b). An example ^{31}P NMR spectrum is shown in Figure 4, where a key observation is noted: the maximum intensity of the new ^{31}P line at 27.05 ppm never exceeds 50% of the theoretical yield of the diamagnetic product from an $\bullet\text{OH}$ adduct. Reactions (3) and (4) in Scheme C, may explain this observation. Reaction (3) shows that the nitron product of disproportionation (in Scheme B) is in tautomeric equilibrium with the more stable N-hydroxypyrrolidone, NA-OH, to which we have attributed the ^{31}P NMR line at 27.05 ppm. Remarkably, the hydroxylamine of the DEPMPPO/ $\bullet\text{OH}$ adduct can spontaneously eliminate H_2O yielding the starting material DEPMPPO! Hence only 50% of the products of the disproportionation reaction (Scheme B) remain. Overall, these results compare qualitatively with parallel EPR measurements in Figure 3. The prospects for efficient *in vivo* detection of $\bullet\text{OH}$ radical is pessimistic since the hydroxylamine product(s) of the DEPMPPO/ $\bullet\text{OH}$ adduct are favored, which eliminate H_2O yielding no EPR nor new NMR peaks. Similar results were found with superoxide radical. In fact the ^{31}P NMR peaks were identical to that observed with $\bullet\text{OH}$ suggesting conversion of DEPMPPO/ $\bullet\text{O}_2^-$ to DEPMPPO/ $\bullet\text{OH}$ or an analogous transformation of their diamagnetic product(s) occurred. Consequently, it appears that DEPMPPO can be recycled after reaction with $\bullet\text{OH}$ or $\bullet\text{O}_2^-$ without sufficient long-term accumulation for detection by either EPR or NMR. Hence, the search for nitron spin traps with more stable, highly sensitive $\bullet\text{OH}$ or $\bullet\text{O}_2^-$ products are desirable.

Fluorinated DEPMPPO

On the other hand, nitrones that incorporate strategically placed trifluoromethyl groups might be more promising. Figure 5 depicts the synthesis of a new spin trap, FDMPO (4-hydroxy-5,5-dimethyl-2-trifluoromethylpyrroline-1-oxide). The parent nitron yields a single NMR resonance at -66.0 ppm, which gives a signal to noise ratio of 10.4 compared with the ^{31}P NMR sensitivity for DEPMPPO. Figure 6 shows simulated and observed EPR spectra for the $\bullet\text{OH}$ and $\bullet\text{CH}_2\text{OH}$ adducts, respectively (Khramtsov *et al.* 2001b). The EPR signals were stable for over a day since bimolecular decay (as noted in Scheme B) is much less probable with the absence of an alpha carbon. Overall the reactivity of FDMPO was comparable to that of DMPO as measured by competitive kinetics. In order to observe the products by ^{19}F NMR, it was necessary to reduce the paramagnetic

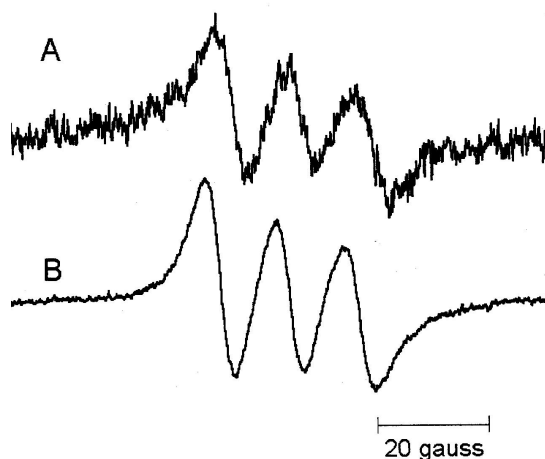


Fig. 6 EPR spectra of $(\text{MGD})_2\text{-Fe(II)-NO}$ in LPS-treated mice. (A) In vivo L-band EPR spectrum of LPS-treated mice. Mice (about 20 g) were injected i.p. with LPS (1 mg in 0.3 ml of saline), followed 6 h later by subcutaneous injection with 0.4 ml of the NO spin-trap, $(\text{MGD})_2\text{-Fe(II)}$, in saline (MGD:100 mM, Fe:20 mM). The EPR spectrum was measured 2 h after administration of the NO spin-trap. (B) Ex-vivo X-band EPR spectrum of an excised liver from a LPS-treated mouse. One hour after spin trap injection, the mice were sacrificed under anesthesia, their organs excised and measured by X-band EPR. This spectrum represents a 12.5 mg liver sample. From Fujii *et al.* (1999) with permission.

adduct(s) with ascorbic acid. However, despite the significantly increased *in vitro* stability of the paramagnetic adducts, it is likely that bioreduction would occur fairly rapidly. Figure 5 depicts ^{19}F results for the $\bullet\text{CH}_3$ adduct of FDMPO in a system containing ascorbate. The inset shows the precursor EPR spectrum.

The $\bullet\text{O}_2^-$ adduct gave an identical spectrum to that with $\bullet\text{OH}$. The conversion of the FDMPO/ $\bullet\text{O}_2^-$ adduct to the FDMPO/ $\bullet\text{OH}$ form was confirmed by the total abolition of the former adduct in the presence of SOD, while catalase had no effect (Khramtsov *et al.* 2001b). Again, the products detected by ^{19}F NMR do not appear to be the initial radical adducts but instead various diamagnetic forms (ie. hydroxylamines). As found for DEPMPPO, the oxygen centered radicals undergo a common decomposition reaction which lead back to the parent nitron. From the perspective of long-term stability, the fluorine containing spin traps are less likely to undergo destructive metabolism. Assuming that toxicity levels are not an obstacle, these NMR spin traps have greater potential.

MRI Spin Trapping

When addressing direct localization and imaging free radical distribution, there is another obstacle

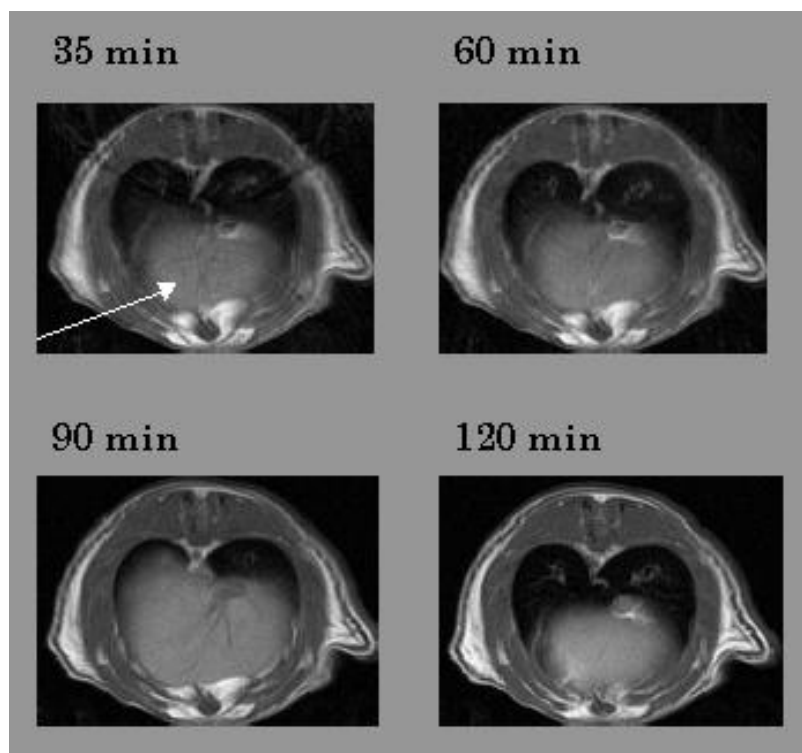


Fig. 7 Imaging of NO in LPS-treated rats. **a:** Transverse T1-weighted MR images focussed on a selected region of the liver in LPS-doped rats. The MR images were measured at the times indicated. Six hours after LPS injection, the NO spin-trap [3 ml of (MGD)₂-Fe(II), MGD: 100 mM, Fe: 20 mM] was administered i.p. Fujii *et al.* (1999) with permission.

with EPR. Despite the higher sensitivity of a free electron vs a proton, the much broader linewidths of EPR resonances places significant limitations on both overall sensitivity and resolution. There are other practical limitations with observing radical distributions in tissue, such as distinguishing tissue boundaries, which are much more difficult to visualize by EPR. On the other hand, MRI is a highly refined, commercialized technology which offers exquisite resolution by virtue of nuclear relaxation times, diffusion rates, oxygen magnetic susceptibility effects, etc. How may one capitalize on this very mature technique to visualize free radical biology, particularly at the site of its generation? Coincidentally, paramagnetic compounds serve as excellent contrast agents by inducing proton relaxation enhancement from rapidly exchanging water molecules.

Nitric Oxide Generation

Nitric oxide can be spin trapped utilizing the dithiocarbamate compounds outlined in Figure 8 which form a stoichiometric 2:1 complex with Fe(II) followed by an axial coordination of NO. For example the water soluble trap, MGD, forms the (MGD)₂Fe(II) NO complex which yields the EPR spectra shown in Figure 6 both *in vitro*, *in vivo* and in septic shock mice. The relaxivity of this com-

plex is relatively frequency independent for both T1 and T2 at 20 and 85 MHz, respectively. As shown in Table 1 (Fujii, Wan, Zhong, Yoshikawa & Berliner, 1999) the uncomplexed MGD, Fe(II) or (MGD)₂ Fe(II) showed an essentially negligible relaxivity. Consequently, only the NO complex should yield any appreciable relaxation enhancement (which should translate to image contrast enhancement) and this should be visualized at the site of NO generation. Figure 7 depicts 1.5T T1 weighted MRI images of septic-shock rats which were pretreated with lipopolysaccharide (LPS) to induce high levels of inducible nitric oxide synthase (NOS) about six hours later. The images show an increased enhancement in the liver with time which could be suppressed by injecting the animals with L-NMMA, a specific iNOS inhibitor. Hence the “biochemistry” of NO generation can be visualized *in vivo* at high resolution at the site of generation and turned on and off with specific enzymatic inhibitors. One may speculate whether this approach has potential for noninvasive imaging of the brain. When the brain is stimulated, NO is generated leaving the possibility for (paramagnetic) nitrosyl hemoglobin and methemoglobin formation.

Since functional MRI (fMRI) is believed to arise from signal enhancement from susceptibility

changes induced by blood flow. However, blood flow independent phenomena may be detected in spin trapped complexes such as above. In particular, the FDA approved pharmaceutical disulfuram is the disulfide dimer of the lipophilic spin trap diethyldithiocarbamate (DETC), which may be introduced orally and complexed with Fe(II) from iron rich stores in the brain. In summary, one can successfully visualize and map NO generation in septic shock rats.

SUMMARY AND CONCLUSIONS

The generalized techniques of NMR and MRI spin trapping offer excellent complementary methods to apply in concert with *in vivo* EPR methods. The NMR approach is applicable when bioreduction/biodestruction of trapped radicals are likely to occur. MRI spin trapping offers all of the unique advantages of NMR imaging with the specificity of locating sites of radical generation and distribution.

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