INTERACTIONS BETWEEN CELLS AND NITROXIDES AND THEIR IMPLICATIONS FOR THEIR USES AS BIOPHYSICAL PROBES AND AS METABOLICALLY RESPONSIVE CONTRAST AGENTS FOR IN VIVO NMR

Harold M. Swartz, University of Illinois
College of Medicine, Urbana, Illinois 61801

The chemical and some physical interactions of nitroxides with cells are usually considered to be experimental drawbacks in the use of nitroxides as biophysical probes. If they are understood, however, these interactions can be used as powerful experimental parameters of redox metabolism.

We are using these interactions for three interrelated measurements: 1) the development of metabolically responsive contrast agents for in vivo NMR techniques; 2) the measurement of redox metabolism in cells in vitro; 3) the measurement of intracellular oxygen of cells in vitro.

The proposed use of nitroxides in vivo as metabolically responsive contrast agents is based principally on the observation that, for many nitroxides, the rate of reduction is up to thirty times faster in severely hypoxic cells (1,2). In principle, regions of the body with hypoxic areas will have lower concentrations of nitroxides because in these regions the nitroxides become reduced to the nonparamagnetic hydroxylamines which do not affect NMR images (paramagnetic molecules affect NMR images by shortening the relaxation times of water protons - the relaxation times of water are the principal imaging parameters for most current in vivo NMR techniques) (3). Thus it may be feasible to use nitroxides to detect and follow processes in vivo that are associated with hypoxia; these include cancer, ischemia (i.e., drastically reduced blood flow) and inflammation.

The measurement of redox metabolism in cells in vitro is based on chemical interactions of nitroxides that lead to their reduction or oxidation in cells. The result of these interactions is to eliminate the ESR spectra of the nitroxides. By understanding the factors that affect the reactions with cells of particular nitroxides one can obtain significant data on the redox metabolism of the cells.

The measurement of intracellular oxygen is based on the physical (magnetic) interactions of oxygen with nitroxides, using the effects of oxygen on the relaxation times of nitroxides via Heisenberg exchange. With this technique we have obtained the first reliable and facile measurements of intracellular oxygen and demonstrated a significant gradient betwen extracellular and intracellular oxygen concentrations in our experimental cell suspension system (4,5). The existence of such a gradient has been debated hotly but not tested directly.

In order to interpret studies using nitroxides in cells and tissues and to exploit these interactions, detailed knowledge of cell-nitroxide interactions are needed. Some data on these interactions are available in the literature but these data are incomplete and often contradictory.

Our experimental approach is to study the interactions of nitroxides with cells and relevant model systems, including liposomes. The nitroxides are especially good tools because of their chemical and physical versatility, the large literature on their use in biological systems and their potential to be minimally toxic (3). We measure the amount of active

nitroxides and their physical characteristics by electron spin resonance. We measure their effective relaxivity by NMR dispersion (NMRD) determinations in which relaxivity is determined over a range of magnetic fields corresponding to Larmor frequencies of .01 to 50 MHz (3,5).

Based on an ongoing series of studies (1-9), along with information and unpublished data from the literature, we have reached the following tentative conclusions on cell-nitroxide interactions:

1. Cells can reduce the paramagnetic nitroxides to the nonparamagnetic hydroxylamines. This is shown by the gradual disappearance of the ESR signal of the nitroxides in suspensions of activity metabolizing cells and its subsequent regeneration to 90-100% of its original value by the addition of a mild oxidizing agent that is capable of reoxidizing the hydroxylamines to nitroxides, but not able to reoxidize derivatives that are reduced beyond the hydroxylamines.

2. For some nitroxides, the rate of reduction depends on the oxygen tension in the cells, being faster at very low oxygen concentrations. This extent of this effect varies with the structure of the nitroxide and varies from essentially no effect to a 20-fold or greater increase in the rate of reduction when cells become hypoxic.

3. The rate of reduction of nitroxides in aqueous environments is first order in regard to nitroxide concentration. This type of kinetics has been observed for cellular reduction in essentially all aqueous soluble nitroxides we have studied.

4. The rate of reduction for nitroxides deep in the membrane is zero order. We have observed this effect most clearly with the 12 doxyl stearate nitroxide. The 5-doxyl stearate analog, which is close to the surface of the membrane disappears with first order kinetics, which suggests that the rate limiting step for reduction of nitroxides located deep within membranes is the diffusion of a reducing substance.

5. Reduction occurs primarily intracellularly. This is shown by the fact that highly charged nitroxides (which do not readily enter cells) are reduced very slowly by intact cells and quite rapidly by cells whose membrane barriers have been breached by freeze-thawing.

6. Both enzymatic and nonenzymatic reduction occurs in cells; for at least some nitroxides the enzymatic mechanism predominates at 37°. We reached this conclusion on the basis of studies of the rate of reduction of nitroxides in cells that were not treated, compared to rates obtained in cells that were heated to 80° for 15 minutes or treated with trichloroacetic acid. At 37°C the cells with intact enzymes reduced the nitroxides approximately 20-fold faster than those whose enzymes were inactivated by heating the cells or exposing them to trichloroacetic acid.

7. Ascorbate effectively can reduce most nitroxides. The reduction of nitroxides by ascorbic acid has been demonstrated previously (10). Some nitroxides should be resistant to this mode of reduction (11).

8. Glutathione is a poor reductant for nitroxides but glutathione-metal complexes are effective reductants. We reached this conclusion on the basis of experiments in aqueous solutions.

9. Some nitroxides are reoxidized by well oxygenated cells. We observed this by incubating nitroxides with hypoxic cells until the ESR signal disappeared and then perfusing the suspension with gases

containing oxygen. Even under optimal conditions the rate of reoxidation was 10-100 times smaller than the rate for reduction. The reoxidation process appears to have both nonenzymatic and enzymatic components.

10. The effects of aqueous nitroxides on relaxation of water protons are weak. This was studied by measuring relaxivity over the frequency range of .01 to 60 MHz. These results confirmed that, in simple aqueous solutions, the nitroxides that we studied do not bind water tightly and therefore relax only by "outer sphere" mechanisms which are, in this case, not very effective because of the short correlation time for the interaction. Enhanced relaxivity of nitroxides in aqueous solution would require development of nitroxides that would bind water molecules more tightly.

11. When bound to proteins some nitroxides are effective relaxers; effective binding occurs spontaneously between doxyl stearates and albumin. These effects were measured by NMRD in simple solutions containing proteins and in plasma. The principal mechanism for the enhanced relaxivity appears to be the slower motion of the nitroxide, due to its binding to the protein. Another possible mechanism, that may occur in some instances, is an enhanced relationship between nitroxide and a water molecule both of which are bound to the protein in close proximity to each other.

12. The nitroxides are effective relaxers of lipid protons. NMRD measurements of the effects of lipid soluble nitroxides on lipid protons demonstrated effective relaxation. This presumably occurs because of the higher viscosity of the lipids, which reduces the rate of diffusion of the nitroxides and the slower motion of the lipids, because of their larger size. The relatively good relaxivity plus the potential to localize the nitroxides in lipid-rich areas by using lipophilic structures, suggests that this may be an effective use of nitroxides may be effective for obtaining contrast in vivo in lipid rich areas.

13. Nitroxides are relatively non-toxic for cells in suspension. This conclusion is based on studies in which we observed no changes in the ability of cells to exclude trypan blue or in their subsequent growth rate following incubation in media containing nitroxides at the concentrations used for achieving relaxation. There also are reports that indicate it is unlikely that nitroxides are mutagenic and that their LD_{50} (in rats) is relatively high (12).

14. The rate of reduction of nitroxides is different in different cell lines. This observation is based on preliminary data from a study of three cell lines. As more detailed information becomes available on the mechanism of reduction of nitroxides by cells, it should be possible to understand and then to predict accurately differences that are likely to be observed in different cell lines and organ systems.

15. The rate of reduction of nitroxides in the same cell line is affected by the metabolic and physiological state of the cell. This effect is seen most easily in changing the oxygen tension. We have also seen changes in the rate of reduction of the nitroxides with the following variables: plateau versus log phase cells; pH; and type of substrate used for cell metabolism.

16. Oxygen is a poor relaxer of protons of water but can cause moderate relaxivity of lipid protons because of its increased solubility in lipids. This observation is based by relaxometry measurements (NMRD) and is consistent with expectations based on physicochemical properties of oxygen.

The effect of oxygen on relaxation of lipid protons is approximately six-fold greater than its effects on water protons due not only to the increased lipid solubility of oxygen but also because of the longer correlation time due to the greater viscosity in lipids. This effect of oxygen may be sufficient to observe changes in vivo in lipid rich areas that have changes in oxygen concentrations of a magnitude that are achievable by hypoxia and/or hyperoxia.

17. Nitroxide-oxygen physical interactions can be used to measure oxygen concentrations within cells. We utilize the oxygen dependent broadening of the ESR spectrum of the nitroxides to make these measurements. The nitroxides within the cell are observed selectively by adding a membrane impermeant broadening agent which effectively removes the ESR spectrum of nitroxides outside the cell. This approach has demonstrated that intracellular oxygen concentrations can be significantly different from the oxygen concentrations in the extracellular media.

CONCLUSIONS

The uses of nitroxides as biophysical probes of viable cells and as metabolically responsive contrast agents remain very attractive and potentially very powerful. Their interactions with cells, while not completely elucidated, seem to be both understandable and capable of extending their usefulness for the purposes under consideration. Our studies of these interactions and their applications are continuing.

ACKNOWLEDGEMENTS

This research was supported by NIH Grants GM-35534, GM-34250, CA-40665, and RR-01811. It was carried out in collaboration with Drs. S. Koenig and R. Brown of the IBM Watson Laboratory, Yorktown Heights, NY; Drs. M. Schara and M. Sentjurc of the Josef Stefan Institute, Ljubljana, Yugoslavia; Dr. G. Bacic of Belgrade, Yugoslavia; Drs. R. Clarkson, R. Magin, P. Morse, D. Nettleton, M. Nilges, and H. Bennett, S. Bernstein, H. Chan, K. Chen, B. Hyslop, and M. Pals of the University of Illinois.

REFERENCES

H. Swartz, K. Chen, M. Pals, M. Sentjurc, and P.D. Morse II. Magnetic Resonance in Medicine 3,169-174(1986).

H.M. Swartz, M. Sentjurc, and P.D. Morse II. Biochimica et Biophysica Acta, in press, 1986.

H.M. Swartz, H. Bennett, R.D. Brown, III, P.D. Morse, II, M. Pals, and S.H. Koenig. Periodic Biologorum **87**,175-183(1985).

P.D. Morse, II and H.M. Swartz. Magnetic Resonance in Medicine **2**,114-127(1985).

H.M. Swartz and M.A. Pals. in press, 1986.

H.F. Bennett, R.D. Brown, III, S.H. Koenig, and H.M. Swartz. Magnetic Resonance in Medicine, in press.

M. Sentjurc, P.D. Morse, II, and H.M. Swartz. Periodic Biologicum, in press.

H.M. Swartz, M. Sentjurc, and P.D. Morse, II. Biochimica et Biophysica Acta, in press, 1986.

H.M. Swartz. Journal of the Chemical Society, in press.

S. Schreier-Muccillo, D. Marsh, and I.C.P. Smith. Archives of Biochemistry and Biophysics **172**,1-11(1976).

J.F. Keana. Spin Labeling II, Academic Press, New York, 1979.

V. Afzal, R.C. Brasch, D.E. Nitecki, and S. Wolff. Investigative Radiology **19**,549-552(1984).