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APPLICATION OF SPIN TRAPS TO BIOLOGICAL SYSTEMS

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Since 1971, when nitroxides were first reported to be bioreduced, several cellular enzymes, in addition to ascorbic acid, have been found to catalyze the reduction of nitroxides to their corresponding hydroxylamines. Numerous studies have demonstrated that cellular bioreduction of nitroxides are both dependent upon the structure of the nitroxide and cell type. For example, pyrrolidinyloxyls are considerably more resistant to bioreduction than their corresponding piperidinyloxyls. In addition, cellular levels of reductases present in freshly isolated rat hepatocytes are considerably greater than concentrations found in freshly isolated rat enterocytes. Thus, through the proper selection of a cell type and an appropriate nitroxide, one can study cellular-mediated free radical processes.

With the discovery that α-hydrogen-containing nitroxides, including 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl (DMPO-OH) decompose rapidly in the presence of superoxide and thiols, the ability to determine if hydroxyl radical is generated during stimulation of human neutrophils, is in doubt. To explore the limits of spin trapping in this context, we have studied the effect of varying the rates of superoxide production, in the presence and absence of thiols, on the decomposition of DMPO-OH. In parallel studies, we have found that *t*-butyl α-methyl-4-pyridinyl-N-oxide nitroxide (4-POBN-CH_3) will not degrade in the presence of superoxide and a thiol. From these studies, we have determined that if hydroxyl radicals were generated as an isolated event in the presence of a continual flow of superoxide, spin trapping might not be able to detect its formation. Otherwise, spin trapping should be able to measure hydroxyl radicals, if continually generated, during activation of human neutrophils.

KEY WORDS: Spin trapping, nitroxides, DMPO, ascorbate.

INTRODUCTION

The study of free radicals in biology is dependent, to a large extent, upon our ability to identify these very reactive species with half-lives usually less than a msec. Of the various techniques available to detect free radicals, spin trapping[1] has received the most attention. This is the result of its unique properties which allow, in the case of 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), the simultaneous detection of superoxide, hydroxyl radical and small carbon-centered free radicals.[2] In addition, spin traps can be synthesized with various lipophilic characteristics, thereby controlling their entry into cells.[3-5] Like all methods, spin trapping is not without its limitations. In recent

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years, these difficulties have been extensively reviewed.[6,7] and will not be further discussed here.

The ability to record ESR spectra of nitroxides, arising from the spin trapping of free radicals by nitrones or nitroso compounds, is a measure of the equilibrium between the rate of spin trap adduct formation and decomposition. In cells, this equilibrium may be shifted, reflecting the metabolic activity of the cell, and in so doing, limit our ability to report accurately the nature of free radicals generated during cellular metabolism of a xenobiotic or stimulation of a phagocytic cell. Because of the importance of these reactions to the field of free radical biology, we have decided to review the literature in the hope of catalyzing interest in the biological reactions of nitroxides.

DISCUSSION

The concept that an enzyme in cells can metabolize nitroxides was first suggested in 1971 by Stier and Reitz[8] who demonstrated that 2,2,6,6-tetramethylpiperidinyloxyl can be reduced by liver microsomes to the corresponding amine, 2,2,6,6-tetramethylpiperidine. They proposed that NADPH-cytochrome P-450 reductase was responsible for this three-electron reduction. Several years later, while we were studying the mechanism by which NADPH transferred electrons to the terminal oxidase, cytochrome P-450, we discovered that this enzyme and not NADPH-cytochrome P-450 reductase, reduces nitroxides to their corresponding hydroxylamines[9,10] (Figure 1). However, only in the presences of a sterically unhindered hydroxylamine, (e.g. N-hydroxynorcocaine) could this enzyme accept an electron, yielding hydrogen peroxide and the corresponding nitroxide[11] (Figure 2). In the intervening years, a number of cytoplasmic NADPH-dependent reductases[12] and ferrocytochrome c[13] have been

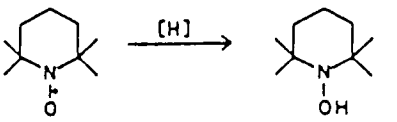

FIGURE 1 The one-electron reduction of nitroxides to their corresponding hydroxylamines has been shown to be catalyzed by cytochrome P-450, cytosolic NADPH-dependent reductases and ascorbic acid.

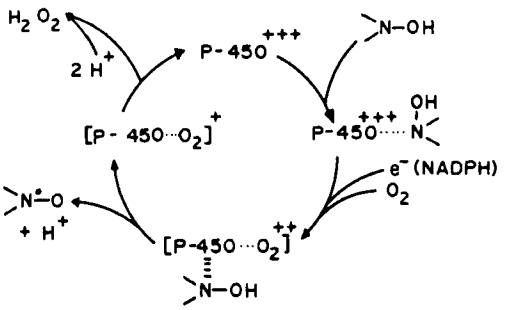

FIGURE 2 A propose mechanism by which cytochrome P-450 accepts an electron from unhindered hydroxylamines, giving nitroxides, hydrogen peroxide and oxidized hemoprotein.

shown to catalyze this reduction. Of interest is the observation that as a class of nitroxides, pyrrolidinyloxyls are considerably more stable than their structural analogs, piperidinyloxyls towards reduction mediated by either cytochrome P-450 or cytoplasmic reductases.[14] At present, we have not been able to determine the reasons for this finding.

Despite the above, there are numerous examples, a few of which are referenced,[15-20] of free radicals being spin trapped, intracellularly, as the result of cellular xenobiotic metabolism. When undertaking these studies, however, one must be cognizant of the potential for nitroxide reduction and choose cell types and experimental conditions with great care. For example, we have been unable to spin trap free radicals reliably from freshly isolated rat hepatocytes, which have high enzymatic content of both cytochrome P-450 and cytoplasmic reductases.[14] When we changed to another cell type such as freshly isolated rat enterocytes, we were able to spin trap free radicals easily under conditions not possible with rat hepatocytes.[16] However, other investigators have noted rapid reduction of α-hydrogen-containing nitroxides by red blood cells and certain tumor cell lines, including chinese hamster ovary and mouse thymus-bone marrow cells.[21]

Several years ago, we observed that nitroxides could be reduced to their corresponding hydroxylamines in the presence of superoxide and thiols; the absence of either component prevented this reaction[22] (Figure 3). Since this mechanism is dependent upon a continued intracellular flux of superoxide, sufficient to overwhelm superoxide dismutase (SOD) as well as a high steady state concentration of intracellular thiols (e.g., glutathione), we doubt that this pathway would account for a significant amount of nitroxide reduction, except in the case of stimulated phagocytic cells.

As a consequence of activation by both soluble and paticulate stimuli, neutrophils undergo a rapid increase in oxygen utilization, termed the "respiratory burst".[23] During the "burst", oxygen metabolism leads to the generation of oxygen reduction products, including superoxide and hydrogen peroxide.[24,25] Although formation of hydroxyl radical by stimulated neutrophils has been reported, problems associated with interpretation of data have limited the usefulness of these investigations. Several years ago, we decided to re-investigate this issue, as it is important to determine if hydroxyl radical plays a role in inflammation and microbicidal activity. We found that when neutrophils were stimulated with either phorbol myristate acetate (PMA) or opsonized zymosan, we spin trapped only superoxide,[26,27] even though under

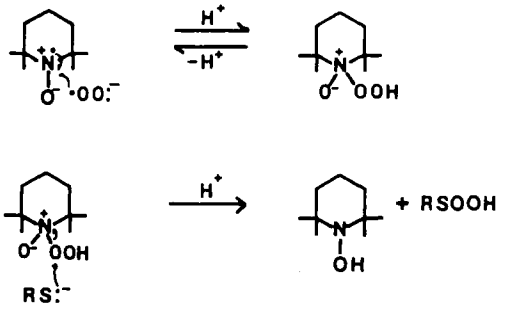

FIGURE 3 A proposed reaction for the decomposition of nitroxides by superoxide and thiols.

certain experimental conditions, the ESR spectrum recorded was that attributal to 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl (DMPO-OH). When we forced PMA-activated neutrophils to produce hydroxyl radical by inclusion of iron-DTPA in the reaction media, hydroxyl radical waned despite continued generation of superoxide.[27] We found[27] that this was the result of the ability of lactoferrin, secreted during stimulation of neutrophils, to remove iron bound to DTPA, thereby preventing the iron-catalyzed Haber-Weiss reaction.

However, it is conceivable that small quantities of hydroxyl radical were still produced and that our experimental design was not sufficient to spin trap such low levels of free radicals. One potential approach to circumvent this problem is to increase cell density, thereby enhancing production of free radicals. When we increased the number of neutrophils from 1×10^7 cells/ml to 4×10^7 cells/ml, we decreased the spin trapping of superoxide, even though under these experimental conditions, the rate of superoxide production, as measured by cytochrome c reduction, was markedly enhanced.[28] This suggested to us that the spin trapped adduct was reduced by superoxide in the presence of a thiol, secreted during stimulation of neutrophils. To test this hypothesis, we incubated stimulated neutrophils with the stable nitroxide, 2,2,6,6-tetramethylpiperidinyoxyl (TEMPO). As shown in Figure 4, reduction rapidly occurred which was blocked by the addition of SOD. To demonstrate that neutrophils secreted a cellular factor which was essential for this reduction, we added cell-free supernatant, obtained following neutrophil stimulation by PMA, to our model superoxide-generating system, hypoxanthine/xanthine oxidase at pH 7.8. Again, rapid reduction of TEMPO was observed which was inhibited in the presence of SOD. We are presently exploring the nature of this cellular factor.

Along the same lines, Samuni, *et al.*[29] recently reported that superoxide enhanced the degradation of α-hydrogen-containing nitroxides, including DMPO-OH and 2,2,5-trimethyl-1-pyrrolidinyloxyl (DMPO-CH_3). Because of the importance of these findings, we have recently completed a study[30] defining the limits of spin trapping with regards to neutrophil-derived free radicals.

Our initial series of experiments involved measuring the decomposition rate of DMPO-OH as a function of superoxide flux, generated by the oxidation of xanthine by xanthine oxidase. DMPO-OH was produced by the UV irradiation of a solution of hydrogen peroxide and DMPO. At 1 μM superoxide/min, the ESR spectral peak height for DMPO-OH decreased only slightly over an 8 min period of time as compared to control (Figure 5A and 5B). At 5 μM/min, the rate of decomposition was markedly enhanced (Figure 5C), but slower than that observed when the rate of

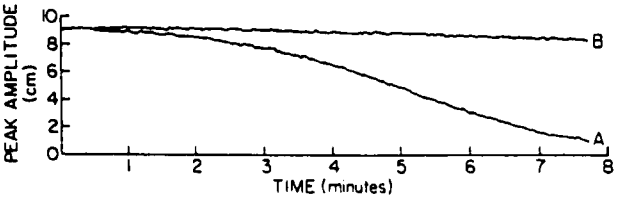

FIGURE 4 Effect of stimulated neutrophils on the rate of TEMPO decomposition. Neutrophils were stimulated with PMA in the presence of TEMPO. The ESR magnetic field was set at the top of the low-field peak of the nitroxide triplet and scanned with time. (A) A short lag time followed by a rapid decrease in TEMPO peak height. (B) In the absence of neutrophils or in the presence of SOD. Experimental details are presented in reference 28.

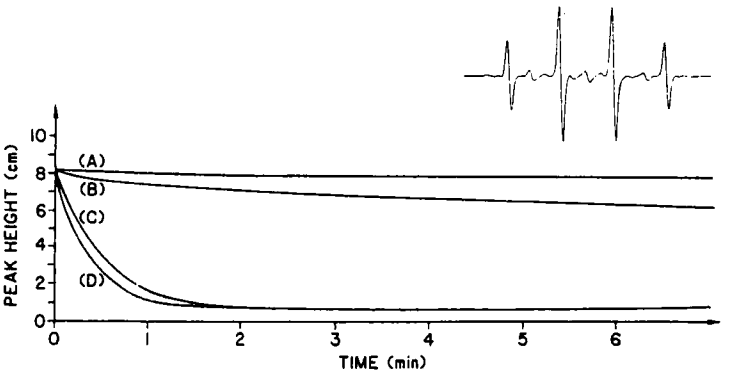

FIGURE 5 Effect of superoxide on the stability of DMPO-OH. The magnetic field was set at the top of the second low-field peak of this spin trapped adduct. (A) depicts the peak height of DMPO-OH in the absence of superoxide. (B) and (C) were generated in the presence of superoxide at 1 μM/min and 5 μM/min, respectively. (D) was identical to (B) except cysteine (200 μM) was added. Experimental details are described in reference 30.

superoxide was 1 μM/min in the presence of cysteine (200 μM) (Figure 5D). Similar results were obtained when DMPO–CH_3, generated by the UV photolysis of hydrogen peroxide in the presence of DMPO and DMSO, was substituted in place of DMPO-OH in the above sequence of reactions. Although these findings support the observations of Samuni and co-workers,[29] they further demonstrate the critical role of a thiol in the destruction of nitroxides.[28]

Given the importance of superoxide to the destruction of DMPO-adducts, decreasing the flux of superoxide should allow increased detection of DMPO–OH and DMPO–CH_3 if hydroxyl radical were, indeed, generated. However, as shown in Figure 6, when neutrophils were stimulated with PMA in the presence of SOD (either 0.1 or 1.0 U/ml), a proportional decrease in DMPO–OOH, DMPO–OH and DMPO–CH_3 was recorded.[30] Finally, when iron was added to the above reaction, the peak height of DMPO–CH_3 dramatically increased. This result, to some degree, comes from protection afforded the nitroxide by decreasing the flow of superoxide. But more importantly, we believe that with the addition of low concentrations of SOD, accelerated production of hydrogen peroxide leads to enhanced generation of hydroxyl radical.

One interesting observation reported by Samuni, *et al.*[31] may shed additional light on whether or not neutrophils generate hydroxyl radical in the presence of a continual flux of superoxide. These investigators reported that *t*-butyl α-methylbenzyl nitroxide (PBN–CH_3), generated by sonolysis, was resistant to the effects of superoxide. We have recently expanded these studies to include *t*-butyl α-methyl-4-pyridinyl-N-oxide nitroxide (4-POBN-CH_3) (unpublished results). As in the case of DMPO–OH, we generated 4-POBN–CH_3 by the UV irradiation of a solution of hydrogen peroxide, DMSO and α-(4-pyridyl-1-oxide)-N-t-butylnitrone (4-POBN). As demonstrated in Figure 7, 4-POBN–CH_3 did not decompose over an eight-minute period of time. In the presence of superoxide (10 μM/min) alone or with the inclusion of cysteine (200 μM) to the superoxide generating system, no loss of ESR signal was observed (Figure 7). Similar results were found with PBN–CH_3 (Unpublished findings). Finally, when phenyl *t*-butyl nitrone (PBN) and DMSO were added to PMA stimulated

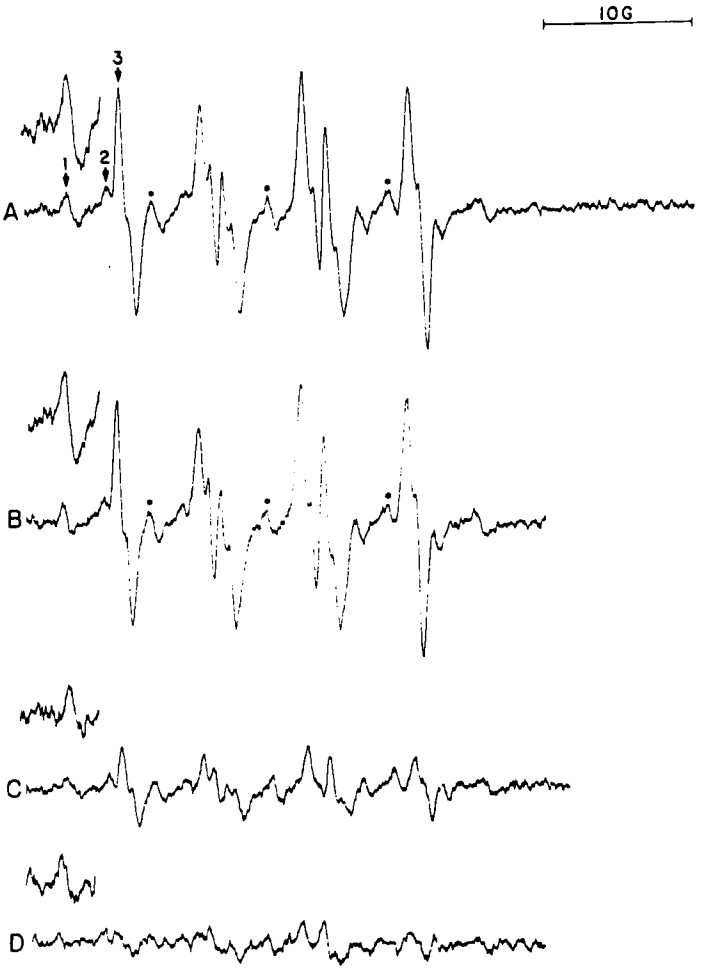

FIGURE 6 ESR spectra generated by stimulation of neutrophils with PMA only (A), with catalase added (B), with SOD (0.1 U/ml) added (C), with SOD (1.0 U/ml) added (D). An amplification (5×10^4) of DMPO-CH_3 was included for comparison. The triplet (*) was not present in the absence of PMA and resulted from the aerobic oxidation of DMPO. Experimental details are given in reference 30.

neutrophils, we were unable to detect any PBN-CH_3, suggesting the absence of hydroxyl radical under these experimental conditions (unpublished results).

These data clearly demonstrate that if a continual flux of hydroxyl radicals were generated during the PMA stimulation of neutrophils, spin trapping could detect its presence. If, however, hydroxyl radical were produced as an isolated event in the presence of a continual flow of superoxide, spin trapping might not be able to detect its generation. Since stimulated neutrophils produce a continuous flux of superoxide, formation of hydroxyl radical should also occur at a steady state level, and therefore be spin trapped at the levels detectable by ESR spectroscopy. At present, we see no evidence for the conclusion that under physiologic conditions, activated neutrophils make hydroxyl radical.

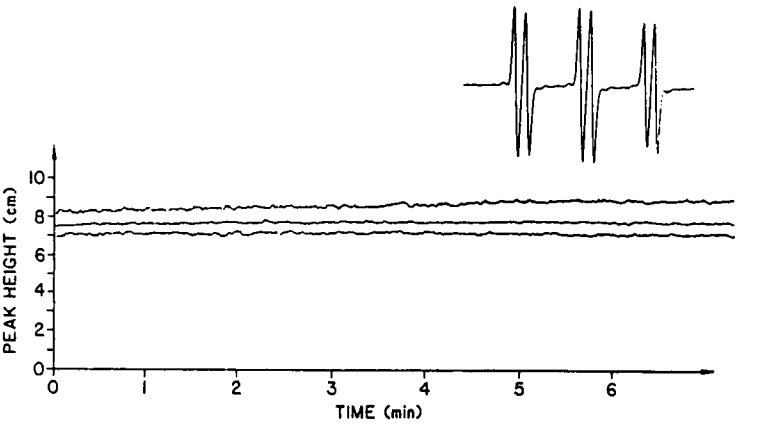

FIGURE 7 Effect of superoxide on the stability of 4-POBN-CH_3. The magnetic field was set at the top of the first low-field peak of 4-POBN-CH_3. The upper scan represents the peak height of 4-POBN-CH_3 in the absence of superoxide. The lower scan represents the peak height in the presence of 10 μM/min. The middle scan represents the peak height in the presence of superoxide (10 μM/min) and cysteine (200 μM). Unlike DMPO-OH or DMPO-CH_3 there is no degradation with time. Experimental details are similar to those described in reference 30.

In this review, we have attempted to make the reader cognizant of many potential problems associated with the spin trapping of free radicals in *in vitro* cell models. For most cells which contain a plethora of enzymes designed to either regulate intracellular levels of free radicals or to reduce the products they form, the detectability of spin trapped adducts is frequently dependent upon the inability of a family of cellular reductases to reduce these nitroxides. For phagocytic cells, which produce a high flux of free radicals in compartments not containing SOD, catalase, glutathione peroxidase and/or reductases, the superoxide-thiol concentration may be one of the most important criterion in determining our ability to detect spin trapped adducts. These and other studies demonstrate the flexibility of spin trapping as well as its limitations. Finally, the application of organic syntheses to free radical biology offers numerous possibilities that should not be overlooked.

Acknowledgments

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