Free Rad. Res. Comms., Vols. 12-13, pp. 39-46
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USE OF A LOW-FREQUENCY ESR SPECTROMETER: IMPLICATIONS FOR SPIN-TRAPPING FREE RADICALS, *IN SITU*

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We have adapted the low-frequency ESR spectrometer, designed and built by H.J. Halpern, to the physiologic needs of organ preparations operating at 250 MHz. Initial studies have allowed us to detect nitroxides in an isolated perfused heart. These *in situ* measurements were made with nitroxides specifically designed to mimic the lipophilic nature of 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl (DMPO-OH). These spin labels provided information about the influence of dynamic factors of the heart, such as flow rate, different cell populations and unequal distribution between compartments on our ability to conduct and interpret spin trapping experiments. They also clarified the sacrifice in sensitivity involved in operating at the lower frequencies. To deal with this later problem, we have increased the sensitivity of the spin trapping method by synthesizing a family of ^{15}N- and deuterium-containing DMPO analogs and by determining their ability to spin trap free radicals generated by the model superoxide system of xanthine/xanthine oxidase. Finally, since activated neutrophils are one of the few cells known to generate free radicals as part of their physiologic function, we used these phagocytic cells, as a source of superoxide.

KEY WORDS: ESR, free radicals, spin trapping.

INTRODUCTION

In recent years, there has been a renaissance in the field of free radical biology as these reactive intermediates appear to mediate a variety of pathologies, including ischemia/reperfusion injury[1] as well as to play an important role in initiating several physiologic responses.[2] Yet our understanding of the mechanism by which free radicals participate in these processes depends, to a large extent, upon our ability to detect and identify these very reactive intermediates with very short half-lives, usually less than a msec. Of the available methods for the identification of free radicals, spin trapping[3] has received the most attention. This is the result of its unique properties, which allow, in the case of DMPO, the simultaneous detection of superoxide, hydroxyl radical and small carbon-centered free radicals.[4] In addition, spin traps can be synthesized with various lipophilic properties, thus, regulating their entry into cells.[5-8]

All of the spin trapping studies, to date, have only been conducted using homogenous solutions, cell fractions and isolated cells.[9-13] This is due to the present generation of commercial ESR spectrometers, which limit our investigations to these rather artificial systems. With the development of a low-frequency ESR spectrometer,[14] we[15] recently have been able to adapt this instrument to the physiologic needs of an isolated

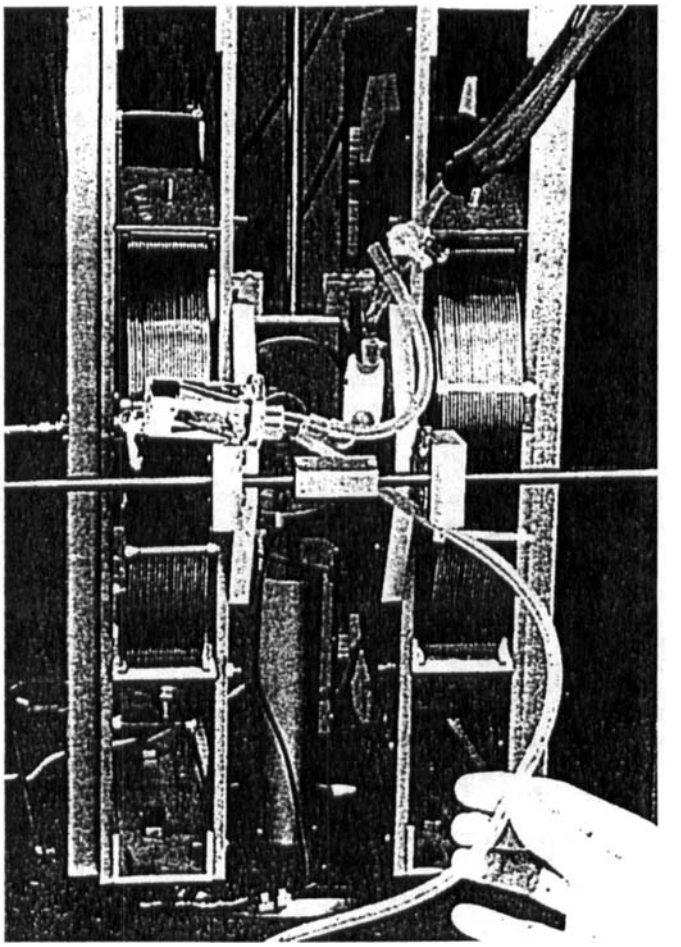

FIGURE 1 The low-frequency electron spin resonance spectrometer is depicted, in which an isolated perfused heart is shown encased in a specially designed cuvette. Details of the instrument design is described in reference 14.

perfused heart (Figure 1). However, before we could attempt to detect and identify specific free radicals, *in situ*, a number of important issues needed to be addressed. These will be elaborated on in the following sections.

DISCUSSION

For an isolated perfused organ, dynamic factors such as flow rate, differential cell populations, unequal distribution between compartments and cellular metabolism make interpretation of spin trapping data extremely difficult. Consider, for example, the typical spin trapping experiment conducted with isolated cells. After addition of the spin trap, equilibrium is rapidly reached between intra- and extracellular compart-

ments. Thus, identification of free radicals in both environments can be verified by addition of free radical scavengers (e.g., SOD for extracellular superoxide or pre-labeled cells with PEG-SOD for intracellular superoxide). However, in the case of an organ, diffusion of the spin trap to the site where free radicals are generated may be more difficult to achieve. For example, let us assume that the epithelial cells of that tissue are where free radicals are generated as the result of a particular pathologic condition. For our model, the spin trap would be added to the perfusate which enters the organ. Then, it needs to transverse the vascular bed, prior to diffusion into the parenchymal cells. Since DMPO and analogs have a very small partition coefficient,[6] the rate of perfusion would regulate DMPO concentration in those cells. Therefore, the flow rate becomes the limiting factor in the ability to spin trap free radicals with this nitrone.

If we now assume that free radicals are also produced by endothelial cells lining the blood vessels of that organ, as one hypothesis suggests,[1] then, we must somehow be able to distinguish between free radical formation at multiple locations. At present, there is no model to assist us in the interpretation of these data.

Finally, one must also consider the metabolic fate of nitroxides, arising from the spin trapping of free radicals by nitrones or nitroso compounds. Although these spin labels are quite stable in solution, lasting in some cases for years, however, in a metabolically active cell, bioreduction may limit our ability to observe spin trapped adducts.[16] For isolated cells, this reaction may be circumvented through the careful selection of a cell, whose reduction of nitroxides is slow, as well as by varying the experimental design. On the other hand, in an isolated perfused organ comprising several cell types with different metabolic properties towards nitroxides, limiting the bioreductive process may present insurmountable difficulties towards accurately determining the distribution of free radicals in the organ under study.

As mentioned, we have spent the last several years addressing many of these issues[15] and will here only briefly highlight our findings. Our initial studies examined how rapidly DMPO entered the myocyte of the isolated perfused heart as a function of flow rate. At 8–10 ml/min, most of the DMPO (0.1 M) added to the perfusate was collected within the initial 15 seconds. Not surprisingly, as the rate of perfusion decreased, the same amount of DMPO obtained with the higher flow, took considerably longer. Since our langendorf heart preparation requires a high perfusion rate to retain physiologic function, a recirculation system would be essential, if spin traps are to have any chance at successfully detecting free radicals in cells other than the endothelium.

To determine what effect the dynamics of an isolated perfused heart would have on the spin trap, DMPO and its spin trapped adducts, we synthesized two nitroxides, whose lipophilic character and structure are similar to DMPO and DMPO-OH.[15] For our initial experiments, we determined how well nitroxides diffused into the heart. Since it took us several minutes to tune the low-frequency ESR spectrometer, determination of the rate of diffusion would be difficult. Therefore, we measured nitroxide concentration in the perfusate as a function of time. For 3-carboxy-2,2,5,5-tretramethyl-1-pyrrolinyloxy (CTMPO), the nitroxide with physical properties similar to DMPO, we observed little diffusion into the myocyte. However, when 2,2,3,3,5,5-hexamethylpyrrolidinyloxyl (HMPO), a mimic for DMPO-adducts, was substituted in the above experiments, this nitroxide rapidly crossed the arterial wall and entered the organ, having a distribution half-life of approximately 2 minutes (Figure 2). We then determined the rate of nitroxide bioreduction in the isolated

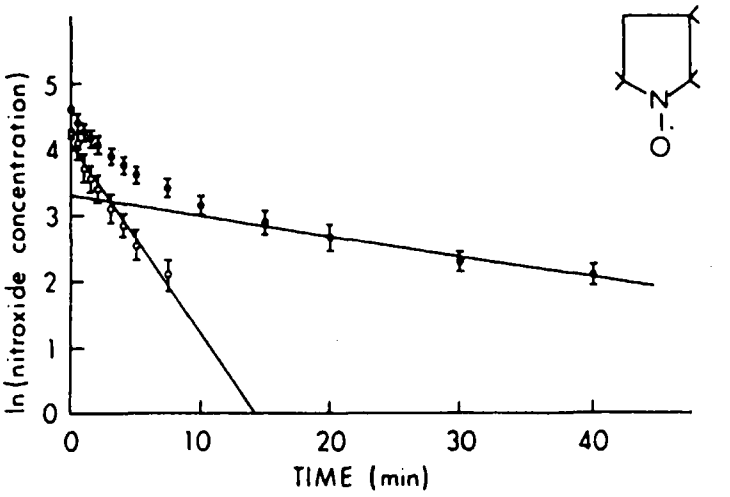

FIGURE 2 Semilogarithmic plot of HMPO concentration versus time after injection of the spin label into an isolated perfused rat heart placed in a modified langendorf apparatus. Samples of perfusate were removed at defined times and nitroxide concentration assayed using a Varian Associates ESR spectrometer model E-9 at room temperature. The distribution half-life was 2.4 minutes, and the metabolic half-life was 22 minutes. For more details, see reference 15.

perfused heart, by measuring the concentration of HMPO in either the perfusate (Figure 2) or the isolated organ (Figure 3). The half-life in both models was, within experimental error, the same. These findings suggest that the low-frequency ESR spectrometer is accurately reporting events taking place in the isolated heart. Finally, these data have allowed us to study the effect of metabolic processes on the spin trapping of free radicals in an isolated perfused organ model.

However, before we can possibly spin trap free radicals in an isolated perfused

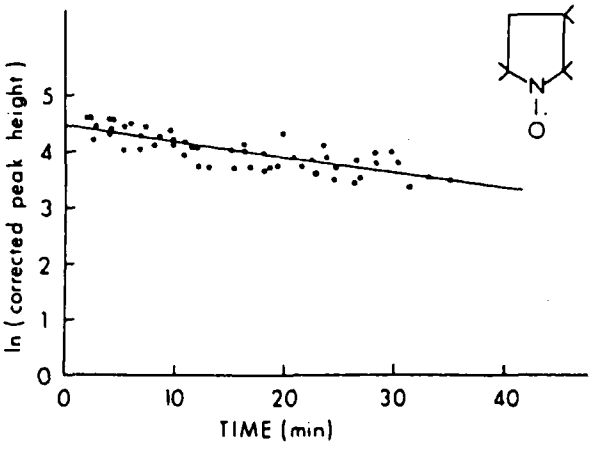

FIGURE 3 Semilogarithmic plot of HMPO concentration versus time after injection of the spin label into an isolated perfused heart housed in the low-frequency ESR spectrometer. From these data, the metabolic half-life was found to be 25 minutes. For more details, see reference 15.

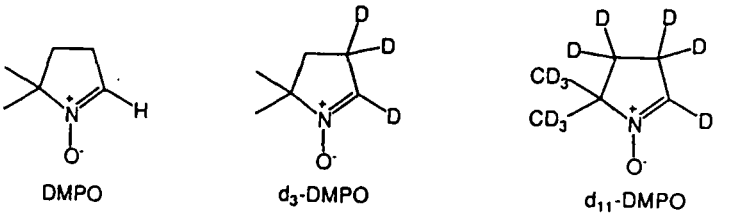

FIGURE 4 The structure of the spin traps DMPO, d_3-DMPO and d_{11}-DMPO is depicted.

organ housed in the low-frequency ESR spectrometer, we must enhance the sensitivity of the spin trapping technique. To begin with, we must attempt to overcome the very poor rate of spin trapping superoxide by DMPO[17] (only 10 M^{-1} sec^{-1} at pH 7.8). One very attractive approach comes from the pioneering work of Beth, *et al.*,[18] in which they found that by substituting ^{15}N- and deuterium for ^{14}N- and hydrogen in spin labels they could improve the signal-to-noise ratio by a factor of ten. Therefore, we synthesized a family of ^{15}N- and deuterium-labeled DMPOs[19] (Figure 4), and compared the peak heights for several of these nitroxides, generated by the spin trapping

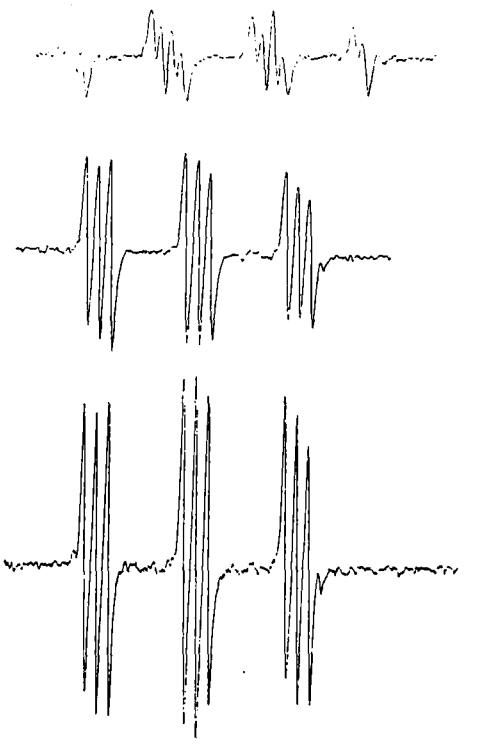

FIGURE 5 ESR spectra obtained from the spin trapping of superoxide by DMPO, d_3-DMPO and d_{11}-DMPO. Experiments were conducted using a Varian Associates model E-9 EPR spectrometer in which the microwave power was 20 mW; modulation frequency was 100 kHz, with an amplitude of 0.2 G; a sweep time of 12.5 G/min and the receiver gain was 1×10^4. The upper scan is DMPO–OOH; the middle scan is d_3-DMPO–OOH; the lower scan is d_{11}-DMPO–OOH.

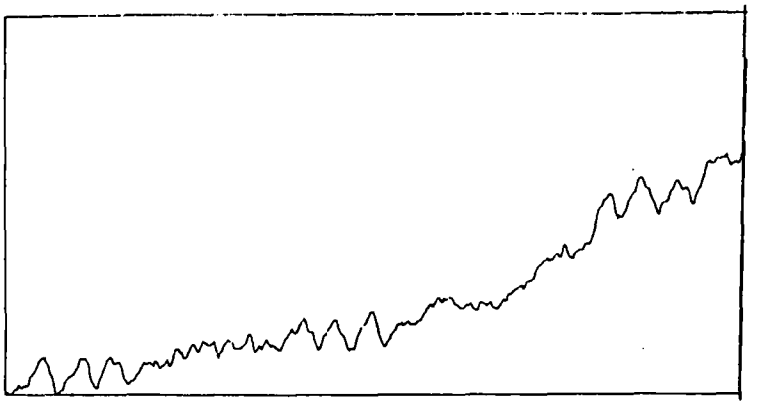

FIGURE 6 ESR spectra obtained from the spin trapping of superoxide (15 μM/min) by d_3-DMPO in the low-frequency ESR spectrometer, in which RF power was 10 dBm, RF frequency 250 mHz, modulation amplitude was 0.6 G. The concentration of d_3-DMPO as 0.1 M in a total volume of 5 ml.

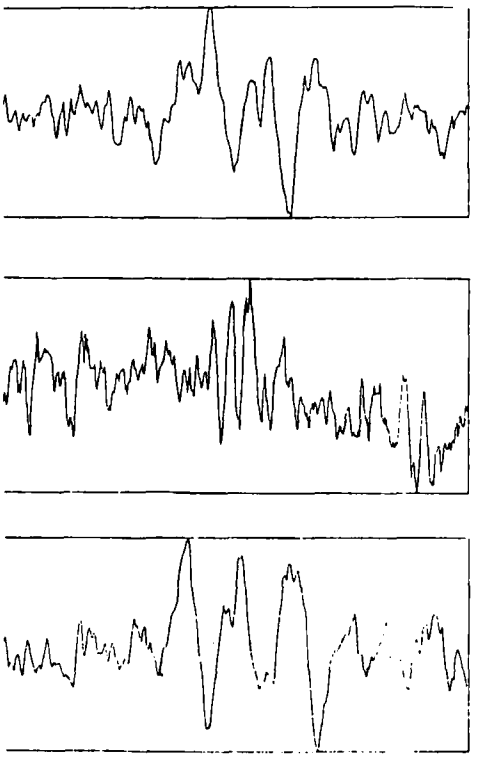

FIGURE 7 ESR spectra obtained from the spin trapping of superoxide, from PMA-activated human neutrophils (1 μM/min) by either d_3-DMPO (0.1 M in 10 ml, upper scan) or d_{11}-DMPO (0.1 M in 5 ml, middle and lower scan). The middle scan is complete spectrum, showing the middle and high frequency triplets. The low frequency triplet is lost in the noise. The upper and lower scans are the middle frequency of d_3-DMPO–OOH and d_{11}-DMPO–OOH. Instrumentation settings are the same as described in Figure 6.

of superoxide, using the model system of xanthine/xanthine oxidase at pH 7.8. As shown in Figure 5, when d_3-DMPO was substituted for DMPO in the above reaction, we noted a two-fold increase in signal-to-noise ratio, which was further enhanced by using d_{11}-DMPO.

With these encouraging results, we have recently conducted a series of experiments, examining our ability to spin trap free radicals in the low-frequency ESR spectrometer. Generating a high flux of superoxide (15 μM/min) by the action of xanthine oxidase on xanthine at pH 7.8, we were able to spin trap superoxide using d_3-DMPO but not DMPO (Figure 6). With this success, we decided to determine if we could spin trap superoxide by PMA-stimulated human neutrophils at a low superoxide flux (1 μM/min). Even with a 5 ml volume buffer, we were able to observe d_3-DMPO-OOH and d_{11}-DMPO-OOH in the low-frequency ESR (Figure 7). Of interest was the finding that we observed the same signal-to-noise ratio when half the number of moles of d_{11}-DMPO was added to the reaction mixture as compared to d_3-DMPO.

In this article we have presented many of the potential problems associated with the spin trapping of free radicals in an isolated tissue housed in a low-frequency ESR spectrometer. Although we have accomplished much towards our goal, nevertheless, there are numerous pitfalls that need to be addressed before we are ready to attempt to detect free radicals generated by an isolated perfused organ.

Acknowledgements

This work was supported in part by grants from the National Science Foundation, The Chemistry of Life Processes Program, DCB 8616115; National Institutes of Health, HL 33550; and The Council for Tobacco Research-U.S.A., Inc.

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