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APPLICATION OF SPIN TRAPPING TO HUMAN PHAGOCYTIC CELLS: INSIGHT INTO CONDITIONS FOR FORMATION AND LIMITATION OF HYDROXYL RADICAL

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In recent years spin trapping techniques have been used extensively to better understand the free radical biology of phagocytic cells. These results demonstrate that spin trapping is of adequate sensitivity to detect superoxide and/or hydroxyl radical generated by these cells, and that spin trapping is capable of measuring phagosomal radicals as well. However, neither neutrophils, monocytes, nor monocyte derived macrophages generate hydroxyl radical in the absence of exogenous iron. Furthermore, neutrophil lactoferrin and myeloperoxidase limit the magnitude (and in the case of lactoferrin the duration) of hydroxyl radical formed by neutrophils in an iron catalyzed system. Since monocytic phagocytes possess no lactoferrin, and limited myeloperoxidase, hydroxyl radical may play an important role in the inflammatory behavior of mononuclear phagocytes.

KEY WORDS: Phagocytes, neutrophils, monocytes, spin-trapping, superoxide, hydroxyl radical.

INTRODUCTION

Phagocytic cells of virtually every species use molecular oxygen to form superoxide.[1] In recent years the electron transport chain allowing single electron reduction of molecular oxygen by phagocytes, and the regulation of this system at the molecular level have been defined.[2] Formation of superoxide will inevitably lead to hydrogen peroxide, and possibly other reactive oxygen intermediates depending on microenvironmental conditions.[3] However it has been extremely difficult to dissect conditions inside the neutrophil phagosome where microorganisms are killed, since this is a closed compartment. We have attempted to optimize the use of spin trapping procedures to address the role of specific free radicals in the microbicidal process and inflammation.[4] The advantages of spin trapping include its ability to specifically and

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concommitantly identify several free radicals,[4] and of some spin traps to enter the phagosome[5] where superoxide, and perhaps hydroxyl radical, are produced.

RESULTS AND DISCUSSION

Application of Spin Trapping to Human Polymorphonuclear Neutrophils

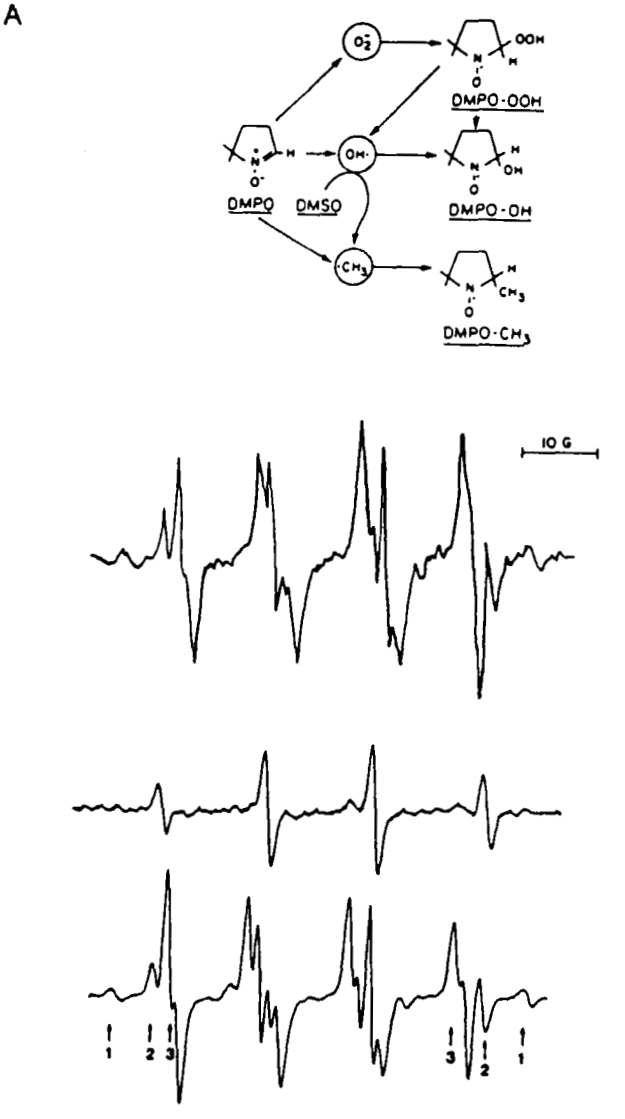
The application of spin trapping methods to phagocyte biology has been summarized in earlier reviews.[4,6] When neutrophils are stimulated with the protein kinase c agonist phorbol myristate actetate in the presence of DMPO (5,5-dimethyl-1-pyrroline-1-oxide) and the iron chelator DETAPAC (diethylenetriaminepentacetic acid) superoxide is detected by the formation of the ESR spectrum corresponding to DMPO-OOH (2,2-dimethyl-5-hydroperoxy-1-pyrrolidinyloxy), which is entirely inhibited by superoxide dismutase and unaffected by catalase (Figure 1A., reference[7]). With phagocytic stimuli such as opsonized zymosan, DMPO-OOH and DMPO-OH (2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxy) are formed, the concentrations of which depend on the particle to cell ratio (Figure 1B). These results have been duplicated in a number of laboratories.[8-10] Although the ESR spectrum characteristic of DMPO-OH might suggest spin trapping of hydroxyl radical,[8] the inhibition of this adduct by SOD but not catalase suggests that this species arises from superoxide and decomposition of DMPO-OOH.[4,6,7,9,10] DMSO is included in the system to allow detection of hydroxyl radical, which would be reported as DMPO-methyl (2,2,5-trimethyl-1-pyrrolidinyloxy, Figure 1, reference[4]). However, this adduct is not detected in substantial concentration in the absence of exogenous Fe^{3+} leading us to the conclusion that *within the limits of spin trapping*,[11] human neutrophils do not generate hydroxyl radical.[5] This conclusion is supported by recent experiments with alternative procedures believed to be more sensitive than spin trapping.[12-14]

Several years ago we noted that the reduction of five- and six-membered ring nitroxides was accelerated by superoxide in the presence of a thiol.[15] Using PMA as a stimulus we noted that DMPO-OH and other nitroxides were reduced by activated neutrophils in the presence of a cellular factor (s), and SOD prevented the reaction.[16] More recently Samuni and coworkers[9] showed that superoxide alone can degrade DMPO-OH and DMPO-methyl and suggested that i) spin trapping in this setting might not allow detection of hydroxyl radical and ii) failure to detect hydroxyl radical

FIGURE 1 A. Diagram of spin adducts of DMPO which would be expected to form in the presence of superoxide and hydroxyl radical when DMSO is present in the reaction mixture. Note that DMPO-OH can form both as a consequence of hydroxyl radical spin trapping *and* decomposition of the superoxide spin adduct DMPO-OOH. (Reproduced with permission of *The Journal of Biological Chemistry*, reference [7]). B. ESR spectrum obtained following stimulation of neutrophils in the presence of DMPO and DMSO. The top scan was obtained with PMA as the stimulus and is dominated by DMPO-OOH. The middle and bottom scans were each obtained following neutrophil stimulation with opsonized zymosan. In the middle scan the zymosan concentration employed was 0.5 mg/ml which resulted in an ESR spectrum comprised nearly entirely of DMPO-OH. In the bottom scan the zymosan concentration was increased to 3 mg/ml and this resulted in an ESR spectrum similar to that observed with PMA (top scan). High and low field peaks corresponding to DMPO-methyl, DMPO-OH, and DMPO-OOH are indicated in the figure as 1, 2, and 3, respectively. For each experiment microwave power was 20 miiliwatts, modulation frequency was 100 kHz with an amplitude of 0.63 G, sweep rate was 12.5 G/min., response time was 1.0 seconds with a gain of 1.25×10^4 (Reprinted with the permission of The Journal of Biological Chemistry, reference[7]).

formation by neutrophils[5,9,10] might result from superoxide-mediated destruction of DMPO–OH or DMPO-methyl, and not failure of neutrophils to generate this radical.

However, there are several lines of evidence to suggest that this is not the case. First, when PMA is used as a stimulus for neutrophils in the presence of DMSO, a small amount of DMPO-methyl is seen as an artifact of DMPO metabolism (Figure 1, reference 4). It is possible, however, that we are only detecting a portion of all the DMPO-methyl that had formed. Accordingly, a series of experiments were conducted to examine this hypothesis.[11] Using preformed DMPO–OH and DMPO-methyl we were able to identify rates of superoxide formation at which these spin trapped adducts were reduced to diamagnetic species, and these rates were considerably higher than required in the presence of a thiol (Figure 2). Next, we identified a concentration



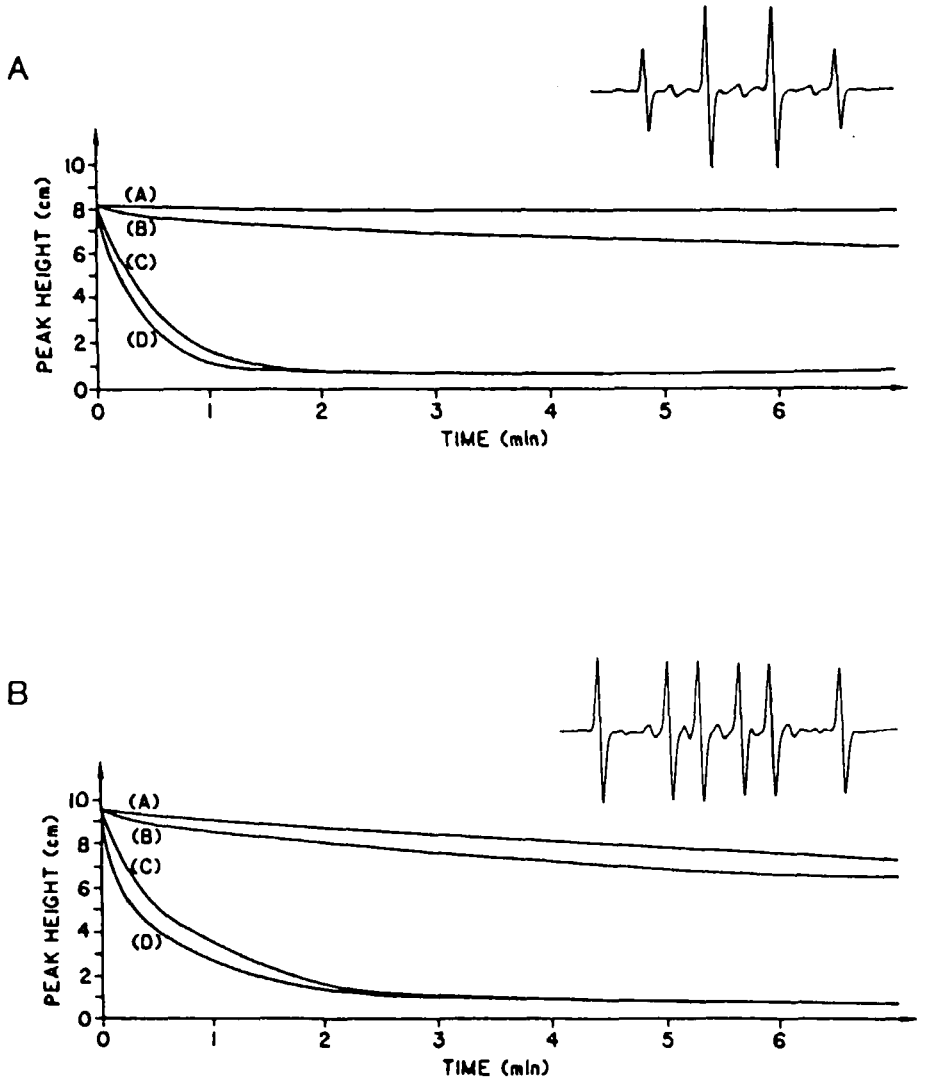


FIGURE 2 A. Effect of superoxide on the stability of DMPO-OH. Tracing (A) represents the peak amplitude of the second low field peak monitoring DMPO-OH formed by UV photolysis of H_2O_2 in the presence of DMPO. In tracings (B) and (C) peak height of DMPO-OH was formed as in A following exposure to superoxide generated at 1 uM/min. (B) and 5 uM/min (C) by the reaction of xanthane oxidase and xanthine. Tracing (D) was obtained under conditions identical to (B) except that cysteine (200 μM) and catalase (300 U/ml) were included. B. Effect of superoxide on the stability of DMPO-methyl. Tracing (A) represents the peak amplitude of the lower field peak of DMPO-methyl formed by UV photolysis of H_2O_2 in the presence of DMPO and DMSO. Tracings (B), (C) and (D) demonstrate the effect of exposure to superoxide as described in Fig 2A. For each set of experiments, microwave power was 20 milliwatts, modulation frequency was 100 kHz with an amplitude of 1 G, sweep rate was 12.5 G/min., response time was 1 second, and receiver gain was 5×10^3 for Figure 2A and 6.3×10^3 for Figure 2B (Reproduced with permission of the *Journal of Biological Chemistry*, reference[11]).

of superoxide dismutase which facilitated spin trapping of hydroxyl radical in an iron catalyzed Haber-Weiss reaction, using a superoxide generating system or human neutrophils. The ability of SOD to enhance spin trapping of hydroxyl radical in this setting appears to be the combined effect of the protection of the appropriate DMPO adducts as well as the accelerated formation of hydrogen peroxide in a setting where Fe^{+2} is available.[5,11] Therefore, we would expect that if hydroxyl radical were produced by stimulated neutrophils suspended in a physiologic (iron free) buffer it would be detected in the presence of an "optimal" concentration of SOD. However, addition of PMA to neutrophils in the presence of varying concentrations of SOD uniformly decreased formation of DMPO-methyl.[11]

More recently it has been suggested that PBN-methyl may not be so susceptible to superoxide-mediated bioreduction,[17] and our results with 4-POBN-methyl (t-butyl alpha-methyl-4-pyrridinyl-N-oxide nitroxide) support this hypothesis.[18] When 4-POBN (1mM) was used to spin trap radicals generated by PMA-stimulated neutrophils in physiologic buffer in the presence of DMSO no ESR spectra was observed, whereas a stable POBN-methyl adduct is generated in an iron catalyzed system.

Since spin trapping allows on-line measurement of radicals, a kinetic view of cellular events can be gleaned. With human neutrophils are stimulated in the presence of Fe^{+3} and DMSO to generate hydroxyl radical, DMPO-methyl is detected in maximal concentration in the initial scan, and decreases thereafter.[19,20] The rate of decrease of DMPO-methyl can be slowed with an anti-lactoferrin antibody. Hydroxyl radical generation is also "prolonged" in PMA stimulated differentiated HL-60 cells which have a neutrophilic oxygen metabolism, but do not make lactoferrin, or in cytoplasts, neutrophils that have been treated to eviscerate their granular contents.[19] Other proteins, such as bovine serum albumin, do not have such an effect. These data suggest that lactoferrin binds iron to prevent hydroxyl radical formation, entirely consistent with recent studies which demonstrate the inability of iron bound to lactoferrin to catalyze hydroxyl radical, except under unusual conditions.[21]

Using deoxyribose oxidation, Winterbourn reported that myeloperoxidase (MPO) was important in limiting neutrophil hydroxyl radical formation.[12] Undertaking a similar study with spin trapping, we noted that azide (which inhibits heme enzymes including MPO) enhanced the magnitude of hydroxyl radical formation in an iron supplemented system.[20] This effect was not observed when azide was added to myeloperoxidase deficient neutrophils eliminating the possibility that the azide effect was related to coincident inhibition of neutrophil catalase. Azide did not effect the duration of hydroxyl radical formation in this system.[20]

Spin Trapping and Human Monocytes

Human monocytes and monocyte-derived macrophages possess a superoxide generating system identical to neutrophils except that one or more components are under dynamic regulation, such that freshly isolated monocytes originally demonstrate vigorous superoxide production which wanes in monocyte-derived macrophages; superoxide production in these cells can be sustained or resuscitated by a variety of activating compounds and cytokines. The most important of these activating cytokines appears to be interferon-gamma.[22] Macrophage activation also drives these cells towards competence to kill a variety of microbidical and tumor targets, some of which appear to be susceptible to reactive oxygen intermediates. Monocytic phagocytes lose their myeloperoxidase in culture, and contain no lactoferrin. However, we have

demonstrated that they acquire a unique isoenzyme of tartrate-resistant acid phosphatase[23] which is structurally similar to uteroferrin and capable of catalyzing hydroxyl radical.[24] DMPO spin trapping experiments similar to those conducted with neutrophils yielded the following results:[25] i) neither monocytes nor macrophages generate hydroxyl radical in the absence of exogenous iron; ii) activation of monocytic phagocytes with interferon-gamma increased the formation of superoxide, manifested as doubling of DMPO-OOH, but hydroxyl radical was not detected; iii) when Fe^{+3} was provided hydroxyl radical was generated. As might be predicted because of the absence of lactoferrin in these cells, the hydroxyl radical signal did not decrease over time as seen with a neutrophil control (Figure 3). These results further emphasize the physiologic importance of myeloperoxidase and lactoferrin to the free radical biology of human phagocytes. Furthermore the results show that the rate of superoxide formation of monocytes was not adequate to interfere with the detection of DMPO-methyl. Using a PBN/DMSO system similar to that described above we have not

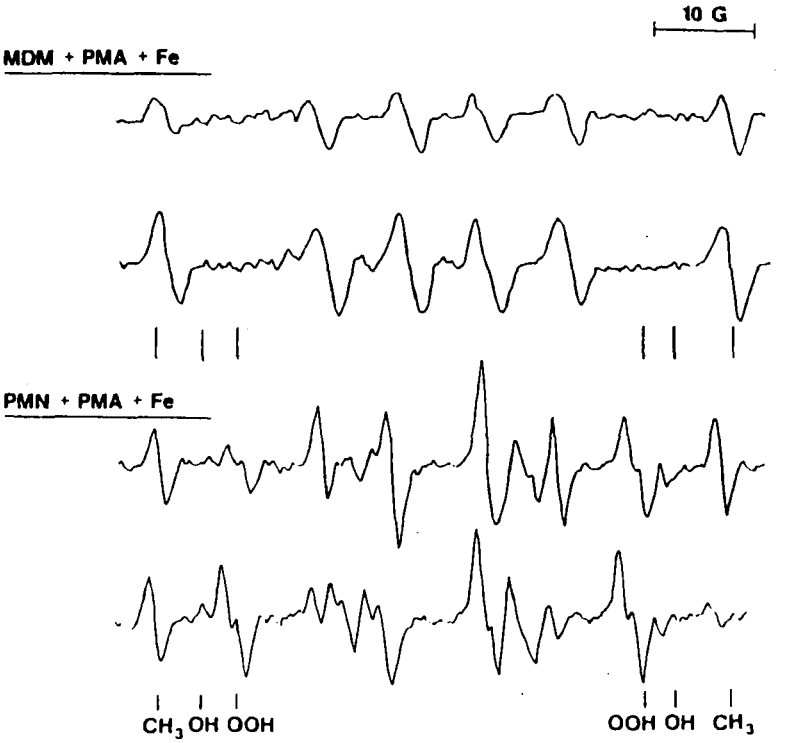


FIGURE 3 Shown are two ESR spectra obtained after stimulation of monocyte-derived macrophages (MDM) with PMA in the presence of iron, DMSO, and DMPO. The top scan was obtained immediately after the addition of PMA and the second seen approximately 30 minutes later. DMPO-methyl remained the only detectable spin adduct consistent with ongoing hydroxyl radical formation. However, when the neutrophils were stimulated under identical conditions, sequential ESR scans obtained over a 12 minute period after PMA addition showed a rapid fall in DMPO-methyl peak amplitudes indicating termination of hydroxyl radical production. In each case microwave power was 20 milliwatts. Modulation frequency was 100 kHz with an amplitude of 1 G. Sweep rate was 12.5 G/min. Response time was 1 s, and the gain was 3.2×10^4 (Reproduced with permission of *The Journal of Experimental Medicine*, reference[25]).

detected PBN-methyl formation by monocytes or monocyte derived macrophages in the absence of exogenous iron.

Spin Trapping Phagocytes and Cellular Compartmentalization

Even though DMPO has a small partition coefficient (0.02, reference[26], several reports have demonstrated its ability to diffuse into cells (reviewed in reference[18]). Phagocyte biologists have been limited in interpretation of intracellular events because of difficulty in studying the phagosome. Generally, studies related to the phagosome involve its disruption with cytochalasin b, which prevents the association of actin filaments required for phagosome closure, but also effects cellular glucose metabolism and one or more components of the respiratory burst. Earlier in our work we used cytochalsin b in our spin trapping studies, and the results suggest that DMPO was entering the phagosome.[5,7] More recently, experiments were conducted to compare the effects of line-broadening agents, ferricytochrome c, and modified SOD on phagosomal spin-trapping.[5] For these experiments we took advantage of the fact that PMA stimulates the extracellular secretion of superoxide, whereas phagocytosed zymosan and other particles would be expected to stimulate the formation of phagosomal superoxide. Neither line-broadening agents nor cytochrome c could be used effectively to eliminate extracellular radicals.[5] With zymosan as a stimulant SOD quenched extra- and intracellular superoxide, consistent with our earlier experience.[7] However, we were able to identify a concentration of polyethylene glycol-modified SOD (PEG-SOD, a large molecular weight compound which retains SOD activity) which eliminated PMA-mediated (extracellular) superoxide, but allowed spin trapping of zymosan stimulated superoxide. Cytochalasin b addition to the system allowed PEG-SOD to eliminate all signals, consistent with our hypothesis that PEG-SOD remains outside the cell, unable to quench the phagosomal superoxide. Although these results confirm the ability of spin trapping to measure cellular events, they do not distinguish between the phagosome and the cytoplasm.

SUMMARY AND CONCLUSION

We believe that the use of spin trapping techniques have made an important contribution to our understanding of the free radical biology of human phagocytes insofar as they demonstrate: 1) that hydroxyl radical is not a physiologic or reactive oxygen intermediate generated by neutrophils or monocuclear phagocytes; 2) that the major role of lactoferrin may be its ability to limit hydroxyl radical formation in tissues during inflammation, by chelating iron that might otherwise become available for catalysis of hydroxyl radical; 3) that monocytic phagocytes have a greater capacity for sustained iron-catalyzed hydroxyl radical generation than do neutrophils. However, it should be noted that monocytes bind lactoferrin,[27] and very recent experiments conducted in our laboratories suggest that exposure to lactoferrin may limit the potential for hydroxyl radical formation by these cells. These studies offer insight into the microbicidal process, and suggest somewhat different strategies for limiting hydroxyl radical mediated inflammation than might be the case if this species were a physiologic product of phagocytic cells.

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