Free Rad. Res., Vol. 34, pp. 81-92
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ESR Detection of Intraphagosomal Superoxide in Polymorphonuclear Leukocytes Using 5-(Diethoxyphosphoryl)-5-Methyl-1-Pyrroline-N- Oxide

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Accepted for publication by N. Taniguchi

(Received June 5, 2000)

We applied a spin trap, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO), to detect $O_2^{\cdot-}$ generation during phagocytosis in human polymorphonuclear leukocytes (PMNs). PMNs were activated with serum-opsonized zymosan (sOZ) in the presence of DEPMPO. The ESR spectra mainly consisted of Cu,Zn-SOD-sensitive DEPMPO-OOH spin adducts. To clarify where these spin-adducts were present, cells after stimulation were separated from extracellular fluid by brief centrifugation and resuspended in Hanks' balanced salt solution. ESR examination showed that DEPMPO-OOH adducts were present in both fractions. When cells were stimulated by phorbol myristate acetate (PMA), the DEPMPO-OOH was detected in extracellular fluid but not in the cell fraction. Furthermore, DEPMPO-OOH adducts were quickly converted into ESR-silent compounds by addition of cell lysate of PMNs. These results indicate that DEPMPO is useful to detect $O_2^{\cdot-}$ of extracellular space including the intraphagosome but not that of intracellular space in sOZ-stimulated phagocytes.

Keywords: ESR, DEPMPO, NADPH oxidase, Phagosome, Polymorphonuclear leukocyte, Superoxide

INTRODUCTION

When polymorphonuclear leukocytes (PMNs) are stimulated with phagocytic particles, bacteria, chemotaxic peptides and phorbol myristate acetate (PMA), they undergo a respiratory burst with an uptake of ambient oxygen resulting in the generation of superoxides ($O_2^{\cdot-}$). This occurs via a single electron transfer mediated by NADPH oxidase[1–8]. It is widely accepted that $O_2^{\cdot-}$ is converted to hydrogen peroxide (H_2O_2) by disproportionation reactions. H_2O_2 is further converted into hydroxyl radicals ($^{\cdot}OH$) by the reactions with trace metals via the Fenton reaction. $^{\cdot}OH$ are harmfully reactive and result in the disruption of the microorganism and host-tissue injury[9].

When PMNs are activated by phagocytotic particles such as opsonized zymosan (OZ) and bacteria, they form phagosomes (phagocytotic

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vacuoles). From histological studies using $CeCl_2$, Ohno *et al.* reported that H_2O_2 accumulated outside the plasma membrane of phagosomes when PMNs were stimulated with OZ or IgG-coated latex particles[10]. The localization of NADPH oxidase components in the phagosome membrane was also reported[11]. The localization of the cytosolic components of NADPH oxidase and the mechanisms of their assembly in the phagosome membrane have been extensively studied[12–14]. Recently, histochemical determination of $O_2^{\cdot-}$ generation using DAB/Mn^{2+} revealed that the soluble stimulants PMA and fMLP produced $O_2^{\cdot-}$ not only on the cell surface but also in intracellular nonphagocytic-vacuoles[15,16]. From these histological and biochemical observations, it seems that $O_2^{\cdot-}$ production in stimulated phagocytes is localized in at least three regions, i.e., the cell surface, phagocytotic vacuole and nonphagocytotic vacuole.

Among several techniques for the detection of transient oxygen radicals in cellular systems, spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) is widely employed to qualitatively and semiquantitatively analyze oxygen radicals produced in phagocytic cells stimulated by OZ, PMA and fMLP[17–24]. However, Finkelstein *et al.* demonstrated that DMPO-OOH adducts generated in OZ-stimulated PMNs are extremely unstable. They found that artificial DMPO-OH adducts are induced by the reactions of DMPO with $^{\cdot}OH$ as their decomposition products[25]. This suggests that it is hard to obtain information about the kinetics of $O_2^{\cdot-}$ and $^{\cdot}OH$ generation as well as their localization in cells by this method, though it is advantageous for the accurate determination of radical structures. Until now, there is no consensus about the place where the spin adducts are produced in activated phagocytes. Recently a new spin trap, the β-phosphorylated nitrone 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO), was developed to trap $O_2^{\cdot-}$, $^{\cdot}OH$, $R^{\cdot}$, $RS^{\cdot}$ and $RO^{\cdot}$[26–29]. A remarkable advantage of this nitrone over DMPO is that the DEPMPO-OOH adduct is much more stable than that of DMPO, with a 15-fold longer half-life.

In the present study, we applied this spin trap to detect $O_2^{\cdot-}$ generated in sOZ-stimulated and PMA-stimulated PMNs. Furthermore, the spin trapping technique combined with separation of the cells by brief centrifugation, described in this communication allowed us to investigate where $O_2^{\cdot-}$ generation occurred in phagocytotic cells.

MATERIALS AND METHODS

Reagents

Zymosan A from *Saccharomyces cervisie*, PMA, Ficol 400 and bovine liver Cu,Zn-SOD were purchased from Sigma Chemical Co., (St. Louis, MO, USA). The spin trap, DEPMPO, was from Oxis International Inc., (Portland, OR, USA). The other chemicals were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Serum was obtained from whole blood of healthy volunteers and the serum-opsonized zymosan (sOZ) was prepared by incubating it in the serum (10 mg in 1.5 ml of serum) at 37°C for 1 h, and then washing it three times with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, HBSS(-). The final concentration of sOZ was adjusted to 10 mg/ml.

PMN Preparation

Peripheral blood of healthy donors was obtained from The Hokkaido Red Cross Blood Center (Sapporo, Japan) and diluted twice by HBSS(-). To collect the leukocyte fraction, 10 ml of 6% dextran was added to 40 ml of diluted blood. The leukocyte-rich supernatant was obtained after incubation at room temperature for 30 min. Thirty milliliters of the leukocyte-rich fraction was placed on 20 ml of a Ficol-Conray solution and centrifuged for 30 min at 2,500 rpm followed by hypotonic lysis of trace erythrocytes. Giemsa stain showed that more than 96% of the cells were PMNs.

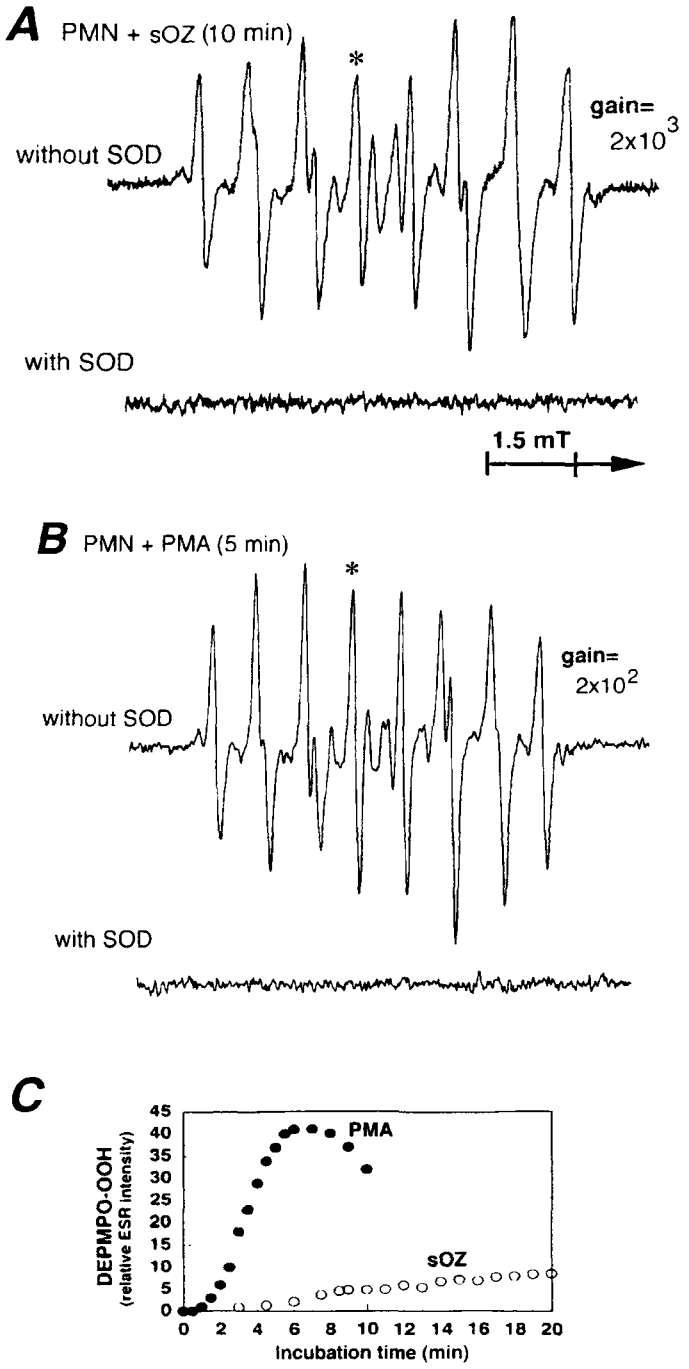

FIGURE 1 ESR spectra from polymorphonuclear leukocytes (PMNs) stimulated with sOZ and PMA. *A*: ESR spectra obtained at 5 min after PMNs ($2x10^7$ cells/ml), sOZ (4 mg/ml) and DEPMPO (10 mM) were incubated without (upper) and with Cu,Zn-SOD (lower). *B*: ESR spectra obtained at 5 min after PMNs ($2x10^7$ cells/ml), PMA (100 mg/ml) and DEPMPO (10 mM) were incubated without (upper) and with Cu,Zn-SOD (lower). *C*: Time course of the intensities of DEPMPO-OOH indicated by asterisk. (•): PMA stimulation, (◦): sOZ stimulation

Spin-Trapping of $O_2^{\cdot-}$ in sOZ-Stimulated and PMA-Stimulated PMNs by DEPMPO

PMN suspension (2.0×10^7 cells/ml) containing 10 mM DEPMPO was stimulated with sOZ (4 mg/ml) or PMA (100 ng/ml) in 100 µl of HBSS containing 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$, HBSS(+), in the presence or absence of 40 mU/ml Cu,Zn-SOD. To distinguish between the extracellular event and the intracellular/intraphagosomal event, brief centrifugation was employed. After PMNs in the presence of DEPMPO were activated in the microcentrifuge tube (1.5 ml) with sOZ or PMA for the indicated periods of time at room temperature, the sample was immediately centrifuged for 30 sec at 6000 rpm. The extracellular fluid was transferred to a new microcentrifuge tube. The cells were resuspended in 200 µl of HBSS(+), and examined together with the extracellular fluid by ESR spectrometry.

ESR Measurements

Cells and the extracellular fluid were separately transferred to a flat quartz ESR cell (LLC-04B, Labotec Co., Tokyo, Japan) fitted to the cavity of an X-band ESR spectrometer (RE-1X, JEOL Co., Tokyo, Japan). The interval from the end of incubation to the start of ESR measurements was 30 sec. The conditions for recording the ESR spectra were follows: time constant = 0.03 sec, scan time = 5.0 mT/min, microwave power = 12 mW, temperature = 23°C. The simulation for the ESR spectrum was performed by using a personal computer (PC9801VM, NEC, Tokyo, Japan) with an isotropic ESR simulation program (ESS-30, Radical Res., Inc., Tokyo, Japan).

Evaluation of the Stability of DEPMPO-OOH

To examine the stability of DEPMPO-OOH, the decay kinetics of DEPMPO-OOH generated by the hypoxanthine/xanthine oxidase (HXn/XOD) system was measured. Phosphate-buffered saline (160 µl) containing 250 µM HXn and 10 mM DEPMPO was prepared and the reaction was started by adding 20 µl of XOD solution (0.5 U/ml). At 2 min after stimulation, 20 µl of 5 mM allopurinol was added to the reaction mixture to inhibit XOD and then the decay of the ESR signal due to DEPMPO-OOH spin adducts was monitored. When the effect of the PMN cell lysate on the decay kinetics of the DEPMPO-OOH adducts was examined, 20 µl of 5 mM allopurinol-containing cell lysate equivalent to $4x10^6$ cells (final conc.: $2x10^7$/ml) was used. Inhibition of XOD activity by allopurinol was confirmed by measurement of oxygen consumption in the sample (YSI 5300., Yellow Spring Instruments, Inc., Yellow Spring, OH, USA).

RESULTS

Spin Trapped Radicals in the Stimulated PMNs in the Presence of DEPMPO

When PMNs ($2x10^7$/ml) were stimulated for 10 min and 5 min by sOZ and PMA in the presence of DEPMPO (10 mM), ESR signals like those shown in the upper panels of Figs. 1A and 1B, were observed. Simulation of these ESR spectra revealed that the major signal observed was that of DEPMPO-OH with a minor amount of DEPMPO-OH (Fig. 2). The hyperfine coupling constants (Hfcs) for DEPMPO-OOH and DEPMPO-OH were quite similar to those reported by Chamulitrat[29]. In the case of OZ stimulation for 10 min, a representative ESR spectrum exhibited 71% DEPMPO-OOH and 29% DEPMPO-OH, Cu,Zn-SOD (40 mU/ml) completely inhibited the production of both DEPMPO-OOH and DEPMPO-OH as shown in lower panels of Figs. 1A and 1B. Neither catalase (1000 U/ml) nor N°-L-monomethyl-arginine (L-NMMA, 100 µM) inhibit the formation of these two adducts (data not shown). The time course of signal intensities indicated by asterisks in upper panels of Figs. 1A and 1B is shown in Fig. 1C. The components indicated by asterisks

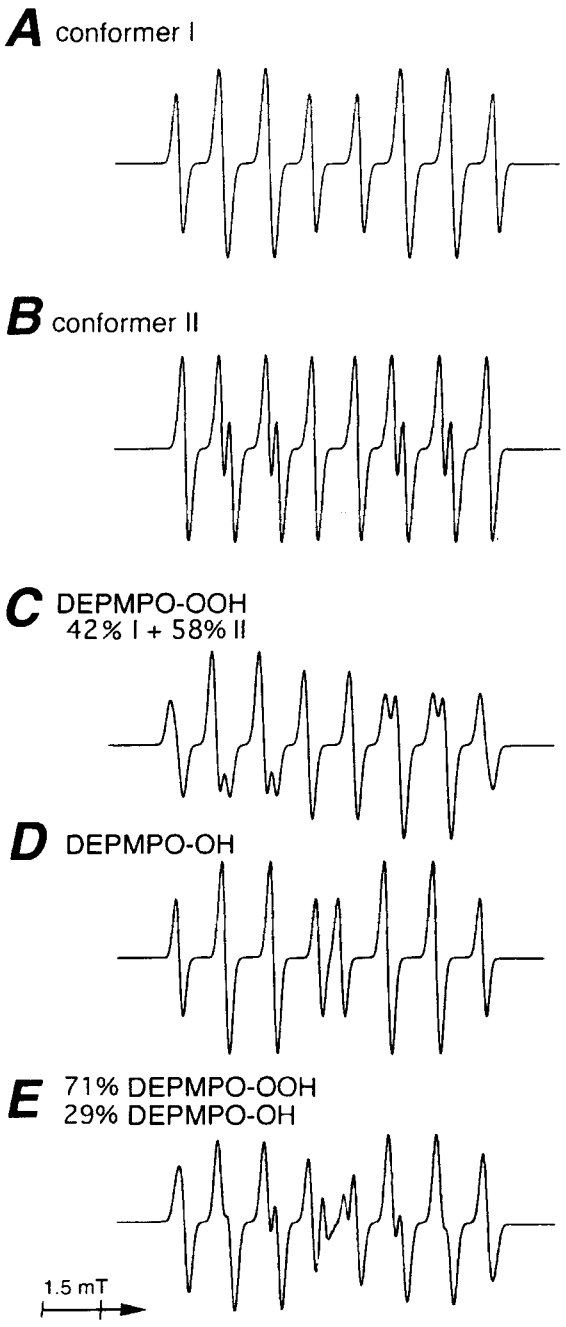

FIGURE 2 Simulated spectra of DEPMPO-OOH and DEPMPO-OH. ***A, B***: Simulated spectra of conformers I and II of DEPMPO-OOH with the following parameters, ***A***: conformer I, a_N=1.34 mT, a_P=5.25 mT, a^{β}_H=1.19 mT. ***B***: conformer II, a_N=1.35 mT, a_P=4.99 mT, a^{β}_H=1.05 mT. ***C***: Simulated spectrum of DEPMPO-OOH, corresponding to an exchange between the two conformers. The reconstruction ratio of the two isomers is 42% conformer I and 58% conformer II. ***D***: Simulated spectrum of DEPMPO-OH with the following parameters, a_N=1.40 mT, a_P=4.74 mT, a^{β}_H=1.30 mT. ***E***: Simulation of experimental spectrum in upper panel of Fig. 1A as a composite of the two species, DEPMPO-OOH (71%) and DEPMPO-OH (29%)

were chosen for time-course analysis because the component due to DEPMPO-OOH did not overlap on that of DEPMPO-OH [29]. The signal intensity of DEPMPO-OOH in sOZ-stimulated PMNs (open circles in Fig. 1C) exhibited a gradual increase for 20 min after stimulation whereas that of PMA-stimulated PMNs (closed circles in Fig. 1C) quickly increased and reached the maximum at 6 min, and then gradually decayed.

Localization of DEPMPO-OOH in the Activated PMNs

To analyze the localization of DEPMPO-OOH adducts in the activated PMNs, we tried to separate the cell fraction and extracellular fluid by short centrifugation (30 sec) after sOZ- or PMA-stimulation of PMNs in the presence of DEPMPO. Figure 3A shows an ESR spectrum obtained when PMNs were stimulated with sOZ for 10 min and oxygen radical species in the presence of DEPMPO. Figures 3B and Fig. 3C show the ESR spectra obtained from the resuspended cell fraction and the extracellular fluid, respectively. About 65% of total spin adducts were observed in the resuspended cell fraction (Fig. 3B), whereas about 35% of spin adducts were present in extracellular fraction (Fig. 3C). On the other hand, when PMNs were incubated for 5 min in the presence of DEPMPO and PMA, an ESR signal that originated from DEPMPO-OOH was mainly produced as shown in Fig. 4A. Figures 4B and 4C show the ESR spectra obtained from the resuspended cell fraction and the extracellular fluid, respectively, after separation by brief centrifugation. DEPMPO-OOH was mainly detected in the extracellular fluid, not in the cell fraction.

Evaluation of the Stability of DEPMPO-OOH

We used HXn/XOD-induced $O_2^{\cdot-}$ adducts as a model system to evaluate the stability of the spin adducts of DEPMPO because the activity of XOD was easily inhibited by adding allopurinol. The oxygen consumption curve of the HXn/XOD system is shown in the left trace of Fig. 5A. The oxygen-consumption rate after addition of XOD was calculated to be about $\Delta[O_2] = -162$ μM/min. Allopurinol (0.5 mM) addition completely inhibited this oxygen consumption after a short time-lag of about 30 sec, suggesting the inhibition of XOD activity (the right trace of Fig. 5A). When DEPMPO was applied to detect HXn/XOD-induced $O_2^{\cdot-}$, the production of DEPMPO-OOH immediately occurred and reached the maximal level at 2 min after addition of XOD (Fig. 5B and upper panel of Fig. 5C). To examine the stability of DEPMPO-OOH, $O_2^{\cdot-}$ production was stopped by adding 0.5 mM allopurinol at 2 min after reaction. The ESR spectrum (in the middle panel of Fig. 5C) at 5 min after allopurinol treatment hardly decayed in comparison with that before allopurinol treatment (in the upper panel of Fig. 5C). During the decay experiments of DEPMPO-OOH, the intensity of DEPMPO-OH was constant for at least 80 min and no DEPMPO-OH was newly produced (data not shown). The decay kinetics showed that the half-life of DEPMPO-OOH was about 68 min (closed circles in Fig. 5D). By contrast, the DEPMPO-OOH was unstable in the presence of PMN cell lysate equivalent to 2×10^7/ml. The intensity of the ESR signal at 5 min after the addition of allopurinol+cell lysate decreased to 24% in comparison with that without cell lysate (the middle and lower panels of Fig. 5C) and the half-life of DEPMPO-OOH in the presence of the PMN cell lysate was estimated to be 2.3 min (open circles in Fig. 5D).

DISCUSSION

In the present experiments combining conventional spin-trapping and separation by brief centrifugation, we detected the DEPMPO-OOH adducts in the extracellular fluid and in the cell

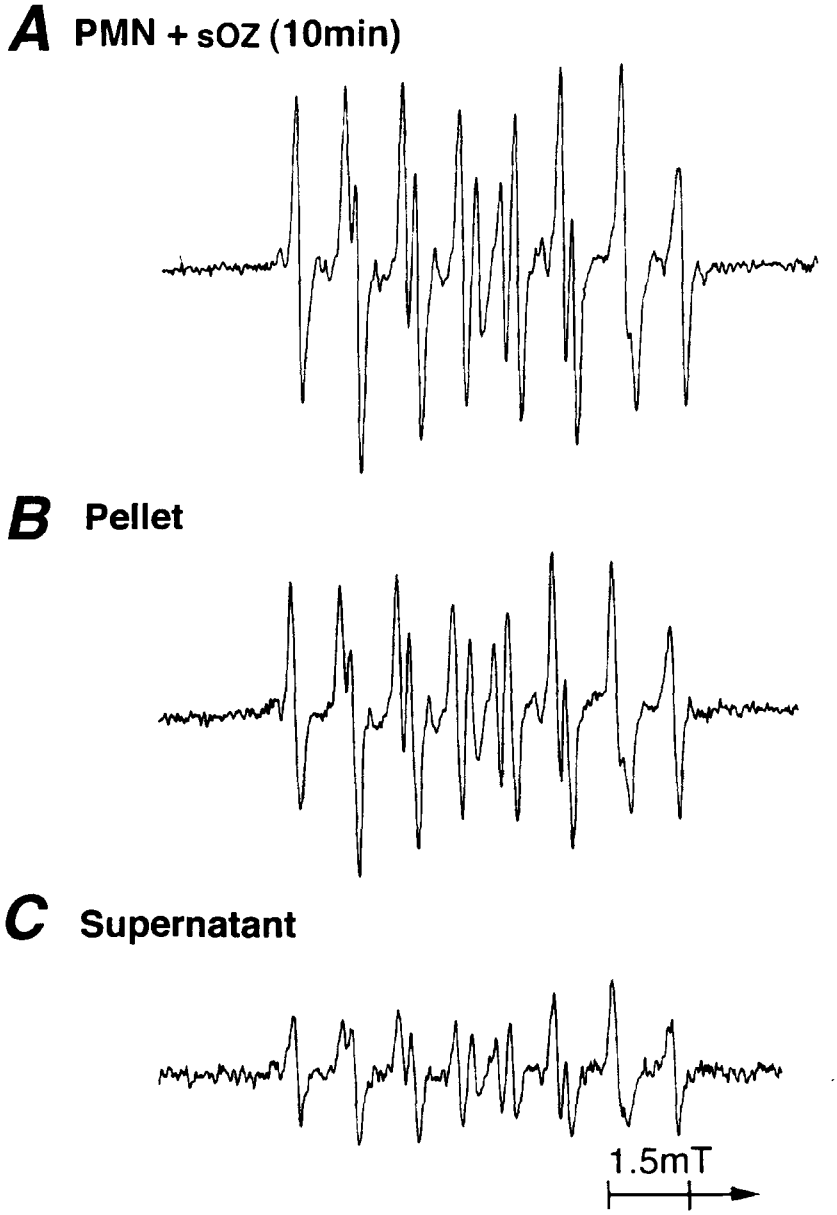

FIGURE 3 Localization of DEPMPO-OOH and DEPMPO-OH adducts in the intraphagosome and extracellular space of sOZ-stimulated PMNs. Cells were separated by brief centrifugation (for 30 sec at 6000 rpm) at 10 min after incubation of PMNs with sOZ (4 mg/ml) and DEPMPO (10 mM). The lag-time between centrifugation and ESR measurement was 30 sec. ESR spectrum *A* was recorded in the same conditions as for the upper panel in Fig. 1A before separation. ESR spectra *B* and *C* were obtained from the cell pellet and the supernatant fraction, respectively. Recording conditions: receiver gain; 2×10^3, modulation width; 0.1 mT, microwave power; 12 mW

fraction in sOZ-stimulated PMNs. It has been reported that the $O_2^{\cdot-}$ forming enzyme, NADPH oxidase, is located on the plasma membrane after stimulation by particles like sOZ, and that

H_2O_2 or $O_2^{\cdot-}$ production is observed at the membrane adherent to the particles inside the phagosome[10–16]. The phagosome is formed in particle-stimulated PMNs, and NADPH oxidase components such as $p47^{phox}$, $p67^{phox}$, $p22^{phox}$ and $gp91^{phox}$ assemble on the phagosomal membrane[13]. Therefore, it is likely that $O_2^{\cdot-}$ is trapped by DEPMPO at the outer surface of the phagosomal membrane as soon as $O_2^{\cdot-}$ emerges from the hydrophobic membrane to hydrophilic intraphagosomal space but not to cytoplasm. The origin of DEPMPO-OOH adducts (35%) observed in extracellular fluid (Fig. 3C) is still unclear, but this extracellular portion of DEPMPO-OOH may be due to trapping of $O_2^{\cdot-}$ from NADPH oxidase activated on the cell surface when attached to sOZ or release of the DMPO-OOH adducts from intraphagosome when degranulated.

Figures 1A and 1B show that not only DEPMPO-OOH adducts but also minor DEPMPO-OH adducts were produced in the stimulated PMN. One possible explanation for the existence of DEPMPO-OH is $^{\cdot}OH$ production by a Fenton-type reaction of H_2O_2. However, this was ruled out because Cu,Zn-SOD, to produce H_2O_2 by disproportionation reaction of $O_2^{\cdot-}$, did not enhance the signal intensity of DEPMPO-OH but completely diminished the ESR signals, and catalase and L-NMMA did not influence the ESR signals. This indicated that the origin of DEPMPO-OH was $O_2^{\cdot-}$ generated by NADPH oxidase and this spin-trapping reaction was independent of nitric oxide. Therefore, it seems possible that the DEPMPO-OH adducts were generated by secondary trapping of $^{\cdot}OH$ formed through the decomposition of DEPMPO-OOH by a reaction similar to that reported in DMPO-OOH by Finkelstein *et al.*[25].

To confirm that superoxides in sOZ-stimulated PMN were produced in phagosomes but not in cytoplasm, we employed a soluble chemical stimulant, PMA, which is a direct activator of protein kinase C and induces NADPH oxidase activation through phosphorylation of $p47^{phox}$ without the formation of phagosomal vesicles[6,15,16]. After stimulation of PMNs with PMA, the spin adducts were observed in the extracellular fluid but not in the cell fraction (Fig. 4). Since soluble stimuli, PMA and fMLP, were reported to produce $O_2^{\cdot-}$ not only on the cell surface but also in the intracellular nonphagosomal vacuoles[15,16] and nitroxide radicals were reported to be easily reduced in cytoplasm[30], our observation seemed to be explained by instability of intracellular DEPMPO-OOH in nonphagosomal vacuoles. To clarify the existence of cytosolic reductants, we compared the stability of DEPMPO-OOH without and with PMN cell lysate. The decay kinetic analysis for DEPMPO-OOH showed that the cell lysate had highly reductive activity for DEPMPO-OOH and converted the spin-adducts to ESR-silent compounds. These results led us to conclude that spin-trapping using DEPMPO could detect oxygen radicals generated in extracellular space including phagosomal vacuoles but not in intracellular nonphagosomal vacuoles in intact cells.

DEPMPO was reported to have an advantage because of its high stability in comparison with other spin traps[26,27], and several investigators successfully detected $O_2^{\cdot-}$, $^{\cdot}OH$, $SO_3^{\cdot-}$, $SO_4^{\cdot-}$ and carbon-centered radicals from various biological systems in vitro by X-band ESR[25–29] and detected in vivo $^{\cdot}OH$ of 5-aminolevulinic acid / Fe^{3+}-treated mice by L-band ESR[31]. In our recent experiments, when DMPO was used instead of DEPMPO to distinguish between phagosomal $O_2^{\cdot-}$ and extracellular $O_2^{\cdot-}$ in sOZ-stimulated PMNs using a similar technique, we observed that DMPO-OOH completely diminished for 30 sec during separation and the DMPO-OH adducts appeared as side products by secondary trapping of OH formed by the quick decomposition of DMPO-OOH[32]. Furthermore, during the decay experiments shown in Fig. 5D and separation experiments presented in Figs. 3 and 4, the intensity of DEPMPO-OH was constant and no more DEPMPO-OH was

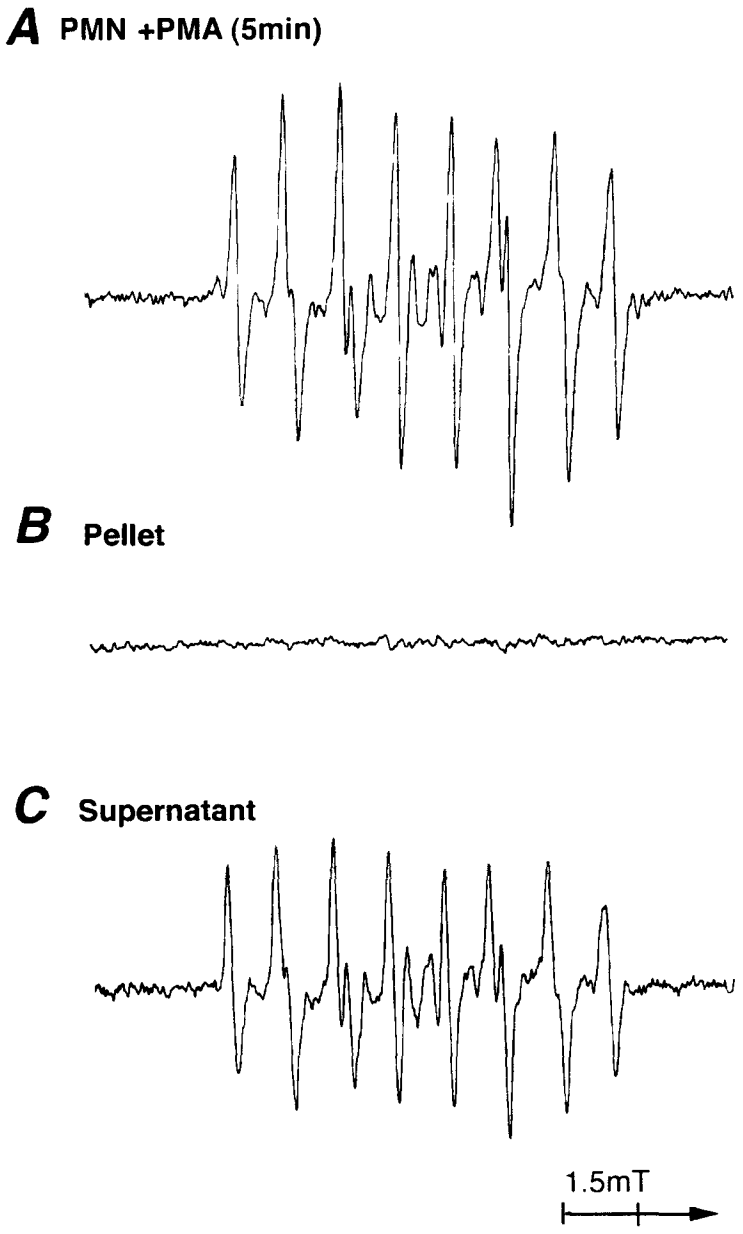

FIGURE 4 Localization of DEPMPO-OOH and DEPMPO-OH adducts in extracellular fluid in PMA-stimulated PMNs. Cells were separated by brief centrifugation (for 30 sec at 6000 rpm) at 5 min after incubation of PMNs with PMA (100 mg/ml) and DEPMPO (10 mM). The lag-time between centrifugation and ESR measurement was 30 sec. ESR spectrum *A* was recorded in the same conditions as for the upper panel in Fig. 1B before separation. ESR spectra *B* and *C* were obtained from the cell pellet and the supernatant fraction, respectively. Recording conditions: receiver gain; 2×10^2, modulation width; 0.1 mT, microwave power; 12 mW

produced, suggesting that the rate of $^{\cdot}OH$ production by the decomposition of DEPMPO-OOH was quite low. Roubaut *et al.* showed that about 50% of superoxides assessed by oxymetry of

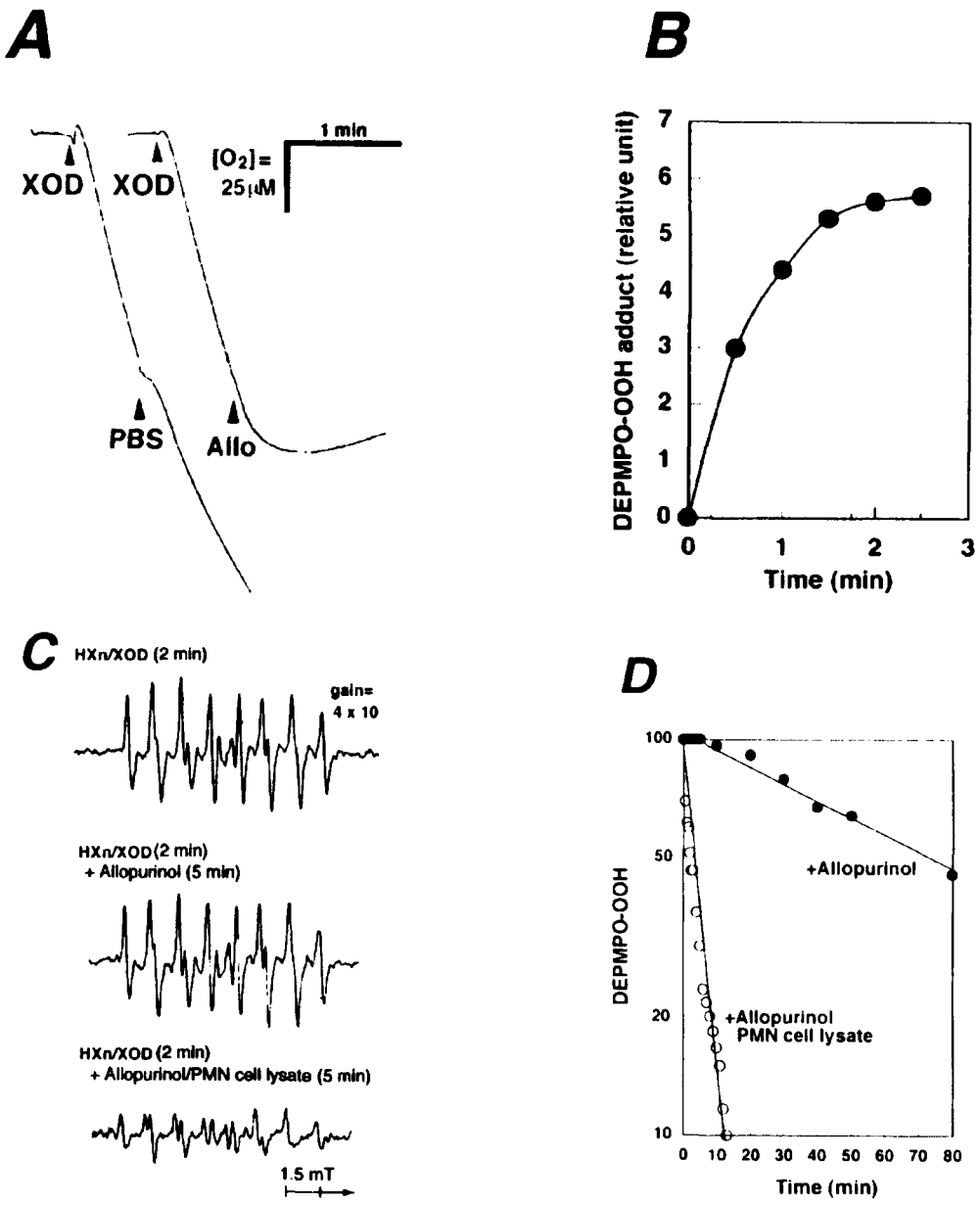

FIGURE 5 Stability of DEPMPO-OOH. *A*: Consumption of O_2 during superoxide generation from the HXn/XOD system. Left trace: PBS as sham control (one-tenth of total volume) was added at 30 sec after the beginning of the reaction. Right trace: 20 μl of 5 mM allopurinol (Allo; one-tenth of total volume; final conc., 0.5 mM) was added 30 sec after the beginning of the reaction. The HXn/XOD system contained hypoxanthine (200 μM) and xanthine oxidase (0.05 units/ml) in PBS. *B*: Time course of production of DEPMPO-OOH in the HXn/XOD system. HXn/XOD reaction mixture containing 10 mM DEPMPO was incubated at room temperature and the intensity of the ESR center signal of DEPMPO-OOH was plotted. *C*: ESR spectra from the HXn/XOD system in the presence of 10 mM DEPMPO. Upper panel: HXn/XOD reaction mixture containing 10 mM DEPMPO was incubated at room temperature for 2 min. Middle panel: Allopurinol was added to HXn/XOD reaction mixture containing 10 mM DEPMPO incubated at room temperature for 2 min and incubated for 5 min. Lower panel: Allopurinol + PMN cell lysate was added to HXn/XOD reaction mixture containing 10 mM DEPMPO incubated at room temperature for 2 min and incubated for 5 min. *D*: Decay kinetics of DEPMPO-OOH adducts. Allopurinol (•) or allopurinol + cell lysate (◦) was added to HXn/XOD reaction mixture containing 10 mM DEPMPO incubated at room temperature for 2 min and the time course of decay of the intensity of the ESR center signal of DEPMPO-OOH was plotted

nitroxide radicals in stimulated PMNs were detected by DEPMPO[28], although DEPMPO-OOH produced inside cells is quickly destroyed by intracellular reductants as shown in this experiment. These facts mean that DEPMPO has high spin-trapping efficiency and high stability in biological systems, and is superior to other spin-trapping reagents such as DMPO, although this spin trap also has a weak point in that the DEPMPO adducts as well as the other nitroxide radicals are susceptible to cytosolic reductants. In summary, we clearly demonstrated that intraphagosomal $O_2^{\cdot-}$ produced in sOZ-stimulated PMNs could be detected by a new spin-trapping technique. The present approach combining DEPMPO and separation by brief centrifugation will be useful to provide unique information about the localization of radical production sites in phagocytes.

Acknowledgements

We thank Dr. O. Yamato (Lab. Vet. Veterinary Internal Medicine, Hokkaido Univ.) and The Hokkaido Red Cross Blood Center (Sapporo, Japan) for helpful support in the preparation of PMNs. This work was supported by Grants-in-Aid for Basic Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, No. 12034203 (to O.I.), No. 11460146 (to H.N.) and No. 12460135 (to M.K.) and by a Grants-in-Aid to Cooperative Research in the Rakuno Gakuen University.

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