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Nitric oxide decreases the stability of DMPO spin adducts

Krzysztof J. Reszka [a,e,*], Michael L. McCormick [b], Garry R. Buettner [c], C. Michael Hart [d], Bradley E. Britigan [a,e]

[a] VA Medical Center-Cincinnati, OH, USA
[b] Department of Internal Medicine, University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA, USA
[c] The Free Radical and Radiation Biology Program of the Department of Radiation Oncology, University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, IA, USA
[d] Department of Internal Medicine, Emory University School of Medicine and Atlanta VA Medical Center, Atlanta, GA, USA
[e] Department of Internal Medicine, University of Cincinnati, 231 Albert Sabin Way, ML 0557, Cincinnati, OH 45267-0557, USA

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Abstract

The effect nitric oxide ($NO^{\bullet}$) on the stability of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) adducts has been investigated using EPR spectroscopy. We report that the DMPO/$HO^{\bullet}$ adduct, generated by porcine pulmonary artery endothelial cells in the presence of H_2O_2 and DMPO, or by a Fenton system (Fe(II) + H_2O_2) is degraded in the presence of the $NO^{\bullet}$-donor, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEANO) or by bolus addition of an aqueous solution of $NO^{\bullet}$. A similar effect of DEANO was observed on other DMPO adducts, such as DMPO/$^{\bullet}CH_3$ and DMPO/$^{\bullet}CH(CH_3)OH$, generated in cell-free systems. Measurements of the loss of DMPO/$HO^{\bullet}$ in the presence of DEANO in aerated and oxygen-free buffers showed that in both of these settings the process obeys first-order kinetics and proceeds with similar efficacy. This indicates that direct interaction of the nitroxide with $NO^{\bullet}$, rather than with $NO_2^{\bullet}$ (formed from $NO^{\bullet}$ and O_2 in aerated media), is responsible for destruction of the spin adduct. These results suggest that the presence of $NO^{\bullet}$ may substantially affect the quantitative determination of DMPO adducts. We also show that $NO_2^{\bullet}$ radicals, generated by a myeloperoxidase/H_2O_2/nitrite system, also degrade DMPO/$HO^{\bullet}$. Because DMPO is frequently used to study generation of superoxide and hydroxyl radicals in biological systems, these observations indicate that extra caution is required when studying generation of these species in the presence of $NO^{\bullet}$ or $NO_2^{\bullet}$ radicals.
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The formation of oxygen-centered free radicals and other highly reactive oxygen species has been implicated in the pathogenesis of many disease states [1–3]. The ability to reliably identify and quantitate such short-lived oxidant species is critical to determining their role in human disease. Over the last three decades, spin trapping, in conjunction with electron paramagnetic resonance spectrometry (EPR), has proven to be a very important experimental technique to accomplish this goal [1,3,4]. Spin trapping involves the addition of a nitrone or nitroso compound (spin trap) to a reaction system [1], which combines with a free radical to form a spin adduct, a nitroxide free radical, with sufficient stability to be detected by EPR. Typically, the EPR spectrum of the spin adduct possesses the hyperfine splitting pattern characteristic of the radical trapped allowing its identification; the concentration of the spin adduct generated is reflected in the EPR spectral peak amplitude [1,5]. Since spin adduct concentration is the net result of not only the rate of spin adduct formation, but also its decomposition, experimental conditions that modify spin adduct stability can confound data interpretation if not adequately addressed in the experimental design.

Since the recognition of the major role of nitric oxide ($NO^{\bullet}$) as a major contributor to both normal human phys-

* Corresponding author. Fax: +1 513 558 0852.
E-mail address: reszkakj@ucmail.uc.edu (K.J. Reszka).

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iology and disease, there has been increasing interest in the consequences of the interaction of NO· with various reactive oxygen species (e.g., superoxide and H_2O_2) in biological systems [6–9]. Both protective and deleterious roles for these reaction products have been identified. Many investigators have turned to the use of chemical compounds that spontaneously decompose to NO· over time (termed NO·-donors) to examine the biologic impact of continuous fluxes of exogenous NO· that likely occur in vivo. NO· is not detected by the spin trapping agents routinely employed in the study of oxygen-centered free radicals such as superoxide and hydroxyl radical (e.g., DMPO, PBN, and POBN) [10,11]. Thus, these spin trapping systems have been employed to investigate the impact of NO· on the generation of these oxygen radicals in biological systems, as well as to detect and quantitate oxygen radicals in systems where NO· is also generated [12–15]. Surprisingly, little attention has been paid to the impact of NO· and/or NO·-donors on the stability of various spin adducts.

We and others have previously shown that H_2O_2 damages endothelial cells [16]. This process appears to involve the generation of hydroxyl radical via the Fenton reaction in which the H_2O_2 reacts with intracellular iron [17]. Previously, one of us found that prior exposure of the cells to NO· results in their protection from subsequent exposure to H_2O_2 [16,18]. Given the link between hydroxyl radical production and H_2O_2-mediated cell injury, we set out to utilize spin trapping methodology to test the hypothesis that the protective effect of NO· resulted from a decrease in H_2O_2-induced formation of hydroxyl radical. The results of these experiments yielded dichotomous results and prompted subsequent experiments that identified important effects of NO· and NO·-donors on the stability of critical spin adducts. The results reported herein identify a major problem in the use of conventional spin traps, particularly 5,5-dimethyl-pyrroline-1-oxide (DMPO), to detect hydroxyl radical and other oxygen-centered free radicals in experimental systems in which NO· and/or NO·-donor compounds are also present.

Materials and methods

Reagents

As the NO·-donor we employed 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEANO)[1] (Molecular Probes, Eugene, OR). The stock solution of DEANO was prepared in 10 mM NaOH and its concentration was checked using $\varepsilon_{250} = 6500\ M^{-1}\,cm^{-1}$ [19]. The spin traps used were DMPO and 4-POBN (Aldrich, Milwaukee, WI). Stock solutions (10 mM each) of hemoglobin (from bovine blood, consisting predominantly of methemoglobin) and $NaNO_2$ (Sigma, St. Louis, MO) were prepared in deionized water. The stock solution of ammonium ferrous sulfate (MCB, Cincinnati, OH) was prepared immediately before use in nitrogen-purged water. MPO, isolated from human neutrophils was a generous gift from Dr. Jerrold Weiss (University of Iowa). Final concentration of MPO in samples was determined to be 3.7 nM based on the absorption of the enzyme stock solution at the Soret band (430 nm) and $\varepsilon_{430} = 9.1 \times 10^4\ M^{-1}\,cm^{-1}$ [20]. The concentration of H_2O_2 (Sigma) was determined using $\varepsilon_{240} = 39.4\ M^{-1}\,cm^{-1}$ [21].

Spin trapping systems

Hydroxyl radicals were spin trapped using DMPO [22]. The DMPO/HO· spin adduct ($a_N = a^{\beta}{}_H = 14.9$ G) is the primary product of the reaction of DMPO with HO· [1,23]. To validate formation of the DMPO/HO· adduct via reaction of DMPO with free HO· radicals, specific HO· scavengers, DMSO and ethanol (CH_3CH_2OH), were used. When HO· radicals react with DMSO or ethanol, methyl ($^{\cdot}CH_3$) and α-hydroxyethyl ($^{\cdot}CH(CH_3)OH$) radicals are formed, respectively. These in turn are trapped by DMPO to yield DMPO/$^{\cdot}CH_3$ ($a_N = 16.4$ G, $a^{\beta}{}_H = 23.6$ G) and DMPO/$^{\cdot}CH(CH_3)OH$ ($a_N = 15.8$ G, $a^{\beta}{}_H = 22.8$ G) spin adducts [1,23]. Thus, detection of DMPO/$^{\cdot}CH_3$ in the presence of DMSO or DMPO/$^{\cdot}CH(CH_3)OH$ in the presence of ethanol, confirms generation of HO· radicals in the system.

To detect HO· radicals, we also employed the 4-POBN system, consisting of 10 mM 4-POBN and 170 mM ethanol [1,24]. Similar to the DMPO system, the $^{\cdot}CH(CH_3)OH$ radicals react with 4-POBN to form a stable spin adduct, 4-POBN/$^{\cdot}CH(CH_3)OH$ with a characteristic splitting pattern ($a_N = 15.5$ G and $a_H = 2.6$ G).

Generation of hydroxyl radical-derived DMPO and 4-POBN spin adducts and assessment of spin adduct stability

In the spin trapping systems described above, hydroxyl radical was generated by combining the spin trapping system with Fe(II) [as $Fe(NH_4)_2(SO_4)_2$] and a 2-fold excess of and H_2O_2 (1:2 mole ratio) in 50 mM potassium phosphate buffer, pH 7.4. All EPR spectra were obtained at room temperature using a Brüker ESP 300 or EMX EPR spectrometers. Unless otherwise noted, instrument settings were: microwave power, 20 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 0.89 G; sweep rate, 0.238 G/min; time constant, 81.92 ms; conversion time, 40.96 ms.

For the time-course experiments, buffer solutions, pH 7.4, containing 38 mM DMPO and 80 μM Fe(II) were initially combined, and H_2O_2 (160 μM final concentration) was added at $t = 0$. After 1 min reaction DEANO (10 or 20 μM final concentration) was added. When required, N_2 gas was bubbled through the sample in the flat EPR

[1] *Abbreviations used:* DEANO, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMPO/HO·, 2,2-dimethyl-5-hydroxy-1-pyrrolidinyloxyl; Hb, hemoglobin; MPO, myeloperoxidase; PBN, α-phenyl-*N*-*tert*-butylnitrone; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl-nitrone; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxyl.

cuvette for 1 min prior to DEANO addition; the DEANO stock solution (2 mL) was de-aerated with N_2 gas for 2 min. EPR scans were executed every 2 min using: microwave power 20 mW, modulation amplitude 1 G, time constant 81.92 ms, conversion time 40.96 ms, and sweep rate 80 G/41.94 s. The receiver gain was 2×10^5.

To study the effect of reagent NO$^\bullet$ on the DMPO/HO$^\bullet$ EPR signal, the latter adduct was prepared as described above. The only difference was that the concentration of DMPO used was 25 mM. One minute after preparation of the DMPO/HO$^\bullet$ adduct, an aliquot (5–50 μL) of the NO$^\bullet$ stock solution in water was added and the sample was incubated for another minute, after which it was transferred to the EPR cell, and its spectrum was measured in 1 min intervals.

NO$^\bullet$ stock solution in deoxygenated water (1.48 mM) was prepared using NO$^\bullet$ gas (99.5%, AGA Specialty Gas, Cleveland, OH). The concentration of NO$^\bullet$ and that of the contaminating nitrite (~260 μM) in the stock solution were checked using luminescence method [25,26]. Under applied conditions, this amount of nitrite should not interfere with the determinations of DMPO/HO$^\bullet$ EPR signals. The data presented in Fig. 6 are from single scans using amplitudes of the second component of the DMPO/HO$^\bullet$ signal, recorded 4 min after the start of the reaction (H_2O_2 addition). Samples for these experiments were prepared in vials with none or minimal head-space to prevent escape of the introduced NO$^\bullet$ from solution.

Generation of $NO_2^\bullet$ radicals

$NO_2^\bullet$ was generated by oxidation of NO_2^- by a MPO/H_2O_2 system [27]. To examine the possible effect of $NO_2^\bullet$ on the DMPO/HO$^\bullet$ EPR signal, the adduct was first generated using the Fenton system, Fe(II) 50 μM and a stoichiometric excess of H_2O_2 (100 μM), in the presence of DMPO (48 mM) over the period of 1 min, after which MPO (3.7 nM) and NO_2^- (50 μM) were added. H_2O_2 remaining after the Fenton reaction was complete was used by MPO to oxidize NO_2^- to $NO_2^\bullet$. The sample was then transferred to the EPR flat cell and the scans were executed in 2-min intervals. Spectra shown are average of two scans. Experiments were repeated at least twice.

Endothelial cells

Porcine pulmonary artery endothelial cells were obtained and maintained in culture as previously described. Briefly, cells were obtained from the main pulmonary artery of slaughterhouse pigs and were placed in 60 mm plastic culture dishes coated with 0.2% gelatin and maintained in culture at 37 °C, 5% CO_2, using MEM-α (Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS, Sigma), 100 U/mL penicillin–streptomycin (GIBCO), 20 μg/mL gentamicin (SoloPak Laboratories, Elk Grove Village, IL) and 2 μg/mL amphotericin B (Pharmacia, Columbus, OH), until primary confluence was reached. Thereafter, the cells were serially passaged. After release into suspension by a brief incubation with trypsin, they were inoculated into new culture dishes and maintained in the same culture medium except that the FBS concentration was decreased to 4%. In all experiments the cells were studied 3–5 days post-confluence. Generation of hydroxyl radical in the cell system was measured upon the addition of H_2O_2 (100 μM) to cells in the presence of DMPO (100 mM). This was assessed following a 30 min incubation period with DEANO (100 μM) or media only prior to the addition of the H_2O_2. The media were then harvested and flash frozen in liquid nitrogen and then stored at −80 °C. Samples remained frozen until the time of EPR measurements.

Results

Effect of NO$^\bullet$-donors on hydroxyl radical formation resulting from the addition of H_2O_2 to endothelial cells

Previous work from one of our laboratories (CMH) has shown that endothelial cells exposed to NO$^\bullet$ via the addition of NO$^\bullet$-donors exhibit a decrease in cellular injury resulting from subsequent exposure to H_2O_2 [16,18]. Among the possible explanations for this observation was that NO$^\bullet$ produced cellular and/or biochemical alterations that resulted in a decrease in H_2O_2-induced hydroxyl radical production. In order to test this hypothesis, we chose to quantitate hydroxyl radical formation using spin trapping methodology. Formation of DMPO/HO$^\bullet$ was assessed 30 min following the addition of 100 μM H_2O_2 to endothelial cell monolayers suspended in HBSS containing DMPO (100 mM) in the presence or absence of 100 μM DEANO. As shown in Fig. 1B, upon the addition of H_2O_2 but in the absence of DEANO, the cells generated a DMPO spin adduct whose hyperfine splitting constants identified it as DMPO/HO$^\bullet$ ($a_N = a^\beta_H = 14.9$ G), the spin adduct expected from the reaction of hydroxyl radical and DMPO. When H_2O_2 was omitted, the DMPO/HO$^\bullet$ EPR signal from cells and DMPO alone was approximately 2.5-fold less intense (Fig. 1A), and no signal was observed from DMPO in a cell free system (Fig. 2, spectrum A). Incubation of the cells in the presence of DMPO, H_2O_2, and DEANO resulted in a dramatic decrease in the magnitude of detectable DMPO/HO$^\bullet$ (Fig. 1D). Also DEANO alone, without the externally added peroxide, when added to cells and DMPO completely abolished the signal (Fig. 1C, compare with 1A).

Effect of an NO$^\bullet$-donor, DEANO, on spin adduct stability

One possible explanation of the effect of DEANO on the EPR signal intensity of DMPO/HO$^\bullet$ in the cellular system was that NO$^\bullet$, released during decomposition of DEANO, shortened the half-life of the DMPO/HO$^\bullet$ spin adduct. In order to test this possibility, DMPO/HO$^\bullet$ was generated using a chemical system (H_2O_2 plus ferrous iron in the

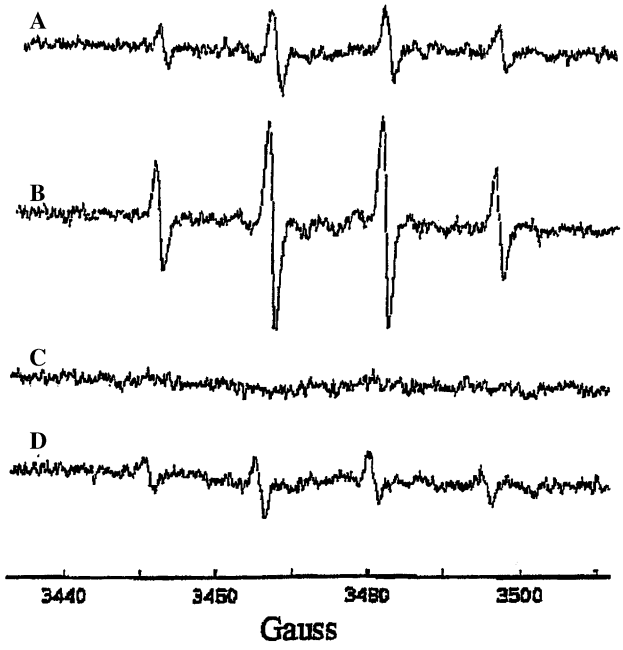

Fig. 1. EPR spectra of a DMPO/HO· adduct generated by endothelial cells. (A) Spectrum from cells and DMPO alone; (B) spectrum generated by incubation of cells in the presence of DMPO (100 mM) and H_2O_2 (100 μM); (C) same as A + DEANO (100 μM); (D) same as B but in the presence of DEANO (100 μM). Cells were incubated at 37 °C for 30 min in the presence of specified reactants, after which EPR spectra were measured (instrument settings as in Material and Methods). Shown are typical results.

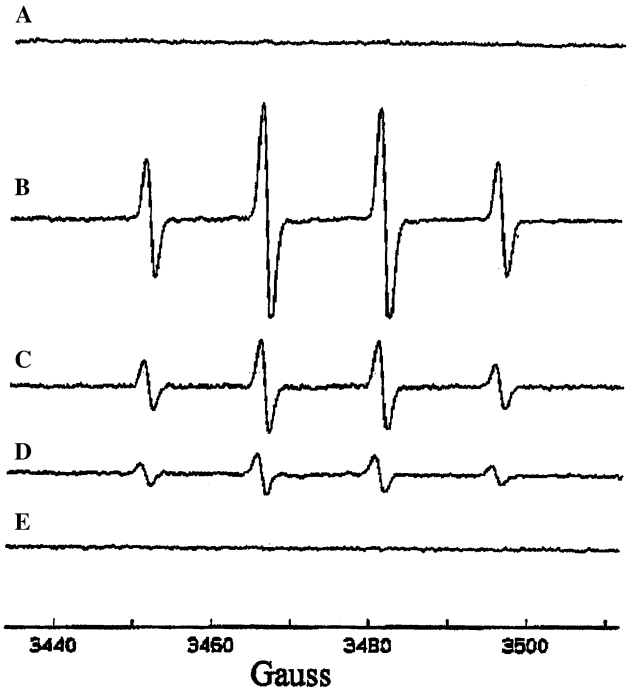

Fig. 2. Effect of DEANO on the EPR spectra of a preformed DMPO/HO· adduct. (A) Control, DMPO (100 mM) alone in pH 7.4 buffer (phosphate, 50 mM); (B) spectrum of DMPO/HO· generated by the Fenton system (40 μM $Fe(NH_4)_2(SO_4)_2$ + 50 μM H_2O_2) in the presence of 100 mM DMPO; (C–E) same as B after the addition of 5, 20, and 100 μM DEANO to the preformed DMPO/HO· adduct. Instrumental settings are given in Materials and Methods. Shown are typical results.

presence of DMPO) and the effect of DEANO on DMPO spin adduct stability was assessed. The presence of DEANO markedly, and in a concentration-dependent manner, decreased the intensity of the DMPO/HO· EPR signal (Figs. 2B–E).

To verify these results, similar experiments were carried out in the presence of DMSO and ethanol as competitive HO· radical scavengers. When DMSO was exposed to the Fenton system in the presence of DMPO, but with DEANO omitted, the EPR spectrum shown in Fig. 3A was detected. It contains contribution from two species identified as DMPO/HO· (labeled "*," $a_N = 14.9$ G, $a^{\beta}_H = 14.9$ G) and DMPO/·CH_3 (labeled "×," $a_N = 16.4$ G, $a^{\beta}_H = 23.6$ G). The presence of the DMPO/HO· component in the spectrum suggests that not all HO· radicals were scavenged by DMSO, but that some escaped and reacted with DMPO to form the adduct. When this experiment was repeated in the presence of DEANO, the EPR spectrum of DMPO/HO· was almost completely abolished and only traces of DMPO/·CH_3 were detected (Fig. 3B). This indicates that not only DMPO/HO·, but also DMPO/·CH_3 and possibly DMPO adducts of other carbon-centered radicals can be affected by DEANO. No EPR-detectable species were observed in the absence of preformed DMPO/HO·, DMPO/·CH_3, or DMPO/·CH(CH_3)OH. Also, when DEANO was added to DMPO no EPR signal was observed (not shown), indicating that DMPO does not form any stable adducts with DEANO or DEANO degradation products.

Ethanol is another efficient HO·-scavenger. The reaction is the source of the α-hydroxyethyl radical (·CH(CH_3)OH), which adds to spin traps (DMPO or 4-POBN) to produce characteristic EPR spectra. Because the combination of 4-POBN (10 mM) in conjunction with ethanol (170 mM) has been previously shown to be more sensitive to HO· than the DMPO and ethanol trapping system [24], we used the former combination (4-POBN and ethanol) as an alternative approach to examine the effect of DEANO on detection of HO· radicals. We found that in the presence of 0.1 mM DEANO the signal amplitude of the 4-POBN/·CH(CH_3)OH adduct decreased by approximately 50% (Figs. 4A and B). In contrast, under similar conditions, the corresponding DMPO/·CH(CH_3)OH adduct

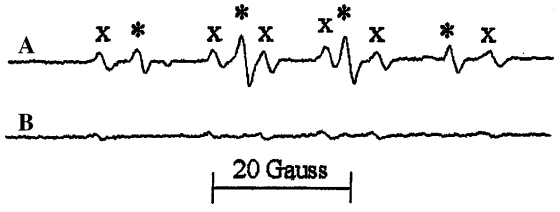

Fig. 3. Effect of DEANO on the EPR spectra of preformed DMPO/HO· and DMPO/·CH_3 adducts. (A) Spectra generated by the Fenton system ($Fe(II) + H_2O_2$) in the presence of DMSO (140 mM) and DMPO (100 mM) in pH 7.4 buffer (phosphate, 50 mM); (B) same as A following the addition of DEANO (100 μM) to the system containing the preformed DMPO adducts. Shown are typical results.

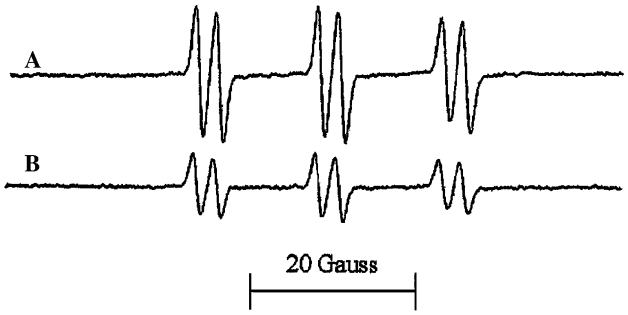

Fig. 4. Effect of DEANO on the EPR spectra of preformed POBN/·CH(CH_3)OH. (A) Spectrum generated by the Fenton system (Fe(II) + H_2O_2) in the presence of ethanol (170 mM) and POBN (10 mM) in pH 7.4 buffer (phosphate, 50 mM); (B) same as A following the addition of DEANO (100 μM) to the system containing the preformed POBN adduct. Shown are typical results.

was almost completely eliminated (not shown). Thus, it appears that DEANO had less effect on the decay rate of 4-POBN/·CH(CH_3)OH then on the decay of DMPO/·CH(CH_3)OH.

Mechanism of DMPO spin adduct decomposition mediated by DEANO

If DEANO was first allowed to totally decompose ($t > 7$ half-lives) before being added to the preformed DMPO spin adducts, it no longer had the ability to alter DMPO spin adduct stability (data not shown). Thus, the stable breakdown product(s) of DEANO was not responsible for the effect. This supported the possibility that NO·, produced continuously during DEANO thermal decomposition could be responsible for the loss of DMPO adducts. Accordingly, we investigated the effect of a NO· trap on DMPO spin adduct stability. Inclusion of the NO· scavenger hemoglobin (50 μM) in the reaction mixture offered a substantial protection against the DEANO-mediated destruction of DMPO/HO· (Fig. 5). Furthermore, as shown in Fig. 6, addition of pure NO· (aqueous solution), resulted in a concentration-dependent decrease of the DMPO/HO· EPR signal intensity.

Because in aerated media NO· reacts with O_2 to form the nitrogen dioxide radical (NO_2·) [28], which also reacts with nitroxides (see below), a possibility existed that this radical too may play a role in DMPO/HO·-degradation. Tests with hemoglobin or NO· gas described above could not eliminate such a possibility. Because in de-aerated buffers formation of NO_2· should be substantially suppressed, we measured time course of the DMPO/HO· EPR signal decay in aerated and N_2-bubbled buffers (pH 7.4), following the addition of DEANO (20 μM). It may be seen in Fig. 7 that when DEANO was omitted, the EPR signal intensities of DMPO/HO· in aerated (■) and N_2-bubbled (▲) buffers were similar and they did not change appreciably during 15 min observation. However, when DEANO was present, the DMPO/HO· EPR signal decreased in both aerated (●) and air-free (▼) buffers. The experimental amplitude versus time data fitted the first-order kinetics with k', the observed first-order rate constant, of 0.20 and 0.23 min^{-1} for air-saturated and air-free solution, respectively. This shows that under conditions where NO_2· could be formed, the loss of the adduct is nearly the same as in air-free buffer where NO_2· could not be generated, and in fact it is even slightly faster under N_2. This result suggests that in both aerated and deaerated buffers the loss of DMPO/HO· is chiefly due to a direct interaction between NO· and the nitroxide, and that NO_2·, if formed, plays a minor role in this process. Based on electrochemical measurements in the absence of nitroxide we estimated that upon introduction of 5 or

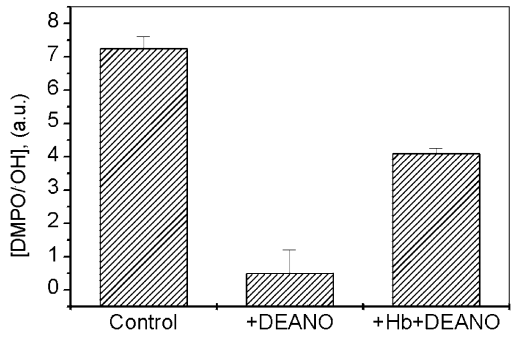

Fig. 5. Hemoglobin protects DMPO/HO· adduct against its degradation by DEANO. DMPO/·OH was generated using the Fenton system followed by the addition of DEANO (100 μM) to the preformed adducts in the absence and presence of 50 μM hemoglobin (columns B and C, respectively). Column A shows the EPR amplitude from a control sample (DEANO and Hb omitted). All signals were recorded 280 s after the start of the reaction. $n = 2$.

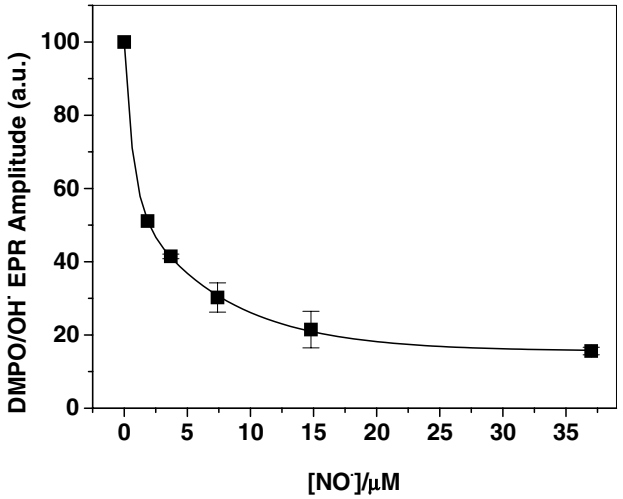

Fig. 6. Effect of NO· on the EPR signal of the DMPO/HO· adduct. DMPO/HO· was generated using the Fenton system in pH 7.4 buffer (phosphate, 50 mM), (Fe(II) 50 μM + H_2O_2 70 μM) in the presence of DMPO (25 mM). One minute after the start of the reaction (H_2O_2 addition), NO· (from 1.48 mM NO· stock solution in water) was added to final concentrations of 1.85, 3.70, 7.40, 14.8, and 37.0 μM NO·. The graph was prepared using the EPR amplitudes of the second component of the spectra whose recording started 2 min after addition of NO·. $n = 2$. a.u., Arbitrary units.

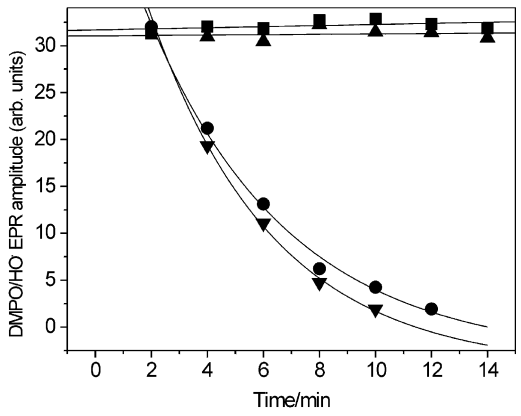

Fig. 7. Time course of decay of the DMPO/HO· EPR signal: in aerated pH 7.4 buffer in the absence (■) and presence (●) and of DEANO, and in de-aerated buffer in the absence (▲) and presence (▼) of DEANO. DMPO/HO· was generated by addition to buffer containing DMPO (38 mM) of Fe^{2+} (80 μM) and H_2O_2 (160 μM). This was followed by addition of DEANO (20 μM). Plotted are the low field amplitudes (arbitrary units) versus time. $n = 2$. Error bars are omitted for clarity.

20 μM DEANO the concentration of NO· increased and within ~5 min reached a steady-state level of approximately 0.7 or 1.4 μM, respectively. This near steady-state condition for [NO·] is the consequence of the reaction of released NO· with dissolved O_2 to produce NO_2·.[2]

Reaction of DMPO/HO· with NO_2·

To find out whether DMPO/HO· may be degraded by NO_2·, we generated this adduct chemically and then exposed it to the NO_2·-generating system consisting of MPO, H_2O_2, and NO_2^- [27]. Very low concentrations of MPO, H_2O_2, and NO_2^- had to be used, to avoid a rapid generation of DMPOX, a DMPO oxidation product [29,30]. Fig. 8 shows that this NO_2· generating system causes a gradual decrease of DMPO/HO· signal intensity. Within less then 4 min after the start of the exposure to NO_2·, the signal of DMPO/HO· was abolished and was replaced by a signal from DMPOX ($a_N = 7.30$ G, $a^{\beta}_H = 4.06$ G, 2H) (Figs. 8D and E). Fig. 9 shows the time course of the DMPO/HO·-decay in the absence (line a) and presence of either MPO (NO_2^- omitted) (line b), or MPO and NO_2^- (line c). It can be seen that while the signal amplitude of DMPO/HO· alone was stable during 15 min observation period (Fig. 9, line a), MPO caused some decay of DMPO/HO· (Fig. 9, line b), apparently using H_2O_2 remaining from the Fenton system to oxidize the nitroxide. This observation is in agreement with earlier reports on the effect of MPO and MPO/H_2O_2 on the stability of DMPO adducts [30]. However, the addition of nitrite markedly accelerated the process of DMPO/HO·-decay (line c). This

[2] These NO· levels are estimates based on measurements of NO· in aerated PBS buffer (pH 7.4) at 22 °C using a WPI Apollo 4000 electrode system over the period of 20 min after the addition of 10 μM DEANO (data not shown).

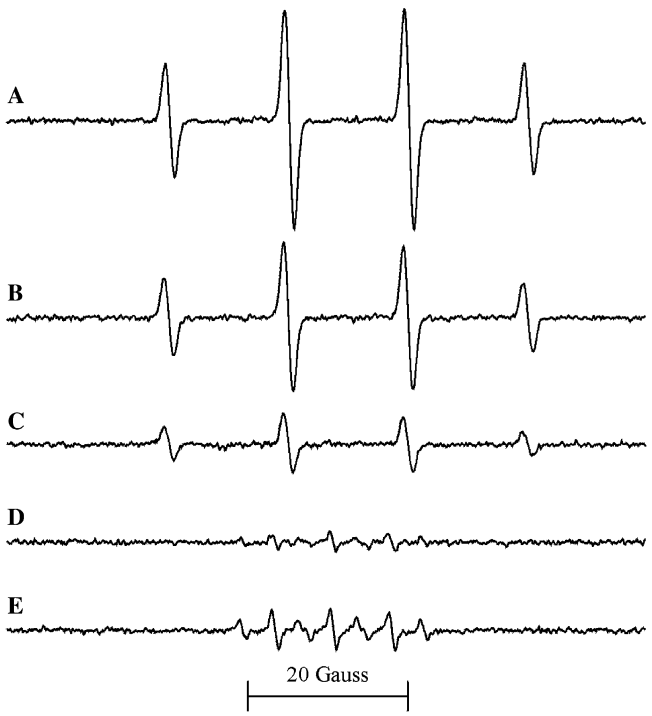

Fig. 8. Effect of NO_2· on the stability of DMPO/HO·: (A) EPR spectrum generated by the Fenton system (Fe(II) 50 μM + H_2O_2 100 μM) in the presence of DMPO (48 mM). (B–E) Same as A but after the addition of MPO (3.7 nM) and NO_2^- (50 μM) to the preformed DMPO/HO·, 1 min after the start of the reaction (H_2O_2 addition). Spectra shown (average of 2 scans) were recorded 1 min after the start of the generation of NO_2· (spectrum B) and then in 2 min intervals (C–E) and represent typical results. The decreasing intensity of the EPR signal amplitude (spectra A–C) indicates that NO_2· causes a gradual loss of the DMPO/HO· adduct. Spectra D and E show that when DMPO/HO· was completely degraded, a new radical, DMPOX, was formed.

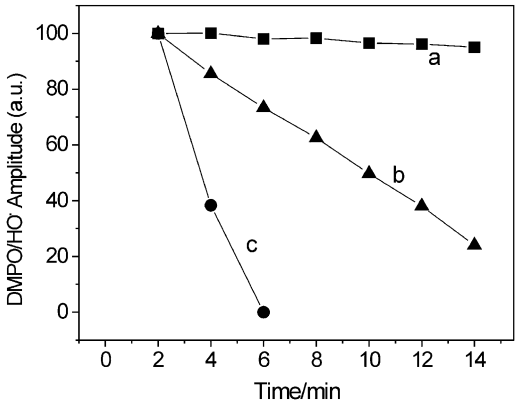

Fig. 9. Effect of NO_2· on the stability of DMPO/HO·: time course of the adduct decay. Data are from experiments similar to those described in Fig. 8. Line (a) –■–: spontaneous decay of DMPO/HO·; line (b) –▲–: decay of DMPO/HO· after MPO (3.7 nM) addition. The faster rate of the DMPO/HO· decay suggests that the nitroxide is metabolized to an EPR-silent product by MPO using remaining H_2O_2; line (c) –●–: decay of DMPO/HO· after MPO and NO_2^- (50 μM) addition. The large increase in the rate of decay of DMPO/HO· after the addition of NO_2^- suggests that NO_2·, generated by the MPO/H_2O_2/NO_2^- system, also degrades the nitroxide. $n = 2$. a.u., Arbitrary units.

result strongly suggests that the $NO_2^{\bullet}$ may be involved in degradation of DMPO/HO$^{\bullet}$ and, perhaps, other DMPO adducts as well.

Discussion

Reactive oxygen and nitrogen species are generated simultaneously at many tissue sites in vivo. Hence, there has been much interest in the chemical reactions that can occur among these various chemical species, as well as their impact on biological systems [12–15]. Inherent in investigation of these events is the need to detect and quantitate oxygen-centered free radical species such as hydroxyl radical in these systems. Among the most commonly used methods for oxygen-centered free radical detection is spin trapping [1,3,4].

The results of the current study indicate that the sensitivity of spin trapping systems, exploiting DMPO for the detection of hydroxyl radicals, may be dramatically decreased in the presence of an NO$^{\bullet}$-donor (DEANO), an agent commonly used in experimental biology to generate continuous fluxes of NO$^{\bullet}$ and, possibly, during the metabolic generation of NO$^{\bullet}$. Additional experiments indicated that it is the production of NO$^{\bullet}$ itself that is directly responsible for the rapid decay of the DMPO spin adducts. This conclusion is supported by the following observations: (1) the loss of DMPO adducts showed dependence on (DEANO); (2) degraded DEANO was unable to affect preformed DMPO adducts; (3) hemoglobin, a NO$^{\bullet}$-scavenger, partially protected DMPO/HO$^{\bullet}$ from degradation by DEANO; (4) bolus addition of a NO$^{\bullet}$ aqueous solution caused the DMPO/HO$^{\bullet}$ spin adduct to decay in a concentration-dependent manner; and (5) in the presence of DEANO, the time course of the loss of DMPO/HO$^{\bullet}$ was nearly the same in aerated and oxygen-free buffers.

In the present work, we show that not only DMPO adducts generated in model systems (Fenton system + DMSO/ethanol), but also POBN adducts appear to be sensitive to DEANO, although to a lesser extent (Fig. 4). The ability of DEANO to affect the detection of the 4-POBN spin adduct in the cell system could not be assessed, since we previously found that addition of H_2O_2 to endothelial cells in the presence of 4-POBN and ethanol (or DMSO) does not generate the respective spin adducts [31]. Therefore, these experiments have not been carried out.

We do not know the exact mechanism whereby NO$^{\bullet}$ leads to the loss of spin-adduct signal intensity. We assume that it might involve a direct reaction between NO$^{\bullet}$ and the spin-adducts' nitroxide moiety, since NO$^{\bullet}$, being a radical tends to react avidly with other radical species. There is some precedence for the possibility of the reaction of NO$^{\bullet}$ with the >NO$^{\bullet}$ moiety of the spin adducts. For example, nitronyl nitroxides, compounds frequently used to detect NO$^{\bullet}$, react in such fashion, forming a different nitroxide and $NO_2^{\bullet}$ radical [32,33]. In this study, we found that addition of DEANO to DMPO/HO$^{\bullet}$ in N_2-bubbled, pH 7.4, buffer also caused loss of the DMPO/HO$^{\bullet}$ EPR signal with efficacy similar to that in aerated buffer. This suggests that direct interaction of the nitroxide with NO$^{\bullet}$ may be responsible for consumption of the spin adduct. However, other studies show that under anaerobic conditions NO$^{\bullet}$ does not react chemically with some cyclic nitroxides (such as 3-carbamoyl-2,2,5,5,-tetramethyl-3-pyrroline-1-yloxy, CTPO), but causes line broadening similar to that induced by molecular oxygen [34]. Thus, the structure of the nitroxide may predetermine its susceptibility to degradation by NO$^{\bullet}$. That in our systems the loss of the EPR signal of the DMPO/HO$^{\bullet}$ adduct, following its reaction with NO$^{\bullet}$, is due to degradation of the adduct and not merely due to the line broadening, was evidenced by the observation that bubbling N_2 gas through the sample did not recover the EPR signal (not shown), which would be expected if NO$^{\bullet}$ interacts only physically with the nitroxide.

We also considered the possibility of degradation of DMPO/HO$^{\bullet}$ by $NO_2^{\bullet}$. In aerated solutions NO$^{\bullet}$ reacts with O_2 to give $NO_2^{\bullet}$, a powerful oxidant, that is known to convert nitroxides to EPR silent oxoammonium cations [35,36]. It was therefore likely that $NO_2^{\bullet}$, produced in aerated buffers, could react with DMPO/HO$^{\bullet}$ causing its degradation. We verified this hypothesis by exposing preformed DMPO/HO$^{\bullet}$ adduct to the $NO_2^{\bullet}$-generating system consisting of MPO, H_2O_2, and NO_2^- [27]. We found that, indeed, in the presence of $NO_2^{\bullet}$, the lifetime of the adduct was dramatically shortened. The DMPO/OH$^{\bullet}$ EPR signal decreased over the period of ~4 min reaction time, after which it was replaced by a signal from DMPOX (Figs. 8 and 9). This supports the speculation, that $NO_2^{\bullet}$, if generated in our systems from NO$^{\bullet}$ and O_2, could contribute to the spin adduct degradation. We emphasize, however, that results of our anaerobic experiments with DEANO clearly show that NO$^{\bullet}$ alone suffices to consume DMPO/HO$^{\bullet}$ adducts.

The ability of NO$^{\bullet}$ and/or NO$^{\bullet}$-derived products to suppress the detection by EPR and DMPO adducts has been reported. For example, it was found that peroxynitrite, the reaction product of NO$^{\bullet}$ and superoxide, shortens the half-life of DMPO spin adducts [37]. However, DEANO is not capable of generating peroxynitrite on its own. Also superoxide, although capable of decreasing DMPO spin adduct' stability [38–41], is not generated by DEANO. A similar inhibition by nitrogen oxides of the formation of DMPO/HO$^{\bullet}$ was reported to occur during the photolytic degradation of nitrite, a known source of HO$^{\bullet}$ [42].

Regardless of the chemical reactions responsible for the above observations, our data reiterate the need for careful attention to the effects of chemical reagents on spin adduct stability in order for proper interpretation of spin trapping data. As reflected by our endothelial cell experiments, the presence of NO$^{\bullet}$ and, as described by others, peroxynitrite and nitrogen dioxide [37], can profoundly decrease the ability of DMPO spin trapping systems to detect the formation of hydroxyl radical. Although not studied in this work, it could be speculated that NO$^{\bullet}$ also shortens the half-life of the superoxide-derived DMPO spin adduct, DMPO/HOO$^{\bullet}$,

either directly, via interaction with the nitroxide, or indirectly, through peroxynitrite, which would likely be formed from $NO^{\bullet}$ and $O_2^{-\bullet}$. If so, then there would be requirement for low arginine substrate availability (and resulting decreased rate of $NO^{\bullet}$-production) in order to spin trap superoxide generated by the various $NO^{\bullet}$ synthases [14,43,44].

In the present study, hydroxyl radical-derived spin adducts of 4-POBN (POBN/$^{\bullet}CH(CH_3)OH$) were less susceptible to $NO^{\bullet}$ donor-mediated decomposition. The spin adducts of the newer nitrone spin trap DEPMPO are also reportedly more stable than DMPO spin adducts [45]. Hence, 4-POBN, and perhaps DEPMPO, spin trapping systems should be preferentially employed for the detection of hydroxyl radical in biological systems in which $NO^{\bullet}$ production is also likely to be occurring. Both the literature and our own data suggest that the effect of $NO^{\bullet}$ on the stability of a nitroxide depends on the structure of the latter, with DMPO adducts being particularly sensitive.

We emphasize that although our study demonstrates the effect of $NO^{\bullet}/NO_2^{\bullet}$ on pre-formed DMPO and POBN adducts, their effect on the detection by spin trapping of radicals generated in cells in situ is also likely to be inhibitory given the very high rate constants for the reaction of $NO^{\bullet}/NO_2^{\bullet}$ with many radicals of biological interest.

Summary and conclusions

DMPO, the spin trap commonly used for the detection of hydroxyl radical gives rise to adducts, which are readily converted to diamagnetic species by $NO^{\bullet}$ most likely through a direct interaction of $NO^{\bullet}$ with the nitroxide moiety of the adducts. This shortens half-life of the DMPO spin adducts, which results in a marked reduction of the spin trapping system's sensitivity for hydroxyl radical detection, and underestimation of the yield of the radical generation. We also demonstrate that $NO_2^{\bullet}$, a product of aerobic oxidation of $NO^{\bullet}$, also degrades DMPO/$HO^{\bullet}$. The importance of the careful assessment of spin trap adduct stability under experimental conditions employed in free radical biology studies is often under-appreciated. Failure to control for this potential in experimental design can lead to misinterpretation of data.

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