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NITRIC OXIDE INTERACTION WITH LACTOFERRIN AND ITS PRODUCTION BY MACROPHAGE CELLS STUDIED BY EPR AND SPIN TRAPPING

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The production of nitrate (NO_3^-) and nitrite (NO_2^-) from macrophage-derived NO· was studied using EPR and spin trapping. The formation of NO_3^- was determined via EPR in reactions involving the iron-binding protein, lactoferrin. The formation of NO_2^- was determined via EPR/spin trapping in the reaction between NO_2^- and H_2O_2. Dissolved nitric oxide (NO·) was reacted with lactoferrin yielding an EPR spectrum (77° K) different from the normal EPR spectrum obtained for lactoferrin, suggesting that NO· interacts with the ferric ions bound to lactoferrin forming a ferric-nitrosyl type complex. The EPR spectrum (77° K) of this ferric-nitrosyl type complex was also observed in the supernatant fluid of macrophage cell suspensions following their stimulation with lipopolysaccharide (LPS). During LPS *stimulation of macrophages*, these cells generate NO· which in turn produces NO_3^- and NO_2^-. The ferric-nitrosyl type complex is formed in a reaction mixture containing apolactoferrin and bicarbonate following the reaction of Fe^{+2} with NO_3^-, generated from macrophage-derived NO·, to produce Fe^{+3} and NO·. Furthermore, in an acidic medium, NO_2^- reacts with H_2O_2 forming peroxynitrous acid (HOONO) which rapidly decomposes into hydroxyl radicals (·OH) and the nitrogen dioxide ($NO_2^·$) radical. In the supernatant fluid of LPS-stimulated macrophage suspensions, the production of ·OH was verified by spin trapping using 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) as the spin trap and ethanol as the ·OH scavenger. The EPR spectra corresponding to the DMPO-OH and the DMPO-hydroxyethyl adducts were identified. These results suggest that the peroxynitrous acid decomposes via the formation of ·OH and $NO_2^·$ and that NO_2^- was formed from macrophage-derived NO·.

KEY WORDS: Nitric oxide, Lactoferrin, Macrophage, EPR, Spin Trapping.

INTRODUCTION

The production of nitric oxide (NO·) by various types of cells and its importance in biology and pharmacology was a focus in recent reviews.[1-3] It is generally thought that the biological synthesis of NO· originates from the N-terminal guanidino group of L-arginine.[3] Although the exact role of NO· in cells remains uncertain, several properties of this molecule are known. First, NO· relaxes vascular smooth muscles in a fashion similar to endothelial-derived relaxing factor (EDRF). Therefore, EDRF and NO· are thought to be identical.[4] However, NO· production by macrophages appears to be involved in cytotoxic or cytostatic

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mechanisms directed toward microorganisms and tumor cells.[5] These mechanisms are thought to be mediated through interactions of NO with transition metals. Electron paramagnetic resonance (EPR) studies have shown that NO binds iron avidily forming an iron-nitrosyl complex in the iron-sulfur centers of various enzymes.[6] However, the cytotoxic selectivity of the macrophage-derived NO toward cells is a central question that remains unclear. One purpose of this work is to determine nitrate (NO_3^-) and nitrite (NO_2^-) production by EPR and spin trapping in suspensions of macrophages producing NO. It is also the purpose of this work to study, using EPR, the interaction of NO with lactoferrin. Lactoferrin, is a bacteriostatic non-heme protein containing two sites with a strong affinity for iron-(III).[7] Lactoferrin was chosen for this study for two reasons: (i) it may provide a simple method for identifying NO production in cells; and (ii) several properties of lactoferrin suggest that this protein may be involved in macrophage-derived NO actions. For instance, peritoneal macrophages contain lactoferrin specific receptors with a large affinity for this protein.[8] Furthermore, although lactoferrin is commonly found in most external secretions of certain mammals, it is also present in neutrophils which are known to produce NO.[9-11] Lactoferrin is found at increased levels in these cells near abscesses and in inflamed tissues.[12-14] Macrophages and neutrophils also produce superoxide (O_2^-) which is known to rapidly react with NO and thought to produce the peroxynitrite anion ($OONO^-$).[15-19] In an acidic environment the peroxynitrous acid (HOONO) is suspected to decompose forming hydroxyl radicals (OH) and the nitrogen dioxide (NO_2) radical.[16] These species are known to be detrimental to cells. Furthermore, $OONO^-$ is also produced in the reaction of H_2O_2 with NO_2^-. H_2O_2 and NO_2^- are, respectively, the product of O_2^- dismutation and a by-product of macrophage-derived NO. Although it has been reported that lactoferrin increases the dismutation of O_2^-,[20] subsequent reports suggest that this may not be the case.[21]

The presence of lactoferrin at inflammatory sites and mucosal surfaces where it may interact with endothelial cell and macrophage-derived NO, in addition to the presence of lactoferrin in neutrophils, its affinity for macrophage cells and its reaction with NO, suggest that this protein may be involved in the mechanism of macrophage-derived NO selective toxicity.

MATERIALS AND METHODS

NO gas was purchased from Matheson Gas Products, Inc. (Fairfield, NJ). Lipopolysaccharide (LPS), Cu,Zn-superoxide dismutase (SOD), bovine lactoferrin, L-arginine and dithizone were obtained from Sigma (St. Louis, MO). Ferrous sulfate, ferric ammonium sulfate, sodium bicarbonate and hydrogen peroxide were purchased from Fisher Scientific Co. (Fair Lawn, NJ). The concentration of hydrogen peroxide was determined by titration with potassium permanganate.[22a] The spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was purchased from Aldrich (Milwaukee, WI) and was verified to be free of radical impurities by EPR. The concentration of DMPO was measured spectrophotometrically ($\lambda = 227$ nm; $\varepsilon = 8 \times 10^3\ M^{-1}\,cm^{-1}$).[23]

Macrophage cells ($P388D_1$) were obtained from American Type Culture Collection (ATCC; Rockville, MD) and were cultured until confluency (37°C) in RPMI-1640 medium (GIBCO; Grand Island, NY). The medium was supplemented with 10% heat inactivated (56°C, 30 min) fetal calf serum. Cells were collected,

centrifuged (1000 rpm, 10 min) and then resuspended in Hanks' balanced salt solution containing Ca^{+2} and Mg^{+2}.

Iron was removed from the lactoferrin by dialysis against citric acid (0.1 M) and the apolactoferrin was then washed against several changes of metal-free water. The concentration of apolactoferrin was determined spectrophotometrically ($\lambda = 280$ nm, $\varepsilon = 1.09 \times 10^5\ M^{-1}\ cm^{-1}$)[24,25]. Metal-free water was prepared by further treating, in a separatory funnel, water obtained from a Sybron/Barnstead NANO-pure system with a solution of dithizone (0.001%) in carbon tetrachloride until the green color persists. HEPES buffer was prepared in the same manner. Following the dithizone treatment the water and buffer were brought to boiling temperature in a water bath to eliminate residual carbon tetrachloride. To eliminate trace metals, all glassware was kept permanently soaking in a 1:1 mixture of concentrated nitric and sulfuric acids prior to use.

Control experiments were done by adding ferric ammonium sulfate (2:1 mole ratio) to apolactoferrin (100 μM) in HEPES buffer (5 mM, pH 7.8) containing an excess carbonate. This anion is required for iron binding to lactoferrin. For the interaction of NO$^{\cdot}$ with lactoferrin, NO$^{\cdot}$ gas was first dissolved by bubbling (1 min) through 2 ml of deaerated water (nitrogen bubbling, 1 hr). An aliquot of this NO$^{\cdot}$ solution was then added to a solution of lactoferrin (100 μM) which was previously prepared in deaerated HEPES buffer (or cell suspension media) and then stirred under a constant stream of nitrogen for several hours. The EPR spectra for the lactoferrin and the lactoferrin/NO$^{\cdot}$ mixture were recorded at 77° K.

Macrophage cells (1×10^7 cells/ml) were first incubated (5 min, 37°C) with L-arginine (500 μM), then 100 Units/ml SOD was added prior to stimulation with 20 μg/ml LPS (10 min, 37°C). The cells were centrifuged and the supernatant fluid was made strongly acidic with sulfuric acid. Ferrous sulfate (100 μM) was added to the solution and allowed to mix for 1–2 min. The pH of the solution was rapidly increased to approximately pH 8 and sodium bicarbonate (200 μM) was added immediately prior to addition of apolactoferrin (50 μM). Following addition of the apolactoferrin, an aliquot was immediately transferred to a quartz EPR tube (1 mm, ID) and frozen in liquid nitrogen (77° K).

For the spin trapping experiments the macrophage cells were stimulated and centrifuged as described above. DMPO (0.2 M, final concentration) and H_2O_2 (1×10^{-4} M, final concentration) were added to the supernatant fluid. The reaction mixture was acidified (pH 3–3.5) with an aliquot of HCl (1 N), rapidly transferred to an EPR quartz flat cell (60 × 10 × 0.25 mm) and the EPR spectrum was immediately recorded. In the experiments requiring the $^{\cdot}OH$ scavenger, ethanol (1.7 M, final concentration) was added to the supernatant fluid containing DMPO and H_2O_2 prior to acidification.

All EPR spectra were recorded on a Varian E-109 X-band spectrometer at 100 KHz magnetic field modulation. The magnetic field was set at: 370.0 mT (lactoferrin) and 338.0 mT (spin trapping); microwave frequency: 9.057 GHz (lactoferrin) and 9.510 GHz (spin trapping); microwave power: 50 mW (lactoferrin) and 20 mW (spin trapping); modulation amplitude: 1.0 mT (lactoferrin) and 0.2 mT (spin trapping); scan range: 200.0 mT (lactoferrin) and 10.0 mT (spin trapping); time constant: 1 s (lactoferrin) and 0.5 s (spin trapping); scan time: 16 min (lactoferrin) and 4 min (spin trapping). Hyperfine coupling constants were obtained by computer simulation generating theoretical EPR spectra matching the experimental spectra including intensity and line widths. This allows accurate computer manipulation of the experimental results.

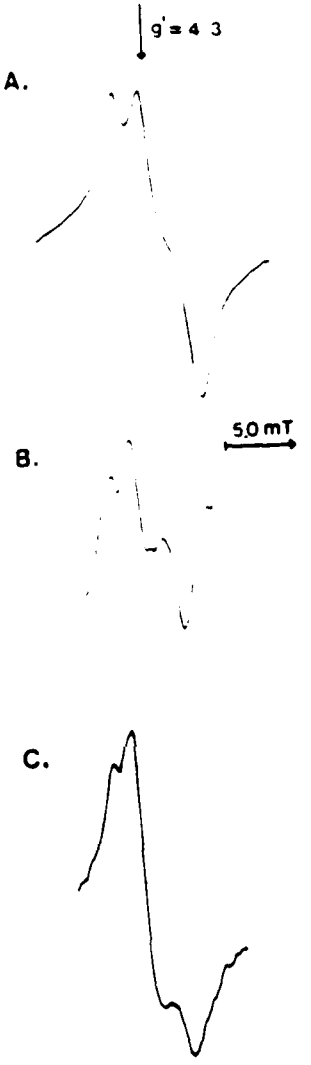

FIGURE 1 EPR spectra of lactoferrin recorded at 77° K. (A) Lactoferrin (5 mM HEPES, pH 7.8); (B) Lactoferrin/NO mixture (5 mM HEPES, pH 7.8); (C) Lactoferrin in supernatant fluid after activation of macrophages (pH 7.9). Receiver gain: 1.25×10^3 for A; 5×10^3 for B; and 8×10^4 for C.

RESULTS AND DISCUSSION

Addition of a 2:1 mole ratio of ferric ammonium sulfate to a solution of apolactoferrin (100 μM) at pH 7.8 (5 mM HEPES) containing excess carbonate, yields at 77° K the EPR spectrum shown in Figure 1A. This spectrum, showing a component at $g' = 4.3$ characteristic of high spin Fe(III) in a rhombic environment, is the typical EPR spectrum observed for lactoferrin.[7] When an aliquot of dissolved NO in deaerated water is added to a deaerated solution of lactoferrin (100 μM) at pH 7.8 (5 mM HEPES), the EPR spectrum in Figure 1A undergoes significant changes indicating that the iron centers in lactoferrin have in some manner been altered.

Although due to the broad nature of the EPR lines and the lack of superhyperfine structure, which would allow a conclusive structural identification of the iron centers, this result (Figure 1B) suggests the interaction of NO· with the iron bound to lactoferrin possibly forming an iron-nitrosyl type complex.

Oxygen in macrophages converts NO· generated by these cells following stimulation to NO_3^- and NO_2^-. Therefore, any NO_3^- present or formed in macrophage suspensions following LPS stimulation can be assumed to result from NO· production. Ferrous ions in acidic solution convert NO_2^- to NO· by the following reaction [Eqn. (1)]:[22b]

$$NO_2^- + Fe^{+2} + 4H^+ \longrightarrow NO\cdot + 3Fe^{+3} + 2H_2O \quad (1)$$

Therefore, supernatants from stimulated macrophage suspensions made acidic with H_2SO_4 should produce Fe^{+3} and NO· upon addition of ferrous ions. Apolactoferrin has no affinity for ferrous ions and in the presence of a synergistic anion (eg. carbonate) only binds ferric ions. Thus, addition of bicarbonate and apolactoferrin to the supernatant fluid after an increase in pH to approximately pH 8 should result in the binding of ferric ions, in addition to, the NO· interaction with the ferric-lactoferrin complex. The results obtained after stimulation of macrophage cells in the presence of L-arginine (500 μM) with LPS (20 μM/ml) are shown in Figure 1C. This figure consists of the EPR spectrum (77° K) obtained after the addition of ferrous sulfate (100 μM) to the acidified supernatant fluid, followed by the addition of bicarbonate (200 μM) and apolactoferrin (50 μM) at approximately pH 8. The EPR spectrum in Figure 1C is identical to the EPR spectrum in Figure 1B originating from the reaction between NO· and the ferri-lactoferrin complex. This result strongly suggests that NO· was generated by the macrophage cells during their stimulation with LPS. It must be noted that if only L-arginine is added to the macrophage suspension, the EPR spectrum in Figure 1C is also observed. However, this EPR spectrum is approximately 50% less intense than the spectrum obtained from the suspensions of stimulated macrophages and is possibly due to the natural production of NO· by these cells.

Spin trapping was employed in order to verify the presence of NO_2^- originating from macrophage-derived NO· after stimulation of these cells with LPS. It is known that H_2O_2 reacts with NO_2^- according to the following equation [Eqn. (2)]:[16]

$$NO_2^- + H_2O_2 \longrightarrow OONO^- + H_2O \xrightarrow{H^+} OH + NO_2 \quad (2)$$

The results in Figure 2 show the EPR spectra obtained after adding DMPO (0.2 M) and H_2O_2 (1×10^{-4} M) to the supernatant fluid from stimulated cells made acidic (pH 3-3.5) with HCl. The EPR spectrum in Figure 2A is the control in which the cell suspension medium containing DMPO and H_2O_2 was acidified. This EPR spectrum shows the formation of a residual amount of DMPO-OH. However, when the DMPO and H_2O_2 are added to the supernatant fluid from macrophage cells which were not stimulated with LPS, a well defined EPR spectrum is observed consisting of a 1:2:2:1 quartet characteristic of the DMPO-OH spin adduct (Figure 2B). The intensity of this EPR spectrum is increased significantly (Figure 2C) when the macrophage cells are stimulated prior to centrifugation and addition of DMPO, H_2O_2 and acid. Figure 2D is the computer simulation obtained using hyperfine coupling constants, $a_N = a_H^\beta = 1.49$ mT, confirming the EPR spectra in Figure 2 correspond to the DMPO-OH adduct. In addition, the computer generated EPR spectrum in Figure 2D was obtained by subtracting

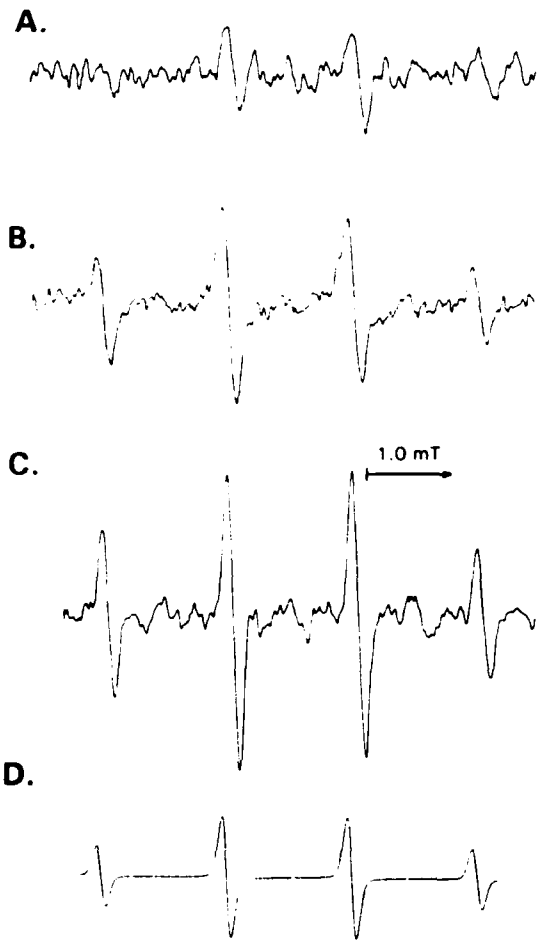

FIGURE 2 DMPO-OH spin adduct EPR spectra obtained at pH 3-3.5 in the supernatant fluid of macrophage cell suspensions after addition of H_2O_2. (A) Control, no cells; (B) Non-stimulated macrophage cells; (C) LPS-stimulated macrophage cells; (D) Computer generated difference between (B) and (C). Receiver gain: 1.25×10^5.

the computer generated EPR spectrum that exactly matches (intensity and linewidths) the EPR spectrum in Figure 2B from the computer generated spectrum that exactly matches the EPR spectrum in Figure 2C. Therefore, Figure 2D represents the DMPO-OH adduct formed possibly from OH produced in reaction 2 following LPS stimulation of macrophages.

In order to verify that the DMPO-OH adduct formed (Figures 2C and 2D) originates from OH radicals generated via decomposition of peroxynitrous acid (Eqn. 2), a similar experiment was done in the presence of ethanol. The results obtained in this experiment are shown in Figure 3. Figure 3A is the EPR spectrum obtained when H_2O_2 (1×10^{-4} M, final concentration) is added to the supernatant fluid from LPS-stimulated macrophages containing DMPO (0.2 M), excess of ethanol (1.7 M) and acidification. This spectrum is composed mainly of the superimposition of two spin adducts. One spin adduct yields an EPR spectrum consisting

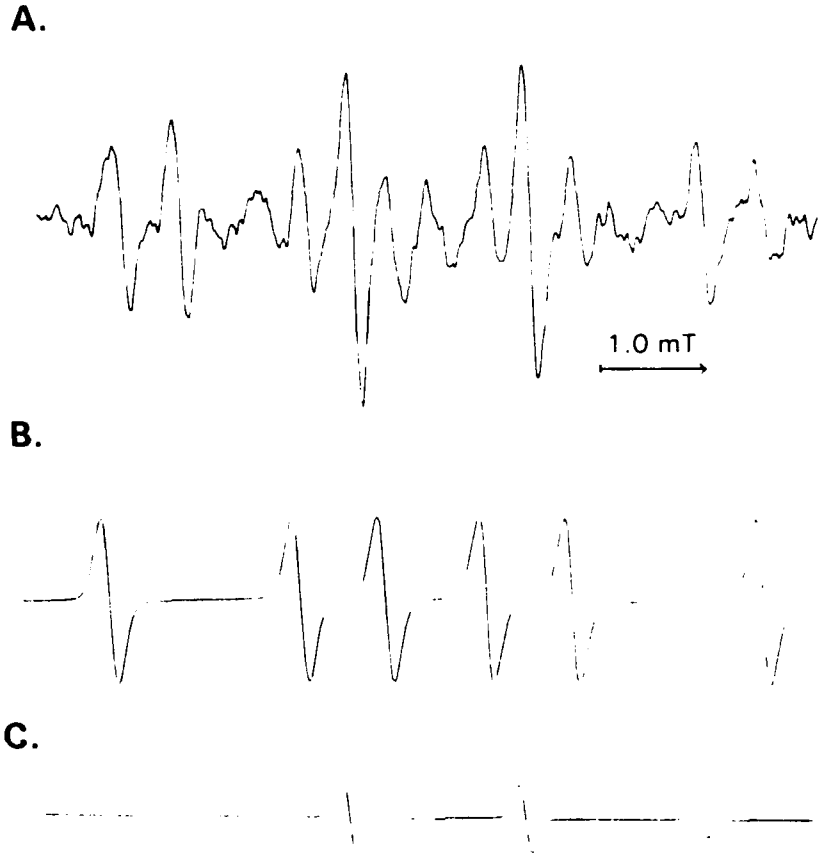

FIGURE 3 DMPO-OH and DMPO-hydroxyethyl adducts obtained at pH 3-3.5 after addition of H_2O_2 to the supernatant fluid of LPS-stimulated macrophages containing ethanol (1.7 M). (A) Experimental EPR spectrum; (B) EPR computer simulation of the DMPO-hydroxyethyl adduct; (C) Computer generated difference between DMPO-OH EPR spectrum in (A) and in Figure 2B. Reciever gain: 1.25×10^5.

of a triplet of doublets. The second spin adduct EPR spectrum consist of a 1:2:2:1 with hyperfine coupling constants $a_N = a_H^\beta = 1.49\,mT$ corresponding to the DMPO-OH adduct. The triplet of doublets can be computer simulated (Figure 3B) using hyperfine coupling constants, $a_N = 1.58\,mT$ and $a_H^\beta = 2.28\,mT$. These parameters are consistent with hyperfine coupling constants for the DMPO-hydroxyethyl adduct obtained following the reaction between OH and ethanol.[26] The EPR spectrum corresponding to the DMPO-hydroxyethyl adduct (Figure 3B) was computer generated to exactly match the experimentally obtained DMPO-hydroxyethyl adduct (Figure 3A) in peak heights and linewidths. Figure 3C is the EPR spectrum obtained corresponding to the residual DMPO-OH spin adduct remaining after subtracting the computer generated EPR spectra that exactly match, in peak heights and linewidths, the DMPO-OH EPR spectra obtained experimentally and shown in Figures 2B and 3A. Since the DMPO-OH EPR spectrum in Figure 2B was obtained in an experiment using non-stimulated macrophage cells, in addition to, the difference between this EPR spectrum and the DMPO-OH EPR

spectrum in Figure 3A consisting only of a residual quantity of DMPO-OH, suggests that the DMPO-OH formed in the experiments using non-stimulated macrophage cells does not originate from OH addition to DMPO. Furthermore, the DMPO-OH EPR spectrum in Figure 3C is approximately three times less intense than the DMPO-OH EPR spectrum in Figure 2D. This suggests that the DMPO-hydroxyethyl adduct was formed at the expense of the additional DMPO-OH spin adduct obtained following macrophage stimulation (Figure 2D) and adding H_2O_2 to the supernatant fluid. The origin of the residual DMPO-OH adduct formed (Figure 3C) is unclear. It could possibly be formed by oxidation mechanisms involving NO_2^- produced following the reaction betweeen NO_2^- and H_2O_2. Spin trapping studies involving the reaction between H_2O_2 and NO_2^- forming the peroxynitrous acid and confirming its subsequent decomposition to OH and NO_2 are described in another report (*ibid.*).[27] In these studies it was shown that the reaction between H_2O_2 and NO_2^- produced the DMPO-OH adduct and another less intense DMPO adduct yielding an EPR spectrum consisting of a triplet of triplets ($a_N^{'} = 1.415$ mT, $a_N^{''} = 0.35$ mT), suggesting the addition of a nitrogen center to DMPO. Although weak, the EPR lines of a similar triplet of triplets is observed between the DMPO-hydroxyethyl and DMPO-OH EPR signals in Figure 3A. This additional evidence supports the formation of NO_2^- from macrophage-derived NO during LPS-stimulation of these cells.

The reaction [Eqn. (2)] between H_2O_2 and NO_2^- is important because it verifies the production of NO_2^- from macrophage-derived NO. It is also important because H_2O_2 and NO_2^- are, respectively, the dismutation product of O_2^- and a by-product of $NO^{\cdot}$ decomposition in cells. Therefore, in cell systems such as endothelial cells, neutrophils and macrophages that are known to produce O_2^- and $NO^{\cdot}$, the reaction between H_2O_2 and NO_2^- could occur generating the same product as the product generated in the reaction between O_2^- and $NO^{\cdot}$. This may be, in part, a possible explanation as to why antioxidant compounds or SOD mimics that effectively react with O_2^- in aqueous systems, do not completely eliminate or prevent damage to cells, tissues or organs during, or immediately after, certain dysfunctions in which O_2^- is known to be implicated.

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