Free Rad. Res., Vol. 23, No. 4, pp. 379–390
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EFFECTS OF SUPEROXIDE ON NITRIC OXIDE-DEPENDENT N-NITROSATION REACTIONS

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(Received December 18th, 1994; in revised form, January 13th, 1995)

Recent studies have demonstrated that nitric oxide (NO) in the presence of superoxide (O_2^-) may mediate mutagenesis via the N-nitrosation of DNA bases followed by nitrosative deamination to yield their hydroxylated derivatives. We have found that phorbol myristate acetate (PMA)-activated extravasated rat neutrophils (PMNs) will N-nitrosate 2,3-diaminonaphthalene (DAN) to yield its highly fluorescent nitrosation product 2,3- naphthotriazole (triazole) via the L-arginine dependent formation of NO. Addition of SOD enhanced triazole formation suggesting that O_2^- production may inhibit the N-nitrosating activity and thus the mutagenic activity of inflammatory PMNs. The objective of this study was to assess the role of superoxide as a modulator of NO-dependent N-nitrosation reactions using PMA-activated PMNs as well as a chemically defined-system that generates both NO and superoxide. We found that PMA-activation of PMNs reduced the amount of N-nitrosation of DAN by approximately 64% when compared to non- stimulated cells (450 vs. 1250 nM). Addition of SOD but not inactivated SOD or catalase to PMA-activated PMNs enhanced the formation of triazole by approximately 4-fold (1950 nM). In addition, we found that the NO-releasing spermine/NO adduct (Sp/NO; 50 μM) which produces approximately 1.0 nmol NO/min generated approximately 8000 nM of triazole whereas the combination of Sp/NO and a superoxide generator (hypoxanthine/xanthine oxidase) that produces approximately 1.0 nmol O_2^-/min reduced triazole formation by 90% (790 nM). Addition of SOD but not catalase restored the N-nitrosating activity. We conclude that equimolar fluxes of superoxide react rapidly with NO to generate products that have only limited ability to N-nitrosate aromatic amino compounds and thus may have limited ability to promote mutagenesis via the nitrosative deamination of DNA bases.

KEY WORDS: nitric oxide, superoxide, N-nitrosation, polymorphonuclear leukocytes

INTRODUCTION

It is known that chronic inflammation of epithelioid tissue is associated with an increased risk of cancer (1). Although the mechanisms by which inflammation promotes malignant transformation remain to be defined, it has been suggested that the leukocytes known to accumulate within the chronically inflamed tissue produce potent mutagens and carcinogens (1). For example, a variety of investigators have demonstrated that certain reactive oxygen metabolites produced by macrophages,

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monocytes and neutrophils (PMNs) promote mutagenesis and carcinogenesis in vitro (reviewed in 1). More recent data suggest that cytokine-activated phagocytic leukocytes also produce large quantities of oxidized metabolites of nitrogen initially derived from nitric oxide (NO) (2). In the presence of molecular oxygen (O_2), NO spontaneously autoxidizes to yield one or more N-nitrosating agents such as dinitrogen trioxide (N_2O_3) and possibly dinitrogen tetroxide (N_2O_4) (3). These compounds are known to N-nitrosate primary aromatic amines resulting in their nitrosative deamination via the formation of nitrosamine and diazonium intermediates (4). Nitrosative deamination of DNA-associated bases by these NO-derived N-nitrosating agents yields a variety of different base substitution mutations and has been suggested to account for the mutagenic and carcinogenic activity of NO in oxygenated solutions (5–7). Furthermore, the nitrosating intermediates produced from the autoxidation of NO have been shown to generate S-nitrosothiol adducts of proteins (8,9). The formation of protein adducts has been shown to inhibit enzyme activity such as alkyl transferase (10) and results in self ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase (11). The nitrosation of thiols may be important in cell cytotoxicity as well as genotoxicity.

We, as well as others, have demonstrated that extravasated but not circulating PMNs and macrophages possess potent NO-dependent N-nitrosating activity (12–14). During the course of these studies, we found that certain nonenzymatic antioxidants such as ascorbate, reduced glutathione (GSH), trolox (water soluble α-tocopherol analog) and 5-aminosalicylic acid inhibited PMN-mediated N-nitrosation of a model aromatic amine (2,3-diaminonaphthalene) raising the possibility that the N-nitrosating species are also oxidizing agents (12). Support for this hypothesis has recently been obtained by Wink *et al.* who demonstrated that the N-nitrosating agent produced from the interaction between NO and O_2 is a potent oxidant (15). Interestingly, we found that superoxide dismutase (SOD) actually enhanced N-nitrosating activity suggesting that superoxide may modulate NO-dependent N-nitrosation reactions (12). Therefore, the objective of this study was to assess the role of superoxide as a modulator of NO-dependent N-nitrosation reactions using PMA-activated PMNs as well as a more chemically defined-system that generates both NO and superoxide.

MATERIALS AND METHODS

Chemicals and Reagents

Hypoxanthine (HX), 2,3-diaminonaphthalene (DAN), phorbol myristate acetate (PMA) and oyster glycogen were purchased from Sigma. Xanthine oxidase (XO) was obtained from Calibiochem Corp. Human recombinant Cu-Zn superoxide dismutase (SOD; 3000 units/mg) was obtained from Kabi-Pharmacia AB (Uppsala) and bovine liver catalase (65,000 units/mg) was purchased from Boehringer-Mannheim (Germany). The nitric oxide-releasing compounds spermine-NO (Sp/NO) and diethylamine-NO (DEA/NO) adducts were provided by Dr. Larry Keefer (National Cancer Institute, Frederick, MD). Purified 2,3-napthotriazole was prepared according to the method of Wheeler *et al.* (16).

Cell Preparations

Elicited (extravasated) PMNs were obtained from male Sprague-Dawley rats (300–400g) following an intraperitoneal injection (IP) of 20 mL of 1% (wt/vol) oyster

glycogen in phosphate-buffered saline (PBS) pH 7.4 (12). Cells were harvested 5–6 hours after IP injection by peritoneal lavage using 50 mL of heparinized PBS. Cells were washed twice with cold PBS and contaminating erythrocytes were removed by hypotonic lysis. The neutrophil-rich pellet was then washed three times with cold PBS, counted and placed on ice. Cells were routinely found to be>95% pure and>95% viable as judged by trypan blue exclusion.

N-Nitrosation of DAN

Elicited PMNs were suspended in Dulbeccos modified phosphate-buffered saline supplemented with 20 mM HEPES (pH 7.4), 20 mM glucose, 0.2 mM DAN, 4 mM glutamine, 1 mM L-arginine and 50 Units/mL each of penicillin and streptomycin. DAN is a primary aromatic diamine that can be N-nitrosated to yield the highly fluorescent 2,3-napthotriazole derivative (12, 16–20; Figure 1). For some experiments PMA (1.0 μM) and/or certain antioxidant enzymes (e.g. SOD at 100 μg/ml and/or catalase at 15 μg/ml) were included in the PMN suspension. Cell suspensions (0.5 mL containing 4×10^6 cells) were added to fetal bovine serum (FBS)-coated microtiter wells (1 cm^2) and incubated from 0 to 4 hrs at 37°C. Coating tissue culture plastic with FBS has been shown to minimize the nonspecific metabolic activation of PMNs by the plastic and presents a surface more closely related to the tissue interstitium (21). Following the incubation period the microtiter plates were centrifuged 400×g for 5 min, the supernatants (0.5 mL) removed and placed into glass tubes for triazole determinations. To the 0.5 mL supernatants, 2.5 mL of 10 mM NaOH was added and the fluorescence of each sample determined using an excitation wavelength of 375 nm and an emission wavelength of 415 nm (16–19). Alkalinization of these solutions enhances dramatically the fluorescence of the triazole such that we were able to detect as little as 30 pico mols/mL of the N-nitrosated product. Concentrations of the triazole were calculated from a standard plot using known concentrations of the purified triazole. Fluorescence emission spectra and static measurements were made on a SLM Instruments Amino/Bowman-2 luminescence spectrometer. Routinely, these were made

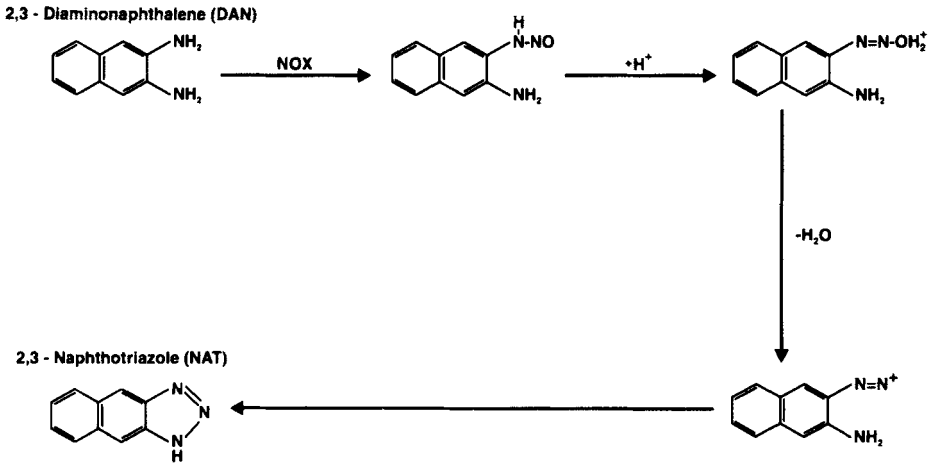

FIGURE 1 *N-nitrosation of 2,3-diaminonaphthalene(DAN) to 2,3-naphthotriazole (NAT).* Reaction of DAN with NO-dependent N-nitrosating agents (NO_x) produces the highly fluorescent triazole derivative, NAT via N-nitrosoamine and diazonium intermediates.

employing 1 cm pathlength quartz cuvettes with excitation at 375 nm (bandpass 4 nm) and emissions monitored at 415 nm (bandpass 8 nm).

Spermine/NO and Hypoxanthine/Xanthine Oxidase System

Spermine-NO (Sp/NO) is a bis-nitric oxide adduct of the polyamine spermine. This compound is unstable at neutral pH and decomposes ($t_{1/2}$ = 39 min) to yield 2 mol of NO per mol of adduct (22). Stock solutions of Sp/NO (10 mM) were prepared in ice-cold 10 mM NaOH in order to minimize premature decomposition. Superoxide was generated enzymatically via xanthine oxidase (XO)-catalyzed oxidation of hypoxanthine (HX). Hypoxanthine was used as the substrate for XO rather than xanthine to minimize the production of uric acid, a potent antioxidant and one of the primary products of the xanthine/xanthine oxidase reaction.

Five hundred microliter reaction volumes containing PBS buffer (pH 7.4), 0.2 mM DAN, 0.5 mM HX, 1.0 mUnit/mL XO and 0.05 mM Sp/NO were incubated in the absence or presence of 100 μg/mL SOD and/or 15 μg/mL catalase for 60 minutes at 37°C. For some experiments, HX and XO were omitted from the reaction mixture. Following the incubation period, 2.5 mL of 10 mM NaOH was added to each sample and triazole determined by fluorescence spectroscopy as described above. Preliminary experiments determined that the HX/XO system produces approximately 1.0 nmole/min of O_2^- using the SOD-inhibitable reduction of cytochrome c (23). Using the World Precision Instruments (WPI) NO sensing electrode, we found that the decomposition of 0.05 mM Sp/NO at pH 7.4 produces approximately 1 nmole/min of NO. Examination of XO incubated in the presence of either 1.0 atmosphere of NO for 3 hrs (anaerobically), 1.0 mM DEA/NO (aerobic and anaerobically) for 1 hr, or 1.0 mM Sp/NO for 3 hrs did not effect the rate of XO reduction of ferricytochrome c.

Quantitation of PMN-generated Nitrate and Nitrate

Following the 4-hour incubation period in the presence or absence of 1.0 μM PMA, total nitrite (i.e., NO_3^- plus NO_2^-) in the 0.5 mL aliquots of supernatant was quantified by first reducing nitrate to nitrite using *Aspergillus* nitrate reductase (24). Samples were then diluted with addition 1.0 mL of Griess reagent (0.1% naphthalene diamine dihydrochloride and 1% sulfanilamide in 2.5% H_3PO_4), incubated for 10 min at 25°C and the absorbance measured at 543 nm. The concentration of NO_2^- was calculated from a standard curve using sodium nitrite as the standard.

N-nitrosation by Peroxynitrite

The ability of peroxynitrite ($ONOO^-$) and its conjugate acid peroxynitrous acid (ONOOH) to N-nitrosate primary aromatic amines was assessed using the DAN assay as described above or the sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride coupled reaction (SULF/NEDD) performed at pH 7.4 recently described by Nims *et al.* (25). Peroxynitrite was prepared according to the method of Beckman *et al.* (26).

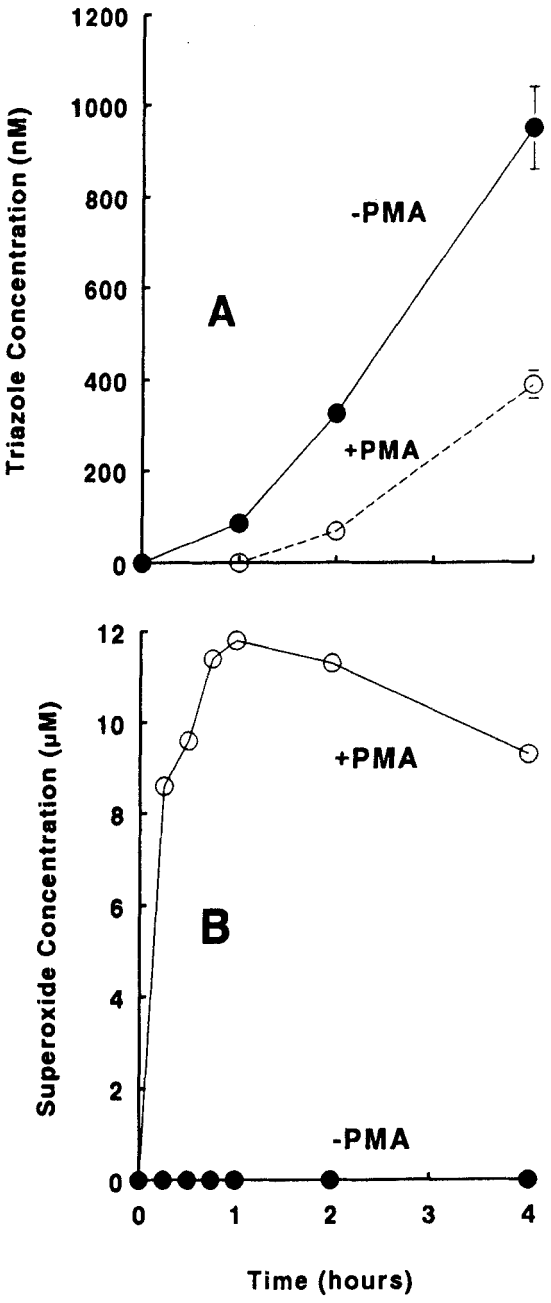

FIGURE 2 *Time-course of triazole and superoxide production by elicited PMNs.* A) Elicited PMNs (4×10^6 cells/well) were incubated in the presence of 0.2 mM DAN and in the absence or presence of PMA for varying times at 37°C. Triazole formation was quantified as described in the Methods Section. B) Elicited PMNs (4×10^6 cells/well) were incubated in the presence of 2 mg/mL cytochrome c and in the absence or presence of 1.0μM PMA at 37°C. Superoxide production was quantified using the SOD-inhibitable reduction of cytochrome c.

RESULTS

Figure 2 demonstrates the time course of triazole and superoxide (O_2^-) formation by extravasated rat PMNs in the absence and presence of PMA. It can be seen that activation of the oxidative metabolism of these cells by PMA (i.e., superoxide production) significantly reduced triazole formation over a 4 hr incubation period. Interestingly, PMA-induced O_2^- production rapidly increased within one hr and then began to decline over the next 3 hours (Figure 2B). Figure 3 demonstrates that addition of PMA to these cells inhibited triazole formation by approximately 64% suggesting that reactive O_2 species inhibited the N-nitrosative reaction. Addition of SOD (100 μg/ml) but not catalase (15 μg/ml) to PMA-activated PMNs enhanced dramatically the N-nitrosation of DAN such that triazole formation increased to 1950±20 (Figure 3). The addition of inactivated SOD did not enhance the N-nitrosating activity of PMA-activated PMNs suggesting that O_2^- and not H_2O_2 (or hydroxyl radical) inhibited the N-nitrosation reaction (data not shown). Subsequent studies revealed that PMNs incubated for 4 hours with PMA produced approximately 30% more NO than did cells incubated in the absence of PMA as measured by the increases in NO_3^-/NO_2^- (Figure 4).

In addition to extravasated PMNs, we also assessed the ability of superoxide to modulate NO-dependent N-nitrosation of DAN using a more chemically-defined system. Figure 5 demonstrates that the NO-releasing Sp/NO adduct is very effective at promoting the N-nitrosation of DAN such that 0.05 mM Sp/NO promotes the

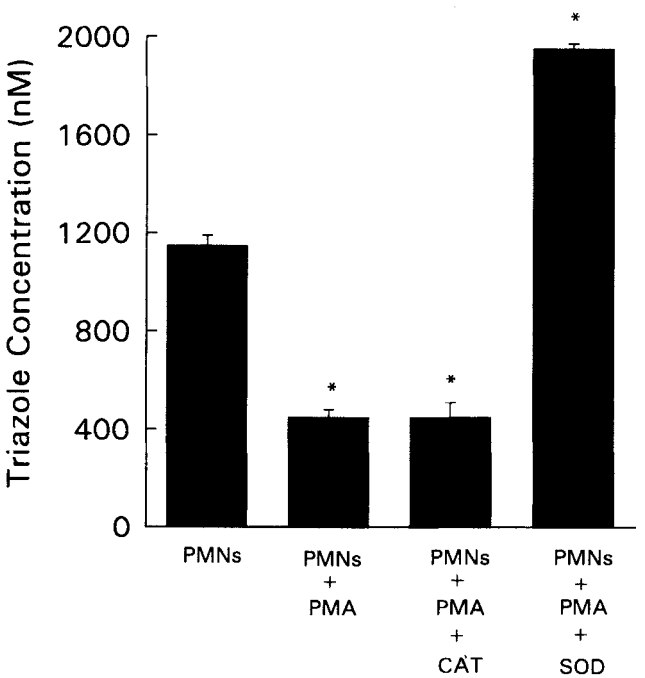

FIGURE 3 *N-nitrosation of DAN by elicited PMNs.* Elicited rat PMNs (4×10^6 cells/well) were incubated for 4 hours at 37°C in the presence of 0.2 mM DAN. For some experiments, 1 μM PMA, 100 μg/ml SOD and/or 15 μg/ml catalase (CAT) were included in the reaction mixtures. Triazole formation was quantified as described in the Methods Section. *represents $p<0.001$ compared to PMNs and + represents $p<0.001$ compared to PMN + SOD group.

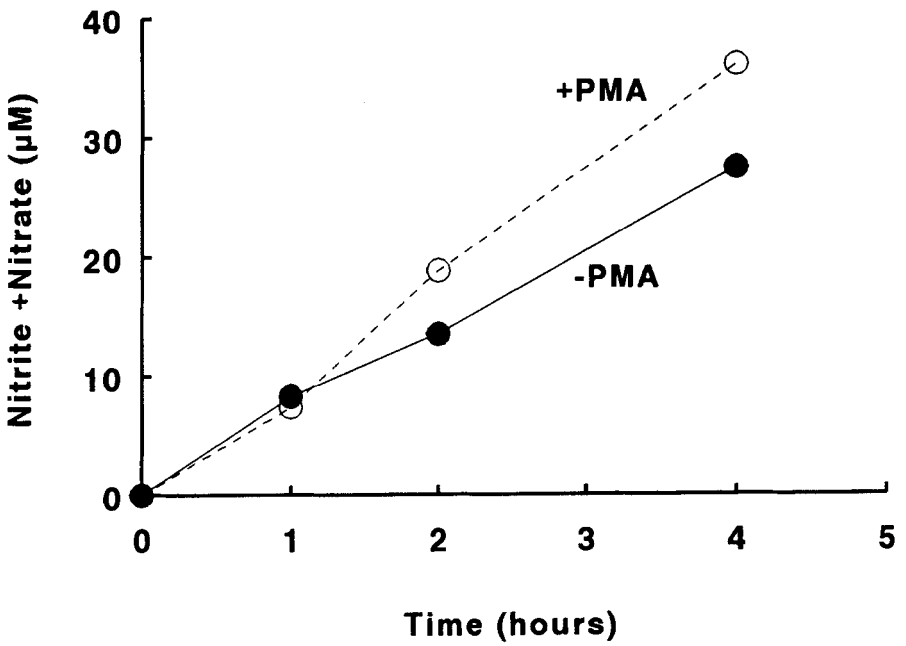

FIGURE 4 *Time-course of Nitrite/Nitrate production by elicited PMNs* Elicited PMNs (4×10^6 cells/well) were incubated in the absence or presence of 1.0 μM PMA for varying lengths of time at 37°C. Total nitrite was determined as described in the Methods Section.

formation of 8080±902 nM triazole. Addition of a superoxide and hydrogen peroxide generator (HX/XO) to this system significantly inhibited the N-nitrosation reaction such that triazole formation was reduced greater than 90% to 790±160 nM. Addition of SOD to the Sp/NO + HX/XO system restored triazole formation to values similar to those obtained with Sp/NO alone whereas addition of catalase did not reverse the inhibition produced by addition of HX/XO (Figure 5).

Figure 6 demonstrates that increasing the concentrations of XO and thus the flux of O_2^- dramatically inhibited Sp/NO-mediated N-nitrosation of DAN such that virtually all N-nitrosation of DAN was inhibited when fluxes of O_2^- and NO were approximately equal (i.e., 1.0 nmol/min). Omission of HX but inclusion of XO did not inhibit the nitrosation reaction. The possibility that NO and O_2^- may interact to yield $ONOO^-$/ONOOH which could then N-nitrosate primary aromatic amines was assessed using the DAN and SULF/NEDD assays. We found that $ONOO^-$/ONOOH did not N-nitrosate these aromatic amines (data not shown).

DISCUSSION

Nitric oxide is a particularly unstable free radical in oxygenated solutions. It will very rapidly interact with O_2 to generate a variety of oxidized metabolites of nitrogen (3,15):

$$2NO + O_2 \rightarrow 2NO_2$$
$$2NO + 2NO_2 \rightarrow 2N_2O_3$$
$$2N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+$$

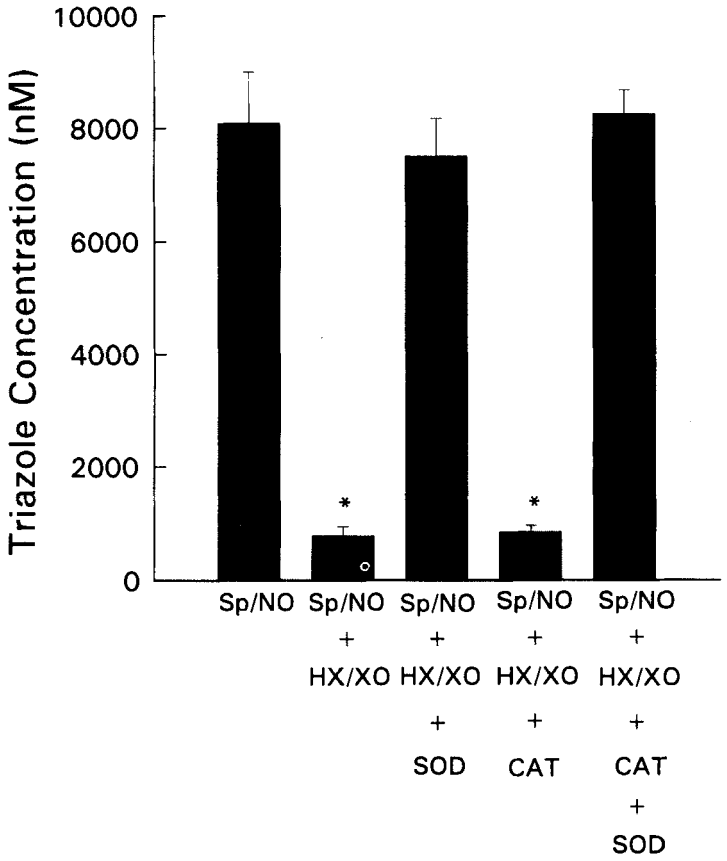

FIGURE 5 *Spermine/NO (Sp/NO)-Mediated N-nitrosation of DAN.* Sp/NO (0.05 mM) was incubated for 60 min at 37°C in the absence or presence of hypoxanthine (HX; 0.5 mM)/xanthine oxidase (XO; 1.0 mU/mL) and 0.2 mM DAN in PBS (pH 7.4) at 37°C. Triazole formation quantified as described in the Methods Section. *represents $p < 0.001$ compared to Sp/NO.

Nitrogen oxides such as N_2O_3 are potent nitrosating agents (NO_x; 4) capable of N-nitrosating primary and secondary amines (27). It has been known for many years that NO_x promotes nitrosative deamination of aromatic primary amines ($ArNH_2$) via the formation of nitrosamine and diazonium intermediates (4):

$$ArNH_2 + NO_x \rightarrow ArNHNO$$
$$ArNHNO + H^+ \rightarrow ArN_2^+ + H_2O$$
$$ArN_2^+ + H_2O \rightarrow ArOH + N_2 + H^+$$

Recent work by Wink *et al.* (5) and Nguyen *et al.* (6) have demonstrated that these types of reactions may be very important in the NO-dependent formation of several different types of base substitution mutations in vitro. In addition, Mordan *et al.* (7) have demonstrated that polycyclic aromatic hydrocarbon-induced malignant transformation in vitro is dependent upon an active NO synthase suggesting that NO or some NO-derived metabolite may be important in mediating chemically-induced malignant

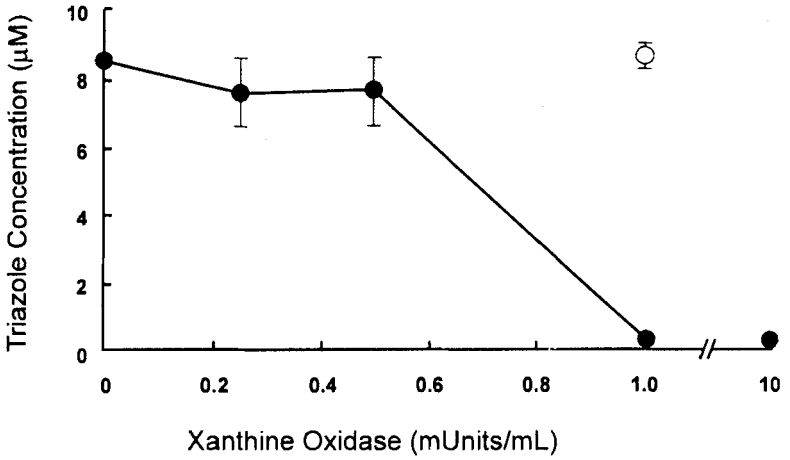

FIGURE 6 *Effect of varying concentrations of Xanthine Oxidase on NO-mediated N-nitrosation of DAN.* Varying concentrations of XO were incubated with 0.05 mM Sp/NO and 0.2 mM DAN in PBS (pH 7.4) at 37°C Triazole formation was quantified as described in the Methods section. The open circle represents XO + Sp/NO in the absence of HX.

transformation. It has been proposed that nitrosative deamination may account for much of the mutagenic and carcinogenic activity induced by NO (5–7). Furthermore, S-nitrosothiol protein adducts formed from the nitrosating intermediate in the NO/O_2 reaction can cause mark inhibition of enzyme activity and may play a role in cytotoxicity as well as genotoxcity (9).

One of the most avid producers of NO in vivo are the phagocytic leukocytes (e.g. PMNs, monocytes, macrophages) (2,3). Furthermore, we have demonstrated that the process of extravasation up regulates the expression of the inducible NOS (iNOS) in PMNs such that these cells may produce large amounts of NO (6–10 μM/hr) (14). However, activated phagocytes also produce reactive oxygen species such as O_2^-, that may be produced in 10 times the amount of NO (60–100 μM/hr; 28). Thus, the steady state levels of NO and NO_x may be greatly influenced by the production of one or more of these reactive oxygen species. Indeed, we found PMA-induced activation of O_2^- production reduced triazole formation (Figure 2A). Interestingly, PMA-induced O_2^- production was maximal at 1 hr but then declined modestly over the next 3 hr (Figure 2B). The reasons for this apparent inhibition of O_2^- formation at the later times is not apparent however it may be that significant levels of oxidants such as hypochlorous acid (or $ONOO^-$) are formed at these times which oxidize small amounts of reduced cytochrome c. We also found that the addition of SOD to PMA-activated PMNs enhanced dramatically (~ 4-fold increase) NO-mediated N-nitrosation of our model primary aromatic amine (Figure 3). Furthermore, we found that PMA increased NO production as measured by increases in NO_2^-/NO_3^- (Figure 4). The reason for this apparent enhancement in NO production by PMA-activated PMNs is not clear, however, recent studies have demonstrated that PMA will up regulate the expression of iNOS in other rodent cells such as hepatocytes (29,30). The fact that neither inactivated SOD (data not shown) nor catalase (Figure 3) restored the N-nitrosating activity of PMA-activated PMNs suggests that O_2^- and not H_2O_2 (or OH•) interacts with NO to generate products that are ineffective at N-nitrosating DAN. This conclusion is supported by other experiments outlined in the present study in which a more chemically defined-system generates both O_2^- and NO. The simultaneous production

of O_2^- and NO at approximately equivalent rates (1 nmole/min) resulted in almost complete inhibition of NO-mediated N-nitrosation of DAN (Figures 5). Elimination of O_2^- by addition of SOD but not catalase restored triazole formation suggesting an inhibitory effect of O_2^- on NO-dependent N- nitrosation reactions.

The mechanism by which O_2^- inhibits NO-dependent N-nitrosation reactions is not entirely clear however there appears to be at least two possibilities. First, O_2^- may scavenge the nitrosating agent by acting as an antioxidant i.e., it may donate its odd electron to NO_x thereby decomposing this oxidizing agent. A more probable mechanism involves the very rapid reaction between O_2^- and NO in a radical-radical coupled reaction to produce peroxynitrite anion ($ONOO^-$).

$$NO + O_2^- \rightarrow OONO^-$$

Recently, Huie and Padmaja (31) reported a diffusion limited second order rate constant of $6.7\times10^9 M^{-1}s^{-1}$ for the reaction between O_2^- and NO. Peroxynitrite exists in equilibrium with its conjugate acid, peroxynitrous acid (ONOOH, pKa 6.8) (32,33):

$$NO + O_2^- \rightarrow ONOO^- + H^+ \rightleftarrows ONOOH$$

which is unstable and at physiological pH decomposes to form potent oxidants with reactivity similar to hydroxyl radical (33). Although ONOOH is capable of mediating nitration of phenolic compounds (34), it does not appear to N-nitrosate primary aromatic amines as determined by is inability to N-nitrosate DAN or sulfanilamide in the SULF/NEDD system (data not shown). Furthermore, $ONOO^-$ mediated oxidation of ferrocyanide is not quenched by the nitrosating scavenger azide, indicating that this anion will oxidize substrate rather than nitrosate (15). It should be noted that although O_2^- may inhibit the potentially mutagenic N-nitrosation of various primary and secondary amines, the formation of $ONOO^-$ could promote the oxidative modification (hydroxylation, oxidation) of DNA bases resulting in a different pattern of mutagenic reactions (DNA strand breaks and 8-OH guanosine) (35,36). The reaction of NO with oxygen in an aqueous environment is at least three orders of magnitude slower (second order with respect to NO) exhibiting a third order rate constant of $2\times10^6 M^{-2}s^{-1}$ (37). Furthermore, the rate constant for the spontaneous dismutation of O_2^- (at neutral pH) is approximately four orders of magnitude slower than the rate for the interaction between NO and O_2^- (38). In contradistinction, SOD-catalyzed dismutation of O_2^- is characterized by a comparatively high rate constant of $2\times10^9 M^{-1}s^{-1}$ (38). Based upon these considerations, we expect that in the absence of SOD, O_2^- will inhibit N-nitrosation reactions by rapidly interacting with NO to generate products that are inactive as nitrosating agents.

The interplay between the oxidative and nitrosative character of reactive nitrogen oxide intermediates may play a critical role in the types of mutagenic and cytotoxic properties of NO. In the presence of O_2^-, NO will nitrosate amines and thiols representing one toxicological spectrum. However, in the presence of superoxide, nitrosation is suppressed where toxicity due to oxidation may be dominant. Either oxidation or nitrosation will have different cellular targets hence different effects on cytotoxicity. The fundamental understanding of the interplay between these two chemistries gives us insight into the chemical role NO can play in the immune surveillance system. We

suggest that O_2^- is an effective modulator of NO-dependent N-nitrosation and may serve to protect against possible mutations caused by NO-induced nitrosative deamination of DNA bases.

Acknowledgements

This work was supported by grants from the National Institutes of Health (DK47663 and CA63641).

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