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Cytotoxicity and Site-specific DNA Damage Induced by Nitroxyl Anion (NO^-) in the Presence of Hydrogen Peroxide

IMPLICATIONS FOR VARIOUS PATHOPHYSIOLOGICAL CONDITIONS*

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Nitroxyl anion (NO^-), the one-electron reduction product of nitric oxide ($NO^\cdot$), is formed under various physiological conditions. We have used four different assays (DNA strand breakage, 8-oxo-deoxyguanosine formation in calf thymus DNA, malondialdehyde generation from 2′-deoxyribose, and analysis of site-specific DNA damage using ^{32}P-5′-end-labeled DNA fragments of the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene) to study the effects of NO^- generated from Angeli's salt on DNA damage. It was found that strong oxidants are generated from NO^-, especially in the presence of H_2O_2 plus Fe(III)-EDTA or Cu(II). $NO^\cdot$ released from diethylamine-NONOate had no such effect. Distinct effects of hydroxyl radical ($HO^\cdot$) scavengers and patterns of site-specific DNA cleavage caused by Angeli's salt alone or by Angeli's salt, H_2O_2 plus metal ion suggest that NO^- acts as a reductant to catalyze the formation of the $HO^\cdot$ from H_2O_2 plus Fe(III) and formation of Cu(I)-peroxide complexes with a reactivity similar to $HO^\cdot$ from H_2O_2 and Cu(II). Angeli's salt and H_2O_2 exerted synergistically cytotoxic effects to MCF-7 cells, determined by lactate dehydrogenase release assay. Thus NO^- may play an important role in the etiology of various pathophysiological conditions such as inflammation and neurodegenerative diseases, especially when H_2O_2 and transition metallic ions are present.

Excess production of nitric oxide ($NO^\cdot$) has been implicated as a cause of diverse pathophysiological conditions such as inflammation, neurodegenerative diseases, cardiovascular disorders, and cancer. These detrimental effects of $NO^\cdot$ have been attributed to reactive nitrogen species such as NOx and peroxynitrite ($ONOO^-$), which are formed by the reaction of $NO^\cdot$ with oxygen and superoxide, respectively. Reactive nitrogen species can oxidize, nitrate, and nitrosate biomolecules such as proteins, DNA, and lipids, thus altering their functions. We have recently reported that NO^-, which is the one-electron reduction product of $NO^\cdot$, can also cause strand breakage and oxidative damage in DNA *in vitro* (1). We have proposed that a highly toxic hydroxyl radical ($HO^\cdot$) generated from the reaction between NO^- and $NO^\cdot$ is responsible for the oxidation reactions (Equations 1 and 2).

$$NO^- + NO^\cdot \rightarrow N_2O_2^- \quad \text{(Eq. 1)}$$

$$N_2O_2^- + H^+ \rightarrow N_2O + HO^\cdot \quad \text{(Eq. 2)}$$

NO^- has been also reported to be cytotoxic, reducing intracellular glutathione levels and causing DNA strand breakage in cultured cells (2). However, it can also be converted under physiological conditions *in vitro*, as well as in cells, to $NO^\cdot$ and other reactive oxygen and nitrogen species including superoxide, hydrogen peroxide (H_2O_2), and peroxynitrite (3–5), and the actual mechanisms and reactive species responsible for the cytotoxic effects of NO^- have not been established.

Three recent publications have suggested that $NO^\cdot$ synthase generates NO^-, which can be then converted to $NO^\cdot$ by superoxide dismutase and other electron acceptors (6–8). NO^- can also be produced from *S*-nitrosothiols in the presence of thiols (9–11). It has been reported that, in the absence of oxygen, nitrosylhemoglobin liberates NO^- in a reaction producing methemoglobin (12). Ferrocytochrome *c* also reacts with $NO^\cdot$ to form ferricytochrome *c* and NO^-, which may have implications for inhibition of mitochondrial oxygen consumption by $NO^\cdot$ (13). In our previous reports, NO^- was proposed as one of the possible agents responsible for DNA strand breakage induced by $NO^\cdot$ and catechol-type compounds such as catecholamines, catechol-estrogens, and certain flavonoids (14). NO^- can be formed by one-electron reduction of $NO^\cdot$ by the quinone/hydroquinone redox system in a manner similar to that of the formation of O_2^- from oxygen (14).

In the present study, we have studied the effects of NO^- generated from Angeli's salt (sodium trioxodinitrate, $Na_2N_2O_3$) on DNA strand breakage and DNA base modifications *in vitro* mediated by H_2O_2 in the presence of metallic ions. At physiological pH, Angeli's salt exists predominantly in the form of the monoanion $HN_2O_3^-$, which decomposes to NO^- and nitrite (NO_2^-) (Equation 3) (15). As HNO is a weak acid (pK_a = 4.7), NO^- is the predominant form in aqueous solution at neutral pH (Equation 4) (15).

$$HN_2O_3^- \rightarrow HNO + NO_2^- \quad \text{(Eq. 3)}$$

$$HNO \leftrightarrow NO^- + H^+ \quad \text{(Eq. 4)}$$

We have found that NO^- generated from Angeli's salt dramatically enhances DNA damage mediated by H_2O_2 in the presence of the ferric ion (Fe(III))-EDTA or copper ion (Cu(II)), indicating that NO^- acts as an endogenous reductant to catalyze formation of strong oxidants. Furthermore, Angeli's salt and H_2O_2 cooperatively exerted cytotoxic effects toward human breast cancer cells. We discuss possible implications of our

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findings as a cause of diverse pathophysiological conditions mediated by activation or overexpression of NO· synthase.

EXPERIMENTAL PROCEDURES

Chemicals—Angeli's salt and diethylamine-NONOate (DEA-NO)[1] were obtained from Cayman Chemical Co. (Ann Arbor, MI). Plasmid pBR322 was purchased from Amersham Pharmacia Biotech. [γ-^{32}P]ATP (222 TBq/mmol) was supplied by NEN Life Science Products. Bathocuproinedisulfonic acid, and 1H-imidazol-1-yloxy,2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide, potassium salt (carboxy-PTIO) were from Dojin Chemicals Co., Kumamoto, Japan. All other chemicals including EDTA, ferric chloride, cuprous chloride, diethylenetriamine pentaacetic acid (DTPA), 8-oxo-2′-deoxyguanosine, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy free radical (4-OH-TEMPO), potassium ferricyanide (III) [$K_3Fe(CN)_6$], superoxide dismutase (from bovine erythrocytes), catalase (from bovine liver, thymol-free), and 2-thiobarbituric acid were obtained from Sigma, Aldrich, or Wako Chemical Industries, Ltd., Osaka, Japan.

Measurement of Malondialdehyde (MDA) Produced from Oxidation of Deoxyribose by Angeli's Salt—MDA formed from the oxidation of 2′-deoxyribose was measured as a marker of HO· generation, according to the method of Hogg *et al.* (16). The reactions were carried out in 100 mM sodium phosphate buffer, pH 7.4, containing 10 μM DTPA, 1 mM 2′-deoxyribose, 500 μM H_2O_2, 50 μM either $FeCl_3$-EDTA or $CuCl_2$, an appropriate amount of HCl to neutralize the NaOH present in the Angeli's salt solution, and 200 μM Angeli's salt prepared in 0.01 N NaOH at 37 °C (final volume, 1 ml; final pH, ~7.5). The MDA content was determined after reaction with 2-thiobarbituric acid using HPLC with a fluorescence detector, as reported previously (1, 17). All experiments were carried out in triplicate.

Analysis of 8-oxo-2′-Deoxyguanosine (8-oxo-dG) in Calf Thymus DNA Incubated with Angeli's Salt—Angeli's salt prepared in 0.01 N NaOH (0–5 mM, 100 μl) was added to a reaction mixture (final volume, 1 ml) containing 0.1 M sodium phosphate buffer, pH 7.5, calf thymus DNA (1 mg), 10 μM DTPA, 500 μM H_2O_2, either 50 μM $FeCl_3$-EDTA or $CuCl_2$, and an appropriate amount of HCl to neutralize the NaOH present in the Angeli's salt solution (final pH ~7.5), and the solution was incubated at 37 °C for 30 min. After the reaction, ethanol-precipitated DNA was hydrolyzed enzymatically, and 8-oxo-dG and 2′-deoxyguanosine were analyzed by HPLC with a Coulochem II electrochemical detector (ESA Inc., Chelmsford, MA) and a Shimadzu UV spectrophotometer (model SPD-2A), respectively, according to a modification of the method of Yamaguchi *et al.* (18). All experiments were carried out in duplicate or triplicate.

Induction and Analysis of DNA Single Strand Breaks—The experiments were carried out by incubating plasmid pBR322 DNA (100 ng) at 37 °C for 30 min in 100 mM sodium phosphate buffer, pH 7.4, containing 10 μM DTPA, 500 μM H_2O_2, either 50 μM $FeCl_3$-EDTA or $CuCl_2$, 200 μM Angeli's salt prepared in 0.01 N NaOH, and an appropriate amount of HCl to neutralize the NaOH present in the Angeli's salt solution (final volume, 10 μl; final pH, ~7.5). After the reaction, electrophoresis was carried out as described previously (1, 14, 19–21). The average number of single strand breaks/pBR322 DNA molecule was calculated according to Epe and co-workers (22, 23), taking into account that the relaxed form (form II) when stained with ethidium bromide gives a fluorescence intensity 1.4-fold higher than the supercoiled form (form I) and that a relaxation is caused by one single strand break/DNA molecule. All experiments were carried out in triplicate, and statistical significance was calculated using the Student's *t* test.

Site-specific DNA Damage—^{32}P-5′-end-labeled DNA fragments of the human *p53* tumor suppressor gene[2] and the c-Ha-*ras*-1 protooncogene (25) were prepared as previously reported (26, 27). The standard reaction mixture in a microtube (1.5-ml, Eppendorf) contained 100 μM Angeli's salt, 20 μM $CuCl_2$, 20 μM H_2O_2, 20 μM/base of sonicated calf thymus DNA, and a ^{32}P-5′-end-labeled DNA fragment in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37 °C for 30 min, the DNA fragments were heated at 90 °C in 1 M piperidine and treated as described previously (26). The treated DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel, and the autoradiogram was obtained by exposing x-ray film to the gel. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the procedure of Maxam and Gilbert (28) using a DNA-sequencing system (LKB 2010 Macrohor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

Lactate Dehydrogenase (LDH) Cytotoxicity Assay—Human breast cancer cells (MCF-7) were cultured in Dulbecco's modified Eagle's medium, (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 10% CO_2. Cells were seeded into 96-well microculture plates one day before the treatment, at a density of 10^5 cells/well with 100 μl of Dulbecco's modified Eagle's medium without phenol red containing 5% fetal bovine serum. Cells were treated with Angeli's salt or DEA-NO prepared in 0.01 N NaOH in combination with H_2O_2 prepared in water (0–10 mM). The NaOH present in the Angeli's salt or DEA-NO solutions was neutralized with the same volume of 0.01 N HCl. The LDH assay was performed using the CytoTox Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). To obtain maximal LDH release, nontreated cells were incubated in the presence of lysis solution (9% Triton X-100) for 45 min at 37 °C. After 4.5 h of incubation in the presence of the different products, the plate was centrifuged at 250 × *g* for 4 min, and 50 μl of supernatant was used for the LDH assay. The percentage of cytotoxicity was calculated according to the following equation,

$$\%\ \text{LDH release} = (\text{exp} - \text{back}_1)/(\text{max} - \text{back}_2) \times 100 \quad \text{(Eq. 5)}$$

where exp = experimental value, max = mean of cell maximum LDH release, back_1 = mean of cell culture background, and back_2 = mean of cell culture background plus lysis solution. All experiments were carried out at least in triplicate, and the results are expressed as means ± S.D.

RESULTS

Four different assays were used to study the effects of Angeli's salt on oxidation reactions mediated by H_2O_2 in the presence of Fe(III)-EDTA or Cu(II).

MDA Production from 2′-Deoxyribose—The first assay was based on oxidation of 2′-deoxyribose leading to the formation of MDA, which has been measured as a marker of HO· generation (29). The formation of MDA in the presence of Angeli's salt, H_2O_2, and Fe(III)-EDTA was very rapid and reached a plateau in 10 min, whereas H_2O_2 and Fe(III)-EDTA alone catalyzed the formation of MDA linearly up to 60 min of incubation (Fig. 1*A*). Lower concentrations of MDA were formed when the reaction was carried out in the presence of H_2O_2 plus Cu(II) than with H_2O_2 plus Fe(III)-EDTA. MDA was also formed dose dependently with different concentrations of Angeli's salt in the presence of H_2O_2 and metallic ions (Fig. 1*B*). However, its formation was inhibited by a high concentration (2 mM) of Angeli's salt, especially when the reaction was carried out in the presence of Fe(III)-EDTA. Fig. 2 compares the levels of MDA formation mediated by H_2O_2 and metallic ion in the presence of Angeli's salt, DEA-NO, or some reducing agents. NO^- generated from Angeli's salt catalyzed MDA formation from 2′-deoxyribose, as did other reducing agents such as ascorbic acid, glutathione, and NAD(P)H. In contrast, NO· generated from 200 μM DEA-NO inhibited MDA formation mediated by 500 μM H_2O_2 and 50 μM Fe(III)-EDTA or Cu(II) by 43 and 19%, respectively (Fig. 2). Formation of MDA from 2′-deoxyribose mediated by 200 μM Angeli's salt, 500 μM H_2O_2, and 50 μM Fe(III)-EDTA was also inhibited by 83 and 80% by the inclusion of 200 μM ferricyanide or 4-OH-TEMPO (electron acceptors), respectively (data not shown).

Formation of 8-oxo-dG in Calf Thymus DNA—As shown in Fig. 3, the levels of 8-oxo-dG increased dose dependently in calf thymus DNA incubated with Angeli's salt in the presence of H_2O_2 and metallic ions. As previously reported for other reducing agents such as ascorbic acid and NADH (27), Angeli's salt

[1] The abbreviations used are: DEA-NO, diethylamine-NONOate; carboxy-PTIO, 1H-imidazol-1-yloxy,2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; DTPA, diethylenetriamine pentaacetic acid; LDH, lactate dehydrogenase; MDA, malondialdehyde; 4-OH-TEMPO, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy free radical; 8-oxo-dG, 8-oxo-2′-deoxyguanosine; HPLC, high pressure liquid chromatography; bp, base pair(s).

[2] P. Chumakov, GenBank™/EBI Data Bank accession number X54156, 1990.

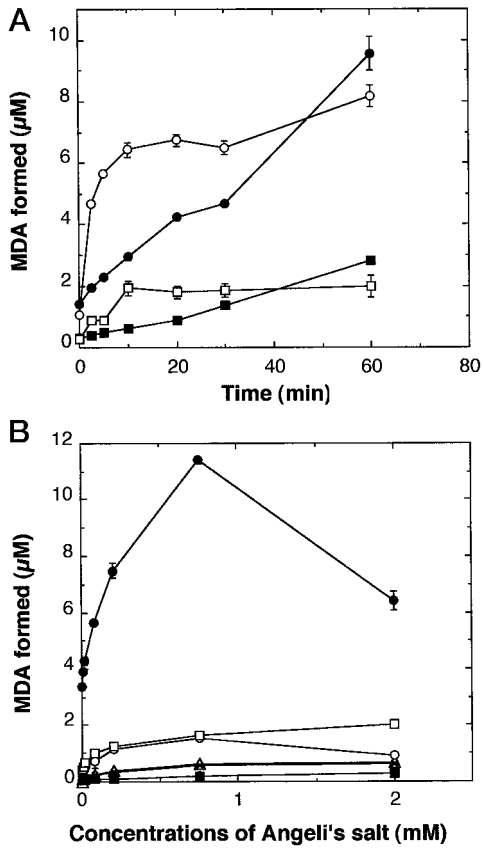

FIG. 1. **Effect of incubation time (*A*) and Angeli's salt concentrations (*B*) on MDA formation from 2′-deoxyribose by H_2O_2 and Fe(III)-EDTA or Cu(II).** *A,* the reactions were carried out in 100 mM sodium phosphate buffer, pH 7.4, containing 10 μM DTPA, 1 mM 2′-deoxyribose at 37 °C (final volume, 1 ml; final pH, ~7.5) in the presence of 200 μM Angeli's salt, 500 μM H_2O_2, and 50 μM $FeCl_3$-EDTA (○); 500 μM H_2O_2 and 50 μM $FeCl_3$-EDTA (●); 200 μM Angeli's salt, 500 μM H_2O_2, and 50 μM $CuCl_2$ (□); and 500 μM H_2O_2 and 50 μM $CuCl_2$ (■). *B,* the reactions were carried out at 37 °C for 10 min in the presence of Angeli's salt alone (△) and Angeli's salt plus 50 μM $FeCl_3$-EDTA (○); Angeli's salt plus 500 μM H_2O_2 (x); Angeli's salt plus 50 μM $FeCl_3$-EDTA and 500 μM H_2O_2 (●); Angeli's salt plus 50 μM $CuCl_2$ (■); and Angeli's salt plus 50 μM $CuCl_2$ and 500 μM H_2O_2 (□). The MDA contents were determined after the reaction with 2-thiobarbituric acid using HPLC with a fluorescence detector, as reported previously (1, 17). All experiments were carried out in triplicate.

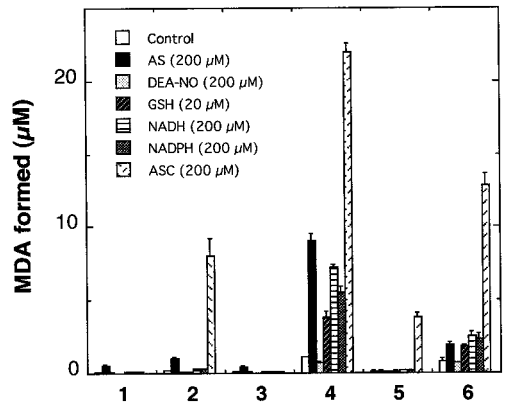

FIG. 2. **Comparison of the effect of Angeli's salt and DEA-NO with that of other reducing agents on MDA formation from 2′-deoxyribose by H_2O_2 and Fe(III)-EDTA or Cu(II).** The reactions were carried out in 100 mM sodium phosphate buffer, pH 7.4, containing 10 μM DTPA, 1 mM 2′-deoxyribose at 37 °C for 10 min (final volume, 1 ml; final pH, ~7.5). *1,* reductant alone; *2,* reductant plus 50 μM $FeCl_3$-EDTA; *3,* reductant plus 500 μM H_2O_2; *4,* reductant plus 500 μM H_2O_2 plus 50 μM $FeCl_3$-EDTA; *5,* reductant plus 50 μM $CuCl_2$; *6,* reductant plus 500 μM H_2O_2 plus 50 μM $CuCl_2$. The compounds tested were: H_2O (none, *Control*), Angeli's salt (*AS*), DEA-NO, glutathione (*GSH*), NADH, NADPH, and ascorbic acid (*ASC*). The concentrations were 200 μM, except for GSH, which was 20 μM.

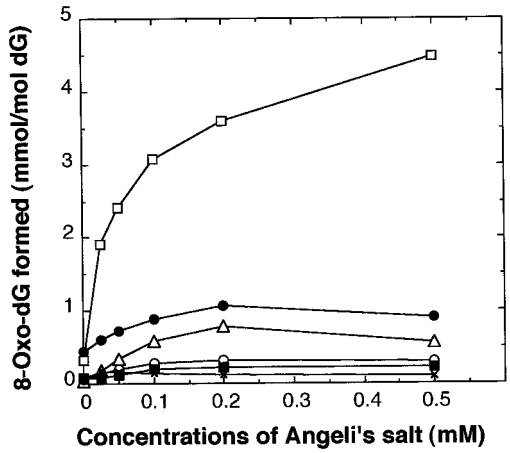

FIG. 3. **Effect of Angeli's salt concentration on 8-oxo-dG formation in calf thymus DNA.** The reactions were carried out in 0.1 M sodium phosphate buffer, pH 7.5, containing calf thymus DNA (1 mg) and 10 μM DTPA at 37 °C for 30 min (final volume, 1 ml) in the presence of Angeli's salt alone (△) and Angeli's salt plus 50 μM $FeCl_3$-EDTA (○); Angeli's salt plus 500 μM H_2O_2 (x); Angeli's salt plus 50 μM $FeCl_3$-EDTA and 500 μM H_2O_2 (●); Angeli's salt plus 50 μM $CuCl_2$ (■); and Angeli's salt plus 50 μM $CuCl_2$ and 500 μM H_2O_2 (□). After the reaction, ethanol-precipitated DNA was hydrolyzed enzymatically, and 8-oxo-dG was analyzed by HPLC with an electrochemical detector, according to a modification of the method of Yamaguchi *et al.* (18). Means of duplicate analyses are shown.

catalyzed the hydroxylation of 2′-deoxyguanosine in DNA more efficiently in the presence of Cu(II) than in the presence of Fe(III)-EDTA. As shown in Table I, hydroxyl radical scavengers (ethanol, D-mannitol, Me_2SO) inhibited 8-oxo-dG formation mediated by Angeli's salt, H_2O_2, and Fe(III)-EDTA more effectively than that mediated by Angeli's salt, H_2O_2, and Cu(II). Two electron acceptors, ferricyanide and 4-OH-TEMPO, also inhibited the formation of 8-oxo-dG by H_2O_2 and either Fe(III)-EDTA or Cu(II). $NO^{\cdot}$ generated from 0.02, 0.2, or 2 mM DEA-NO did not increase 8-oxo-dG levels in DNA induced with 500 μM H_2O_2 and 50 μM Fe(III)-EDTA or Cu(II), but rather reduced the hydroxylation of deoxyguanosine mediated by H_2O_2 and Fe(III)-EDTA or Cu(II) (40–55% inhibition) (data not shown).

DNA Strand Breakage—The pBR322 plasmid DNA was incubated with 200 μM Angeli's salt in the presence or absence of 500 μM H_2O_2 plus 50 μM Fe(III)-EDTA or Cu(II), and the percentages of form I (supercoiled form), form II (open ring form), and form III (linear form) were measured (Table II). As we previously reported (1), incubation of plasmid pBR322 with Angeli's salt alone formed 52.6% of form II, corresponding to ~1.25 single strand breaks/10^4 bp. Increased levels of DNA strand breakage were also observed when plasmid DNA was incubated with metallic ions (Fe(III)-EDTA or Cu(II)) alone or in combination with H_2O_2 and Fe(III)-EDTA or Cu(II) compared with nontreated plasmid. However, the addition of Angeli's salt dramatically enhanced strand breakage induced by H_2O_2 plus Fe(III)-EDTA or Cu(II). In particular, when the reaction was carried out in the presence of Angeli's salt, H_2O_2, and Cu(II), none of forms I, II, and III were clearly detected, indicating that the DNA was completely fragmented. When the plasmid was incubated with Angeli's salt, H_2O_2, and Fe(III), only forms II and III were formed, indicating that in addition to single strand breakage, double strand breaks were also induced.

Effects of $^{\cdot}OH$ Scavengers and Bathocuproine on DNA Dam-

TABLE I

Effects of hydroxyl radical scavengers and electron acceptors on 8-oxo-dG formation in calf thymus DNA incubated with Angeli's salt plus H_2O_2 in the presence of Fe(III)-EDTA or Cu(II)

Calf thymus DNA (1 mg/ml) was incubated with 200 μM Angeli's salt, 500 μM H_2O_2, and 50 μM Fe(III)-EDTA or Cu(II) in the presence of HO· scavengers and electron acceptors in 0.1 M sodium phosphate buffer (pH 7.4) containing 10 μM DTPA at 37 °C for 30 min. Mean ± S.D. (n = 3) are presented.

	8-Oxo-dG (μmol)/dG (mmol)	
	Fe(III)-EDTA	Cu(II)
Control[a]	0.427 ± 0.070 (100)[b]	3.12 ± 0.15 (100)[b]
Ethanol (0.2 M)	0.039 ± 0.009 (9)	3.71 ± 0.12 (119)
Me_2SO (0.2 M)	0.036 ± 0.010 (9)	2.68 ± 0.27 (86)
D-Mannitol (0.1 M)	0.075 ± 0.009 (18)	2.74 ± 0.16 (88)
Ferricyanide (0.02 M)	0.172 ± 0.055 (40)	0.90 ± 0.17 (29)
4-OH-TEMPO (0.02 M)	0.021 ± 0.008 (5)	1.17 ± 0.22 (38)

[a] Control; in the absence of scavengers.
[b] Numbers in parentheses are the percentages of controls (100%).

TABLE II

DNA strand breakage induced by Angeli's salt in the absence or presence of H_2O_2 and/or Fe(III)-EDTA or Cu(II)

Plasmid pBR322 DNA (100 ng) was incubated in 100 mM sodium phosphate buffer, pH 7.4, containing 10 μM DTPA at 37 °C for 15 min. Concentrations of compounds used: Angeli's salt (200 μM), H_2O_2 (500 μM), $FeCl_3$-EDTA (50 μM), and $CuCl_2$ (50 μM). Mean ± S.D. (n = 3) are presented. AS, Angeli's salt.

	Percentages of form		
	I (Supercoiled)	II (Ring open)	III (Linear)
Control	94.8 ± 1.6	5.2 ± 1.6	—[a]
H_2O_2	91.7 ± 1.7	8.3 ± 1.7	—
Fe(III)-EDTA	80.7 ± 1.8	19.3 ± 1.8	—
Cu(II)	89.2 ± 3.1	10.8 ± 3.1	—
AS	47.4 ± 3.2	52.6 ± 3.2	—
AS + H_2O_2	40.9 ± 1.3	59.1 ± 1.3	—
AS + Fe(III)-EDTA	24.2 ± 5.7	75.8 ± 5.7	Trace
AS + Cu(II)	49.5 ± 2.8	50.5 ± 2.8	—
H_2O_2 + Fe(III)-EDTA	47.1 ± 3.2	52.9 ± 3.2	Trace
H_2O_2 + Cu(II)	—	80.7 ± 3.8	19.3 ± 3.8
AS + H_2O_2 + Fe(III)-EDTA	—	64.7 ± 8.6	35.3 ± 8.6
AS + H_2O_2 + Cu(II)	—	—	—

[a] Not detected.

age Induced by Angeli's Salt—Fig. 4 shows that incubation of the ^{32}P-5′-end-labeled 261-bp fragment (*Ava*I* 1645-*Xba*I 1905) of the human c-Ha-*ras*-1 protooncogene with 200 μM Angeli's salt alone can induce DNA damage (*lane 2*). Hydroxyl radical scavengers such as ethanol, D-mannitol, and sodium formate inhibited the damage induced by Angeli's salt (*lanes 3–5*). Carboxy-PTIO, an NO·-trapping agent, which may also scavenge other oxidants (20, 30), inhibited the Angeli's salt-mediated DNA damage (*lane 6*), whereas bathocuproine, a Cu(I)-specific chelating agent, did not affect it (*lane 7*). On the other hand, 40 μM Angeli's salt in the presence of 20 μM $CuCl_2$ and 40 μM H_2O_2 exerted much stronger effects on the DNA than Angeli's salt alone (*lane 8*). This DNA damage was not inhibited by hydroxyl radical scavengers (*lanes 9–11*), whereas it was inhibited by carboxy-PTIO and bathocuproine almost completely (*lane 12* and *13*).

Site Preference of DNA Cleavage—The DNA cleavage sites were examined using ^{32}P-5′-end-labeled DNA fragments of the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene by the procedure of Maxam and Gilbert (28). As seen in Fig. 5, *B* and *D*, Angeli's salt alone caused DNA cleavage at every nucleotide position without marked site preference. On the other hand, Angeli's salt in the presence of H_2O_2 and Cu(II) induced piperidine-labile sites frequently at thymine residues

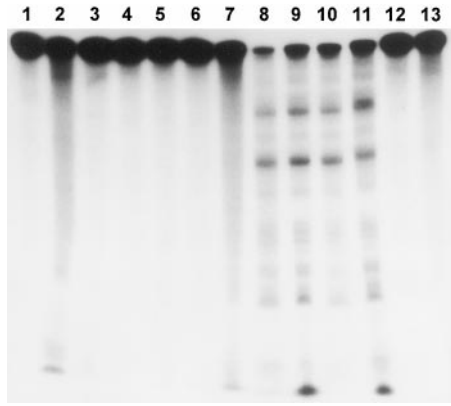

FIG. 4. **Effects of ·OH scavengers and bathocuproine on DNA damage induced by Angeli's salt alone (*lanes 2–7*) or Angeli's salt in the presence of H_2O_2 and Cu(II) (*lanes 8–13*).** The ^{32}P-5′-end-labeled 261-bp fragment (*Ava*I* 1645-*Xba*I 1905) of the human c-Ha-*ras*-1 was incubated in 200 μl of 10 mM sodium phosphate buffer at pH 7.8 containing 5 μM DTPA with Angeli's salt and $CuCl_2$, H_2O_2 and 20 μM/base of sonicated calf thymus DNA in the presence of the scavenger indicated at 37 °C for 30 min. After piperidine treatment, DNA fragments were analyzed by the method described under "Experimental Procedures." *Lane 1,* control; *lane 2,* 200 μM Angeli's salt alone; *lane 3,* + 0.8 M ethanol; *lane 4,* + 0.2 M D-mannitol; *lane 5,* + 0.2 M sodium formate; *lane 6,* + 500 μM carboxy-PTIO; *lane 7,* + 50 μM bathocuproine; *lane 8,* 40 μM Angeli's salt, 20 μM $CuCl_2$, and 40 μM H_2O_2; *lane 9,* + 0.8 M ethanol; *lane 10,* + 0.2 M D-mannitol; *lane 11,* + 0.2 M sodium formate; *lane 12,* + 500 μM carboxy-PTIO; *lane 13,* + 50 μM bathocuproine.

(Fig. 5, *A* and *C*). The most preferred site was the thymine residue, especially in the 5′-CTG-3′, 5′-GTG-3′, and 5′-GTA-3′ sequences.

Cytotoxicity—Human breast cancer cells (MCF-7) were incubated for 4.5 h with various concentrations of H_2O_2 in the presence or absence of 500 μM Angeli's salt or DEA-NO (Fig. 6*A*). Either 500 μM Angeli's salt or DEA-NO or 0–0.5 mM H_2O_2 alone did not elicit significant cytotoxic effects in MCF-7 cells. However, in the presence of Angeli's salt or DEA-NO, LDH release was increased by H_2O_2 dose dependently with the increase reaching 48.6 ± 5.2% and 30.3 ± 0.2%, respectively, at the 500 μM concentration. On the other hand, Angeli's salt alone showed weak cytotoxic activity after 4.5 h of incubation. However, increased cytotoxicity was observed when the cells were analyzed after 8 h of incubation (data not shown). The presence of H_2O_2 increased dramatically the cytotoxicity mediated by Angeli's salt. The increases in LDH release induced by 500 μM H_2O_2 alone or 2 mM Angeli's salt alone were only 9.0 ± 2.0% and 17.0 ± 0.3%, respectively, but up to 72.4 ± 1.7% when both compounds were incubated together. Although no cytotoxic effects were found with even the highest concentration (2 mM) of DEA-NO alone, the presence of H_2O_2 also enhanced cytotoxicity mediated by DEA-NO (Fig. 6*B*). However, the cytotoxic effect with DEA-NO plus H_2O_2 was, in general, weaker than that with Angeli's salt plus H_2O_2. It should be noted that Angeli's salt and DEA-NO have similar half-lives (~2.5 min) under physiological conditions.

DISCUSSION

Four different assays (DNA strand breakage, MDA formation from oxidation of 2′-deoxyribose, hydroxylation of 2′-deoxyguanosine in DNA, and analysis of site-specific DNA damage using ^{32}P-5′-end-labeled DNA fragments of the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene) have been used to study the effects of Angeli's salt, an NO^- generating compound (2–5), on DNA damage. It was found that Angeli's salt alone produced oxidants as previously reported (1), whereas the presence of H_2O_2 and either Fe(III)-EDTA or

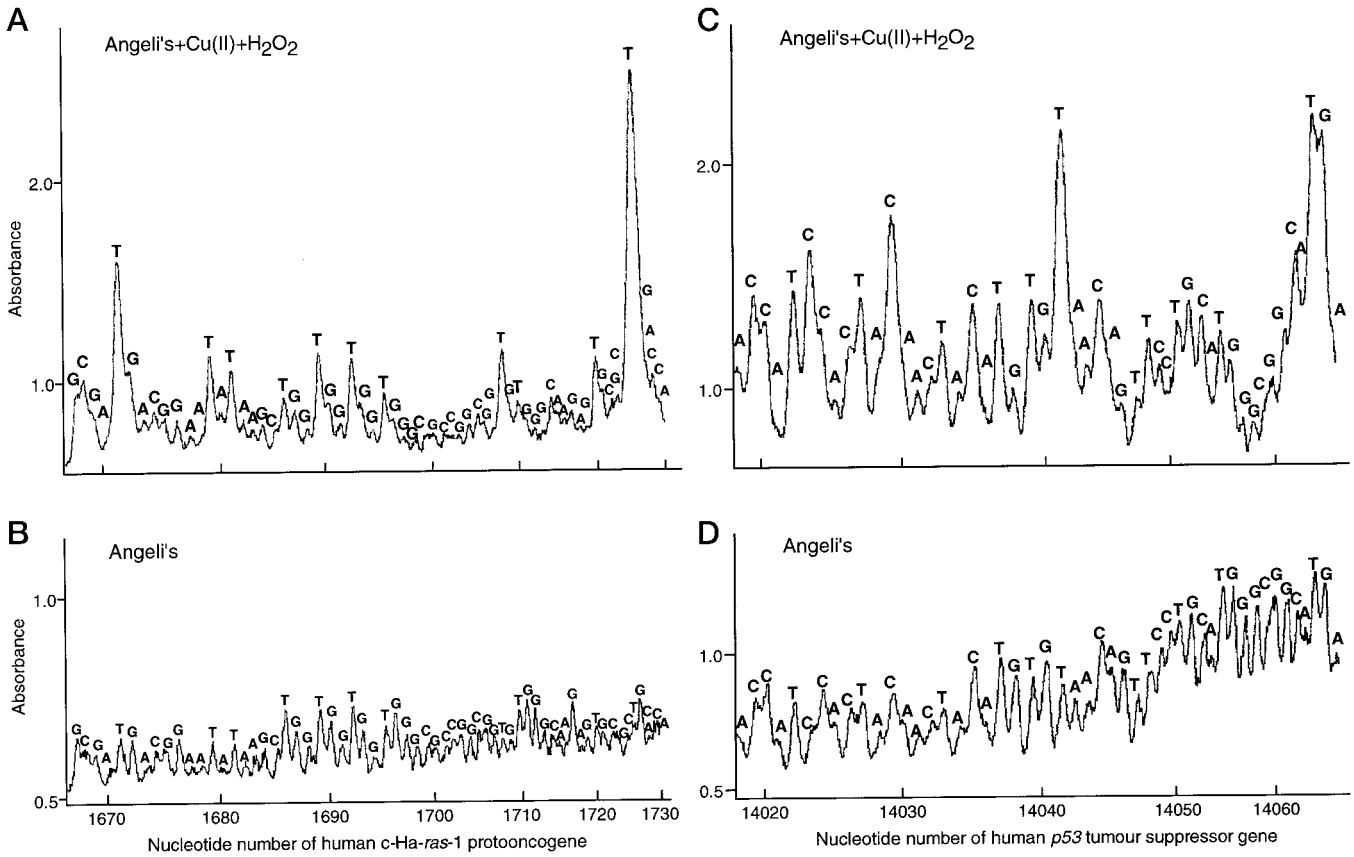

FIG. 5. **Site preference of DNA cleavage induced by Angeli's salt alone (*B* and *D*) or Angeli's salt in the presence of H_2O_2 and Cu(II) (*A* and *C*).** The ^{32}P-5′-end-labeled 261-bp fragment (*Ava*I* 1645-*Xba*I 1905) from c-Ha-*ras*-1 (*A* and *B*) or the 211-bp fragment (*Apa*I 13972-*Hin*dIII* 14 182) from *p53* (*C* and *D*) in 200 μl of 10 mM sodium phosphate buffer at pH 7.8 containing 5 μM DTPA and 20 μM sonicated calf thymus was incubated with Angeli's salt alone or Angeli's salt in the presence of H_2O_2 and Cu(II) at 37 °C for 30 min. After piperidine treatment, DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel using a DNA-sequencing system, and the autoradiogram was obtained by exposing x-ray film to the gel. The relative amounts of oligonucleotides produced were measured using a laser densitometer (LKB 222) UltroScan XL. The piperidine-labile sites of the treated DNA were determined by direct comparison with the same DNA fragment after undergoing DNA sequencing reactions according to the Maxam-Gilbert procedure (28). The horizontal axis shows the nucleotide number of the c-Ha-*ras*-1 protooncogene (25) (*A* and *B*) or the *p53* tumor suppressor gene[2] (*C* and *D*). *A* and *C,* 100 μM Angeli's salt + 20 μM $CuCl_2$ + 20 μM H_2O_2; *B* and *D,* 100 μM Angeli's salt.

Cu(II) dramatically enhanced the production of oxidants mediated by Angeli's salt. $NO^{\cdot}$ generated from DEA-NO did not enhance the formation of oxidants even in the presence of H_2O_2 and metallic ions but rather inhibited it. Electron acceptors, ferricyanide and 4-OH-TEMPO, which have been reported to convert NO^- to $NO^{\cdot}$ (2), inhibited Angeli's salt-mediated oxidation reactions even in the presence of H_2O_2 and metallic ions, suggesting that NO^-, but not $NO^{\cdot}$, is responsible for the oxidation reactions. As previously reported for other reducing agents such as ascorbic acid and NADH (27), Angeli's salt induced DNA strand breakage and catalyzed the hydroxylation of 2′-deoxyguanosine in DNA more efficiently in the presence of Cu(II) than in the presence of Fe(III)-EDTA. Distinct effects of $HO^{\cdot}$ scavengers on the oxidation reactions mediated by Fe(III)-EDTA and Cu(II) were also similar to those reported for ascorbic acid, glutathione, and NADH (27). The DNA cleavage sites examined using ^{32}P-5′-end-labeled DNA fragments of the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene indicate that Angeli's salt alone caused DNA cleavage at every nucleotide position without marked site preference. This cleavage pattern was similar to that reported for DNA damage induced by $HO^{\cdot}$ (with reductants such as ascorbic acid, glutathione, and NADH in the presence of H_2O_2 and Fe(III)-EDTA) (27). On the other hand, Angeli's salt in the presence of H_2O_2 and Cu(II) induced damage frequently at thymine residues, which was also similar to the pattern reported for DNA damage induced by the reductant, H_2O_2, and Cu(II) (27). These findings together lead us to conclude that NO^- can act as a reducing agent to generate strong oxidants in the presence of H_2O_2 and transition metallic ions.

It has been reported that reducing agents such as ascorbic acid and NADH can reduce transition metallic ions (M^{n+1}) to their reduced forms (M^n), which stimulate production of reactive oxygen species from H_2O_2 (Equation 6).

Reducing agent[ascorbic acid, glutathione, NADH, etc.]

$$+ M^{n+1}[\text{Fe(III), Cu(II), etc.}]$$

$$\rightarrow \text{oxidized reducing agent} + M^n[\text{Fe(II), Cu(I), etc.}] \quad \text{(Eq. 6)}$$

Similarly NO^- can act as a reducing agent to reduce transition metallic ions (Equation 7).

$$NO^- + M^{n+1} \rightarrow NO^{\cdot} + M^n \quad \text{(Eq. 7)}$$

In the case of Fe(III)-EDTA, the Fenton reaction mediated by Fe(II) forms $HO^{\cdot}$, which is responsible for DNA damage (Equation 8), because $HO^{\cdot}$ scavengers effectively inhibit the oxidation reactions. Conversely, Cu(II)-mediated DNA damage is not inhibited by $HO^{\cdot}$ scavengers, suggesting that a Cu(I)-peroxide complex, which exhibits $HO^{\cdot}$-like activities, may be responsible for the DNA damage (Equation 9) (27, 31–33).

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^{\cdot} + OH^- \quad \text{(Eq. 8)}$$

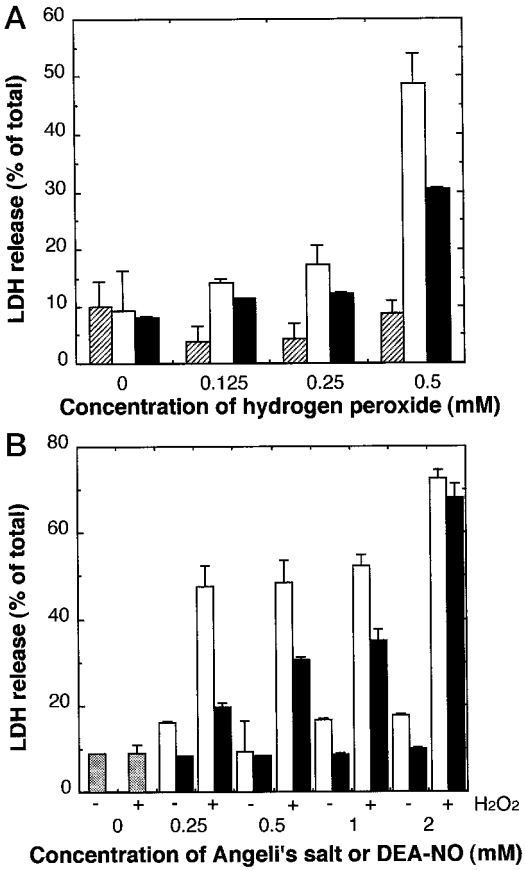

FIG. 6. **Effects of concentrations of H_2O_2 (*A*) and Angeli's salt or DEA-NO (*B*) on the cytotoxicity toward MCF-7 cells.** *A*, the cells were incubated for 4.5 h with various concentrations of H_2O_2 (0–500 μM) in combination either with 500 μM Angeli's salt or DEA-NO. *B*, Angeli's salt or DEA-NO at various concentrations (0–2 mM) was incubated in the presence or absence of 500 μM of H_2O_2. Results are expressed as the percentage (mean ± S.D.) of total LDH release into supernatant. *Hatched bar,* controls without Angeli's salt or DEA-NO; *open bar,* with Angeli's salt; *closed bar,* with DEA-NO.

$$Cu(I) + H_2O_2 \rightarrow Cu(I)\text{-}OOH + H^+ \qquad \text{(Eq. 9)}$$

In a previous study (1), we demonstrated that Angeli's salt and Piloty's acid (NO^--generating compounds) can produce strong oxidant(s) capable of inducing DNA strand breakage and oxidizing 2′-deoxyribose and calf thymus DNA to form MDA and 8-oxo-dG, respectively. These results led us to propose that NO^- is a possible endogenous source of $HO^\cdot$, which may be formed either directly from the reaction product of NO^- with $NO^\cdot$ ($N_2O_2^-$) (Equations 1 and 2) or indirectly through H_2O_2 formation (Equations 10 and 11).

$$NO^- + O_2 \rightarrow NO^\cdot + O_2^- \qquad \text{(Eq. 10)}$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(Eq. 11)}$$

In the present study, we observed that Angeli's salt alone induced DNA damage in the absence of H_2O_2 and transition metallic ions, confirming our previous findings (1). The inclusion of both H_2O_2 and transition metallic ions, however, synergistically enhanced DNA damage induced by Angeli's salt. On the other hand, the DNA damage induced by Angeli's salt was suppressed by the addition of either Fe(III)-EDTA or Cu(II) (Fig. 3), suggesting that NO^- was converted to $NO^\cdot$, which was inactive in generating oxidant(s) through the above mechanisms via either Equations 1 and 2 or Equations 10 and 11. In addition, $NO^\cdot$ possibly formed complexes with reduced transition metallic ions, inhibiting the generation of O_2^- and also the formation of oxidants from H_2O_2 (19, 34).

Several groups have recently reported that H_2O_2 and $NO^\cdot$ cooperatively enhance their cytotoxic activity toward hepatoma cells (35), lymphoma cells (36), ovarian cancer cells (37), and *Escherichia coli* (38). Using aromatic hydroxylation of salicylate as an indicator, the reaction of H_2O_2 with $NO^\cdot$ generated from DEA-NO was shown to produce an $HO^\cdot$-like oxidant (39). Farias-Eisner *et al.* (37) also reported that $NO^\cdot$, H_2O_2, and ferric ion in combination produce a potent oxidant, which can oxidize benzene to produce phenol, and they proposed the following mechanism for $HO^\cdot$ generation (Equations 12–14).

$$NO^\cdot + Fe(III) \rightarrow [Fe(III)\text{-}NO \leftrightarrow Fe(II)\text{-}{}^+NO] \qquad \text{(Eq. 12)}$$

$$[Fe(III)\text{-}NO \leftrightarrow Fe(II)\text{-}{}^+NO] + H_2O \rightarrow Fe(II) + NO_2^- + 2H^+ \qquad \text{(Eq. 13)}$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^\cdot + OH^- \qquad \text{(Eq. 14)}$$

$$\text{Overall: } NO^\cdot + H_2O_2 \rightarrow NO_2^- + HO^\cdot + H^+ \qquad \text{(Eq. 15)}$$

In contrast, our results demonstrated inhibitory effects of $NO^\cdot$ on the Fenton reaction and no production of oxidants, at least under our experimental conditions using DEA-NO, H_2O_2, and Fe(III)-EDTA or Cu(II) *in vitro*. This $NO^\cdot$-mediated inhibition of the Fenton reaction was in agreement with results from our previous study (19) and others (34, 40, 41).

On the other hand, Angeli's salt alone at higher concentrations (1 and 2 mM) exerted weak cytotoxicity, as reported for cultured Chinese hamster V79 lung fibroblasts by Wink *et al.* (2), whereas no cytotoxic effects were observed with even the highest concentration (2 mM) of DEA-NO alone under our experimental conditions. However, the presence of H_2O_2 increased cooperatively the cytotoxicity mediated by Angeli's salt. These results are in good agreement with those from our present *in vitro* study. This cytotoxic effect is probably because of the generation of $HO^\cdot$ through the Fenton reaction, which could occur in our cell culture system because $Fe(NO_3)_3$ was present in the medium. Conversely, synergistic cytotoxic effects of DEA-NO and H_2O_2 against MCF-7 cells were also observed, although the effects were, in general, lower than with Angeli's salt plus H_2O_2. Similar cooperative effects of $NO^\cdot$ and H_2O_2 on cytotoxicity were reported for other types of cells (35–38). These results are not in agreement with our *in vitro* study, which showed that DEA-NO did not generate oxidants even in the presence of H_2O_2 and metallic ions. There are several possible explanations for this discrepancy between *in vitro* and *in vivo* results. One possibility is that DEA-NO plus H_2O_2 induced cytotoxicity by a mechanism independent of $HO^\cdot$ formation. For example, $NO^\cdot$ inhibits the mitochondrial respiratory chain reaction, and H_2O_2 further enhances its toxicity. Alternatively, $NO^\cdot$ may be converted in cells to NO^-, which exerts toxic effects with H_2O_2. $NO^\cdot$ has been reported to be converted to NO^- by ferrocytochrome *c* (13).

In conclusion, we have demonstrated that the NO^--releasing compound, Angeli's salt, can catalyze the formation of strong oxidants in the presence of H_2O_2 and metallic ions, inducing DNA strand breakage, oxidation of DNA to form 8-oxo-dG *in vitro,* and exerting cytotoxic effects toward human breast cancer cells. Recent studies have demonstrated that NO^- may be formed *in vivo* under a variety of physiological conditions, including by $NO^\cdot$ synthase (6–8) and from *S*-nitrosothiols (9–11) and nitrosylhemoglobin (12). $NO^\cdot$ can be also converted to NO^- in the presence of biomolecules such as superoxide dismutase (42) and ferrocytochrome *c* (13) and by the quinone/hydroquinone redox system in a manner similar to that of the formation of O_2^- from oxygen (14). As stimulated immune cells including neutrophils and macrophages can produce H_2O_2, one

can expect that, during an inflammatory process, the formation of both NO^- and H_2O_2 could enhance dramatically the antimicrobial and anti-tumoricidal activity. In addition to the inflammatory process, under a number of pathological conditions (*e.g.* ischemia reperfusion injury, etc.), increased production of reactive oxygen species and activation of $NO^\cdot$ synthase have been shown to occur (24). Activated $NO^\cdot$ synthase produces NO^-, which may then encounter H_2O_2 to generate strong oxidants, as shown in this study. Thus NO^- may also play an important role as a cause of diverse pathophysiological conditions such as inflammation, neurodegenerative diseases, and cardiovascular disorders, especially when H_2O_2 and transition metallic ions are present together.

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