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), 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 32P-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 H₂O₂ plus Fe(III)-EDTA or Cu(II). NO⁻ released from diethylamine-NONOate had no such effect. Distinct effects of hydroxyl radical (HO•) scavengers and patterns of site-specific DNA cleavage caused by Angeli’s salt alone or by Angeli’s salt, H₂O₂ plus metal ion suggest that NO⁻ acts as a reductant to catalyze the formation of the HO• of H₂O₂ plus Fe(III) and formation of Cu(I)-peroxide complexes with a reactivity similar to HO• from H₂O₂ and Cu(II). Angeli’s salt and H₂O₂ 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 H₂O₂ and transition metallic ions are present.

Excess production of nitric oxide (NO) has been implicated as a cause of diverse pathophysiological conditions such as inflammation, neurodegenerative diseases, cardiovascular disorders, and cancer. These detrimental effects of NO have been attributed to reactive nitrogen species such as NOx and peroxynitrite (ONOO⁻), which are formed by the reaction of NO 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, can also cause strand breakage and oxidative damage in DNA in vitro (1). We have proposed that a highly toxic hydroxyl radical (HO•) generated from the reaction between NO⁻ and NO is responsible for the oxidation reactions (Equations 1 and 2).

NO⁻ + NO⁺ → N₂O₃⁻⁻ (Eq. 1)

N₂O₃⁻⁻ + H⁺ → N₂O + HO• (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 and other reactive oxygen and nitrogen species including superoxide, hydrogen peroxide (H₂O₂), 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 synthase generates NO⁻, which can be then converted to NO 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). Ferricytochrome c also reacts with NO to form ferriytochrome c and NO⁻, which may have implications for inhibition of mitochondrial oxygen consumption by NO⁻ (13). In our previous reports, NO⁻ was proposed as one of the possible agents responsible for DNA strand breakage induced by NO and catechol-type compounds such as catecholamines, catechol-estrogens, and certain flavonoids (14). NO⁻ can be formed by one-electron reduction of NO by the quinone/hydroquinone redox system in a manner similar to that of the formation of O₂ from oxygen (14).

In the present study, we have studied the effects of NO⁻ generated from Angeli’s salt (sodium trioxodinitrate, Na₂N₂O₅) on DNA strand breakage and DNA base modifications in vitro mediated by H₂O₂ in the presence of metallic ions. At physiological pH, Angeli’s salt exists predominantly in the form of the monoanion HN₂O₅⁻, which decomposes to NO⁻ and nitrite (NO₂⁻) (Equation 3) (15). As HNO is a weak acid (pKₐ = 4.7), NO⁻ is the predominant form in aqueous solution at neutral pH (Equation 4) (15).

HN₂O₅⁻ → HNO + NO₂⁻ (Eq. 3)

HNO → NO⁻ + H⁺ (Eq. 4)

We have found that NO⁻ generated from Angeli’s salt dramatically enhances DNA damage mediated by H₂O₂ 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 H₂O₂ cooperatively exerted cytotoxic effects toward human breast cancer cells. We discuss possible implications of our

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Synergism of NO− and H2O2 on Cytotoxicity and DNA Damage

findings as a cause of diverse pathophysiological conditions mediated by activation or overexpression of NO− synthases.

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. [5-3H]Thymidine (50 Ci/mmol) was supplied by NEN Life Science Products. Bathocuproinedisulfonic acid, and 1H-imidazol-1-yl-oxo-2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide 3-oxide, potassium salt (carboxy-PTIO) were from Dojin Chemicals Co., Kumamoto, Japan. All other chemicals including EDTA, ferric chloride, cuprous chloride, diethylenthretriamine pentaaetate complex (DTPA), 8-oxo-2′-deoxyguanosine, 4-hydroxy-2,2,6,6-tetramethylpiperidinoxyl free radical (4-OH-TEMPO), potassium ferricyanide (III) [K3Fe(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 mM DTPA, 1 mM 2′-deoxyribose, 500 μM H2O2, 50 μM either FeCl3-EDTA or CuCl2, an appropriate amount of HCl to neutralize the NaOH present in the Angeli’s salt solution, and 200 μg 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 mM sodium phosphate buffer, pH 7.5, calf thymus DNA (1 mg), 10 mM DTPA, 500 μM H2O2, 50 μM either FeCl3-EDTA or CuCl2, 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 Coulomach 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 45 min in 10 mM sodium phosphate buffer, pH 7.4 containing 10 mM DTPA, 500 μM H2O2, either 50 μM FeCl3-EDTA or CuCl2, 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 fluorescence detector to detect any nicked DNA, as described previously (1, 17).

Results

Four different assays were used to study the effects of Angeli’s salt on oxidation reactions mediated by H2O2 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, H2O2, and Fe(III)-EDTA was very rapid and reached a plateau in 10 min, whereas H2O2 and Fe(III)-EDTA alone catalyzed the formation of MDA linearly up to 60 min of incubation (Fig. 1A). Lower concentrations of MDA were formed when the reaction was carried out in the presence of H2O2 plus Cu(II) than with H2O2 plus Fe(III)-EDTA. MDA was also formed dose dependently with different concentrations of Angeli’s salt in the presence of H2O2 and metallic ions (Fig. 1B). 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 H2O2 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 H2O2 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 H2O2, and 50 μM Fe(III)-EDTA was also inhibited by 83 and 80% by the inclusion of 200 μM ferrocyanide 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 H2O2 and metallic ions. As previously reported for other reducing agents such as ascorbic acid and NAD(P)H (27), Angeli’s salt...
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, Me2SO) inhibited 8-oxo-dG formation mediated by Angeli’s salt, H2O2, and Fe(III)-EDTA more effectively than that mediated by Angeli’s salt, H2O2, and Cu(II). Two electron acceptors, ferricyanide and 4-OH-TEMPO, were far more efficient in the presence of Cu(II) than in the presence of Fe(III)-EDTA or Cu(II). NO generated from 0.02, 0.2, or 2 mM DEA-NO did not increase 8-oxo-dG levels in DNA induced with H2O2 and Fe(III)-EDTA or Cu(II), but rather reduced the hydroxylation of deoxyguanosine mediated by Fe(III)-EDTA or Cu(II), but rather reduced the hydroxylation of deoxyguanosine mediated by H2O2 and Fe(III)-EDTA or Cu(II) (18) (Fig. 2, panel E).

DNA Strand Breakage—The pBR322 plasmid DNA was incubated with 200 μM Angeli’s salt in the presence or absence of 500 μM H2O2 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/104 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 H2O2 and Fe(III)-EDTA or Cu(II) compared with nontreated plasmid. However, the addition of Angeli’s salt dramatically enhanced strand breakage induced by H2O2 plus Fe(III)-EDTA or Cu(II). In particular, when the reaction was carried out in the presence of Angeli’s salt, H2O2, 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, H2O2, 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 OH Scavengers and Bathocuproine on DNA Dam-

Fig. 1. Effect of incubation time (A) and Angeli’s salt concentrations (B) on MDA formation from 2′-deoxyribose by H2O2 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 for 10 min (final volume, 1 ml; final pH, ~7.5). I, reductant alone; 2, reductant plus 50 μM FeCl3-EDTA; 3, reductant plus 500 μM H2O2; 4, reductant plus 500 μM H2O2 plus 50 μM CuCl2; 5, reductant plus 50 μM CuCl2; 6, reductant plus 500 μM H2O2 plus 50 μM CuCl2. The compounds tested were: H2O (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. 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 H2O2 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). I, reductant alone; 2, reductant plus 50 μM FeCl3-EDTA; 3, reductant plus 500 μM H2O2; 4, reductant plus 500 μM H2O2 plus 50 μM CuCl2; 5, reductant plus 50 μM CuCl2; 6, reductant plus 500 μM H2O2 plus 50 μM CuCl2. The compounds tested were: H2O (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 mM 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 FeCl3-EDTA (○); Angeli’s salt plus 500 μM H2O2 (x); Angeli’s salt plus 50 μM FeCl3-EDTA and 500 μM H2O2 (●); Angeli’s salt plus 50 μM CuCl2 (■); and Angeli’s salt plus 50 μM CuCl2 and 500 μM H2O2 (□). The 8-oxo-dG contents were determined after the reaction with 2-thiobarbituric acid using HPLC with a fluorescence detector, as reported previously (1, 17). Me2SO has been added as a reductant plus 500 μM CuCl2 to avoid DNA precipitation. When the precipitated DNA was hydrolyzed enzymatically, and 8-oxo-dG was generated from 0.02, 0.2, or 2 mM DEA-NO, no increase in 8-oxo-dG levels in DNA induced with H2O2 and Fe(III)-EDTA or Cu(II) (data not shown).
age Induced by Angeli’s Salt—Fig. 4 shows that incubation of the \(^{32}\)P-5’-end-labeled 261-bp fragment (AvdI\^1 1645-XbaI 1905) of the human c-Ha-ras-1 protooncogene with 200 \(\mu\)M Angeli’s salt alone can induce DNA damage (lane 2). Hydroxyl radical scavengers such as ethanol, \(\nu\)-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 \(\mu\)M Angeli’s salt in the presence of 20 \(\mu\)M CuCl\(_2\) and 40 \(\mu\)M \(\mathrm{H}_2\mathrm{O}_2\) 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 \(\mathrm{H}_2\mathrm{O}_2\) and Cu(II) induced piperidine-labile sites frequently at thymine residues (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 \(\mathrm{H}_2\mathrm{O}_2\) in the presence or absence of 500 \(\mu\)M Angeli’s salt or DEA-NO (Fig. 6A). Either 500 \(\mu\)M Angeli’s salt or DEA-NO or 0–0.5 mM \(\mathrm{H}_2\mathrm{O}_2\) 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 \(\mathrm{H}_2\mathrm{O}_2\) dose dependently with the increase reaching 48.6 ± 5.2% and 30.3 ± 0.2%, respectively, at the 500 \(\mu\)M 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 \(\mathrm{H}_2\mathrm{O}_2\) increased dramatically the cytotoxicity mediated by Angeli’s salt. The increases in LDH release induced by 500 \(\mu\)M \(\mathrm{H}_2\mathrm{O}_2\) 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 incuated together. Although no cytotoxic effects were found with even the highest concentration (2 mM) of DEA-NO alone, the presence of \(\mathrm{H}_2\mathrm{O}_2\) also enhanced cytotoxicity mediated by DEA-NO (Fig. 6B). However, the cytotoxic effect with DEA-NO plus \(\mathrm{H}_2\mathrm{O}_2\) was, in general, weaker than that with Angeli’s salt plus \(\mathrm{H}_2\mathrm{O}_2\). 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 \(\mathrm{H}_2\mathrm{O}_2\) and either Fe(III)-EDTA or

![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 \(\mathrm{H}_2\mathrm{O}_2\) and Cu(II) (lanes 8–12). The \(^{32}\)P-5’-end-labeled 261-bp fragment (AvdI\^1 1645-XbaI 1905) of the human c-Ha-ras-1 was incubated in 200 \(\mu\)l of 10 mM sodium phosphate buffer at pH 7.8 containing 5 \(\mu\)M DTPA with Angeli’s salt and CuCl\(_2\), \(\mathrm{H}_2\mathrm{O}_2\), and 20 \(\mu\)M of sonicated calf thymus DNA in the presence of the scavenger indicated at 37 °C for 30 min. After piperdine treatment, DNA fragments were analyzed by the method described under “Experimental Procedures.” Lane 1, control; lane 2, 200 \(\mu\)M Angeli’s salt alone; lane 3, 200 \(\mu\)M Angeli’s salt and CuCl\(_2\); lane 4, 200 \(\mu\)M CuCl\(_2\); lane 5, 200 \(\mu\)M CuCl\(_2\) and Cu(II); lane 6, 200 \(\mu\)M CuCl\(_2\) and Cu(II); lane 7, 200 \(\mu\)M CuCl\(_2\) and Cu(II); lane 8, 40 \(\mu\)M Angeli’s salt, 20 \(\mu\)M CuCl\(_2\), and 40 \(\mu\)M Cu(II); lane 9, 200 \(\mu\)M Angeli’s salt, 20 \(\mu\)M CuCl\(_2\), and 20 \(\mu\)M Cu(II); lane 10, 200 \(\mu\)M CuCl\(_2\) and Cu(II); lane 11, 200 \(\mu\)M CuCl\(_2\) and Cu(II); lane 12, 40 \(\mu\)M CuCl\(_2\) and Cu(II); lane 13, 40 \(\mu\)M CuCl\(_2\) and Cu(II).](image-url)
Cu(II) dramatically enhanced the production of oxidants mediated by Angeli’s salt. NO\textsubscript{2} generated from DEA-NO did not enhance the formation of oxidants even in the presence of H\textsubscript{2}O\textsubscript{2} and metallic ions but rather inhibited it. Electron acceptors, ferricyanide and 4-OH-TEMPO, which have been reported to convert NO\textsubscript{2} to NO\textsubscript{z} (2), inhibited Angeli’s salt-mediated oxidation reactions even in the presence of H\textsubscript{2}O\textsubscript{2} and metallic ions, suggesting that NO\textsubscript{2}, but not NO\textsubscript{z}, 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\textsuperscript{9}-deoxyguanosine in DNA more efficiently in the presence of Cu(II) than in the presence of Fe(III)-EDTA. Distinct effects of HO\textsubscript{z} 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 32P-5\textsuperscript{9}-end-labeled DNA fragments of the human c-Ha-ras-1 protooncogene and the p53 tumor suppressor gene 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 the reductant, H\textsubscript{2}O\textsubscript{2}, and Cu(II) (27). These findings together lead us to conclude that NO\textsuperscript{−} can act as a reducing agent to generate strong oxidants in the presence of H\textsubscript{2}O\textsubscript{2} and transition metallic ions.

It has been reported that reducing agents such as ascorbic acid and NADH can reduce transition metallic ions (M\textsuperscript{n+1}) to their reduced forms (M\textsuperscript{n}), which stimulate production of reactive oxygen species from H\textsubscript{2}O\textsubscript{2} (Equation 6).

\[
\text{Reducing agent} + \text{ascorbic acid, glutathione, NADH, etc.} \rightarrow \text{oxidized reducing agent + M}^{n+1} [\text{Fe(III), Cu(II), etc.}] \quad \text{(Eq. 6)}
\]

Similarly NO\textsuperscript{−} can act as a reducing agent to reduce transition metallic ions (Equation 7).

\[
\text{NO}^{−} + \text{M}^{n+1} \rightarrow \text{NO}^{+} + \text{M}^{n} \quad \text{(Eq. 7)}
\]

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

\[
\text{Fe(II) + H}_2\text{O}_2 \rightarrow \text{Fe(III) + HO}^{−} + \text{OH}^{−} \quad \text{(Eq. 8)}
\]
Several groups have recently reported that H$_2$O$_2$ and NO$^-$ 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$_2$O$_2$ with NO$^-$ generated from DEA-NO was shown to produce an HO$^-$-like oxidant (39). Farias-Eisner et al. (37) also reported that NO$^-$, H$_2$O$_2$, and ferric ion in combination produce a potent oxidant, which can oxidize benzene to produce phenol, and they proposed the following mechanism for HO$^-$ generation (Equations 12–14).

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

\[
[\text{Fe(III)}-\text{NO} \leftrightarrow \text{Fe(II)-NO}] + \text{H}_2\text{O} \rightarrow \text{Fe(II)} + \text{NO}_2^- + 2\text{H}^+ \quad \text{(Eq. 13)}
\]

\[
\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{HO}^- + \text{OH}^- \quad \text{(Eq. 14)}
\]

Overall: \[\text{NO}^- + \text{H}_2\text{O}_2 \rightarrow \text{NO}_2^- + \text{HO}^- + \text{H}^+ \quad \text{(Eq. 15)}\]

In contrast, our results demonstrated inhibitory effects of NO$^-$ on the Fenton reaction and no production of oxidants, at least under our experimental conditions using DEA-NO, H$_2$O$_2$, and Fe(III)-EDTA or Cu(II) in vitro. This NO$^-$-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$_2$O$_2$ 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$^-$ 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$_2$O$_2$ against MCF-7 cells were also observed, although the effects were, in general, lower than with Angeli’s salt plus H$_2$O$_2$. Similar cooperative effects of NO$^-$ and H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ induced cytotoxicity by a mechanism independent of HO$^-$ formation. For example, NO$^-$ inhibits the mitochondrial respiratory chain reaction, and H$_2$O$_2$ further enhances its toxicity. Alternatively, NO$^-$ may be converted in cells to NO, which exerts toxic effects with H$_2$O$_2$. NO$^-$ has been reported to be converted to NO by ferrocyanochrome $c$ (19).

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$_2$O$_2$ and metallic ions, inducing DNA strand breakage, oxidation of DNA to form 8-oxo-dG, 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$^-$ synthase (6–8) and from S-nitrosothiols (9–11) and nitrosylhemoglobin (12). NO$^-$ can be also converted to NO by the presence of biomolecules such as superoxide dismutase (42) and ferrocyanochrome $c$ (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$_2$O$_2$, one...
can expect that, during an inflammatory process, the formation of both NO\(^-\) and \(\text{H}_2\text{O}_2\) could enhance dramatically the anti-microbial 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\(^-\) synthase have been shown to occur (24). Activated NO\(^-\) synthase produces NO\(^-\), which may then encounter \(\text{H}_2\text{O}_2\) to generate strong oxidants, as shown in this study. Thus NO\(^-\) may also play an important role as a cause of diverse pathophysiological conditions, as inflammation, neurodegenerative diseases, and cardiovascular disorders, especially when \(\text{H}_2\text{O}_2\) and transition metallic ions are present together.

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