The Chemistry and Tumoricidal Activity of Nitric Oxide/Hydrogen Peroxide and the Implications to Cell Resistance/Susceptibility*

(Received for publication, July 27, 1995, and in revised form, November 20, 1995)

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The mechanism of cytotoxicity of the NO donor 3-morpholino-sydnonimine toward a human ovarian cancer cell line (OVCAR) was examined. It was found that the NO-mediated loss of cell viability was dependent on both NO and hydrogen peroxide (H₂O₂). Somewhat surprisingly, superoxide (O₂⁻) and its reaction product with NO, peroxynitrite (·ONO), did not appear to be directly involved in the observed NO-mediated cytotoxicity against this cancer cell line. The toxicity of NO/H₂O₂ may be due to the production of a potent oxidant formed via a trace metal, H₂O₂, and NO-dependent process. Because the combination of NO and H₂O₂ was found to be particularly cytotoxic, the effect of NO on cellular defense mechanisms involving H₂O₂ degradation was investigated. It was found that NO was able to inhibit catalase activity but had no effect on the activity of the glutathione peroxidase (GSHPx)-glutathione reductase system. It might therefore be expected that cells that utilize primarily the GSHPx-glutathione reductase system for degrading H₂O₂ would be somewhat resistant to the cytotoxic effects of NO. Consistent with this idea, it was found that ebselen, a compound with GSHPx-like activity, was able to protect cells against NO toxicity. Also, lowering endogenous GSHPx activity via selenium depletion resulted in an increased susceptibility of the target cells to NO-mediated toxicity. Thus, a possible NO/H₂O₂/metal-mediated mechanism for cellular toxicity is presented as well as a possible explanation for cell resistance/susceptibility to this NO-initiated process.

The cytotoxic actions of activated macrophages against human cancer cell lines both in vitro and in vivo have been, at least partially, attributed to their ability to generate nitric oxide (NO)² (for example see Hibbs et al., 1988). Although the cytotoxic/cytostatic activity of NO is well established, the chemical mechanism by which NO elicits its cytotoxic action is less well understood. NO is capable of degrading certain iron-containing prosthetic groups, which results in an inhibition of the mitochondrial respiratory chain, DNA synthesis, and aconitase activity (Hibbs et al., 1988). Along with NO generation, activated macrophages also produce superoxide (O₂⁻). The reaction of NO with O₂⁻ is extremely rapid (Huike and Padmaja, 1993) and results in the generation of peroxynitrite (·ONO), which is a potent chemical oxidant when in the protonated form (Koppell et al., 1992). It has been demonstrated that ·ONO can be formed from macrophage-derived NO (Ioannidis and de Groot, 1993) and is capable of, for example, lipid peroxidation (Radi et al., 1991a), oxidation of sulfhydryl functions (Radi et al., 1991b) and aconitase inhibition (Hausladen and Fridovich, 1994; Castro et al., 1994). It has therefore been proposed that ·ONO is responsible for a significant portion of macrophage-derived cytotoxicity through a direct reaction of ·ONO with critical cellular components (Koppell et al., 1992). However, a recent report utilizing NO donor compounds indicated that NO was particularly tumoricidal in the presence of hydrogen peroxide (H₂O₂). Thus, it has been suggested that ·ONO may not be the only mechanism responsible for the cytotoxic actions of NO (at least in the hepatoma cell line utilized in that study) (Ioannidis and de Groot, 1993).

H₂O₂ is formed as an indirect product of macrophage activation (via the dismutation of O₂⁻). Therefore, we have performed a detailed examination of the chemistry and enzymology of possible NO/H₂O₂ interactions with tumor cell components in order to evaluate the possible role of NO/H₂O₂ in macrophage-mediated tumoricidal activity. Herein, we present evidence confirming the original observations of Ioannidis and de Groot (1993) indicating that the combination of NO and H₂O₂ was particularly cytotoxic to a human ovarian cancer cell line, and we propose a mechanism, based on a novel chemical process involving both NO and H₂O₂, for macrophage and NO-mediated tumoricidal activity. Furthermore, we present evidence supporting a hypothesis that may explain cell susceptibility/resistance toward NO-mediated cytotoxicity.

EXPERIMENTAL PROCEDURES

Enzymes, Chemicals, and Solutions—Superoxide dismutase (bovine erythrocytes), catalase (bovine liver), glutathione peroxidase (bovine erythrocytes) (GSHPx), glutathione reductase (Bakers' yeast), glutathione (GSH), NADPH, sodium azide, EDTA, 30% hydrogen peroxide (H₂O₂), epidermal growth factor, transferrin, insulin, and endothelial cell growth supplement were all purchased from Sigma. Selenious acid (H₂SeO₃) and all organic chemicals were obtained from Aldrich and were of the highest purity available. Ebselen was purchased from Biomol Research Labs (Plymouth Labs, PA). 3-Morpholino-sydnonimine (SIN-1) was obtained from Cayman Chemical Co. (Ann Arbor, MI). 5-Nitroso-N-acetylpenicillamine (SNAP) was synthesized by the method of Field et al. (1978). Potassium phosphate, ferric chloride, ferrous sulfate, and H₂O₂ (30%) were purchased from Fisher. NO gas was purchased from Matheson (Cucamunga, CA) and passed through aqueous base prior to utilization. All chemical reactions and manipulations were performed under strict anaerobic conditions. Oxygen was purged from all systems by utilizing several vacuum-N₂ purge cycles on a high vacuum line, and manipulations of gas and liquid samples were
carried out using N<sub>2</sub>-purged gas-tight syringes. Introduction of reactants to reaction solutions was achieved via injection through rubber septa. Water was purified by distillation in an all-glass apparatus. H<sub>2</sub>O<sub>2</sub> concentrations in stock solutions were determined by iodometric titration (Jeffery et al., 1989).

Cells and Media—The human epithelial ovarian cancer cell line Ni (Hendrix, 1989) and OVCAR (Van Leersum, 1992) was included in this experiment, the rate of NADPH loss was monitored for 2 min in the absence of NO. Then 50 µl of NO gas was added, and the rate of NADPH loss was monitored afterwards. As controls, spontaneous NADPH loss in both the presence and the absence of NO was determined by monitoring the decrease in absorbance at 366 nm in the absence of GSHPx and was subtracted from values obtained in the presence of GSHPx. Note: The addition of 50 µl of NO to the control experiments either prior to the addition of H<sub>2</sub>O<sub>2</sub> or after 2 min of incubation did not significantly alter the rate of spontaneous NADPH loss.

The Effect of NO on Ebselen-Mediated H<sub>2</sub>O<sub>2</sub> Degradation—The same procedure described above for measuring GSHPx activity was utilized except that a 50 µM solution of ebselen in buffer was utilized in place of GSHPx (final ebselen concentrations in the incubations was 28 µM). Ebselen was solubilized by first dissolving it in a minimum amount of methanol, followed by the addition of the requisite amount of phosphate buffer solution, pH 7.0, containing 2.5 mM EDTA and 2.5 mM sodium azide (Me<sub>2</sub>SO concentration in the incubations was always <0.25%). As described above, all solutions were degassed on a vacuum line prior to the initiation of the reaction. Reactions were initiated by the addition of 200 µl of a 12.5 mM degassed solution of H<sub>2</sub>O<sub>2</sub>. In incubations carried out in the presence of NO, 50 µl of pure NO gas was injected into the headspace of the degassed cuvette containing all the reaction components except H<sub>2</sub>O<sub>2</sub>. The cuvette was then vigorously shaken before the addition of H<sub>2</sub>O<sub>2</sub>. The reactions were allowed to proceed for 90 s by monitoring the absorbance at 366 nm. Control experiments were performed in the absence of ebselen to determine the spontaneous rate of NADPH degradation. These control values were subtracted from the values obtained in the presence of ebselen to determine the change in NADPH value.

Effect of Ebselen on NO-Mediated Loss in Cell Viability—OVCAR cell viability was determined by performing the above experiments measuring lactate dehydrogenase activity in the presence and the absence of ebselen (10 µM). This concentration of ebselen was chosen on the basis of prior observations by others (Leurs et al., 1990; Chaudhrie et al., 1994).

The Effect of Selenium Depletion on GSHPx Activity and on NO/H<sub>2</sub>O<sub>2</sub>/Iron-mediated Cytotoxicity—Following a general method previously described by Jullian et al. (1992), cells were cultured in the absence of serum using an artificial serum extender. Thus, minimum essential medium was utilized in the presence of the following medium supplements: epidermal growth factor (2 ng/ml), transferrin (5 µg/ml), insulin (2.5 µg/ml), and selenium supplements (note: selenious acid has been previously determined to be a bioavailable and relatively nontoxic form of selenium at this concentration (Hocman, 1988)). The exposure of these cells to SIN-1 was performed identically to those described above for the serum-cultured OVCAR cells. Cytotoxicity was monitored by measuring lactate dehydrogenase release from these cells as described above. The determination of GSHPx activity in these cells was performed using the method of Paglia and Valentine (1967). Possible Reaction between NO and H<sub>2</sub>O<sub>2</sub> as Determined by Loss of NO—Into three 25-ml round bottom flasks equipped with septum—locked glass stopper tops, solutions in which the effect of added selenium was examined, selenious acid was added to the artificial serum extender at 25 µM concentration (note: selenious acid has been previously determined to be a bioavailable and relatively nontoxic form of selenium at this concentration (Hocman, 1988)). The exposure of these cells to SIN-1 was performed identically to those described above for the serum-cultured OVCAR cells. Cytotoxicity was monitored by measuring lactate dehydrogenase release from these cells as described above. The determination of GSHPx activity in these cells was performed using the method of Paglia and Valentine (1967). Spectroscopic Analysis of NO-Iron Species in Solution—Spectroscopic studies were carried out on a UV/visible spectrophotometer (Talas, San Diego, CA) operating at 200–800 nm. In a typical experiment, 2 ml of a 1 mM solution of either FeCl<sub>2</sub>-H<sub>2</sub>O or FeSO<sub>4</sub>-H<sub>2</sub>O in N<sub>2</sub>-degassed distilled water was placed into a 3-ml quartz cuvette equipped with a gas tight rubber septum. The solution was then degassed, and the cuvettes were left under N<sub>2</sub> for the duration of the experiments. Pure NO gas and/or H<sub>2</sub>O<sub>2</sub> were added to the cuvettes, and changes in absorbance of these solutions were recorded on a scan chart recorder.

The determination of Nitrite (NO<sub>2</sub>−) and Nitrate (NO<sub>3</sub>−) determinations were performed as described previously (Bush et al., 1992) using the general methods of Branan and Hendrix (1989). Briefly, measurement of NO<sub>2</sub>− levels were made by monitoring NO evolution via chemiluminescence detection from a measured sample placed into a refluxing solution of iododeacetic acid (this solution will only reduce NO<sub>2</sub>− and not NO<sub>3</sub>− to NO). Total NO<sub>2</sub>− plus NO<sub>3</sub>− determinations were made by monitoring NO evolution from a measured sample placed into a boiling VCl<sub>2</sub>HCl solution (this solution will reduce both NO<sub>2</sub>− and NO<sub>3</sub>− to NO). The determination of NO<sub>3</sub>−
levels was made by simply subtracting the value for NO\textsubscript{3} from the NO\textsubscript{2} value from duplicate samples analyzed by both methods. Quantitation was accomplished using a standard curve made up of known amounts of NO\textsubscript{2} and NO\textsubscript{3}.

Thus, in a typical experiment, the appropriate metal salt was placed into a 10-ml flask equipped with a serum-capped stopcock. The salt was then taken up in 2 ml of water and degassed. Then NO gas was injected into the reaction headspace. When required, the addition of H\textsubscript{2}O\textsubscript{2} to these solutions was also made via injection of a degassed stock solution in water. Reaction times were chosen on the basis of the spectroscopic studies described above. That is, because the reduction of Fe(III) by NO was found to be slow, such reactions were allowed to run for 15 min, whereas the reaction between H\textsubscript{2}O\textsubscript{2} and Fe(II), which was found to be extremely fast, was run for only 1 min. Reactions were quenched by the addition of strong base (NO\textsubscript{2}) in the presence of H\textsubscript{2}O\textsubscript{2}. The reaction flask was then degassed again to remove any unreacted NO. Aliquots of the solution were then analyzed immediately. Control experiments were performed to assure the stability of measured species, NO\textsubscript{2} and NO\textsubscript{3}, under the assay and reaction conditions.

Oxidation of Benzene by the NO/H\textsubscript{2}O\textsubscript{2}/Metal System—In a typical experiment, 0.3 mmol of benzene in 15 ml of purified water (20 m\textsuperscript{-3}), the approximate maximum solubility in water) containing 0.3 mmol of H\textsubscript{2}O\textsubscript{2} (20 m\textsuperscript{-3}) and 3.6 \mu mol of ferric chloride (0.2 m\textsuperscript{-3}) was oxidized in the presence of 0, 0.1, 0.5, and 1 equivalent of NO (based on benzene) for the appropriate time under anaerobic conditions. The reactions were terminated by immediate extraction of the organic products and reactant with 2 × 25 ml of ethyl ether containing internal standard (phenethyl alcohol). The organic extract was then dried with sodium sulfate and concentrated on a rotary evaporator. Gas chromatographic analysis of the reaction products was accomplished using a Hewlett-Packard 5880 gas chromatograph utilizing a 25 meter, 5% phenyl methyl silicone column, 0.32 micron film, 0.2-mm inner diameter operating at a flow of approximately 0.8 ml/min with the following temperature program: initial temperature, 100°C; initial time, 5 min; program rate, 10°C/min; final temperature, 225°C; final time, 5 min. Reaction products were identified on the basis of the comparison of the retention time with authentic standards (phenol, 4.77 min). Reaction products were also characterized by gas chromatography-mass spectral analysis on a Hewlett-Packard 5971A, operating at 70 eV and utilizing a 12.5-m HP-1 column. Reaction product identification was confirmed by comparison of the mass spectra with published spectra (McLafertry and Stauffer, 1988). Quantitation of reaction products was accomplished by standard curve analysis using phenethyl alcohol as an internal standard.

RESULTS

The Effect of NO on OVCAR Cell Viability—SIN-1 is a well known NO donor (for example, see Ioannidis and de Groot (1993)) and was found to cause a significant loss of OVCAR cell viability (as measured by lactate dehydrogenase release into the media) (Fig. 1). 5 mM SIN-1 resulted in a release of over 75% of total lactate dehydrogenase into the cell media. Similar results were also observed using another NO donor, SNAP, as well (data not shown). Thus, these data indicate that NO derived from these NO donors is indeed cytotoxic to OVCAR cells. It should be noted that NO and NO donors were determined to have no effect on the lactate dehydrogenase assay (data not shown). Also, the time course for toxicity was determined for 5 mM SIN-1, and little or no toxicity was observed at 6, 12, 18, and 24 h following initial exposure to SIN-1. Significant toxicity was only observed after 48 h. Therefore, cell viability was determined 48 h after initial exposure to SIN-1. The duration of exposure of the OVCAR cells to NO released from SIN-1 at 2.5 and 5 mM initial concentrations is somewhat difficult to determine because previous kinetic studies of NO release from SIN-1 show a nonlinear relationship with concentration (Ioannidis and deGroot, 1993; Fedisch and Noack, 1987). However, the most conservative estimate using the available kinetic data would indicate that NO release from SIN-1 at either 2.5 or 5 mM should be >94% complete after 24 h.

The Effect of Superoxide Dismutase and Catalase on SIN-1-mediated Cytotoxicity—Superoxide dismutase was found to enhance the cytotoxic effect of 2.5 mM SIN-1 (Fig. 1), whereas protection against SIN-1-mediated cytotoxicity was observed when catalase was added. The addition of only H\textsubscript{2}O\textsubscript{2} (100 \mu M in the absence of SIN-1) to the cell incubation did not result in a significant loss of cell viability. However, 100 \mu M H\textsubscript{2}O\textsubscript{2} was found to be cytotoxic in the presence of SNAP (that is, SNAP cytotoxicity was significantly enhanced in the presence of 100 \mu M H\textsubscript{2}O\textsubscript{2}; data not shown).

The above data implicate both NO and H\textsubscript{2}O\textsubscript{2} as being involved in the observed cytotoxicity. Because cells are normally able to keep H\textsubscript{2}O\textsubscript{2} levels at a minimum by utilizing enzymes that specifically degrade H\textsubscript{2}O\textsubscript{2} to innocuous species, the effect of NO on these enzymes, catalase and the GSH Px-glutathione reductase system, was examined.

The Effect of NO on Catalase Activity—Under anaerobic conditions (to assure a significant lifetime for NO under the experimental conditions), the effect of NO on catalase activity was determined. When approximately 0.45 \mu mol of pure NO gas was introduced to an incubation consisting of 5 units of catalase (0.25 \mu g) in 3 ml of 18 mM H\textsubscript{2}O\textsubscript{2}, the enzyme activity decreased to approximately 23% of the control value (Table I). The concentration of NO in the catalase/H\textsubscript{2}O\textsubscript{2} solution was determined to be 15.5 \mu M (which is in close agreement with the value calculated from Henry's law of 20 \mu M).

The Effect of NO on GSH Px Activity—The catalytic reduction of H\textsubscript{2}O\textsubscript{2} by GSH requires two enzymes, GSH Px, which reduces H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O using GSH, and glutathione reductase, which converts the GSH disulfide (glutathione) back to the reduced form at the expense of NADPH (Wendel, 1980). Because NADPH is consumed in this system, the activity of the GSH Px-glutathione reductase system can be monitored by measuring NADPH consumption. Thus, the effect of NO on NADPH con-
The Reaction of NO with H$_2$O$_2$ is particularly cytotoxic to OVCAR cells, studies were conducted to determine the possible interactions of NO and iron in solution. Thus, the addition of excess NO gas to a 1 mM Fe(III) (FeCl$_3$) solution resulted in the gradual formation of an apparent NO adduct as indicated by the appearance of absorbances at 436 and 578 nm. Significantly, the addition of NO to a 1 mM Fe(II) (FeSO$_4$) solution resulted in the rapid formation of the same adduct as evidenced by the identical UV-visible spectrum. The absorbance spectrum of the apparent NO adduct is identical to that of the so-called “brown ring” complex, Fe(II)NO(H$_2$O)$_6$ with $\lambda_{max}$ = 436 and 578 nm (Littlejohn and Chang, 1982). Thus, it is apparent that NO is able to reduce Fe(III) to Fe(II) in water to generate an “NO (or equivalent) species. Reaction of NO with water should yield, as the nitrogen oxide product, NO$_2$ (Reactions 1 and 2). Complexation of Fe(II) by another equivalent of NO would then give the observed brown ring complex (Reaction 3).

NO + Fe(III) $\rightarrow$ [Fe(III)-NO $\leftrightarrow$ Fe(II)-NO]

\textbf{REACTION 1.}

[Fe(III)-NO $\leftrightarrow$ Fe(II)-NO] + H$_2$O $\rightarrow$ Fe(II) + NO$_2$ + 2H$^+$

\textbf{REACTION 2.}
The oxidation of the complex as evidenced by the immediate loss of the 
H2O2 to the brown ring complex results in a rapid disappear-
ance of Fe(II) species in the presence of an oxidizing agent like H2O2 
would be the terminal product. However, the formation of an 
Fe(II) species in the presence of an oxidizing agent like H2O2 
should lead to the formation of a potent oxidizing species like 
·OH via a Fenton process (Reaction 4). (Note: the addition of 
Fe(III), NO, and H2O2 should be capable of reacting by the process defined by the sum of Reactions 1, 
2, and 4 (Reaction 5).

Thus, Reaction 5 represents a process by which NO and H2O2 
can react, in the presence of a catalytic amount of a trace metal, 
to generate a potent oxidizing species like ·OH.

Analysis of the Reaction Mixtures for Nitrite (NO2) and Nitrate (NO3)—As indicated in Reaction 2, the reduction of Fe(III) to Fe(II) should result in NO2 generation exclusively. This was tested when 5 ml of NO gas (approximately 220 μmol) was introduced into the headspace of a 10-ml flask containing a 2-ml solution of Fe(III)Cl3 (2 mM) in water under anaerobic conditions, followed by the addition of NaOH after 30 min (to quench the reaction and stabilize the nitrogen oxide products) and degassing (to remove the excess NO). Analysis of the inorganic nitrogen products showed exclusive generation of NO2. Only trace amounts of NO3 were detected. Also, a 2 mM, anaerobic solution of Fe(III)Cl3 in water was reacted with NO gas (5 ml in the reaction headspace as described above) and 1 equivalent of H2O2. After 15 min, the reaction was quenched with base, degassed and the levels of NO2 and NO3 determined. Again, NO2 was the primary nitrogen oxide product with only trace amounts of NO3 detected. If, indeed, ·OH were generated in solution, it may be expected that excess NO could trap this reactive species to give NO3 (Reaction 6).

This is apparently the case under the conditions of these experiments because a 0.2 mM solution of Fe(II)SO4 in water was found to react under anaerobic conditions with NO (excess) and 
H2O2 (1 equivalent) to give NO3 as the primary nitrogen oxide 
product in only trace amounts. The results of these studies are summarized in Table II.

The Oxidizing Potential of NO/H2O2 in the Presence of a 
Trace Metal—Aromatic ring hydroxylation is an established 
reaction of ·OH (Kaur and Halliwell, 1994). Therefore, the 
oxidation of benzene by the NO/H2O2/iron system was exam-
ined. As expected, it was found that benzene could be oxidized 
to phenol by the NO/H2O2/iron system, and the yield of 
phenol increased when NO addition was increased from 0.1 to 
0.5 equivalents (Table III). Longer reaction times resulted in a 
new total loss of substrate and the further oxidation of phenol 
to highly oxidized products as evidenced by a darkening of the 
reaction solution. No oxidation products were found in the 
absence of NO. There is no doubt that the oxidation of benzene 
to phenol was catalytic in ferric ion because a >2000% yield 
based on iron was obtained. Also, a greater than 40% yield of 
phenol based on NO could be attained when 0.5 equivalents 
of NO were used. Interestingly, when higher concentrations of 
NO are utilized, the yield of phenol was not increased. This is 
likely due to a combination of effects including an increased 
importance of Reaction 6 at high NO levels, a more rapid rate 
of phenol destruction (to further oxidized and undetected spe-
cies) and trapping of other radical intermediates by NO. Thus, 
in this system it appears that NO can act as a reducing agent 
for the generation of Fe(II) and thus initiate radical mediated 

effect of added ferric ion on the decomposition of NO in 
the presence of hydrogen peroxide. ● NO + H2O2, trace Fe3+
added at 35 min; □ NO without H2O2, trace Fe3+ added at 35 min; ▲ NO without H2O2 or added Fe3+.

Fe(II)+(H2O)3 + NO → Fe(II)-NO(H2O)3 (brown ring complex)

In the absence of any oxidizing agents, the Fe(II)-NO complex 
would be the terminal product. However, the formation of an 
Fe(II) species in the presence of an oxidizing agent like H2O2 
should lead to the formation of a potent oxidizing species like 
·OH via a Fenton process (Reaction 4). (Note: the addition of 
H2O2 to the brown ring complex results in a rapid disappear-
ance of the complex as evidenced by the immediate loss of the

absorbances at 436 and 578 nm; data not shown.)

Fe(II) + H2O2 → Fe(III) + NO− + ·OH

REACTION 4.

Therefore, a solution of Fe(III), NO, and H2O2 should be capable 
of reacting by the process defined by the sum of Reactions 1, 
2, and 4 (Reaction 5).

overall NO + H2O2 → NO2 + ·OH + H+

REACTION 5.

Fig. 3. The effect of selenium (Se) depletion on the susceptibility 
of OVCAR cells to SIN-1 mediated cytotoxicity. The cells 
without selenium were determined to have approximately 28% less 
GSHPx activity compared with the cells with selenium. *, p < 0.0005 
when compared with cells cultured in the presence of 25 nM selenious 
acid. All values represent the mean of at least two experiments per-
formed in triplicate. LDH, lactate dehydrogenase.

Fig. 4. Effect of added ferric ion on the decomposition of NO in 
the presence of hydrogen peroxide. ● NO + H2O2, trace Fe3+
added at 35 min; □ NO without H2O2, trace Fe3+ added at 35 min; ▲ NO without H2O2 or added Fe3+.

Fe(II)+(H2O)3 + NO → Fe(II)-NO(H2O)3 (brown ring complex)

Reaction 3.

In the absence of any oxidizing agents, the Fe(II)-NO complex 
would be the terminal product. However, the formation of an 
Fe(II) species in the presence of an oxidizing agent like H2O2 
should lead to the formation of a potent oxidizing species like 
·OH via a Fenton process (Reaction 4). (Note: the addition of 
H2O2 to the brown ring complex results in a rapid disappear-
ance of the complex as evidenced by the immediate loss of the
cytotoxic is not obviously immediate, because unlike the NO and O$_2$, they would not be expected to directly react with each other to generate a chemically destructive species. However, we have shown that NO and H$_2$O$_2$, in the presence of trace metals, are capable of generating a potent oxidant (Reactions 1, 2, and 4), possibly hydroxy radical (-OH), which should be capable of indiscriminate damage to cellular components. There is chemical precedence for the individual steps in the reaction sequence leading to -OH generation. The reduction of Fe(III) to Fe(II) by NO (Reactions 1 and 2) has been reported previously (Wayland and Olsen, 1974; Wade and Castro, 1990; Gwost and Coulton, 1973), and we have obtained spectroscopic evidence that this can occur with simple iron salts. The reduction of H$_2$O$_2$ by Fe(II) to generate -OH (Reaction 3) is a well known process generally referred to as the Fenton reaction (for an example see Goldstein et al. (1993)). Also, analysis of the nitrogen oxide products from the reaction of NO with Fe(II), NO with Fe(III)/H$_2$O$_2$, and NO with Fe(II)/H$_2$O$_2$ indicate that NO$_2$ is the primary species generated (Table II) and is generally consistent with the proposed chemistry (Reactions 1, 2, 4, and 6). That is, the reaction of Fe(III) with NO should give, maximally, 1 equivalent of NO$_2$ per Fe(II) via Reaction 2 (0.5 equivalents found). The reaction of NO with Fe(III) and 1 equivalent of H$_2$O$_2$ (based on Fe(III)) should give maximally 3 equivalents of NO$_2$ via the sequence of Reactions 2, 4, 6, and 2 (2.3 equivalents found). Finally, the reaction of NO with Fe(II) and 1 equivalent of H$_2$O$_2$ (based on Fe(II)) should give, maximally, 2 equivalents of NO$_2$ via the reaction sequence 4, 6, and 2 (although the stoichiometry for this reaction was unexplainably higher than expected, 2.3, only NO$_2$ was detected indicating again that Reaction 6 predominates under these conditions).

It is possible that -OONO could have been generated in our chemical system via Reaction 7,

$$[\text{Fe(III)-NO} \rightarrow \text{Fe(II)-}^{-}\text{NO} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(II)+}^{-}\text{OONO} + 2\text{H}^+]$$

Reaction 7.

or possibly by the combination of Reactions 8 and 9.

$$\cdot\text{OH + H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \cdot\text{OOH}$$

Reaction 8.

$$\cdot\text{OOH + NO} \rightarrow \cdot\text{OONO} + \text{H}^+$$

Reaction 9.

It should be mentioned that referring to the product of the Fenton reaction as -OH may not be entirely correct because this subject remains a matter of some controversy (for example, see Wink et al. (1991, 1994)). However, it is clear that the combination of Fe(II) and H$_2$O$_2$ results in the generation of a potent oxidant with reactivity similar to that of -OH. Therefore, for the sake of convenience and simplicity, we will refer to the Fenton product as -OH.
However, because NO\textsubscript{3} (the thermodynamically stable decomposition product of \textsuperscript{\textsuperscript{15}}\textsuperscript{\textsuperscript{15}}ONOO) was not generated to any significant extent under the conditions of our experiments, we believe that \textsuperscript{\textsuperscript{15}}\textsuperscript{\textsuperscript{15}}ONOO is not the likely oxidant in our chemical systems.

Therefore, based purely on chemical studies, it is not unreasonable that the metal catalyzed reduction of H\textsubscript{2}O\textsubscript{2} by NO (Reaction 5) can occur. In fact, we have demonstrated that this chemical system is capable of oxidizing organic substrates, such as benzene, via a process that is consistent with -OH formation. Therefore, our results establish chemical precedence for a process by which NO and H\textsubscript{2}O\textsubscript{2} in the presence of trace metals, can lead to the generation of potent oxidants which, if formed, would be deleterious to cells.

The above reactions are reminiscent of the well known Haber-Weiss process that instead of NO utilizes O\textsubscript{2} as the reducing agent (Reactions 10, 11, and 12).

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\text{O}_2 + \text{Fe}^{(III)} \rightarrow \text{O}_2 + \text{Fe}^{(II)} \]

**REACTION 10.**

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

**REACTION 11.**

\[
\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{HO}^- + \cdot \text{OH} \]

**REACTION 12.**

Hydroxy radical generated via the Haber-Weiss reaction has been proposed to be responsible for some of the cytotoxicity associated with O\textsubscript{2}. However, detractors from this idea have noted several points that would suggest that the Haber-Weiss reaction is an unlikely mechanism of O\textsubscript{2}-mediated cytotoxicity (for example, see Freeman, 1994). For example, because intracellular iron concentrations are kept low by iron binding proteins and the levels of both O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} are kept low by the presence of degradative enzymes such as superoxide dismutase, catalase and GSHPx, it is thought that conditions capable of supporting significant Haber-Weiss chemistry are not physiologically attainable. Some of these same criticisms can be raised as well against the proposal that the NO/H\textsubscript{2}O\textsubscript{2}/iron system (reaction 5) was responsible for the observed cytotoxicity. However, NO may influence cellular conditions and processes to allow such chemistry to occur in the cell. For example, activated macrophages are capable of liberating significant portion of the bound iron in target tumor cells (Nibbs et al., 1984; Lancaster and Hibbs, 1990) (although it is known that O\textsubscript{2} can also release iron from proteins as well (for example see Ryan and Aust (1992) and Fridovich (1995)). Also, NO is able to increase intracellular H\textsubscript{2}O\textsubscript{2} generation through inhibition of mitochondrial respiration (Bolanos et al., 1994) and possibly through the inhibition of catalase (Table I). Thus, NO is capable of both releasing the iron required for catalysis of Reaction 5 as well as increase the intracellular H\textsubscript{2}O\textsubscript{2} levels required for the generation of the presumed toxic entity. Although it is clear that NO is responsible for Fe\textsuperscript{II} reduction to Fe\textsuperscript{III} in the purely chemical systems, under physiological conditions other reducing agents, such as ascorbate, may be serving to reduce the released metals.

Because NO is known to have a high affinity for the iron in heme proteins (for example, see Hoshino et al., 1993), it was not surprising that catalase, a heme protein, was inhibited by NO.\textsuperscript{3} Based on the equilibrium constant between NO and catalase of 1.8 \times 10\textsuperscript{5} M\textsuperscript{-1} (Hoshino et al., 1993) and assuming that NO inhibition was primarily competitive, the concentration of NO in our experiment (15.5–20 \mu M) would predict approximately 73–80% inhibition of catalase activity. This calculated value is consistent with the experimentally obtained value of 77% enzyme inhibition. Therefore, in cells that utilize catalase as its primary method for H\textsubscript{2}O\textsubscript{2} degradation, NO may be expected to raise intracellular H\textsubscript{2}O\textsubscript{2} levels. Under pathophysiological conditions, it may not be unreasonable to reach micromolar concentrations of NO near activated macrophages, which should result in substantial catalase inhibition. It should be noted that the observed inhibition of catalase by NO is not incongruous with our finding that catalase protects cultured cells from NO/H\textsubscript{2}O\textsubscript{2}-mediated cytotoxicity. In these in vitro experiments, catalase was used at a relatively high concentration (400 units/ml),\textsuperscript{4} and some residual activity should have remained in spite of the presence of NO.

Significantly, we found that NO does not inhibit GSHPx (or the GSHPx-glutathione reductase system).\textsuperscript{5} Thus, cells that primarily utilize GSHPx to keep H\textsubscript{2}O\textsubscript{2} levels low may be expected to be somewhat resistant to NO-mediated cytotoxicity. Interestingly, it has been demonstrated that GSHPx levels in macrophages increase when the cells are cytokine-activated (conditions that also result in the induction of NO biosynthesis) (jun et al., 1993).\textsuperscript{6} Moreover, it has also been shown that GSHPx activity plays an important role in macrophage functions under oxidative stress (Rokutan et al., 1988) and endothelial cells, which synthesize NO, rely heavily on GSHPx to degrade H\textsubscript{2}O\textsubscript{2} (Harlan et al., 1984).

If indeed GSHPx activity were important in protecting cells from the ravages of NO/H\textsubscript{2}O\textsubscript{2}/iron chemistry, it would be expected that ebselen, a compound with GSHPx-like activity (Muller et al., 1984), would offer some protection. This was found to be the case (Fig. 2). It should be noted that ebselen also has antioxidant properties, which are unrelated to its ability to mimic GSHPx, and this may also play a role in the observed protective effect (Muller et al., 1984). In view of this, we also examined the effect of selenium depletion on the susceptibility of the OVCAR cells to NO-mediated cytotoxicity. As expected, selenium depletion decreases the activity of the selenium-dependent GSHPx in OVCAR cells and consequently renders them more susceptible to SIN-1 mediated toxicity (Fig. 3). Thus, based on the results of this study, it appears that GSHPx may be vital to the viability of cells when exposed to significant levels of NO. The protective effect of GSHPx may be due, in part, to reduction of intracellular NO/H\textsubscript{2}O\textsubscript{2}/iron-oxidizing chemistry through the elimination of one of the critical reactants, H\textsubscript{2}O\textsubscript{2}. These studies are consistent with previous observations by others who found that tumor cells high in GSHPx activity were more resistant to activated macrophage-mediated oxidant injury (no correlation with catalase levels was observed, however) (Nathan, 1982). Also, endothelial cells rich in GSHPx were found to be resistant to activated neutrophil damage, whereas cells that were catalase-rich and GSHPx-poor were highly susceptible to activated neutrophil-mediated cyto-

\textsuperscript{3} During the review of this manuscript, a report was published that describes reversible inhibition of catalase by NO with a K\textsubscript{i} of 0.18 \mu M (Brown, 1995).

\textsuperscript{4} Previous work by Ionidis and DeGroot (1993) indicates that 5 \mu M of catalase was capable of measurably diminishing the cytotoxicity of 5 \mu M SIN-1 toward hepatoma cells. Therefore, even if catalase were inhibited by \textgreater 98% in our experiments, some protection should still have been observed.

\textsuperscript{5} During the review of this manuscript Asahi et al. (1995) published that NO can inhibit GSHPx. However, their study differs from our experiments in that SNAP was used as the NO source, whereas we used authentic NO. It should be recognized that SNAP is not just an NO source but can also transnitrosate protein thiols as well (for example, see Arnelle and Stannier, 1995). Thus, the difference between that study and this one may be due to the source of NO utilized.

\textsuperscript{6} R. Farias-Eisner, G. Chaudhuri, E. Aeberhard, and J. M. Fukuto, unpublished results.
toxicity (Vercellotti et al., 1988). Like macrophages, neutrophils are known to generate NO (Wright et al., 1989).

Thus, it is proposed that NO or activated macrophage-mediated cytotoxicity can be attributed to the generation of reactive radical species, such as -OH, through a chemical process involving a trace redox active metal, H$_2$O$_2$, and NO. Of particular importance is the possibility that this hypothesis may also be the basis for explaining the differential susceptibility of cells to NO cytotoxicity. Because NO does not affect the GSHPx-glutathione reductase system, cells that rely heavily on these enzymes for handling intracellular H$_2$O$_2$ would have increased resistance to NO cytotoxicity.

Acknowledgments—We are indebted to Dr. Waldemar Radziszewski for his helpful suggestions and advice and to Russ Byrns for his technical assistance.

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