THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 11, Issue of March 15, pp. 6144–6151, 1996
Printed in U.S.A.


# 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)

**Robin Farias-Eisner‡, Gautam Chaudhuri‡§, Ernesto Aeberhard¶, and Jon M. Fukuto§‖**

*From the Departments of ‡Obstetrics-Gynecology, §Pharmacology, and ¶Pediatrics, University of California at Los Angeles School of Medicine, Los Angeles, California 90095-1735*

**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 ($H_2O_2$). Somewhat surprisingly, superoxide ($O_2^-$) and its reaction product with NO, peroxynitrite ($^-$OONO), did not appear to be directly involved in the observed NO-mediated cytotoxicity against this cancer cell line. The toxicity of NO/$H_2O_2$ may be due to the production of a potent oxidant formed via a trace metal-, $H_2O_2$-, and NO-dependent process. Because the combination of NO and $H_2O_2$ was found to be particularly cytotoxic, the effect of NO on cellular defense mechanisms involving $H_2O_2$ 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 $H_2O_2$ 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/$H_2O_2$/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)[1] (for example see Hibbs *et al.* (1988), Stuehr and Nathan (1989), and Farias-Eisner *et al.* (1994)). 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 ($O_2^-$). The reaction of NO with $O_2^-$ is extremely rapid (Huie and Padmaja, 1993) and results in the generation of peroxynitrite ($^-$OONO), which is a potent chemical oxidant when in the protonated form (Koppenol *et al.*, 1992). It has been demonstrated that $^-$OONO can be formed from macrophage-derived NO (Ischiropoulus *et al.*, 1992) 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 $^-$OONO is responsible for a significant portion of macrophage derived cytotoxicity through a direct reaction of $^-$OONO with critical cellular components (Koppenol *et al.*, 1992). However, a recent report utilizing NO donor compounds indicated that NO was particularly tumoricidal in the presence of hydrogen peroxide ($H_2O_2$) and not $O_2^-$. Thus, it has been suggested that $^-$OONO 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).

$H_2O_2$ is formed as an indirect product of macrophage activation (via the dismutation of $O_2^-$). Therefore, we have performed a detailed examination of the chemistry and enzymology of possible NO/$H_2O_2$ interactions with tumor cell components in order to evaluate the possible role of NO/$H_2O_2$ 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 $H_2O_2$ 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 $H_2O_2$, for macrophage and/or 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 ($H_2O_2$), epidermal growth factor, transferrin, insulin, and endothelial cell growth supplement were all purchased from Sigma. Selenious acid ($H_2SeO_3$) 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). *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was synthesized by the method of Field *et al.* (1978). Potassium phosphate, ferric chloride, ferrous sulfate, and $H_2O_2$ (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-$N_2$ purge cycles on a high vacuum line, and manipulations of gas and liquid samples were

* This work was supported by Palomba Weingarten, the Allegra Charach Memorial Cancer Research Fund, Prof. T. Roy Fukuto, and the Jonsson Cancer Center Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work is dedicated to the memory of Prof. T. Roy Fukuto formally of the Departments of Entomology and Chemistry at the University of California at Riverside. His financial support and guidance made much of this work possible.

‖ To whom correspondence should be addressed: Dept. of Pharmacology, UCLA School of Medicine, Center for the Health Sciences, Los Angeles, CA 90095-1735. Tel.: 310-206-7151.

[1] The abbreviations used are: NO, nitric oxide; GSH, glutathione; GSHPx, glutathione peroxidase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SIN-1, 3-morpholino-sydnonimine.

carried out using $N_2$-purged gas-tight syringes. Introduction of reactants to reaction solutions was accomplished via injection through rubber septa. Water was purified by distillation in an all-glass apparatus. $H_2O_2$ concentrations in stock solutions were determined by iodometric titration (Jeffery *et al.*, 1989).

*Cells and Media*—The human epithelial ovarian cancer cell line NIH:OVCAR-3 (OVCAR) was obtained from the American Type Culture Collection. Cell manipulation was performed as described previously (Farias-Eisner *et al.*, 1994).

*The Effect of NO Donors on Cell Viability*—Cell viability was determined by measuring lactate dehydrogenase activity in the cell media by a method previously described (Wroblewski and LaDue, 1955). Briefly, lactate dehydrogenase activity was measured in cell culture supernatants after a 48-h incubation in the absence and the presence of the NO donors. Cytotoxicity was expressed as the percentage of total lactate dehydrogenase activity present in the $0.5 \times 10^6$ OVCAR cells/well incubated with 1 ml of 1% Triton X-100 for 30 min at 23 °C. SIN-1 solutions were made up and manipulated as described previously (Kroncke *et al.*, 1993).

The time course of toxicity was determined for 5 mM SIN-1. Thus, cell viability was monitored by the method described above over a time period of 48 h with time points at 6, 12, 18, 24, and 48 h.

*The Effect of Catalase, Superoxide Dismutase, and $H_2O_2$ on NO-mediated Loss in Cell Viability*—OVCAR cell viability was determined as described above in the presence and the absence of catalase (400 units/ml), superoxide dismutase (200 units/ml), and $H_2O_2$ (100 $\mu$M). The concentration of superoxide dismutase was chosen on the basis of previous observations by Darley-Usmar *et al.* (1992), who demonstrated that superoxide dismutase was able to compete with NO for $O_2^-$ at a similar SIN-1/superoxide dismutase ratio.

*Effect of NO on Catalase Activity*—Catalase-mediated degradation of $H_2O_2$ in the presence and the absence of NO was determined. $H_2O_2$ loss was measured by the general method of Beers and Sizer (1952). Thus, 2.9 ml of an 18 mM solution of $H_2O_2$ in 0.05 M phosphate buffer, pH 7.0, was added to a 4-ml cuvette. The cuvette was then degassed. To start the reaction, 0.100 ml of a degassed solution of catalase (2.5 $\mu$g/ml) in 0.05 M phosphate buffer, pH 7.0, was introduced to the $H_2O_2$ solution. The loss of $H_2O_2$ in solution was then determined by monitoring the loss of absorbance at 240 nm over a 1-min period. In the sample containing NO, 10 $\mu$l of pure NO gas (approximately 0.45 $\mu$mol) was introduced into the 1-ml headspace of the degassed $H_2O_2$ solution. The solution was then shaken vigorously prior to the introduction of catalase. Catalase activity was calculated by the method recommended by the supplier (Sigma). Briefly, the time ($t$) required for a decrease in absorbance, at 240 nm, of 0.05 absorbance units was recorded (in the linear portion of the curve). This corresponds to the decomposition of 3.45 $\mu$mol of $H_2O_2$. Thus, 3.45 $\mu$mol/t/mg catalase = the reported enzyme activity.

The concentration of NO in the catalase solution was determined by analyzing a 1-ml aliquot from the cuvette using a previously described procedure (Fukuto *et al.*, 1992). Briefly, the 1-ml sample was introduced into a 10-ml flask, the gaseous contents of the solution were sparged with $N_2$, and the gas stream was passed into a chemiluminescence detector (Antek 720, Houston, TX). Quantitation of the NO evolved was accomplished by comparison of the detector response with that of known standards of authentic NO. Also, the expected concentration of NO in the catalase solution was calculated using Henry's law and Henry's coefficient for NO previously reported (Shaw and Vosper, 1977). The two values were found to be in close agreement, 15.5 $\mu$M (experimentally determined) and 20 $\mu$M (calculated).

*Effect of NO on the Activity of the GSHPx-Glutathione Reductase System*—The activity of GSHPx was determined by the general method of Wendel (1981). Thus, 1 ml of a 0.3 units/ml solution of GSHPx in 0.25 M phosphate buffer, pH 7.0, containing 2.5 mM EDTA and 2.5 mM sodium azide, was placed into a 4-ml cuvette. Then 200 $\mu$l each of the following solutions were added to the cuvette: glutathione reductase (6 units/ml in the above buffer solution), GSH (10 mM in distilled water), and NADPH (2.5 mM in 0.1% $NaHCO_3$ solution). The cuvette was sealed with a rubber septum and degassed. The solution was allowed to stand at room temperature for 5 min (to allow reduction of GSHPx by GSH). The reaction was run at room temperature and started by the addition of 200 $\mu$l of a degassed 12 mM solution of $H_2O_2$. The decrease in the NADPH level was then determined by monitoring the absorbance at 366 nm. The effect of NO on NADPH consumption was determined in two ways. First, 50 $\mu$l of NO gas (approximately 2.2 $\mu$mol) was introduced to the enzyme solution prior to the addition of $H_2O_2$. The solution was then shaken, and then hydrogen peroxide was added. In another experiment, the rate of NADPH loss was monitored for 2 min in the absence of NO. Then 50 $\mu$l 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 $\mu$l of NO to the control experiments either prior to the addition of $H_2O_2$ or after 2 min of incubation did not significantly affect the rate of spontaneous NADPH loss.

*The Effect of NO on Ebselen-mediated $H_2O_2$ Degradation*—The same procedure described above for measuring GSHPx activity was utilized except that a 50 $\mu$M solution of ebselen in buffer was utilized in place of GSHPx (final ebselen concentrations in the incubations was 28 $\mu$M). Ebselen was solubilized by first dissolving it in a minimum amount of $Me_2SO$ prior to the addition to the 0.25 M phosphate buffer solution, pH 7.0, containing 2.5 mM EDTA and 2.5 mM sodium azide ($Me_2SO$ 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 $\mu$l of a 12.5 mM degassed solution of $H_2O_2$. In incubations carried out in the presence of NO, 50 $\mu$l of pure NO gas was injected into the headspace of the degassed cuvette containing all the reaction components except $H_2O_2$. The cuvette was then vigorously shaken before the addition of $H_2O_2$. The reactions were followed over 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.

*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 $\mu$M). This concentration of ebselen was chosen on the basis of prior observations by others (Leurs *et al.*, 1990; Chaudiere *et al.*, 1984).

*The Effect of Selenium Depletion on GSHPx Activity and on SIN-1 Cytotoxicity*—Following a general method previously described by Julian *et al.* (1992), cells were cultured in the absence of serum using instead 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 $\mu$g/ml), insulin (2.5 $\mu$g/ml), and endothelial cell growth supplement (7.75 $\mu$g/ml). In instances where the effect of added selenium was examined, selenious acid was added to the artificial serum extender at a 25 nM 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).

*Possible Reaction between NO and $H_2O_2$ as Determined by Loss of NO*—Into three 25-ml round bottom flasks equipped with septum-sealed stopcocks were added 15 ml of purified water. To two flasks was then added 0.1 mmol of $H_2O_2$. Nothing was added to the third flask. All three flasks were then degassed. To all flasks 2.4 ml of pure NO gas was then added. 200 $\mu$l of headspace gas was then sampled from each flask at 5–10-min intervals and analyzed by injection into the chemiluminescence detector. After 35 min, 0.01 mmol of ferric chloride taken up in a minimal amount of degassed water was added. Monitoring of headspace NO levels then continued for another 30 min.

*Spectroscopic Analysis of NO-Iron Species in Solution*—Spectroscopic studies were carried out on a Uvikon 810 spectrophotometer (San Diego, CA) operating at 200–800 nm. In a typical experiment, 2 ml of a 1 mM solution of either $FeCl_3 \cdot 6H_2O$ or $FeSO_4 \cdot 7H_2O$ in $N_2$-degassed water (final pH values of these solutions were approximately 3.1 and 5.4, respectively) 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 $N_2$ for the duration of the experiments. Pure NO gas and/or $H_2O_2$ were added to the cuvettes, and changes in absorbance of these solutions were recorded on a strip chart recorder.

*Measurement of Nitrite ($NO_2^-$) and Nitrate ($NO_3^-$) Concentrations*—Nitrite and nitrate determinations were performed as described previously (Bush *et al.*, 1992) using the general methods of Braman and Hendrix (1989). Briefly, measurement of $NO_2^-$ levels were made by monitoring NO evolution via chemiluminescence detection from a measured sample placed into a refluxing solution of iodide/acetic acid (this solution will only reduce $NO_2^-$ and not $NO_3^-$ to NO). Total $NO_x$ ($NO_2^-$ plus $NO_3^-$) determinations were made by monitoring NO evolution from a measured sample placed into a boiling $VCl_3$/HCl solution (this solution will reduce both $NO_2^-$ and $NO_3^-$ to NO). The determination of $NO_3^-$

levels was made by simply subtracting the value for $NO_2^-$ from the $NO_x$ value from duplicate samples analyzed by both methods. Quantitation was accomplished using a standard curve made up of known amounts of $NO_2^-$ and $NO_3^-$.

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 $H_2O_2$ 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 $H_2O_2$ 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 ($NO_2^-$ is unstable under acidic conditions, especially in the presence of $H_2O_2$). 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, $NO_2^-$ and $NO_3^-$, under the assay and reaction conditions.

*Oxidation of Benzene by the NO/$H_2O_2$/Metal System*—In a typical experiment, 0.3 mmol of benzene in 15 ml of purified water (20 mM, the approximate maximum solubility in water) containing 0.3 mmol of $H_2O_2$ (20 mM) and 3.6 $\mu$mol of ferric chloride (0.2 mM) 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% phenylmethyl 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 (McLafferty 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/or the 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; Feelisch 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 $H_2O_2$ (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 $H_2O_2$ was found to be cytotoxic in the presence of SNAP (that is, SNAP cytotoxicity was significantly enhanced in the presence of 100 $\mu$M $H_2O_2$; data not shown).

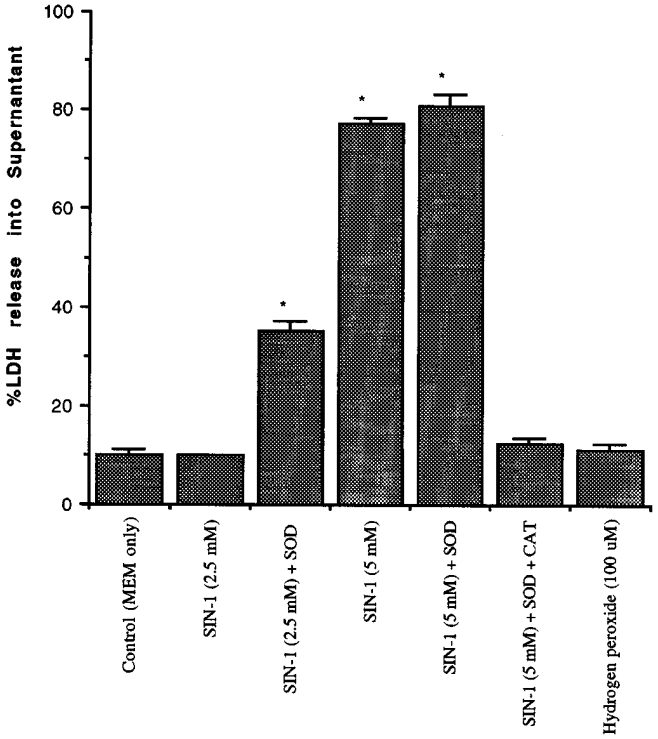


FIG. 1. **The effect of superoxide dismutase (200 units/ml), $H_2O_2$, and catalase (400 units/ml) on SIN-1-mediated loss of OVCAR cell viability.** Each *shaded column* represents the mean (± S.E.) value (percentage) of the total lactate dehydrogenase (*LDH*) activity in the supernatant of 0.5 × $10^6$ OVCAR cells/well. *, $p < 0.05$ compared with control (minimum essential medium (*MEM*) alone), SIN-1 (2.5 mM), and SIN-1 (5.0 mM) + superoxide dismutase + catalase. All values represent the mean of at least two experiments performed in triplicate.

The above data implicate both NO and $H_2O_2$ as being involved in the observed cytotoxicity. Because cells are normally able to keep $H_2O_2$ levels at a minimum by utilizing enzymes that specifically degrade $H_2O_2$ to innocuous species, the effect of NO on these enzymes, catalase and the GSHPx-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 $H_2O_2$, the enzyme activity decreased to approximately 23% of the control value (Table I). The concentration of NO in the catalase/$H_2O_2$ 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 GSHPx Activity*—The catalytic reduction of $H_2O_2$ by GSH requires two enzymes, GSHPx, which reduces $H_2O_2$ to $H_2O$ 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 GSHPx-glutathione reductase system can be monitored by measuring NADPH consumption. Thus, the effect of NO on NADPH con-

TABLE I
*The effect of NO on catalase activity*

| Conditions[a] | Enzyme activity[b] |
|---|---|
| Catalase + $H_2O_2$ | 780 |
| Catalase + $H_2O_2$ + NO[c] | 180 |

[a] All incubations performed under anaerobic conditions. 5 units of catalase in 3 ml of 18 mM $H_2O_2$. The loss of $H_2O_2$ determined over 1 min.
[b] Enzyme activity defined as $\mu$mol $H_2O_2$ consumed/s/mg protein. These values represent the average of two separate experiments.
[c] NO added as a pure gas into the cuvette headspace (see "Experimental Procedures"). Actual NO concentrations in the incubation solution was determined to be approximately 15.5 $\mu$M.

sumption by the GSHPx-glutathione reductase system was examined. To assure a significant lifetime for NO under the experimental conditions, the incubations were carried out under anaerobic conditions. When NO was added to the GSHPx-glutathione reductase system (containing NADPH and GSH) either prior to or 2 min after initiating the reaction with hydrogen peroxide addition, no effect on the rate of NADPH consumption was observed when compared with controls run in the absence of NO (data not shown). Significantly, in control experiments where the rate of NADPH consumption was monitored in the absence of GSHPx (a value subtracted from the values obtained in the complete system), the addition of NO did not alter the spontaneous rate of NADPH degradation. This indicates that the decrease in NADPH was not due to a reaction with the NO/$H_2O_2$ but rather was due to the GSHPx-glutathione reductase enzyme system. It is also worth noting that in reactions carried out aerobically, NO did not affect GSHPx activity. Thus, NO does not affect the ability of the GSHPx-glutathione reductase system to degrade $H_2O_2$.

*The Effect of the GSHPx Mimic, Ebselen, on NO-mediated Cytotoxicity of OVCAR Cells*—The effect of ebselen was examined for its ability to protect against SIN-1/superoxide dismutase. As expected, the addition of ebselen to OVCAR cells resulted in a dramatic increase in resistance to SIN-1-mediated cytotoxicity (Fig. 2). Also, like the GSHPx-glutathione reductase system, NO was found to have no effect on the peroxidase activity of ebselen.

*The Effect of Selenium Depletion on GSHPx Activity and the Susceptibility of OVCAR Cells of NO-mediated Toxicity*—Because GSHPx is a selenium-dependent enzyme, depletion of selenium from cultured cells results in a decrease in their GSHPx activity. In tissue culture, the source of selenium is serum. Therefore, OVCAR cells were cultured using an artificial serum mixture in which the concentration of selenium (in the form of selenious acid) could be manipulated. It was found that cells cultured in the absence of selenium exhibited a 28% decrease in GSHPx activity compared with cells cultured with 25 nM selenium. Significantly, the selenium deplete cells also exhibited an increased susceptibility to the cytotoxicity associated with SIN-1 compared with cells cultured in the presence of selenium (Fig. 3). Selenium depletion appeared to have little or no effect on the overall viability of the cells in the absence of SIN-1. Because it appears that the combination of NO and $H_2O_2$ is particularly cytotoxic to OVCAR cells, studies were then undertaken to attempt to determine if chemistry between NO and $H_2O_2$ exists that may be responsible for these observations.

*The Reaction of NO with $H_2O_2$*—Under anaerobic conditions, NO did not react with $H_2O_2$. That is, when 2.4 ml of gaseous NO (approximately 0.1 mmol) was added to a flask containing 15 ml of 6.7 mM $H_2O_2$, no loss of NO from the reaction headspace was found after 35 min. However, the addition of 0.01 mmol of ferric chloride resulted in a rapid consumption of NO as evidenced by a drop in NO levels in the reaction headspace.

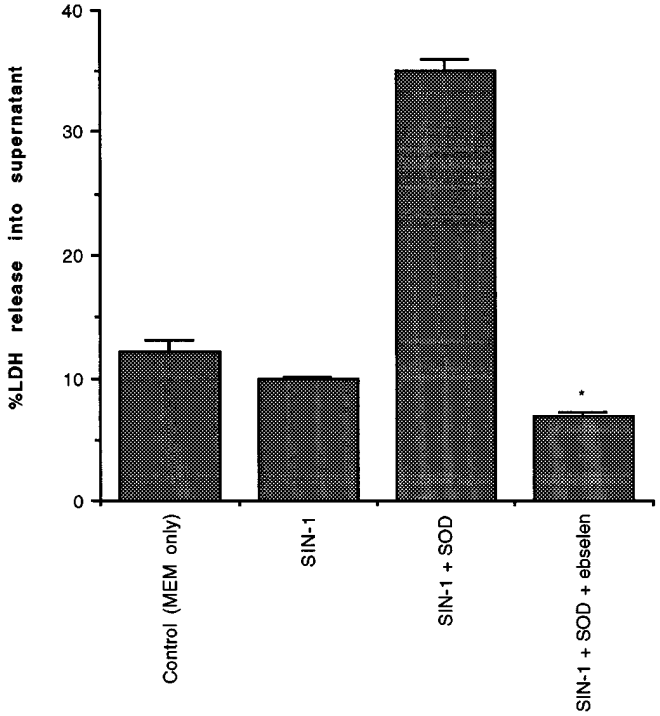


FIG. 2. **The effect of ebselen on the SIN-1/superoxide dismutase-mediated loss of OVCAR cell viability.** Each column represents the mean (± S.E.) value (percentage) of total lactate dehydrogenase (*LDH*) activity in the supernatant of $0.5 \times 10^6$ cells/well. *, $p < 0.05$ compared with SIN-1 (2.5 mM) with superoxide dismutase. The final concentrations of the enzymes and reagents were: SIN-1, 2.5 mM; superoxide dismutase, 200 units/ml; catalase, 400 units/ml; and ebselen, 10 $\mu$M. All values represent the mean of at least two experiments performed in triplicate. *MEM*, minimum essential medium; *SOD*, superoxide dismutase.

25 min after the addition of ferric ion, over 50% of the NO was consumed (Fig. 4). Moreover, analysis of the aqueous phase of the reaction mixture showed that no NO remained in solution (data not shown). The loss of NO exceeded the amount of iron added, indicating that the reaction was catalytic in ferric ion. Control reactions that lacked either hydrogen peroxide or ferric ion addition showed no loss of NO gas in the headspace (Fig. 4) and a significant amount of dissolved NO (data not shown).

Spectroscopic studies were then performed 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, $Fe(II)NO(H_2O)_5$ 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).

$$NO + Fe(III) \rightarrow [Fe(III)\text{-}NO \leftrightarrow Fe(II)\text{-}{}^+NO]$$

REACTION 1.

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

REACTION 2.

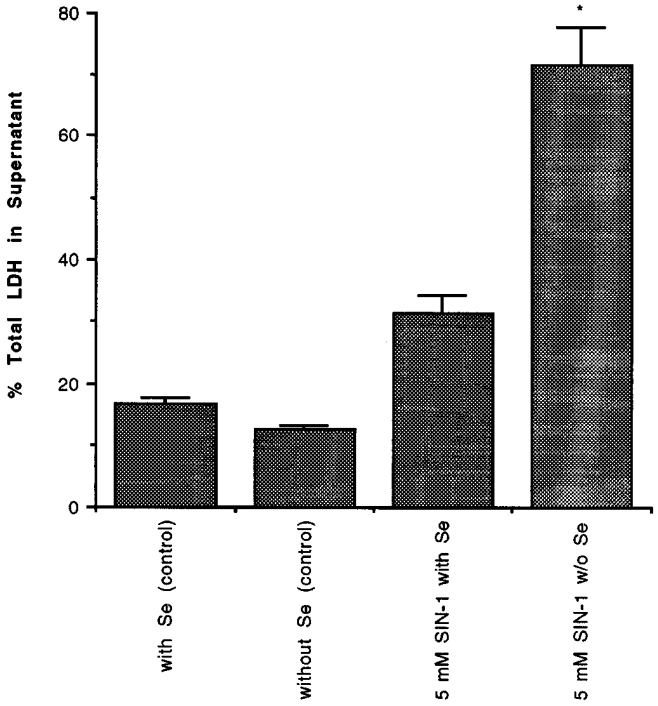


FIG. 3. **The effect of selenium (Se) depletion on the susceptibility of OVCAR cells to SIN-1 mediated cytotoxicity.** The cells without selenium were determined to have approximately 28% less GSHPx activity compared with the cells with selenium. *, $p < 0.0005$ when compared with cells cultured in the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

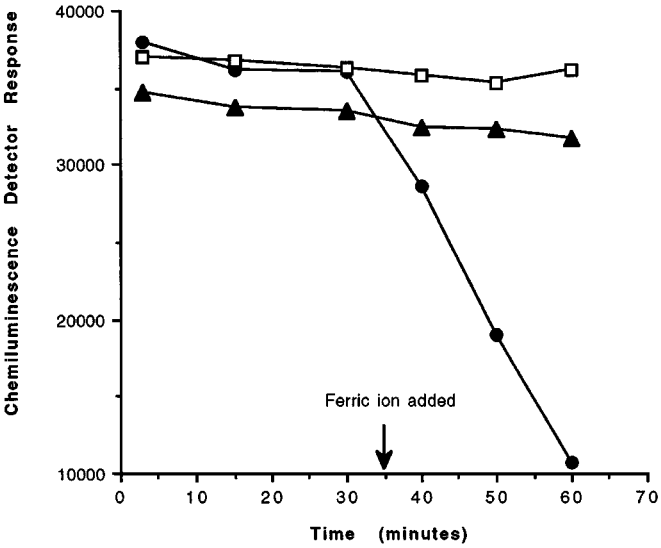


FIG. 4. **Effect of added ferric ion on the decomposition of NO in the presence of hydrogen peroxide.** ●, NO + $H_2O_2$, trace $Fe^{+3}$ added at 35 min; □, NO without $H_2O_2$, trace $Fe^{+3}$ added at 35 min; ▲, NO without $H_2O_2$ or added $Fe^{+3}$.

$$Fe(II)\text{-}(H_2O)_5 + NO \rightarrow Fe(II)\text{-}NO(H_2O)_5 \text{ (brown ring complex)}$$

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 $H_2O_2$ should lead to the formation of a potent oxidizing species like ·OH via a Fenton process (Reaction 4). (Note: the addition of $H_2O_2$ to the brown ring complex results in a rapid disappearance of the complex as evidenced by the immediate loss of the absorbances at 436 and 578 nm; data not shown.)

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^- + \cdot OH$$

REACTION 4.

Therefore, a solution of Fe(III), NO, and $H_2O_2$ should be capable of reacting by the process defined by the sum of Reactions 1, 2, and 4 (Reaction 5).

$$\text{overall } NO + H_2O_2 \rightarrow NO_2^- + \cdot OH + H^+$$

REACTION 5.

Thus, Reaction 5 represents a process by which NO and $H_2O_2$ 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 ($NO_2^-$) and Nitrate ($NO_3^-$)*—As indicated in Reaction 2, the reduction of Fe(III) to Fe(II) should result in $NO_2^-$ generation exclusively. This was tested when 5 ml of NO gas (approximately 220 $\mu$mol) was introduced into the headspace of a 10-ml flask containing a 2-ml solution of $Fe(III)Cl_3$ (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 $NO_2^-$. Only trace amounts of $NO_3^-$ were detected. Also, a 2 mM, anaerobic solution of $Fe(III)Cl_3$ in water was reacted with NO gas (5 ml in the reaction headspace as described above) and 1 equivalent of $H_2O_2$. After 15 min, the reaction was quenched with base, degassed and the levels of $NO_2^-$ and $NO_3^-$ determined. Again, $NO_2^-$ was the primary nitrogen oxide product with only trace amounts of $NO_3^-$ detected. If, indeed, ·OH were generated in solution, it may be expected that excess NO could trap this reactive species to give $NO_2^-$ (Reaction 6).

$$\cdot OH + NO \rightarrow NO_2^- + H^+$$

REACTION 6.

This is apparently the case under the conditions of these experiments because a 0.2 mM solution of $Fe(II)SO_4$ in water was found to react under anaerobic conditions with NO (excess) and $H_2O_2$ (1 equivalent) to give $NO_2^-$ as the primary nitrogen oxide with $NO_3^-$ present in only trace amounts. The results of these studies are summarized in Table II.

*The Oxidizing Potential of NO/$H_2O_2$ 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/$H_2O_2$/iron system was examined. As expected, it was found that benzene could be oxidized to give phenol by the NO/$H_2O_2$/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 near 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 species) 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

TABLE II
*The nitrogen oxide products from the reactions of metal salts with NO and NO/$H_2O_2$*

| Reaction conditions[a] | Yield of $NO_x$[b] | |
|---|---|---|
| | $NO_2^-$ (maximum yield)[c] | $NO_3^-$ |
| | μmol | |
| Fe(III) (0.4 μmol) + NO (excess), 30 min[d] | 0.2 (0.4) | Trace[e] |
| Fe(III) (0.4 μmol) + NO (excess) + $H_2O_2$ (0.4 μmol), 15 min[d] | 0.94 (1.2) | Trace[e] |
| Fe(II) (0.4 μmol) + NO (excess) + $H_2O_2$ (0.4 μmol), 1 min[d] | 0.92 (0.8) | Trace[e] |

[a] All reactions were carried out under anaerobic conditions in a 10-ml flask with 2 ml of a 2 mM solution of the metal salt.
[b] Values represent the average of two separate experiments.
[c] Maximum yield based on reactions 1, 2, 4, and 6 in the text. Due to the possibility of the reaction, $\cdot OH + Fe(II) \rightarrow {}^-OH + Fe(III)$, the maximum stoichiometry may not be realized.
[d] 5 ml of gaseous NO was added to the reaction headspace. Typically, this procedure results in a 1–2 mM solution of NO.
[e] Equal to or below the levels found in control reactions with no NO added.

TABLE III
*The effect of reaction conditions on the conversion of benzene to phenol by NO/$H_2O_2$/iron*

| Reaction conditions[a] | Yield of phenol | |
|---|---|---|
| | Based on Fe(III) | Based on NO |
| | % | |
| 20 mM $H_2O_2$, 0.2 mM Fe(III), 20 mM benzene (no NO added) | <0.1 | |
| Same as above except 0.1 equivalent of NO added[b] | 145 | 14 |
| Same as above except 0.5 equivalent of NO added[b] | 2066 | 41 |
| Same as above except 1.0 equivalent of NO added[b] | 2055 | 21 |

[a] Reactions were carried out anaerobically in 15 ml of purified water for 15 min.
[b] NO equivalents based on benzene. NO gas was added to the reaction headspace and allowed to diffuse into the reaction solution.

oxidations or can act as a radical trap, which terminates oxidative radical chain processes.

## DISCUSSION

SIN-1 is cytotoxic toward OVCAR cells (Fig. 1). It might be expected that the NO donor SIN-1 would be an especially cytotoxic NO donor because it is capable of stoichiometric generation of $O_2^-$ along with NO, and many studies have implicated the NO-$O_2^-$ reaction product, ${}^-OONO$, as being a particularly potent and destructive oxidant (Koppenol *et al.*, 1992). If this were the case, superoxide dismutase should attenuate the cytotoxicity of SIN-1. However, we found that superoxide dismutase did not attenuate SIN-1-mediated cytotoxicity and, in some cases, actually potentiated it. This observed potentiation by superoxide dismutase could be attributed to an increase in the relative rate of $H_2O_2$ generation from $O_2^-$ dismutation *versus* trapping by NO, provided that $H_2O_2$ contributes to the overall cellular toxicity of NO. Furthermore, our results suggest that NO-mediated cytotoxicity is somewhat dependent on the presence of $H_2O_2$ because catalase was able to protect against the toxicity of SIN-1 (Fig. 1). These results are consistent with the previous work of Ioannidis and de Groot (1993), who originally found that $H_2O_2$ enhances the toxicity of NO toward a hepatoma cell line. Interestingly, they also found that ${}^-OONO$ did not appear to play a role in the observed cytotoxicity in their system. The above mentioned results indicate that $H_2O_2$ may be an important mediator of the tumoricidal activity of NO. Significantly, it has been previously demonstrated that activated macrophage-mediated cytotoxicity toward tumor cells could be inhibited by catalase, whereas superoxide dismutase had no effect (Weiss and Slivka, 1982) (also, preliminary results in our laboratory with OVCAR cells confirm this observation). Thus, on the basis of the effects of both catalase and superoxide dismutase, SIN-1 appears to serve as reasonable model for the activity exhibited by activated macrophages.

The chemical mechanism(s) by which NO and $H_2O_2$ are cytotoxic is not immediately obvious, 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 $H_2O_2$, 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 $H_2O_2$ 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)).[2] Also, analysis of the nitrogen oxide products from the reaction of NO with Fe(III), NO with Fe(III)/$H_2O_2$, and NO with Fe(II)/$H_2O_2$ 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(III) via Reaction 2 (0.5 equivalents found). The reaction of NO with Fe(III) and 1 equivalent of $H_2O_2$ (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 $H_2O_2$ (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,

$$[Fe(III)\text{-}NO \leftrightarrow Fe(II)\text{-}{}^+NO] + H_2O_2 \rightarrow Fe(II) + {}^-OONO + 2H^+$$

REACTION 7.

or possibly by the combination of Reactions 8 and 9.

$$\cdot OH + H_2O_2 \rightarrow H_2O + \cdot OOH$$

REACTION 8.

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

REACTION 9.

[2] 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 $H_2O_2$ 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 $NO_3^-$ (the thermodynamically stable decomposition product of $^-OONO$) was not generated to any significant extent under the conditions of our experiments, we believe that $^-OONO$ 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 $H_2O_2$ 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 $H_2O_2$, 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 $O_2^-$ as the reducing agent (Reactions 10, 11, and 12).

$$O_2^- + Fe(III) \rightarrow O_2 + Fe(II)$$

REACTION 10.

$$H_2O_2 + Fe(II) \rightarrow Fe(III) + HO^- + \cdot OH$$

REACTION 11.

$$\text{overall } O_2^- + H_2O_2 \rightarrow O_2 + HO^- + \cdot OH$$

REACTION 12.

Hydroxy radical generated via the Haber-Weiss reaction has been proposed to be responsible for some of the cytotoxicity associated with $O_2^-$. However, detractors from this idea have noted several points that would suggest that the Haber-Weiss reaction is an unlikely mechanism of $O_2^-$-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 $O_2^-$ and $H_2O_2$ 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/$H_2O_2$/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 (Hibbs *et al.*, 1984; Lancaster and Hibbs, 1990) (although it is known that $O_2^-$ can also release iron from proteins as well (for examples see Ryan and Aust (1992) and Fridovich (1995)). Also, NO is able to increase intracellular $H_2O_2$ 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 $H_2O_2$ levels required for the generation of the presumed toxic entity. Although it is clear that NO is responsible for Fe(III) reduction to Fe(II) 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 hemeproteins (for example, see Hoshino *et al.*, 1993), it was not surprising that catalase, a hemeprotein, was inhibited by NO.[3] Based on the equilibrium constant between NO and catalase of $1.8 \times 10^5$ $M^{-1}$ (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 $H_2O_2$ degradation, NO may be expected to raise intracellular $H_2O_2$ 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/$H_2O_2$-mediated cytotoxicity. In these *in vitro* experiments, catalase was used at a relatively high concentration (400 units/ml),[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).[5] Thus, cells that primarily utilize GSHPx to keep $H_2O_2$ 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).[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 $H_2O_2$ (Harlan *et al.*, 1984).

If indeed GSHPx activity were important in protecting cells from the ravages of NO/$H_2O_2$/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/$H_2O_2$/iron-oxidizing chemistry through the elimination of one of the critical reactants, $H_2O_2$. 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-

[3] During the review of this manuscript, a report was published that describes reversible inhibition of catalase by NO with a $K_i$ of 0.18 $\mu$M (Brown, 1995).

[4] Previous work by Ionnidis and DeGroot (1993) indicates that 5 units/ml of catalase was capable of measurably diminishing the cytotoxicity of 5 mM SIN-1 toward hepatoma cells. Therefore, even if catalase were inhibited by > 98% in our experiments, some protection should still have been observed.

[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 Stamler, 1995). Thus, the difference between that study and this one may be due to the source of NO utilized.

[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, $H_2O_2$, 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 $H_2O_2$ 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.

REFERENCES

Arnelle, D. R., and Stamler, J. S. (1995) *Arch. Biochem. Biophys.* **318,** 279–285
Asahi, M., Fujii, J., Suzuki, K., Seo, H., Kuzuya, T., Hori, M., Tada, M., Fujii, S., and Taniguchi, N. (1995) *J. Biol. Chem.* **270,** 21035–21039
Beers, R. F., and Sizer, I. W. (1952) *J. Biol. Chem.* **195,** 133–140
Bohn, H., and Schonafinger, K. (1989) *J. Cardiovasc. Pharmacol.* **14,** Suppl. 11, S6–S12
Bolanos, J. P., Peuchen, S., Heales, S. J. R., Land, J. M., and Clark, J. B. (1994) *J. Neurochem.* **63,** 910–916
Braman, R. S., and Hendrix, S. A. (1989) *Anal. Chem.* **61,** 2715–2718
Brown, G. C., (1995) *Eur. J. Biochem.* **232,** 188–191
Bush, P. A., Gonzalez, N. E., Griscavage, J. M., and Ignarro, L. J. (1992) *Biochem. Biophys. Res. Commun.* **185,** 960–966
Castro, L., Rodriguez, M., and Radi, R. (1994) *J. Biol. Chem.* **269,** 29409–29415
Chaudiere, J., Wilhemsen, E. C., and Tappel, A. L. (1984) *J. Biol. Chem.* **259,** 1043–1050
Darley-Usmar, V. M., Hogg, N., O'Leary, V. J., Wilson, M. T., and Moncada, S. (1992) *Free Rad. Res. Commun.* **17,** 9–20
Farias-Eisner, R., Sherman, M. P., Aeberhard, E., and Chaudhuri, G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9407–9411
Feelisch, M., and Noack, E. A. (1987) *Eur. J. Pharmacol.* **139,** 19–30
Field, L., Dilts, R. V., Ravichandran, R., Lenhert, P. G., and Carnahan, G. E. (1978) *J. Chem. Soc. Chem. Commun.* 249–250
Freeman, B. (1994) *Chest* **105,** (suppl.) 79S–84S
Fridovich, I. (1995) *Annu. Rev. Biochem.* **64,** 97–112
Fukuto, J. M., Wallace, G. C., Hszieh, R., and Chaudhuri, G. (1992) *Biochem. Pharmacol.* **43,** 607–613
Goldstein, S., Meyerstein, D., and Czapski, G. (1993) *Free Rad. Biol. & Med.* **15,** 435–445, and references therein
Gwost, D., and Caulton, K. G. (1973) *Inorg. Chem.* **12,** 2095–2099
Harlan, J. M., Levine, J. D., Callahan, K. S., Schwartz, B. R., and Harker, L. A. (1984) *J. Clin. Invest.* **73,** 706–713
Hausladen, A., and Fridovich, I. (1994) *J. Biol. Chem.* **269,** 29405–29408
Hibbs, J. B., Jr., Taintor, R. R., and Vavrin, Z. (1984) *Biochem. Biophys. Res. Commun.* **123,** 716–723
Hibbs, J. B., Taintor, R. R., Vavrin, Z., and Rachlin, E. M. (1988) *Biochem. Biophys. Res. Commun.* **157,** 87–94
Hocman, G. (1988) *Int. J. Biochem.* **20,** 123–132
Hoshino, M., Ozawa, K., Seki, H., and Ford, P. C. (1993) *J. Am. Chem. Soc.* **115,** 9568–9575
Huie, R. E., and Padmaja, S. (1993) *Free Rad. Res. Commun.* **18,** 195–199
Ioannidis, I., and de Groot, H. (1993) *Biochem. J.* **296,** 341–345
Ischiropoulus, H., Zhu, L., and Beckman, J. S. (1992) *Arch. Biochem. Biophys.* **298,** 446–451
Jeffery, G. H., Bassett, J., Mendham, J., and Denney, R. C. (eds) (1989) *Vogel's Textbook of Quantitative Chemical Analysis*, 5th Ed., p. 394, Longman, London
Julian, J., Carson, D. D., and Glasser, S. R. (1992) *Endocrinology* **130,** 68–78
Jun, L., Mei, Z., and Yuan, C. (1993) *Int. J. Immunol.* **15,** 429–433
Kaur, H., and Halliwell, B. (1994) *Methods Enzymol.* **233,** 67–82
Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropolous, H., and Beckman, J. S. (1992) *Chem. Res. Toxicol.* **5,** 834–842
Kroncke, K.-D., Brenner, H-H., Rodriguez, M.-L., Etzkorn, K., Noack, E. A., Kolb, H., and Kolb-Bachofen, V. (1993) *Biochim. Biophys. Acta* **1182,** 221–229
Lancaster, J. R., and Hibbs, J. B., Jr. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1223–1227
Leurs, R., Bast, A., and Timmerman, H., (1990) *Eur. J. Pharmacol.* **179,** 193–199
Littlejohn, D., and Chang, S. G. (1982) *J. Phys. Chem.* **86,** 537–540
McLafferty, F. W., and Stauffer, D. B. (1988) *The Wiley/NBS Registry of Mass Spectral Data*, Vol. 1, John Wiley & Sons, Inc., New York
Muller, A., Cadenas, E., Graf, P., and Sies, H. (1984) *Biochem. Pharmacol.* **33,** 3235–3239
Nathan, C. F. (1982) *Fed. Proc.* **41,** 2206–2211, and references therein
Paglia, D. E., and Valentine, W. N. (1967) *J. Lab. Clin. Med.* **70,** 158–169
Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991a) *Arch. Biochem. Biophys.* **288,** 481–487
Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991b) *J. Biol. Chem.* **266,** 4244–4250
Rokutan, K., Hosokawa, T., Nakamura, K., Koyama, K., Aoike, A., and Kawai, K. (1988) *Int. Arch. Allergy Appl. Immunol.,* **87,** 113–119
Ryan, T. P., and Aust, S. D. (1992) *Crit. Rev. Toxicol.* **22,** 119–141
Shaw, A. W., and Vosper, A. J. (1977) *J. Chem. Soc. Faraday Trans.* **73,** 1239–1244
Stuehr, D. J., and Nathan, C. F. (1989) *J. Exp. Med.* **169,** 1543–1555
Vercellotti, G. M., Dobson, M., Schorer, A. E., and Moldow, C. F. (1988) *Proc. Soc. Exp. Biol. Med.* **187,** 181–189
Wade, R. S., and Castro, C. E. (1990) *Chem. Res. Toxicol.* **3,** 289–291
Wayland, B. B., and Olson, L. W. (1974) *J. Am. Chem. Soc.* **96,** 6037–6041
Weiss, S. J., and Slivka, A. (1982) *J. Clin. Invest.* **69,** 255–262
Wendel, A. (1980) *Enzymatic Basis of Detoxication* (Jakoby, W. B., ed) Chapter 16, pp. 333–354, Academic Press, New York
Wendel, A. (1981) *Methods Enzymol.* **77,** 325–333
Wink, D. A., Nims, R. W., Desrosiers, M. F., Ford, P. C., and Keefer, L. K. (1991) *Chem. Res. Toxicol.* **4,** 510–512
Wink, D. A., Nims, R. W., Saavedra, J. E., Utermahlen, W. E., Jr., and Ford, P. C (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6604–6608
Wright, C. D., Mulsch, A., Busse, R., and Osswald, H. (1989) *Biochem. Biophys. Res. Commun.* **160,** 813–819
Wroblewski, F., and LaDue, J. S. (1955) *Proc. Soc. Exp. Biol. Med.* **90,** 210–213