Nitric Oxide Potentiates Hydrogen Peroxide-induced Killing of Escherichia coli

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Summary

Previously, we reported that nitric oxide (NO) provides significant protection to mammalian cells from the cytotoxic effects of hydrogen peroxide (H_{2}O_{2}). Murine neutrophils and activated macrophages, however, produce NO, H_{2}O_{2}, and other reactive oxygen species to kill microorganisms, which suggests a paradox. In this study, we treated bacteria (Escherichia coli) with NO and H_{2}O_{2} for 30 min and found that exposure to NO resulted in minimal toxicity, but greatly potentiated (up to 1,000-fold) H_{2}O_{2}-mediated killing, as evaluated by a clonogenic assay. The combination of NO/H_{2}O_{2} induced DNA double strand breaks in the bacterial genome, as shown by field-inverted gel electrophoresis, and this increased DNA damage may correlate with cell killing. NO was also shown to alter cellular respiration and decrease the concentration of the antioxidant glutathione to a residual level of 15–20% in bacterial cells. The iron chelator desferrioxamine did not stop the action of NO on respiration and glutathione decrease, yet it prevented the NO/H_{2}O_{2} synergistic cytotoxicity, implicating metal ions as critical participants in the NO/H_{2}O_{2} cytocidal mechanism. Our results suggest a possible mechanism of modulation of H_{2}O_{2}-mediated toxicity, and we propose a new key role in the antimicrobial macrophagic response for NO.

Materials and Methods

Hypoxanthine (HX), oxyypurinol, Hepes buffer, superoxide dismutase (SOD) (Cu/Zn, Mn, and Fe), desferrioxamine (DF), DEA, sodium nitrite, glutathione, nicotinamide dinucleotide phos-
phate, glutathione peroxidase, hydrogen peroxide, dihydroxyphenyl-
adamine, and lucigenin were purchased from Sigma Chemical Co. (St. Louis, MO); catalase and xanthine oxidase (XO) were purchased from Boehringer Mannheim Corp. (Indianap-
olis, IN). Agar and soft agar were purchased from GIBCO BRL (Gaithersburg, MD). Nitric oxide gas and nitrogen were purchased from Matheson Gas Products (East Rutherford, NJ). Argon came from M.G. Industries (Malvern, PA). PBS and Luria-Bertani (LB) medium were purchased from Quality Bio-
logical Inc. (Gaithersburg, MD). All water was distilled and sub-
sequently purified further through a water purification system (Milli-Q; Millipore Corp., Bedford, MA). DEA/NO (C2H5
N((O)NO)-Na+) was provided as a generous gift from Joseph Saavedra (National Cancer Institute, Frederick, MD) (synthesized and purified as previously described [10]). A Bradford protein re-
genent kit was purchased from Bio-Rad Laboratories (Hercules,
CA). The vials used in the anaerobic experiments were pur-
chased from Wheaton Industries (Millville, NJ). A spectropho-
tometer (model DU 65; Beckman Instruments, Inc., Fullerton,
CA) was used for bacterial OD measurements. The E. coli strains JM 101, K-12 RW 193, K-12 RW B19, and HB101 were ob-
tained from American Type Culture Collection (Rockville,
MD). The E. coli strains 1157, KL-16, and its xth A mutant were obtained from E. coli Genetic Stock Center (Yale University, New Haven, CT).

Cytotoxicity Assay. Bacteria were grown in LB medium in a shaker incubator, 250 revolutions/min, at 37°C for the cell density registered an absorbance at 600 nm of 0.2 (for the log phase assay) or 1.4 (for the plateau phase assay). The cells were suspended in 5 ml of PBS-20 mM Hepes buffer at a concentration of 10^6/ml and exposed for 30 min at 37°C to either different concentrations of H2O2 (0.1, 0.5, 1, and 2 mM) or 5 mM HX + 0.2 U/ml XO with or without DEA/NO (0.01, 0.1, and 1.0 mM). For control, 1 mM DEA and 1 mM sodium nitrite (NaNO2) (end products after NO release from DEA/NO) were used instead of the DEA/NO, together or separately, in the presence of H2O2. Catalase (for H2O2) at a final concentration of 260 U/ml or cata-
lase and oxyprurinol (for HX/XO) at a final concentration of 0.1 mM were used to stop the reactions. After treatment, the cells were rinsed and diluted in PBS, and an aliquot of 0.5 ml (with the appropriate dilution to yield a scorable number of colonies on plates) was plated in triplicate on LB agar plates and placed in a 37°C incubator in an air atmosphere. After an overnight incubation, the plates were removed, and the colonies were counted. All experiments were conducted a minimum of three times, and data points are in triplicate. The data shown in the figures are re-
results from one of the triplicate experiments, and error bars (shown when larger than symbol) represent standard deviations. Linear regression and t test analyses as conducted in Fig. 2.A were per-
formed using the program Systat 5.2 (SYSTAT Inc., Evanston, IL).

Some experiments were performed under hypoxic conditions by bubbling argon for 30 min into 2 ml of PBS bacteria suspen-
sion (10^6/ml) in a 5-ml vial. The bacterial suspension was then exposed to either an argon-bubbled H2O2 (1 mM) or DEA/NO (1 mM) solution or to both for 30 min. The reactions were ter-
minated by the addition of catalase, and the bacteria were plated as described above.

Experiments with DF were performed as follows. The bacteria (5-10 x 10^8/ml) were grown for 2 h (OD at 600 nm = 0.15-
0.25) in LB broth with DF (0.5 or 1 mM) at 37°C in the shaker incubator. The cells were then suspended in PBS-20 mM Hepes buffer with DF (1 mM) and treated with DEA/NO and H2O2 as described above.

For the NO gas experiments, LB medium-20 mM HEPES was bubbled for 30 min with nitrogen to remove oxygen, with NO for 1 h, and then stored in sealed glass bottles and used for the experiments with the cells and H2O2. The bacteria (10^6/ml) were suspended in the gassed media and incubated for 30 min with or without 1 mM H2O2. At the end of the experiment, cata-
lase was added for 1 min, and then the bacteria were plated as described above. For the anoxic NO gas experiments, the bacte-
ria were suspended (10^6/ml) in 2 ml PBS containing 20 mM Heps in a 5-ml vial, and argon gas was bubbled into the solu-
tion for 5 min. Aliquots (2 ml) of anoxic bacterial suspension were then treated in three separate ways: (a) H2O2 solution (15 
ml) was injected to produce a final concentration of 0.5 mM ar-
gon-bubbled H2O2; (b) 0.5 ml NO gas alone; and (c) both H2O2 and NO gas together. The treatment conditions were maintained for 20 min at 37°C, after which the samples were bubbled for 3 min with argon to remove any residual NO gas, and then the vi-
als were opened and 4 µl of catalase (260 U/ml) was added. The bacteria were then plated as described above.

Cu/Zn SOD was used at concentration up to 5,000 U/ml in the cell toxicity experiments, and catalase was used at 260 U/ml. The enzymes were added before drug treatment.

SOD Activity Evaluation. The activity of the three different SOD isoenzymes (Cu/Zn, Fe, and Mn) was evaluated as de-
scribed by McCord and Fridovich (11) after 30-min exposure to either H2O2 (0.1 or 1 mM) or DEA/NO (1 mM) or both.

In bacteria, the relative SOD activity was determined by mea-
suring the loss of chemoluminescence produced from the reac-
tion of superoxide anion (O2-) with lucigenin (12) when an ali-
quot of sonicated bacteria extract was added to the reaction. Chemoluminescence measurements were made using a spectro-
fluorometer (model 8000; SLM-Aminco, Urbana, IL), with a photomultiplier tube in the photon counting mode. The reaction mixture consisted of 50 mM phosphate buffer, pH 8.0, containing 5 mM HX, 200 µM lucigenin, 200 U/ml catalase, and 50 
µM DTPA. Chemoluminescence was produced by the addition of 0.03 U/ml XO. After 3-5 min (to allow the light output to reach a plateau), 20 µl of the sonicated bacterial extract was added to the light-producing mixture. Counts before and after the addi-
tion of the bacterial extract were taken, and the percentage of loss of chemoluminescence was calculated. All treated samples were then compared with the light loss from the untreated control sample (as light intensity decreases, SOD inhibition increases). Bacterial extracts were also heated in boiling water for 1 h and tested for SOD activity; no SOD activity in these samples could be measured. The experiments were performed in triplicate.

Field-inverted Gel Electrophoresis (FIGE). Bacteria (10^7/ml) were treated as described above in LB broth, and the DNA was labeled by incubating the cells with 0.02 µCi/ml [3H]thymidine for 2 h before and during the exposure. DNA was prepared for electro-
phoresis by the method of Schwartz and Cantor (13) as modified by Ager et al. (14) and Stamato and Denko (15). After treatment, the bacteria were resuspended in PBS at 10^7/ml. An equal vol-
ume of 2% low-melting-temperature agarose was added, and the cell suspension was drawn with a syringe into 3/32-in. (internal diameter) silicone tubing. Both ends of the tubing were clamped, and the tubing was immersed in an ice bath to solidify the agarose rapidly. The agarose was then extruded from the tubing and cut into 5-mm lengths; these "plugs" were placed into 1.5-ml centrifuge tubes. The cell wall was digested, incubating the plugs in 1 mg/ml lysozyme in TE buffer (10 mM Tris, 1 mM EDTA) for 1 h. DNA was released by incubating at 55°C in lysis buffer (Tris-
NaCl, 1% Sarkosyl, and 1 mg/ml protease K) for 24 h. The
plugs were then rinsed in TE buffer for 24 h with three buffer changes. RNA was digested by incubation with 0.1 μg/ml boiled RNAse A in TE buffer for 2 h at 37°C. The 0.8% agarose gels were cast in 0.5 × TBE (1 × TBE is 90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA). Agarose plugs were loaded into 2 × 6 × 5-mm wells, which were sealed with melted agarose. Electrophoresis was performed for 24 h at 56 V (4 V/cm), with a 3:1 ratio of forward/reverse pulse time. The initial forward pulse was 7.5 s (reverse pulse 2.5 s), increasing to a final forward pulse time of 90 s (final reverse pulse 30 s). The running buffer (0.5 × TBE) was recirculated and cooled to 12–15°C, as previously described by Stamato and Denko (15), to keep the released DNA concentrated in a narrow band to facilitate quantitation (see below). After electrophoresis, the gels were soaked in 0.5 μg/ml ethidium bromide for 30 min, destained for 30 min with distilled water, and photographed on a UV light box. The lanes were separated from one another, and the well containing the plug was separated from the portion of the lane containing the released DNA. These small pieces of agarose were put into separate scintillation vials, and, after adding 50 μl of concentrated HCl to prevent resolubification of the agarose, the agarose was melted by placing each vial on a hot plate. To each vial, 15 ml of Hydrofluor® (National Diagnostics Inc., Atlanta, GA) was added, and radioactivity was determined using a scintillation counter (model 1217; LKB Instruments, Bromma, Sweden). The data are expressed as a percentage of DNA remaining in the well and were calculated as follows:

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\text{Percent of DNA remaining} = \frac{\text{CPM in the well}}{\text{CPM in the lane} + \text{CPM in the well}} \times 100.
\]

Oximetry. Log phase bacteria were suspended (5 × 10^7) in LB medium and incubated with or without 1 mM DEA/NO at 37°C. After 15 min, an aliquot (2 ml) of each sample was tested for oxygen consumption using an oxygraph (model 5/6; Gilson Medical Electronics, Inc., Middleton, WI) equipped with a Clark-type electrode.

H_2O_2 Consumption. The levels of H_2O_2 in the presence of different bacterial cell concentrations were measured by spectrophotometrically monitoring the formation of I^- from I^- as previously described (16).

Glutathione (GSH) Measurements. For aerobic evaluation, log phase bacteria (10^9/ml) were incubated in 20 ml of LB broth in the presence of DEA/NO (1 mM) for 30 min and then pelleted and resuspended in 1 ml of 0.6% sialofolic acid. For anaerobic evaluation, 10 ml of LB bacteria suspension (200 × 10^9/ml) were bubbled for 1 h with argon and then treated with an argon-bubbled DEA/NO solution. After 30 min, the bacterial suspension was bubbled again with argon to remove all the NO, and the sealed vial was cooled to 4°C before opening. The amount of GSH was calculated by a modification of Tietze's method (17) and expressed in micrograms GSH/milligrams protein. Protein concentrations were assessed by the Bradford method (18). GSH levels were also measured in mammalian cells (Chinese hamster V79 lung fibroblasts). Log phase V79 cells were grown in F12 medium plus 10% FCS. Cells were treated with 1 mM DEA/NO for 30 min, and GSH levels were determined as described above.

Asays for Glutathione Peroxidase and Glutathione Transferase. Glutathione peroxidase activity was measured by following the decrease in reduced nicotinamide dinucleotide phosphate absorbance at 340 nm after the addition of 0.17 U/ml of human glutathione peroxidase isolated from human red blood cells (Sigma Chemical Co.), glutathione reductase and 1 mM H_2O_2. Glutathione transferase activity was measured by following absorbance changes at 340 nm using the transferase substrate chlorodinitrobenzene. The reaction mixture included 5 U/ml of the human placental transferase (Sigma Chemical Co.) with 1 mM chlorodinitrobenzene and 1 mM GSH.

Electron Microscopy. Log phase bacteria (2 × 10^7/ml) were exposed for 30 min to 1 mM DEA/NO, 1 mM H_2O_2, or both. The cells were pelleted and fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) at room temperature for 24 h, postfixed in OsO_4, and embedded in Maraglas 655 (Ladd Research Industries Inc., Burlington, VT). Sections were stained with uranyl acetate–lead citrate and examined with an electron microscope (model 400; Philips Technologies, Cheshire, CT).

Results and Discussion

DEA/NO + HX/XO Cytotoxicity. Upon host invasion by bacteria, neutrophils and macrophages are activated and begin to consume oxygen rapidly (19). During this "oxygen burst," murine macrophage release, among other moieties, NO, O_2^- and H_2O_2 (2, 7). To stimulate these conditions, we produced NO, O_2^- and H_2O_2 in a continuous manner, as might be expected by bacterially activated macrophages. E. coli strain JM101 (20) was treated with DEA/NO that releases NO and/or HX/XO, which produces in an aerobic environment O_2^- and H_2O_2. When these cells were exposed for 30 min to NO generated in situ from DEA/NO (0.01, 0.1, and 1 mM), minimal or no toxicity was observed. O_2^- and H_2O_2 produced in situ enzymatically with HX/XO caused 50–90% cell killing. Using 1 mM DEA/NO in the presence of H_2O_2 and O_2^- in a cytotoxicity was enhanced by 10,000-fold (Fig. 1). Peroxynitrite (ONOO^-), the product of the reaction of O_2^- with NO, has been implicated in the NO-mediated bactericidal effect of NO (8). We therefore tried to elucidate the actual species responsible for the DEA/NO bactericidal potentiation by removing either O_2^- or H_2O_2 from the reaction.

SOD and Catalase Effects on Cytotoxicity. We attempted to eliminate O_2^- from the reaction mixture by adding 5,000 U of Cu/Zn-SOD (~5 μM). Cu/Zn-SOD was not deactivated by NO (data not shown). Considering the concentration of NO and SOD as well as their respective rate constants of reaction with O_2^- (21–23), 5,000 U/ml SOD should have been sufficient to compete effectively for O_2^- and diminish the ONOO^- formation. As is seen in Fig. 1, the addition of SOD did not decrease the DEA/NO potentiation. As further proof that extracellular ONOO^- was not participating as an active intermediate in the observed potentiation, we placed catalase in the HX/XO mixture to remove all H_2O_2. In the absence of H_2O_2, where only NO, O_2^- and potentially ONOO^- would be formed, no potentiation was observed (Fig. 1). The failure of SOD and the success of catalase to prevent the DEA/NO cytotoxicity only exclude O_2^- and by extension ONOO^- anion from having importance extracellularly but do not rule out the possibility that ONOO^- anion may still function as a key toxic intermediate intracellularly.
If intracellular $O_2^{-}$ were elevated, it is conceivable that the different response to NO/H$_2$O$_2$ exposure in mammalian cells compared with *E. coli* may result from the bacteria having different isoforms of SOD that are susceptible to inactivation by the NO/H$_2$O$_2$. Bacterial cells contain Fe-SOD, possibly trace quantities of periplasmic Cu/Zn-SOD, and an oxygen-inducible Mn-SOD (24, 25), whereas mammalian cells have Cu/Zn cytoplasmically and Mn-SOD intramitochondrially (24). Therefore, the three purified isoforms of SOD were treated in vitro individually for 30 min with DEA/NO (1 mM) and H$_2$O$_2$ (1 mM). DEA/NO plus H$_2$O$_2$ or H$_2$O$_2$ alone inactivated the Fe-SOD but not the other isoforms (data not shown). To test whether the endogenous Fe-SOD was inactivated, JM 101 cells were treated with H$_2$O$_2$ plus DEA/NO, then the cell lysate was assayed for total SOD activity. The treated cells lost ~50% of the SOD activity found in untreated cells (Table 1). These results do not allow us to categorically conclude that the loss in SOD activity alters the sensitivity of the cells to an experimental oxidative stress. However, we decided to simplify the experimental system by removing $O_2^{-}$ from the extracellular reaction conditions by using only H$_2$O$_2$ and DEA/NO.

**DEA/NO + H$_2$O$_2$ Cytotoxicity.** Treatment of *E. coli* with bolus H$_2$O$_2$ (0.1, 0.5, 1, and 2 mM) for 30 min resulted in cell killing between 10 and 90% (Fig. 2 A). DEA/NO and H$_2$O$_2$ were applied simultaneously, the results were similar to those obtained with HX/XO; DEA/NO potentiated toxicity in a concentration-dependent manner (Fig. 2 A). To confirm that it was indeed the NO generated from DEA/NO that potentiated H$_2$O$_2$ cytotoxicity and not the decomposition products of DEA/NO, cells were treated with H$_2$O$_2$ and the decomposition products of DEA/NO (sodium nitrite and diethylamine). As seen in Fig. 2 B, only intact DEA/NO potentiated the cytocidal effects of H$_2$O$_2$, whereas the decomposition products did not. An additional experiment was conducted to rule out the possible contribution of metabolically generated $O_2^{-}$ to the H$_2$O$_2$ potentiation by DEA/NO by treating cells with H$_2$O$_2$ under hypoxic conditions. As shown in Fig. 3 A, H$_2$O$_2$ cytotoxicity was also enhanced under hypoxic conditions by DEA/NO treatment. To eliminate any possibility that a DEA/NO decomposition intermediate was responsible for the observed potentiation, we conducted a further experiment using NO gas in combination with H$_2$O$_2$, as described in Materials and Methods. As shown in Fig. 3 B, potentiation of H$_2$O$_2$ cytotoxicity by NO gas treatment was also observed. Collectively, these results not only implicated the role of NO in the potentiation but support our use of DEA/NO as an NO delivery compound.

To assure ourselves that these results were not specific for JM 101 cells, we tested the combination of NO and H$_2$O$_2$ in five other *E. coli* strains. In all cases, *E. coli* were synergistically killed by the combination of NO and H$_2$O$_2$ (Fig. 4). Likewise, we examined the effects of cell density at the time of H$_2$O$_2$ exposure for JM 101 cells. Over a cell density range from $10^6$ to $10^9$/ml, potentiation of H$_2$O$_2$ cytotoxicity was observed that was similar to the potentiation observed in Fig. 2 A (data not shown).

To determine whether bacterial cells had to be actively dividing and metabolizing for there to be potentiation between NO and H$_2$O$_2$, plateau phase cells were compared with those in exponential growth. Potentiation of plateau phase cells was observed with the combination of DEA/NO and H$_2$O$_2$ (Fig. 5), but the cytotoxicity was decreased.

Table 1. Total SOD Activity of JM 101 Cells Treated with H$_2$O$_2$ ± DEA/NO

| Condition | Relative percent of SOD activity |
|-----------|---------------------------------|
| Control   | 100 ± 11                        |
| 1 mM DEA/NO | 63 ± 1                         |
| 1 mM H$_2$O$_2$ | 96 ± 2                        |
| 1 mM H$_2$O$_2$ + 1 mM DEA/NO | 32 ± 7                        |
compared with the exponentially growing cultures, presumably because of a decreased metabolism rate of plateau phase cells. To elucidate further the requirements of the bacterial potentiation, we examined whether both reagents must be present simultaneously, or if NO or H$_2$O$_2$ alone cause a lasting cellular dysfunction that is unmasked by the other agent. To this end, bacteria were first exposed to either DEA/NO (1 mM) for 30 min and then H$_2$O$_2$ (1 mM), or first to H$_2$O$_2$ (1 mM) for 30 min followed by a 1-min incubation with catalase to remove any residual unmetabolized H$_2$O$_2$ and then DEA/NO (1 mM). In neither case was potentiation observed (data not shown). The H$_2$O$_2$ concentration was monitored to determine the H$_2$O$_2$ consumption during the 30-min exposure. When cell densities of $\geq 10^8$ cells/ml were used, the H$_2$O$_2$ levels were quickly depleted; however, below $10^7$ cells/ml, no significant depletion of H$_2$O$_2$ levels was observed (Fig. 6 A).

Oxygen Consumption. It has been reported that NO may interfere with respiration (26), which may in part explain the different effect NO has on peroxide-mediated cytotoxicity in bacterial and mammalian cells. Clark electrode oximetry showed that NO profoundly decreases oxygen utilization by E. coli (Fig. 6 B) and suggested that this may be a cause of the observed potentiation. NO itself reacts with oxygen to form other nitrogen oxide species (NO$_x$), which have been argued to be important in biological toxicities. These reactive NO$_x$ have been shown to modify DNA chemically and to inhibit various enzymes (27-31). Therefore, elevated intracellular levels of oxygen could result in the formation of reactive NO$_x$. However, as noted above, when bacteria were treated with H$_2$O$_2$ in a hypoxic environment, there was potentiation by DEA/NO (Fig. 3). We cannot rule out the possibility that oxygen is produced during catalase metabolism of H$_2$O$_2$ and, therefore, cannot absolutely exclude that oxygen functions in some manner to increase potentiation between NO and H$_2$O$_2$. However, the hypoxic experiments do suggest a minor role in the potentiation for NO$_x$ and ONOO$^-$. Effect of Metal Chelators on Cytotoxicity. Numerous examples suggest that H$_2$O$_2$ is not cytotoxic until reduced by either Fe$^{2+}$ or Cu$^{+1}$ ions to yield either 'OH or hypervalent metal ions (19). Previous reports have shown that iron and other metal ions may be released from sulfide-iron clusters or sulphydryl-iron chelates when treated with NO (31). If NO were responsible for releasing intracellular redox-active iron during H$_2$O$_2$ treatment, and this were a
mechanism involved in the observed synergy, then chelation of the redox-active iron released by NO should eliminate the observed potentiation. This hypothesis was tested by preincubating cells with the iron-chelating agent DF for varying times to allow time for sequestration of mobilized intracellular iron ions. After incubation, the cells were treated with DEA/NO plus H₂O₂. Fig. 7 demonstrates that DF preincubation for 2 hr eliminated the potentiating effect of DEA/NO plus H₂O₂. Furthermore, when cells were not preincubated with the chelator, and DF was present only during the treatment, the potentiating toxicity was not affected. The lack of protection in this last condition indicates that the potentiation may result from redox-active iron ion being released intracellularly. Furthermore, DF did not interfere with NO-induced ablation of respiration (Fig. 6 C). This is evidence that DF can protect against cytotoxicity without restoring oxygen consumption and, therefore, rules out the possibility that DF is reacting directly with NO or some of its bioproducts as previously proposed (see Fig. 6, B and C) (32). Therefore, we conclude that DF must be protecting by chelating transition metals, particularly iron ion, and as a corollary the observed potentiation is mediated, at least in part, by transition metal ions. The protective effect of DF excludes the possibility that
the NO/H$_2$O$_2$ potentiation results by releasing iron and inactivating metal-containing enzymes, such as aconitase (33, 34) or DNA repair enzymes (30, 35). Had NO inactivated such enzymes by iron release, the enzymes would remain inactivated by NO even in the presence of DF. DF reacts to chelate adventitious Fe$^{3+}$ ions; it does not stop NO from releasing iron from sulfur-iron clusters. Therefore, NO may still inactivate respiration in the presence of DF, as is seen in Fig. 6B. DF would tightly bind adventitious Fe$^{3+}$ ions and thereby prevent formation of Fe$^{2+}$ and stop the subsequent reaction with H$_2$O$_2$ to produce highly oxidizing species that may damage critical cellular targets such as DNA (36).

**Molecular Target of DEA/NO + H$_2$O$_2$ Cytotoxicity.** To delineate further how DEA/NO enhances H$_2$O$_2$ damage, we treated the cells with DEA/NO and either tert-butyl hydroperoxide or cumene hydroperoxide—two agents known to cause oxidation of lipid membranes (37, 38). DEA/NO did not potentiate the oxidative effect of either agent (data not shown). Electron microscopy was used to ascertain if there was gross damage to the bacterial cell wall/membrane by DEA/NO and H$_2$O$_2$. No such damage was noted (data not shown). Our inability to demonstrate membrane damage by electron microscopic evaluation does not rule out more subtle disruption of the membrane function. However, the results do not support the hypothesis that bacterial protoplasmic membrane is the organelle structure damaged by the combination of DEA/NO and H$_2$O$_2$.

Because H$_2$O$_2$ is known not to cause observable double-strand breaks of genomic DNA (15), we performed FIGE (13–15) on JM 101 cells to ascertain if NO altered the effect of H$_2$O$_2$ on bacterial genomic DNA double-strand breaks. Table 2 shows that treatment with H$_2$O$_2$ does not result in observable double-strand scissions beyond that found in non-H$_2$O$_2$-treated cells (87.7 vs. 85.9% undamaged DNA). However, when DEA/NO is present during the H$_2$O$_2$ exposure, an increased number of double-strand DNA breaks is observed (26.9 vs. 87.7% undamaged DNA). To be consistent with survival studies performed with JM
Table 2. DNA Double-stranded Break for H$_2$O$_2$ ± DEA/NO

|          | Control | DEA/NO | H$_2$O$_2$ | DEA/NO + H$_2$O$_2$
|----------|---------|---------|-----------|-----------------|
| DF (-)   | 85.9    | 87.0    | 87.7      | 26.9           |
| DF (+)   | 86.6    | 89.9    | 90.4      | 63.9           |

The numbers represent the percentage of DNA that was not damaged and are the expression of the intact genomic DNA that was in the well of the FIGE. DF(-), without DF; DF(+), with DF at 0.5 mM.

101 cells, DF, which provided substantial protection of E. coli from DEA/NO/H$_2$O$_2$, should protect E. coli against these double-strand breaks. Table 2 demonstrates that DF treatment does indeed protect E. coli (63.9% undamaged DNA) from DEA/NO/H$_2$O$_2$ double-strand break induction when compared with the non-DF-treated cells (26.9%). As noted, protection was not complete because DF did not restore the DNA to the level of the DF/H$_2$O$_2$ treatment (90.4%).

Because GSH is known to be an important detoxifier of H$_2$O$_2$ (39) and metal complexes of NO can react with GSH (31), we examined whether intracellular GSH was depleted by treatment with DEA/NO in both V79 cells (mammalian cells that NO protected against H$_2$O$_2$ [9]) and JM 101 cells. DEA/NO decreased the intracellular GSH levels in mammalian cells by only 35%, whereas in JM 101 cells, intracellular GSH levels decreased by 75–85% (Table 3). Decreasing the intracellular GSH levels by 75–85% of control during an oxidative stress may well contribute to the bacteriocidal potentiation from NO and H$_2$O$_2$. We tested some of the enzymes in which GSH is a cofactor and found that DEA/NO does not inactivate GSH peroxidase or GSH transferase (data not shown).

Conclusions. In summary, our data illustrate the following points regarding the release of NO during an oxidative burst: (a) NO potentiated the bacteriocidal effect of H$_2$O$_2$; (b) a target of the potentiation appears to be DNA; (c) superoxide, ONOO$^-$, or NO$_x$ species seem to have a minor role in the potentiation; (d) transition metal ions are involved in the synergistic cytotoxicity; and (e) GSH levels are decreased by DEA/NO. These findings suggest that the enhancement of H$_2$O$_2$-induced DNA strand scission and cytotoxicity by NO may be due to several factors, as depicted in Fig. 8.

Fig. 8 illustrates that in E. coli, the respiratory enzyme complexes are found within the inner membrane of the cell envelope (40). In a normal oxygen environment, the respiratory enzymes probably consume the majority of oxygen arriving at the bacterial inner membrane, as depicted by step I in Fig. 8. Hence, the concentration of oxygen that reaches the cytoplasm is low, and the portion that does enter into the cell is more than likely reduced to superoxide.

Table 3. GSH (μg/mg protein) in Four Different E. coli Strains and Chinese Hamster V79 Cells Treated with 1 mM DEA/NO

| Strain          | JM 101 | KL-16 | 1157   | 9101 xth | V79 cells |
|-----------------|--------|-------|--------|----------|-----------|
| Control         | 12.14  | 16.52 | 40.80  | 13.72    | 10.2      |
| + DEA/NO       | 1.54 (87%)* | 3.44 (79%) | 18.12 (56%) | 2.20 (84%) | 6.6 (35%) |

The bacteria strains and the mammalian cell line (V79) were treated and assayed as described in Materials and Methods.

*The percentages in brackets represent depletion of GSH by DEA/NO.
which would be detoxified by the two isoforms of SOD. We have shown that oxygen consumption by JM 101 cells is almost completely stopped by NO (Fig. 6 B). Inhibition of oxygen consumption should allow more oxygen to cross into the intracytoplasmic space and form high levels of the superoxide anion, as denoted by a bold, dashed arrow in Fig. 8. Diminution of GSH would then result from the reaction of NO and RSH (cellular thiols) compounds (Fig. 8, step IV), which would permit Fenton reactions to produce reactive oxygen species (ROS) (Fig. 8, step V) that would go on to oxidatively damage intracellular targets. Fenton reactions should accelerate as a result of NO-induced release of labile, redox-active transition metals (Fig. 8, step III). Such an increase should result in more DNA damage, and ultimately this would lead to the enhanced cytotoxicity that we have observed in the JM 101 cells. Metal mobilization (Fig. 8, step III) and increased intracellular iron have been shown to facilitate both cell killing and DNA damage by H₂O₂ (42). Previous studies implicated NO in the bactericidal activity of macrophages, since inhibition of NOS greatly reduced cytotoxicity (43–45). Lastly, it is known that nonheme iron proteins (Fig. 8, step II) are in greater abundance in bacteria than in mammalian cells and as such may be a ready source for iron mobilization as well as deactivation by NO. Iron–sulfur proteins such as aconitase are particularly sensitive to damage by reactive nitrogen oxide species (Fig. 8, step II) (34, 46, 47), which would then result not only in enzymic deactivation but also in an increased cytoplasmic low-molecular-weight, redox-active iron pool. Were redox-labile iron ions to occur in the near vicinity of bacterial DNA, damage to DNA would be expected to be more facile (Fig. 8, step V). In mammalian cells, enzymes such as aconitase are not in close proximity to DNA. The differences in compartmentalization and the higher amount of iron–sulfur proteins in bacteria compared with mammalian cells appear to explain the different response to oxidative stress.

The differential response of mammalian cells (9) and bacteria to NO/H₂O₂ may afford a unique means to combat invading microorganism invasion with minimal damage to host cells. Our results provide a framework to better understand how the production of NO can enhance the immune response to E. coli invasion and yet limit toxicity to the host cells.
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