Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 9813–9817, November 1993
Neurobiology

Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species

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Communicated by Louis Sokoloff, July 30, 1993

ABSTRACT **Nitric oxide, NO, which is generated by various components of the immune system, has been presumed to be cytotoxic. However, NO has been proposed to be protective against cellular damage resulting during ischemia reperfusion. Along with NO there is often concomitant formation of superoxide/hydrogen peroxide, and hence a synergistic relationship between the cytotoxic effects of nitric oxide and these active oxygen species is frequently assumed. To study more carefully the potential synergy between NO and active oxygen species in mammalian cell cytotoxicity, we utilized either hypoxanthine/xanthine oxidase (a system that generates superoxide/hydrogen peroxide) or hydrogen peroxide itself. NO generation was accomplished by the use of a class of compounds known as "NONOates," which release NO at ambient temperatures without the requirement of enzyme activation or biotransformation. When Chinese hamster lung fibroblasts (V79 cells) were exposed to hypoxanthine/xanthine oxidase for various times or increasing amounts of hydrogen peroxide, there was a dose-dependent decrease in survival of V79 cells as measured by clonogenic assays. However, in the presence of NO released from $(C_2H_5)_2N[N(O)NO]^-Na^+$ (DEA/NO), the cytotoxicity resulting from superoxide or hydrogen peroxide was markedly abrogated. Similarly, primary cultures of rat mesencephalic dopaminergic cells exposed either to hydrogen peroxide or to hypoxanthine/xanthine oxidase resulted in the degradation of the dopamine uptake and release mechanism. As was observed in the case of the V79 cells, the presence of NO essentially abrogated this peroxide-mediated cytotoxic effect on mesencephalic cells.**

Nitric oxide, NO, is one of the proposed cytotoxic species produced by the immune surveillance system (1, 2). Many reports also suggest that during the ischemia reperfusion event, NO mediates tissue injury (3–12). However, it has been suggested that NO can function as a protective agent on the basis of two lines of evidence: (*i*) NO synthase (NOS) inhibitors increase tissue damage during *in vivo* ischemia reperfusion within the cerebral cortex, and (*ii*) in more direct evidence, NO has been shown to prevent damage during ischemia reperfusion events in both brain and heart (13–18). Additional studies provide evidence that NO, though present, plays a minimal role in the pathological effects associated with ischemia reperfusion injury (19, 20) or tumor necrosing factor-mediated cytotoxicity (21). Unfortunately, the exact role(s) NO plays in cytotoxicity *in vivo* is not clear because there are a number of different physiological functions NO or the NOS inhibitors could affect simultaneously. It has been proposed that NO or reactive nitrogen oxide species can directly cause cell death. However, primary neuronal cell cultures exposed to concentrations of NO as high as 1 mM show no adverse effects (22, 23). Many of the biological events in which NO has been proposed as a toxin occur concurrently with the production of reactive oxygen species—e.g., immune response and ischemia reperfusion injury.

To clarify the relationship between the cytotoxic properties of the reactive oxygen species and NO, it is necessary to simplify the system. We now show that NO released from a series of compounds known as the "NONOates" (24), which are $R_1R_2N[N(O)NO]^-$, is capable of abrogating the hydrogen peroxide-mediated cytotoxic effects in Chinese hamster lung fibroblasts and mesencephalic dopaminergic neurons.

MATERIALS AND METHODS

Chemicals. Hypoxanthine (HX) and xanthine oxidase (XO) were purchased from Boehringer Mannheim. Sodium nitrite, diethylamine, sulfinamide, diethylenetriaminepentaacetic acid, and *N*-(1-naphthyl)ethylenediamine dihydrochloride were purchased from Aldrich. Cytosine β-D-arabinofuranoside and ferricytochrome *c* were purchased from Sigma. $(C_2H_5)_2N[N(O)NO]^-Na^+$ (DEA/NO; Chemical Abstracts Service Registry Number 86831-65-4) and $(H_2N)(CH_2)_3N[N(O)NO]^-(CH_2)_4NH_2^+(CH_2)_3NH_2$ (SPER/NO; Chemical Abstract Service Registry Number 136587-13-8) were synthesized and assayed for NO production via chemiluminescence technique as described (24).

Enzyme Analysis/Controls. The activity of XO was monitored in the absence and presence of 1 mM DEA/NO by two different assays. (*i*) Superoxide-induced reduction of ferricytochrome *c* to ferrocytochrome *c* was monitored spectrophotometrically at 550 nm (25). The reaction was carried out in a 1-ml volume in aerated 50 mM phosphate buffer (pH 7.8) containing 50 μM diethylenetriaminepentaacetic acid. HX was maintained at 2.5 mM and ferricytochrome *c* at 20 μM. The reactions were initiated with the addition of XO (final concentration 0.2 units/ml). (*ii*) The activity of XO in the absence and presence of 1 mM DEA/NO was directly monitored by measuring the production of uric acid spectrophotometrically at 305 nm for 10 min. All conditions were identical to that used above except for the deletion of ferricytochrome *c*. All enzymatic assays and chemical reactions were performed at 37°C.

Saturated NO solutions were made as described (26). Anaerobic solutions of 1 mM hydrogen peroxide in 10 mM phosphate buffer (pH 7.4) were mixed with 1 mM NO. No rapid formation of nitrate/nitrite (<1000 s) was observed as monitored at 210 nm by previously described stopped-flow

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Abbreviations: HX, hypoxanthine; XO, xanthine oxidase; NOS, nitric oxide synthase; DEA/NO, $(C_2H_5)_2N[(O)NO]^-Na^+$; SPER/NO, $(H_2N)(CH_2)_3N[N(O)NO]^-(CH_2)_4NH_2^+(CH_2)_3NH_2$.

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techniques (26). In addition, the nitrosation of sulfinamide in an aerobic solution (100 mM phosphate buffer, pH 7.4) by intermediates of the NO/O_2 reaction followed by subsequent diazotization with *N*-(1-naphthyl)ethylenediamine dihydrochloride to form the azo dye was not inhibited in the presence of 1 mM hydrogen peroxide; thus, the consumption of NO by hydrogen peroxide was not significant under these conditions. The rate of decomposition of DEA/NO was unaffected by the presence of 1 mM hydrogen peroxide; likewise, hydrogen peroxide was not consumed by DEA/NO or intermediates of the DEA/NO decomposition reaction as measured by the production of I_3^- as follows (27). A 1 mM DEA/NO solution was allowed to decompose in the presence of 1 mM hydrogen peroxide in 50 mM phosphate buffer (pH 7.4) containing 0.1 mM diethylenetriaminepentaacetic acid at 37°C. There was no difference in the H_2O_2 levels in the presence and absence of DEA/NO as measured by spectrophotometrically monitoring the formation of I_3^- from I^- as described (27).

Cell Culture. *Chinese hamster V79 cells.* Chinese hamster V79 lung fibroblasts were cultured in F-12 medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics. Cell survival was assessed by clonogenic assay, with the plating efficiency ranging between 85% and 95%. Stock cultures of exponentially growing cells were trypsinized, rinsed, and plated (7×10^5 cells per dish) into a number of 100-cm^2 Petri dishes and incubated 16 hr at 37°C prior to experimental protocols. Cells were exposed to HX/XO (final concentration, 0.5 mM/0.08 unit/ml, respectively) as a function of time or to various concentrations of hydrogen peroxide for 1 hr. DEA/NO, SPER/NO, nitrite, or diethylamine (final concentration, 0.1 or 1 mM) were added to parallel cultures immediately prior to addition of HX/XO or hydrogen peroxide. Additionally, for some experiments 1 mM DEA/NO was added to medium (without cells) and incubated at 37°C for either 60 min or 16 hr. These solutions were then added just prior to the addition of hydrogen peroxide to evaluate the effects of DEA/NO, which had released NO. After treatment the cells were washed twice with phosphate-buffered saline, trypsinized, counted, and plated in triplicate for macroscopic colony formation. Each dose determination was plated in triplicate, and experiments were repeated a minimum of two times. Plates were incubated 7 days, after which colonies were fixed with methanol/acetic acid, 3:1 (vol/vol), stained with crystal violet, and counted. Colonies containing >50 cells were scored. Error bars represent the SD of the mean and are shown when larger than the symbol.

Primary cultures of mesencephalic neurons. The ventral tegmental mesencephalon was dissected from 14-day-old embryos (precisely timed pregnant Sprague–Dawley rats; Zivic–Miller) under sterile conditions and mechanically dissociated in complete culture medium. The culture medium consisted of 1:1 mixture of modified minimal essential medium and nutrient mixture F-12 supplemented with 6 mg of D-glucose per ml, 2 mM glutamine, 0.5 unit of penicillin G per ml, 0.5 mg of streptomycin per ml (all from GIBCO), and 15% (vol/vol) equine serum (HyClone Sterile Systems). Cells were plated at a density of 40,000 cells per cm^2 into multiwell plates (Costar) that had been coated with poly(D-lysine) (15 μg/ml) and laminin (10 μg/ml). The cells were maintained 5–7 days at 37°C in an atmosphere of 95% air and 5% CO_2 saturated with H_2O. To inhibit glial cell growth, 1 μM cytosine β-D-arabinofuranoside was added on day 5 in culture.

[^{3}H]Dopamine Uptake. The cells in each well were washed three times with 1 ml of phosphate-buffered saline containing 6 mg of D-glucose per ml and thereafter Dulbecco's modified Eagle's medium (Quality Biological, Framingham, MA) containing D-glucose at 6 mg/ml and 50 μM ascorbic acid. [^{3}H]Dopamine (New England Nuclear; specific activity, 45 Ci/mmol) was added to 50 nM, and the mixture was incubated for 15 min at 37°C. [^{3}H]Dopamine uptake was stopped by aspirating the incubation solution and washing the cells three times with ice-cold phosphate-buffered saline containing D-glucose at 6 mg/ml. The cells were removed by washing the wells with equal volumes of 0.2 M NaOH and 0.2 M HCl containing 0.02% Triton X-100. The residual intracellular radioactivity was determined by scintillation spectroscopy.

RESULTS

To test the cellular effects of NO released by the NONOates in the presence of reactive oxygen species, Chinese hamster V79 cells were exposed to the HX/XO reaction (which generates superoxide and hydrogen peroxide) or hydrogen peroxide alone in the absence or presence of DEA/NO. Treatment with 1 mM DEA/NO alone was not cytotoxic. In the absence of DEA/NO, exposure of V79 cells for various time intervals to HX/XO resulted in cell killing (Fig. 1 *Upper*). However, in the presence of 1 mM DEA/NO, the cytotoxic effects resulting from exposure to HX/XO were markedly inhibited. A lower DEA/NO concentration (0.1 mM) provided only marginal protection (Fig. 1 *Upper*). The decomposition products of 1 mM DEA/NO—namely, 1 mM diethylamine or 1 mM nitrite—did not afford the protection observed for DEA/NO (Fig. 1 *Lower*). SPER/NO (1 mM) which releases NO at a rate 0.06 times that of DEA/NO also provided protection in V79 cells against HX/XO-induced cytotoxicity.

It was independently verified that neither 1 mM DEA/NO nor its decomposition products, 1 mM diethylamine or 1 mM

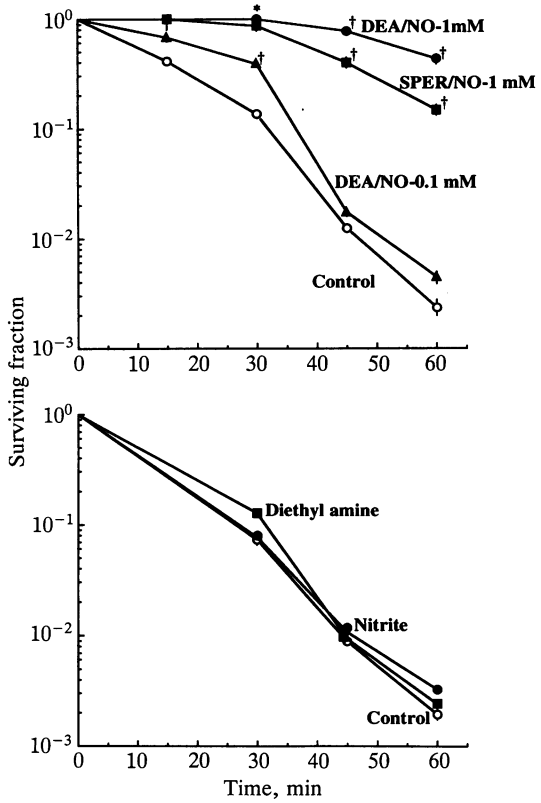

FIG. 1. (*Upper*) Survival of Chinese hamster cells exposed to HX/XO as a function of time in the absence (○) or presence of 1 mM DEA/NO (●), 0.1 mM DEA/NO (▲), or 1 mM SPER/NO (■). (*Lower*) Survival of Chinese hamster cells exposed to HX/XO as a function of time in the absence (○) or presence of 1 mM diethylamine (■) or 1 mM nitrite (●). *, $P < 0.05$; or †, $P < 0.005$ (both when compared with respective controls by Student's *t* test).

nitrite, preincubated with the enzyme inhibited the activity of XO as measured by the production of superoxide (ferricytochrome *c* reduction). Furthermore, the uric acid production was not inhibited by the presence of DEA/NO as monitored by UV absorption changes at 305 nm (data not shown). These data indicate that substrate turnover is not reversibly or irreversibly inhibited. However, the presence of DEA/NO did inhibit the superoxide dismutase-sensitive ferricytochrome *c* reduction, suggesting that either reduction of oxygen to form superoxide was inhibited or that NO scavenged the HX/XO-generated superoxide to form peroxynitrite anion, $OONO^-$, which was then rapidly converted to nitrate.

Fig. 2 *Upper* shows survival curves for V79 cells exposed to various concentrations of hydrogen peroxide for 1 hr. DEA/NO (1 mM) provided essentially complete protection against hydrogen peroxide cytotoxicity. Diethylamine (1 mM) had no effect on hydrogen peroxide cytotoxicity, while nitrite (1 mM) slightly potentiated hydrogen peroxide cytotoxicity by hydrogen peroxide concentrations > 0.5 mM (Fig. 2 *Lower*). In a separate experiment, V79 cells were exposed to a solution of 1 mM DEA/NO for 1 hr prior to addition of hydrogen peroxide. This resulted in only modest protection of the cells from the cytotoxic effects of added hydrogen peroxide (Fig. 2 *Upper*) and was certainly not protective to the extent found when DEA/NO was added just prior to hydrogen peroxide addition. However, DEA/NO incubated for 16 hr in medium prior to addition of hydrogen peroxide enhanced the cytotoxicity of hydrogen peroxide (Fig. 2 *Upper*) similar to that seen for nitrite (Fig. 2 *Lower*). In addition, cells first treated for 1 hr with hydrogen peroxide followed by a 1-hr treatment with 1 mM DEA/NO did not afford any protection (data not shown). Chemical controls demonstrated that the rate of formation of NO due to the decomposition of DEA/NO was not altered by the presence of hydrogen peroxide. Conversely, hydrogen peroxide was not consumed in the presence of DEA/NO (data not shown). These results show that the NO-generating compounds must be present during hydrogen peroxide exposure to be protective.

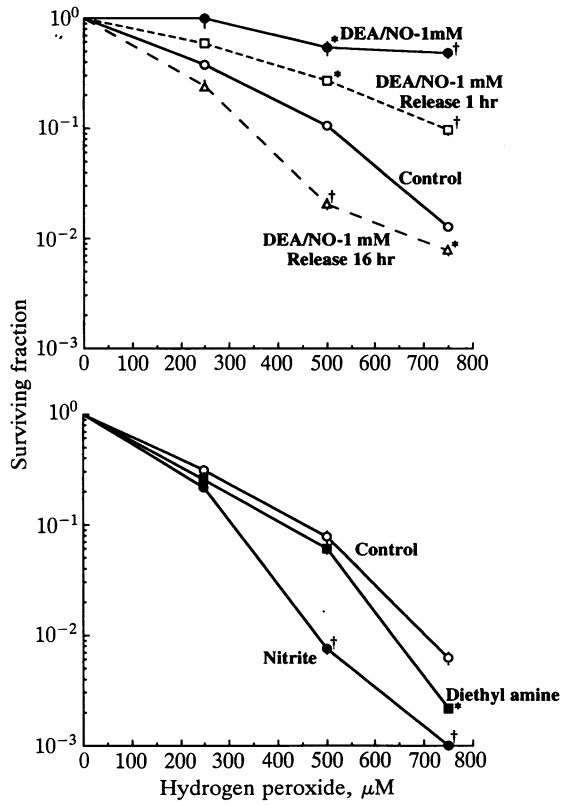

FIG. 2. (*Upper*) Survival of Chinese hamster cells exposed to various concentrations of hydrogen peroxide for 1 hr in the absence (○) and presence of 1 mM DEA/NO (●) or 1 mM DEA/NO allowed to release NO 60 min (□) or 16 hr (△) prior to hydrogen peroxide addition. (*Lower*) Survival of Chinese hamster cells exposed to various concentrations of hydrogen peroxide for 1 hr in the absence (○) and presence of 1 mM diethylamine (■) or 1 mM nitrite (●). *, $P < 0.05$; or †, $P < 0.005$ (both when compared with respective controls by Student's *t* test).

It has also been noted that inhibitors of XO reduce the toxicity induced by kainate in cerebellum neurons, suggesting the intermediacy of reactive oxygen species (28, 29). Since NO protects against superoxide and hydrogen peroxide-mediated cytotoxic effects in V79 cells, it may be plausible that during an ischemia reperfusion event, where reactive oxygen species are generated, cells capable of generating NO are protected. To test this hypothesis, we assayed the effects of hydrogen peroxide and HX/XO on ^{3}[H]dopamine uptake of rat mesencephalic embryonic cells. Exposure of these cells to 50 μM hydrogen peroxide for 1 hr or to 0.04 unit of HX/XO per ml for 5 or 10 min resulted in the loss of the cell's ability to take up ^{3}[H]dopamine as shown in Table 1. In contrast, when 50 μM DEA/NO was added together with hydrogen peroxide or HX/XO, complete protection occurred. Since radiolabeled dopamine uptake can be used as a measure for neurite viability, it can be inferred that NO protects neurons from damage induced by reactive oxygen species. A 1-hr exposure of cells to hydrogen peroxide resulted within the next 18 hr to the formation of large swellings in neurites and swelling of the soma (Fig. 3*B*). Exposure of cells to 100 μM DEA/NO for 60 min failed to cause morphological changes (Fig. 3*D*). The appearance of neurites and soma was similar as in nontested cells (Fig. 3*A*). Exposure to HX/XO for as short as 5 min elicited similar morphological changes in mesencephalic neurons (Fig. 3*C*). In the presence of 100 μM DEA/NO, there was marked protection of cells from the damage induced by exposure to hydrogen peroxide (Fig. 3*E*) or HX/XO (Fig. 3*F*) because the abnormalities in the neurites and cell bodies were prevented.

DISCUSSION

The role that nitric oxide plays in cytotoxic events is unclear. Some reports have portrayed NO as a toxic agent and suggested a role for this agent in mediating neurotoxicity in ischemia reperfusion injury (3–12). Yet, other reports suggest that NO may have a protective effect during ischemia reper-

Table 1. ^{3}[H]Dopamine uptake in primary cultures of mesencephalic neurons exposed to H_2O_2 or to HX/XO in the presence or absence of 100 μm DEA/NO

Addition to Incubation Medium		Time of exposure,	[^{3}H]Dopamine uptake, pmol per well per 15 min	
Agent	Conc.	min	Control	100 μM DEA/NO
None		—	2.9 ± 0.20	2.7 ± 0.11
H_2O_2	50 μM	60	0.63 ± 0.15*	2.8 ± 0.11
	100 μM	60	0.15 ± 0.014*	2.6 ± 0.22
HX/XO	0.04 unit/ml	5	1.1 ± 0.21*	2.6 ± 0.15
	0.04 unit/ml	10	0.36 ± 0.21*	2.3 ± 0.10

Cells were cultured for 7 days and then exposed to H_2O_2 or HX/XO for the length of time indicated. The cells were washed with phosphate-buffered saline containing 6 mg of D-glucose per ml; culture medium was added to each well, and the incubation was continued for 18 hr. ^{3}[H]Dopamine uptake was determined in six wells per group (450,000 cells per well). Values are means ± SD for six replicate experiments. Conc., concentration.

*$P < 0.01$ when compared with the nontreated group by Student's *t* test.

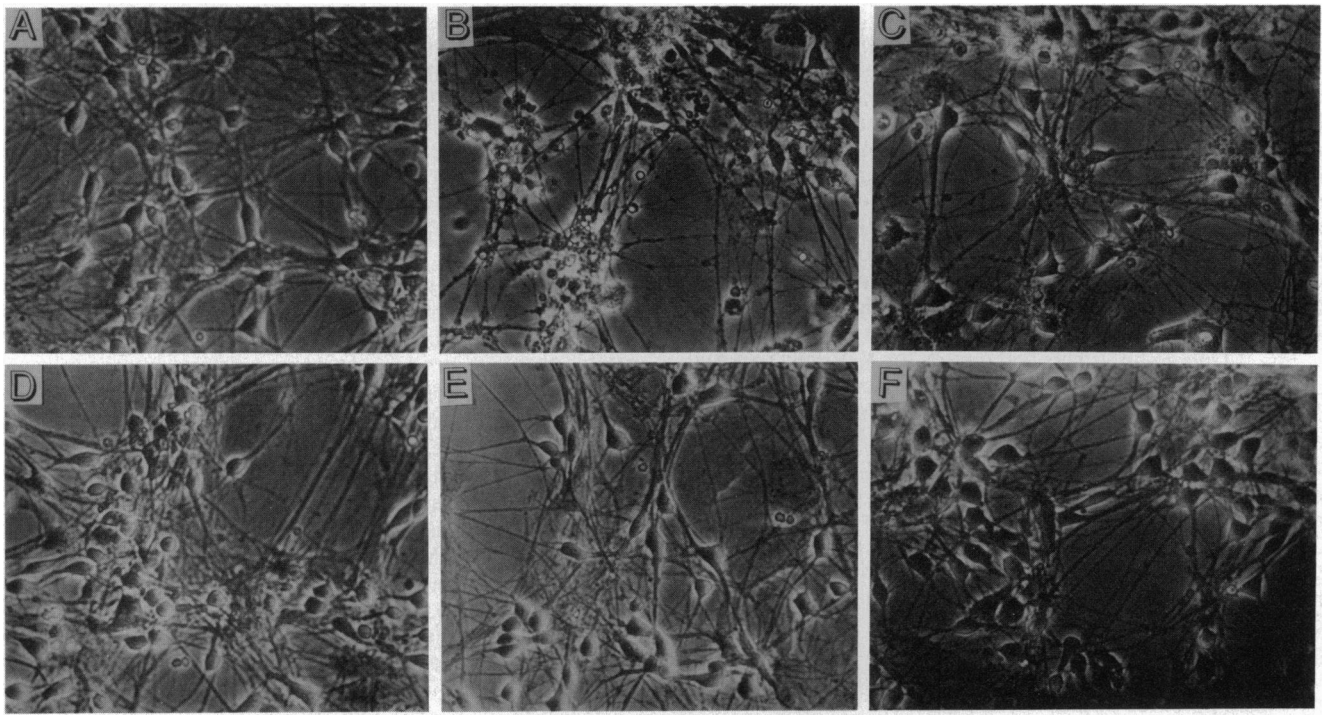

FIG. 3. Primary cultures of mesencephalic neurons exposed to hydrogen peroxide or to HX/XO. Photos were taken 18 hr after initial treatments. (*A*) Control; (*B*) 100 μM hydrogen peroxide alone (1-hr exposure); (*C*) HX/XO (10-min exposure); (*D*) 100 μM DEA/NO alone (1-hr exposure); (*E*) 100 μM hydrogen peroxide plus 100 μM DEA/NO (1-hr exposure); (*F*) HX/XO plus 100 μM DEA/NO (10-min exposure).

fusion injury (13–18, 30). Primary cell cultures of various types such as the two presented here and others reported elsewhere clearly demonstrate that cells exposed to high concentrations of NO are not adversely affected (22, 23). These observations demonstrate that NO or the intermediates derived from the NO/O_2 reaction are not appreciably cytotoxic under the conditions employed. Under biological conditions where NO has been speculated to be a toxic substance (i.e., cytolytic action of the immune system and ischemia reperfusion), other potent toxic agents such as hydrogen peroxide and superoxide also are present. This underscores the importance of investigating the interplay between NO and reactive oxygen species in the mediation of cytotoxicity.

Exposure of V79 cells to hydrogen peroxide or HX/XO has been shown to be a good model for the study of the biological effects of reactive oxygen species (31, 32). It has been shown that hydrogen peroxide exposure to V79 cells results in dose-dependent cytotoxicity (31). Furthermore, cytotoxicity of HX/XO was not abated in the presence of superoxide dismutase. In contrast, the protection resulting from the presence of catalase clearly demonstrated that hydrogen peroxide was the predominant toxin (31). The major difficulty in accurately assessing the properties of NO in the presence of reactive oxygen species has been the lack of a continuous source of NO as would be expected for NOS *in vivo*. For example, when a bolus of aqueous NO is administered to a solution, the NO concentration decreases rapidly because of the oxidation by O_2 (26). A series of compounds containing a $[N(O)NO]^-$ functional group, known as the NONOates, can serve as NOS mimics releasing NO over a period of time in a controlled and predictable fashion (24). When V79 cells were treated with HX/XO or hydrogen peroxide in the presence of DEA/NO, the cytotoxicity was substantially reduced. These results clearly show that hydrogen peroxide-mediated cytotoxicity can be prevented by the presence of a NO-generating compound. When the experiments were carried out in the presence of the NONOate SPER/NO, which releases NO at a rate 0.06 times that of DEA/NO, less protection against HX/XO cytotoxicity was observed. Since the amount of NO produced over any time interval by SPER/NO is less than DEA/NO, this suggests that NO released from these complexes is responsible for the protection.¶ Further, a series of control experiments using decomposition products of DEA/NO showed that the latter were ineffective in protecting cells from hydrogen peroxide- or HX/XO-induced cytotoxicity. The decomposition of DEA/NO did not consume hydrogen peroxide or affect XO substrate turnover. The biological and chemical controls clearly indicate that NO is mediating the protection observed.

Another possible toxic agent in mammalian cells is peroxynitrite anion ($OONO^-$), which would be expected to form in the presence of O_2^- that is generated from XO and NO (11, 33–35). The reaction rate constant for NO and O_2^- is reported to be $5.6 \times 10^7\ M^{-1}\cdot s^{-1}$, with the product being the potent oxidant ($OONO^-$) (36). This anion has been speculated to play a critical role in potentiating the toxic effects of NO, although $OONO^-$ is rapidly converted at physiological pH to nitrate. The quenching of O_2^- reduction of ferricytochrome *c* by DEA/NO can be explained by the scavenging of the O_2^- by NO to form peroxynitrite anion. However, any peroxynitrite anion which might be formed under the above conditions does not induce cytotoxicity as shown in Fig. 1.

The most dramatic protection of hydrogen peroxide-mediated cell damage was seen with the mesencephalic dopaminergic cells (Table 1 and Fig. 3). Exposure of these cells to 10% of the hydrogen peroxide used for V79 cells or HX/XO resulted in a marked decrease of the ability of these neurons to take up the neurotransmitter dopamine (Table 1). As was seen for V79 cells, the presence of the NO-releasing

¶Since direct reaction of reactive oxygen intermediates with the NONOates would be expected to occur at a similar rate, it appears that direct scavenging of the oxidizing intermediates is not taking place. The products of DEA/NO—i.e., diethylamine and nitrite—react with ·OH at near diffusion controlled rates, yet show no cytoprotection against hydrogen peroxide.

agent DEA/NO completely eliminated the neurotoxic effects. Even more dramatic was the attenuation of the "torpedo structures" (swellings) on the neurites (Fig. 3 *E* and *F*). The presence of either hydrogen peroxide or HX/XO resulted in the formation of these structures (Fig. 3 *B* and *C*); however, in the presence of DEA/NO, this compromise of the structural integrity of the fibers was prevented. It should be emphasized that nitrogen oxide intermediates derived from the NO/O_2 reaction did not result in observable changes in the fiber structure (Fig. 3*D*). These data indicate that membrane damage mediated by hydrogen peroxide was prevented by NO.

The results of our study suggest a positive role for NO in preventing the damage associated with reactive oxygen species. Two biological end points with two sources of reactive oxygen species clearly showed that NO serves to eliminate the adverse effects associated with the oxygen species. Hydrogen peroxide toxicity results from the formation of oxidants derived from the reaction with metals or metalloproteins (37). A plausible explanation of our findings is that NO blocks the formation of the hydroxyl radical equivalents that result from the metal-mediated reduction of hydrogen peroxide. *In vitro* experiments show NO to be an antioxidant in Fenton-type reactions, although the chemical mechanism is unclear (38). Preliminary data in our laboratory suggest that an additional possibility might be that NO terminates free-radical chain reactions within the lipid membrane (unpublished data).

It has been stated that neurons that express NOS survive an ischemia reperfusion event, whereas surrounding neurons not expressing NOS die (3). The mechanism of the neuronal cell death has been attributed to the migration of NO to neighboring cells; however, since NO is a readily diffusible gas in tissue (39), the highest concentration of NO would be expected at the source with exponential dilution as the NO migrates away from the cell. Therefore, if NO is a potent toxin, the greatest cytotoxic effect would be expected to be at the cells containing the NOS. From our results, it is more reasonable to propose that the higher concentrations of NO protect the NOS-containing cells from hydrogen peroxide-mediated cell death and that cells further away from the source would be perfused by exponentially decreasing amounts of NO, and therefore would be protected to a lesser extent.

Conclusions. The above results clearly show that NO protects against hydrogen peroxide-mediated cell damage and death. Our study also allows the comparison of cytotoxic effects of a number of proposed toxic reactive small molecules, including hydrogen peroxide-derived oxidants O_2^-, NO, and NO/O_2-derived intermediates. Results from this study and others (31, 40) clearly indicate that hydrogen peroxide-derived oxidants are the most cytotoxic of the above agents. The presence of NO and HX/XO have been suggested to form $OONO^-$. Though $OONO^-$ may be formed, the contribution of this anion to HX/XO-induced cytotoxicity was negligible in the present studies. Our observations suggest that NO may play a critical role *in vivo* in protecting mammalian cells from toxic oxygen reactive species.

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