Nitric Oxide- and Superoxide-Mediated Toxicity in Cerebral Endothelial Cells

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ABSTRACT

Nitric oxide and superoxide are free radicals that appear to contribute to the pathogenesis of a number of brain disorders, and cerebral endothelial cells are a potential target of these agents. Because of the capacity for these two agents to combine, it has been suggested that nitric oxide might either enhance or inhibit the toxic effects of superoxide. To establish the effect of the generation of superoxide and nitric oxide alone and in combination, cerebral endothelial cells were exposed to sodium nitroprusside, a source of nitric oxide, and/or paraquat, a source of superoxide. Paraquat enhanced the toxicity of sodium nitroprusside, as did diethyldithiocarbamate, an inhibitor of superoxide dismutase, which supports the hypothesis that enhanced levels of superoxide can combine with nitric oxide to form a more toxic product. Also, the toxicity of paraquat could be partially inhibited by blocking endogenous nitric oxide synthesis using N\textsuperscript{G}-monomethyl-L-arginine. When ascorbate was administered along with sodium nitroprusside to increase nitric oxide generation, as little as 5 μM sodium nitroprusside was toxic when superoxide dismutase was inhibited. Whereas concentrations of 50 to 500 μM sodium nitroprusside and 0.4 mM ascorbate caused ~100% toxicity, there was no measurable toxicity when these doses were accompanied by 2 mM glutathione or 50 U/ml of catalase; this suggests that peroxides may also contribute to nitric oxide toxicity. These results suggest that the simultaneous generation of nitric oxide and superoxide is synergistic, resulting in enhanced toxicity.

Nitric oxide and superoxide are free radicals that appear to play critical roles in the development of brain injury after stroke (Chan et al., 1987; Chan et al., 1990; Kinouchi et al., 1991; Lipton et al., 1993; Nishikawa et al., 1994; Zhang et al., 1994) and may also contribute to a number of other disorders of the CNS, including Parkinson’s disease, Alzheimer’s disease and amyotrophic lateral sclerosis (Olanow and Ardesh, 1994). However, the specific effects of these free radicals and the mechanism by which they modify CNS injury are not fully understood. Superoxide generally appears to exacerbate ischemic injury, and treatments that decrease the level of superoxide radicals can effectively reduce ischemic brain injury (Chan et al., 1987; Kinouchi et al., 1991; Yang et al., 1994). The effects of nitric oxide on brain injury are less clear than those of superoxide and seem to depend on whether the molecule is derived from neurons or endothelial cells (Huang et al., 1994) and on the exact chemical milieu in which the molecule resides and the redox state of the molecule (Lipton et al., 1993). The oxidized form of nitric oxide, the nitrosion ion (NO\textsuperscript{+}), can protect neurons from NMDA-mediated toxicity, apparently by reacting with a modulatory site on the NMDA receptor, whereas the reduced form of nitric oxide is neurotoxic (Lipton et al., 1993). In addition, nitric oxide can be beneficial after brain injury, apparently because it can induce vasodilation (Palmer et al., 1987) and might thereby increase flow to regions critically deprived of blood.

A critical question has been how the interaction of nitric oxide and superoxide might affect the toxic properties of these free radicals. Because nitric oxide can combine with superoxide, it has been suggested that nitric oxide production might protect the brain from the damaging effects of superoxide (Feigl, 1988). Nitric oxide can reduce the production of cytotoxic superoxide radicals by leukocytes (Rubanyi et al., 1991) and by endothelial cells (Heim et al., 1991). However, the product of the combination of superoxide and nitric oxide is peroxynitrite, a potentially toxic anion that is capable of generating highly damaging hydroxyl radicals and nitration of tyrosine residues, leading to protein damage (Beckman et al., 1990; Ischiropoulos et al., 1992). Although it now appears

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ABBREVIATIONS: ANOVA, analysis of variance; BCA, bathocuproinedisulfonic acid; DDC, diethyldithiocarbamate; GSHpx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; LDH, lactate dehydrogenase; NMDA, N-methyl-D-aspartate; NMMA, N\textsuperscript{G}-monomethyl-L-arginine; PBS, phosphate-buffered saline; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; SOD, Cu,Zn-superoxide dismutase; SNAP, S-nitroso-N-acetyl-DL-penicillamine.
clear that nitric oxide and superoxide have the potential in vitro to combine to form peroxynitrite, and although peroxynitrite is toxic to cells (Lipton et al., 1993), the capacity of separate sources of nitric oxide and superoxide to combine in vivo and induce toxicity in a synergistic manner has not been fully established.

It is also not clear which cells might play a critical role in the response to superoxide and nitric oxide. However, it has been suggested that cerebral endothelial cells may be an important target of these free radicals and peroxynitrite (Beckman et al., 1990). Indeed, there are several sources of both superoxide and nitric oxide in these cells. For example, endothelial cells synthesize superoxide in response to stimulation by bradykinin (Holland et al., 1990), during enzymatic activity of xanthine oxidase (Terao et al., 1991) and during normal respiration. In addition, endothelial cells can synthesize nitric oxide directly, and adjacent cells, such as smooth muscle cells, may also synthesize nitric oxide as a response to inflammatory mediators (Koide et al., 1993). Endothelial cell damage certainly occurs after a variety of insults, including cerebral ischemia (Kuroiwa et al., 1988; Yang and Betz, 1994), brain trauma (Cortez et al., 1987) and radiation (Gobbel et al., 1991), resulting in breakdown of the blood-brain barrier, edema, tissue swelling and exacerbation of injury. The finding that reduction of superoxide levels during and after cerebral ischemia leads to a reduction in blood-brain barrier breakdown and cerebral edema (Chan et al., 1987; Kinouchi et al., 1991) supports the assertion that endothelial cells are a probable target of free radicals.

In the present study, we have measured the effects of superoxide and nitric oxide generation on cerebral endothelial cells, and we have determined the role of the interaction of these free radicals in endothelial cell injury. We postulated that the simultaneous administration of independent sources of these two free radicals might have synergistic toxic effects. The goals of this study were to determine 1) whether the severity of injury induced by a nitric oxide generator, SNP, is altered by a superoxide source, paraquat, 2) whether the severity of injury due to superoxide or nitric oxide production is modulated by alteration of the endogenous production of nitric oxide or superoxide, and 3) whether the severity of injury due to nitric oxide is modulated by alteration of the concentration or activity of antioxidant enzymes, such as cytosolic SOD or catalase.

Materials and Methods

Isolation of cerebral endothelial cells. Cerebral endothelial cells were isolated from 14-day-old Sprague-Dawley rats of both sexes as previously described (Gobbel et al., 1994). Cells were grown on plastic, 24-well dishes coated with collagen (25 μg/cm²) of type VII collagen from rat tail, Sigma Chemical Co., St. Louis, MO) in M199 with Earle’s salts supplemented with 10% horse serum, heat-inactivated 5% fetal calf serum, 20 mM HEPES buffer, 0.1 mg/ml heparin sulfate, 0.1 mg/ml endothelial cell growth supplement (Sigma Chemical Co.), 2.0 mM glutamine, 2.5 mM glucose, 2.5 μg/ml fungizone and 0.1 mg/ml gentamicin. The cells were incubated at 37°C in 5% CO₂ and the cultivation medium was changed every 2 to 3 days. Only primary cultures were used at confluence, 5 to 9 days after isolation.

Drug treatment. Paraquat, SNP, carbachol, NMMA, DDC, ascorbic acid, SOD, catalase, GSH and BCA, a copper chelator, were obtained from Sigma Chemical Co. SIN-1 was a gift from Dr. Joseph Beckman. All agents were dissolved directly in a solution of M199 with Earle’s salts, 5% horse serum, 2.5 μg/ml fungizone and 0.1 mg/ml gentamicin just before treatment, and the solution was passed through a 0.22-μm filter to remove any bacterial contamination. To expose the cells to the various agents, the cultivation medium was changed to M199 with Earle’s salts, 5% horse serum, 2.5 μg/ml fungizone and 0.1 mg/ml gentamicin, and the cells were incubated for 18 hr in the various drug-containing solutions.

Determination of cell viability. Cell viability was determined by measuring the release of the intracellular enzyme LDH by nonviable cells with increased membrane permeability. The amount of LDH released was determined by measuring the concentration of LDH in an aliquot of the medium overlaying the cells. The LDH was measured spectrophotometrically using a commercially available kit (Sigma Chemical Co.) that is based on the increase in absorption at 340 nm due to the reduction of NAD by LDH. The amount of LDH remaining in the cells was determined by replacing the treatment medium of the cells with an equal volume of PBS, disrupting the cells by sonication for 10 sec and measuring LDH within the PBS. The amount of LDH released was normalized to the total LDH present in the culture (released and intracellular) to quantify the fraction of LDH released, which should approximate the fraction of nonviable cells in the culture. The LDH concentration in untreated control cells is typically around 0.7 U/mg protein, and each well of a 24-well plate contains approximately 0.06 mg of protein when the cells reach confluence.

Statistical analysis. The results are presented as the mean of the data ± S.D. One-way ANOVA followed by Dunnett’s test was used to compare the viability of cells treated with multiple doses of SNP or paraquat to the viability of cells treated with no drug (controls). Two-way ANOVA was used to evaluate the effect of various drugs or drug combinations on the viability of cells after treatment with SNP, paraquat or both paraquat and DDC. The factors included in the two-way ANOVA were dose of paraquat or SNP and dose of additional drugs or drug combinations. Analyses with P < .05 were considered significant.

Results

Enhancement of toxicity by simultaneous generation of superoxide and nitric oxide. Treatment of cerebral endothelial cells with SIN-1, which simultaneously generates both superoxide and nitric oxide radicals, caused a pronounced increase in toxicity as measured by LDH release, and the effect was dose-dependent over a narrow range of concentrations. The percentages of LDH released after 18 hr of treatment with 0, 1 and 5 mM SIN-1 were 12.6 ± 1.4, 13.9 ± 1.8 and 99.4 ± 0.7 (n = 4), respectively.

To determine the effects of nitric oxide and superoxide alone and in combination, we added SNP and/or paraquat as separate sources of these two free radicals (fig. 1). Cerebral endothelial cells were adversely affected by increasing doses of either paraquat (fig. 2A) or SNP (fig. 2B). There was a significant increase in the percentage of LDH released by the cells after exposure to >0.2 mM paraquat, and 96.7 ± 0.6% of the LDH was released after treatment with 5 mM paraquat, which indicated that virtually all cells were killed by this dose. A significant increase in LDH release relative to untreated controls was detected after exposure to 1 mM SNP, and the high level of LDH release after exposure to 10 mM (100% for all samples) indicated virtually complete killing by these doses.

The presence of paraquat enhanced the toxicity of SNP (fig. 2B). Although a dose of 0.1 mM paraquat had no effect on SNP toxicity, a dose of 0.2 mM paraquat, which caused minimal toxicity alone (fig. 2A), caused a significant leftward shift of the curve describing the percentage of LDH release after
at lower doses of paraquat (10–100 μM) and paraquat (fig. 3B). When further studies were carried out in the present set of experiments. Chemicals used are underlined; enzymes are noted in italics. The stimulation and the inhibition of enzyme activity by an agent are indicated by + and –, respectively. SNP generates nitric oxide (NO) after reduction with ascorbate or another agent. NO can also be generated physiologically by the conversion of arginine to citrulline by nitric oxide synthase. Nitric oxide synthase activity is stimulated by carbachol and inhibited by the arginine analog NMMA. Reduced paraquat reacts with oxygen to generate superoxide (O$_2^-$). Superoxide can also be converted in the presence of hydrogen ions into oxygen and hydrogen peroxide (H$_2$O$_2$) by SOD, whose activity is inhibited by DDC. In turn, hydrogen peroxide can be broken down by catalase into water and oxygen, or it can be converted by GSHpx into water, resulting in the oxidation of GSH to GSSG.

Effect of modulation of endogenous superoxide or nitric oxide on toxicity of exogenous superoxide or nitric oxide. The effect of DDC alone on the toxicity of paraquat and SNP, we added 1 mM DDC to some cultures to inhibit SOD activity (fig. 1). As anticipated, treatment with DCC enhanced the toxicity due to paraquat (fig. 3A). Whereas a dose of 0.5 mM was necessary in the absence of DDC to cause significant increases in LDH release, a finding consistent with our previous one, in the presence of DDC, a dose of 0.1 mM paraquat caused significant increases in LDH release. Combined treatment with DDC and paraquat (0.1 mM), which resulted in 49.9 ± 10.7% LDH release, caused a dramatic increase in the toxicity of SNP. A dose of 0.5 mM SNP, which caused only 19.1 ± 1.9% LDH release compared with 14.7 ± 2.8% in untreated controls, caused complete toxicity (100% LDH release) in the presence of DDC and paraquat (fig. 3B). When further studies were carried out at lower doses of paraquat (10–100 μM) and lower doses of SNP (5–50 μM) to determine the minimally toxic dose of SNP in the presence of DDC, we found that as little as 5 μM SNP could cause a significant increase in toxicity in the presence of 100 μM paraquat (fig. 4). Although there was no apparent effect of any dose of SNP when administered in the presence of 10 to 30 μM PQ, 5 to 50 μM SNP caused >90% LDH release when administered in the presence of 100 μM PQ.

To confirm the role of superoxide radicals in the combined toxicity of paraquat and SNP, we added 1 mM DDC to some cultures to inhibit SOD activity (fig. 1). As anticipated, treatment with DDC enhanced the toxicity due to paraquat (fig. 3A). Whereas a dose of 0.5 mM was necessary in the absence of DDC to cause significant increases in LDH release, a finding consistent with our previous one, in the presence of DDC, a dose of 0.1 mM paraquat caused significant increases in LDH release. Combined treatment with DDC and paraquat (0.1 mM), which resulted in 49.9 ± 10.7% LDH release, caused a dramatic increase in the toxicity of SNP. A dose of 0.5 mM SNP, which caused only 19.1 ± 1.9% LDH release compared with 14.7 ± 2.8% in untreated controls, caused complete toxicity (100% LDH release) in the presence of DDC and paraquat (fig. 3B). When further studies were carried out at lower doses of paraquat (10–100 μM) and lower doses of SNP (5–50 μM) to determine the minimally toxic dose of SNP in the presence of DDC, we found that as little as 5 μM SNP could cause a significant increase in toxicity in the presence of 100 μM paraquat (fig. 4). Although there was no apparent effect of any dose of SNP when administered in the presence of 10 to 30 μM PQ, 5 to 50 μM SNP caused >90% LDH release when administered in the presence of 100 μM PQ.

Effect of modulation of endogenous superoxide or nitric oxide production on toxicity of exogenous superoxide or nitric oxide. The effect of DDC alone on the toxicity of SNP was tested to determine whether increases in endogenous superoxide production resulting from inhibition of SOD might enhance nitric oxide toxicity and partially account for the results shown in figure 4. There was a significant increase in SNP toxicity to cerebral endothelial cells in the presence of DDC (ANOVA; P < .0001; fig. 5). In contrast, modification of endogenous production of nitric oxide did not substantially affect the toxicity of paraquat as measured by LDH release. Endogenous nitric oxide production was stimulated using the ACh receptor agonist carbachol at 0.1 mM and inhibited using the nitric oxide synthase blocker NMMA at 0.1 mM (fig. 1). There was no apparent significant effect of NMMA (fig. 6A). Although the presence of carbachol did slightly alter the toxicity due to paraquat (ANOVA; P < .05), the only notable increase in paraquat toxicity was at 0.2 mM with slightly greater amounts of LDH release (28.3 ± 7.0%) in the presence of carbachol compared with the LDH release in the absence of carbachol (21.4 ± 0.4%) (fig. 6A). This effect was not present when the cells were treated with paraquat and DDC in combination (fig. 6B), and NMMA also had no impact on the effect of paraquat and DDC in combination.

In contrast to the minimal effects of carbachol and NMMA, SNP once again caused a dramatic increase in the toxicity due to paraquat and DDC (fig. 6B). Although there was no increase in toxicity due to either SNP alone (19.8 ± 0.5% LDH release) or the combination of DDC and 0.02 mM para-
paraquat (13.5 ± 6.8% LDH release) compared with controls treated with no drugs (27.3 ± 8.2% LDH release), the combination of all three caused a 59.6 ± 4.0% release of LDH. We added 25 μM BCA to chelate copper and to test whether the enhanced toxicity of paraquat and SNP by DDC might be mediated by the reported ability of DDC to increase intracellular copper (Nobel \textit{et al.}, 1995). However, BCA had no effect on the apparent synergism of paraquat and SNP in the presence of DDC.

**Effect of a reducing agent and antioxidant enzymes on SNP toxicity.** Ascorbic acid was added to our cultures to act as a reducing agent and increase the generation of nitric oxide (Harrison and Bates, 1993; Lipton \textit{et al.}, 1993) (fig. 1). This caused a significant (ANOVA; P < .001) and relatively dramatic increase in the toxicity of SNP, which suggested that the release of nitric oxide was important for the toxicity of SNP. In the absence of ascorbic acid, the addition of 0.5 mM SNP increased LDH release only from 22.8 ± 1.5% to 25.9 ± 1.2%; in the presence of 0.4 mM ascorbate, the addition of 0.5 mM SNP increased LDH release from 18.8 ± 3.3% to 89.7 ± 5.6%. Similar increases in LDH release in response to the addition of ascorbate were seen after treatment with 2 mM SNP.

The addition of DDC to inhibit SOD significantly increased the toxicity of the combination of ascorbic acid and SNP (ANOVA; P < .001; figs. 7 and 8). As much as 15 μM SNP caused only minimal increases in LDH release in the absence of DDC, but in the presence of DDC, as little as 5 μM SNP significantly enhanced LDH release and led to increased cell disruption and loss as judged by phase-contrast microscopy (fig. 8). Thus it appeared that micromolar concentrations of SNP were toxic in the presence of a reducing agent to enhance nitric oxide generation.

We added the copper-zinc form of SOD (50 U/ml) exogenously to determine whether the toxicity of combined ascorbic acid and SNP was reversible by inhibition of superoxide generation (fig. 1). Catalase was added to some wells as a
control for any possible, nonspecific effects of exogenous enzyme. Surprisingly, although the SOD had no or minimal effect on toxicity, catalase caused a significant and complete inhibition of the LDH released in response to ascorbate and SNP (ANOVA; \( P < 0.001 \); fig. 7A). Phase-contrast microscopy demonstrated that the total cellular disruption and degeneration induced by 0.5 mM SNP and 0.4 mM ascorbic acid could be completely inhibited by catalase (fig. 8). To test whether catalase might be acting indirectly to enhance SOD activity by removing peroxides generated by the latter enzyme (fig. 1), we examined the effect of catalase on toxicity when SOD activity was inhibited by DDC. As before, DDC enhanced toxicity due to nitric oxide generation (fig. 7A, and B), and catalase blocked toxicity. However, in addition, catalase protected against nitric oxide toxicity even when SOD activity was blocked by DDC (fig. 7B), although this protection could be overcome by sufficiently high doses of SNP.

The protection of cells from SNP toxicity by catalase suggested that peroxides might be contributing to the damage. Because excessive peroxides could be generated after depletion of GSH and the resultant loss of GSHpx activity (fig. 1), we added glutathione (2 mM) to determine whether such treatment might inhibit SNP toxicity. GSH, like catalase, significantly inhibited toxicity of SNP (ANOVA; \( P < 0.001 \); fig. 9).

**Discussion**

An unanswered question has been whether the co-production of superoxide and nitric oxide would reduce or enhance the toxic effects of these individual free radicals. This is important to consider, because the inadvertent combination of nitric oxide and superoxide from diverse sources might exacerbate certain diseases. For example, there may be excess production of both superoxide and nitric oxide after cerebral ischemia; superoxide might be formed because of increased metabolism of ATP and breakdown of the resultant hypoxanthine by xanthine oxidase (Terada et al., 1991). Simultaneously, excess production of nitric oxide could occur either in an attempt to bring about vasodilation or because of cytokine stimulation of smooth muscle cells (Koide et al., 1993). In the present experiments, we used SNP and paraquat to generate nitric oxide and superoxide, respectively. Each molecule of SNP generates one molecule of nitric oxide after activation by light or chemical reduction (Harrison and Bates, 1993), whereas each molecule of paraquat can gener-
ate many superoxide molecules by a redox cycle involving oxidation of an agent, such as NADPH, followed by reduction of \( \text{O}_2 \) (Bus and Gibson, 1984). The concentrations of nitric oxide and superoxide achieved in the cells by given concentrations of SNP and paraquat, respectively, thus depend on a number of factors, including the availability of reducing agents and the rate of subsequent reaction and degradation of the free radicals generated. Because the availability of reducing agents and the rate of degradation are unknown, it is impossible to predict accurately the actual concentration of free radicals generated by SNP and by paraquat. However, in the present experiments, it appears that the toxicity of SNP and paraquat is due to generation of nitric oxide and superoxide rather than to some other secondary reaction. The reason we draw this conclusion is that the toxicity of SNP was enhanced by a reducing agent to increase the rate of nitric oxide release. In addition, toxicity of paraquat was enhanced by DDC, an inhibitor of the SOD enzyme responsible for superoxide degradation. Our results strongly sup-port the concept that independent sources of nitric oxide and superoxide can act in a synergistic fashion to enhance rather than ameliorate toxicity. In the first place, the toxic effects of SNP and paraquat were significantly and consistently exacerbated by the presence of the other molecule, even when SNP was present at concentrations that would cause minimal toxicity when administered alone (fig. 4). Second, merely inhibiting SOD enhanced the toxicity of SNP (fig. 5).

One reason why nitric oxide and superoxide could be synergistic is that they can combine to form peroxynitrite, a highly toxic compound (Lipton et al., 1993). We would predict that, if superoxide is combining with nitric oxide to form peroxynitrite, then treatment with paraquat and SNP should increase nitrotyrosine formation (Beckman et al., 1992). In a preliminary experiment examining the effect of the treatment of endothelial cells with SNP and paraquat, there was no evidence of proteins containing nitrotyrosine, judging on the basis of Western blotting. Our ability to detect nitrotyrosine may have been hampered by a currently poor understanding of the lifetime of nitrotyrosine-containing proteins and of the amount of such proteins that may be formed. Nevertheless, this result suggests that it is important to consider whether reactions other than the formation of peroxynitrite might account for our results. For example, we considered whether the enhancement of the synergism between paraquat and SNP by DDC might be related to the reported ability of DDC to increase free copper concentrations by transport of this metal ion across the cell. Copper could then catalyze a Haber-Weiss type of reaction to produce increased levels of highly damaging hydroxyl radicals:

\[
\text{Cu}^{2+} + \text{O}_2^- + H_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^\cdot
\]

However, this reaction does not appear to account for our results. We added to our cultures BCA, a compound that has been previously used to demonstrate the involvement of, and to counteract, copper-mediated toxicity by dithiocarbamates such as DDC (Mohindru et al., 1983; Nobel et al., 1995). BCA does not readily cross cell membranes (Harmon and Crane, 1976), so it should not interfere with SOD activity, but it
should interfere with transport of copper into the cell by DDC. Although the concentration of BCA that we used has been reported to be effective in other systems (Mohindru et al., 1983), this concentration did not alter the DDC-mediated enhancement of paraquat and SNP toxicity in our studies.

We also considered whether superoxide might act as ascorbate does to reduce SNP and increase the release of nitric oxide (fig. 1). However, if superoxide does act in this fashion, it appears to do so rather poorly in that there was enhancement of the toxicity of SNP only when paraquat was added at toxic or near toxic concentrations (figs. 2 and 4). If the reactivity of superoxide with SNP is lower than its reactivity with SOD, then the ability to generate nitric oxide may be limited unless SOD activity is inhibited. This could explain why inhibiting SOD to increase superoxide formation increases the toxicity of SNP down to concentrations of around 5 μM (fig. 4). Although we cannot completely rule out the possibility that superoxide increases nitric oxide generation by SNP and that nitric oxide alone is toxic, another finding strongly supports the hypothesis that the combination of nitric oxide and superoxide is more toxic than either molecule alone. This finding is that inhibition of SOD by DDC enhanced toxicity even when ascorbate was already present to effectively enhance the production of nitric oxide from SNP (figs. 7 and 8).

Considering that superoxide appears to enhance nitric oxide toxicity, the potentially high endogenous production of superoxide by cerebral endothelial cells could make them particularly susceptible to nitric oxide toxicity. For example, the mitochondrial content of cerebral endothelial cells is 2- to 5-fold higher than that of other cells (Oldendorf et al., 1977), and mitochondria are a potential source of superoxide (Sanders et al., 1993). Furthermore, the activity of xanthine oxidase, a superoxide-generating enzyme, is relatively high within these cells (Terada et al., 1991). Indeed, the data that exist support the notion that cerebral endothelial cells are sensitive to nitric oxide. Treatment with 150 μM SNP in the presence of 0.4 mM ascorbate caused a >90% decrease in viability of the endothelial cells in our experiments (fig. 7A). In comparison, treatment of cortical neurons with 0.4 mM SNP and 0.4 mM ascorbate for 18 hr produced only a 20% to 30% decrease in viability (Lipton et al., 1993). However, it may be difficult to compare directly results taken from individual studies done in separate laboratories. Furthermore, the role of xanthine oxidase in nitric oxide toxicity is not clear. Inactivation of xanthine oxidase activity within endothelial cells does not change the basal level of production of oxygen-derived free radicals (Paler-Martinez et al., 1994) and does not reduce the amount of damage induced by cerebral ischemia (Lindsay et al., 1991). Nevertheless, our findings suggest that further investigation into the role of supporting cells such as endothelial cells may be useful in exploring the pathogenesis of disorders in which nitric oxide appears to play a role.

The observation that inhibition of SOD by DDC enhanced the toxicity of nitric oxide suggested that exogenous application of SOD might modulate the toxicity of exogenous nitric oxide. However, SOD was ineffective, possibly because the large size of SOD (M_r = 32,000) may limit its ability to cross the cell membrane and enter the cytoplasm and because the negative charge on the superoxide anion may limit its movement out of the cell. We also found that blockade of nitric oxide synthetase by NMMA failed to ameliorate paraquat toxicity judged on the basis of LDH release. This result is in contrast to the report that NMMA can block death in PC12 cells due to superoxide produced after SOD down-regulation (Troy et al., 1996). However, cell viability in that report was based on the number of cells remaining on the plate, whereas LDH release, as used in our study, was determined by changes in membrane permeability. When we reevaluated our data and used total LDH remaining on the plate as a measure of number of cells remaining after treatment, there was significantly greater viability in the NMMA-treated population (ANOVA; P < 0.005). In the absence of NMMA, after treatment with 20, 50 and 100 μM paraquat, the remaining amounts of LDH were 78.6 ± 4.8, 60.8 ± 12.0 and 40.4 ± 19.5 mU, respectively; in the presence of 0.1 mM μM NMMA, the remaining amounts of LDH were 90.4 ± 2.1, 76 ± 2.7 and 55.2 ± 9.8 mU, respectively. Although we cannot rule out the possibility that the increase in remaining LDH was due to protection of the enzyme from inactivation by free radicals, the result is consistent with a 15% to 35% sparing of cells by NMMA. This effect of NMMA on remaining LDH was observed only when DDC was present. In contrast to the apparent effects of NMMA on paraquat toxicity in the presence of SOD inhibition, stimulation of nitric oxide synthesis by carbachol did not induce any changes in survival as evaluated either by percentage of LDH released or by amount of remaining LDH.

The inability of carbachol to enhance the toxicity of paraquat may be related to the concentrations of endogenous nitric oxide and the degree of change in nitric oxide production induced by carbachol. Although we used a dose that should effectively stimulate nitric oxide production (Dowell et al., 1993), the change in concentrations may be too low to have a measurable effect on toxicity. In addition, endogenous nitric oxide concentrations are reported to be around 100 nM (Shibuki, 1990), which is 50-fold less than the 5 μM concentration of nitric oxide generators that our results suggest is necessary to enhance toxicity.

Although exogenous SOD had no detectable effect on toxicity of SNP, catalase alone had rather dramatic protective effects. This result extends the report that SOD and catalase in combination can reduce the damage induced in cortical neurons by nitric oxide- and peroxynitrite-generating chemicals, because our results suggest that much of the protection may be due to catalase (Lipton et al., 1993). This finding is consistent with the report that catalase can protect against the toxicity of the combination of SNAP, a nitric oxide generator, and hypoxanthine/xanthine oxidase, a superoxide-generating system (Volk et al., 1995). That report suggested that the toxicity of nitric oxide might be due to an interaction with hydrogen peroxide resulting from the dismutation of superoxide. Our results show that catalase is protective even when a superoxide generator is not present, which suggests that endogenous production of peroxides due to treatment with nitric oxide generators is sufficient to cause toxicity. We tested the possibility that catalase, by enzymatically scavenging the peroxides formed upon dismutation of superoxide, our results show that catalase is protective even when a superoxide generator is not present, which suggests that endogenous production of peroxides due to treatment with nitric oxide generators is sufficient to cause toxicity. We tested the possibility that catalase, by enzymatically scavenging the peroxides formed upon dismutation of superoxide (fig. 1), might be enhancing SOD activity and reducing superoxide concentrations. However, catalase was protective even when SOD was inhibited by DDC, which suggests that peroxides themselves are responsible for the toxicity. Asahi and colleagues have indicated that SNAP can inactivate GSHpx and that administration of SNAP can enhance per-
Oxide formation (Asahi et al., 1995). Thus the primary cause of excess peroxide generation may be inactivation of GSHpx. Interestingly, the IC50 for inactivation of GSHpx by SNAP is reported to be 2 μM (Asahi et al., 1995), which is close to the minimal toxic concentration of SNP found in our experiments. Nitric oxide may also deplete GSH, a substrate in the GSHpx reaction (fig. 1), by reacting with the sulfhydryl group to form S-nitrosoglutathione (Clancy et al., 1994). Indeed, our results show that adding exogenous GSH can block the toxicity of sodium nitroprusside (fig. 9). Catalase may protect against excess peroxides generated upon inactivation of GSHpx and/or depletion of glutathione, because unlike GSHpx, catalase is not affected by nitric oxide (Asahi et al., 1995) and does not require GSH as a substrate (fig. 1).

In conclusion, our findings support the hypothesis that independent sources of nitric oxide and superoxide may act in concert in a synergistic fashion to produce toxicity. They further suggest that cerebral endothelial cells are particularly susceptible to toxicity induced by nitric oxide when SOD activity is inhibited. Finally, considering that peroxide-scavenging molecules can block damage produced by nitric oxide, the toxicity appears to be mediated by the generation of peroxides.

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