Nitric oxide (NO) challenge to human neuroblastoma cells (SH-SY5Y) ultimately results in apoptosis. Tumor suppressor protein p53 and cell cycle inhibitor p21 accumulate as an early sign of N-nitrosogluthathione-mediated toxicity. Cytochrome c release from mitochondria and caspase 3 activation also occurred. Cells transfected with either wild type (WT) or mutant (G93A) Cu,Zn-superoxide dismutase (Cu,Zn-SOD) produced comparable amounts of nitrite/nitrate but showed different degree of apoptosis. G93A cells were the most affected and WT cells the most protected; however, Cu,Zn-SOD content of these two cell lines was 2-fold the SH-SY5Y cells under both resting and treated conditions. We linked decreased susceptibility of the WT cells to higher and more stable Bcl-2 and decreased reactive oxygen species. Conversely, we linked G93A susceptibility to increased reactive oxygen species production since simultaneous administration of N-nitrosogluthathione and copper chelators protects from apoptosis. Furthermore, G93A cells showed a significant decrease of Bcl-2 expression and, as target of NO-derived radicals, showed lower cytochrome c oxidase activity. These results demonstrate that resistance to NO-mediated apoptosis is strictly related to the level and integrity of Cu,Zn-SOD and that the balance between reactive nitrogen and reactive oxygen species regulates neuroblastoma apoptosis.

Apoptosis has first been recognized during organ development, as part of a natural process aimed at removing superfluous or used up cells. Crucial roles of apoptosis have thus been extended to morphogenesis, tissue homeostasis, immune regulation, and the elimination of infected, mutated, or damaged cells (1–3). In this context, the brain represents one of the most intriguing systems since apoptotic cell death is involved both in organ remodeling during development and also in the most widespread neurodegenerative diseases (4).

Programmed cell death proceeds through a well established program consisting of initiation and execution phases involving intrinsic, evolutionarily highly conserved molecular components (5–7). On the contrary, the triggering signals and the metabolic requirements necessary for the initiation step may vary in diverse cell types. NO, a potentially oxidant radical, may be considered unique among the regulators of apoptosis since it can function either as a pro- or an antiapoptotic agent (8). NO is physiologically produced by NO synthases in various cellular types including endothelial cells and neurons, and it acts as a pleiotropic messenger molecule regulating blood flow and cellular signaling (9, 10). It is also a component of the nonspecific host defense against tumor cells and pathogens (11, 12). However, many studies suggested that NO production is associated with several neuropathological processes (13). The molecular mechanisms underlying NO-mediated toxicity are not yet clearly understood, and it is suggested that they differ from those involved in its physiological actions and may involve multiple pathways depending on the cell type and on the mode of cell death, i.e. necrosis versus apoptosis.

NO-mediated apoptosis involves the same interplay between the regulatory proteins that seems to control the execution of mammalian apoptosis (14). Among these, the protein product of bcl-2 protooncogene plays a pivotal role in apoptosis by regulating cytochrome c efflux from mitochondria, which in turn activates cysteine proteases (caspases) ultimately leading to specific DNA fragmentation. Constitutive expression of high Bcl-2 protein levels by transfection experiments has proven that Bcl-2 or related family members can protect from NO-mediated apoptosis (15, 16). The tumor suppressor gene product p53 is able to modulate apoptosis in DNA-damaged cells (17), through a mechanism scarcely understood, and it has been suggested to play a functional role during NO-induced apoptosis (18, 19).

NO-mediated toxicity may rely on secondary products derived from a large variety of nonenzymatic reactions between NO and cellular components such as thiols, metals, or superoxide (O2·−). Several studies suggest that the proapoptotic effect of NO is a result of the formation of peroxynitrite from the nearby diffusion-controlled reaction between NO and O2·− (20–22). In neurons the target of peroxynitrite has been identified...
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only partially; possible cytotoxic mechanisms involve DNA and mitochondrial damage (13). However, in cell systems such as mesangial cells or macrophages, it was demonstrated that whereas the balanced and simultaneous generation of both radicals is non-destructive, the unopposed generation of either NO or O2 induces apoptosis and, at high concentrations, necrotic cell death (23). From these observations it is possible to hypothesize that the milieu of intracellular antioxidants may modulate NO toxicity.

The enzyme superoxide dismutase (SOD) may play a fundamental role in modulating NO toxicity, since it acts as an antioxidant, dismutating O2−. As matter of fact, cells producing an increased amount of SOD were resistant to NO-mediated toxicity (24). In contrast, increase of O2 release by down-regulation of Cu,Zn-SOD led to NO-mediated apoptotic cell death (25). Furthermore, it has been demonstrated that Cu,Zn-SODs carrying mutations associated with the familial form of amyotrophic lateral sclerosis (FALS) are pro-apoptotic agents, although they retain enzymatic SOD activity (26).

In an attempt to understand the role of Cu,Zn-SOD as a determinant of NO-mediated toxicity in neuronal cells, we studied the susceptibility of a human neuroblastoma cell line (SH-SY5Y) to NO donors in comparison with the same cells transfected with a fully active mutant or with a wild type Cu,Zn-SOD. In particular, we study the molecular mechanisms involved in GSNO-mediated apoptosis, since it has been demonstrated recently that it can be physiologically produced from commercially available pure NO-releasing agents (27). The results obtained showed that GSNO-mediated apoptosis in these cells is associated with a canonical sequence of events including Bcl-2, p53, and p21 modulation, cytochrome c release in the cytosol, and caspase 3 activation. Cells carrying wild type SOD are protected from NO-mediated apoptosis, whereas cells transfected with the mutant SOD are more susceptible.

Our results point to the antioxidant activity of Cu,Zn-SOD as a regulator of NO-mediated apoptosis in neuronal cells; however, the gain of other functions due to mutations overwhelms beneficial effect amplifying NO toxicity. The overall results provide an explanatory mechanism for the etiology of neurodegenerative diseases showing apoptosis and in particular of amyotrophic lateral sclerosis.

EXPERIMENTAL PROCEDURES

Materials

Puromycin (3’-[(a-amino-p-methoxydiphenylamino)-3’-deoxy-N, N-dimethiladenosine) dihydrochloride, DCI- (N-pentyl-l-2-amino-3-mercaptop-3-methylbutanoic acid hydrochloride), Hepes, CHAPS, PIPES, MeSO, dimethyl sulfoxide), b-mannitol, EGTA, protease inhibitor mixture, dithiothreitol (DTT), sulfinamide (N-paminobenzensulfonamide), phenylmethylsulfonyl fluoride, N-(1-naphtyl)ethylenediamine dihydrochloride, bathocuproinedisulfonic acid, monoclonal anti-Bcl-2 clone Bcl-2, monoclonal anti-p53 clone BP53-12, and cytochrome c (horse heart) were obtained from Sigma. Ac-DEVD-AMC, caspase 3 inhibitor I (Ac-DEVD-CHO), Hoechst 3342, diethylenetriamine NONOate (NONO) and NOR-4 were purchased from Calbiochem. Anti-cytochrome c monoclonal antibody (clone 7H8.2c12) was from Pharmingen (San Diego, CA). Anti-p21 (C-29) G polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-mouse IgG (H + L) horseradish peroxidase conjugate was from Bio-Rad. Supersignal substrate chemiluminescence reagent was from Pierce. 2’,7’-Dichlorodihydrofluorescein diacetate (DCF-DA) was from Molecular Probes Inc. (Eugene, OR). Reduced and oxidized glutathione were from Roche Molecular Biochemicals. All other chemicals were obtained from Merck.

Cell Culture

Human neuroblastoma cells SH-SY5Y were purchased from the European Collection of Cell Culture and grown in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum, at 37 °C in an atmosphere of 5% CO2 in air. Monoclonal cell lines transfected either with human wild type Cu,Zn-SOD (named WT) or with a mutated SOD G93A (named G93A) were obtained as described previously (28). Transfected cells had the same antioxidant pattern, such as of Mn-SOD, as SH-SY5Y cells. Cells were routinely trypsinized and plated at 20 × 107/75-cm2 flasks. Cell viability for all cell lines was assessed by trypan blue exclusion.

Treatments

S-Nitrosoglutathione was synthesized as described previously (29). Briefly, glutathione was dissolved in HCl at 4 °C and treated with an equimolar amount of NaNO2. The mixture was stirred for 40 min and subsequently treated with cold acetone. The precipitate was collected, washed, and dried under vacuum. freshly synthesized GSNO was characterized by UV spectroscopy and sterilized by filtration. GSNO solutions were prepared just before the experiment. Treatments were performed with different amount of GSNO ranging from 0.025 to 1 mM for 48 h at 37 °C in medium supplemented with serum. The effect was dose-dependent between 0.1 and 1 mM (not shown). The concentration of 0.2 mM GSNO was selected for all the experiments since at this concentration the degree of apoptosis was well quantified for the three cell lines and avoided accumulating cells that underwent secondary necrosis in G93A cells. Diethylamine NONOate (NONO) and NOR-4 were used at concentration of 0.5 mM, and due to the rapid half-life of these compounds (16 min for NONO and 60 min for NOR-4) we treated the cells for 15 h. Moreover, analysis of apoptosis at 24 or 48 h did not show a further increase of the extent of apoptosis.

 Puromycin was used at concentration of 5 μM in medium supplemented with serum for 24 h at 37 °C. p-Penicillamine or bathocuproine was used at concentration of 100 μM for 24 h. This concentration was selected because it was not toxic for the cells during the treatment period, as stated by staining with the vital dye trypan blue.

For detection of intracellular ROS, cells were incubated with 50 μM DCF-DA (dissolved in MeSO) for 30 min at 37 °C. In the case of GSNO, cells were treated with GSNO for 15 h at 37 °C and then incubated with DCF-DA for an additional 30 min at 37 °C. After chilling on ice, cells were washed with ice-cold phosphate-buffered saline (PBS, 10 mM phosphate buffer, 2.7 mM KC1, 137 mM NaCl pH 7.4), scraped from the plate, and resuspended in PBS containing 10 mM EDTA. The fluorescence intensities of 2’,7’-dichlorofluorescein formed by the reaction of DCF-DA with ROS of more than 100 viable cells from each sample were analyzed by recording FL-1 fluorescence by a flow cytometer. Prior to data collection, propidium iodide was added to the sample for gating out dead cells. 100 μM tert-butylhydroperoxide treatment was used as a positive control. Experiments were repeated at least four times with similar results. The data are expressed as one representative histogram. The time course of these experiments was shortened at 15 h to avoid GSNO-treated cells from detaching from the plates.

Analysis of Apoptosis

p-Buffered saline was used for nuclei staining and cells were analyzed by flow cytometry. Cells were fixed in 4% paraformaldehyde and stained with 50 μM 7-amino-4-triazololamidine glucouridine (PI) by propidium iodide. The fraction of cells with fragmented nuclei among the total cell population was calculated in the Hoechst 3342-stained cells, counting at least 300 cells in at least 10 random-selected fields as described (30). Attached apoptotic cells were detected with the fluorescence microscope directly on the flasks by analysis of nuclear fragmentation after staining with Hoechst 3342 dye as described (30). Alternatively, attached (after trypsinization) and detached cells were combined, washed in PBS, and stained with 50 μM propidium iodide prior to analysis by a FACSScan instrument (Becton Dickinson, San José, CA).

Preparation of Cell Lysates and Western Blots Analyses

Cytosolic Cytochrome c Determination—Cells (detached and attached) were washed with PBS and collected by centrifugation at 700 × g for 5 min at 4 °C. The cell pellet was resuspended in extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KC1, 5 mM EGTA, 2 mM MgCl2, 1 mM diithiothreitol (DTT), and protease inhibitors (31). After 30 min incubation on ice, cells were homogenized with a glass Dounce. Cell homogenates were spun at 14,000 × g for 15 min at 4 °C, and supernatants were removed and stored at −80 °C, until analysis by gel electrophoresis. 50 μg of cytosolic protein extracts were loaded onto each lane of a 12% SDS-polyacrylamide gel, separated, and then blotted to nitrocellulose membrane.
Cu,Zn-SOD and NO-mediated Neuronal Apoptosis

**Fig. 1. Apoptosis in SH-SY5Y cells and its dependence on intracellular Cu,Zn-SOD.** A, typical cell cycle plot of SH-SY5Y cells before and after GSNO treatment. The sub-G1 region is integrated by cells undergoing apoptosis. Cells were treated with 0.2 mM GSNO (B), 0.5 mM NO-4 or 0.5 mM NONO (C), 5 μg/ml puromycin (D) for 48, 15, and 24 h, respectively. Attached and detached cells were combined and treated with propidium iodide prior to FACS analysis as described under "Experimental Procedures." B and D, lane 1, SH-SY5Y cells; lane 2, GSNO or puromycin-treated; lane 3, 93A cells; lane 4, GSNO or puromycin-treated; lane 5, WT cells; lane 6, GSNO or puromycin-treated. C, lane 1, SH-SY5Y cells; lane 2, NO-4-treated; lane 3, NONO-treated; lane 4, 93A cells; lane 5, NO-4-treated; lane 6, NONO-treated; lane 7, WT cells; lane 8, NO-4-treated; lane 9, NONO-treated. Data are expressed as means ± S.D., n = 4, for NONO and NONO-4; n = 6 for GSNO and puromycin. * p < 0.01 with respect to SH-SY5Y cells; **, p < 0.001 with respect to SH-SY5Y cells.

**Fig. 2. Cu,Zn-SOD protein levels in cell lines treated with GSNO.** Cu,Zn-SOD immunoreactive protein was detected by Western blotting using a polyclonal antibody, as described under "Experimental Procedures." 50 μg of protein of cell extracts was loaded on each lane. Lane 1, SH-SY5Y cells; lane 2, GSNO-treated; lane 3, 93A cells; lane 4, 93A cells; lane 5, WT cells; lane 6, GSNO-treated; lane 7, purified human Cu,Zn-SOD.

(Bio-Rad). Purified cytochrome c from horse heart was used as standard. Purified mouse anti-cytochrome c monoclonal antibody was used as primary antibody (1:5000). The specific protein complex formed upon appropriate secondary antibody treatment (1:10,000) was identified using the "SuperSignal" substrate chemiluminescence reagent.

**Enzyme Activities and Other Assays**

*Cytochrome c Oxidase Activity*—Cells (attached and detached) were washed and collected by centrifugation. Cell pellets were resuspended in PBS and sonicated. Lysates were centrifuged at 25,000 × g for 20 min at 4 °C. Supernatants were used for SOD activity and protein content assay. Cu,Zn-SOD activity was measured by a polarographic method (32) with an AMEL (Milano, Italy) model 466 polarographic analyzer, in tetraborate buffer at pH 9.6. Under these conditions the activity of Mn-SOD is almost fully inhibited (33). Data were expressed in μg/mg protein with reference to purified human Cu,Zn-SOD. For immunoblot analyses, 50 μg of proteins from supernatants were applied to each lane of a 12% polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane. Cu,Zn-SOD was localized after incubation with a specific antibody as described previously (34).

*Cu,Zn-Superoxide Dismutase Activity*—Cells (attached and detached) were washed and collected by centrifugation. Cell pellets were resuspended in PBS and sonicated. Lysates were centrifuged at 25,000 × g for 20 min at 4 °C. Supernatants were used for SOD activity and protein content assay. Cu,Zn-SOD activity was measured by a polarographic method (32) with an AMEL (Milano, Italy) model 466 polarographic analyzer, in tetraborate buffer at pH 9.6. Under these conditions the activity of Mn-SOD is almost fully inhibited (33). Data were expressed in μg/mg protein with reference to purified human Cu,Zn-SOD. For immunoblot analyses, 50 μg of proteins from supernatants were applied to each lane of a 12% polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane. Cu,Zn-SOD was localized after incubation with a specific antibody as described previously (34).

*Lysozyme Assay*—Lysozyme activity was determined by measuring the reduction in turbidity of 0.1% suspension of Micrococcus luteus in 0.05 M Tris/HCl, pH 7.5, by following the optical density at 540 nm. The enzyme activity was calculated from a standard curve of lysozyme concentrations and is expressed as units per mg of protein.

*Glutathione Assay*—Glutathione was determined by the method of Beutler et al. (39) using the reagent mixture containing NADPH, dithiothreitol, and glutathione reductase. The reaction was initiated by the addition of NADPH and followed by measuring the increase in absorbance at 340 nm. The results are expressed as μM glutathione per mg of protein.

*Cu,Zn-Superoxide Dismutase Activity*—Cells (attached and detached) were washed and collected by centrifugation. Cell pellets were resuspended in PBS and sonicated. Lysates were centrifuged at 25,000 × g for 20 min at 4 °C. Supernatants were used for SOD activity and protein content assay. Cu,Zn-SOD activity was measured by a polarographic method (32) with an AMEL (Milano, Italy) model 466 polarographic analyzer, in tetraborate buffer at pH 9.6. Under these conditions the activity of Mn-SOD is almost fully inhibited (33). Data were expressed in μg/mg protein with reference to purified human Cu,Zn-SOD. For immunoblot analyses, 50 μg of proteins from supernatants were applied to each lane of a 12% polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane. Cu,Zn-SOD was localized after incubation with a specific antibody as described previously (34).

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*Cu,Zn-Superoxide Dismutase Activity*—Cells (attached and detached) were washed and collected by centrifugation. Cell pellets were resuspended in PBS and sonicated. Lysates were centrifuged at 25,000 × g for 20 min at 4 °C. Supernatants were used for SOD activity and protein content assay. Cu,Zn-SOD activity was measured by a polarographic method (32) with an AMEL (Milano, Italy) model 466 polarographic analyzer, in tetraborate buffer at pH 9.6. Under these conditions the activity of Mn-SOD is almost fully inhibited (33). Data were expressed in μg/mg protein with reference to purified human Cu,Zn-SOD. For immunoblot analyses, 50 μg of proteins from supernatants were applied to each lane of a 12% polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane. Cu,Zn-SOD was localized after incubation with a specific antibody as described previously (34).

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*Glutathione Assay*—Glutathione was determined by the method of Beutler et al. (39) using the reagent mixture containing NADPH, dithiothreitol, and glutathione reductase. The reaction was initiated by the addition of NADPH and followed by measuring the increase in absorbance at 340 nm. The results are expressed as μM glutathione per mg of protein.

*Cu,Zn-Superoxide Dismutase Activity*—Cells (attached and detached) were washed and collected by centrifugation. Cell pellets were resuspended in PBS and sonicated. Lysates were centrifuged at 25,000 × g for 20 min at 4 °C. Supernatants were used for SOD activity and protein content assay. Cu,Zn-SOD activity was measured by a polarographic method (32) with an AMEL (Milano, Italy) model 466 polarographic analyzer, in tetraborate buffer at pH 9.6. Under these conditions the activity of Mn-SOD is almost fully inhibited (33). Data were expressed in μg/mg protein with reference to purified human Cu,Zn-SOD. For immunoblot analyses, 50 μg of proteins from supernatants were applied to each lane of a 12% polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane. Cu,Zn-SOD was localized after incubation with a specific antibody as described previously (34).
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Fig. 3. Molecular markers of apoptosis under GSNO treatment. Cells were treated with 0.2 mM GSNO for 48 h. A, cytochrome c released in the cytosol was detected by Western blotting using a monoclonal antibody as described under “Experimental Procedures.” 50 μg of cytosolic protein were loaded on each lane. Lane 1, SH-SY5Y cells; lane 2, GSNO-treated; lane 3, G93A cells; lane 4, GSNO-treated; lane 5, WT cells; lane 6, GSNO-treated; lane 7, purified cytochrome c. B, caspase 3 activity was measured fluorometrically with Ac-DEVD-AMC as substrate. Data are expressed as arbitrary units. I, SH-SY5Y cells; 2, GSNO-treated; 3, G93A cells; 4, GSNO-treated; 5, WT cells; 6, GSNO-treated; **, p < 0.001, n = 6. Immunoreactive p53 protein (C) and immunoreactive p21 protein (D) were measured by Western blotting using monoclonal antibody as detailed under “Experimental Procedures.” 50 μg of protein of cell extracts was applied to each lane. Lane 1, SH-SY5Y cells; lane 2, GSNO-treated; lane 3, G93A cells; lane 4, GSNO-treated; lane 5, WT cells; lane 6, GSNO-treated; last lane shows molecular weight markers.

RESULTS

Modulation of NO-induced Apoptosis by Cu,Zn-SOD—Human neuroblastoma cells SH-SY5Y underwent apoptosis upon treatment with different NO-donors (Fig. 1). Cells began to acquire apoptotic features, such as nuclear fragmentation, while still attached; detachment followed and apoptotic cells were floated in the culture medium before undergoing secondary necrosis. Fig. 1A shows a typical cell cycle plot of SH-SY5Y cells obtained by flow cytometry (propidium iodide staining) after GSNO treatment. Cells in the sub-G1 region were considered apoptotic. The effect of GSNO was dose-dependent, between 0.1 and 1 mM; 0.2 mM GSNO at 48 h were the conditions applied throughout the experiments, unless otherwise specified (see under “Experimental Procedures”). We also tested the response to the treatment with GSNO of SH-SY5Y cells transfected either with a wild type Cu,Zn-SOD (WT) or with a FALS-associated pro-apoptotic mutant Cu,Zn-SOD (G93A). As shown in Fig. 1B, the three cell types tested responded to the apoptogenic stimulus in a different manner. In fact, FACS analysis of treated cells showed that G93A cells were the most susceptible ones (51 ± 7% apoptotic cells), whereas the WT were the most resistant (15 ± 3% apoptotic cells), with an intermediate behavior for the SH-SY5Y cells (34 ± 3%). These results were also confirmed by counting apoptotic cells by optical microscopy. We also tested the susceptibility of SH-SY5Y cells and of transfected cell lines to pure NO donors such as NONO and NOR-4. Fig. 1C shows that the behavior of the cells was equivalent to that observed with GSNO, even though the extent of apoptosis was lower. This was not due to the shorter time applied in these experiments; in fact, we observed no change in the extent of apoptosis between 15 and 24 or 48 h. It is possible that the very fast release of NO from these compounds can be responsible for this result. Neuroblastoma cells were also exposed to puromycin (5 μg/ml) for 24 h. This agent, which inhibits protein synthesis, is a well-established NO-independent apoptotic inducer in many cellular systems (30). Similar results were obtained as for NO-treated cells, in that G93A cells were significantly more affected by the treatment compared with the other two cell types (Fig. 1D). However, cells transfected with normal SOD were much less protected than against NO-mediated apoptosis. On the basis of these results and of the recent knowledge suggesting that GSNO can be produced in cultured cells upon treatment with commercially available pure NO donors, such as NONO and NONO (27), we studied in detail the molecular mechanisms involved in GSNO-mediated apoptosis.

The different extent of apoptosis observed in the three cell lines was neither due to an altered GSNO handling nor to a different Cu,Zn-SOD content. As shown in Fig. 2, the G93A cells obtained by flow cytometry (propidium iodide staining) after GSNO treatment, gave an average value of 45.95 ± 5.17 μM/ml for the three cell lines tested. SOD activity (not shown) and protein levels of WT and G93A (Fig. 2) are comparable and twice as much as that of the SH-SY5Y cells. Furthermore, SOD activity of the three cell lines was unaffected by GSNO treatment (not shown) as well as...
of G93A cells than in SH-SY5Y and WT cells (Fig. 3B). Caspase-3 activation followed the same trend as cytochrome c release, which was highest for G93A cells. Cytochrome c accumulated after GSNO treatment, the release was highest for G93A cells and almost at the limit of detection for WT cells.

Proteolytic activity associated to caspases was measured by testing the cytosolic extracts for their ability to cleave the fluorimetric substrate Ac-DEVD-AMC, which is specific for caspase-3. Caspase-3 activation is important in the cascade of proteolytic events of apoptosis in that it is considered one of the points of no return in the process leading to cell destruction (40). Caspase-3 activation followed the same trend as cytochrome c release, with higher activity in the cytosolic extracts of G93A cells than in SH-SY5Y and WT cells (Fig. 3B).

The tumor suppressor gene p53 is known to be a member of the DNA damage-response pathway. It has been demonstrated that p53 protein increases in response to NO donors and that it efficiently senses NO intoxication at an early stage of cellular damage (14). Fig. 3C shows that the p53 protein was stably expressed in neuroblastoma cells to a varying extent; in particular, WT cells showed a lower p53 protein level with respect to SH-SY5Y cells and G93A cells, which showed the higher level. Upon GSNO treatment, p53 significantly accumulated even in the WT cells, reaching comparable values, and this result indicates that this protein is an upstream sensor of NO toxicity. Furthermore, a monoclonal anti-p53 antibody detected two bands, which likely represent the phosphorylated or dephosphorylated form of the protein. Interestingly, the lower molecular weight form almost disappeared after GSNO treatment, supporting the current opinion that p53 activation involves phosphorylation of the protein. In response to DNA damage, human p53 is phosphorylated within its transactivation domain at serine 15, and this leads to its stabilization (41). In order to confirm further a role for p53 activation in the GSNO-mediated toxicity, we analyze the expression of p21, which is a potent inhibitor of cell cycle kinases also implied in the DNA damage response (42). It has been demonstrated that ectopic expression of p53 alone could induce p21 expression, followed by p21 cleavage and apoptosis (43). Fig. 3D shows that p21 is expressed in SH-SY5Y cells and that the protein accumulated upon GSNO treatment. Furthermore, a cleavage product was observed in the SH-SY5Y cells and at higher extent in the G93A cells. This result provides a 2-fold indication as follows: first it is confirmatory of p53 activation, and second it indicates that G93A (at higher rate) and SH-SY5Y cells are committed to apoptosis.

Bcl-2 protein modulation is responsible for cell survival or suicide; constitutive expression of high Bcl-2 protein levels by transfection experiments has proven that Bcl-2 or related family members can protect cells from NO-mediated apoptosis (44, 45). In our experiments, as evidenced by Western blot analysis, untreated SH-SY5Y and WT cells express high levels of Bcl-2 protein, whereas G93A cells show a much lower content (Fig. 4). 48 h of GSNO treatment decreased Bcl-2 expression almost to a different extent; in particular, Bcl-2 content of G93A cells was at the limit of detection.

Markers of Oxidative Stress in GSNO-induced Apoptosis—
The data reported in Figs. 1 and 2, as well as previous reports (26, 46), clearly show that the increased susceptibility to apoptosis of G93A cells cannot be explained in terms of SOD level. However, we found evidence for different levels of ROS in the three cell lines used during the GSNO treatment. Cells were loaded with the peroxide- and hydroxyl radical-sensitive fluorophore DCF-DA and assayed by FACS analysis. Fig. 5 shows that G93A cells had markedly higher levels of DCF fluorescence than SH-SY5Y cells and WT cells, the latter one having the lowest level. Furthermore, this trend was more pronounced upon 15 h of treatment of the cells with GSNO (Fig. 5).

Evidence for increased peroxidase activity or hydroxyl radical formation has been demonstrated for fully active SOD mutants associated with FALS (47). Therefore, we treated the cells with copper chelators to test whether such a pro-oxidant effect may be associated with looser copper coordination by such mutants, still compatible with catalytically adequate redox cycling. The addition of deferoxamine or bathocuproine (100 μM), in the culture medium of G93A cells, significantly inhibited cell apoptosis induced by GSNO (Fig. 6). However, in our
Cells were treated with 100 μM penicillamine or bathocuproine 1 h prior to and throughout GSNO treatment (24 h). Cells were scored for apoptosis by FACS analysis as described under "Experimental Procedures." Lane 1, SH-SY5Y cells; lane 2, GSNO-treated; lane 3, penicillamine-treated; lane 4, bathocuproine-treated; lane 5, G93A cells; lane 6, GSNO-treated; lane 7, penicillamine-treated; lane 8, bathocuproine-treated; lane 9, WT, GSNO-treated; lane 10, GSNO-treated; lane 11, penicillamine-treated; lane 12, bathocuproine-treated. Data are expressed as means ± S.D., n = 6, for GSNO and penicillamine; n = 4 for bathocuproine. *, p < 0.01 with respect to GSNO-treated; **, p < 0.001 with respect to GSNO-treated. □, SH-SY5Y; ■, G93A; □, WT.

**FIG. 6. Effect of copper chelators on GSNO-induced apoptosis.** Cells were treated with 100 μM penicillamine or bathocuproine 1 h prior to and throughout GSNO treatment (24 h). Cells were scored for apoptosis by FACS analysis as described under "Experimental Procedures." Lane 1, SH-SY5Y cells; lane 2, GSNO-treated; lane 3, penicillamine-treated; lane 4, bathocuproine-treated; lane 5, G93A cells; lane 6, GSNO-treated; lane 7, penicillamine-treated; lane 8, bathocuproine-treated; lane 9, WT, GSNO-treated; lane 10, GSNO-treated; lane 11, penicillamine-treated; lane 12, bathocuproine-treated. Data are expressed as means ± S.D., n = 6, for GSNO and penicillamine; n = 4 for bathocuproine. *, p < 0.01 with respect to GSNO-treated; **, p < 0.001 with respect to GSNO-treated. □, SH-SY5Y; ■, G93A; □, WT.

An increased flux of ROS may lead to increased peroxynitrite levels. This molecule exerts cytotoxicity via its reaction with various molecular targets as follows: it can nitrate or hydroxylate protein residues (48); it can oxidize thiols (49); and it can inhibit components of the mitochondrial transport chain (50). In the absence of direct specific methods for peroxynitrite measurement, we studied its potential molecular targets. Glutathione, the most abundant low molecular weight thiol, was not significantly affected either by the transfection with the mutant SOD or by the GSNO treatment. Fig. 7A shows reduced and oxidized glutathione contents of cells after GSNO treatment that were not significantly different from untreated cells. Among the above-mentioned molecular target of NO toxicity, the hemoprotein cytochrome c oxidase appears to be the only one strongly affected by GSNO insult and protected by native Cu,Zn-SOD (Fig. 7B). In fact, its activity was significantly decreased in SH-SY5Y and G93A cells, whereas no change was observed for WT cells.

**FIG. 7. Molecular markers of oxidative stress upon GSNO treatment. A, levels of reduced (odd numbers) or oxidized (even numbers) glutathione. Lanes 1 and 2, SH-SY5Y cells; lanes 3 and 4, G93A cells; lanes 5 and 6, WT cells. Glutathione was determined by high pressure liquid chromatography as described under "Experimental Procedures." B, activity of cytochrome c oxidase. Spectrophotometric determination of cytochrome c oxidase activity was performed as described under "Experimental Procedures." Lane 1, SH-SY5Y cells; lane 2, GSNO-treated; lane 3, G93A cells; lane 4, GSNO-treated; lane 5, WT cells; lane 6, GSNO-treated. Data are expressed as means ± S. D. *, p < 0.05 with respect to untreated; **, p < 0.01 with respect to untreated; n = 6. □, SH-SY5Y; ■, G93A; □, WT.

**mutant (G93A), as active as the wild type, were more susceptible to NO toxicity. Therefore, we suggest that O$_2^-$ removal is not sufficient to protect from NO-mediated apoptosis and that additional properties of the G93A mutant SOD potentiate apoptogenic stimuli of NO.

Our study demonstrated that apoptosis was the mode of cell death of SH-SY5Y cells upon NO insult, independently of the NO donor used. In fact, it has recently been demonstrated that GSNO can be physiologically produced in rat brain slices and that in cultured cells it is formed following treatment with commercially available NO donors (27). The reaction between GSH and NO has been suggested to be catalyzed by ceruloplasmin, a multicopper oxidase, involved in iron metabolism. This protein is present in plasma as a glycoprotein and is bound to plasma membrane in astrocytes, where it functions as an important antioxidant agent (53).

In our model, apoptosis was characterized by an induction of p53 protein, which is able to induce either growth arrest or apoptosis in DNA-damaged cells (17). Induction of a G1 arrest is dependent upon sequence-specific DNA binding and transcriptional activation of p53 target genes such as p21, which is an inhibitor of cyclin-dependent kinases and thereby blocks cell cycle progression (42). p53 increase was an early response to GSNO treatment; in fact, whereas a small percentage of WT

**DISCUSSION**

Apoptosis elicited by endogenous or exogenously produced NO may play a role in brain pathological conditions, including brain ischemia and neurodegeneration (13, 14). Human neuroblastoma cells in culture respond to NO challenge by altering intrinsic machinery that ultimately leads to apoptotic cell death (51, 52). NO toxicity may be strengthened by O$_2^-$ via formation of peroxynitrite, and SOD that catalytically removes O$_2^-$ is therefore indicated as a protecting agent. In the present report, we succeeded in creating a model of neuroblastoma cells capable of surviving NO treatment by transfecting SH-SY5Y cells with Cu,Zn-SOD. However, cells transfected with a SOD
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In vitro with peroxynitrite to catalyze the nitration of phenolic group to severe forms of FALS (47). In addition Cu,Zn-SOD can react with peroxidase activity derived radical, is also dependent on the activity of Cu,Zn-SOD. This indicates that Cu,Zn-SOD has a causative role for Bcl-2 in the increased susceptibility observed in G93A cells. In fact, the extent of apoptosis, observed under physiological conditions, is similar to that observed in the WT cells and lower with respect to that of SH-SY5Y cells.

A specific marker of NO toxicity was the decrease of cytochrome c oxidase activity, which paralleled the extent of apoptosis. This is likely to generate impairment of mitochondrial function, which in turn may potentiate apoptotic commitment.

An alternative model for protein export from mitochondria depends on Bax and/or Bid, two members of the Bcl-2 protein family (57). It involves the translocation of Bid or Bax from cytosol to mitochondria, where they may possibly form a pore, as shown for the related protein Bcl-XL (58). In our experiments Bcl-2 was down-regulated by the GSNO treatment in SH-SY5Y cells, whereas the resistance of WT cells to apoptosis was associated with a higher level of this protein in the treated cells. In this connection, it has to be pointed out that G93A cells showed the lowest basal concentration of this protein. Since they were the most prone to NO-induced apoptosis, we propose that anti-apoptotic effects of Bcl-2 be, at least in part, due to its antioxidant activity (59). Although the mechanism of this putative function is not known, it is established that the cytosolic steady-state of reactive oxygen species, which are potentially threatening species and modulators of physiological signal transduction (60), is increased when Bcl-2 is down-regulated.

The concentration of $O_2^-$, which is the primary dioxygen-derived radical, is also dependent on the activity of Cu,Zn-SOD. Recently, it has been demonstrated that this enzyme also possesses peroxidase activity in vitro (61) and that this activity is increased in fully active mutants like G93A that are associated to severe forms of FALS (47). In addition Cu,Zn-SOD can react with peroxynitrite to catalyze the nitration of phenolic group in vitro (62) and can buffer copper toxicity in yeast (63, 64). These alternative activities make the enzyme a key factor in putative mechanisms of cell damage related to NO and/or $O_2^-$ and/or redox metal ions.

The results described in the present report suggest that native Cu,Zn-SOD actually represents a factor protecting neurons from NO-induced apoptosis, whereas the G93A SOD mutant, despite high dismutating activity, increased intracellular flux of ROS. In fact, G93A cells treated with DCF-DA showed significant increase in fluorescence intensity as compared with both WT and SH-SY5Y cells. The DCF-DA molecule has been extensively utilized to demonstrate intracellular production of ROS. However, it has recently been suggested from in vitro studies that DCF-DA can be also oxidized by some peroxidase activity (65). Consequently, in our model the increase in the fluorescent dye can be also due to increased peroxidase activity of the mutant enzyme.

Protection exerted by copper chelation may also indicate a role for aberrant copper chemistry of the mutant enzyme both in ROS production and in NO-increased apoptosis in G93A cells, as already shown for another Cu,Zn-SOD mutant (H46R) (66). It has to be pointed out that the protection from GSNO-induced apoptosis exerted by copper chelators accounts for the increased susceptibility observed in G93A cells. In fact, the extent of apoptosis, observed under physiological conditions, is similar to that obtained with the WT cells and lower with respect to that of SH-SY5Y cells. It seems that G93A cells behave as WT when copper is properly chelated.

In conclusion, a higher level of Cu,Zn-SOD activity that leads to protection of human neuroblastoma cells against GSNO-dependent apoptosis can be damage-mediator in the same cells when other activities associated with this enzyme become operative. A vicious circle, including down-regulation of Bcl-2 and deviating redox activity of copper in the G93A mutant, may be the amplification factor leading to ROS unbalance in G93A cells and may explain how and why an ALS mutant cause a pro-apoptotic response. This means that dysoxidation of enzymatic superoxide/peroxo handling is actually a key factor in NO toxicity and suggests that a proper balance between reactive nitrogen and oxygen species controls the apoptogenic activity of endogenously generated radicals in neurodegenerative disease.

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