Downregulation of Cu/Zn Superoxide Dismutase Leads to Cell Death via the Nitric Oxide–Peroxynitrite Pathway

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We previously showed that the downregulation of Cu/Zn superoxide dismutase (SOD1) activity in PC12 cells by exposure to an appropriate antisense oligonucleotide causes their apoptotic death. In this report, we used this model to examine the pathways by which SOD1 downregulation leads to death and to compare these pathways with those responsible for death caused by withdrawal of trophic support. To improve delivery of the SOD1 antisense oligonucleotide, we coupled it to a carrier "vector" peptide homologous to the third helix of the *Drosophila* Antennapedia homeodomain. This caused not only efficient cellular uptake even in the presence of serum, but also inhibition of SOD1 activity and promotion of apoptosis at 100-fold lower concentrations of oligonucleotide. Death induced by SOD1 downregulation appeared to require the reaction of superoxide with nitric oxide (NO) to form peroxynitrite. In support of this, inhibitors of NO synthase, the enzyme responsible for NO synthesis, blocked death in our experiments, whereas NO generators and donors accelerated cell death. *N*-Acetylcysteine and chlorophenylthiol cAMP, which rescue PC12 cells and neurons from the withdrawal of nerve growth factor and other forms of trophic support, did not protect PC12 cells from SOD1 downregulation. In contrast, overexpression of *bcl-2*, which also rescues these cells from loss of trophic support, was equally effective in saving the cells in the SOD1 downregulation paradigm. Taken together with past findings, such observations suggest that SOD1 downregulation and withdrawal of trophic support trigger apoptosis via distinct initial mechanisms but may utilize a common final pathway to bring about death. Our findings may be relevant to the causes and potential amelioration of neuronal degenerative disorders caused by impaired regulation of cellular levels of NO and superoxide.

Key words: Cu/Zn superoxide dismutase (SOD1); free radicals; neuronal cells; PC12 cells; antisense oligonucleotides; superoxide; peroxynitrite; NO; apoptotic death

Free radicals represent a class of biologically generated species that pose a potential threat to neuronal survival. Cu/Zn superoxide dismutase (SOD1) is among the key cellular enzymes by which neurons and other cells detoxify free radicals and protect themselves from damage (McCord and Fridovich, 1969; Fridovich, 1986). The observation that a subset of cases of familial amyotrophic lateral sclerosis (FALS) is associated with mis-sense mutations of SOD1 has provided the first molecular basis for involvement of this enzyme in neuronal degenerative disorders (Rosen et al., 1993).

One way to study the role of SOD1 in neuronal maintenance is to assess the effects of reducing its activity in living cells. This was achieved in a past study by treating PC12 rat pheochromocytoma cells with SOD1 antisense oligonucleotides under conditions that led to a 50–60% decrease in SOD1 activity. This caused death, via an apoptotic mechanism, of ~50% of the cell population within 24 hr (Troy and Shelanski, 1994). Similar results were achieved by Rothstein et al. (1994) with motor neurons in explant cultures from spinal cord. In both cases, antioxidants such as vitamin E prevented the death, which is consistent with an action of free radicals.

The present study addresses two issues. The first regards the molecular pathway by which SOD1 downregulation and consequent increases in superoxide lead to apoptotic death. Two such pathways have been suggested (for review, see Deby and Goutier, 1990; Halliwell and Gutteridge, 1990; Olanow, 1993; Brown, 1995; Rowland, 1995). One involves superoxide purely as a reducing agent for transition metal ions such as Fe^{3+}. In this scheme, the reduced metal ion catalyzes the conversion of hydrogen peroxide to the highly reactive and destructive hydroxyl radical (Halliwell and Gutteridge, 1990). The other pathway invokes the interaction of superoxide with nitric oxide (NO), leading to formation of peroxynitrite. Peroxynitrite then can be protonated and rapidly decomposed to a strong oxidant (Beckman et al., 1990). We present experiments here designed to distinguish whether either or both of these pathways are involved in apoptotic death caused by SOD1 downregulation.

A second issue addressed here pertains to the relationship, if any, between the mechanisms by which SOD1 downregulation and trophic factor withdrawal lead to apoptosis. Our initial study suggested that the two causes of death are initially divergent, but use a common final pathway to bring about death (Troy and Shelanski, 1994). The present work provides further data to support this point of view.

To carry out our studies, we have chosen the PC12 cell line as

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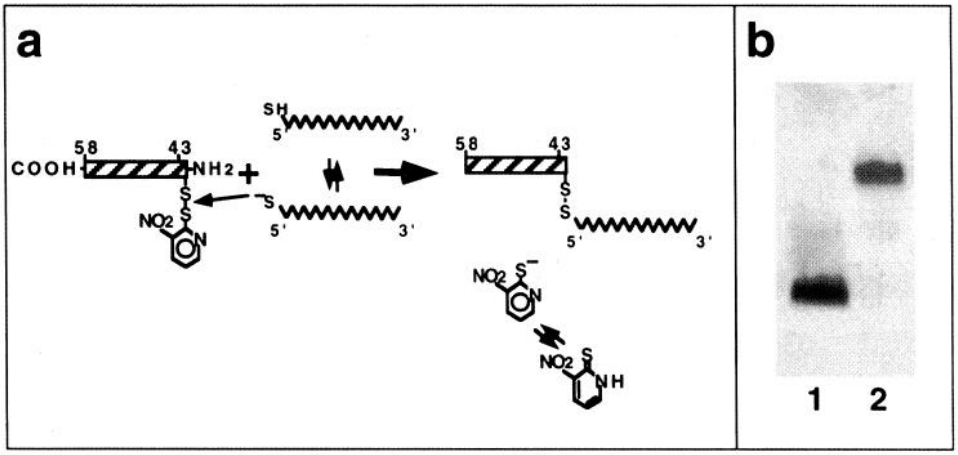


Figure 1. Coupling of Antennapedia peptide to ASOD1. *A*, Schematic illustration of coupling of Antennapedia peptide (vector peptide) with antisense oligonucleotide. *B*, PAGE of coupled and free vector peptide. Equimolar concentrations of oligonucleotide and peptide were incubated in deionized water for 1 hr at 37°C. An aliquot of the free peptide (*lane 1*) or coupled peptide (*lane 2*) was analyzed by 20% SDS-PAGE.

a model system. This line has become widely used as a model for neuronal cell survival and death, and responds both to downregulation of SOD1 (Troy and Shelanski, 1994) and to withdrawal of trophic factor support (Batistatou and Greene, 1991, 1993; Rukenstein et al., 1991). To facilitate our investigations further, we have exploited a recently developed, highly efficient method to deliver antisense oligonucleotides.

MATERIALS AND METHODS

Peptide synthesis. Peptide synthesis of $pAntp_{43-58}$ was carried out as described by Derossi et al. (1994). Boc-Cys-(NPyS)OH was coupled directly to the peptide as the last (N-terminal) amino acid.

Coupling reaction. Oligonucleotides bearing an SH group at their 5′ end and an NH group at their 3′ end were purchased from Appligene (Nice, France). Oligonucleotides were resuspended in deionized water, an equimolar ratio of $NPyS\text{-}pAntp_{43-58}$ peptide was added, and the mixture was incubated at 37°C for 1 hr. The yield of the reaction, estimated by SDS-PAGE followed by Coomassie blue staining, was routinely >50%.

Cell culture. PC12 cells were grown as previously described (Greene and Tischler, 1976) on rat-tail collagen-coated dishes in RPMI 1640 medium containing 5% fetal calf serum (FCS) and 10% heat-inactivated horse serum (complete media). Nerve growth factor (NGF)-primed cells were grown for at least 7 d in RPMI with 10% heat-inactivated horse serum containing NGF (100 ng/ml). For incubation with unmodified oligonucleotides, cells were washed three times with serum-free RPMI 1640 and then plated on fresh collagen-coated dishes in RPMI 1640 supplemented with insulin (3 μM) (Troy et al., 1992; Troy and Shelanski, 1994). For incubation with Antennapedia peptide-linked oligonucleotides, cells were replated in complete media or were treated as for unmodified oligonucleotides.

Oligonucleotide internalization and visualization. Fluorescent ASOD1 oligonucleotide coupled to $pAntp_{43-58}$ peptide (penetratin 1) was purchased from Appligene. Oligonucleotide was uncoupled by preincubation with 100 mM dithiothreitol (DTT) for 15 min at 37°C. The coupled oligonucleotide or the uncoupled oligonucleotide was diluted in complete media, and the volume added to cultured cells was equal to the volume present in the culture dish. After 2 hr, the cells were washed three times with complete media, fixed for 5 min at −20°C in ethanol/CH_3COOH (95/5), dried, and mounted in Moviol before examination.

Confocal microscopy. Data were obtained with a confocal scanning laser microscope (Sarastro 2000, Molecular Dynamics, Sunnyvale, CA). Excitation was obtained with an argon laser set at 488 nm for fluorescein isothiocyanate (FITC), and the emitted light was filtered with an appropriate long-pass filter (510 nm). The sections shown were taken approximately at mid-height level of the cells. Photomultiplier gain and laser power were identical within each experiment.

Cell viability. Cells were grown in 24-well dishes and lysed in 200 μl of a solution that lyses the cell membrane but leaves the nuclei intact (Soto and Sonnenschein, 1985). The nuclei were counted in a hemocytometer.

SOD specific activity. Cells were extracted with 0.5% Nonidet NP-40, and protein was measured by the Bradford method (Bradford, 1976). SOD1 levels were determined as described previously (Troy and Shelanski, 1994), with a modification of the xanthine–xanthine oxidase system (Beauchamp and Fridovich, 1971), measuring the reduction of nitroblue tetrazolium (NBT) at 560 nm in the presence and absence of KCN (Elroy-Stein et al., 1986). Briefly, cell extracts or SOD (Sigma, St. Louis, MO) were incubated in 50 mM sodium carbonate buffer at pH 10.2 containing 0.1 mM EDTA, 1×10^{-4} M xanthine, 1 mM KCN, 2.5×10^{-5} M NBT, and 2.2×10^{-9} M xanthine oxidase in a volume of 1 ml. Reduction of NBT was measured at 560 nm. SOD1 activity was determined from an SOD standard curve and is reported as the KCN-sensitive activity.

RESULTS

Downregulation of SOD1 by peptide-linked antisense oligonucleotides

In a previous study, we showed that exposure of PC12 cells to unmodified SOD1 antisense oligonucleotides leads to loss of SOD1 activity and apoptotic death (Troy and Shelanski, 1994). However, this approach requires a relatively high level of oligonucleotide, frequent readditions, and the use of serum free medium. To extend our observations, we have used a more efficient method to deliver oligonucleotides to cultured cells. The Antennapedia homeodomain protein translocates across biological membranes (Joliot et al., 1991), and directed mutagenesis has defined a 16-amino-acid peptide of the third helix (amino acids 43–58), defined as the vector peptide, that is responsible for the translocation (Le Roux et al., 1993; Derossi et al., 1994). This smaller sequence does not bind to cognate sequences in potential target promoters, thus avoiding other possible activities inherent in the 60-amino-acid homeodomain protein (Derossi et al., 1994; Allinquant et al., 1995). To facilitate cellular uptake of the SOD1 antisense oligonucleotide (designated ASOD1), it was linked to the vector peptide by a disulfide bond (Fig. 1). As illustrated in Figure 2, the peptide-linked oligonucleotide (V-ASOD1) is taken up by PC12 cells grown in serum-containing medium and localizes within the nucleus within 2 hr. Uptake is seen in all cells examined. Under the same conditions, there is no apparent uptake of uncoupled ASOD1. It is likely that once V-ASOD1 is taken up by the cell, the disulfide bond is reduced to release free ASOD1. In support of this, Figure 2 shows an experiment in which incubation of V-ASOD1 with the reducing agent (DTT) causes loss of uptake of the oligonucleotide in serum-containing media. Uncoupling of the vector and oligonucleotide was confirmed by SDS-PAGE (data not shown).

We next compared the relative potencies of ASOD1 and V-ASOD1 in promoting PC12 cell death and depressing SOD1 activity. Figure 3*A* shows that 50% of the cells die within 24 hr of

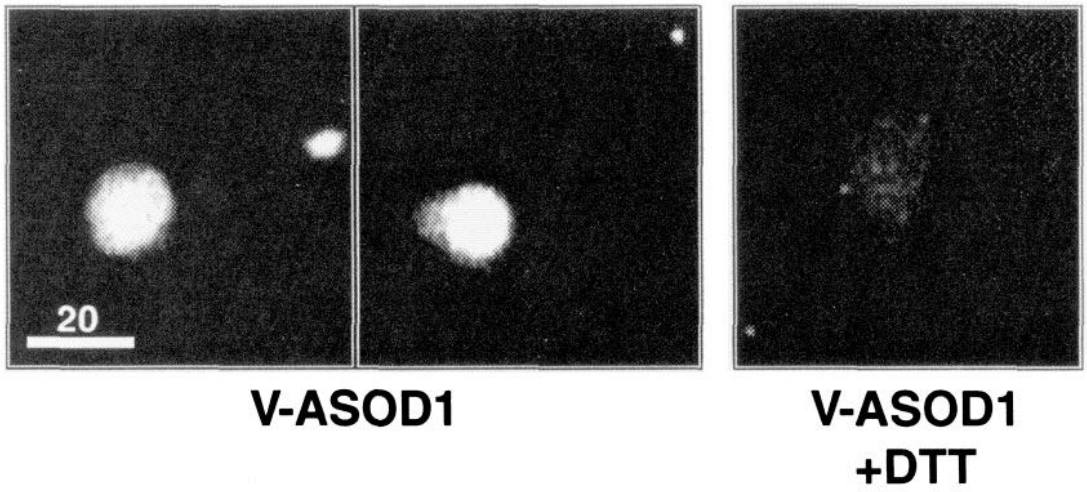


Figure 2. Nuclear accumulation of ASOD1 in serum-containing media requires coupling to Antennapedia peptide. Analysis by confocal microscopy of internalization of FITC-labeled ASOD1 (100 nM) coupled (*left* and *middle*) or uncoupled (*right*) to the vector peptide. Incubation with the antisense was for 2 hr in RPMI, 5% FCS, and 10% horse serum. Uncoupling was achieved by preincubating V-ASOD1 with 100 mM DTT for 15 min at 37°C.

exposure to ASOD1 and V-ASOD1 concentrations of 4 μM and 50 nM, respectively. In contrast, incubation with vector alone or with the vector linked to the sense sequence of ASOD1 did not alter cell viability. Our previous work (Troy and Shelanski, 1994) showed that the action of ASOD1 is sequence-specific with neither a scrambled oligonucleotide nor a sense oligonucleotide affecting either SOD1 enzymatic activity or the intensity of cellular SOD1 staining. Figure 3*B* shows the results of an experiment in which PC12 cells were exposed to 4 μM ASOD1 and 50 nM V-ASOD1 for various times up to 6 hr (time period before cell death was evident) and then evaluated for SOD1 activity. At these concentrations, there was a similar rate of loss of activity down to a level of about one-third that in control cells. Thus, V-ASOD1 effectively decreases SOD1 activity, but does so at concentrations approximately two orders of magnitude lower than ASOD1. V-ASOD1 was equally efficacious in serum-containing medium (data not shown) and in serum-free medium supplemented with insulin (Fig. 3).

NO synthase (NOS) inhibitors protect cells from V-ASOD1-induced death

Recent findings indicate that PC12 cells contain low, but detectable, levels of noninducible NOS (Peunova and Enikolopov, 1995). One potential mechanism by which superoxides may lead to death is by interaction with cellular NO to form peroxynitrite (Beckman et al., 1990; Oury et al., 1993). To test this possibility, PC12 cells were incubated with V-ASOD1 in the presence of NOS inhibitors and cell viability was evaluated. Figure 4*A* shows that *N*-nitro-L-arginine methyl ester (L-NAME) completely protected the cells from death at a concentration of 10 μM. This protective effect was eliminated in the presence of excess L-arginine (100 μM) (data not shown). Similar results were found with PC12 cells that had been neuronally differentiated (primed) by NGF. However, in this case full protection required 30 μM L-NAME (Fig. 4*B*). This difference in L-NAME sensitivity may reflect the higher levels of NOS in primed cells (Peunova and Enikilopov, 1995). Some toxicity of L-NAME became apparent in both control and V-ASOD1-treated cultures at 20 μM for naive cells and at 100 μM for the primed cells. However, in both instances, L-NAME maintains viability equal to that seen in control cells without V-ASOD1 treatment.

We also evaluated responses to two additional NOS inhibitors, *N*-monomethyl-L-arginine (MMA) and *N*-nitro-L-arginine (NA). Figure 4, *C* and *D*, shows that MMA (30–100 μM) and NA (100 μM) fully prevent death caused by exposure to V-ASOD1.

NO generators potentiate and accelerate cell death

If the formation of peroxynitrite from superoxide and NO is a major pathway by which V-ASOD1 induces cell death, then increasing the available NO should alter the rate and/or extent of this process. The data in Figure 5*A* show that the NO generators *S*-nitroso penicillamine (SNAP) and sodium nitroprusside (SNP) both increase cell death achieved in the presence of V-ASOD1. This was observed for primed and for naive cultures, although primed cells showed greater sensitivity and fewer surviving cells. The NO generators increase the rate of V-ASOD1-induced cell death when cell survival at 5–6 and 24 hr of treatment in the presence and absence of SNP, SNAP, and the NO adduct DETA-NO (all at 100 μM) is compared. At 5–6 hr there was no loss of viability in the presence of V-ASOD1 alone, whereas the generators plus V-ASOD1 resulted in significant losses. Only by 24 hr did viability in the cultures treated with V-ASOD1 alone decrease to the levels in cultures treated with the combination of antisense construct plus NO generator. These data indicate that NO generators increase both the rate and extent of cell death induced by downregulation of SOD1.

Free radical scavengers/transition metal chelators inhibit V-ASOD1-induced death

We next examined the capacities of several iron chelators/free radical scavengers for their effect on V-ASOD1-induced death (Fig. 6). Desferrioxamine, an iron chelator that also scavenges free radicals, including peroxynitrite (Beckman et al., 1990), protected the cells completely at a concentration of 10 μM (Fig. 6*A*). Mimosine, another iron chelator that scavenges free radicals and that has antimitotic activity (Lalande, 1990), provided complete rescue at doses of 10 and 100 μM. However, at 400 μM this drug was without protective effect (Fig. 6*B*). A third iron chelator, diethylenetriaminepentaacetic acid (DTPA), gave complete protection from death at 10 and 30 μM, but not at 100 μM (Fig. 6*C*).

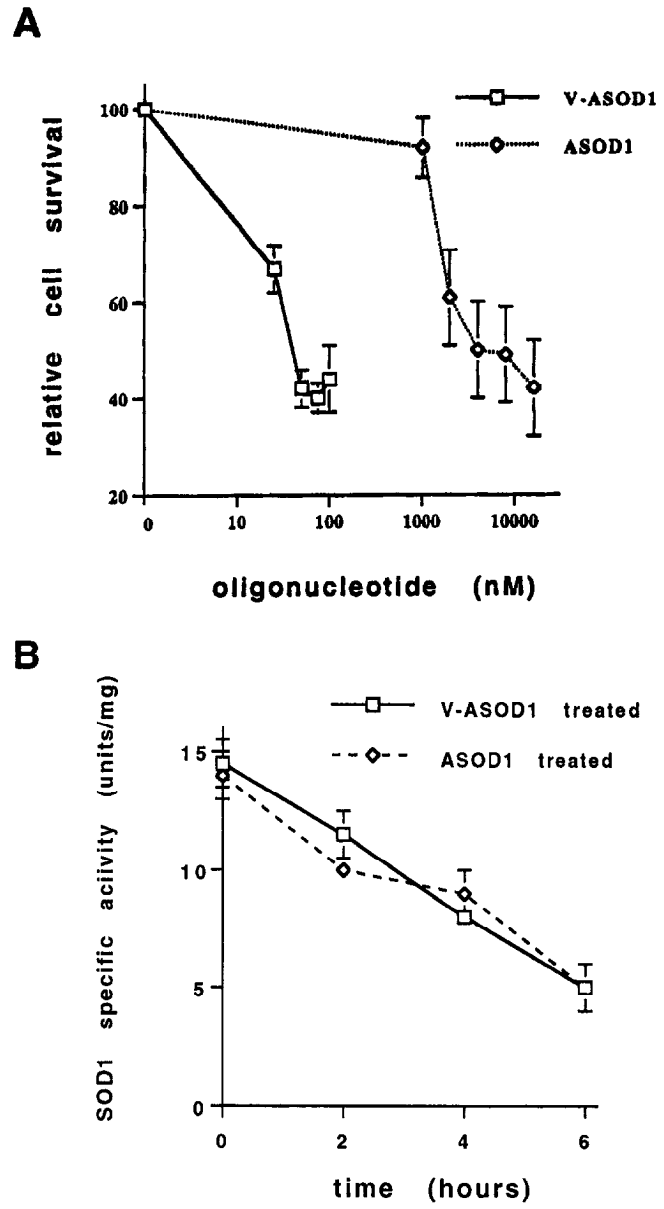


Figure 3. Antennapedia peptide-linked ASOD1 is more efficient than unmodified ASOD1. *A*, Dose-response with V-ASOD1 or ASOD1. Naive PC12 cells were washed and plated in serum free RPMI 1640 supplemented with 3 μM insulin and then incubated with indicated concentration of the different oligonucleotides. Cells were lysed at 24 hr treatment, and nuclei were counted. The number of surviving cells is expressed relative to the number present without oligonucleotide (designated as 100). *B*, SOD1 specific activity over 6 hr with V-ASOD1 or ASOD1. Naive PC12 cells were incubated with 50 nM V-ASOD1 or with 4 μM ASOD1. Cells were extracted with 0.5% NP-40, and protein was measured by the Bradford method. SOD1 levels were determined with the xanthine–xanthine oxidase system, with measurement of the reduction of nitrobluc tetrazolium at 560 nm in the presence and absence of KCN, at the indicated times. SOD1 activity was determined from a SOD standard curve and is reported as the KCN-sensitive activity.

DTPA has been reported to have no interaction with peroxynitrite (Beckman et al., 1990).

N-Acetylcysteine (NAC) and cAMP derivatives do not inhibit V-ASOD1-induced death

NAC is an antioxidant compound that is reported to protect cells from oxidative stress and that rescues PC12 cells and neurons from apoptotic death caused by loss of trophic support (Ferrari et al., 1995). NAC increases intracellular levels of glutathione (Meister and Anderson, 1983; Issels et al., 1988) which, in turn, is involved in destruction of cellular hydrogen peroxide. Despite these activities, NAC (0.1–60 mM) did not prevent V-ASOD1-induced cell death (Fig. 7), even at concentrations that rescue serum- and NGF-deprived sympathetic neurons (Ferrari et al., 1995).

Permeant AMP analogs, such as CPT-cAMP, protect PC12 cells and cultured sympathetic neurons from withdrawal of support by trophic factors (Rydel and Greene, 1988; Rukenstein et al., 1991). However, 100 μM CPT-cAMP, which protects PC12 cells from apoptosis induced by serum and NGF withdrawal, did not rescue the cells from death caused by exposure to V-ASOD1 (data not shown). These results with NAC and with cAMP analogs point to distinct differences in the pathways by which SOD1 downregulation and trophic factor withdrawal lead to cell death. They point away from a role for hydroxyl radical and glutathione-mediated death.

bcl-2 protects PC12 cells from V-ASOD1-induced cell death

The proto-oncogene *bcl-2* inhibits cellular apoptotic death under a variety of circumstances (Korsmeyer, 1992a,b; Hockenbery et al., 1993; Kane et al., 1993). For instance, PC12 cells and sympathetic neurons overexpressing *bcl-2* show resistance to death after withdrawal of trophic support (Garcia et al., 1992; Batistatou et al., 1993). To determine whether *bcl-2* also offers protection from loss of SOD1, we tested the effect of V-ASOD1 on a line of PC12 cells that overexpress this gene. Figure 8 shows that, although *bcl-2* expression does not affect the ability of V-ASOD1 to reduce SOD1 activity, it protects the cells from death at V-ASOD1 concentrations of up to 480 nM. This finding indicates that *bcl-2* can protect cells from apoptotic death triggered by either loss of trophic support or diminution of SOD1 activity.

DISCUSSION

Use of Antennapedia peptide for delivery of antisense oligonucleotides

We have shown that linking ASOD1 to an Antennapedia peptide enhanced its potency by 100-fold and facilitated its uptake, causing nuclear localization within 2 hr. Moreover, in contrast to our experience with unmodified oligonucleotides (Troy et al., 1992; Troy and Shelanski, 1994), the peptide-linked oligonucleotide could be used effectively in serum-containing medium. This approach has the potential for enabling the use of antisense oligonucleotides in a broader array of cell culture conditions and potentially in intact organisms. Several other approaches have been used to modify oligonucleotides to increase their utility (for review, see Colman, 1990); the most widely used is the phosphorothioate (P-S) modification of the backbone. The P-S oligonucleotides are nuclease-resistant, but the modification alters the chirality of the molecules which can result in suboptimal binding to mRNA. In contrast, for the peptide delivery system used here, interaction with mRNA is unimpeded, because the oligonucleotide is released in an unmodified form by cytoplasmic reducing agents.

Pathways of ASOD1-induced cell death

Our experiments explored the mechanism by which SOD1 downregulation leads to apoptotic cell death. Past observations, that antioxidants protect cells from SOD1 loss, pointed to oxidative

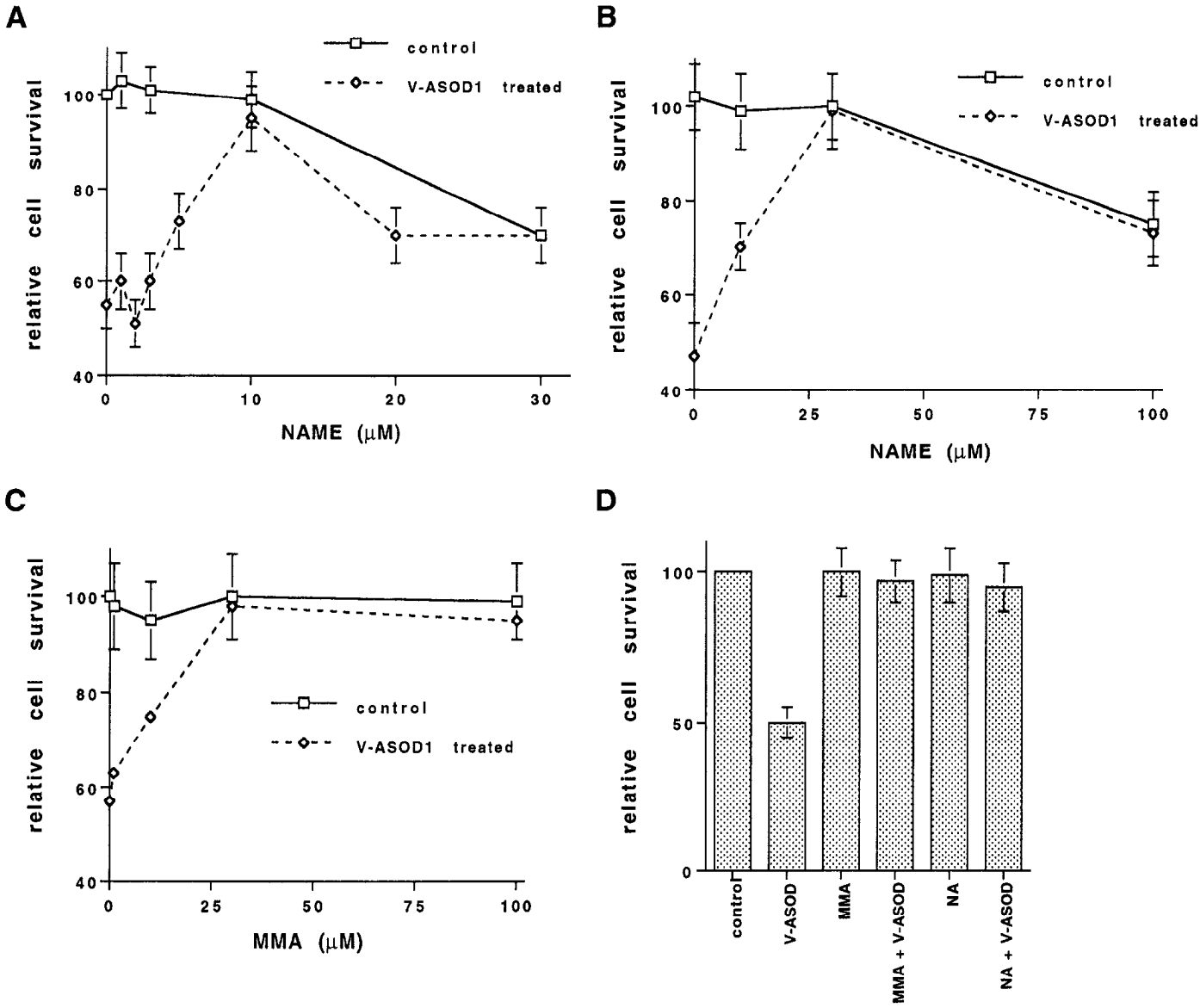
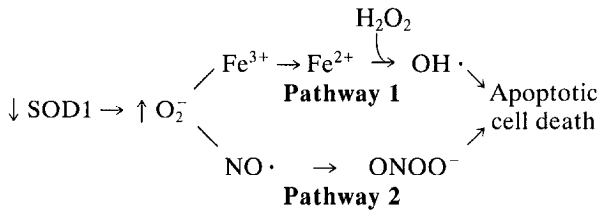


Figure 4. NOS inhibitors protect cells from death induced by downregulation of SOD1. *A*, Protection of naive cells by L-NAME. Naive PC12 cells were incubated with the indicated concentrations of L-NAME in the presence or absence of V-ASOD1 (50 nM). *B*, Protection of NGF-primed cells by L-NAME. NGF-primed PC12 cells were incubated with the indicated concentrations of L-NAME in the presence or absence of V-ASOD1 (50 nM). *C*, Protection of PC12 cells by MMA. Naive PC12 cells were incubated with the indicated concentrations of MMA in the presence or absence of V-ASOD1 (50 nM). *D*, Protection of cells by other NOS inhibitors. Naive PC12 cells were incubated with MMA or NA (100 μM) in the presence or absence of V-ASOD (50 nM). Relative cell survival was determined as described in Figure 3.

damage as the initiating cause of death, presumably via excess accumulation of superoxides (Rothstein et al., 1994; Troy and Shelanski, 1994; Greenlund et al., 1995). The present findings with metal chelators/free radical scavengers further support this possibility. As illustrated below, we have examined two alternative pathways that have been proposed to account for the death promoting effects of superoxide.



In pathway 1, superoxide promotes hydroxyl radical production by the iron-mediated Haber–Weiss reaction, wherein superoxide reduces Fe^{+3}, which then reacts with hydrogen peroxide to form hydroxyl radicals (Halliwell and Gutteridge, 1990). The release of reduced iron from ferritin by superoxide can also lead directly to lipid peroxidation (Thomas et al., 1985; Deby and Goutier, 1990; Halliwell and Gutteridge, 1990). The protective actions of metal chelators that we observed point to a possible role for Fe and/or other transition metals. However, the chelators may have multiple actions and may also scavenge free radicals or interact directly with peroxynitrite (Beckman et al., 1990; Radi et al., 1991). The inability of NAC to protect the cells from SOD1 downregulation also argues against a primary role for the reduction pathway. Although NAC does not scavenge superoxide, it does scavenge

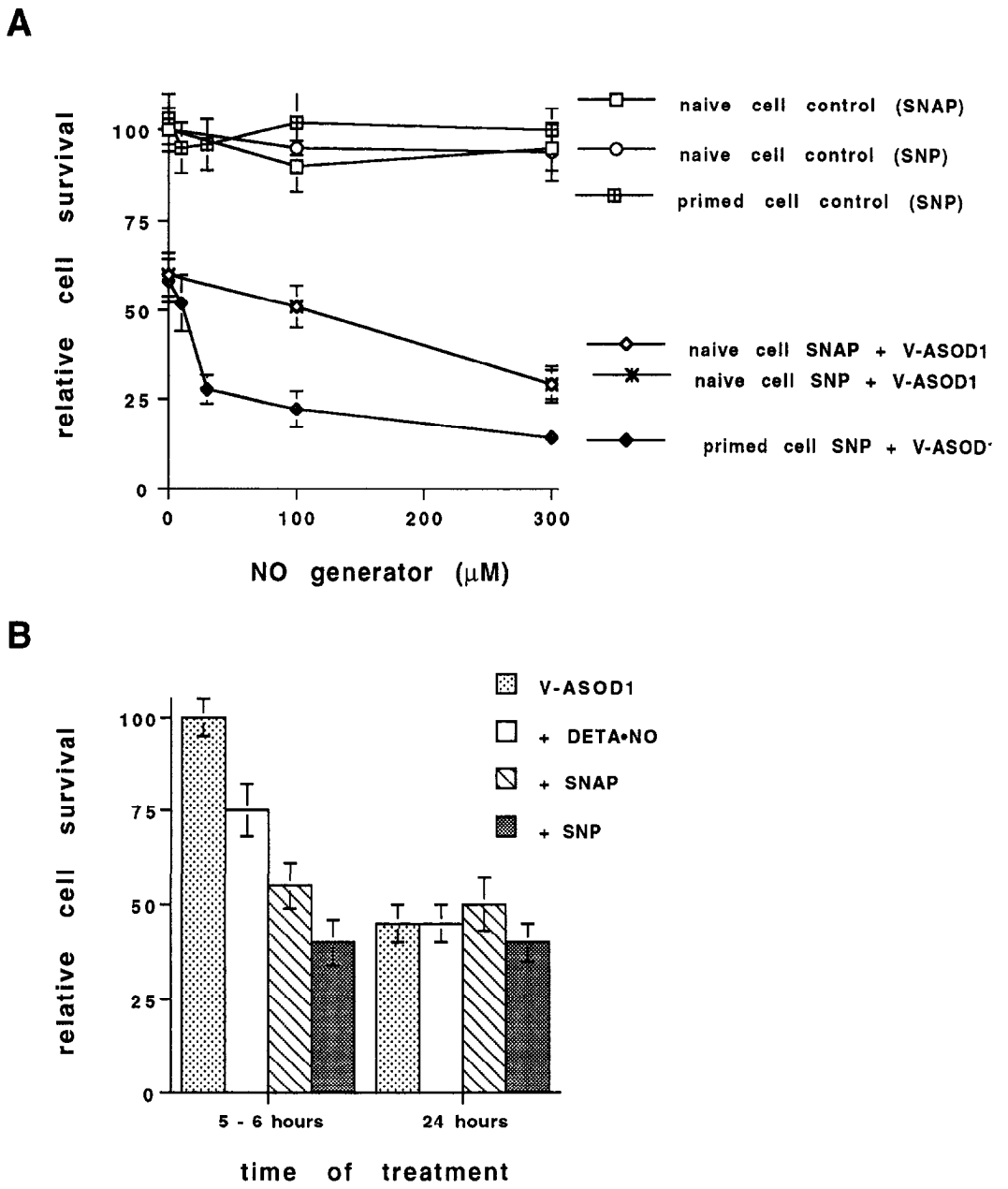


Figure 5. Enhancement and acceleration of cell death by NO generators. *A*, Naive or NGF-primed PC12 cells were incubated with the indicated concentrations of the NO generators in the presence or absence of V-ASOD1 (50 nM). *B*, PC12 cells were incubated with the indicated NO generators (all at 100 μM) in the presence of V-ASOD1 (50 nM). Surviving cells were counted at the times noted. Relative cell survival was determined as described in Figure 3.

hydroxyl radicals (Aruoma et al., 1989). Furthermore, this compound increases intracellular levels of glutathione, which should enhance clearance of hydrogen peroxide (Issels et al., 1988). In contrast to our observations, Rothstein et al. (1994) reported that NAC prevented death induced by inhibition of SOD1 in organotypic spinal cord cultures. The presence of multiple cell types in the primary cultures may account for this apparent discrepancy in results.

In pathway 2, superoxide interacts with NO to form peroxynitrite. The protective effects of NOS inhibitors and the acceleration and increase of V-ASOD1-promoted death by NO generators strongly suggest that damage from downregulation of SOD1 involves NO. Recent findings have demonstrated that PC12 cells possess a detectable level of constitutively active NOS (Peunova and Enikilopov, 1995). Thus, our findings are consistent with the notion that diminution of cellular SOD1 activity permits the formation of death-promoting reaction products between NO and superoxide. One somewhat surprising outcome of our past work was that NGF-pretreated PC12 cells show enhanced sensitivity to ASOD1 (Troy and Shelanski, 1994). The recent demonstration that NGF treatment substantially increases PC12 cell levels of constitutively active NOS (Peunova and Enikilopov, 1995) might account for this phenomenon. Thus, the increased sensitivity of NGF-primed cells to ASOD1 could be a result of their enhanced capacity to synthesize NO and, therefore, to generate increased amounts of peroxynitrite. This is supported by our finding that primed cells require higher doses of NOS inhibitors for full protection against V-ASOD1-induced death and are more sensitive to the effects of NO generators.

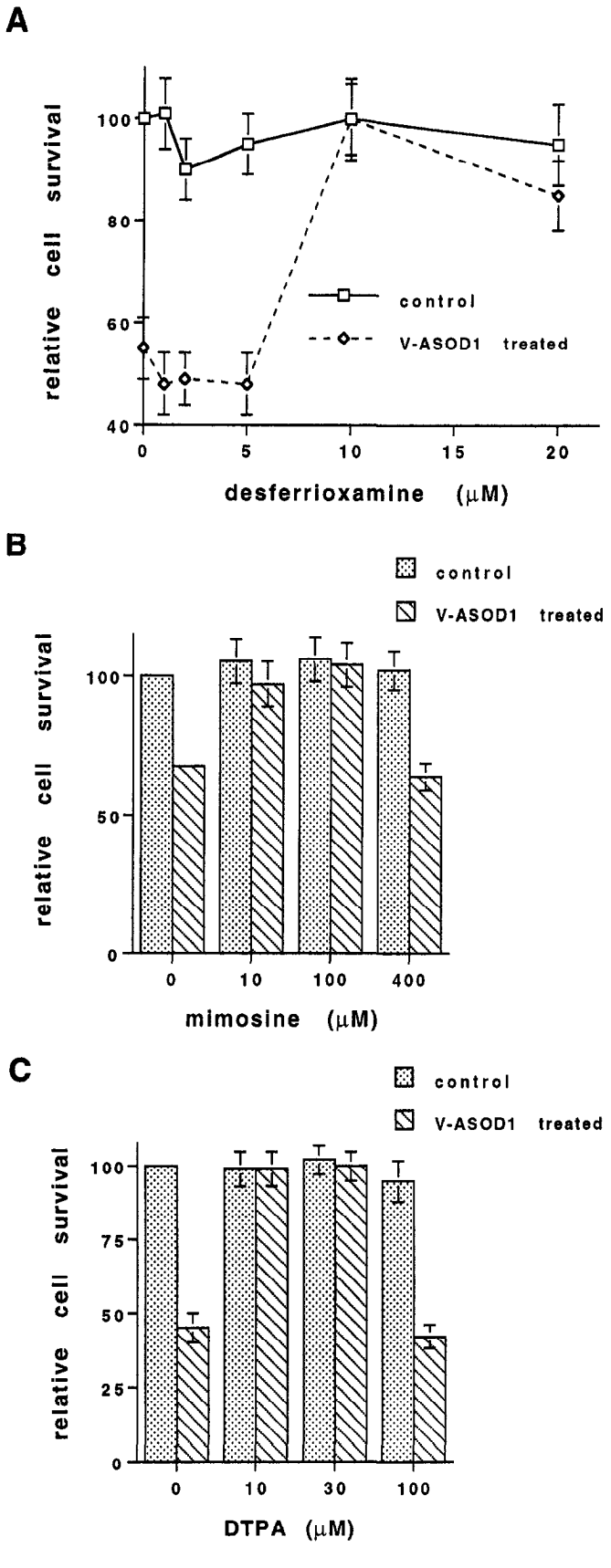


Figure 6. Iron chelators protect from V-ASOD1-induced cell death. Cells were incubated with the indicated concentrations of the respective chelators, with and without V-ASOD1 (50 nM). *A*, Desferrioxamine; *B*, mimosine; *C*, DPTA. Relative cell survival was determined as described in Figure 3.

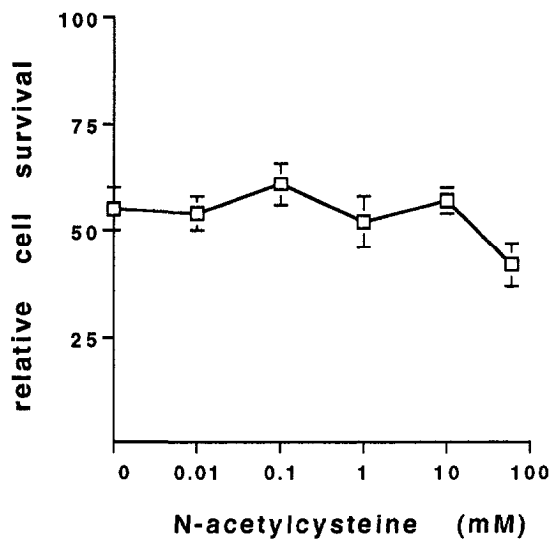


Figure 7. NAC does not protect the cells. PC12 cells were incubated in the presence of 50 nM V-ASOD1 and the indicated concentrations of NAC. At 24 hr, cells were lysed and nuclei were counted. The number of surviving cells is expressed relative to the number present without oligonucleotide and without NAC (designated as 100).

The mechanism by which peroxynitrite may lead to cell death is presently unclear and requires further study. Death could be triggered by nonenzymatic decomposition of peroxynitrite to hydroxyl radical and nitrogen dioxide (Beckman et al., 1990). However, the ineffectiveness of NAC in our V-ASOD1 experiments argues against a major role for the hydroxyl radical. An alternative possibility worthy of further consideration is that peroxynitrite could lead to cell death by a mechanism involving nitrosylation of critical tyrosine residues (Stammler, 1994).

Taken together, our results point to the NO–peroxynitrite pathway as a major contributor to the cell death caused by SOD1 downregulation. Our data, however, do not rule out entirely a role for the pathway involving reduction of iron. Thus, both pathways may participate in triggering cell death, and inhibition of either may be sufficient to promote survival.

Comparison of apoptotic death induced by SOD1 downregulation and by serum or trophic factor deprivation

One advantage of the PC12 cell system is that it has been used to model death caused both by SOD1 downregulation and by withdrawal of trophic support. This has permitted comparison of the mechanisms involved in the two events. The present observations support and extend the view that death induced by these two causes involves initially independent pathways. This was suggested in part by the observation that, although insulin and long-term NGF treatment promote PC12 cell survival, they did not protect them from SOD1 downregulation (Troy and Shelanski, 1994). Also consistent with this was the converse finding that, although vitamin E protects PC12 cells from SOD1 downregulation, it does not prevent their death when it is brought about by withdrawal of NGF or other trophic support (Ferrari et al., 1995). Furthermore, as shown here, CPT-cAMP and NAC, two agents that effectively replace NGF as a survival-promoting factor for neurons and serum-deprived PC12 cells, are ineffective in preventing death in the SOD1 downregulation paradigm. Finally, our findings point to an oxidative mechanism for initiation of death by downregulation of SOD1, whereas it has been argued that death caused by NGF

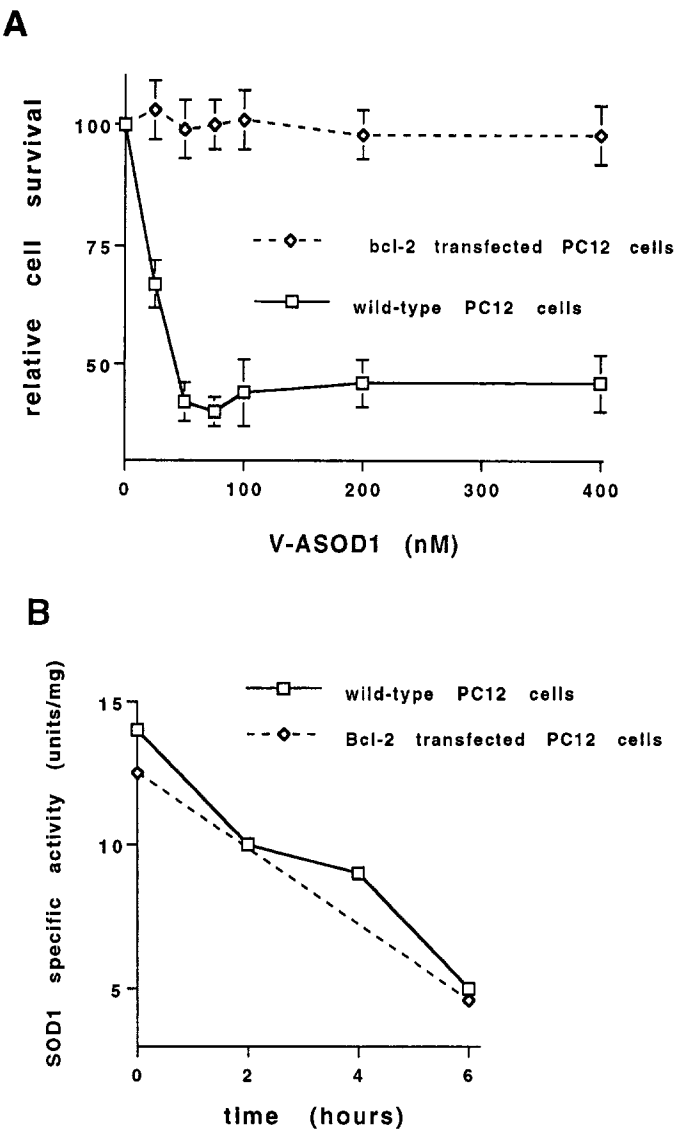


Figure 8. *bcl-2* protects cells from downregulation of SOD1. *A*, Relative cell survival of wild-type and *bcl-2*-transfected PC12 cells incubated with V-ASOD1 at indicated concentrations. Relative cell survival was determined as described in Figure 3. *B*, SOD1 specific activity of wild-type and *bcl-2*-transfected PC12 cells over 6 hr with 50 nM V-ASOD1. Cells were extracted with 0.5% NP-40, and protein was measured by the Bradford method. SOD1 levels were determined with the xanthine–xanthine oxidase system, with measurement of the reduction of nitroblue tetrazolium at 560 nm in the presence and absence of KCN, at the indicated times. SOD1 activity was determined from an SOD standard curve and is reported as the KCN-sensitive activity.

and trophic factor withdrawal is related to a cell cycle-related mechanism (Ferrari and Greene, 1994; Rubin et al., 1993).

Our findings call attention to the possibility that trophic factor deprivation and SOD1 downregulation, despite the utilization of initially separate mechanisms, lead to apoptosis by a common final pathway. This was suggested by the observation that aurintricarboxylic acid, an inhibitor of apoptotic death which rescues PC12 cells and neurons from NGF deprivation (Batistatou and Greene, 1991), also rescues PC12 cells from loss of SOD1 activity (Troy and Shelanski, 1994). In the present experiments, we found that PC12 cells transfected with a *bcl-2* expression construct, which are resistant to serum and NGF withdrawal, are also protected from downregulation of SOD1. These observations thus provide further support for the existence of a common final pathway.

SOD1 and neuronal degeneration

Our findings indicate that interfering with the destruction of superoxide and increasing NO levels accelerates death of neuronal cells. It is presently controversial whether the SOD1 defects associated with certain cases of FALS lead to death by a mechanism associated with loss or gain of function (Brown, 1995; Rowland, 1995). If the former occurs, then our findings are relevant to this disorder. In this case, diminution of SOD1 may enhance susceptibility of motor neurons to the formation of peroxynitrite, thereby leading to degeneration and death. There is evidence that some forms of amyotrophic lateral sclerosis may involve impaired uptake of glutamate (Choi, 1988; Rothstein et al., 1992). Excess glutamate in turn can lead to enhanced levels of intracellular Ca^{2+} which, in turn, can activate Ca^{2+}-sensitive forms of NOS and increase NO synthesis (Coyle and Puttfarcken, 1993). Thus, there may be a critical threshold for levels of NO and superoxide in neurons that, if exceeded for either species, leads to excessive peroxynitrite formation and, ultimately, apoptosis. Our findings suggest several agents that may be effective in blocking this damage.

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