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ACCELERATED COMMUNICATION

Expression of Human Copper/Zinc-Superoxide Dismutase Inhibits the Death of Rat Sympathetic Neurons Caused by Withdrawal of Nerve Growth Factor

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SUMMARY

Rat superior cervical ganglion neurons require the presence of nerve growth factor (NGF) to develop and survive in culture. If NGF is removed from the culture medium, then the neurons die of programmed cell death. We investigated the potential role of Ca^{2+} and reactive oxygen species in this process. We found that overexpression of human wild-type copper/zinc-superoxide dismutase in cultured superior cervical ganglion neurons, using an adenovirus-based vector, substantially protected the cells from the effects of NGF withdrawal, although overexpression of the Ca^{2+}-binding protein calbindin D_{28k} or the enzyme β-galactosidase did not. We also observed that treatment of the cells with the cytokine transforming growth factor-β_1, which has been shown to protect neurons against oxidative injury, delayed cell death produced by NGF withdrawal. These data suggest a role for reactive oxygen species in triggering programmed cell death of rat sympathetic neurons upon growth factor withdrawal.

Elucidation of the molecular basis of nerve cell degeneration is of critical importance to an understanding of neuronal development and disease. Recently, several important areas of investigation have started to provide us with some possible general principles for understanding why neurons live or die under different conditions. For example, the concepts of excitotoxicity (1, 2) and of PCD (3) have proven to be extremely useful ways of thinking about the problem.

The idea that neurons can die by initiating a program of genetically directed cell suicide and that this is a process involved in the normal development of the nervous system is now well established (4). The possibility that the same pathway might be inadvertently activated under pathological conditions, leading to inappropriate neural degeneration, has now been widely discussed (5). It is clearly important to consider the relative roles of PCD and necrosis in different types of neuronal degeneration.

Although PCD and necrosis are usually discussed as being different types of degenerative process, there are also some common features to be considered. These include possible roles for several intracellular mediators, including Ca^{2+} and reactive oxygen species (1, 6, 7). The role of reactive oxygen species in apoptosis and neurodegeneration has recently been highlighted by the idea that the antiapoptotic protein Bcl-2 may act by inhibiting the formation or reducing the effects of reactive oxygen radicals (8, 9) and by the discovery that down-regulation of the enzyme SOD induces apoptotic death of cultured neurons (10). Moreover, in humans, mutations in the same enzyme are directly linked to familial amyotrophic lateral sclerosis (11). In the present series of experiments we have used a well established model of PCD in the nervous system, i.e., the removal of NGF from sympathetic neurons in culture (12), and investigated the possible

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ABBREVIATIONS: PCD, programmed cell death; NGF, nerve growth factor; SCG, superior cervical ganglion; SOD-1, copper/zinc-superoxide dismutase; BHK, baby hamster kidney; TGF, transforming growth factor; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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roles of some of these factors in the resulting nerve cell degeneration. To do this, we have used a novel adenovirus-based system for the efficient expression of genes of interest in sympathetic neurons. Our results indicate that reactive oxygen species may play a key role in this type of neuronal death.

Materials and Methods

Cell culture. SCGs were isolated from 1–5-day-old Holzman rats (13). Rat pups were killed by a cut made into the thorax and through the heart, under anesthesia. This procedure reduced the bleeding from the carotid artery during dissection of the ganglia. After dissection, ganglia were incubated for 40 min, at 37°, in Ca^{2+}- and Mg^{2+}-free Hanks' solution containing 0.2% trypsin (Worthington, Freehold, NJ) and were then dissociated into single cells by gentle trituration. Dissociated cells were washed twice with L-15 culture medium containing 0.1% (w/v) bovine serum albumin (Calbiochem-Novabiochem, La Jolla, CA) and were plated on poly-L-lysine- and laminin-treated glass coverslips. Glass coverslips were prepared by incubation for a minimum of 15 min with 0.5 mg/ml poly-L-lysine (Sigma Chemical Co., St. Louis, MO) dissolved in borate buffer. The coverslips were then rinsed three times with water and covered with 0.02 mg/ml laminin in L-15 medium, for a minimum of 1 hr, before the cells were replated. Cells were maintained in L-15 culture medium supplemented with 1 μg/ml NGF (United States Biochemicals, Cleveland, OH), 5% rat serum (GIBCO, Grand Island, NY), glucose, bicarbonate, penicillin-streptomycin, and vitamins, as described (14).

Construction of recombinant, replication-defective adenovirus containing wild-type copper/zinc-SOD (SOD-1). A SOD-1 cDNA clone was obtained from the American Type Culture Collection (15). A blunt-ended *Pst*I-*Nhe*I fragment was inserted into the *Eco*RV site of the transfer vector pAdKN downstream of the elongation factor 1-α promotor and upstream of the cellular heavy chain enhancer (4F2) and the bovine growth hormone polyadenylation site (pA+), to generate pAdKN.SODWT plasmid. The pAdKN plasmid also contains 0–1 and 9–16 map units of DNA sequence of adenovirus type 5. To construct a replication-defective adenovirus (AdEF.SODWT) containing SOD-1 cDNA, *Nhe*I-digested pAdKN.SODWT and *Xba*I- and *Cla*I-digested adenovirus type 5 sub360 were co-transfected into human embryonic kidney 293 cells using a calcium phosphate-DNA precipitation method. The recombinant AdEF.SODWT was plaque purified three times to isolate a homogeneous population of the virus. The virus used in the studies was further purified by CsCl isopycnic ultracentrifugation and was dialyzed against HEPES-buffered saline (10 mM HEPES, 140 mM NaCl, 2 mM $MgCl_2$, pH 7.5) containing 10% glycerol. Purified virus was stored at −70° in small aliquots and titered by plaque assay. Adenoviruses carrying the genes for calbindin D_{28k} (AdCABP-1) and β-galactosidase (AdBAc.LacZ) have been described (16, 17).

Infection protocol. After 3 days *in vitro* the cultures were infected with the respective virus at a multiplicity of infection of 1500. For this purpose, aliquots of the high titer viruses were added to the culture medium and distributed by gentle agitation. After 2 hr, the infection medium was exchanged with regular, NGF-supplemented, culture medium. After an additional 3 days, cultures either were subjected to NGF deprivation (see NGF withdrawal) or were fixed, and expression of the respective proteins was verified by immunocytochemistry. No nonspecific toxic effects of the viruses were apparent under the conditions used in these studies. The percentage of neurons showing positive staining for each antigen was >95% in every experiment and was usually not significantly different from 100%. In contrast, no neurons were stained for the antigens under noninfected conditions (see Fig. 2).

Western blot analysis. Sympathetic neurons were infected with either AdBAc.LacZ, AdCABP-1, or AdEF.SODWT virus at a multiplicity of infection of 1500 or were not infected. Four days after infection, neurons were washed with cold PBS and scraped. Neurons were resuspended in 15 μl of $H_{10}K_{10}D_1$ buffer (10 mM HEPES, 10 mM KCl, 1 mM dithiothreitol, pH 7.4) and lysed on ice with 1% Nonidet P-40 for 15 min. Cytoplasmic protein (10 μg), obtained after centrifugation at 12,000 rpm for 10 min at 4°, was analyzed for the presence of 19-kDa SOD-1 protein by Western blotting using horseradish peroxidase-conjugated anti-SOD-1 polyclonal antibody (catalogue no. PP077; Binding Site) and an enhanced chemiluminescence Western blotting detection system (Amersham).

SOD activity gels. BHK-21 cells were either infected with AdEF.SODWT or not infected, and 96 hr after infection cytoplasmic extracts were prepared in $H_{10}K_{10}D_1$ buffer containing 0.5% Nonidet P-40. One milligram of protein was electrophoresed on a 7.5% non-denaturing polyacrylamide gel at 4°, and SOD enzyme activity was determined by using the nitroblue tetrazolium method (18).

Immunohistochemistry. In each experiment successful expression of proteins was verified by immunohistochemistry. For this purpose cultures were fixed with 4% paraformaldehyde in culture medium at 37° for 15 min. After cells were washed three times with 0.1 M PBS (0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.2 M NaCl), pH 7.4, they were permeabilized with 0.1% Triton X-100 (Eastman Kodak, Rochester, NY) in PBS for 2.5 min. The coverslips were then incubated in blocking medium [0.1% Tween-20 (Sigma) and 4% bovine serum albumin (Sigma) in 0.1 M PBS] for 1 hr at room temperature. Incubations with primary antibodies were performed overnight at 4°, using murine monoclonal anti-human SOD-1 (1/300), anti-calbindin D_{28k} (1/1000), and anti-β-galactosidase (1/1000) antibodies (Sigma), diluted in blocking medium. Monoclonal antibodies were detected using anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1/2000 in blocking solution, and a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The peroxidase was visualized using 3,3′-diaminobenzidine (Sigma) as the chromogenic substrate. Coverslips were mounted in 90% glycerol with 0.1 M phosphate buffer containing 0.01% NaN_3.

TGF-β_1 treatment. Twenty-four hours before NGF withdrawal, cultures received 10 ng/ml TGF-β_1 (recombinant human form; R & D Systems, Minneapolis, MN) from a 1000 ng/ml stock solution (in saline containing 1 mg/ml ovalbumin and 4 mM HCl). Controls were treated with an equal volume of the vehicle; TGF-β_1 or vehicle was also present during the period of NGF withdrawal.

NGF withdrawal. For induction of PCD, the original culture medium was exchanged for fresh culture medium with (control conditions) or without (NGF withdrawal) the NGF supplement. Neuronal degeneration was analyzed by phase-contrast microscopy after 24, 48, and 72 hr. Neurons exhibiting a rough appearance with irregularly shaped cell bodies, blebs, and vacuoles, followed by cell shrinkage, neurite fragmentation, and loss of phase-brightness, were considered to be damaged. Frequently, nuclear fragmentation could be observed by phase-contrast microscopy. Healthy neurons were identified as having round to oval, smooth, phase-bright cell bodies and intact neurites. A total of 100–200 neurons were examined in three to five randomized subfields of the coverslips. Similar numbers of neurons were examined under all experimental conditions. Each condition was represented by six coverslips. All experiments were performed in quadruplicate.

Results

It has been widely demonstrated that the development and survival of rat sympathetic neurons in cell culture are dependent on the presence of the appropriate growth factors in the culture medium. In most paradigms the major important factor is thought to be NGF. When NGF is removed from the medium, neurons die over a period of a few days, by a mechanism that shows many of the hallmarks of the "apoptotic" form of PCD (12). We readily confirmed this to be the case.

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Neurons in our cultures died over a 3-day period after NGF withdrawal (Fig. 1) and exhibited many of the morphological criteria suggestive of apoptosis, incuding shrunken somata and membrane blebbing. Forty-eight hours after NGF withdrawal, neurons also stained positively for double-stranded DNA breaks, using the TUNEL staining procedure.

We examined the effects of expression of the proteins calbindin D_{28k}, human copper/zinc-SOD-1, and β-galactosidase on the neuronal death observed after NGF removal. These proteins were chosen because they represent relatively physiological ways of perturbing cell handling of Ca^{2+} (calbindin D_{28k}) (17, 19) and reactive oxygen species such as superoxide anions (copper/zinc-SOD-1). β-Galactosidase expression was used as an additional control. To achieve efficient expression of these proteins in cultured neurons, we used adenovirus-based vectors (17). Using these vectors, approximately 100% of the neurons could be made to express the appropriate protein 3 days after viral infection (Fig. 2) (17). Evidence of expression of each antigen was obtained by immunohistochemistry 24 hr after infection. The intensity of staining increased over the next 24–48 hr and then stabilized (Fig. 2). No diminution in staining intensity was observed over the next 7 days.

We observed a striking effect of overexpression of SOD-1 in these neurons (Figs. 1 and 2). In contrast to the control situation, in which >90% of the neurons had died by 72 hr after NGF withdrawal, only 10% of the copper/zinc-SOD-1-overexpressing cells had died by that time. The degree of cell death in SOD-1-overexpressing neurons in the presence of NGF was not different from control conditions (i.e., using the calbindin D_{28k} β-galactosidase-expressing virus) (Figs. 1 and 2). Copper/zinc-SOD-1 expression protected neurons at earlier time points (24 and 48 hr) as well (Fig. 1). In contrast to the effects of SOD-1 expression, cells expressing calbindin D_{28k} or β-galactosidase died as rapidly as did control cells not treated with any virus (Fig. 1).

Although the small number of cells in the SCG neuron cultures did not allow us to perform conventional enzyme assays, we think that overexpression of immunoreactive human SOD-1 protein in SCG neurons was associated with an increase in enzyme activity in these cells, for the following reasons. First, Western blots clearly demonstrated the ex-

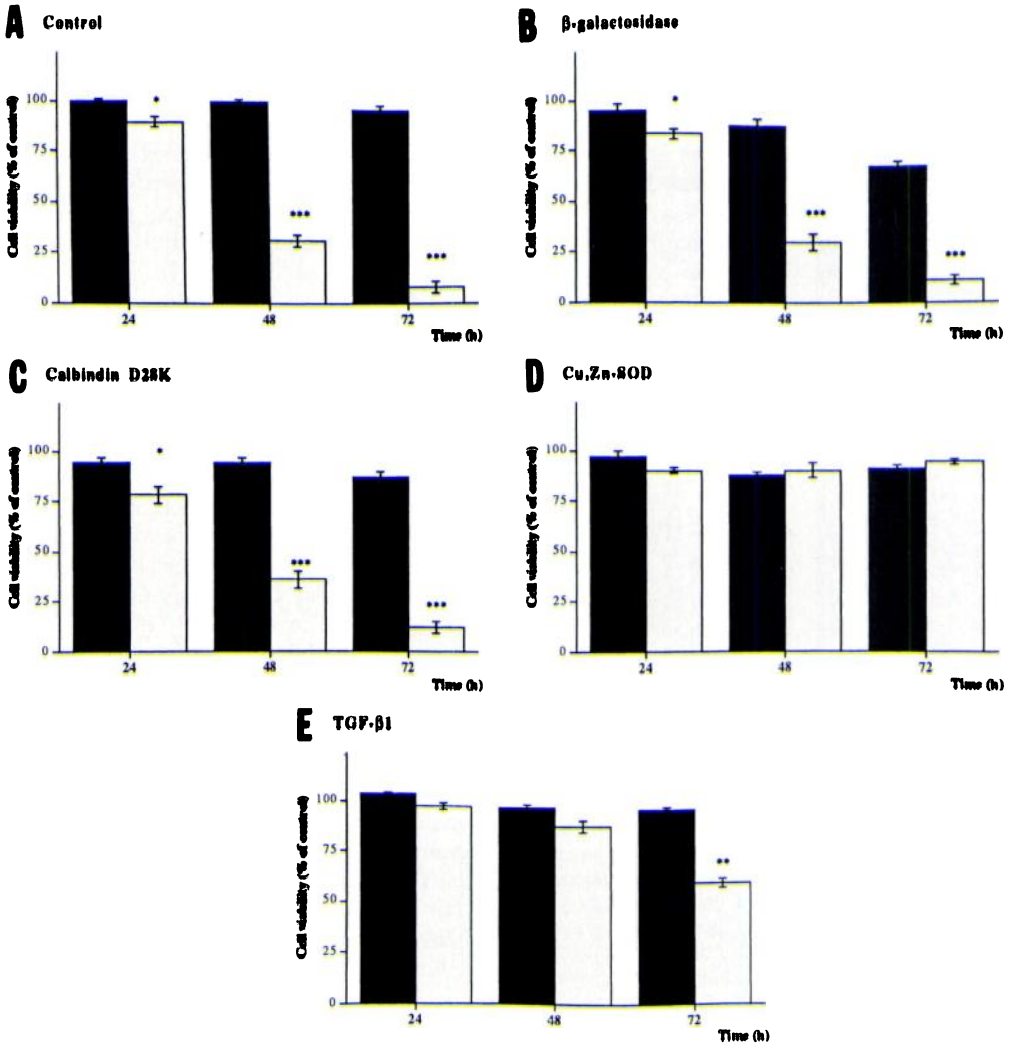

Fig. 1. Histograms representing the time course of survival of rat SCG neurons grown in the presence (■) or absence (□) of NGF. A, Control conditions, without any treatment; B, cells overexpressing β-galactosidase; C, cells overexpressing calbindin D_{28k}; D, cells overexpressing human SOD-1; E, treatment with TGF-β_1 24 hr before the start of NGF withdrawal (four experiments in each case; see Materials and Methods). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's t test.

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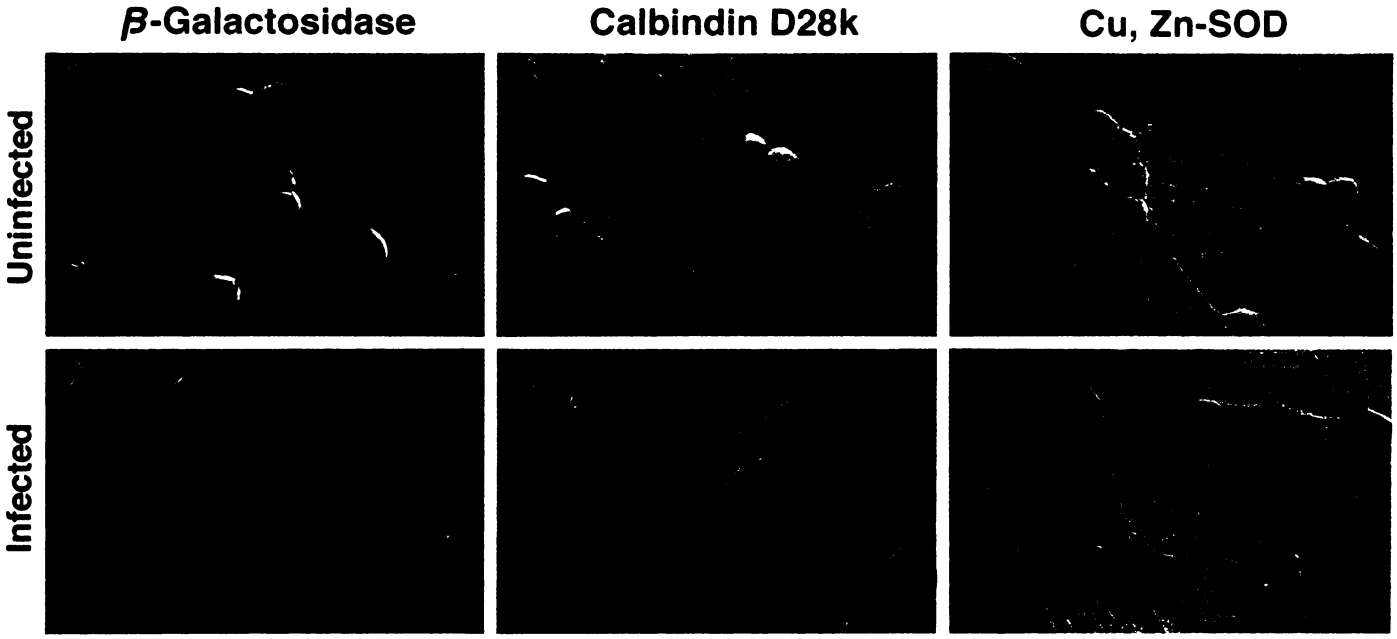

Fig. 2. D–F, Expression of β-galactosidase (D), calbindin D_{28k} (E), and human SOD-1 (F) in neurons infected with adenoviruses containing cDNAs. Expression of proteins was examined 3 days after infections, using monoclonal antibodies specific for the corresponding proteins. A–C, Lack of β-galactosidase (A), calbindin D_{28k} (B), and SOD-1 (C) expression in uninfected cells.

pression of the human protein in cultures of rat SCG neurons (Fig. 3A). Second, when the protein was overexpressed in BHK cells using the same adenovirus it was clearly active (Fig. 3B). We also observed an increase in total SOD activity when the protein was expressed in PC-12 cells, a cell line that is related to sympathetic neurons. Under conditions where 100% of the PC-12 cells were positively stained for human SOD-1, we observed a 1.53-fold increase in total SOD activity, using the procedure of Elstner and Heupel (20). Finally, preliminary imaging studies suggest that SOD-1-overexpressing neurons have reduced rates of endogenous superoxide production.[2] We also recently demonstrated the biological activity of the calbindin D_{28k} protein expressed using the AdCABP-1 virus employed in these studies (17). We demonstrated that the calbindin D_{28K} expressed using this virus had the expected $^{45}Ca^{2+}$-binding activity and immunoreactivity (17).

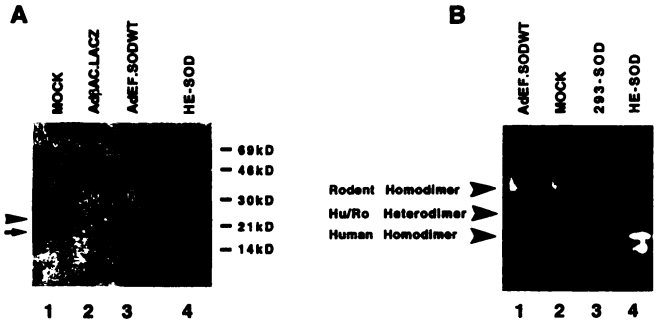

Fig. 3. A, Western blot analysis of SOD-1 expression in cultured sympathetic neurons after infection with AdEF.SODWT virus. AdEF-.SODWT-infected cell extracts show expression of human SOD-1 (*lane 3*, *arrowhead*) with the same electrophoretic mobility as recombinant human erythrocyte SOD (*lane 4*), whereas uninfected (*lane 1*) and AdBAc.LacZ-infected (*lane 2*) cell extracts show no evidence of bands at the same position. The immunostained protein product with greater electrophoretic mobility (*arrow*) presumably represents cross-reactive rodent SOD. *Numbers to the right*, protein molecular weight markers. B, SOD activity in AdEF.SODWT-infected BHK-21 cells. One band, corresponding to rodent homodimer SOD activity, is seen in uninfected BHK-21 cells (*lane 2*). BHK-21 cells infected with AdEF.SODWT (*lane 1*) show two additional bands. The band with greatest electrophoretic mobility corresponds, in mobility, to the human homodimer SOD activity band in uninfected human 293 cells (*lane 3*) and recombinant human erythrocyte SOD (*lane 4*). The band with electrophoretic mobility between those of the rodent SOD homodimer and the human SOD homodimer, in AdEF.SODWT-infected BHK-21 cells (*lane 1*), represents human/rodent (*Hu/Ro*) SOD heterodimers.

Because of the observed effects of SOD-1 overexpression, we also compared the effect of SOD-1 expression with that obtained by treatment of the cells with TGF-β_1. We demonstrated that this cytokine was able to partially prevent the PCD of hippocampal pyramidal neurons in culture after the removal of conditioned medium or exposure to oxidative stress (21). Thus, we were interested in determining whether such an antioxidant effect was also seen using sympathetic neuron cultures. TGF-β_1 significantly slowed the death of sympathetic neurons after NGF withdrawal, although not as profoundly as overexpression of SOD-1 (Fig. 1).

Discussion

There are now numerous examples of the ability of growth factors and cytokines to enhance the survival of different populations of neurons both *in vivo* and *in vitro* (22, 23). Removal of these growth factors is often associated with the death of particular neurons. In many cases this appears to occur by PCD (24). Several types of PCD are thought to occur in the nervous system (25). In the case of NGF withdrawal from sympathetic neurons in culture, intensive studies have shown the PCD to be similar to that which has been designated apoptosis (5, 12, 25).

Several factors, including Ca^{2+} (6) and reactive oxygen species (26–28), have been previously identified as possibly

[2] V. P. Bindokas and R. J. Miller, unpublished observations.

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being important in the apoptotic pathway in sympathetic neurons. For example, it has been demonstrated that modest depolarization of these neurons with high K^+ concentrations inhibits neuronal death after NGF withdrawal. This effect has been shown to be blocked by dihydropyridine L-type Ca^{2+} channel antagonists, implying that it is due to influx of Ca^{2+} by this route (6). It has been suggested that Ca^{2+} concentrations must be within a specific range for sympathetic neurons to survive and that the cells die if the concentration falls below or rises above these levels (6). It has also been shown that generation of reactive oxygen species can induce apoptosis in a number of neuronal types, including sympathetic neurons and related cells (9, 29, 30). Furthermore, inhibition or down-regulation of SOD activity causes neuronal cells to undergo apoptosis (10). This is also seen in NGF-differentiated PC-12 cells, which are related to sympathetic neurons (27). NGF withdrawal also results in the up-regulation, in sympathetic neurons, of several genes that are also turned on by reactive oxygen species (26, 31). On the other hand, injection of the Bcl-2 protein, which may act as a free radical scavenger, blocks the effects of NGF withdrawal (28), and the death of sympathetic neurons induced by hypoxia can be reduced by stimulating cellular uptake of SOD added to the culture medium (32).

The results obtained in the present series of experiments clearly support a role for reactive oxygen species in NGF withdrawal-induced apoptosis. Thus, increasing SOD expression in these neurons had a profound inhibitory effect on cell death. Similar protective effects were recently shown after microinjection of SOD-1 into NGF-deprived sympathetic neurons (31). Thus, it may be that one mechanism for the survival-enhancing effects of NGF involves growth factor activation of processes that allow the neurons to scavenge potentially toxic free radicals more efficiently (26). It is interesting to note that we also found that the cytokine TGF-β_1, which has been shown to have protective "antioxidant" effects on some central neurons (21), delayed the death of SCG neurons caused by NGF withdrawal. In the case of hippocampal pyramidal neurons, this action was associated with a large induction of Bcl-2 protein, which has been suggested to have a free radical-scavenging activity (8, 9). However, it is not clear whether this is true in SCG neurons, where endogenous levels of Bcl-2 are relatively high even under normal conditions (33). It is also possible that the increased survival observed in neurons overexpressing SOD-1 is not due to the enzymatic effects of this protein *per se* but is due to secondary effects on other constituents of the neurons, which can then affect their viability.

In contrast to the effects of SOD expression, we observed no protection resulting from overexpression of the Ca^{2+}-binding protein calbindin D_{28k} in the cells. We have previously demonstrated that this protein can effectively buffer Ca^{2+} signals in neurons (19) and that its expression can produce changes in the characteristics of excitatory synaptic transmission in the hippocampus (17). However, as discussed above, the intracellular Ca^{2+} concentration may have to fall into a quite narrow range to be compatible with neuronal survival (6). It is possible that expression of the Ca^{2+}-binding protein does not alter Ca^{2+} homeostasis in these neurons in such a way as to produce this Ca^{2+} window in the absence of NGF or that other changes produced by NGF removal are the predominant influences.

Although our results do suggest the involvement of reactive oxygen species in apoptosis in this case, they do not reveal at which point this influence is exerted. Thus, it is possible to distinguish factors that can trigger cells to undergo apoptosis because they make the cells "dysfunctional" in some way from those factors that are actually involved in the genetic program designed to carry out the events involved in cell suicide (24). The studies presented here demonstrate the potential use of adenovirus-mediated gene expression in the elucidation of such issues. These virus vectors provide an efficient means and valuable neurobiological tool for the delivery of foreign genes to neurons.

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References

1. Choi, D. W. Calcium mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* **11**:465–469 (1988).
2. Rothman, S. M., and J. W. Olney. Excitotoxicity and the NMDA receptor. *Trends Neurosci.* **10**:299–302 (1987).
3. Wyllie, A. H., J. F. R. Kerr, and A. R. Currie. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68**:251–306 (1980).
4. Oppenheim, R. S. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* **14**:453–501 (1991).
5. Wyllie, A. H. Death from the inside out: an overview. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **345**:237–241 (1994).
6. Franklin, J. L., and E. M. Johnson. Block of neuronal apoptosis by a sustained increase of steady state free Ca concentration. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **345**:251–256 (1994).
7. McConkey, D. I., and S. Orrenius. Signal transduction pathways to apoptosis. *Trends Cell Sci.* **4**:370–374 (1994).
8. Hockenberry, D. M., Z. N. Oltvai, X. M. Yin, C. L. Milliman, and S. J. Korsmeyer. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**:241–251 (1993).
9. Kane, D. J., T. A. Sarafian, R. Anton, H. Hahn, E. B. Gralla, J. S. Valentine, T. Ord, and D. E. Bredesen. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science (Washington D. C.)* **262**:1274–1277 (1993).
10. Rothstein, J. D., L. A. Bristol, B. Hosler, R. H. Brown, and R. W. Kuncl. Chronic inhibition of superoxide dismutase produces apoptotic death of spinal neurons. *Proc. Natl. Acad. Sci. USA* **91**:4155–4159 (1994).
11. Deng, H. Z., A. Hentati, J. A. Tainer, Z. Iqbal, A. Cayabyab, W. Y. Hung, E. D. Getzoff, P. Hu, B. Herfeldt, R. P. Roos, C. Warner, G. Deng, E. Soriano, C. Smyth, H. E. Parge, A. Ahmed, A. D. Roses, R. A. Hallewell, M. A. Pericak-Vance, and T. Siddique. Amyotrophic lateral sclerosis and structural defects in Cu/Zn superoxide dismutase. *Science (Washington D. C.)* **261**:1047–1051 (1993).
12. Edwards, S. N., and A. M. Tolkovsky. Characterization of apoptosis in cultured rat sympathetic neurons after nerve growth factor withdrawal. *J. Cell Biol.* **124**:537–546 (1994).
13. Higgins, D., P. J. Lein, D. J. Osterhout, and M. I. Johnson. Tissue culture of mammalian autonomic neurons, in *Culturing Nerve Cells* (G. Barker and K. Goslin, eds.). MIT Press, Cambridge, MA, 177–204 (1991).
14. Mains, R. E., and P. H. Patterson. Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differential properties. *J. Cell. Biol.* **59**:329–345 (1973).
15. Sherman, L., N. Dafni, J. Lieman-Hurwitz, and Y. Groner. Nucleotide sequence and expression of human chromosome 21-encoded superoxide dismutase mRNA. *Proc. Natl. Acad. Sci. USA* **80**:5465–5469 (1983).
16. Barr, E., J. Carroll, S. Tripathy, K. Kozarsky, J. Wilson, and J. M. Leiden. Percutaneous transluminal gene transfer into the heart using replication defective recombinant adenovirus. *Gene Therapy* **1**:51–58 (1994).
17. Chard, P. S., J. Jordan, C. J. Marcuccilli, R. J. Miller, J. H. Leiden, R. P. Roos, and G. D. Ghadge. Regulation of excitatory transmission at hippocampal synapses by calbindin D_{28k}. *Proc. Natl. Acad. Sci. USA*, in press.
18. Beauchamp, C., and I. Fridovich. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**:817–827 (1971).
19. Chard, P. S., D. Bleakman, S. Christakos, C. S. Fullmer, and R. J. Miller. Calcium buffering properties of calbindin D_{28k} and parvalbumin in rat sensory neurones. *J. Physiol. (Lond.)* **472**:341–357 (1993).
20. Elstner, E. F., and A. Heupel. Inhibition of nitrite formation from hydroxylammonium chloride: a simple assay for superoxide dismutase. *Anal. Biochem.* **70**:616–620 (1976).
21. Prehn, J. H. M., V. P. Bindokas, C. J. Marcuccilli, S. Krajewski, J. C. Reed,

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and R. J. Miller. Regulation of neuronal Bcl-2 protein expression and calcium homeostasis by transforming growth factor type β confers wide ranging protection on rat hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **91**:12599–12603 (1994).
22. Thoenen, H. The changing scene of neurotrophic factors. *Trends Neurosci.* **14**:165–170 (1991).
23. Henderson, C. E., W. Camu, C. Mettling, A. Gouin, K. Poulsen, M. Karihariloo, J. Rullmans, T. Evans, S. B. McMahon, M. P. Armanini, L. Berkmeier, M. P. Phillips, and A. Rosenthal. Neurotrophins promote motor neuron survival and are present in embryonic limb bud. *Nature (Lond.)* **363**:266–270 (1993).
24. Raff, M. C. Social controls on cell survival and cell death. *Nature (Lond.)* **356**:397–399 (1992).
25. Clarke, P. G. H. Development cell death: morphological diversity and multiple mechanisms. *Anat. Embryol. (Berl.)* **181**:195–213 (1990).
26. Estus, S., W. J. Zaks, R. S. Freeman, M. Gruda, R. Bravo, and E. M. Johnson. Altered gene expression in neurons during programmed cell death: identification of c-*jun* as necessary for neuronal apoptosis. *J. Cell Biol.* **127**:1717–1727 (1994).
27. Troy, C. M., and M. L. Shelanski. Down-regulation of copper/zinc superoxide dismutase causes apoptotic death in PC-12 neuronal cells. *Proc. Natl. Acad. Sci. USA* **91**:6384–6387 (1994).
28. Garcia, I., I. Martinou, Y. Tsujimoto, and J. C. Martinou. Prevention of programmed cell death of sympathetic neurons by the *bcl*-2 proto-oncogene. *Science (Washington D. C.)* **258**:302–304 (1992).
29. Behl, C., J. B. David, R. Lesley, and D. Schubert. Hydrogen peroxide mediates amyloid β protein toxicity. *Cell* **77**:817–827 (1994).
30. Sendtner, M., and H. Thoenen. Oxidative stress and motor neuron disease. *Curr. Biol.* **4**:1036–1039 (1994).
31. Greenlund, L. J. S., T. L. Deckworth, and E. M. Johnson. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed cell death. *Neuron* **14**:303–315 (1995).
32. Rosenbaum, D. M., J. Kalberg, and J. A. Kessler. Superoxide dismutase ameliorates neuronal death from hypoxia in culture. *Stroke* **25**:857–863 (1994).
33. Merry, D. E., D. J. Veis, W. F. Hicky, and S. J. Korsmeyer. Bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. *Development* **120**:301–311 (1994).

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