Superoxide Dismutase Delays Neuronal Apoptosis: A Role for Reactive Oxygen Species in Programmed Neuronal Death

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Summary

Sympathetic neurons in culture die by apoptosis when deprived of nerve growth factor (NGF). We used this model of programmed cell death to study the mechanisms that mediate neuronal apoptosis. Cultured sympathetic neurons were injected with copper/zinc superoxide dismutase protein (SOD) or with an expression vector containing an SOD cDNA. In both cases apoptosis was delayed when the neurons were deprived of NGF. The delay was similar to that seen when a bcl-2 expression vector was injected. SOD, injected 8 hr after NGF deprivation, provided no protection, indicating that superoxide production may occur early in response to trophic factor deprivation. We have demonstrated, with a redox-sensitive dye, an increase in reactive oxygen species (ROS) that peaked at 3 hr after sympathetic neurons were deprived of NGF. If NGF was added back to the culture medium after the period of peak ROS generation, apoptosis was completely prevented, suggesting that ROS production serves as an early signal, rather than a toxic agent, to mediate apoptosis.

Introduction

Widespread neuronal death occurs during the normal development of the nervous system. It is believed that access to sufficient neurotrophic factors during a critical developmental period is one of the deciding factors of neuronal life or death, such that those neurons that receive adequate amounts of trophic factor will live, and those that do not will die (Oppenheim, 1991). This programmed neuronal death is thought to match neuronal number to target size and to rid the nervous system of inappropriate connections. The characteristics of this death, such as shrinkage of the neuronal soma with intact organelles, nuclear condensation, fragmentation of the DNA into oligonucleosomes, and blebbing of the plasma membrane, are hallmarks of apoptosis, a mode of death seen during both development and normal tissue maintenance (Kerr et al., 1972). In sympathetic and other neurons, the death is dependent on the synthesis of new RNA and protein, suggesting that this death is an active process in which the neuron participates in its own demise (Martin et al., 1988). Evidence from Caenorhabditis elegans suggests that a specific genetic program is executed for apoptosis to occur. Specific cells invariantly undergo programmed cell death (PCD) during the natural development of the worm.

The expression of two genes, ced-3 and ced-4, is required for PCD to occur (Yuan and Horvitz, 1990, 1992), and the overexpression of ced-9 can block the death (Hengartner et al., 1992; Hengartner and Horvitz, 1994). Recently, the cysteine protease interleukin 1β-converting enzyme (ICE), mouse nedd2, and human ich-1ₗ (Wang et al., 1994) have been identified as mammalian homologs of ced-3 (Yuan et al., 1993). Overexpression of these gene products induces apoptosis in fibroblasts (Miura et al., 1993; Kumar et al., 1994; Wang et al., 1994). These findings indicate that proteolytic events are necessary for apoptosis. Whether the expression of ICE, nedd2, or ich-1ₗ is required for apoptosis to occur in any cell type is unclear; however, when dorsal root ganglion neurons are injected with a vector expressing the cowpox virus gene crmA, an inhibitor of ICE, apoptosis in response to nerve growth factor (NGF) deprivation is prevented (Gagliardini et al., 1994). bcl-2 and bcl-xₗ are mammalian homologs of ced-9; when either is overexpressed in a number of cell types, PCD can be delayed (Hockenbery et al., 1990; Boise et al., 1993; Hengartner and Horvitz, 1994). bcl-2 is clearly important in regulating the viability of cells in the immune system (Veis et al., 1993). These pieces of evidence, and the fact that inhibitors of protein and RNA synthesis block PCD in many systems, suggest that a genetic program regulates apoptosis in mammals as well.

During development of the rat, approximately 40% of the sympathetic neurons in the superior cervical ganglion undergo apoptosis during the first few days after the animal is born (Wright et al., 1983), apparently owing to an insufficient supply of NGF. NGF deprivation also triggers apoptosis in sympathetic neurons in vitro (Martin et al., 1988). In addition, treatment of sympathetic neurons with cytosine arabinoside (Martin et al., 1990; Deckwerth and Johnson, 1993) or with the cytokines LIF (leukemia inhibitory factor) or CNTF (ciliary neurotrophic factor; Kessler et al., 1993) can also trigger apoptosis. The overexpression in sympathetic neurons of bcl-2, which has recently been shown to have antioxidant properties (Hockenbery et al., 1993; Kane et al., 1993), delays the apoptosis in response to NGF deprivation (Garcia et al., 1992). This suggests a role for reactive oxygen species (ROS) in neuronal apoptosis. In other systems, stimuli that cause oxidative stress, including culture in high oxygen (Enokido and Hatanaka, 1993), exposure to β-amyloid (Forloni et al., 1993; Loo et al., 1993; Behl et al., 1994), and transient ischemia (Linnik et al., 1993; Okamoto et al., 1993; MacManus et al., 1994), have been reported to trigger the apoptotic process in other neuronal types. A role for ROS in motor neuron death has been suggested by the identification of mutations in copper/zinc superoxide dismutase (SOD) linked to familial amyotrophic lateral sclerosis (Deng et al., 1993; Rosen et al., 1993; Robberecht et al., ˙1994). ROS are also thought to be important in lymphocyte PCD, since the overexpression of either bcl-2 or glutathione peroxidase delays the IL-3 deprivation–induced apoptosis of a B-cell line (Hockenbery et al., 1990). Glutathione
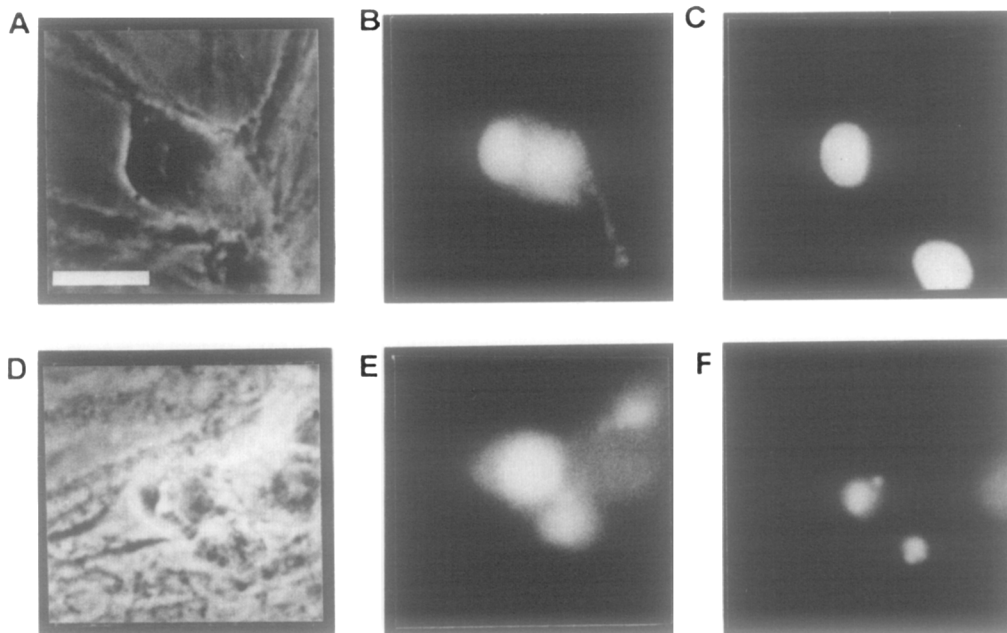
Figure 1. Apoptosis of Neurons Injected with SOD is Delayed

Sympathetic neurons were injected with SOD or boiled SOD, deprived of NGF for 24 hr, then fixed and stained with Hoechst 33,258 to examine the nuclear morphology.

(A) A phase-contrast photomicrograph of a neuron injected with SOD.

(B) This neuron was identified as injected by rhodamine-dextran staining.

(C) The chromatin was uncondensed as examined by Hoechst 33,258 staining. This is representative of a cell that was scored as live in these experiments.

(D and E) A phase-contrast photomicrograph of two neurons injected with denatured SOD (D), as demonstrated by rhodamine-dextran positivity (E).

(F) Hoechst 33,258 staining shows badly clumped DNA. These are representative of cells that were scored as dead. Bar, 25 μm.

peroxidase catalyzes the reduction of hydrogen peroxide and organic peroxides by glutathione, and thereby has a key role in the detoxification of ROS. These data led us to investigate further whether ROS are involved in the NGF deprivation–induced death of sympathetic neurons. By using an in vitro model of the developmental death of sympathetic neurons, we have shown that microinjection of copper/zinc SOD protein or a SOD expression vector delays the apoptosis of sympathetic neurons. Injection of an antisense SOD expression vector enhanced the death of neurons deprived of NGF. In addition, using a redox-sensitive dye to analyze the production of ROS after NGF deprivation, we saw a peak in fluorescence intensity 3 hr after deprivation. These data indicate that in sympathetic neurons ROS are important mediators of apoptosis induced by NGF deprivation.

Results

Injection of SOD Protein Protects Sympathetic Neurons from Programmed Cell Death

We examined whether ROS, in particular superoxide, are involved in apoptosis of sympathetic neurons induced by NGF deprivation. After 7 days in culture, sympathetic neurons were injected with a 2.5 mg/ml solution of bovine erythrocyte SOD. Rhodamine-labeled dextran was included in the injection solution so that injected neurons

could be identified. As a control, the SOD solution was heat denatured before injection. Immediately after injection with SOD or denatured SOD, neurons were deprived of NGF, and the number of rhodamine-positive neurons was scored. At 24–40 hr after NGF deprivation, the number of remaining rhodamine-positive neurons was scored by a blinded observer. The viability of rhodamine-positive neurons was assessed both by the morphology of the cell by phase-contrast light microscopy and by using the fluorescent DNA stain Hoechst 33,258 to examine chromatin morphology (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994; Estus et al., 1994). Figure 1 shows representative examples of neurons from an experiment in which cells were injected with SOD or with denatured SOD. The nuclear and cellular morphology of the SOD-injected cell (Figures 1A–1C) is typical of a healthy neuron; the cell membrane is smooth, and the chromatin is stained uniformly across the nucleus. Neurons injected with denatured SOD (Figures 1D–1F) displayed a morphology typical of dying cells; the membrane was rough, and the chromatin was fragmented and clumped. In nine independent experiments, injection of SOD showed a clear protective effect (Figure 2A). An average of 56% of SOD-injected neurons (n = 257) were alive at the time of scoring, whereas only 27% of neurons injected with denatured SOD (n = 269) were alive. Protection by SOD was dose dependent; 40 hr after NGF deprivation, 67% (n = 15) of
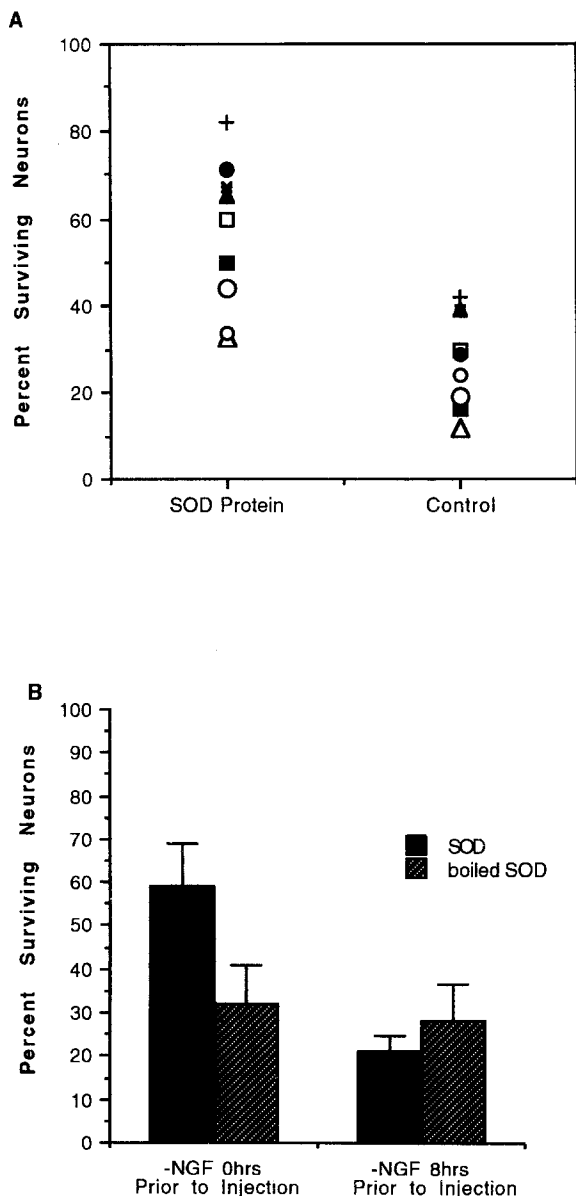
A

B

Figure 2. Injection of SOD at the Time of NGF Deprivation Delays Neuronal Death

(A) Cultured sympathetic neurons were injected with SOD (2.5 mg/ml) or a control solution and deprived of NGF. After 24–48 hr of deprivation, cultures were fixed and scored as living or dead by the phase-contrast appearance and the nuclear morphology as shown in Figure 1. A total of nine independent experiments showed that sympathetic neurons were protected from death by SOD (n = 257), but not by a control injection (n = 269). Each pair of data (SOD and its control) is represented by a pair of symbols. Plus signs and closed circles represent experiments with water controls, and controls in the other seven experiments are boiled SOD.

(B) Neurons were injected at the time of NGF deprivation or 8 hr after NGF deprivation. After the deprivation period, cultures were fixed and scored as alive or dead. Neurons injected with SOD at the time of NGF deprivation were protected from death as compared with control (p < .001, Student's t test), whereas neurons injected with SOD 8 hr after NGF deprivation were not (p > .5, Student's t test). Error bars represent SEM from three independent experiments.

neurons injected with 2.5 mg/ml SOD were alive, 54% (n = 24) injected with 1.25 mg/ml were alive, and 35% (n = 34) of neurons injected with 0.25 mg/ml SOD were alive, which was not different from the denatured SOD control (n = 31) in the experiment. In some experiments, neither the SOD nor boiled SOD showed any protective effect. This may have been caused by limited protein stability, excessive cellular damage during injection, or injection of insufficient amounts of SOD protein. However, when these experiments were included in the analysis, the p value for the paired Student's t test was less than 0.05. If bovine copper/zinc SOD, up to 1200 U/ml, was added to the extracellular medium, no protective effect was seen. There was variability in the number of neurons protected from apoptosis by SOD protein injection, partly because of technical reasons. However, the data indicate that copper/zinc SOD, when injected into sympathetic neurons immediately before NGF deprivation, protects neurons from apoptosis.

Neurons Injected with Copper/Zinc SOD after 8 hr of NGF Deprivation Are Not Protected from Apoptosis

We next examined whether SOD would be protective if injected into neurons that were already deprived of NGF. Readdition of NGF after 22 hr of deprivation saves 50% of the cultured sympathetic neurons in our system; we have defined this as the commitment point for NGF (Deckwerth and Johnson, 1993). Commitment of a neuron to die suggests that an irreversible step in the death process has taken place. To examine how early the commitment point for SOD occurred, in three independent experiments neurons were injected with SOD after they had been deprived of NGF for 8 hr, or just prior to NGF deprivation. If neurons were injected with SOD after being deprived of NGF for 8 hr, there was no protective effect (25% survival, n = 140) in comparison with denatured SOD (27% survival, n = 130; p > .5, Student's t test; Figure 2B). In contrast, in all three experiments, cultures maintained in NGF prior to injection were protected from apoptosis by SOD (59% survival, n = 121) but not by denatured SOD (31% survival, n = 153; p < .001, Student's t test). This indicates that SOD was effective in delaying apoptosis only when injected within the first 8 hr after NGF deprivation, and at later times there was no protection. These data suggest that there may be a burst of superoxide early after NGF deprivation of sympathetic neurons. To test the validity of this idea, we analyzed the production of ROS after NGF deprivation.

Staining with a Redox-Sensitive Dye Indicates the Presence of Increased ROS 3 hr after NGF Deprivation

The protective effect of SOD suggests that superoxide is generated within the first 8 hr after NGF deprivation. To detect ROS production, we used the redox-sensitive dye 5(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate bis(acetoxymethyl) ester (C-DCDHF-DA), which is readily taken up by cells. After uptake, intracellular ester-
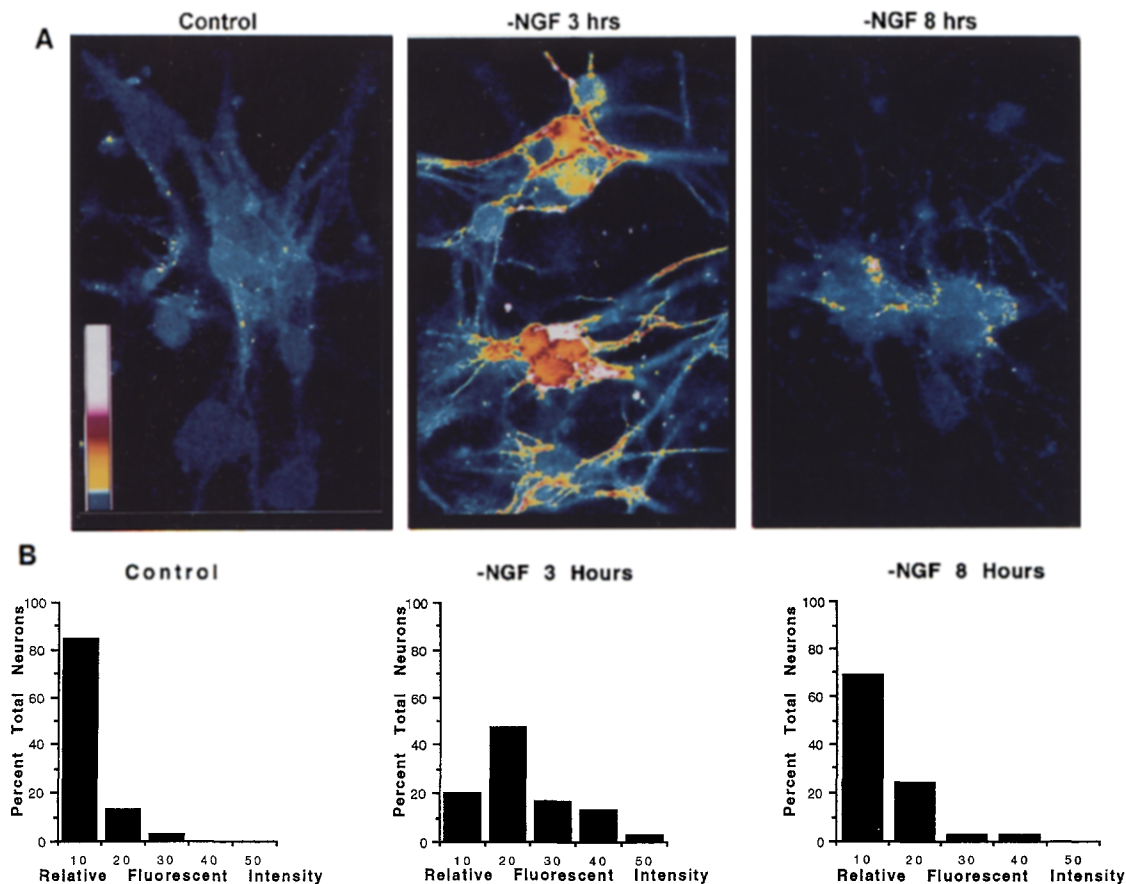
Figure 3. Staining with C-DCDHF-DA Indicates an Increase in ROS Production Peaking about 3 hr after NGF Deprivation

After 6 days in culture, sympathetic neurons were either maintained in NGF (control) or deprived of NGF for 3 or 8 hr. Cultures were stained for 20 min and analyzed by fluorescent confocal microscopy.

(A) Pseudocolor fluorescent images of representative control, 3 hr-deprived, and 8 hr-deprived cultures from one experiment. Neurons deprived of NGF for 3 hr were consistently brighter than control or 8 hr-deprived neurons. The pseudocolor scale represents a range of 0–255 relative fluorescent units. For the purposes of illustration, the color range was narrowed to enhance differences between control and 3 hr-deprived samples; however, this does not alter the absolute relative fluorescent intensity value.

(B) Absolute relative fluorescent intensities for every neuron in each field were determined and compiled. The data from one experiment are shown. The relative fluorescent intensity for neurons deprived for 3 hr was significantly shifted to the right as compared with control neurons (p < .001, Mann-Whitney U test) and 8 hr-deprived neurons (p < .001, Mann-Whitney U test). Neurons deprived of NGF for 8 hr were slightly brighter than control neurons (p < .04, Mann-Whitney U test). Approximately 50 neurons per time point were scanned and quantified.

ases hydrolyze the ester bonds, releasing the intact, non-fluorescent substrate. This reduced substrate is oxidized by superoxide and other ROS to the fluorescent species, carboxydichlorofluorescein, which is retained by living cells. After 7 days in culture, sympathetic neurons were either maintained in the presence of NGF or deprived of NGF for 3 or 8 hr and then loaded for 20 min with 10 μM C-DCDHF-DA in serum-free medium containing no NGF. Neurons were examined for fluorescence intensity initially by viewing under a fluorescent microscope. In seven of nine experiments, neurons that had been deprived of NGF for 3 hr appeared more intense than the control or 8 hr-deprived neurons. In a separate experiment, neurons were deprived of NGF for 2, 3, 5, or 8 hr. At 2 hr after deprivation, there was no increase in fluorescent intensity as compared with control, but at 3 hr, there was a clear increase, at 5 hr, a fall, and a further decrease at 8 hr (data not shown).

We next sought to quantitate this increase in fluores-

cence by confocal image analysis. In eight independent experiments, approximately ten fields of neurons were scanned in each sample, and the fluorescent intensity of every neuron in each field was quantified. Figure 3A shows an example of confocal images of neurons after C-DCDHF-DA staining. Neurons maintained in the presence of NGF showed a low intensity of fluorescence. In contrast, in seven of eight experiments, neurons deprived of NGF for 3 hr showed a clear increase in the level of fluorescence (the average fluorescent intensity ranged from 1.3- to 2.8-fold of control in the seven experiments), and by 8 hr after NGF deprivation, the fluorescent intensity decreased nearly to control levels. Figure 3B shows the results of an experiment in which confocal imaging was used to quantitate the relative fluorescence intensity in control, 3 hr-deprived, and 8 hr-deprived neuronal cultures. This result indicates that NGF deprivation leads to the production of ROS, peaking about 3 hr after deprivation.
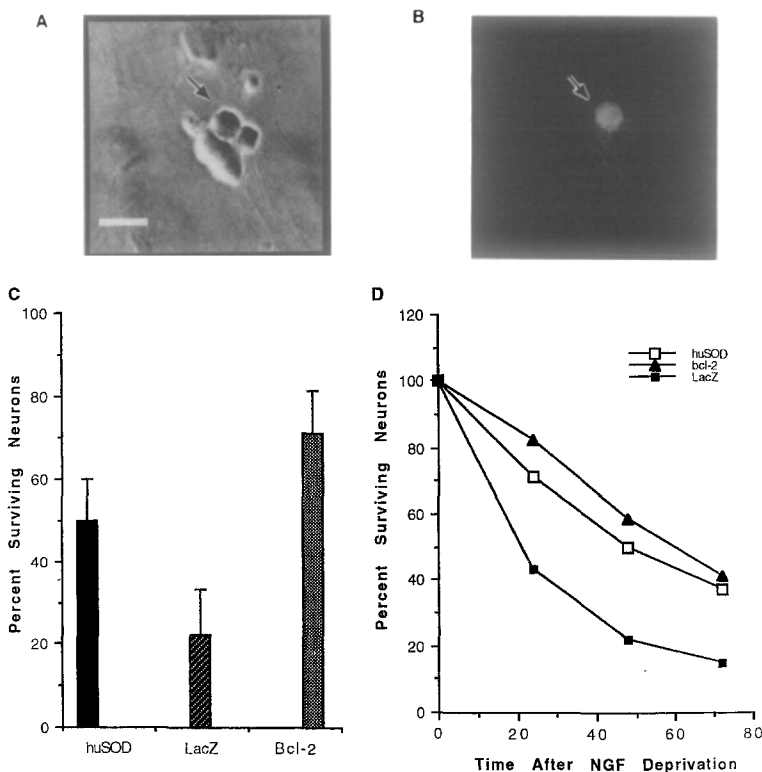
Figure 4. Neurons Injected with a *huSOD* Expression Vector Produce the Protein and Are Protected from Apoptosis

(A and B) Neurons were injected with the *huSOD* expression vector and maintained in NGF for 36 hr, then fixed and immunostained. (A) shows a phase-contrast image of a group of neurons, one of which was injected. (B) shows the injected neuron stained with an antibody against SOD. Bar, 25 μm.

(C) After 6 days in culture, sympathetic neurons were injected with the *huSOD* (n = 277), *bcl-2* (n = 154), or *lacZ* (n = 226) expression vector and maintained in NGF for 24 hr. All cultures were then deprived of NGF for 24–48 hr, fixed, and scored as described in Figure 1. In seven independent experiments, the expression of human SOD had a significant protective effect compared with LacZ expression (p < .001, Student's t test). In four of the seven experiments, bcl-2 was included as a positive control. The protection seen with bcl-2 was better than that seen with SOD (p < .01, Student's t test). Error bars represent SEM.

(D) Data from a representative experiment are shown, in which neurons were injected with the *huSOD* (n = 22), *bcl-2* (n = 23), or *lacZ* (n = 10) expression vector, then maintained in NGF for 24 hr. Neurons were deprived of NGF and scored at the indicated number of hours after deprivation.

Overexpression of Human Copper/Zinc SOD Delays Neuronal Apoptosis

To confirm the protection from apoptosis by SOD protein injections, to control for protein stability, and to rule out artifacts caused by the presence of contaminating substances, we next determined whether injection of a vector expressing human copper/zinc SOD would protect NGF-deprived neurons from death. We generated a human copper/zinc SOD (*huSOD*) cDNA by reverse transcription–polymerase chain reaction (RT-PCR) and confirmed the clone by sequencing. This cDNA was cloned into an expression vector. A human *bcl-2* cDNA was also cloned into the expression vector for use as a positive control, and *lacZ* was used as a negative control. After 6 days in culture, sympathetic neurons were injected with one of the expression vectors (*huSOD*, *bcl-2*, or *LacZ*) in a solution containing rhodamine dextran, so injected cells could be identified. After injection, cultures were scored for the number of rhodamine-positive cells and returned to NGF-containing medium to allow time for expression of the gene products. Figures 4A and 4B show immunostaining that confirmed the expression of the human copper/zinc SOD in injected cells. The expression of human *bcl-2* was confirmed by immunostaining and that of *lacZ* by X-gal histochemistry (data not shown).

To assess the effect of SOD expression on survival, in seven independent experiments, sympathetic neurons cultured for 6 days were injected with the *huSOD* expression vector or the *lacZ* expression vector. In four of the seven experiments, the human *bcl-2* expression vector was injected as a positive control. After 24 hr in NGF-containing medium, cultures were scored for the number of rhodamine-positive neurons and then deprived of NGF. After 24–40 hr of NGF deprivation, the number of live rhodamine-positive neurons was determined (Figure 4C). Both *SOD* and *bcl-2* were protective, with 50% of cells alive after injection of *huSOD* and 71% alive after injection of *bcl-2*, whereas only 22% remained alive in the *lacZ*-expressing controls. Results with the expression vector were much more reproducible than with injection of the bovine SOD protein. The increase in survival of neurons expressing huSOD was highly statistically significant in comparison with LacZ-expressing neurons (p < .001, ANOVA).

The length of time that *SOD* or *bcl-2* expression protected neurons from apoptosis was also determined. Neurons were injected with *huSOD*, human *bcl-2*, or *lacZ* expression vectors, maintained in NGF for 24 hr, and then deprived of NGF. The number of surviving neurons was scored at 24, 48, and 72 hr after NGF deprivation (Figure 4D). Both SOD and bcl-2 showed significant protective effects even 3 days after trophic factor deprivation; both proteins delayed the time course of death but did not completely block apoptosis.

Overexpression of SOD Does Not Prevent the Fall in Neuronal Protein Synthesis after NGF Deprivation

The rate of neuronal protein synthesis, as assayed by [^{35}S]methionine incorporation, decreases rapidly (to 50% of NGF control in 10 hr) after deprivation, and this decrease can be largely prevented by potassium depolarization or increased cyclic AMP, each of which prevents apoptosis (Deckwerth and Johnson, 1993; Franklin et al., 1995).

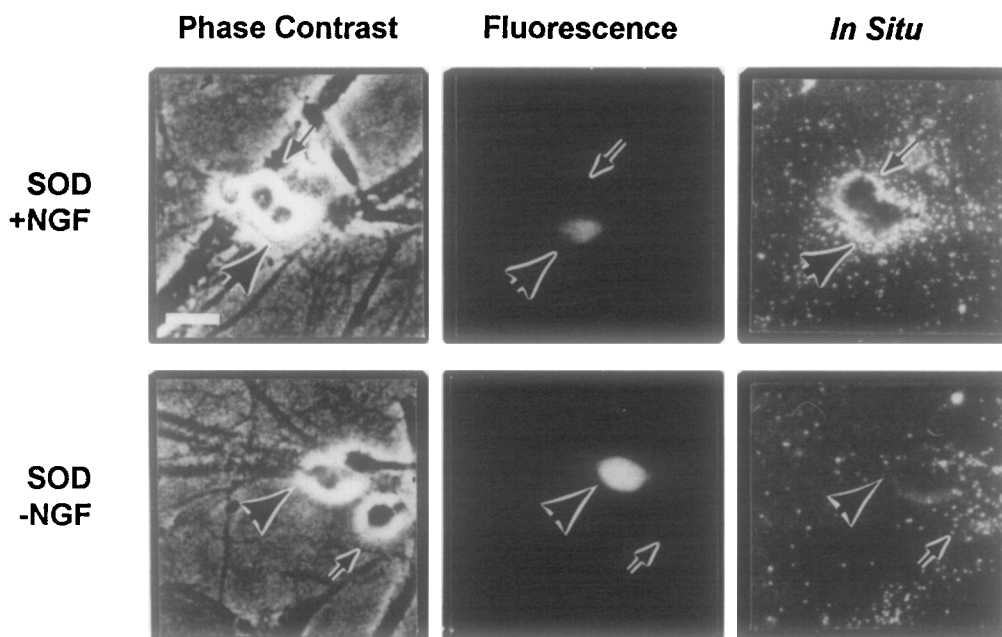## Phase Contrast   Fluorescence   *In Situ*

Figure 5. Expression of SOD Does Not Prevent the Fall in Protein Synthetic Rates after NGF Deprivation.

Sympathetic neurons were injected with the *huSOD* expression vector, maintained in NGF for 24 hr, and then either deprived of NGF or maintained in the presence of NGF. After 14 hr of NGF deprivation or maintenance, cultures were labeled with [^{35}S]methionine/cysteine, then exposed to emulsion. The first panels show phase-contrast images of injected (large arrows) and noninjected neurons (small arrows) in the presence or absence of NGF. The second panels show fluorescent images of the same fields of neurons; injected neurons were identified by the presence of the fluorescent label within the cell (identified by large arrows). The third panels show the in situ protein synthetic assay with silver grains overlying neurons. Scale bar, 25 μm.

Since either bcl-2 or SOD overexpression delayed death but did not produce long-term prevention, we tested whether their expression would significantly block this fall in protein synthesis.

We developed an in situ protein synthesis assay that allowed the comparison of [^{35}S]methionine/cysteine incorporation in individual neurons. Using this assay, neurons that were injected and expressed the foreign gene product were compared with uninjected neighboring neurons. Neurons were injected with the *huSOD*, *bcl-2*, or *lacZ* expression vectors and maintained in NGF-containing medium for 24 hr and then deprived of NGF. At the end of the NGF deprivation period, neuronal cultures were pulsed with [^{35}S]methionine/cysteine. Following exposure to emulsion, the number of grains overlying neurons was counted under dark-field microscopy. When injected neurons that were maintained in NGF were compared with neighboring uninjected cells, there was no difference in the number of grains overlying the neurons (lowest, p > .7, Student's t test) irrespective of the vector injected (Figure 5; Table 1). This indicates that the rate of [^{35}S]methionine/cysteine incorporation, and hence overall protein synthetic rates, was unaffected by the injection process. Neurons deprived of NGF (18 hr total) had, on average, 77% fewer silver grains over them than those maintained in NGF. In NGF-deprived cultures, neurons overexpressing SOD (Figure 5; Table 1), bcl-2, or LacZ (data not shown) had no difference in the number of grains overlying them as compared with uninjected neighboring neurons (lowest,

Table 1. Expression of SOD Does Not Prevent the Decrease in Protein Synthesis after NGF Deprivation

	n	Grains Counted	Average/Neuron	p value Student's t test
Experiment 1				
Control + NGF	9	648	72	
SOD + NGF	9	673	75	p > .8
Control − NGF	29	479	17	
SOD − NGF	22	303	14	p > .3
Experiment 2				
Control + NGF	8	397	50	
SOD + NGF	8	447	56	p > .7
Control − NGF	14	196	14	
SOD − NGF	13	165	12	p > .9

Neurons were treated as described in Figure 5. Silver grains overlying neurons were counted under dark-field microscopy.

p > .3, Student's t test). This indicates that the dramatic decrease (75%–85%) in protein synthesis that occurs with NGF deprivation is not prevented by SOD or bcl-2 overexpression. Because this crucial cellular function is not maintained by the expression of these protective proteins, it is not surprising that injected neurons do not remain viable for a prolonged period of time. This failure of SOD and bcl-2 to maintain protein synthetic rates may indicate that, although they retard the loss of viability, they do not substitute for the other stimulatory functions of NGF.
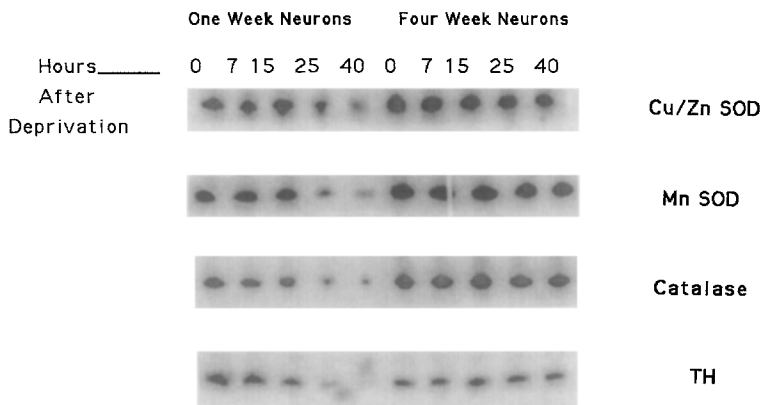
One Week Neurons   Four Week Neurons

Hours_____ 0 7 15 25 40   0 7 15 25 40
After
Deprivation

Cu/Zn SOD

Mn SOD

Catalase

TH

Figure 6. mRNA Levels of Redox-Related Messages Decrease as Neurons Die and Remain Relatively Constant as Neurons Mature mRNA was isolated from 1-week-old and 4-week-old sympathetic neuronal cultures that had been deprived of NGF for the indicated times. cDNA was prepared, and messages were analyzed by PCR. Several representative messages are shown along with the neuron-specific message, tyrosine hydroxylase (TH).

RNA Levels of Redox-Related Genes Decrease during Apoptosis and Remain Constant as Neurons Mature

Since increased ROS were generated after NGF deprivation, we determined whether this increase was produced by a selective decrease in the transcription of endogenous SOD RNA or other genes known to function in the detoxification of ROS. We also assessed whether in response to increased ROS neurons mounted a later protective response by increasing RNA levels of detoxifying genes. To evaluate gene expression in a small number of cultured sympathetic neurons, a semiquantitative RT-PCR assay was used (Freeman et al., 1994; Estus et al., 1994). A group of individual cultures containing the same number of neurons was prepared at a single time and divided into two sets. The first set of neurons was deprived of NGF after 6 days in culture. We examined RNA levels of several redox-related genes including copper/zinc SOD, catalase, manganese SOD (Figure 6), glutathione reductase, and selenium glutathione peroxidase (data not shown) as neurons underwent apoptosis. Over the time course of NGF deprivation, the level of all the redox-related messages examined fell with a rate similar to that of the neuron-specific message, tyrosine hydroxylase. The abundance of most RNA species examined in this system is decreased by 15 hr after NGF deprivation, a time well before the neurons are committed to die (Freeman et al., 1994; Estus et al., 1994). A fall in mRNA levels could be caused by a combination of decreased synthesis and increased degradation. Because many messages, which under normal circumstances have different stabilities, fell with similar time courses, degradation may have an important role in neuronal apoptosis. None of the redox-related mRNAs fell with a particularly rapid time course. Thus, when ROS appeared to be generated, 3 hr after NGF deprivation, there was no decrease in redox-related mRNA abundance. Thus, it does not appear that decreased production of redox-related gene products causes the increased ROS. Importantly, none of the mRNA species was increased in dying neurons, indicating that the neurons did not mount, at least by transcriptional activation, an antioxidant defense response via these enzymes.

The second set of sympathetic neuronal cultures were

maintained for 4 weeks prior to NGF deprivation. As the newborn animal matures, sympathetic (and other) neurons become progressively less dependent on trophic factor for survival (Jacobson, 1991). This phenomenon can be reproduced with sympathetic neurons in vitro by maintaining them for 4 weeks in culture in medium containing NGF (Lazarus et al., 1976). If NGF is then removed, most neurons remain viable for at least a week, suggesting that they are much less trophic-factor dependent. We evaluated whether this decrease in trophic factor dependence was correlated with an increase in redox-related gene expression and, hence, an increased ability to detoxify ROS. Examination of the redox-related mRNA levels in mature neurons, after NGF deprivation, showed no significant fall (Figure 6). This is similar to what is seen with tyrosine hydroxylase and is consistent with the observation that the cells are not dying. None of the messages were dramatically increased with aging, suggesting that increased expression of genes for these enzymes is not a likely explanation for the gain of trophic factor independence.

Expression of Antisense SOD Message Sensitizes Mature Neurons to NGF Deprivation

The fact that mature neurons die more slowly allowed us to determine whether a decrease in endogenous neuronal SOD enhanced the susceptibility to NGF deprivation. Decreasing the amount of SOD protein in the neuron would be expected to increase the amount of superoxide present and potentially increase the susceptibility to death. After 13 days in culture, neurons are significantly less acutely dependent on NGF for survival than are neurons cultured for 7 days, but are more dependent than 4-week-old neurons (unpublished data). This intermediate state of NGF dependence provided a good model in which to test the effect of antisense SOD expression. Neurons maintained in the presence of NGF for 11 days were injected with an antisense SOD expression vector or control vectors, including an antisense cyclin D1 expression vector, an antisense tyrosine hydroxylase expression vector, or a LacZ expression vector, and then returned to NGF for 2 days. To determine whether antisense SOD expression decreased the amount of SOD protein in the neurons, cells were injected with the antisense vector, and 2 days later,
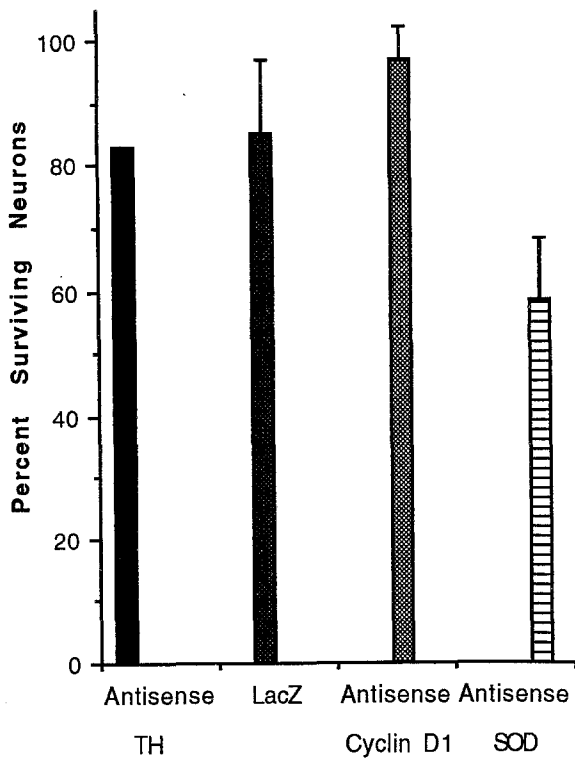
Figure 7. Anti-Sense SOD Expression Sensitizes Mature Neurons to NGF Deprivation

Eleven-day-old sympathetic neurons were injected with an antisense SOD expression vector or the control vectors and maintained in NGF for 48 hr. Cultures were then deprived of NGF for 24 hr, and survival was determined. Compared with any of the controls, more antisense SOD-injected neurons died (all $p < .02$, in Student t test). None of the controls were statistically different from each other ($p > 0.3$, ANOVA test). Data for antisense SOD data are from four independent experiments ($n = 216$), each with at least one control. LacZ was used in three experiments ($n = 212$), antisense cyclin D1 in two ($n = 89$), and antisense tyrosine hydroxylase in one ($n = 29$).

were stained for endogenous SOD. In a representative experiment, 34% of the neurons injected with the antisense SOD vector ($n = 32$) showed a clear decrease in the intensity of SOD immunofluoresence, whereas none of the neurons injected with the antisense tyrosine hydroxylase ($n = 28$) and 6% of the neurons injected with the antisense cyclin D1 ($n = 16$) vector showed a decrease in SOD staining. In addition, we analyzed the specificity of the antisense SOD expression by staining with an antibody against tyrosine hydroxylase. No decrease was seen in tyrosine hydroxylase staining of neurons injected with antisense SOD (data not shown). To determine whether this decrease in SOD protein affected neuronal survival, we again injected 11-day-old neurons with the expression vectors; the number of rhodamine-positive neurons was scored, and neurons were maintained for 2 days in the presence of NGF. The cells were then deprived of NGF for 24 hr, and the number of remaining rhodamine-positive neurons was then determined. In four independent experiments, significantly more (42%) antisense SOD-injected neurons died than neurons injected with antisense cyclin

D1 (3%), antisense tyrosine hydroxylase (17%), or LacZ (15%; Figure 7). The increased number of neurons that died from antisense SOD was very similar to the number of neurons that showed a clear decrease in SOD protein levels. These experiments show that a specific decrease in SOD protein was achieved by the injection of the antisense expression vector and indicate that this decrease led to an increased susceptibility of neurons to programmed cell death.

Discussion

In this paper, we present data consistent with the hypothesis that ROS, in particular superoxide, have a significant role in neuronal apoptosis after trophic-factor deprivation. These data include the protective effect of increased SOD levels and the sensitization to death by decreased SOD levels. The early increase in fluorescence detected by the redox-sensitive dye C-DCDHF-DA and the early commitment point for SOD suggest that ROS may act within the first several hours to mediate downstream events that eventually lead to the apoptosis of the cell.

Increasing SOD Levels Delays, but Does Not Block, Apoptosis of Sympathetic Neurons after NGF Deprivation

Injection into sympathetic neurons of either SOD protein or a SOD expression vector protected against apoptosis induced by NGF deprivation. The fact that SOD supplied at varying levels extracellularly was not protective indicates that superoxide generated within an individual neuron, and acting intracellularly, is a critical event in apoptosis. We used the injection of a bcl-2 expression vector as a positive control for protection from apoptosis, and we obtained results very similar to Garcia et al. (1992). The protective effect of SOD overexpression was very similar to that observed with bcl-2.

Injection of SOD or bcl-2 expression vectors effectively delayed neuronal death in a portion of the injected cells, but did not block the process. This may be due to three factors. First, this may reflect the delivery of varying amounts of material into cells by microinjection. Immunostaining of injected cells revealed varying intensities, demonstrating that varying amounts of protein are expressed by injected neurons. Apoptosis may be delayed only in the neurons expressing the highest levels of protective proteins.

Second, the delay in apoptosis rather than a block seen with SOD or bcl-2 overexpression may be caused by a failure to mimic the trophic effects of NGF. This would lead to the loss of vital cellular functions and, potentially, to the engagement of catabolic processes after NGF deprivation. Analysis of neuronal protein synthetic rates showed a dramatic decrease after NGF deprivation, and this was not blocked by overexpression of SOD or bcl-2. This fall in protein synthetic rates in the absence of NGF would also decrease the amount of expressed SOD or bcl-2 because they could no longer be made. Although the decrease in protein synthesis may not be directly fatal if other catabolic processes induced by NGF deprivation

are not prevented and the protective proteins are no longer made, the neuron would be expected to die eventually. In contrast to SOD, we have noted that more effective saving agents, such as depolarization or increased cyclic AMP, do prevent the fall in protein synthesis after NGF deprivation (Deckwerth and Johnson, 1993). The failure of SOD and bcl-2 to maintain protein synthesis after NGF deprivation may, indeed, indicate a failure to mimic the trophic effects of NGF and a failure to block catabolic processes that occur after NGF deprivation.

Third, the delay in apoptosis rather than a block may reflect multiple, independent parallel processes that occur during programmed neuronal death after trophic factor deprivation. These could include the increased production of cytotoxic molecules, the decreased production of protective molecules, the decreased production of ATP, and the activation of catabolic processes including the degradation of cellular RNA, DNA, and protein. The delay in apoptosis imparted by SOD or bcl-2 could reflect a block in the pathway that is normally rate limiting in the apoptotic process, but a failure to block all pathways. Our data cannot distinguish between the above possibilities, but they provide strong evidence that superoxide is important, at some early point, in mediating apoptosis.

Decreasing SOD Levels Increases Susceptibility to Apoptosis

Injection of an antisense SOD expression vector into sympathetic neurons decreased the amount of SOD protein in 34% of the cells and enhanced the sensitivity to apoptosis in a similar percentage of cells. Rothstein et al. (1994) have shown a similar phenomenon in cultured spinal neurons. Superoxide dismutase activity was either inhibited pharmacologically or by antisense oligonucleotides, and, over several weeks, caused the death of motor neurons. Troy and Shelanski (1994) have shown that within 24 hr, antisense SOD oligonucleotides decrease SOD protein levels, decrease SOD activity, and induce apoptosis in PC12 cells. In contrast, we did not observe a direct induction of death by antisense SOD expression, but an increased sensitivity to a death inducing stimulus (NGF deprivation). This difference may be explained by the limited period of observation; however, because the expression of antisense message decreases over time, an extended period of observation is not possible. The fact that decreasing SOD levels sensitized neurons to apoptosis further supports a role for superoxide in the process.

mRNA Levels for Redox-Related Genes Fall during Apoptosis and Do Not Increase with Age

The analysis of mRNA levels for several enzymes important for the detoxification of ROS demonstrated that there was a fall in sympathetic neurons undergoing apoptosis. The decrease in abundance of the redox-related mRNAs showed a pattern very similar to the neuron-specific message, tyrosine hydroxylase. Since each of the messages analyzed, which under normal circumstances have different stabilities, fell with very similar time courses, it is likely that there was a generalized degrada-

tion of mRNA. This fall in mRNA abundance occurred largely after the time that peak levels of ROS appear to be produced (~ 3 hr after NGF deprivation), and indicates that the fall did not cause the increased ROS. In addition, since increasing SOD levels was protective against apoptosis, we determined whether there was a dramatic increase in the abundance of SOD and other redox-related messages as neurons matured and became less trophic-factor dependent. We saw no dramatic differences between the abundance of redox-related messages in sympathetic neurons cultured for 1 week or 4 weeks. This indicates that sympathetic neurons do not achieve trophic factor independence by increasing mRNA levels of redox-related genes, but does not rule out increases in redox-related enzyme stability or catalytic activity as neurons mature. This leaves the possibility open that maturity increases the capacity for ROS detoxification and, hence, decreases susceptibility to apoptosis.

Increased Production of ROS Occurs Early in Apoptosis

Increased SOD protein did not delay neuronal apoptosis if injected after 8 hr of NGF deprivation. In contrast, other long-term saving agents, such as potassium depolarization, inhibitors of macromolecular synthesis, and cyclic AMP analogs, can be added up to 12 hr after NGF deprivation and still completely block the death of all of the neurons (Martin et al., 1988; Rydel and Greene, 1988; Edwards et al., 1991; Martin et al., 1992; Deckwerth and Johnson, 1993; Franklin et al., 1995). The data indicate that the relevant effect of superoxide had already occurred by 8 hr after trophic factor deprivation and that after this time, the dismutation of superoxide was no longer able to stop or retard the apoptotic pathway. However, this did not distinguish between two possibilities: first, that after 8 hr of NGF deprivation, massive amounts of superoxide had formed and damaged the neuron to the point that increased SOD was not protective, or second, that superoxide production was a transient event that signaled other downstream events to occur, and that once the signal had been transduced, increased SOD was not protective. The former possibility appears less likely since many saving agents completely reverse death even after 8 hr of NGF deprivation.

To distinguish between the two possibilities, we used the redox sensitive dye C-DCDHF-DA to analyze ROS production during neuronal apoptosis. Staining with C-DCDHF-DA indicated that there was an increase in the formation of ROS, peaking at 3 hr after NGF deprivation and declining thereafter. It is possible that an increase in dye uptake or esterase activity trapping additional dye in the cell could lead to a similar result; however, there are other events taking place in the neurons after NGF deprivation that are also suggestive of an increase in ROS formation. The transcription of c-jun is increased in sympathetic neurons by 5 hr after trophic factor deprivation, and by 10 hr, there is a significant increase in junB, fosB, c-fos, and mkp-1 transcription (Estus et al., 1994). The transcription of c-jun (Devary et al., 1991; Manome et al., 1993), c-fos (Crawford et al., 1988; Shibanuma et al., 1988), and mkp-1 (Keyse

and Emslie, 1992) are induced when cells are exposed to oxidative stress and is consistent with a cellular response to increased ROS. In addition, the level of cellular glutathione, the most abundant water-soluble cellular antioxidant, is significantly elevated after 6 hr of NGF deprivation (T. L. D., unpublished data), which also may be a cellular response to an early increase of ROS. In conjunction with the protective effect of SOD only at the time of NGF deprivation and not 8 hr after deprivation, the increase in fluorescence in sympathetic neurons observed with C-DCDHF-DA 3 hr after NGF deprivation is most likely reflective of an increase in ROS. This result indicates that there is a transient increase in ROS soon after NGF deprivation and suggests that superoxide may be serving as a signal, rather than a toxic agent, in apoptosis. ROS may signal by modulating transcription. This modulation may be direct or through known redox-sensitive proteins like REF-1, which reduces and induces the dimerization of c-jun with c-fos (Xanthoudakis and Curran, 1992) or NFkB, a transcription factor that can be activated by oxidative stress to bind DNA (Adcock et al., 1994).

It is also possible that superoxide, or other ROS, could act to modulate other cellular functions. Nitric oxide, also a free-radical gas, has been shown to act as a signaling molecule by modulating a variety of processes including guanylyl cyclase activity and N-methyl-D-aspartate-receptor activity. Nitric oxide may modulate function by reacting with functional groups or cofactors (for review see Stamler et al., 1992) to alter their activity. Because superoxide has a similar capacity, it may function in an analogous manner.

Conclusions

Recent data have begun to outline a series of events that are important for sympathetic neuronal PCD. These include a series of biochemical changes, such as decreases in protein and RNA synthesis, degradation of RNA and DNA, generation of ROS, and the expression of certain genes. The expression of c-jun is a critical event in apoptosis, as demonstrated by the ability of neutralizing antibodies to block the process (Estus et al., 1994). Likewise, the protective effect of crmA expression, an inhibitor of ICE, in dorsal root ganglion neurons implies that an ICE-like proteolytic event is necessary for death (Gagliardini et al., 1994). Herein, we present data that superoxide generation is also an important mediator of apoptosis. Current data are consistent with a series of events involving first the generation of ROS, followed by increased c-jun expression. This induction, accompanied by decreased expression of other genes and the initiation of catabolic events, leads to the activation of an ICE-like protease and eventual apoptosis. This scheme, although incomplete, provides a framework upon which to insert other events in the pathway. Hopefully, a more complete understanding of the apoptotic process will lead to selective methods to control it.

Experimental Procedures

Sympathetic Neuronal Culture
Primary cultures of superior cervical ganglion neurons were prepared by a modification (Martin et al., 1988) of the method of Johnson and

Argiro (1983). Approximately 3000 neurons were plated in the center of collagen-coated, 35 mm dishes and maintained for several days in NGF-containing medium (AM50). The medium was Eagle's minimal essential medium with Earle's salts (MEM; Life Technologies, Gaithersburg, MD), with the addition of 50 ng/ml NGF (prepared by the method of Bocchini and Angeletti, 1969), 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxy-uridine (an antimitotic), and 20 μM uridine. Neurons were deprived of NGF by incubation in the same medium, but without NGF (AM0) and with goat polyclonal anti-mouse NGF antiserum added.

Microinjection of SOD Protein
Bovine erythrocyte copper/zinc SOD (Calbiochem, San Diego, CA) was solubilized at a concentration of 5 mg/ml in either water or a 100 mM KCl/10 mM KP$_i$ buffer (pH 7.4). Solubilization in buffer gave more reproducible results. A dye solution containing 8 mg/ml rhodamine dextran (Sigma, St. Louis, MO) and 100 mM KCl/10 mM KP$_i$ was combined 1:1 with the enzyme; solutions were centrifuged for 10 min. Femtotip injection pipettes (Eppendorf, Madison, WI) were used for all injections, and approximately 50 femtoliters per cell was injected. During injections, the medium on the neurons was Leibovitz's L-15 (Life Technologies), which is phosphate buffered. Proteins were injected into the cytoplasm of neurons, taking care to avoid disturbing the nucleus. Scoring of experiments was by a blinded observer. The unit activity indicated for SOD was determined by the cytochrome c reduction assay (McCord and Fridovich, 1969).

Construction and Injection of Expression Vectors
The base expression vector was constructed by cloning into pUC19, a 695 bp hCMV IE-1 promoter (620 bp upstream of the transcription start site and 75 bp downstream; Boschart et al., 1985) and a 355 bp portion of the mouse protamine-1 gene (Kleene et al., 1985), including the polyadenylation signal. Specific expression vectors were constructed by cloning cDNAs into the BamHI site between the promoter and polyadenylation signal of the base vector. The human bcl-2 cDNA was generously provided by Dr. Stanley Korsmeyer (Washington University), and the rat tyrosine hydroxylase cDNA was kindly provided by Dr. Karen O'Malley (Washington University). The cyclin D1 antisense expression vector was provided by Mitotics (Boston, MA). The expression is also driven by the hCMV promoter in this vector. The human copper/zinc SOD cDNA was cloned by PCR using the forward primer 5'-GCGGATCCTTCCGTTGCAGTCCTCGGAA-3' and the reverse primer 5'-GCGGATCCTTCTACAGCTAGCAGGATAA-3'. Both primers were flanked by BamHI sites to facilitate cloning of the cDNA into the base expression vector. After cloning, the huSOD cDNA was sequenced to ensure no mutations had been introduced. DNA was prepared by column purification (Qiagen, Chatsworth, CA) and resuspended at 0.18 μg/μl in sterile-filtered, deionized water. For injections, DNA was combined 1:1 with a dye solution containing 8 mg/ml rhodamine dextran, 100mM KCl, and 10mM KP$_i$. Neuronal culture medium was changed to Leibovitz's L-15 (Life Technologies), and injections were made into the nucleus of each neuron. It was noted that concentrations of DNA above 0.5 μg/μl produced a delay in apoptosis irrespective of the cDNA in the vector. We demonstrated by the in situ protein synthesis assay (see below) that injection of DNA at concentrations as low as 0.2 μg/μl occasionally caused an inhibition of protein synthesis in the presence of NGF, which presumably explains the delay in death. Experiments were scored by a blinded observer.

In Situ Protein Synthesis Assay
Neurons were injected with solutions containing 0.09 μg/μl expression vector DNA, 2.25 mg/ml CY3-labeled donkey anti-sheep antibody as a fixable tracer (Chemicon, Temecula, CA), 50 mM KCl, and 5 mM KP$_i$, and then maintained in AM50 for 24 hr. Cultures were deprived of NGF for 14 hr before beginning a 4 hr labeling period. During the labeling period, neurons were incubated at 35°C in MEM with 10% fetal bovine serum containing 10 μM unlabeled L-methionine and cysteine, 10 μCi/ml Tran[^{35}S]-label (ICN, Irvine, CA), and 50 ng/ml NGF or polyclonal goat anti-NGF antibody. After labeling, cultures were washed three times with PBS to eliminate free label and then fixed for 20 min at room temperature in fresh 4% paraformaldehyde in PBS. Cells were dehydrated through a 70%, 95%, 100% ethanol step-

gradient and dipped in Kodak emulsion. After an overnight exposure, dishes were developed in D-19 developer (Kodak, Rochester, NY), fixed, and rinsed in water. Dishes were coverslipped with water as the mounting fluid and then photographed. The number of grains overlying neurons were counted under dark-field microscopy. Only grains lying within the borders of the cell body of each neuron were counted.

C-DCDHF-DA Staining

Neuronal cultures were maintained in AM50 for 7 days after dissection and then deprived of NGF in AM0 with goat polyclonal anti-NGF antibody for the indicated times. After deprivation, cultures were incubated for 20 min at 35°C in prewarmed serum-free medium consisting of 1:1 Dulbecco's Modified Eagle Medium and Ham's F12 Medium (Life Technologies), with 20 nM progesterone (Sigma), 30 nM selenium (Pflatz and Bauer, Waterbury, CT), 100 μM putrescine (Sigma), 5 mg/liter insulin, 10 mg/liter rat transferrin (Jackson Immunoresearch Labs, West Grove, PA), 2 mM glutamine (Sigma), and 10 μM C-DCDHF-DA (#C-2938, Molecular Probes, Eugene, OR). Cultures were viewed with fluorescence microscopy and photographed. This dye is very sensitive to illumination with the excitation wavelength, and emitted fluorescence rapidly increases with illumination. Great care was taken to view cells within seconds to avoid fluorescence generated by illumination of the sample.

Quantitative fluorescence imaging was done on a Molecular Dynamics (Sunnyvale, CA) confocal microscope using Image Space software. Each sample was independently stained so samples were exposed to the dye for the same time. The dye solution was freshly prepared in prewarmed serum-free medium for each sample. Following staining for 20 min at 35°C, samples were rinsed twice with serum-free medium containing no dye, coverslipped, and scanned on the confocal microscope. Fields at 400× magnification were rapid scanned (~3 seconds per scan) once, the optimal vertical position was set, and then the field was rapid scanned a second time. Since illumination with the excitation wavelength (488 nm) caused increased fluorescence because of oxidation of the dye, each field was exposed to light for exactly the same time, and the laser intensity was set for only 10% of maximum. Any light emitted over 510 nm during scanning was quantified. The pixel size for scanning was 0.6 μm. After scanning, the average relative fluorescence intensity for every neuron in each field was determined using Image Space software. The Mann-Whitney U test (rank sum test) was used for statistical analysis.

Hoechst 33,258 Staining

Neuronal cultures were fixed for 20 min in freshly prepared 4% paraformaldehyde in PBS, then rinsed with PBS, stained for 15 min in 1 μg/ml Hoechst 33,258 (Molecular Probes) in PBS. Cultures were then rinsed twice with PBS and examined under UV illumination on a Nikon Microphot fluorescence microscope.

cDNA Preparation

Primary cultures (approximately 25,000 neurons per dish), which had been preplated for 2 hr on plastic to remove most nonneuronal cells before final plating on collagen-coated dishes, were maintained in AM50 for 6 days. Cultures were deprived of NGF for the indicated times, and then RNA was harvested. Polyadenylated RNA was isolated by using an oligo(dT)-cellulose mRNA purification kit, as directed by the manufacturer (QuickPrep Micro kit, Pharmacia, Piscataway, NJ). Half of the poly(A) RNA was converted to cDNA by reverse transcription with Moloney murine leukemia virus reverse transcriptase (Superscript, Life Technologies) with random hexamers (16 μM) as primers. The 30 μl reaction contained 50 mM Tris (pH 8.3), 40 mM KCl, 6 mM MgCl₂, 1 mM dithiothreitol, 500 μM dATP, 500 μM dTTP, 500 μM dCTP, 500 μM dGTP, and 20 U RNAsin (Promega, Madison, WI). After 10 min at 20°C, the samples were incubated for 50 min at 42°C, and the reaction was then terminated by adding 70 μl water and heating to 94°C for 5 min.

PCR Analysis

Rather than normalizing the quantity of cDNA put into each PCR, we normalized to the percent of total RNA isolated from the culture. Since all RNA isolations and reverse transcriptions from the individual cultures were done in the same volumes, this normalization translated into using equal volumes of the RT reaction to input into the PCR. Using

this method, there is a clear fall in the abundance of neuronal-specific messages when neurons die over the 40 hr period of NGF deprivation, whereas messages specific for Schwann cells (S100b) remain constant (Freeman et al., 1994; Estus et al., 1994). Because the efficiency of RT is gene specific and the efficiency of PCR is primer set specific, this method cannot be used to directly compare levels of two or more gene products; however, this technique is useful for comparing relative changes in the expression of a single gene in a series of similar samples. Oligonucleotide primer sequences were synthesized by the Washington University Protein Chemistry Laboratory. Reactions for PCR amplification of specific cDNAs were prepared on ice. Each reaction contained 50 μM dCTP, 100 μM dGTP, 100 μM dATP, 100 μM dTTP, 15 μCi [α-³²P]dCTP (3000 Ci/mmol), 1.5 mM MgCl₂, 50mM KCl, 10mM Tris (pH 9.0), 0.1% Triton X-100, 1 μM of each primer, 1 U Taq polymerase (Life Technologies), and 1% of the cDNA synthesized in the RT reaction. Each redox-related gene product was amplified for 25 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C in a Perkin Elmer Cetus (Norwalk, CT) thermocycler. Tyrosine hydroxylase was amplified 17 cycles under the same conditions. After amplification, products were separated on a 10% polyacrylamide gel, which was dried and visualized on a PhosphorImager (Molecular Dynamics). The copper/zinc SOD forward primer sequence was 5'-GGGAATTCAAGG-CCGTGTGCGTGCTGAA-3'; the reverse was 5'-GCGGATCCCCA-TATTGATGGACATGGAA-3' (the pair generated a 145 bp fragment). The manganese SOD forward primer sequence was 5'-GGGAATTCA-GCGTGACTTTGGGTCTTTT-3'; the reverse was 5'-GCGGATCCGAG-CAGGCGGCAATCTGTAA-3' (the pair generated a 129 bp fragment). The glutathione reductase forward primer was 5'-GGGAATTCCCA-AGTTGTGAGGGTAATT-3'; the reverse was 5'-GCGGATCCTTTTTC-CCACTGACCTCTAT-3' (the pair generated a 175 bp fragment). The selenium glutathione peroxidase forward primer was 5'-GGGAATTCT-GGCACAGTCACCGTGTAT-3'; the reverse was 5'-GCGGATCCGA-GGGACGCGACATTCTCAA-3' (the pair generated a 113 bp fragment). The catalase forward primer was 5'-GGGAATTCGACCAGATGAAG-CAGTGGAA-3'; the reverse was 5'-GCGGATCCCAAAAGCACCTGC-TCCCTTT-3' (the pair generated a 209 bp fragment).

Immunostaining

The conditions of staining were similar for detection of human superoxide dismutase, rat superoxide dismutase, rat tyrosine hydroxylase, and human bcl-2. Human superoxide dismutase was detected 36 hr after injection of the expression vector, human bcl-2 was detected 24 hr after injection, and rat SOD and tyrosine hydroxylase were detected 48 hr after injection of the antisense expression vectors. In each case, neurons were fixed in fresh 4% paraformaldehyde in PBS at room temperature for 30 min, permeabilized 20 min in Tris-buffered saline containing 0.1% Triton X-100, 1% bovine serum albumin, and 1% normal goat serum, and then incubated with the primary antibody diluted in the permeabilization solution (1:1000 rabbit polyclonal anti-bovine SOD antibody [Rockland, Gilbertsville, PA], 1:1000 mouse monoclonal anti-rat tyrosine hydroxylase antibody [generously provided by Dr. Karen O'Malley], 1:100 6C8 hamster monoclonal anti-human bcl-2 [courtesy of Dr. Stanley Korsmeyer]). For the detection of overexpressed human SOD, incubation with the primary rabbit polyclonal anti-bovine SOD antibody was for 1 hr at room temperature, and for the detection of endogenous rat SOD, the incubation was at the same dilution, but overnight at 4°C. Incubation with the anti-rat tyrosine hydroxylase antibody was also overnight at 4°C. Incubation with the anti-human bcl-2 was for 1 hr at room temperature. Samples were rinsed twice and then washed twice for 10 min in permeabilization solution. Secondary antibodies were diluted in permeabilization solution and incubated for 30 min at room temperature with the samples. Fluorescein isothiocyanate–labeled goat anti-rabbit antibody (1:1000; ICN, Costa Mesa, CA) was used to detect rat and human SOD, and fluorescein isothiocyanate–labeled goat anti-mouse (Boehringer Mannheim, Indianapolis, IN) or hamster (Southern Biotechnology, Birmingham, AL) antibody was used to detect rat tyrosine hydroxylase and human bcl-2, respectively. After three 10 min washes in the permeabilization solution and one in PBS, samples were viewed under UV on a Nikon Microphot fluorescence microscope and photographed.

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